THE UNCONVENTIONAL ROLE OF AKT1 IN ADVANCED PROSTATE CANCER: FUNCTIONAL AND MOLECULAR CHARACTERIZATION

BY

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(Under the Direction of Somanath Shenoy)

ABSTRACT

Despite the reduced incidence and improved 5-year survival rate of localized PCa patients, there is a marked increase in the incidence of metastatic PCa (mPCa) and associated mortality. Decades of clinical trials targeting Akt oncogene for cancer have not yielded any desirable results. A growing body of evidence, including a prostate cancer study from our laboratory, have demonstrated that genetic or pharmacological Akt inhibition in the advanced cancers promotes metastasis. In our laboratory, although Akt1 deficiency in TRansgenic Adenocarcinoma of the Mouse Prostate (TRAMP) mice inhibited prostatic inter-epithelial neoplasia and tumor development, pharmacological suppression or genetic deletion of Akt1 in the advanced PCa promoted epithelial-to-mesenchymal transition (EMT) and metastasis. The overall objective of this thesis is to understand how Akt1 suppression mechanistically promotes PCa EMT and metastasis. *First*, our results from immunohistochemistry, as well as the cBioPortal patient genomic database analyses, demonstrated reduced Akt phosphorylation (activity) in high Gleason Score compared to low Gleason Score PCa tissues. *Second*, we identified that the promotion of PCa EMT with Akt1 gene deletion is due to increased Nodal expression. Pharmacological inhibition of Nodal pathway mitigated PCa EMT and metastasis in athymic nude mice. Genomic database analysis confirmed increased expression of TGFβ1 and Nodal in

high Gleason Score compared to low Gleason Score PCa tissues. *Third*, we profiled the microRNAs and mRNAs comparing Akt1 intact with Akt1 deficient human PCa cells using the NanoString® technology. We identified increased miR-199a-5p and decreased let-7a-5p in Akt1-silenced PCa cells correlating to the TGFβ1 pathway activation. Treatment of PCa cells with miR-199a-5p inhibitor and/or let-7a-5p mimic inhibited TGFβ-RI expression and affected their motility and invasion *in vitro*. Genomic data analysis from cBioPortal and Real-Time PCR analysis of human PCa tissues demonstrated elevated miR-199a-5p and reduced let-7a-5p in high vs. low Gleason Score tissues. Taken together, our study has demonstrated that targeting Akt1 in the advanced PCa promotes Nodal expression through FoxO and TGFβ pathway activation by microRNAs, presents FoxO and Nodal as two druggable targets for mPCa, and suggest that miR-199a-5p and let-7a-5p may potentially be targeted or utilized for the early detection of mPCa.

INDEX WORDS: EMT; Prostate Cancer; Nodal; TGFβ; microRNA; Metastasis; N-cadherin

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The University of Georgia
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DEDICATION

All praises are to the almighty, graceful God for giving me the strength, health, and knowledge to complete my Ph.D. dissertation

I dedicate this thesis…

… To my beloved parents, ***NORAH & MOHAMMED****, who taught me the value of education*

… To my beloved brothers, ***ABDULAZIZ*** *and* ***ABDULLAH****, who stood next to me in the time of difficulties and frustration*

… To my beloved son, ***MOHAMMED****, and daughter,* ***NORAH,*** *who filled my life with love and happiness*

…To my beloved soulmate, ***EMAN****, the sincere and blessed wife, who always put me first and made countless sacrifices and whom I couldn't achieve anything without her support*

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CHAPTER 1

1. Introduction and literature review

1.1. Prostate gland

The prostate is a male sexual organ situated on the anterior wall of the rectum; between the bladder and penis. It is about a walnut-size in young males and grows slowly up to an

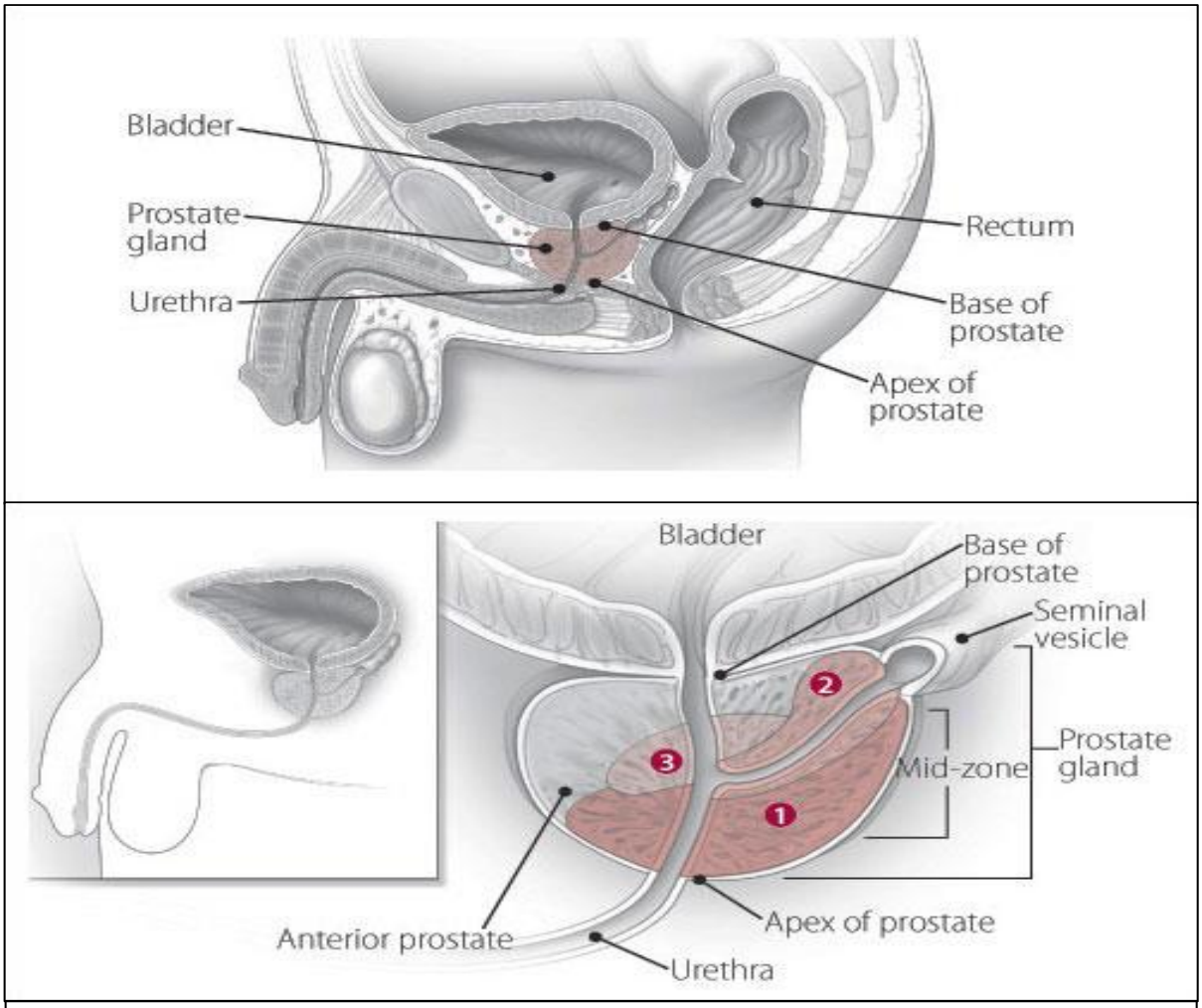


FIGURE 1: Adapted from http://www.harvardprostateknowledge.org

average weight of 40 grams in adults. The prostate surrounds the urethra, which runs from the bladder through the penis carrying the urine out of the body, and secrets fluid into the urethra

during ejaculation. This prostatic fluid is an essential component of the semen, as it nourishes and supports sperms when they are expelled out. Human prostate is divided into two lobes (right and left), which are held together and surrounded by fibromuscular and connective tissue. Each lobe is divided into four regions or zones including 1) Peripheral zone (PZ), 2) Central zone (CZ) 3) Transitional zone (TZ) and 4) Anterior fibromuscular zone (Figure 1). With the exception of the anterior zone that is mainly made of muscle fibers and connective tissue, all zones contain multiple glands connected to ductal channels that deliver the secreted prostatic fluid into the urethra [1]. Although prostate cancer (PCa) can develop in any zone, it mainly originates from the peripheral zone [2].

1.2. Prostate cancer epidemiology and risk factors

Prostate cancer is estimated to account for 20% of all cancer cases and 10% of cancer-

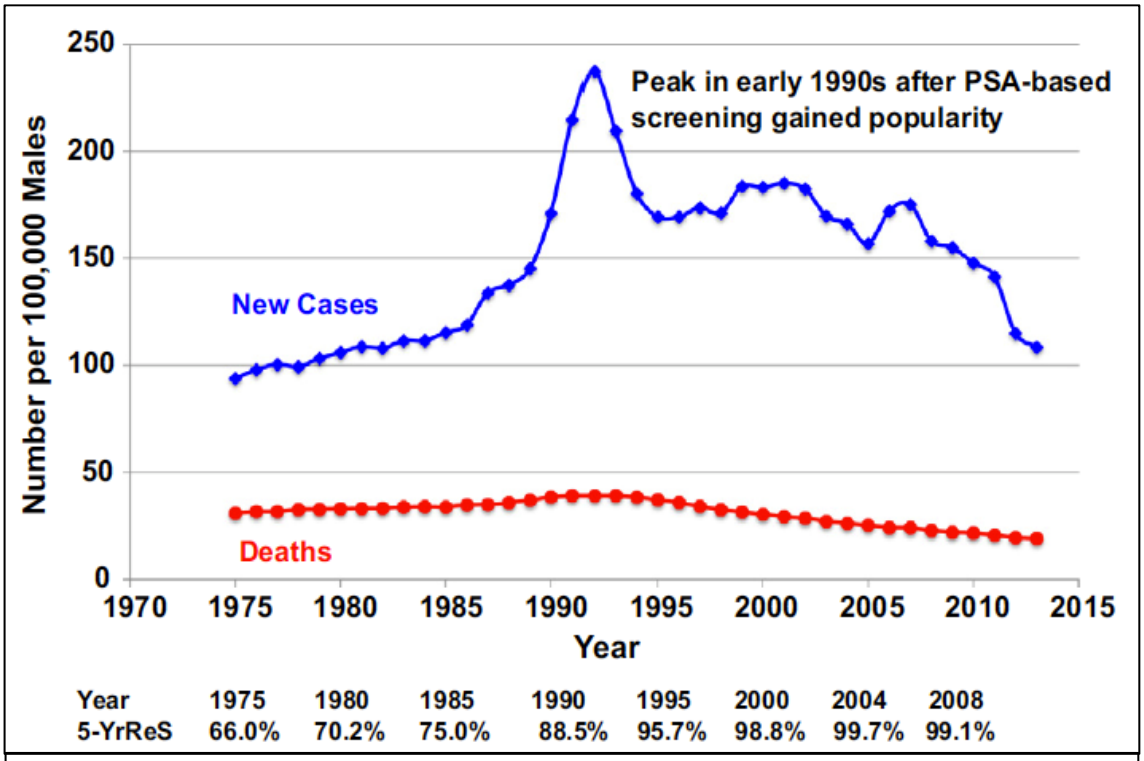


FIGURE 2: Adapted from Stewart, R.W., et al., *Screening for prostate cancer.* Semin Oncol, 2017. 44(1): p. 47-56.

related deaths in men in the United States [3]. In details, around 174,650 newly diagnosed cases and 31,620 deaths from PCa are expected to occur 2019, making it the most common cancer and the second cause of death after lung cancer among the American men according to the American Cancer Society (ACS) [3].

Incidence and mortality rates of PCa have been influenced by many factors such as screening and treatment approaches, patients' age and race/ethnicity [4, 5]. In the United States, the incidence rate had spiked to 85% in the late 1980s and early 1990s because of the rise in disease awareness and widespread prostate-specific antigen (PSA) testing even in men without cancer symptoms (Figure 2) [6-8]. Moreover, the introduction of transurethral resection of the prostate (TURP) for benign prostatic hyperplasia (BPH) in the early 1980s contributed to the early diagnosis and escalation in PCa cases, which some would have been diagnosed in later years while others would never be identified at all [7]. This spike was followed by a sharp decline of approximately 29% between 1992 and 1995. This was attributed to the pre-identified cases using PSA testing in addition to reducing the use of TURP [6, 7]. Later, the incidence rate started increasing slightly between 1996 and 2000 then reduced thereafter [6]. More importantly, despite the slight increase in 2005, the incidence rate has been steadily decreasing since 2010. This was mainly attributed to the recommendation of

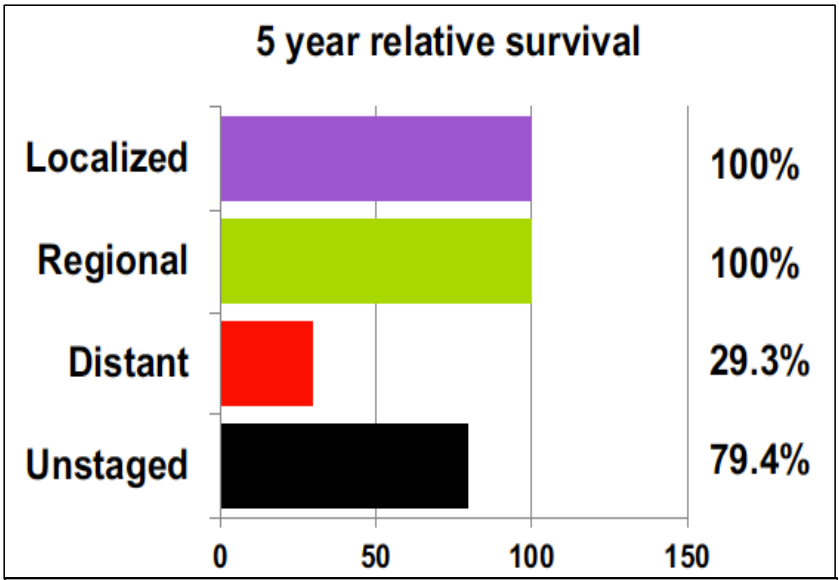
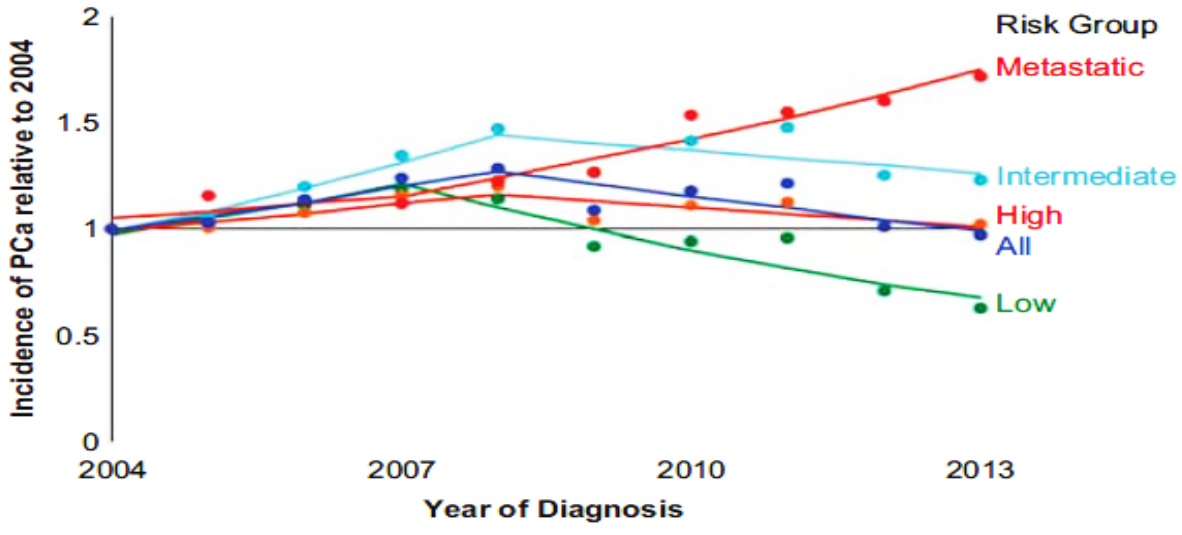


FIGURE 3: Adapted from Stewart, R.W., et al., *Screening for prostate cancer.* Semin Oncol, 2017. 44(1): p. 47-56.

the US Preventive Services Task Force (USPSTF) against the routine PSA screening, as more concerns have been raised due to over-diagnosis and over-treatment of PCa [3]. Although the number of cases has been decreasing, PCa is still considered substantially worrisome such that 1 out of 6 men will be diagnosed with it. In other words, the lifetime risk of developing PCa is about 16%, while 2.9% is the risk of dying from it [4]. The overall mortality rate from PCa gradually increased between 1976 and 1992, and then reduced thereafter [3, 4, 8]. Between 2007 and 2011, the rate decreased by 3.5% per year, however, it was unknown whether this reduction was due to PSA screening or improved treatment [9]. Despite this, careful analysis has revealed that this reduction was mainly driven by reduced number of deaths in patients diagnosed with



Risk Group	Trend 1			Trend 2		
	Interval	APC	p	Interval	APC	p
Low	2004-2007	7.5	0.3	2007-2013	-9.3	<0.05
Intermediate	2004-2008	10	<0.05	2008-2013	-2.7	0.3
High	2004-2008	4.1	0.1	2008-2013	-2.7	0.1
Metastatic	2004-2007	3.3	0.4	2007-2013	7.1	<0.05
All	2004-2008	6.3	0.1	2007-2013	-4.8	0.1

FIGURE 4: Adapted from Weiner, A.B., et al., *Increasing incidence of metastatic prostate cancer in the United States (2004-2013).* Prostate Cancer Prostatic Dis, 2016. 19(4): p. 395-397.

confined and regional PCa, as the 5-year relative survival rate after treatment was 100% (Figure

3) [4]. On the other hand, patients with distant/metastatic PCa had only 29% 5-year relative survival rate with the current treatments. More importantly, a recently published study on PCa showed that the incidence of low-risk PCa from 2007 to 2013 has significantly reduced to 37% relative to 2004, whereas metastatic PCa, which is the most common cause of death from PCa, has significantly increased to 72% more than 2004 (Figure 4) [10]. Cumulatively, this has raised many questions about the current screening and treatment approaches of distant/metastatic PCa and highlighted the urgent need to find new screening and treatment strategies for this type of PCa.

Multiple risk factors have been studied for their correlation to PCa. Some of them are significantly correlated such as age, family history, and race/ethnicity, while others including diet, supplements intake, obesity, smoking, sexual activity and sexually transmitted disease (STDs) may influence the possibility of PCa development and progression. Understanding the relationship between these factors and PCa is necessary in order to choose the appropriate treatment, improve the overall survival and quality life, and to minimize the adverse events associated with the treatment. In general, PCa is rare before the age of 40, while the average age at the time of diagnosis is 66 years according to the ACS, hence it is considered the disease of the elderly. The risk of PCa rapidly rises during the elder ages. Between 2007 and 2011, 0.6% of PCa cases were diagnosed in patients aged 35-45, whereas 36.3% of the cases were found among 65-74 years patients' [11]. Furthermore, age over 60 years was significantly correlated with increased risk of PCa (OR: 5.35, 95% CI: 2.17-13.19; $P<0.0001$) [12]. Many studies suggested an important role of ethnicity in PCa development. It has been shown that black men (Africans, African Americans, and Caribbeans with black ancestries) had higher incidence and mortality rates compared to Caucasians [11, 13]. Other groups including Hispanic, American

Indian/Alaska Native, and Asian/Pacific Islander (A/PI) had a lower risk of PCa, respectively, based on the Centers for Disease Control and Prevention (CDC). Family history is one of the well-established risk factors. Having one of the 1st-degree relatives (father or brother) with PCa increases the risk by 2.3-fold (95% CI: 1.76–3.12) as shown by the Health Professional Follow-Up Study, and the risk becomes higher when several relatives are diagnosed with PCa at a younger age [7]. PCa studies based on ethnicity and family history highlight the potential role of genetic abnormalities in the development and progression of PCa. PTEN, BRCA1 and BRCA2 mutations and P53 loss were found to contribute to PCa development [14]. Moreover, BRCA1 and BRCA2 carriers were found to have a higher risk of PCa compared to the non-carriers [15]. Environmental factors such as herbicides, pesticides, and polycyclic aromatic chemicals have been shown to increase the risk of developing PCa but mechanisms remain unknown [11, 14]. Although results are conflicted and yet to be confirmed, a Western lifestyle characterized by high fat and red meat intake and dairy products/calcium consumption has been associated with increased risk of PCa. On the other hand, diets containing antioxidants and anticancer such as lycopene in tomato and phytoestrogen in Soy, and supplements such as vitamin E, vitamin D, and Selenium were correlated to a reduction in the risk of PCa [5, 7, 11, 16]. Obese men appeared to have a 2-fold increase in the risk of PCa [11]. Moreover, they have a higher risk of developing advanced PCa compared to non-obese patients [11, 16]. Although the risk of smoking on PCa development has to be determined, a meta-analysis showed a significant increase in PCa incidence among heavy smokers (RR: 1.22, 95% CI: 1.01-1.46) [17]. Higher sexual frequency increased the odds of PCa development (OR: 1.2, 95% CI: 1.1-1.3) as shown by a meta-analysis of 12 retrospective studies [18]. Another meta-analysis demonstrated higher

odds of PCa in patients with a history of STDs such as gonorrhea and HPV (OR: 1.48, 95% CI: 1.26–1.73) [19].

1.3. Signs and symptoms of prostate cancer

Patients diagnosed with an early PCa may not have signs and remain asymptomatic. However, during the advanced stages, patients usually suffer from a variety of lower urinary tract symptoms such as dysuria, weakened urinary stream, hematuria, hematospermia, loss of bladder control due to pressure exerted by the tumor on the bladder, frequent urination during a day and night (nocturia), pain in the pelvic area and swelling in the lower extremities, problems with erection and ejaculation. Pain in the hip, back, ribs and legs take place during bone metastasis. Nonetheless, all above-mentioned urinary symptoms are experienced with other prostate diseases such as prostatitis and benign prostatic hypertrophy (BPH). Therefore, further screening tests are necessary to confirm the diagnosis with PCa**.**

1.4. Clinical assessment and staging of prostate cancer

Before the 1990's, the majority of PCa patients were present with locally advanced or metastatic cancers. This is owing to the unpredictable behavior of PCa as it progresses slowly from prostatic intraepithelial neoplasia (PIN) to early-asymptomatic cancer, which then advances to the symptomatic disease. A tremendous effort has been made towards discovering screening tools, which can detect PCa in the early stage. Fortunately, the discovery of PSA in 1979 was a huge revolution in the PCa field [20]. PSA is an androgen-regulated serine protease secreted primarily in an inactive form by the luminal epithelial cells and activated by a trypsin-like human kallikrein enzyme (hK2) [21]. The major function of PSA is to digest semenogelin I and II that maintain gel form of the semen. PSA is secreted in the blood as well. The most accepted cutoff value for normal PSA is 4ng/ml [22] although its level is influenced by age, sexual activity and

other physiological and risk factors. In spite of introducing PSA as a biomarker for cancer progression and recurrence after treatment, ACS guidelines for cancer checkup published in 1993 have recommended using it as a screening tool to detect early PCa, thus PSA levels > 4ng/ml indicate a positive prediction of PCa [23]. Further analysis using a transrectal ultrasound-guided biopsy (TRUS) and, rarely, TURP must be performed to confirm PSA results. However, despite this, low PSA levels were found in some PCa patients [24]. On the other side, high levels were seen in other prostate diseases such as BPH. On top of that, as discussed previously, over-diagnosis and over-treatment have been correlated with the use of PSA [25]. Taken together, this has yielded controversial decisions on its efficiency as a screening biomarker. Other screening tools include, but not limited to, digital rectal examination (DRE) and prostate cancer antigen 3 (PCA3). DRE is used to examine the prostate size and surrounding nodules. Abnormal DRE requires further confirmation through biopsy, even after measuring the PSA level. PCA3 gene is highly expressed in the prostate cancerous compared to the normal or hypertrophic tissue [26]. PCA3 is noncoding mRNA that is detected in the urine after DRE. A ratio of 60-100 times of PCA3 mRNA over PSA mRNA is detected in 95% of prostate cancer cases. Typically, PCA3 is used to decide whether a prostate biopsy is necessary for further analysis or not, especially when PSA levels or DRE usage were insufficient to make such a decision. The results of DRE, PSA and histopathological grading-based on 'Gleason Score' system provide the basis of cancer staging that is the most critical step in guiding treatment decision. The Gleason system scores the primary and secondary patterns in PCa biopsy from 1 to 5 based on the features of cancer cells. Values of the two patterns are summed to give the Gleason score, which ranges from 2 to 10. In 2017, the American Joint Committee on Cancer (AJCC) has released the new updated version of cancer staging/grading system that considers grade group and Gleason score rather than Gleason

score only to determine tumor grade (refer to table 1) [27]. International Society of Urologic Pathology (ISUP) endorsed this change because of better prognostic values observed by considering grade group in the treatment decision [28]. The grade group and Gleason score are related as follows:

- Grade group 1: Gleason score ≤ 6 (3 + 3); only discrete well-formed glands
- Grade group 2: Gleason score = 7 (3 + 4); predominantly well-formed glands with a lesser component of poorly formed/fused glands
- Grade group 3: Gleason score = 7 (4 + 3); predominantly poorly fused glands with a lesser component of well-formed glands
- Grade group 4: Gleason score = 8 (4 + 4, 3 + 5 or 5 + 3); only poorly fused glands, predominantly well-formed glands and lesser component lacking glands or predominantly lacking glands and lesser component well-formed glands
- Grade group 5: Gleason score = 9 and 10; lacking gland formation (or with necrosis) with/out poorly fused glands

Overall, grade group 1 are considered the least aggressive while group 5 are highly aggressive cancers.

Note: The implementation of this new staging system took place in 2018 to ensure that all the cancer care community has the infrastructure in place to document the eight-edition system.

1.5. Treatment of prostate cancer

According to the 2017 National Comprehensive Cancer Network (NCCN) guidelines, there are two treatment approaches available for PCa, the non-pharmacological approach that includes watchful waiting or active surveillance, radiotherapy and surgery, and the pharmacological approach that combines hormonal therapy, immunotherapy, and chemotherapy.

Initial treatment of PCa depends primarily on the presence or absence of symptoms, disease stage, Gleason score, patient age, and life expectancy (refer to table 2). The overall desired outcome of PCa treatment is determined mainly by the disease stage. Since the early stage, PCa is curable, the main goal is to reduce mortality and morbidity of the disease. On the other hand, since the advanced PCa is currently incurable, the current focus is to relieve symptoms and maintain the quality of life. Hormonal therapy or Androgen deprivation therapy is accomplished by using orchiectomy, LHRH agonist, LHRH antagonist, antiandrogenic agents, and androgen synthesis inhibitors. In general, the initial treatment response will last for 2-3 years in most cases after which progression will take place. This will eventually lead to the use of chemotherapy, docetaxel, and prednisone, or immunotherapy such as Sipuleucel-T.

TABLE 1: T: tumor extent; N: lymph node invasion; M: presence or absence of metastasis. Adapted from Prostate Cancer – Major Changes in the American Joint Committee on Cancer Eighth Edition Cancer Staging Manual.

When T is:	& N is:	& M is:	& PSA is:	& Gleason score is:	& Grade is:	& the Stage is:
cT1a-c, cT2a	N0	M0	<10 ng/mL	≤ 6	1	I
pT2	N0	M0	<10 ng/mL	≤ 6	1	I
cT1a-c, cT2a	N0	M0	≥10, <20 ng/mL	≤ 6	1	IIA
pT2	N0	M0	≥10, <20 ng/mL	≤ 6	1	IIA
cT2b-c	N0	M0	<20 ng/mL	≤ 6	1	IIA
T1-2	N0	M0	<20 ng/mL	3 + 4 = 7	2	IIB
T1-2	N0	M0	<20 ng/mL	4 + 3 = 7	3	IIC
T1-2	N0	M0	<20 ng/mL	8	4	IIC
T1-2	N0	M0	≥20 ng/mL	≤ 6 to 8	1-4	IIIA
T3-4	N0	M0	Any	≤ 6 to 8	1-4	IIIB
Any T	N0	M0	Any	9 - 10	5	IIIC
Any T	N1	M0	Any	Any	Any	IVA
Any T	Any	M1	Any	Any	Any	IVB

TABLE 2: EBRT: External beam radiation therapy; RP: Radical prostatectomy; PLND: Pelvic lymph node dissection; ADT: Androgen deprivation therapy. Adapted from NCCN guidelines 2017.

Recurrence Risk	Expected Survival	Initial Therapy
Very Low Risk: • T1c with • Gleason Score ≤ 6 / Grade group 1 and • PSA < 10 ng/ml and • Fewer than 3 biopsy cores positive (< 50% cancer in each core), and • PSA density < 0.15 ng/ml/g	≥ 20 years	Active Surveillance or
		EBRT or brachytherapy or
		RP ± PLND if metastasis ≥ 2%
	10 - 20	Active Surveillance
	< 10	Watchful Waiting
Low Risk: • T1-T2a with • Gleason Score ≤ 6 / Grade group 1 and • PSA < 10 ng/ml	≥ 10 years	Same as very low risk with expected survival ≥ 20 years
	< 10	Watchful Waiting
Intermediate Risk: • T2b-T2c or • Gleason Score 7 / Grade group 2 or 3 or • PSA 10 – 20 ng/ml	≥ 10 years	RP + PLND if metastasis ≥ 2% or
		EBRT ± 4 to 6 months ADT ± brachytherapy or brachytherapy alone
	< 10	EBRT ± 4 to 6 months ADT ± brachytherapy or brachytherapy alone or
		Watchful Waiting
High Risk: • T3a or • Gleason Score 8 / Grade group 4 or • Gleason Score 9 – 10 / Grade group 5 or • PSA 10 – 20 ng/ml		EBRT + 2 to 3 years ADT or
		EBRT + brachytherapy ± 2 to 3 years ADT or
		RP + PLND
Very High Risk: • T3b-T4 or • Primary Gleason Score 5 / Grade group 5 or • More than 4 biopsy cores with Gleason Score 8 – 10 / Grade group 4 or 5		EBRT + 2 to 3 years ADT or
		EBRT + brachytherapy ± 2 to 3 years ADT or
		RP + PLND or
		ADT or
		Watchful Waiting
Regional: • Any T, N1, and M0		EBRT + 2 to 3 years ADT or
		ADT
Metastatic: • Any T, N, and M1		ADT

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CHAPTER 2

The unconventional role of Akt1 in the advanced cancers

Alwhaibi, Abdulrahman et al, **Review article accepted by the Journal *Pharmacological Research,* July 2019. Reprinted here with permission of publisher.**

Abstract

Decades of research have elucidated the critical role of Akt isoforms in cancer as pro-tumorigenic and metastatic regulators through their specific effects on the cancer cells, tumor endothelial cells and the stromal cells. The pro-cancerous role of Akt isoforms through enhanced cell proliferation and suppression of apoptosis in cancer cells and the cells in the tumor microenvironment is considered a dogma. Intriguingly, studies also indicate that the Akt pathway is essential to protect the endothelial-barrier and prevent aberrant vascular permeability, which is also integral to tumor perfusion and metastasis. To complicate this further, a flurry of recent reports strongly indicates the metastasis suppressive role of Akt, Akt1 in particular in various cancer types. These reports emanated from different laboratories have elegantly demonstrated the paradoxical effect of Akt1 on cancer cell epithelial-to-mesenchymal transition, invasion, tumor endothelial-barrier disruption, and cancer metastasis. Here, we emphasize on the specific role of Akt1 in mediating tumor cell-vasculature reciprocity during the advanced stages of cancers and discuss how Akt1 differentially regulates cancer metastasis through mechanisms distinct from its pro-tumorigenic effects. Since Akt is integral for insulin signaling, endothelial function, and metabolic regulation, we also attempt to shed some light on the specific effects of diabetes in modulating Akt pathway in the promotion of tumor growth and metastasis.

Keywords: Akt1; Cancer; Diabetes; Metastasis; Tumor endothelium

2. Scientific Premise, central hypothesis, and the specific aims

The unconventional role of Akt1 in advanced cancers

2.1. Introduction

In the advanced stages, cancer cells become highly invasive and eventually spread to distant organs, resisting treatments and risking the patients' lives (1, 2). Once tumor cells acquire the ability to invade the surrounding tissues, the process of metastasis is instigated, and the cells enter the circulation through the lymphatic or vascular networks (3). Loss of cell-cell adhesion and acquisition of the migratory features allow malignant tumor cells to dissociate from the primary tumor, break the cell-matrix interactions and disintegrate the extracellular matrix (ECM) network that enables their invasion to the surrounding areas (4). Upon reaching a congenial microenvironment, these cells settle and adhere to a new location, start to colonize and profusely proliferate to generate the life-threatening secondary tumors (1, 5). Akin to the primary tumors, secondary tumors must also re-initiate angiogenesis in order for their growth to exceed 1-2 mm^3 in size. Without angiogenesis, these metastasized tumors are deprived of oxygen and nutrients delivered through diffusion and thereby fail to develop further (2). Indeed, these events demonstrate the importance of vascular networks in cancer metastasis. Thus, mutual cooperation between the tumor and vascular compartments ensures the overall growth of tumors, tumor cells-transendothelial migration and invasion (micrometastasis) as well as metastasis and colonization in distant organs (6, 7).

Although the cross-talk between the tumor and vascular compartments is crucial in the regulation of tumor growth and metastasis, less attention has been given to the mechanisms by which tumor and vascular cells reciprocate with each other within the tumor microenvironment.

In a recent review, we outlined the importance of Src family of kinases (SFKs) in the regulation of tumor vascular permeability, endothelial-barrier regulation, tumor growth and metastasis (8). In addition to SFKs, another important molecule that mediates such a cross-talk is protein kinase B (PKB or Akt), a serine-threonine kinase that exists in three isoforms namely Akt1, Akt2 and Akt3 (9). Akts are known to elicit isoform-, cell- and context-specific effects (10-13). In this review, we will shed the light on the experimental and signaling aspect of Akt1 to understand how it orchestrates the interaction between the tumor cells and the vascular compartment in the advanced stages of cancer.

2.2. Phosphoinositide-3-kinase and Akt signaling pathway

The family of PI3Kinase (PI3K) consists of a group of lipid kinases that belong to three different classes (class I, II and III) and have the ability to phosphorylate hydroxyl group of inositol ring in the membranous inositol phospholipids (1-3). Class I PI3Ks are heterodimers that have the catalytic subunit (p110) linked to one of the regulatory subunits (p55, p65, p85 or p101). This class has been well documented in human cancers and can be further divided into 2 subclasses; subclass Ia and Ib. Subclass Ia comprises 3 isoforms (PI3Kα, PI3Kβ, and PI3Kδ) with the catalytic domain (p110) combined to the regulatory domain (p55, p65 or p85, respectively), while subclass Ib has γ isoform (PI3Kγ) with p110 catalytic subunit combined to the p101 regulatory subunit. The catalytic subunit is responsible for adding phosphate group to phosphatidylinositol 4,5-bisphosphate (PIP2) and producing phosphatidylinositol 3,4,5-trisphosphate (PIP3), an important second messenger functioning as a binding site in the inner cellular membrane for many proteins that contain pleckstrin homologies (PH) domain, such as Phosphoinositide-Dependent Kinase-1 (PDK-1), Akt and other serine/threonine kinases. When Akt interacts with PIP3, it transiently localizes to the inner membrane, so PDK-1 can

phosphorylate Akt at threonine residue and activate it. Currently, less is known about class II PI3K enzymes, namely PI3K-C2α, PI3K-C2β and PI3K-C2γ, and Class III PI3K member PI3K-C3, also known as vacuolar protein sorting 34 (Vps34).

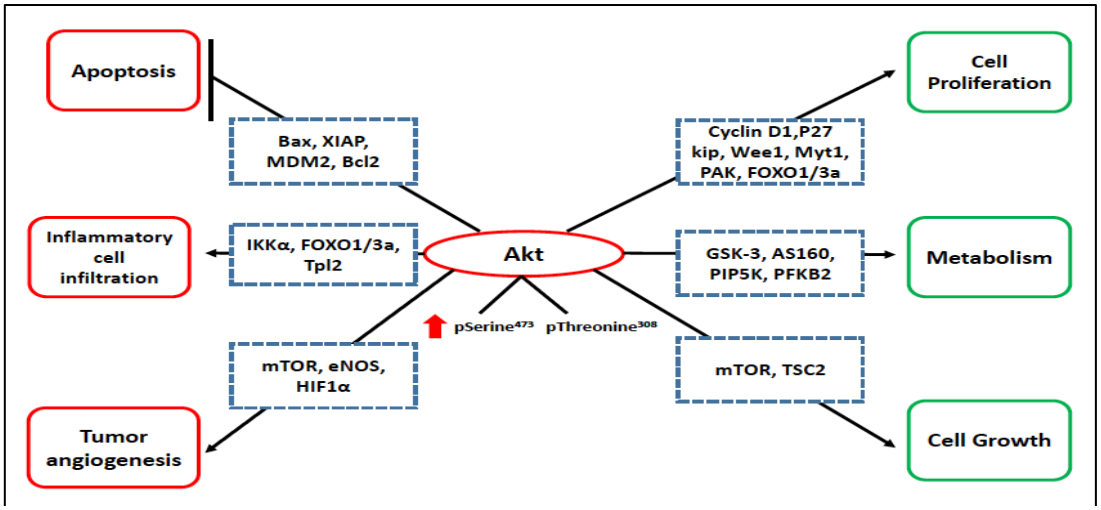

FIGURE 1. Pro-tumorigenic effects of Akt in early stages of cancer. Enhanced Akt expression/activity in cancer cells results in enhanced proliferation, metabolism and cell cycle through various downstream targets including mTOR, GSK3α, GSK3β, FoxO transcription factors, MDM2, BAD, p27KIP1, etc. In addition, Inhibition of cells apoptosis and induction of tumor favorable cytokines, which promotes inflammatory cells infiltration and tumor angiogenesis, further support cancer cells survival and tumor growth. Arrowhead indicates enhanced cell function and flat arrow indicates inhibition of function. *Green:* Direct effect of Akt activity within cancer cell; *Red:* Indirect effects of increased Akt activity to promote tumor growth. Bax- Bcl-2-associated X protein, XIAP- X-linked inhibitor of apoptosis protein, MDM2- mouse double minute 2 homolog, Bcl2- B-cell lymphoma 2, Ikkα- Ikappa B kinase (IKK)-associated protein 1α, FOXO1/3a- Forkhead box 1/3a,Tpl2- tumor progression locus 2, mTOR- mammalian target of rapamycin, eNOS- endothelial nitric oxide synthase, HIF1α- hypoxia-inducible factor 1α, p27Kip1- Cyclin-dependent kinase inhibitor 1B, Myt1- Myelin transcription factor 1, PAK- p21 activated kinase, GSK3- Glycogen synthase kinase 3, AS160- Akt substrate of 160 kDa, PIP5K- Phosphatidylinositol-5-Phosphate kinase, PFKB2- Phosphofructokinase-2, TSC2-Tuberous sclerosis proteins (tuberin).

The family of Akt consists of 3 different isoforms namely Akt1/PKBα, Akt2/PKBβ, and Akt3/PKBγ, which are transcribed from three different chromosomes, and not through alternative splicing (14). These isoforms share structural resemblances to protein kinases A and C, hence called PKB. Although different genes encode these isoforms, they share ~80% homology in their structure and substrate specificities (15, 16). All Akt isoforms have a pleckstrin homology (PH) domain that is located at the N-terminal side and responsible for binding to PIP3. The catalytic domain of Akt has a threonine residue (Thr308, Thr309, and Thr305 in Akt1, Akt2, and Akt3, respectively) that is phosphorylated by PDK-1 for its activation. For complete activation, Akt requires additional phosphorylation at serine residue (Ser473, Ser474 and 472 in Akt1, Akt2, and Akt3, respectively). Although a significant structural similarity is present among all the isoforms as mentioned previously, their functions in physiology and pathology do not appear to be redundant, which could be attributed to its tightly regulated subcellular localization (17).

2.3. Akt1 and tumor development

The Akt pathway has a distinguished role in different cellular processes such as metabolism, proliferation, growth and cell death, and due to its integral role in promoting cell survival and inhibition of apoptosis, it is known as the "survival kinase" (1, 2). Deregulation of Akt pathway builds the elements that are necessary for oncogenic transformation (18), promotion of tumor growth (19, 20) and recruitment of inflammatory cells required in the tumor microenvironment (3). As a result, Akt gene amplification has been observed in many types of human cancers (5). Mouse gene knockout studies focused on the Akt isoforms revealed their tissue and stage-specific expression and function (5, 21). Moreover, the fact that different genes encode different Akt isoforms and certain isoforms are hyper-activated in specific tumors

supports the notion that Akt-isoform specificity determines tumorigenesis and cancer progression differently in various cancers. Since Akt1 is the predominantly expressed and the best-characterized isoform in many cancers, owing to its tissue versatility and context-specific effects, we will focus on the recent advances on its role in various stages of tumor growth with an emphasis on the cross-talk between the tumor and vascular compartments. Figure 1 summarizes the genes and their biological functions that are essential for the pro-tumorigenic effects of Akt1.

In an early study published in 1987, Akt was found to be amplified 20-fold in some of the human gastric carcinoma tissue samples, however, authors thought that was a sporadic event (22). Later, another study conducted on Asian population showed a significantly enhanced level of Ser473 phosphorylated Akt in the tumor compared to normal tissues (23). The phosphorylated Akt was predominantly localized in the cellular membrane and cytoplasm, and occasionally in the nuclei of the cancer cells, while it was restricted to the cytoplasm in the normal cells. Following this, Sun M *et al* reported a predominant activation of Akt1 in many other types of human cancers such as the prostate, breast and ovary carcinomas (24). Interestingly, they showed that phosphorylated Akt1 was restricted to the primary tumor cells and absent in the stromal tissues. Later, we demonstrated the importance of Akt1 and its cooperation with the MAP Kinase pathway on oncogenic transformation (18) and cancer growth in the prostate (25). Our studies have also indicated that pharmacological (26-29) and genetic suppression of Akt activity (25, 30) could inhibit prostate cancer cell function *in vitro* and tumor growth *in vivo*. On the same line, Akt1 upregulation has also been shown in mammary adenocarcinoma developed in Neu and PyMT transgenic mice and its ablation significantly aborted cancer cell survival, thus emphasizing on the major role of Akt1 in tumor initiation and growth in the mammary glands (31). The role of Akt1 has also been demonstrated in lung cancer mouse models. One study

showed that Akt1 deletion in tobacco-induced lung cancer and K-Ras mutant mouse models prevented tumor initiation in the lungs (32). In another study, It was shown that mice overexpressing IGF-IR developed lung cancer and Akt1 ablation significantly suppressed it (33). Moreover, this study showed that selective inhibition of Akt1 using A-674563 enhanced cancer cell apoptosis compared to pan-Akt inhibitor MK-2206 suggesting that targeting Akt1 isoform specifically could be more effective than inhibiting all Akt isoforms in lung cancer. Among the breast cancer studies, Wu Y et al showed that transgenic mice expressing a constitutively active Akt1 (MMTVmyr-Akt1) as such did not develop any neoplasms, however, treatment with 7,12 dimethyl-1,2-benzanthracene (DMBA) induced higher mammary tumorigenesis in the glands of virgin and post-lactating transgenic compared to the wild-type mice (42.9% vs. 7.1%, respectively) indicating that Akt1 activation is a major risk factor in the development of mammary carcinoma secondary to carcinogens exposure (34). Additional details regarding the role of Akt in tumorigenesis have been extensively reviewed elsewhere (35).

2.4. Akt1 in angiogenesis and vascular permeability

Angiogenesis and vascular permeability are essential for the tumor perfusion, growth and transendothelial migration of cancer cells (36). Researchers have identified Akt1 as the predominant Akt isoform in endothelial cells (ECs) that is responsible for their growth and survival (37, 38). The role of Akt1 in the regulation of angiogenesis and vascular tone is also well established (38, 39). Studies on conditional *Akt1*$^{-/-}$ and *Akt2*$^{-/-}$ mice revealed the non-redundant function of Akt1 isoform in angiogenesis, where *Akt1*$^{-/-}$, but not *Akt2*$^{-/-}$, mice had significant inhibition of retinal angiogenesis (40). Moreover, since nitric oxide (NO) is a major modulator of angiogenesis and blood flow and its release is promoted by phosphorylation of

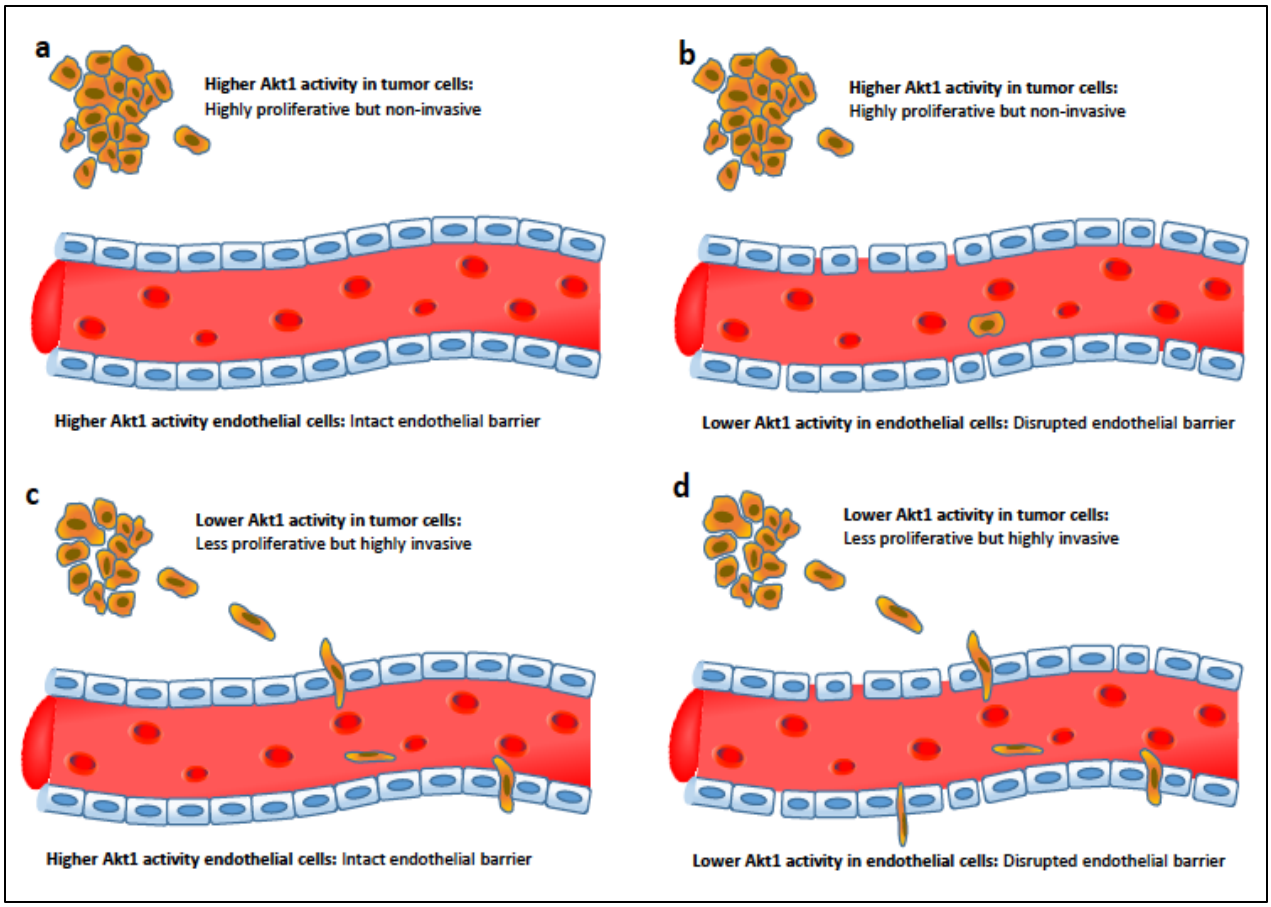

FIGURE 2. Akt1 in angiogenesis and endothelial-barrier function with respect to tumor cell trans-vascular migration and metastasis. Four different scenarios are represented in presence or absence of Akt1 activity in tumor and endothelial cells. **(a)** The tumor cells with higher Akt1 activity proliferates at a higher rate but are less invasive while endothelial barrier becomes impermeable due to the higher Akt1 activity thus blocking intravasation. **(b)** Despite less invasive, Akt1 active tumor cells may cross the Akt1 suppressed endothelial barrier due to the disrupted endothelial barrier. **(c)** Akt1 suppressed tumor cells proliferate at a lower rate but their highly invasive ability helps them to cross the Akt1 active endothelial barrier. **(d)** The highly invasive Akt1 suppressed tumor cells easily penetrate the highly permeable Akt1 suppressed endothelial barrier thereby causing intravasation and extravasation at a higher degree.

eNOS, many researchers reported that inhibition of Akt1 was accompanied by a significant reduction in the levels of phosphorylated eNOS and NO, thus blocking angiogenesis (41, 42). In a hind limb ischemia model elucidating the role of Akt1 in adaptive angiogenesis, a study indicated a significant impairment in vascular regeneration and >50% of the reduction in eNOS phosphorylation was observed in the *Akt1*$^{-/-}$ mice lungs after vascular Endothelial Growth Factor-A (VEGF) administration compared to the wild type animals (43). In support of this, another study in a cutaneous wound healing model also revealed impaired angiogenesis and ECM remodeling in *Akt1*$^{-/-}$ mice (38). More recently, Akt1 has been shown to promote angiogenesis and cardiac remodeling following myocardial infarction (44). These studies indeed demonstrate the ability of Akt1 in the regulation of adaptive angiogenesis, tissue remodeling, and blood flow. Therefore, targeting Akt1 or its pharmacological inhibition would impair the adaptive angiogenesis.

Tumor angiogenesis is a unique process that occurs as a result of interaction between the tumor cells and endothelial cells. VEGF, as discussed previously, is an important cytokine required for initiating and regulating physiological angiogenesis (45). However, the elevation in VEGF levels in many solid tumors beside the hyper-activation of Akt1 confirms the crosstalk between these molecules and their importance in tumor angiogenesis (46-48). One of the underlying mechanisms through which VEGF and Akt1 interplay in regulating angiogenesis are through activation of integrins, that is necessary for migration of endothelial cells (49). In order to recapitulate the tumor-like effect in non-tumor endothelial cells, researchers adopted Akt1 over-expression and knockout in non-tumor endothelial cells. An initial study using this approach reported that the overexpression of active form of Akt1, myristoylated-Akt1 (MyrAkt1), in the endothelial cells of mice resulted in the formation of enlarged blood vessels

compared to the wild type mice (50). The newly formed vessels had features similar to those observed in tumor vessels with a significant increase in permeability. In another study, endothelial myrAkt1 overexpression in mice was lethal with the formation of abnormally tortuous blood vessels in the embryos, similar to the tumor blood vessels (51) suggesting that the endothelial Akt1 is not only promoting tumor vascularization but also enhancing vascular permeability.

The genuine effects of Akt1 in tumor vasculature were elegantly revealed by a study published by Dr. Byzova's group using an Akt1 deficient mouse model (37). Although VEGF-induced cell migration was hampered in *Akt1*$^{-/-}$ endothelial cells, the vasculature supporting melanoma xenografts implanted in *Akt1*$^{-/-}$ mice were significantly leaky compared to vessels surrounding xenografts implanted in the wild type group. Because of that, these vessels were considered young and immature. Strikingly and unlike previous studies, subcutaneous xenografts in these mice were more vascularized compared to the ones in the wild type group. Mechanistically, the increased vascular permeability and enhanced angiogenesis seen in Akt1 null mice were attributed to the decreased levels of anti-angiogenic factors, Thrombospondin 1 and 2 (TSP1 and TSP2, respectively) in both tumor and ECs of these mice compared to the wild-type mice indicating that Akt1 can directly regulate expression of these factors. Further analysis of these mice revealed that constitutive generation of VEGF through Adenovirus in *Akt1*$^{-/-}$ mouse skin also resulted in increased vascular permeability. In support of these observations, a recent study from our group showed that shRNA-mediated Akt1 knockdown in endothelial cells led to a significant reduction in the expression of 20 genes encoding tight-junction proteins, claudins, and mice with tamoxifen-induced VE-CreAkt1 had enhanced VEGF-induced vascular permeability compared to the wild type group (52). Moreover, Akt1 knock out mice had lower

expression of Angiopoietin-1 that is known for mediating vascular protection, thus confirming the indispensable role of Akt1 in vascular maturation. Recently, we showed that although endothelial Akt1 loss in mice with tamoxifen-induced VE-CreAkt1 had not affected xenograft tumor growth, its loss enhanced lung metastasis in these mice, suggesting that targeting Akt1 in ECs could potentiate transmigration of cancer cells through the vascular/endothelial barrier as shown in Figure 2 (53).

Apart from the direct effects of Akt1 on the tumor endothelial cells and cancer cells, a recent study from our group also identified the reciprocal cross-talk between Akt1 and Src, a non-receptor tyrosine kinase, to regulate and maintain vascular homeostasis (54), cancer growth and metastasis (55). In addition, we recently demonstrated that candesartan, an angiotensin receptor blocker, inhibited prostate tumor growth via a tumor endothelium-dependent and tumor cell-independent manner indicating that modulation of Akt1 in tumor endothelial cells via angiotensin-renin system can have therapeutic benefits in cancer (29). Interestingly, although candesartan had no significant effect on various signaling pathways in the tumor cells, it specifically activated endothelial Akt1, promoted vascular normalization and reduced permeability thus inhibiting the growth of prostate tumor xenografts in mice. Similarly, simvastatin with its dual role of activation and inhibition of Akt1 in the endothelial (56) and cancer cells (26, 27), respectively, had demonstrated prevention of prostate cancer metastasis via vascular normalization (28).

Other laboratories have also reported the endothelial-barrier protection offered by activated Akt1. Following the initial findings, where the Akt1 suppression led to increased vascular permeability in mouse tumor xenografts (37), studies from Liao group showed the presence of vascular lesions in Akt1 deficient mice as a result of impaired mTOR signaling (57).

Another study reported the endothelial-barrier protective role of Akt1 as a result of VE-cadherin overexpression and clustering (58). Furthermore, the protective effect of Akt1 on lung edema was reported as a result of stimulation by sphingosine-1-phosphate (59). Interestingly, Pestell group demonstrated that although mammary tumor xenografts in *ErbB2/Akt1*$^{+/+}$mice were larger than *ErbB2/Akt1*$^{-/-}$ mice, the vascular density surrounding tumors in the latter was significantly higher compared to the former (60). Overall, these studies strongly indicate that Akt1 plays a dual and context-specific role in modulating tumor angiogenesis and vascular permeability.

2.5. Akt1 in the advanced cancers and metastasis

Apart from its pro-cell survival and proliferation roles, Akt1 has also been involved in the migration and invasion of cancerous cells (61), highlighting its importance in cancer progression and metastasis. Cancer progression was significantly ameliorated in tumors formed by Akt1 deficient lung cancer cells compared to Akt2 deficient cells (62), thus revealing that silencing Akt1, but not Akt2, can abrogate cancer cell migration *in vitro* and lung invasion *in vivo*. In bladder cancer cells, Akt1 suppression resulted in a significant reduction in their migration, while Akt2 suppression didn't have a significant effect (20). In soft tissue sarcomas, tumor cell migration and invasion were mainly controlled by Akt1 isoform, and its silencing not only abolished these properties but also reduced the expression of specific epithelial to mesenchymal transition (EMT) markers such as vimentin, which is linked to invasive cancers (63). In prostate cancer, cell motility, invasion, and transendothelial migration were promoted through Akt1-mediated integrin activation (61). Expression of the constitutively active Akt1 (CA-Akt1) in prostate cancer cells promoted tumor cell interaction with the endothelial cells and ECM proteins via enhanced cancer cell integrin β_3 affinity, an effect that was reversed with the overexpression of inactive, a dominant negative mutant of Akt1 (DN-Akt1) (61). The role of TGFβ1-Akt1

pathway has also been indicated in melanoma progression and metastasis, in which TGFβ1-mediated Akt1 activation was necessary to induce SKP2 expression, enhance N-cadherin and reduce epithelial marker E-cadherin expression thereby exhibiting mesenchymal cell morphology (64). In a pancreatic ductal adenocarcinoma study, $KRas^{G12D}$ mutant mice expressing myristoylated Akt1 developed early liver and abdominal metastases compared to the control $KRas^{G12D}$ group (65). In a murine model of thyroid cancer, $Akt1^{-/-}$ mice had less invasive thyroid tumors with the absence of lung metastasis compared to the control group (66). In a breast cancer study, $ErbB2/Akt1^{+/+}$ mice that developed mammary tumors also developed lung metastasis; however, this was blunted in $ErbB2/Akt1^{-/-}$ mice (60). This was also supported by a significant reduction of $ErbB2/Akt1^{-/-}$ cells migration and invasion *in vitro* compared to $ErbB2/Akt1^{+/+}$ cells. Interestingly, as previously discussed, in spite of the larger mammary tumors size in $ErbB2/Akt1^{+/+}$mice, $ErbB2/Akt1^{-/-}$ mice tumors were significantly highly vascularized despite their smaller size. In a gastric cancer study, Han Z et al reported that activated Akt1 was significantly higher in the advanced, poorly differentiated gastric tumors compared to the early stages indicating the importance of Akt1 activation in gastric cancer progression (23).

Although a plethora of such reports has been published on the promoting effect of Akt1 activation on cancer metastasis, more recent studies, particularly started on breast cancer, have challenged this concept (Table 1). The first report on the observation that Akt1 activation inhibiting cancer cell migration and invasion came from Muller's group (67). Although ErbB2/activated Akt1 mice (bitransgenic mice) had accelerated mammary tumorigenesis, fewer invasions to the surrounding tissues and a significant reduction in lung metastatic lesions were observed in these mice compared to the control group indicating that tumors developed with

activated Akt1 had less metastatic propensity compared to the tumors with a reduced level of active Akt1. Another study supporting this observation was published by Alex Toker's group (68), where investigators showed that overexpression of activated Akt1 attenuated breast cancer cell migratory and invasive properties *in vitro*, and reduced the formation of actin cytoskeleton stress fibers. On the other hand, siRNA-mediated Akt1 deletion rescued the migratory and invasive phenotype of cancer cells. Mechanistically, ubiquitination and proteasomal degradation of nuclear factor activated T cells (NFAT) mediated by HDM2 (the human homolog of the oncoprotein and E3 ubiquitin ligase MDM2) was observed with the activation of Akt1 and totally reversed with Akt1 gene silencing. Another study on the distinct role of Akt1 and Akt2 in breast cancer cell lines came from Virginia Novaro's group (69). In spite of the reduction in IBH-6 cells proliferation *in vitro* with genetic deletion of Akt1, cells invasion was significantly enhanced compared to their controls. On the other hand, Akt2 expression was crucial for promoting cells migration and invasion with less evident effect on proliferation shown after its deletion. Mechanistically, while Akt1 deletion enhanced β1 integrin and focal adhesion kinase (FAK) expression around the edges of IBH-6 cells supporting their attachment during the invasion, Akt2 deletion reduced Vimentin and F-actin expression. Therefore, Akt1 deletion and Akt2 overexpression are essential for peritumoral invasion and lung metastasis.

A link between Akt1 activity and palladin, an actin-binding protein that anchors other proteins to actin fibers, was demonstrated in supporting the suppressive effects of Akt1 activity on breast cancer cell invasion (70). Palladin, an Akt1 specific substrate phosphorylated at Ser507, is required to maintain spheroid cells structure, prevent invadopodia formation and strengthen cells adhesion. However, upon Akt1 depletion, reduction in palladin phosphorylation destabilized actin filaments and enhanced cell branching and migration as shown in Figure 2.

Another study has shown that over-expression of myrAkt1 inhibited RhoA activity and led to inhibition of breast cancer cell motility and invasion (71). In addition, an alternative mechanism by which Akt1 suppression has a pro-migratory and invasion effect was demonstrated in the human mammary epithelial cells (MCF-10A cells), where Akt1 silencing (but not Akt2) enhanced ERK activation (72). This was accompanied by significant changes in MCF-10A cell morphology shown by losing cuboidal-epithelial shape and acquiring spindle-shaped EMT characteristics, as depicted in Figure 2, along with the increased expression of Vimentin and N-cadherin. Another study on MCF-10A cells demonstrated that Akt1 down-regulation reduced miR-200 abundance, resulting in reduced E-cadherin expression and enhanced TGFβ1-mediated EMT (73). Interestingly, a paradoxical role of Akt1 and Akt2 isoforms in mammary tumorigenesis and metastasis was observed in transgenic models upon co-expression of these isoforms with activated ErbB2 or polyomavirus middle T antigen (PyVmT Y315/322F) (74). Whereas Akt1 promoted mammary gland tumorigenic activity, it did not impact the metastatic phenotype observed in these mice. In contrast, co-expression of Akt2 promoted invasion and lung metastasis in these mice. A more recent study conducted in breast cancer cells highlighted the role of Akt1 as a negative regulator of EMT and metastasis (75). Mechanistically, Akt1 was shown to regulate the function of Twist-1, a transcription factor involved in EMT and promotion of breast cancer metastasis. Akt1 was responsible for the direct phosphorylation of Twist-1 leading to its ubiquitination by β-TrCP and proteolytic degradation, however, inhibition of Akt1 by MK-2206 led to Twist-1 stabilization and enhanced breast cancer migration and invasion *in vitro*, associated with increased N-cadherin and vimentin, and decreased E-cadherin expression. Moreover, stabilization of Twist-1 was associated with enhanced lung metastasis *in vivo*.

Interestingly, context-specific effects of Akt1 on cancer promotion and metastasis in mouse models were reported in a recent study published by Nissim Hay's group. Their study demonstrated that inhibition of hepatic Akt1 in systemic *Akt2*$^{-/-}$ mice led to hepatic carcinogenesis, however, that was not observed with inhibition of either hepatic Akt1 in *Akt2*$^{+/-}$ mice or with inhibition of one allele of hepatic Akt1 in *Akt2*$^{-/-}$ mice (76). Of note, *Akt2*$^{-/-}$ mice did not develop hepatic cancer although Akt2 was the main isoform expressed in the liver. *Akt1*$^{-/-}$ mice treated with diethylnitrosamine (DEN) developed macroscopic tumors via activation of FoxO1 and resultant liver inflammation. Overall, this study not only demonstrated the mutual role of Akt1 and Akt2 in maintaining liver homeostasis but also challenged the dogma that Akt1 is a tumor promoter in all contexts. A reinvestigation on the role of Akt1 in hepatocellular carcinoma (HepG2) and colorectal cancer (HCT-116) cell lines revealed the paradoxical effects of Akt1 activation on cell motility and invasion (77). Whereas Akt1 over-expressing HepG2 cells exhibited enhanced cell migration and invasion, these were impaired in HCT-116 cells. Of note, using PI3 kinase inhibitor wortmannin in both cell lines significantly reversed these results. Surprisingly, while the expression of matrix metalloproteinases MMP2 and MMP9 was elevated in HepG2 with Akt1 activation, the same was decreased in HCT-116 cells upon Akt1 activation, which led to the conclusion that the effect of Akt1 inhibition on motility could be cell-type specific.

In prostate cancer, Akt1 silencing in androgen-sensitive and androgen-resistant prostate cancer cell lines as well as prostate epithelial cells or treatment with pan-Akt inhibitor triciribine in androgen-resistant prostate cancer cell line (PC3) increased integrin β_1 localization at the periphery and induced its activity leading to enhanced focal adhesions to the extracellular matrix, which augmented spreading and invasion ability of these cells (78). Intriguingly, silencing of

Akt2 also exhibited similar effects. However, Akt1 loss promoted migration via enhanced expression and activity of receptor tyrosine kinases such as EGFR and hepatocyte growth factor receptor (cMET), whereas Akt2 loss was associated with induction of miR-200a/b that has been implicated in EMT and cells invasion, suggesting that Akt1 and Akt2 may modulate different pathways in the regulation of cell motility and invasion. Recently, we have identified a new role of Akt1 in prostate cancer. By using the transgenic adenocarcinoma of the mouse prostate (TRAMP) mice, which develop neuroendocrine prostate cancer spontaneously by the age of 24 weeks, we reported that although Akt1 silencing abrogated oncogenic transformation in these mice, inhibition of Akt1 during the advanced stages promoted lung metastasis (30). Moreover, silencing of Akt1 in androgen-resistant prostate cancer cell lines (PC3 and DU145) enhanced EMT, shown by increased N-cadherin, Snail, and reduced E-cadherin. Mechanistically, we demonstrated that suppression of the Akt1-βcatenin pathway during the advanced prostate cancer enhanced TGFβ1-mediated EMT and cancer metastasis. The most recent findings from our laboratory have also identified the microRNA signatures responsible for the early tumorigenic effects of Akt activation and late metastasis promoting effects of Akt suppression in prostate cancer (79), once again supporting the dual, stage-specific effects of Akt1 activity on cancer.

The latest findings in breast, prostate and liver cancers on the reciprocal regulation of cancer growth and metastasis by Akt1 isoform have been extended to the non-small lung cancer (NSCLC) and head and neck squamous cell carcinoma (HNSCC). A study conducted by the Giaccone group showed that Akt1 inactivation in NSCLC cell lines with K-RAS and EGFR mutant background enhanced cancer cell migration and metastasis (80). More importantly, oral administration of MK-2206 enhanced brain metastasis in mice administered A549 cells via intracardial route. Despite the absence of such results with Akt2 or Akt3 inhibition, their finding

highlights the contribution of genetic background on determining the effect of Akt1 inactivation on cancer metastasis. In HNSCC, Picco group reported that Akt1 activation in cancer cell lines is not only essential for tumorigenesis but also for maintaining epithelial phenotype (81). However, inhibition of Akt1 expression by shRNA or suppression of its activity using MK-2206 decreased cell-cell contact and enhanced their invasive capacity, as shown in Figure 2 (81). Altogether these studies demonstrate the inhibitory effect of Akt1 on tumor cell migration and invasion in multiple cancers, thereby challenging the concept that Akt1 activation is necessary for promoting cancer metastasis. A schematic representation of the role of Akt1 in advanced cancer cells and tumor vasculature in a metastatic stage is shown in Figure 2.

In addition to the abundance of studies on the differential role of Akt1 in the early and advanced cancers, few studies on Akt2 have been debating the same concept. As discussed previously (72) although Akt1 silencing induced EMT-like phenotype in MCF-10A cells through activation of the ERK pathway, this was reversed by inhibition of Akt2 in these cells. Moreover, as mentioned early (69), despite the enhanced lung metastasis of the breast cancer cell lines after Akt1 deletion, overexpression of Akt2 promoted EMT in these cells and resulted in a similar effect. Another study showed that upregulation of Akt2 by Twist led to enhanced invasion and migration in the breast cancer cell lines (82). In the same study, this association was also observed in human breast cancer samples in which Akt2 and Twist expression was significantly elevated in the late stage compared to the early stage specimens. Cumulatively, these studies suggest the positive role of Akt2 in promoting breast cancer progression and metastasis. Interestingly, a study published by Sarah Wootton group to dissect the role of Akt isoforms in NSCLC showed that in spite of the necessity of Akt1 for NSCLC initiation and progression in AJEJJenv infected mice, Akt2 and Akt3, to some extent, appeared to have protective role against

tumorigenesis (83) conferring that the role of Akt2 and Akt3 can be a cancer type-specific. The role of Akt3 has also been debated in the literature. Clark and Toker group showed that silencing of Akt3 in triple negative breast cancer (TNBC), MCF10DCIS, MDA-MB468, and BT-549 cells, enhanced their migration *in vitro*, with no effect on invasion, and inhibited MDA-MB231 cells spheroid growth (84). On the other hand, another study from Manfred Jücker group showed that Akt3 downregulation in Balb-neuT mice, a transgenic model for ErbB2-induced breast cancer, reduced cancer progression and enhanced its sensitivity to tamoxifen, by reducing expression and activity of ERBb2 and ERBb3 and enhancing ERα expression (85). Overall, this conflict in Ak2 and Akt3 functions urges for more research to understand their role in different types and stages of cancers.

2.6. Summary and Conclusion

Akt1 has a critical role in modulating tumor angiogenesis and cancer metastasis. Despite the dogma that targeting Akt1 could be a beneficial approach to treat cancer, the paradoxical effect of Akt1 in the advanced cancer stages must be considered in cancer therapy. Although inhibition of Akt1 or targeting its activity can suppress tumorigenesis during the early cancer stages, this could be detrimental on the advanced cancers as it compromises the endothelial-barrier function, enhances EMT in tumor cells, and induces tumor cells-transendothelial migration, thus promoting cancer metastasis. Overall, this review highlights the importance of the role of Akt1 in tumor progression and invasion and accentuates its cell-type and cancer-stage specific effects.

Reference	Studied cancer	Method of Akt1 manipulation in vitro or in vivo	Observation	Mechanism
Hutchinson JN. et al. Cancer Res. 2004;64(9):3171-8.	Breast cancer	• **In vivo:** Bitransgenic mice overexpressing ErbB-2 and activated Myr-Akt1.	Myr-Akt1 overexpression inhibited lung metastasis despite accelerating mammary tumorigenesis.	Unknown
Yoeli-Lerner M. et al. Mol Cell. 2005;20(4): 539-50.	Breast cancer	• **In vitro:** Breast cancer cell lines overexpressing activated Myr-Akt1 or transfected with Akt1-siRNA.	Cells migration and invasion were attenuated with Myr-Akt1 overexpression but enhanced with Akt1-siRNA.	Myr-Akt1 phosphorylated HDM2 that enhanced degradation of migration/ invasion promoting factor-NFAT.
Chen L. et al. Oncology reports. 2014;31(2): 737-44.	Colorectal cancer	• **In vitro:** Colorectal cancer cell line overexpressing Akt1 with/out Wortmannin treatment (Akt1 activity inhibitor).	Akt1 overexpression inhibited cells migration and invasion, which was reversed by Wortmannin treatment.	Akt1 overexpression inhibited the expression of MMP2, MMP9, HIF1α and VEGF that have a critical role in cancer progression. However, Wortmannin rescued their levels.
Li CW. et al. Cancer Res. 2016;76(6): 1451-62.	Breast cancer	• **In vitro:** Mouse embryonic fibroblasts (MEFs) overexpressing activated Myr-Akt1 • **In vivo:** Tumor bearing mice treated with MK-2206 (Akt1 activity inhibitor).	Myr-Akt1 overexpression inhibited EMT in MEFs whereas MK-2206 enhanced lung metastasis of cancer cells.	Myr-Akt1 phosphorylated the migration/ invasion promoting factor-Twist-1 and enhanced its degradation β-TrCP, whereas all reversed by MK-2206.
Rao G. et al. Sci Rep. 2017;7(1):7066.	Non small cell lung cancer (NSCLC)	• **In vitro:** NSCLC cell line overexpressing activated Myr-Akt1 • **In vivo:** Tumor bearing mice treated with MK-2206 (Akt1 activity inhibitor).	Myr-Akt1 overexpression inhibited EMT in NSCLC cell line whereas MK-2206 induced their migration, invasion and bone and brain metastasis	Akt1 inhibition by MK-2206 promoted MARCKS phosphorylation that is required for LAMC2 overexpression and the enhanced migration, invasion and metastasis resulted from Akt1 inhibition, however, this was reversed by Myr-Akt1 overexpression

Gao F. et al. Cancer Lett. 2017;402:177-189.	Prostate cancer (PCa)	• **In vitro:** PCa cell lines with Akt1 downregulation using shRNA
		• **In vivo:**
		  • Mice administered Akt1 silenced cancer cells
		  • Tumor bearing TRAMP mice treated with TCBN (Akt1 activity inhibitor).
			Silencing of Akt1 in PCa cell lines and inhibition of its activity in tumor bearing TRAMP mice induced EMT and lung and liver metastasis, respectively.	Downregulation of Akt1 expression or inhibition of its activity using TCBN suppressed β-catenin and enhanced TGFβ signaling that promoted EMT and cancer metastasis.

Riggio M. et. al. Sci Rep. 2017 Mar 13;7:44244.	Breast cancer	• **In vitro:** Breast cancer cell lines overexpressing Myr-Akt1 or Akt2, or transfected with Akt1 or Akt2 shRNA • **In vivo:** Mice administered Akt1 or Akt2 silenced or overexpressing breast cancer cells.	Silencing of Akt1 enhanced lung metastasis despite the reduction in cells proliferation., while overexpression of Akt2 increased vimentin and F-actin levels with less evident effect on cellular growth.	Inhibition of Akt1 increased β1 integrin and FAK expression that enhanced cells invasions, while overexpression of Akt2 increased vimentin and F-actin levels that enhanced cells migration and invasion.
Brolih S. et al. BMC cancer. 2018;18(1): 249.	Head and neck squamous cell carcinoma (HNSCC)	• **In vitro:** HNSCC cell line with Akt1 downregulation using shRNA or treated by MK-2206 (Akt1 activity inhibitor).	Silencing of Akt1 expression or inhibition of its activity by MK-2206 in HNSCC cell line promoted loss of epithelial morphology, induced EMT-like phenotype, and increased their invasive capacity.	Unknown

2.7. Hypothesis and Specific Aims

Metastatic Prostate cancer (mPCa) remains a fatal disease in men worldwide despite the current therapies and the tremendous amount of research that has been devoted to finding a cure for it. In spite of our understanding of the triggers that initiate oncogenic transformation and tumor growth, the mechanisms that regulate PCa metastasis remain elusive. This represents one of the major obstacles limiting our effort to find a cure for mPCa.

The PI3Kinse-Akt pathway is essential for oncogenic transformation and cancer development and our initial findings confirm the critical role of Akt1 in PCa growth. However, the recent study from our laboratory has revealed a stage-specific effect of Akt1 in PCa, where pharmacological inhibition of its activity in the advanced stage promoted epithelial-to-mesenchymal transition (EMT) that induced lung metastasis in tumor-bearing Transgenic adenocarcinoma of the mouse prostate (TRAMP) mice. More importantly, genetic deletion of Akt1 in human PCa cell lines (PC3 and DU145) promoted EMT and fostered lung metastasis in athymic nude mice. In both models, the Akt1-TGFβ1-βcatenin pathway drove this process.

Our data from mice prostate gene array showed that pharmacological inhibition of Akt1 in TRAMP mice-bearing advanced PCa enhanced Nodal expression. Nodal up-regulation is shown by multiple cancer studies to promote cancer progression and metastasis. The mechanisms through which Nodal is regulated to promote cancer metastasis are **beyond our knowledge.** Driven by **my long-term goal** towards finding a cure for mPCa metastasis, I hypothesize that "Akt1 suppression in the advanced PCa potentiates Nodal expression and deregulates miRNAs subsequently promoting PCa EMT and metastasis" (Figure 3)

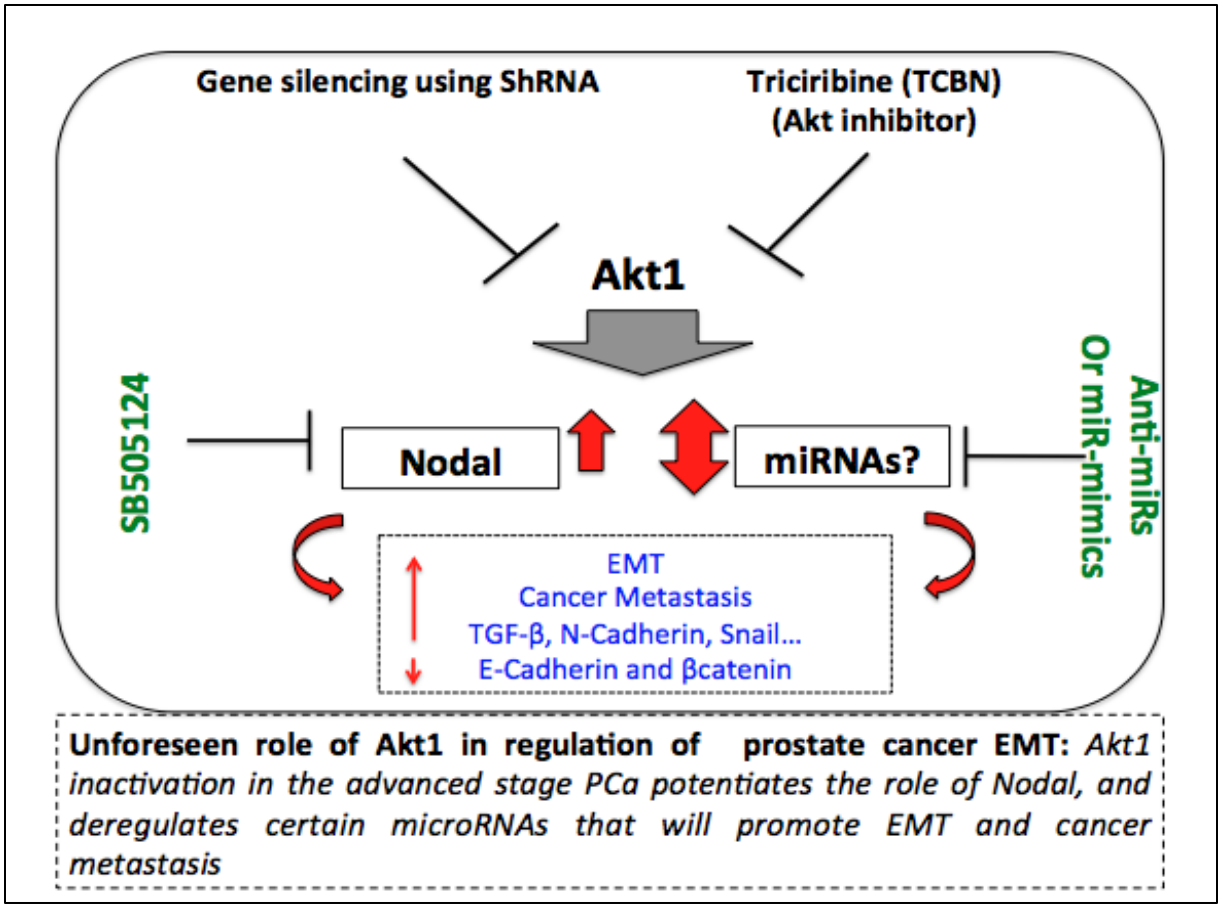

FIGURE 3: Schematic diagram of the Overall hypothesis

I have set three **specific aims** to test my hypothesis:

- **Aim 1**: Determine a causal relationship between Akt1 inhibition and EMT/metastasis in advanced stage PCa
- **Aim 2**: Investigate how Nodal pathway contributes to PCa EMT and metastasis following Akt1-inhibition in the advanced stages
- **Aim 3:** Profile the Akt1-regulated miRNAs in the early and advanced stage PCa and identify the candidate miRNAs for biomarker and therapeutics development

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CHAPTER 3

Genome atlas analysis based profiling of Akt pathway genes in the early and advanced human prostate cancer

Alwhaibi, Abdulrahman et al, **Manuscript accepted by the Journal *Oncoscience,* May 2019.**

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Abstract

Recent studies conducted in the mouse and cellular models suggest a stage-specific, differential effect of Akt activity modulation on tumor growth and metastasis in various cancers. In prostate cancer (PCa), although the deletion of Akt1 gene in a neuroendocrine model of TRansgenic Adenocarcinoma of the Mouse Prostate (TRAMP) blunted oncogenic transformation and tumor growth, Akt1 suppression in the advanced PCa resulted in the activation of transforming growth factor-β pathway and enhanced metastasis to the lungs. Such a dual role for the Akt isoforms and its signaling partners has not been investigated in human PCa. In the current study, we performed genomic database analysis of Akt isoforms and associated pathway molecules in human prostate adenocarcinoma, castration-resistant PCa, neuroendocrine PCa and metastatic PCa for mutations, genetic alterations, mRNA and protein expressions and activating phosphorylations from cBioportal. Results from the protein data analysis from the cBioportal were compared to the results of our data on human PCa tissue analysis and the cellular effects of Akt1 suppression using MK-2206 on PCa cell aggressiveness. Our study indicates the existence of a dual role for Akt1 in PCa and warrants a large-scale analysis of the early and advanced stage PCa clinical samples for further clarity.

Keywords: Akt1, Akt2, Akt3, cBioportal, TCGA, prostate cancer

3.1. Introduction

Metastatic prostate cancer (PCa) is the leading cause of cancer-related deaths in men in the US and Europe [1]. Although slow-growing cancer, PCa that has metastasized to the bone, lungs, and brain becomes difficult to treat [2]. Uncertainties in the molecular mechanisms leading to the switch from early to advanced PCa are the underlying reason for the unreliable screening measures and ineffective treatments in the management of early and metastatic PCa [3].

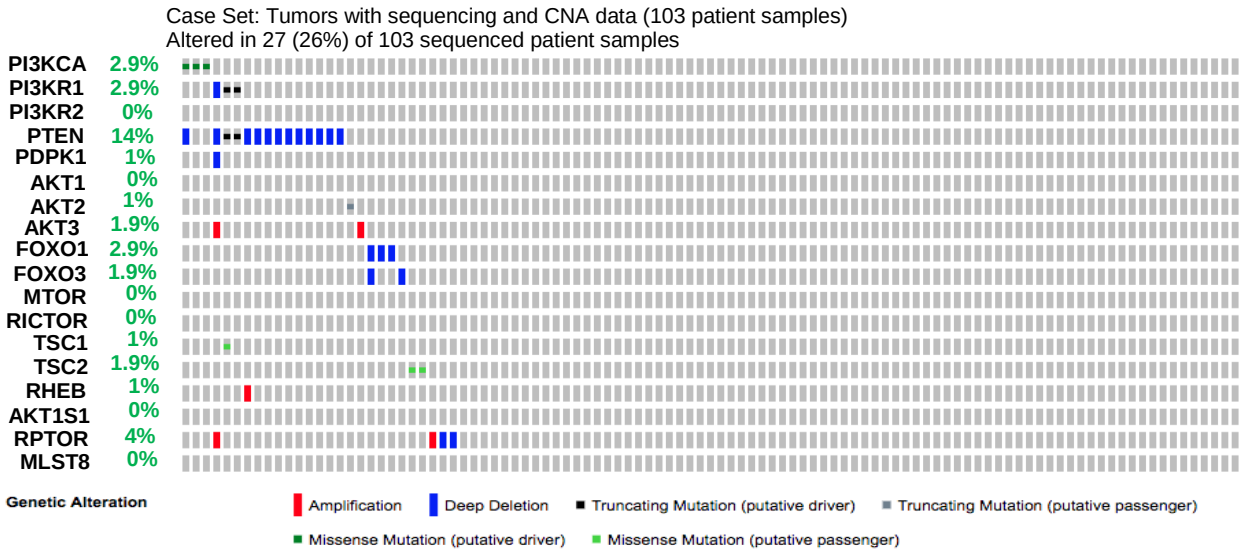
Phosphoinositide-3-Kinase (PI3K)/Akt pathway has a well-established role in the regulation of cellular processes essential for cell survival such as metabolism, proliferation, growth, anti-apoptosis and cytoskeletal reorganization [4]. Aberrant activation of the PI3K/Akt pathway has been recognized as an essential step towards the initiation and progression of many cancers [5]. Activation of this pathway is driven by genetic mutation or activity deregulation of the upstream components such as receptor tyrosine kinases (RTKs) [6], non-RTKs such as Src family kinases [7] or modulation of the downstream components including PTEN inactivation or deletion, PI3K constitutive activation or amplification, Akt hyperactivation and other genetic changes in signaling molecules involved in this pathway [8].

Although Akt pathway has been targeted for cancer therapy for many years, as of today no drugs that target Akt has been approved for any cancer treatments. Recently we showed that Akt1, the predominant Akt isoform in the PCa cells [9] and tumor vascular cells [10-12] plays a dual, reciprocal role in prostate tumor growth and metastasis [13]. Such a finding has also been reported in three other cancer types such as the breast [14, 15], liver [16] and non-small cell lung cancer [17]. Interestingly, our most recent study has indicated the important role of several microRNAs in the dual, stage-specific role of Akt1 in cancer with Akt1 activity suppression in

the early and advanced stages of murine neuroendocrine model of PCa in a Transgenic adenocarcinoma of the mouse prostate (TRAMP) tissues resulting in entirely different set of microRNA expression [18]. Further, a more recent study from our laboratory has demonstrated that endothelial-specific loss of Akt1 in mice promotes PCa metastasis to the lungs [19]. These preclinical studies have identified Akt1 as a molecule that promotes tumor growth but inhibits metastasis in cancer. The above studies also have identified a reciprocal link between Akt1 and TGFβ pathways in promoting cancer cell epithelial-to-mesenchymal transition (EMT) and metastasis. As of today, the genomic and proteomic changes in Akt isoforms and their signaling pathway molecules in the primary and advanced stages of human PCa have not been studied in detail.

In the current study, we performed a genomic and proteomic database analysis (http://www.cbioportal.org) [20, 21] of Akt pathway from human PCa patient studies performed in various types such as the human prostate adenocarcinomas [22-24], castration-resistant PCa [25], neuroendocrine PCa [26] and metastatic PCa [25, 27], and determined the alterations in mRNA, protein expression, and genetic mutations. There were data from a total of 13 studies available in cBioportal performed on PCa patient samples, and 6 of them have the data on mRNA and one has proteomic expression changes in various genes. These include a study on primary PCa, two studies in prostatic adenocarcinoma (both primary and metastatic), a study on neuroendocrine PCa and two studies on castration-resistant metastatic PCa. Genomic data mining analyses from these studies available on cBioportal with respect to the alterations in the Akt pathway molecules are presented in this article. Our results strongly suggest the existence of a dual role for the Akt pathway in the early and advanced PCa and warrants large-scale analysis of PCa patient samples for further clarity on this new information.

A



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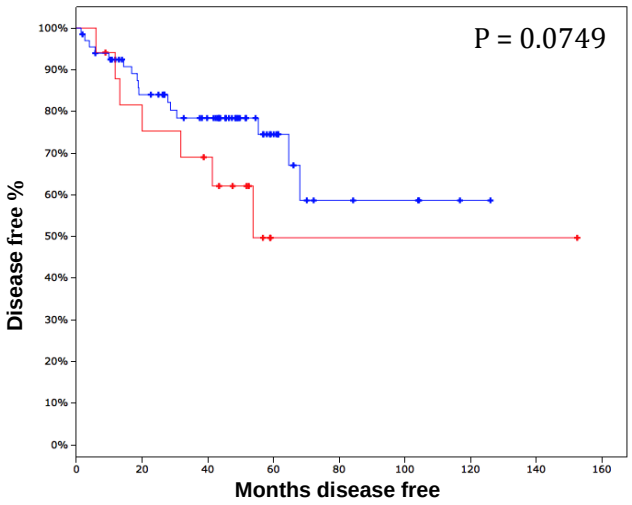

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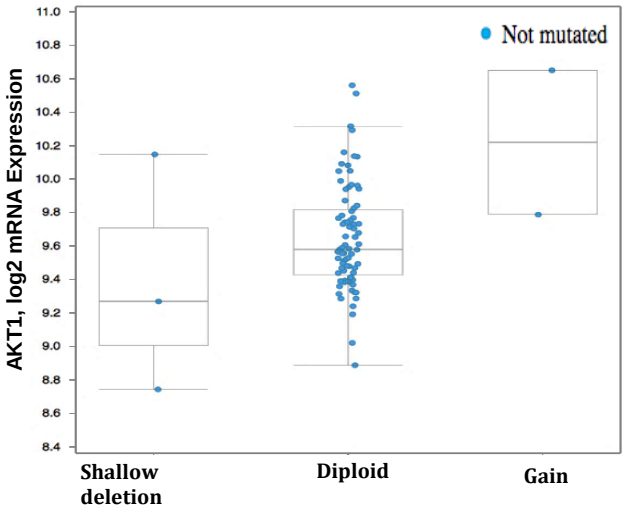

D

Gene	Cytband	Mean ± SD mRNA expression		p-value	q-value
		In altered group	In un-altered group		
AKT1	14q32.33	9.68 ± 0.47	9.64 ± 0.32	0.753↑	0.975↑
AKT2	19q13.2	9.07 ± 0.21	9.07 ± 0.18	0.947↑	0.996↑
AKT3	1q43-q44	7.79 ± 1.37	8.17 ± 0.83	0.263↓	0.810↓

FIGURE 1: Gene alterations in Akt pathway human prostate adenocarcinoma. (A) Oncoprint showing genomic alterations in the Akt pathway genes in human prostate adenocarcinoma samples based on the integrative genomic profiling performed by the MSKCC study (Taylor BS et al, Cancer Cell, 2010). **(B)** Kaplan–Meier survival analysis showing decreased disease-free survival in patients with observed Akt pathway alterations compared to the unaltered group. **(C)** A plot showing the relationship between Akt1 mRNA abundance and copy-number alteration (CAN) in the Akt1 gene in human prostate adenocarcinoma. **(D)** Chart showing the overall mRNA expression of Akt1, Akt2, and Akt3 in the prostatic adenocarcinoma tissues with observed Akt pathway alterations compared to the un-altered group.

3.2. Results

- **CBioportal cancer genome atlas show minimal mutations in the Akt isoforms in PCa**

We first determined the existence of any known gene mutations in the 3 Akt isoforms. Our analysis of the six PCa studies from the cBioportal genome atlas indicated no significant genetic mutations in any of the Akt isoforms that compromised its activity. A very small population of PCa patients exhibited a single mutation in the Akt1 isoform resulting in E17K alteration (Supplemental Figure 1). While this was identified to be a missense mutation, similar mutations in the Akt2 and Akt3 isoforms resulted in the alterations of A214V and A101G residues (Supplemental Figure 1), once again in a very small population of PCa patients. Together, these studies indicated that mutations in the Akt isoforms are not major determinants for its activity deregulation contributing to the onset or aggressiveness of PCa.

- **Analysis of the integrative genomic profiling of human primary tumors and metastatic PCa by the MSKCC identifies alterations in the Akt pathway genes**

Genomic profiling by the MSKCC group is one of the first among the large-scale analysis of genes performed in the primary and advanced PCa patient samples [22]. Out of the 216 patient samples used in the analysis, genomic data on 103 patient samples and 149 control samples are available on cBioportal. In our analysis, approximately 26% (27 out of 103) of the patients exhibited alterations in genes from the PI3K/Akt pathway (Figure 1A) leading to reduced disease-free survival in patients (Figure 1B). Among these, 14 % of the alterations were due to a deep deletion in the PTEN gene (Figure 1A). Whereas 2.9% of the patients exhibited missense mutations (putative driver) in the PI3K catalytic subunit, 2.9% of the patient samples showed a deletion or truncating mutation (putative driver) in the PI3K regulatory subunit-1 (Figure 1A). Deep deletions were also observed in FoxO1 and FoxO3 in 2.9% and 1.9% of the patient

population, respectively (Figure 1A). Interestingly, no genetic alterations were observed in the Akt isoforms except amplification of Akt3 in two patients (Figure 1A). Despite the markedly increased Akt1 mRNA in the screened tumors, the majority did not have amplified the Akt1 gene as shown by a diploid Akt1 (Figure 1C). There were also no significant differences in the mean mRNA expression levels of Akt1, Akt2 and Akt3 isoforms between the altered and un-altered groups (Figure 1D). Together, our analysis indicated that while alterations in many Akt pathway components such as PTEN may contribute to the hyperactivation of Akt isoforms, the evidence on the direct effect of genomic alteration on their activity is unclear.

- **Genomic analysis of the human neuroendocrine PCa samples by the Trento/Broad/Cornell study reveals high alterations in the Akt pathway genes**

A genomic study by the Trento/Broad/Cornell group was primarily focused on the human neuroendocrine PCa [26]. Out of the 107 patient samples used for the analysis, genomic data on 77 patient samples is available on cBioportal. In our analysis, approximately 66% (51 out of 77) of the patient samples showed genetic alterations in genes from the PI3K/Akt pathway (Figure 2A). The highest level of genetic alterations was observed in the case of PTEN deletion or amplification (31%). High amplification of genes such as PI3K catalytic subunit (30%), PI3K regulatory subunit-1 (13%), PI3K regulatory subunit-2 (25%), and the Akt isoforms (25%, 18% and 31% in Akt1, Akt2, and Akt3, respectively) were also observed (Figure 2A). Gene amplifications were observed in the mTOR pathway genes such as mTOR (14%), Raptor (29%), Rictor (21%), Tuberous sclerosis complex-1 (TSC1; 27%), TSC2 (23%) and Rheb (27%) (Figure 2A). Data from the RNA-seq analysis indicated significant gain and amplification of Akt1 mRNA in a large population of these patients (Figure 2B). Interestingly, there were no significant differences in the mean mRNA expression levels of Akt1, Akt2 and Akt3 isoforms

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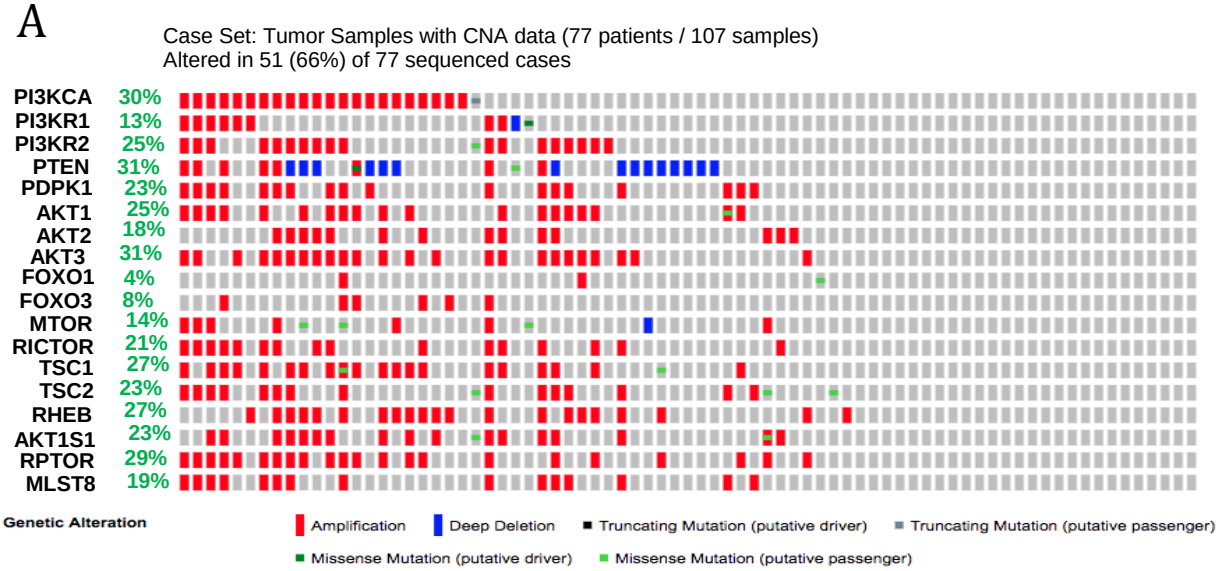

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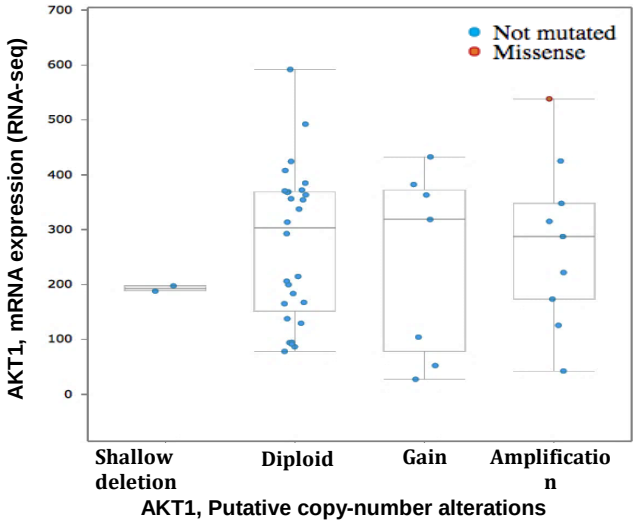

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Gene	Cytband	Mean ± SD mRNA expression		p-value	q-value
		In altered group	In un-altered group		
AKT1	14q32.33	247.91 ± 142.17	300 ± 138.95	0.244↓	0.614↓
AKT2	19q13.2	1.03 ± 3.05	0.98 ± 0.89	0.936↑	0.975↑
AKT3	1q43-q44	0.11 ± 2.12	0.57 ± 1.87	0.457↓	0.731↓

FIGURE 2: Gene alterations in Akt pathway in castration-resistant neuroendocrine PCa. **(A)** Oncoprint showing genomic alterations in the Akt pathway genes in human castration-resistant neuroendocrine PCa samples based on the genomic profiling by the Trento/Cornell/Broad study (Beltran H et al, Nat Med, 2016). **(B)** A plot showing the relationship between Akt1 mRNA abundance and copy-number alteration (CAN) in the Akt1 gene in human castration-resistant neuroendocrine PCa. **(C)** Chart showing the overall mRNA expression of Akt1, Akt2, and Akt3 in neuroendocrine PCa samples with observed Akt pathway alterations compared to the un-altered group.

between the altered and un-altered groups (Figure 2C). Together, our analysis indicated that although deletion of PTEN and amplification many other Akt pathway genes were observed in neuroendocrine PCa samples, there was no significant difference in the mean mRNA levels of the Akt isoforms in the altered group of patients vs. the un-altered group

- **Exome sequence analysis of the human prostate adenocarcinoma samples by the Broad/Cornell study shows very low alterations in the Akt pathway genes**

Exome sequencing by the Broad/Cornell group was primarily focused on the human prostate adenocarcinoma [23]. Genomic data on all the 109 sequenced patient samples are available on cBioportal. In our analysis, only 15% (16 out of 109) of the patient samples showed genetic alterations in genes from the PI3K/Akt pathway (Figure 3A). Like in the other studies, alterations were primarily observed in PTEN (7%) in the form of gene deletion or loss of function mutations (Figure 3A). Although amplification in the PI3K catalytic subunit was found in a single patient, single cases of missense mutations were also observed in PI3K catalytic subunit, Akt1, Akt3, FoxO3, mTOR, Rictor, TSC2, and Raptor (Figure 3A). Data from the RNA-seq analysis indicated no significant gain or amplification of Akt1 mRNA in these prostate adenocarcinoma samples (Figure 3B). Also, there were no significant differences in the mean mRNA expression levels of Akt1, Akt2 and Akt3 isoforms between the altered and un-altered groups (Figure 3C). Overall, this indicates no significant genetic alterations in the PI3K/Akt pathway genes among prostate adenocarcinoma patients.

- **Clinical genomics of the human metastatic PCa reveals high alterations in the Akt pathway genes**

Genome analysis by the Fred Hutchinson group was primarily focused on the human metastatic PCa [25]. Out of the 136 patient samples used for the analysis, genomic data on 54

patients is available on cBioportal. In our analysis, approximately 81% (44 out of 54) of the patient samples exhibited genetic alterations in genes from the PI3K/Akt pathway (Figure 4A). The highest level of genetic alterations was once again observed in the case of PTEN deletion or loss of function mutations (44%). Although high alterations of genes such PI3K catalytic subunit amplification (11%), FoxO1 gene deletion (19%) and Rheb amplification (13%) were also observed, only 4-7% alterations were noted in the Akt isoforms (Figure 4A). Data from the mRNA expression analysis indicated a significant gain of Akt1 mRNA in a large population of the metastatic PCa samples (Figure 4B). However, there were no significant differences in the mean mRNA expression levels of Akt1, Akt2 and Akt3 isoforms between the altered and un-altered groups (Figure 4C).

Another study on metastatic PCa was conducted by the MSKCC group [27]. Genomic data on all the 150 sequenced patient samples are available on cBioportal. In our analysis, approximately 78% (117 out of 150) of the patient samples indicated genetic alterations in genes from the PI3K/Akt pathway (Figure 5A). Similar to the Fred Hutchinson study, 42% of the alterations were found in the PTEN gene deletion, fusion or loss of function mutations (Figure 5A). Minimal alterations (2-12%) were also found in most other genes of the PI3K/Akt pathway in metastatic PCa samples (Figure 5A). Data from the mRNA expression analysis indicated some gain of Akt1 mRNA in a smaller population of the metastatic PCa samples (Figure 5B). However, there were no significant differences in the mean mRNA expression levels of Akt1, Akt2 and Akt3 isoforms between the altered and un-altered groups (Figure 5C). Together, these two studies indicate the importance of PTEN inactivation in the activation of the PI3K/Akt pathway in metastatic PCa.

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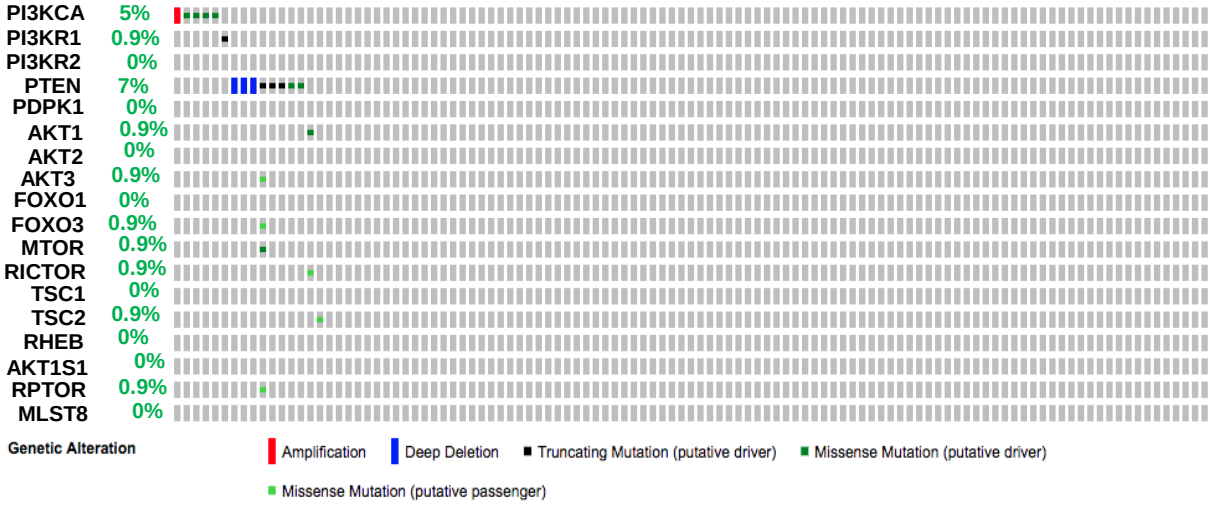

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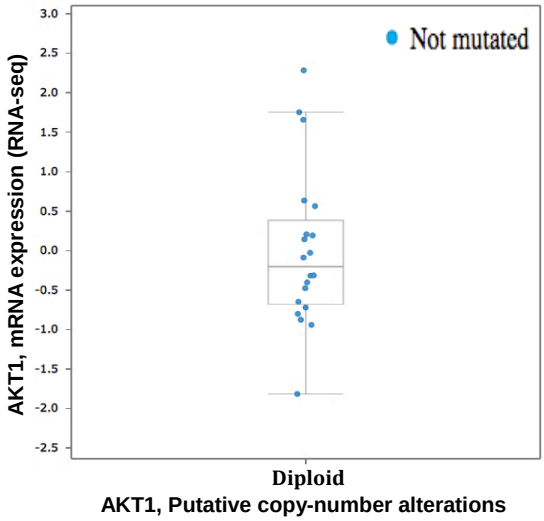

C

Gene	Cytband	Mean ± SD mRNA expression		p-value	q-value
		In altered group	In un-altered group		
AKT1	14q32.33	57.27 ± 29.8	47.53 ± 11.33	0.724↑	0.980↑
AKT2	19q13.2	14.29 ± 8.42	23.59 ± 7.28	0.347↓	0.919↓
AKT3	1q43-q44	10.47 ± 4.32	8.48 ± 3.58	0.631↑	0.973↑

FIGURE 3: Gene alterations in Akt pathway in human non-metastatic prostate adenocarcinoma. (A) Oncoprint showing genomic alterations in the Akt pathway genes in human non-metastatic prostate adenocarcinoma tissues based on the exome sequencing performed by the Broad/Cornell study (Barbieri CE et al, Nat Gen, 2012). **(B)** A plot showing the relationship between Akt1 mRNA abundance and copy-number alteration (CAN) in the Akt1 gene in human non-metastatic prostate adenocarcinoma. **(C)** Chart showing the mean mRNA expression of Akt1, Akt2, and Akt3 in the non-metastatic prostatic adenocarcinoma tissues with observed Akt pathway alterations compared to the un-altered group.

- **TCGA study of the primary PCa identifies genetic alterations in the Akt pathway genes**

Genomic profiling of a large collection of primary PCa samples identified genetic alterations in the Akt pathway genes [24]. Genomic data on all the 492 sequenced patient samples are available on cBioportal and NCI TCGA sites. In our analysis, approximately 51% (252 out of 492) of the patient samples showed alterations in genes from the PI3K/Akt pathway (Figure 6A) leading to reduced disease-free survival (Figure 6B) and overall survival in patients (Figure 6C). Among these, 22 % of the alterations were due to a deep deletion in the PTEN gene, 16% in FoxO1 gene deletion, 14% were in FoxO3 gene deletion and 7% in the PI3K regulatory subunit-1 (Figure 6A). Interestingly, no genetic alterations were observed in the Akt isoforms except isolated cases of amplification or deletion in Akt1 (1.4%), Akt2 (1%) and Akt3 (2%) (Figure 6A). Despite the markedly increased Akt1 mRNA in the screened tumors, the majority did not have amplified the Akt1 gene as shown by a diploid Akt1 (Figure 6D). A significant difference in the protein expression of Akt1 was also not observed in these patients' samples (Figure 6E; Figure 7A-D).

- **Suppression of Akt activity (reduced Akt phosphorylation) is linked to the promotion of EMT in the advanced stage PCa via increased expression of TGFβ1**

We next reviewed the data from www.clinicaltrials.gov on the effects of Akt inhibitor MK-2206 in various cancer clinical trials. Our analysis indicated no significant clinical benefits of MK-2206 on many of the advanced stage, metastatic cancers (Supplemental Table 1). In many trials, particularly the metastatic cancers, MK-2206 treatment showed reduced overall survival and progression-free survival of the cancer patients (E.g. NCT01253447 and NCT01658943). Nevertheless, some benefits of MK-2206 treatment were observed in the early-stage tumors [28-30].

Since none of the 6 genomic studies showed any significant gain of Akt isoform mean mRNA and protein expression levels between the altered and un-altered groups, we next compared the levels of pAktSer473 and pAktThr308 phosphorylation levels (level of Akt activation) with Gleason score. Our analysis of data from the TCGA study (N=352 patients) showed significant in pAktT308 levels between low Gleason score (Score 6-7) versus high Gleason score (Score 8-10; N=149; N=203) samples (Figure 7E). Although not significant, there was a strong trend correlating increased pAktT308 levels between low Gleason score (Score 6-7) versus high Gleason score (Score 8-10) samples (Figure 7F).

In order to further explore this, we determined the effect of Akt activity suppression with MK2206 treatment on epithelial-to-mesenchymal transition (EMT) and aggressiveness of PC3 and DU145 human PCa cells. In our analysis, treatment with 5 μM MK2206 revealed reduced Akt phosphorylation associated with the increased expression of EMT marker N-cadherin in both PC3 and DU145 human PCa cell lines (Figure 8A-B). Similarly, Akt1 gene deletion using shRNA, hence reduction in its activity, also resulted in increased expression of EMT transcription factor Snail and TGFβ-R1 in PC3 and DU145 cells (Figure 8C-D). A stage-specific analysis of TRAMP prostates collected at 12, 24, 32 and 40 weeks indicated an inverse relationship between S473Akt phosphorylation (activity) and TGFβ1 expression (Figure 8E-F), where reduced Akt1 phosphorylation in the advanced PCa is associated with the increased TGFβ1. A similar effect was also observed in the staining of phosphorylated Akt (pSer473Akt) in human PCa tissues, where a decreased expression of pSer473Akt in high Gleason score (5+5) PCa tissues was observed compared to low Gleason score samples (3+3), particularly in the proliferating luminal cells (Figure 8G-H).

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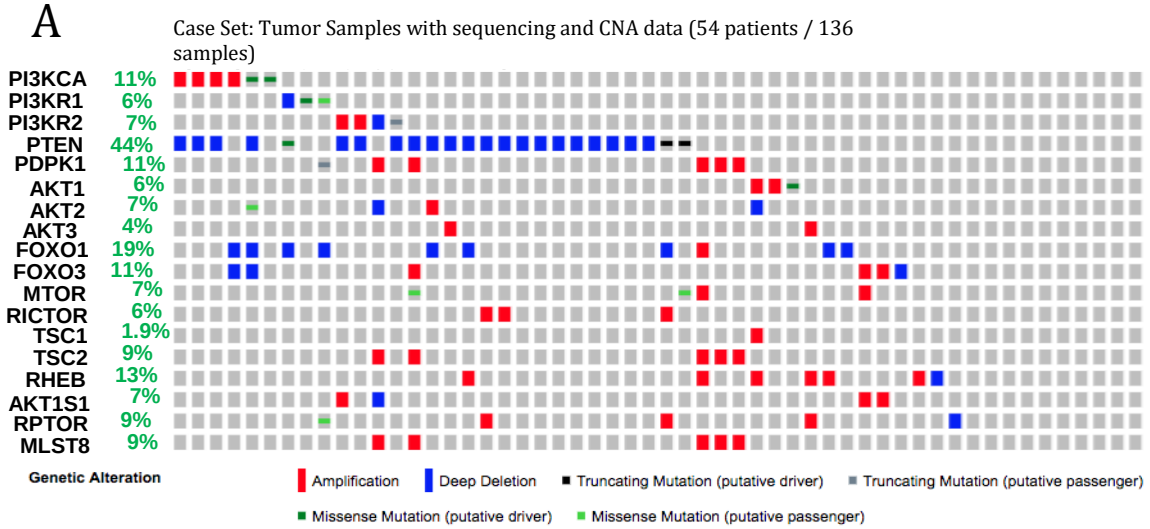

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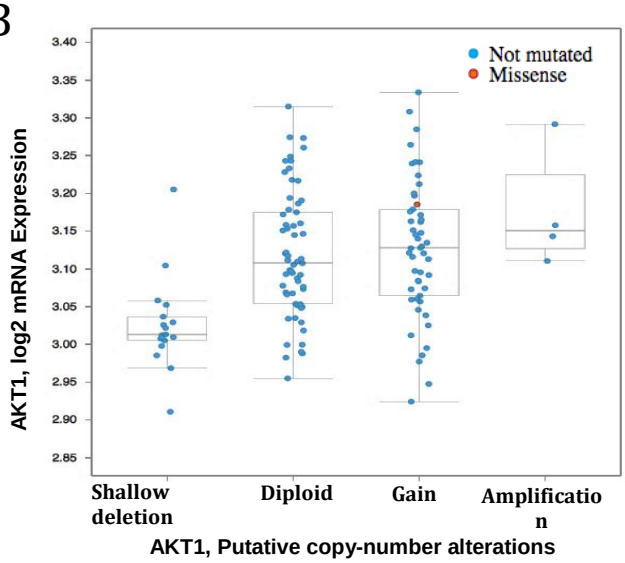

C

Gene	Cytband	Mean ± SD mRNA expression		p-value	q-value
		In altered group	In un-altered group		
AKT1	14q32.33	3.12 ± 0.09	3.09 ± 0.10	0.111↑	0.471↑
AKT2	19q13.2	3.96 ± 0.07	3.97 ± 0.08	0.599↓	0.864↓
AKT3	1q43-q44	3.67 ± 0.22	3.67 ± 0.20	0.992↓	0.998↓

FIGURE 4: Gene alterations in Akt pathway in human metastatic PCa. (A) Oncoprint showing genomic alterations in the Akt pathway genes in human metastatic PCa tissues based on the genomic analysis performed by the Fred Hutchinson study (Kumar A et al, Nat Med, 2016). **(B)** A plot showing the relationship between Akt1 mRNA abundance and copy-number alteration (CAN) in the Akt1 gene in human metastatic PCa. **(C)** Chart showing the mean mRNA expression of Akt1, Akt2 and Akt3 in the metastatic PCa tissues with observed Akt pathway alterations compared to the un-altered group.

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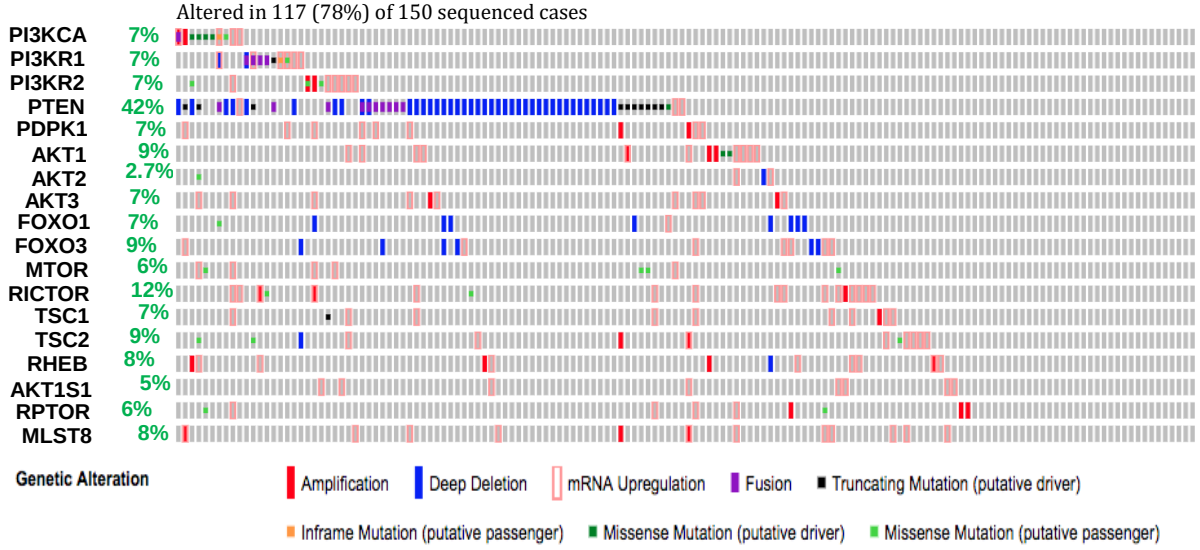

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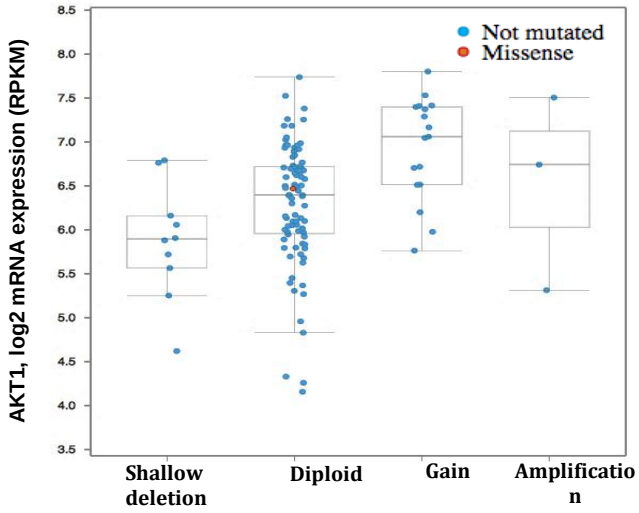

AKT1, Putative copy-number alterations from GISTIC

C

Gene	Cytband	Mean ± SD mRNA expression		p-value	q-value
		In altered group	In un-altered group		
AKT1	14q32.33	6.40 ± 0.76	6.18 ± 0.50	0.103↑	0.459↑
AKT2	19q13.2	6.24 ± 0.76	5.91 ± 0.70	0.0628↑	0.393↑
AKT3	1q43-q44	2.93 ± 1.47	3.01 ± 0.98	0.754↓	0.917↓

FIGURE 5: Gene alterations in Akt pathway in human advanced PCa. (A) Oncoprint showing genomic alterations in the Akt pathway genes in human advanced (metastatic castration-resistant) PCa tissues based on the genomic analysis performed by the MSKCC/UMICH study (Robinson D et al, Cell, 2015). **(B)** A plot showing the relationship between Akt1 mRNA abundance and copy-number alteration (CAN) in the Akt1 gene in human advanced PCa. **(C)** Chart showing the mean mRNA expression of Akt1, Akt2 and Akt3 in the advanced PCa tissues with observed Akt pathway alterations compared to the un-altered group.

In order to illustrate the clinical implications of these results, mRNA data on patients from MSKCC/UMICH (Robinson D et al, Cell, 2015) study were used to determine a correlation between tumor anatomic site and EMT. Strikingly, although Akt1 was not significantly different between the selected cohorts, a trend towards increased TGFβ1, CDH2 (N-cadherin) and Snail were observed in the metastatic tumor sites (N=114) compared to the tumors localized in the prostate (N=4) (Figure 9A-D). Since Snail and TGFβ1 protein levels were not available in the TCGA data, we determined the mRNA levels of these genes and CDH2 (N-cadherin). Intriguingly, although not significant, there was a strong trend correlating increased TGFβ1, Snail and CDH2 levels in the higher (N=206) compared to the lower Gleason score group (N=292) (Figure 9A-D), indicating that the suppression of Akt1 activity in advanced PCa could promote EMT and metastasis.

3.3. Discussion

The serine/threonine kinase Akt has long been known for its role in cell survival and proliferation via modulation of its downstream substrates such as glycogen synthase kinase-3 (GSK3), FoxO, Bad and Bcl2, etc. [12] in promoting tumor growth [9, 13, 31-37]. Many laboratories have demonstrated that Akt isoforms are expressed and activated differentially in tumors [38, 39], thus the notion that they have distinct roles in cancer is well accepted. Intriguingly, the most recent studies *in vitro* and animal models [39-44] on the role of Akt in advanced cancers clearly demonstrate an unexpected, suppressive role of Akt in cancer metastasis. A previous study from our laboratory demonstrated that although Akt1 is essential for oncogenic transformation in a neuroendocrine PCa *TRAMP* mouse model, pharmacological inhibition of Akt using triciribine in advanced PCa bearing *TRAMP* mice or genetic ablation of Akt1 gene in PC3 and DU145 human PCa cells augmented EMT and metastasis [13]. However,

such a negative correlation between Akt activity and metastasis has never been studied in human PCa. Hence, in the current study, we compared the genomic data on the Akt pathway genes based on six studies that have deposited their sequencing data in cBioportal. We also determined the effect of Akt activity suppression by MK-2206, a drug used in clinical trials for cancer, analyzed a small population of human PCa samples for the level of activating Akt phosphorylation in the advanced stage PCa compared to early stage and BPH tissues, and highlighted the correlation between Akt1 mRNA or protein expression/activity and EMT in the advanced PCa based on few selected CBioportal studies

Initial reports on the inhibitory effects of Akt1 activation on cancer cell migration and invasion *in vitro* came from Toker laboratory [45]. In this study, siRNA-mediated Akt1 deletion promoted breast cancer cell invasion via the human homolog of the E3 ubiquitin ligase (HDM2)-mediated ubiquitination and degradation of the nuclear factor of activated T-cells (NFAT). Another study by the same group linked Akt1 activity to palladin, an actin-binding protein that anchors cytoskeletal proteins to actin fibers thus reducing reduced stress fiber formation and attenuating breast cancer cell invasion *in vitro* [46]. Subsequent studies by other laboratories further supported this theory by demonstrating enhanced ERK activation resulting in the loss of cuboidal-epithelial morphology in Akt1, but not Akt2-deficient MCF-10A cells, promoting EMT and invasion *in vitro* [47] leading to miR-200 abundance [14]. Akt1 silencing in either of the androgen-sensitive or androgen-resistant PCa cells induced β_1-integrin activity and their localization in the cell periphery (in PC3 cell line) thus promoting focal adhesion formation and invasion [48]. Interestingly, although Akt1 overexpression in ERbB-2 transgenic mice resulted in the up-regulation of cyclin D1 levels accompanied by accelerated mammary tumorigenesis, tumors developed in these bitransgenic mice were less invasive to the surrounding tissues

compared to the tumors in ERbB-2 strain [49]. More importantly, lung metastatic lesions were significantly less in the bitransgenic mice indicating that tumors developed with activated Akt1 had less metastatic properties compared to the ones with less active Akt1.

After a decade-long hiatus, there has been a renewed interest on the dual role of Akt in cancer after it was discovered that the deletion of Akt1 gene in *Akt2*$^{+/-}$ mice potentiate inflammation-induced hepatic cancer [16]. Following this, Akt1 was identified as a negative regulator of breast cancer metastasis *in vivo* via proteolytic degradation of twist-1, a transcriptional factor that induces EMT [15]. Interestingly, inhibition of Akt by MK-2206 treatment led to twist-1 stabilization, promoting breast cancer cell invasion *in vitro* and lung metastasis *in vivo* accompanied by increased N-cadherin and vimentin, and decreased E-cadherin expressions. Latest in this series are the two parallel reports from NSCLC and PCa. Whereas Akt inhibition by MK-2206 *in vitro* promoted NSCLC invasion and metastasis through the activation of MARCKs-LAMC2 [17], Akt inhibition by triciribine promoted PCa EMT and metastasis in a neuroendocrine mouse model of *TRAMP* [13]. Furthermore, silencing of Akt1 in PCa cell lines (PC3 and DU145) enhanced EMT associated with increased N-cadherin, Snail, and reduced E-cadherin. Overall, these studies demonstrate that Akt(1) suppression in advanced cancers will promote EMT and metastasis.

Although a plethora of information from the cellular and pre-clinical studies have demonstrated the dual role of Akt1 activity in cancer, a correlation between Akt1 activity suppression and promotion metastasis has not been demonstrated in any type of human cancers. Our initial analysis of the cBioportal database revealed the existence of missense mutations in the Akt isoforms that did not modulate its activity thus indicating that genetic mutations in Akt isoforms did not contribute to the onset or aggressiveness of PCa. The MSKCC genomic data

showed alterations in the PI3K/Akt pathway in 26% of the advanced stage PCa patient population linking to reduced disease-free survival [22]. Interestingly, 14% of these alterations were in the PTEN gene that is known to contribute to the hyperactivation of the PI3K/Akt pathway [50, 51]. Interestingly, there were no significant differences in the mean mRNA expression levels of Akt isoforms between the altered and unaltered groups. The Trento/Broad/Cornell genomic data on human neuroendocrine PCa [26], however, had 66% of the patients exhibiting alterations in genes from the PI3K/Akt pathway. Once again, 31% of these were as a result of PTEN deletion or amplification. Gene amplifications were also observed in the Akt isoforms (25%, 18% and 31% in Akt1, Akt2, and Akt3, respectively). Fred Hutchinson genome analysis of the human metastatic PCa [25] revealed approximately 81% of the genes from the PI3K/Akt pathway with genetic alterations. While 44% of genetic alterations were observed in PTEN deletion or loss of function mutations, only 4-7% alterations were noted in the Akt isoforms. Exome sequencing by the Broad/Cornell group on the human prostate adenocarcinoma [23] showed only 15% of the patients with genetic alterations in the PI3K/Akt pathway, out of which 7% alterations were in PTEN and a single case of missense mutations was observed in Akt1 and Akt3 isoforms. Genomic data of metastatic PCa from the MSKCC study [27] showed approximately 78% of the patients showing alterations in the PI3K/Akt pathway genes, with the majority of 42% of the alterations found as the PTEN gene deletion, fusion or loss of function mutations. Interestingly, none of these studies showed any significant differences in the mean mRNA expression levels of Akt isoforms between the altered and un-altered groups, suggesting that while genetic alterations in PTEN may have contributed to Akt hyperactivation, there was no evidence on the direct effect of genomic alterations in the Akt1 isoforms on their activity in these PCa samples.

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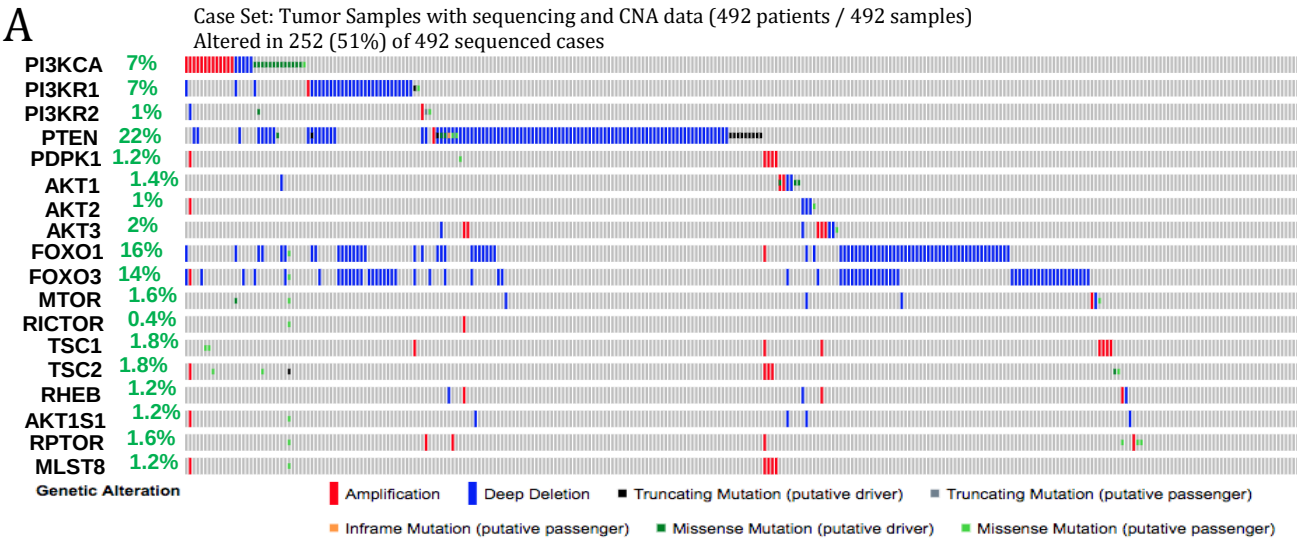

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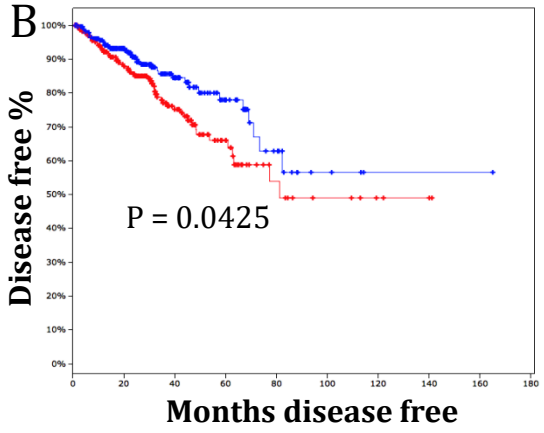

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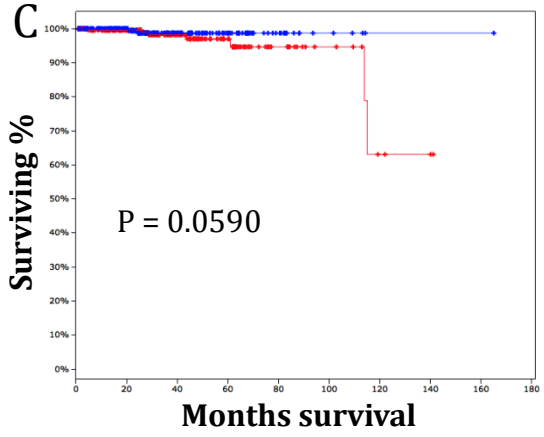

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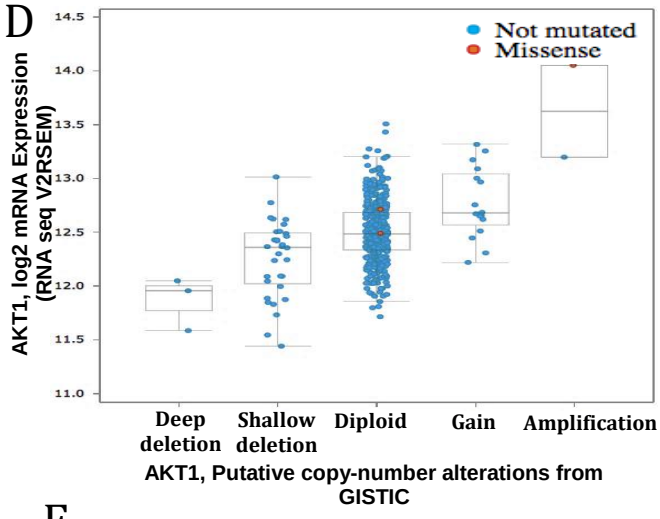

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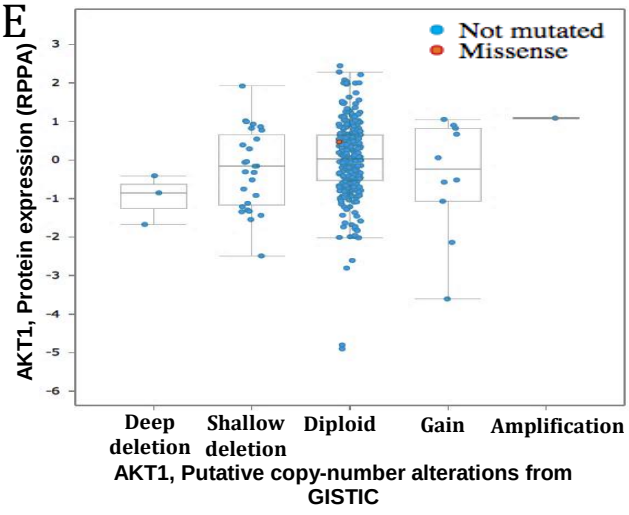

F

Gene	Cytband	Mean ± SD mRNA expression		p-value	q-value
		In altered group	In un-altered group		
AKT1	14q32.33	12.50 ± 0.34	12.50 ± 0.29	0.951↑	0.979↑
AKT2	19q13.2	11.56 ± 0.27	11.57 ± 0.22	0.584↓	0.776↓
AKT3	1q43-q44	10.27 ± 0.68	10.36 ± 0.55	0.117↓	0.313↓

FIGURE 6: Gene alterations in Akt pathway in human primary prostate adenocarcinoma. (A) Oncoprint showing genomic alterations in the Akt pathway genes in human primary (non-metastatic) PCa tissues based on the analysis by the cancer genome atlas research network (TCGA, Cell, 2015). **(B)** Kaplan–Meier survival analysis showing decreased disease-free survival in patients with observed Akt pathway alterations compared to the un-altered group. **(C)** Kaplan–Meier survival analysis showing decreased overall survival in patients with observed Akt pathway alterations compared to the un-altered group. **(D)** A plot showing the relationship between Akt1 mRNA abundance and copy-number alteration (CAN) in the Akt1 gene in human primary PCa. **(E)** A plot showing the relationship between Akt1 protein expression and copy-number alteration (CAN) in the Akt1 gene in human primary PCa. **(F)** Chart showing the mean mRNA expression of Akt1, Akt2 and Akt3 in the PCa tissues with observed Akt pathway alterations compared to the un-altered group.

A

Gene	Mean±SD Protein expression		p-value
	In altered group	In un-altered group	
AKT1-Phospho-T308	0.03 ± 0.55	0.16 ± 0.59	0.0486↓
AKT1	0.46 ± 0.50	0.56 ± 0.25	0.0841↓
AKT1-Phospho-S473	0.22 ± 0.76	0.37 ± 0.77	0.104↓

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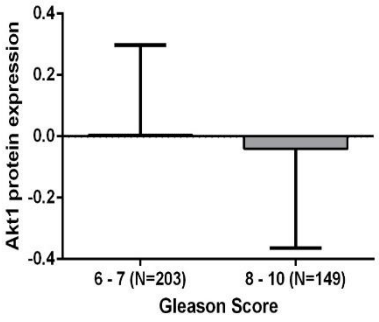

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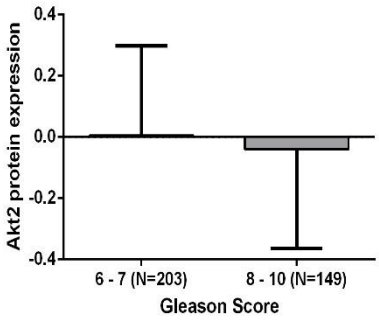

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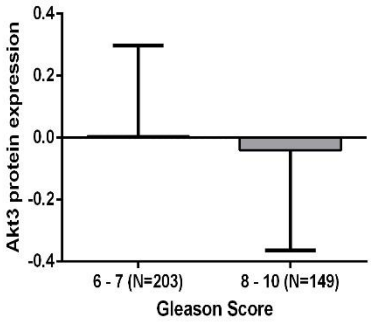

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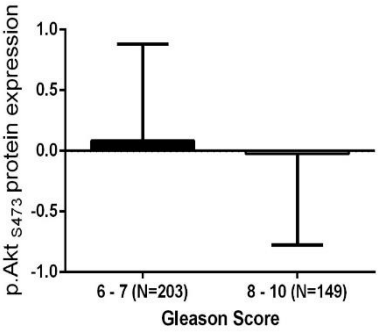

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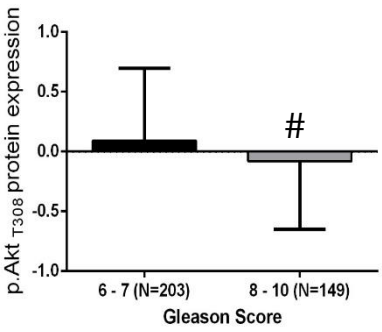


FIGURE 7: Alterations in Akt1 protein expression and its activity in human primary prostate adenocarcinoma. (A) Chart showing a decline in the mean protein expression of Akt1, p.AktS473 and p.AktT308 in the PCa tissues with observed Akt pathway alterations compared to the un-altered group based on the analysis by the cancer genome atlas research network (TCGA, Cell, 2015). **(B-F)** Patients stratification based on Gleason score [higher (8-10) and lower (6-7) group] showed a declining trend in Akt1, Akt2, Akt3, p.AktS473 and a significant reduction in p.AktT308 protein/phosphorylation levels in the higher compared to the lower Gleason score group. $^{\#}P < 0.01$; Unpaired Student t-test for two group analysis (GraphPad Prism 6.01). Data are presented as means ± SD.

The TCGA data was the sole source of information that allowed comparison of genomic, proteomic expressions and activating phosphorylations of Akt isoforms in a large collection of primary prostate adenocarcinoma samples [24]. Although approximately 51% of the patients showed genetic alterations in genes from the PI3K/Akt pathway, only isolated cases of amplification or deletion in Akt1 (1.4%), Akt2 (1%) and Akt3 (2%) genes were noted. Interestingly, despite the amplification of the Akt isoform mRNAs, no significant differences in their expression between the altered and un-altered groups were observed. These studies revealed that although mutations and deletions in PTEN gene lead to PCa, the lack of difference in mRNA and protein expression (data not shown for Akt2 and Akt3) in the Akt isoforms between the altered and un-altered group indicated that the inhibition of PTEN does not contribute further to the already increased Akt in PCa. Intriguingly, further analysis of S473 and T308 activating phosphorylation of Akt1 showed a reduction in their activity in the altered compared un-altered group. This was corroborated with increased gene expression of EMT markers such as TGFβ1, CDH2 and Snail correlating with higher Gleason score and/or metastatic tumor colonies in sites other than the tumors in prostate. Similarly, a significant correlation between reduced Akt phosphorylation (reduced activity) and higher Gleason score was also observed in TCGA analysis indicating Akt de-addiction contributing to cancer aggressiveness.

Activation of PI3K and Akt as a result of PTEN inactivation has been demonstrated to be a contributing factor for PCa oncogenesis [50, 51]. However, as has been critically reviewed by Blanco-Aparicio *et al,* further cancer progression due to PTEN loss occurs as a result of Akt-independent mechanisms [40]. Several mouse models have revealed that Akt activation, although important for oncogenic transformation, alone is not sufficient for tumorigenesis. Expression of myrAkt1 (active) in the prostate [41] or *PTEN*$^{-/-}$ mice [42] lead to prostatic inter-epithelial

neoplasia and tumor, but not metastasis. Expression of myrakt1 did not promote breast cancer metastasis in *P53*$^{-/-}$ mice either [43, 44]. A recent review states that having more Akt in cancer is not always better [39]. While the previous reports from the preclinical studies and the most recent reports from the transgenic mouse models demonstrate increased metastasis in the breast, liver, prostate, and lung (NSCLC) cancers with Akt1 suppression, the cBioportal analysis provides reasonable, if not complete evidence indicating the existence of such a phenomenon in human PCa patients as well. Nevertheless, our data from the human PCa cell lines on the effect of MK-2206 in promoting EMT along with the observation of reduced phosphorylated S473Akt in 5+5 Gleason PCa samples compared to 3+3 Gleason PCa samples, a trend toward elevation of TGFβ1, N-cadherin and Snail mRNA levels in the distant metastatic tumors compared to PCa tumors from MSKCC/UMICH study and reduced phosphorylated S473Akt and T308Akt associated with increased TGFβ1 and N-cadherin mRNA levels in the TCGA study suggest a negative correlation between Akt activity and EMT/metastasis in human the advanced PCa. Recent Phase I/II clinical studies have also reported no significant benefits of using MK-2206 for metastatic cancers [52-57]. However, a large-scale analysis of Akt activity in PCa samples will be needed to further confirm this observation.

3.4. Materials and Methods

- **Genotyping of TRAMP mice**

Genotyping of TRAMP (C57BL/6) transgenic mice (Jackson, Bar Harbor, ME) was performed as described [13]. All experiments were carried out in accordance with guidelines set by VA Medical Center in Augusta and as approved by the institutional animal care and use committee. DNA was extracted from the tails of 10- to 21-day old litters (Qiagen, Valencia, CA). TRAMP transgene (600bp) was detected by PCR with an annealing temperature of 55°C

(forward: 5'-GCGCTGCTGACTTTCTAAACATAAG-3' and reverse: 5'-GAGCTCACGTTAAGTTTTGATGTGT-3'). The internal positive control produced a 324bp fragment (forward: 5'-CTAGGCCACAGAATTGAAAGATCT-3' and reverse: 5'-GTAGGTGGAAATTCAGCATCATCC-3'). TRAMP mouse prostates were collected at 12, 24, 32 and 40 weeks and subjected to Western blot analysis.

- **Cell lines, reagents, and antibodies**

Human PC3 and DU145 cells were obtained from ATCC (Manassas, VA). Cells were maintained in DMEM-G (Hyclone, Logan, UT) with 10% FBS (Atlanta Biologicals, GA), 100 U/ml penicillin, and 100 µg/ml streptomycin in a humidified incubator at 37°C and 5% CO_2, and routinely passaged when 80– 90% confluent. Antibodies for N-cadherin, TGFβ1, Akt1, pS473Akt, panAkt1and Snail1 were purchased from Cell Signaling (Danvers, MA). Anti-β-actin was purchased from Sigma (St. Louis, MO). Akt inhibitor MK2206 was purchased from Selleckchem (Houston, TX).

- **Western blot analysis and immunohistochemistry analysis**

Western blot analysis was performed as described previously [58, 59]. Images were scanned at 600dpi, cropped, contrast/brightness adjusted equally across the entire blot and presented without combining any two or more different blots. Densitometry analysis was performed using the NIH Image J Software. Unedited images are provided in Supplemental Figures 2 and 3. Slides containing benign prostatic hyperplasia (BPH), 3+3 Gleason score and 5+5 Gleason score PCa patient sections were subjected for immunohistochemistry using pS748Akt antibodies and counterstained by hematoxylin as described previously [60, 61].

- **shRNA-mediated gene silencing and generation of stably silenced PCa cells**

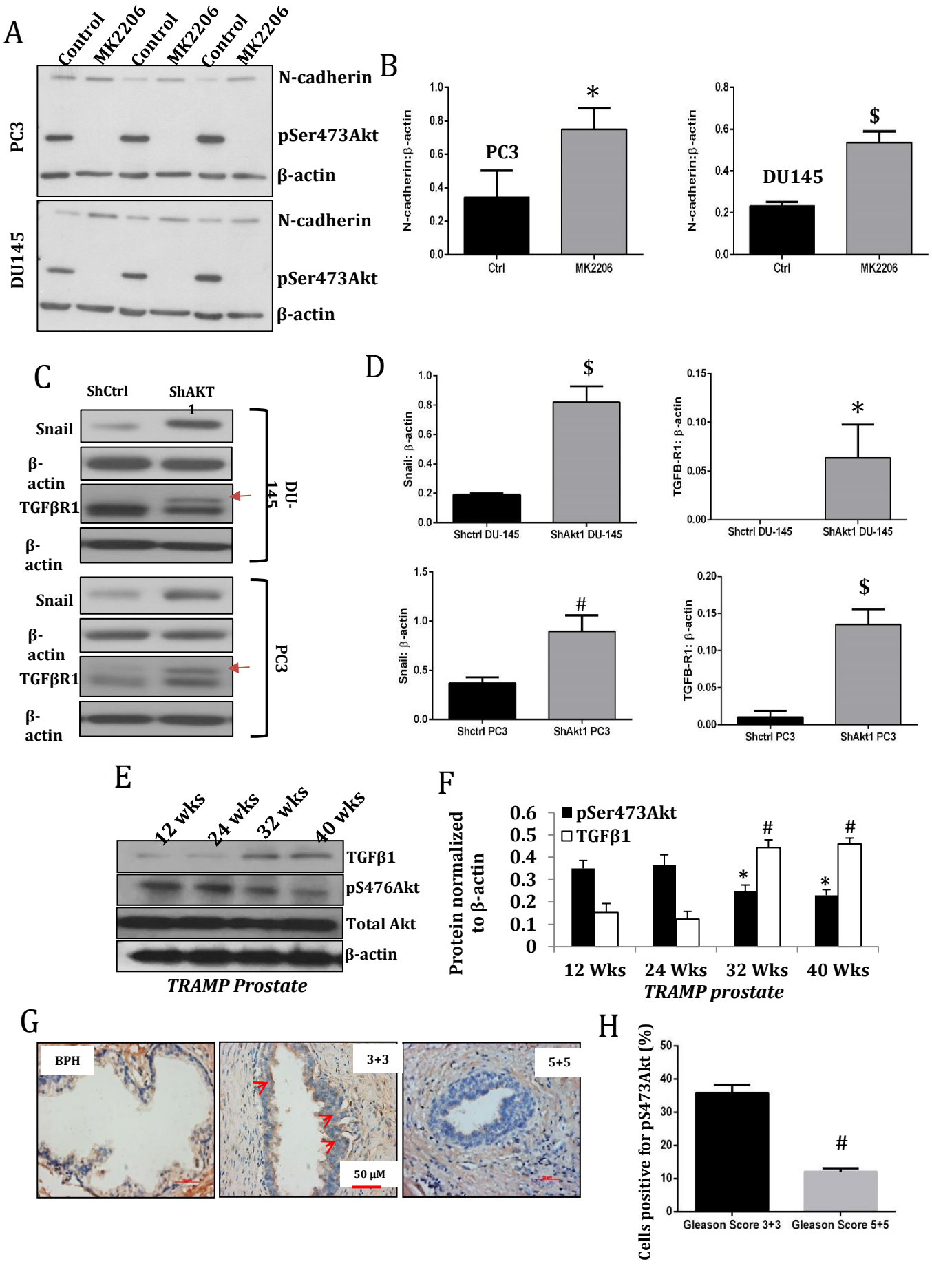
Human PC3 and DU145 cells were transfected with SMARTvector 2.0 Lentivirus ShAkt1 or non-targeting ShControl particles (GE Dharmacon, Lafayette, CO). Lentiviral infections were performed in 6 well plates. Lentiviral particles were mixed with 1ml SFM4 Transfx-293 (GE Hyclone, Lafayette, CO) solution and applied to PC3 and DU145 cells with 10 µg polybrene (American bioanalytical, MA). After 16 hours, the medium was replaced with complete EBM-2. After 3 days, GFP was detected using a confocal imaging microscope (LSM510, Carl Zeiss, Germany). Stable silencing of Akt1 as compared to ShControl cells was achieved by puromycin selection (8 µg/ml, Thermo, Grand Island, NY). Post selection, cells were maintained in complete DMEM high glucose medium with 0.6 µg/ml puromycin.

- **Analysis of clinical trials on Akt1 inhibitor (MK-2206)**

Data from phase 3 clinical trials on the use of Akt inhibitor MK-2206 in the treatment of various cancers were collected from www.clinicaltrials.gov. The basis of the evaluation of MK-2206 efficacy is either the standard therapy or experts' opinions unless otherwise stated. All the single group studies were compared to the first line (standard) therapy from the published literature.

- **Gene expression and alteration analysis from patient databases**

Information regarding protein and mRNA expression and other genetic alterations in Akt pathway molecules were obtained from the publically available cBioportal http://www.cbioportal.org [20, 21]. Before analyzing genomic alterations in the studies of interest, certain genomic profiles that are mutations and copy number alterations were selected for comparative analysis. The studies of interest are metastatic PCa SU2C/PCF Dream team [27], neuroendocrine PCa [26], castration-resistant PCa [25] and two prostate adenocarcinoma studies [22, 23]. "Protein expression" analysis was performed from the only study available in the


A
Control
MK2206
Control
MK2206
Control
MK2206
PC3
N-cadherin
pSer473Akt
β-actin
DU145
N-cadherin
pSer473Akt
β-actin
B
N-cadherin:β-actin
PC3
Ctrl
MK2206
*
N-cadherin:β-actin
DU145
Ctrl
MK2206
$
C
ShCtrl
ShAKT 1
Snail
β-actin
TGFβR1
β-actin
DU-145
Snail
β-actin
TGFβR1
β-actin
PC3
D
Snail: β-actin
Shctrl DU-145
ShAkt1 DU-145
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TGFB-R1: β-actin
Shctrl DU-145
ShAkt1 DU-145
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Snail: β-actin
Shctrl PC3
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TGFB-R1: β-actin
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32 wks
40 wks
TGFβ1
pS476Akt
Total Akt
β-actin
TRAMP Prostate
F
pSer473Akt
TGFβ1
Protein normalized to β-actin
12 Wks
24 Wks
32 Wks
40 Wks
TRAMP prostate
G
BPH
3+3
50 μM
5+5
H
Cells positive for pS473Akt (%)
Gleason Score 3+3
Gleason Score 5+5
#

FIGURE 8: A decrease in Akt phosphorylation (activity), not expression is linked to EMT in PCa. (A) Representative Western blot images of PC3 and DU145 cell lysates treated with DMSO (control) or Akt inhibitor MK2206 for 72 hours (5 μM) showing changes in the phosphorylation of Akt associated with changes in the expression of mesenchymal marker N-cadherin. **(B)** Bar graphs showing changes in N-cadherin expression in PC3 and DU145 cells with MK2206 treatment (n=3). **(C)** Representative Western blot images of PC3 and DU-145 ShControl and ShAkt1 cell lysates showing changes in the expression of TGFβ-R1 and mesenchymal transcription factor Snail. **(D)** Representative bar graph of band densitometry analysis for TGFβR1 and Snail1 of PC3 and DU-145 ShControl and ShAkt1 cell lysates (n=3). **(E-F)** Western blot images and band densitometry analysis of *TRAMP* prostate lysates collected from 12, 24, 32 and 40 wks-old mice, and analyzed for changes in pS473Akt and TGFβ1 expressions, showing an inverse relationship between pS473Akt (decreased) and TGFβ1 (increased) in the high-grade tumor (n=4). **(G-H)** Immunohistochemistry of early stage PCa (Gleason 3+3) showing a higher number of phosphorylated Akt (PSer473, active) positive cells compared to the advanced stage (Gleason 5+5) (n=5) as counted using Image-J software and percentage of pAkt-positive cells were determined. **P*<0.01 compared to pS473Akt on 12 wks; #*P*>0.01 compared to TGFβ1 on 12 wks. $^{\$}P < 0.01$; Unpaired Student t-test for two groups analysis (GraphPad Prism 6.01). Data are presented as means ± SD.

database [24]. For the genes of interest, we chose the user-defined option of "PI3K-Akt-mTOR pathway". Genomic analysis was performed using the OncoPrint option to summarize the genomic alterations of Akt1 from 5 different studies stated above. On the table, rows represented genes and columns represented samples. Genomic alterations including mutations, CNA (amplifications and deletions), and changes in gene expression were analyzed.

3.5. Statistical analysis

All the data are presented as mean ± SD and were calculated from multiple experiments performed in quadruplicates. For the data analyses, Student's two-tailed t-test or one-way ANOVA were used to determine significant differences between treatment and control groups using the GraphPad Prism 4.03 software and SPSS 17.0 software. All the existing statistical analysis data were obtained from the cBioportal. Data with $P<0.05$ were considered significant.

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ETHICAL APPROVAL

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. This article does not contain any studies with human participants. Only de-identified and archived human PCa samples were used for analysis as exempted by the institutional review board.

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SUPPLEMENTAL TABLE 1: Data on clinical trials on Akt inhibitor MK-2206 for various cancers collected from www.clinicaltrials.gov

Clinical trial ID	Cancer type	Size	Treatment groups	Results	Compared to First line /Standard therapy
NCT01283035	Recurrent and platinum-resistant ovarian, fallopian tube, or primary peritoneal cancer	5	Single group; MK-2206 monotherapy PO QW for 4 weeks (one cycle); repeat if patient is benefiting	Not available	
NCT01349933	Recurrent or metastatic head and neck cancer	21	Single group; MK-2206 monotherapy 200 mg PO QW for 4 weeks (one cycle); repeat if patient is benefiting	**OS:**10 months (95% CI; 5.9 - 20); PFS: 3.5 months (95% CI; 0.9 - 7.3)	Cisplatin + Gemcitabine; OS: 29.1 months; PFS: 7.0 months [1]
NCT01253447	Relapsed or refractory acute myeloid leukemia (AML)	19	Single group; MK-2206 monotherapy 200 mg PO QW for 4 weeks (one cycle); repeat if patient is benefiting	Study was terminated due to insufficient drug efficacy	
NCT01802320	Previously treated, metastatic or locally advanced colon or rectal cancer	18	Single group; MK-2206 monotherapy PO QW for 4 weeks (one cycle); repeat if patient is benefiting	Not available	
NCT01186705	Metastatic KRAS-Wild-Type, PIK3CA-Mutated, Colorectal Cancer	1	Single group; MK-2206 monotherapy 200 mg PO QW for 4 weeks (one cycle); repeat if patient is benefiting	Not available	
NCT01277757	Advanced breast cancer with a PIK3CA mutation, or an AKT mutation and/or PTEN loss/PTEN mutation	28	Single group; MK-2206 monotherapy 200 mg PO QW for 4 weeks (one cycle); repeat if patient is benefiting	Not available	
NCT01260701	Advanced Gastric/Gastro-esophageal Junction Cancer	70	Single group; MK-2206 monotherapy 60 mg PO QOD on days 1-28 (one cycle); repeat if patient is benefiting	**OS:** 5.1 months (95% CI; 3.7 - 9.4) **PFS:** 1.8 months (95% CI; 1.7 - 1.8)	Cisplatin + 5-FU; OS: 33 weeks; PFS: 27 week [2]
NCT01169649	Metastatic Neuroendocrine Tumors (NET)	8	Single group; MK-2206 monotherapy 200 mg PO QW for 4 weeks (one cycle); repeat if patient is benefiting	Not available	
NCT01294306	Advanced non-small cell lung cancer progressed after previous response to Erlotinib Hydrochloride therapy	80 (45 EGFR-Mutation and 34 EGFR-WT)	Single group; MK-2206 monotherapy PO QOD on days 1-28; repeat if patient is benefiting	**OS:** EGFR-Mutated: 10.6 months (95% CI; 8.6 - 23.2); EGFR WT: 11.1 months (95% CI; 7.3 - 22.1) **PFS:** EGFR-Mutated: 4.4 months (95% CI; 2.7 - 6.6); EGFR WT: 4.6 months (95% CI; 2.9 - 8.5)	Cisplatin + Gemcitabine or Cisplatin + Docetaxel; OS: 19·5 months; PFS: 5.2 months [3]
NCT01519427	Stage III/IV melanoma that failed prior therapy with Vemurafenib or Dabrafenib	2	Single group; MK-2206 monotherapy PO QW for 4 weeks + selumetinib PO BID on days 1-21 (one cycle); repeat if patient is benefiting	**OS:** 153 days, range (117 to 189); **PFS:** 105 days, range (42 to 168)	Vemurafenib compared to Decarbazine; OS: 13·6 months; PFS: 6·9 months [4] Dabrafenib compared to Decarbazine: OS: favoring Dabrafenib; PFS: 5.1 months [5]
NCT01658943	Previously treated metastatic pancreatic cancer	121 (62 mFOLFOX and 58 MK + Selumetinib)	Two groups: 1st group: mFOLFOX regimen PO QD on days 1-28; 2nd group: MK2206 + selumetinib, MK2206 (135 mg) PO QW and selumetinib PO QD on days 1-28	**1st group: OS:** 6.7 months (95% CI; 6 - 8.3); **FBS:** 2 months (95% CI; 1.8 - 2.9); **2nd group: OS:** 3.9 months (95% CI; 3.5 - 4.6); **FBS:** 1.9 months (95% CI; 1.8 - 2.1)	
NCT01239355	Previously treated advanced liver cancer	15	Single group; MK-2206 monotherapy 200 mg PO QW for 4 weeks (one cycle); repeat if patient is benefiting	**OS:** 6.1 months (95% CI; 3.0 - 8.4); **PFS:** 1.7 months (95% CI; 1.6 - 3.0)	Sorafenib; OS: 10.7 months; PFS: 4.1 months [6]
NCT01466868	Relapsed or refractory diffuse large B Cell Lymphoma (AKTIL)	22	Single group; MK-2206 monotherapy 200 mg PO QW for 4 weeks (one cycle); repeat if patient is benefiting	Not available	
NCT01481129	Relapsed or refractory diffuse large B Cell Lymphoma (AKTIL)	22	Single group; MK-2206 monotherapy PO QW for 4 weeks (one cycle); repeat if patient is benefiting	**OS:** 9.6 months (95% CI; 2.8 to 18.8); **PFS:** 1.71 months (95% CI; 0.8 to 1.8)	Gemcitabine + Dexamethasone + Cisplatin; OS: 8.9 months; FBS: 3.1 months [7]
NCT01307631	Recurrent or advanced endometrial cancer	37 (9 PIK3CA Mutant and 28 PIK3CA WT)	Two groups; All with MK-2206 monotherapy PO QW for 4 weeks (one cycle); repeat if patient is benefiting	**OS:** 8 months; range (0-12 months) **PFS:** PIK3CA Mutant: 1.6 months (90% CI; 0 - 1.6); PIK3CA WT: 1.8 months (90%CI; 0 - 1.8)	Carboplatin + Paclitaxel; OS: 37 months; PFS: 13 months (Miller DS, Filiaci G, Mannel R, et al. Randomized Phase III Noninferiority Trial of First Line Chemotherapy for Metastatic or Recurrent Endometrial Carcinoma: A Gynecologic Oncology Group Study. LBA2. Presented at the 2012 Society of Gynecologic Oncology Annual Meeting, Austin, TX.)
NCT01258998	Relapsed or refractory lymphoma	59	Single group; MK-2206 monotherapy 200 mg PO QW for 4 weeks (one cycle); repeat if patient is benefiting	**OS:** Not available **PFS:** 2.8 months	Gemcitabine + Dexamethasone + Cisplatin; OS: 8.9 months; FBS: 3.1 months [7]. (Cancer. 2004 Oct 15;101(8):1835-42)
NCT01604772	Progressive, recurrent, or metastatic adenoid cyst carcinoma	14	Single group; MK-2206 monotherapy 150 mg PO QW for 4 weeks (one cycle); repeat if patient is benefiting	OS: Not available; PFS: 9.2 months (95% CI; 3.8-11)	No optimal treatment due to inadequate clinical trials. (Uptodate.com; Malignant salivary gland tumors: Treatment of recurrent and metastatic disease)

Clinical trial ID	Cancer type	Size	Treatment groups	Results	Compared to First line /Standard therapy
NCT01333475	Advanced colorectal carcinoma	21 (12 in the 1st group (TAC1) and 9 in the 2nd group (TAC1A))	Two groups: 1st group (TAC1) : MK-2206 + AZD6244, MK-2206 90 mg PO QW and AZD6244 Hydrogen sulfate 75 mg PO QD (one cycle); repeat if patient is benefiting 2nd group (TAC1A): MK-2206 + AZD6244, MK-2206 135 mg PO QW and AZD6244 Hydrogen sulfate 100 mg PO QD (one cycle); repeat if patient is benefiting	This biomarker-driven phase 2 study was conducted to determine the antitumor activity of dual therapy. OS and PFS are not available	
NCT01425879	Advanced refractory biliary cancer that cannot be removed by surgery	8	Single group; MK-2206 monotherapy 200 mg PO QW for 4 weeks (one cycle); repeat if patient is benefiting	**OS:** 3.5 months (95% CI; 2.2 - 6.7); **PFS:** 1.7 months (95% CI; 0.5 - 5.6)	Cisplatin + Gemcitabine; OS: 11.7 months; PFS: 8 months [8]
NCT01239342	Refractory kidney cancer	43 (29 in the MK group and 14 in the Everolimus group) For PFS analysis, 1 patient was excluded from each group	Two groups: 1st group: MK-2206 200 mg PO QW for 4 weeks; repeat if patient is benefiting 2nd group: Everolimus 10 mg PO QD for 4 weeks; repeat if patient is benefiting	**MK-2206 group: OS:** 23.5 months (95% CI; 10.7 - 37.4) **PFS:** 3.68 months (95% CI; 1.77 - 5.75) **Everolimus group:** **OS:** 15.7 months (95% CI; 6.5 - not estimable) **PFS:** 5.98 months (95% CI; 5.08 to not estimable)	
NCT01251861	Patients with previously treated prostate cancer	104	Two groups: 1st group: Observation on weeks 1-12, then bicalutamide PO QD on weeks 13-44. Patients with a PSA decline of >= 50% may continue on bicalutamide until week 72 in the absence of disease progression or unacceptable toxicity. 2nd group: MK2206 PO QW on weeks 1-44 and bicalutamide PO QD on weeks 13-44. Patients with a PSA decline of >= 50% may continue on Akt inhibitor MK2206 and bicalutamide until week 72 in the absence of disease progression or unacceptable toxicity	Not available	
NCT01370070	Recurrent and metastatic nasopharyngeal carcinoma	21	Single group; MK-2206 monotherapy 200 mg PO QW for 4 weeks (one cycle); repeat if patient is benefiting	**OS:** 10 months (95% CI; 5.9 to 20.0) **PFS:** 3.5 months (95% CI; 0.9 to 7.3)	Gemcitabine + Cisplatin; OS: 29.1 months; PFS: 7 months [9]

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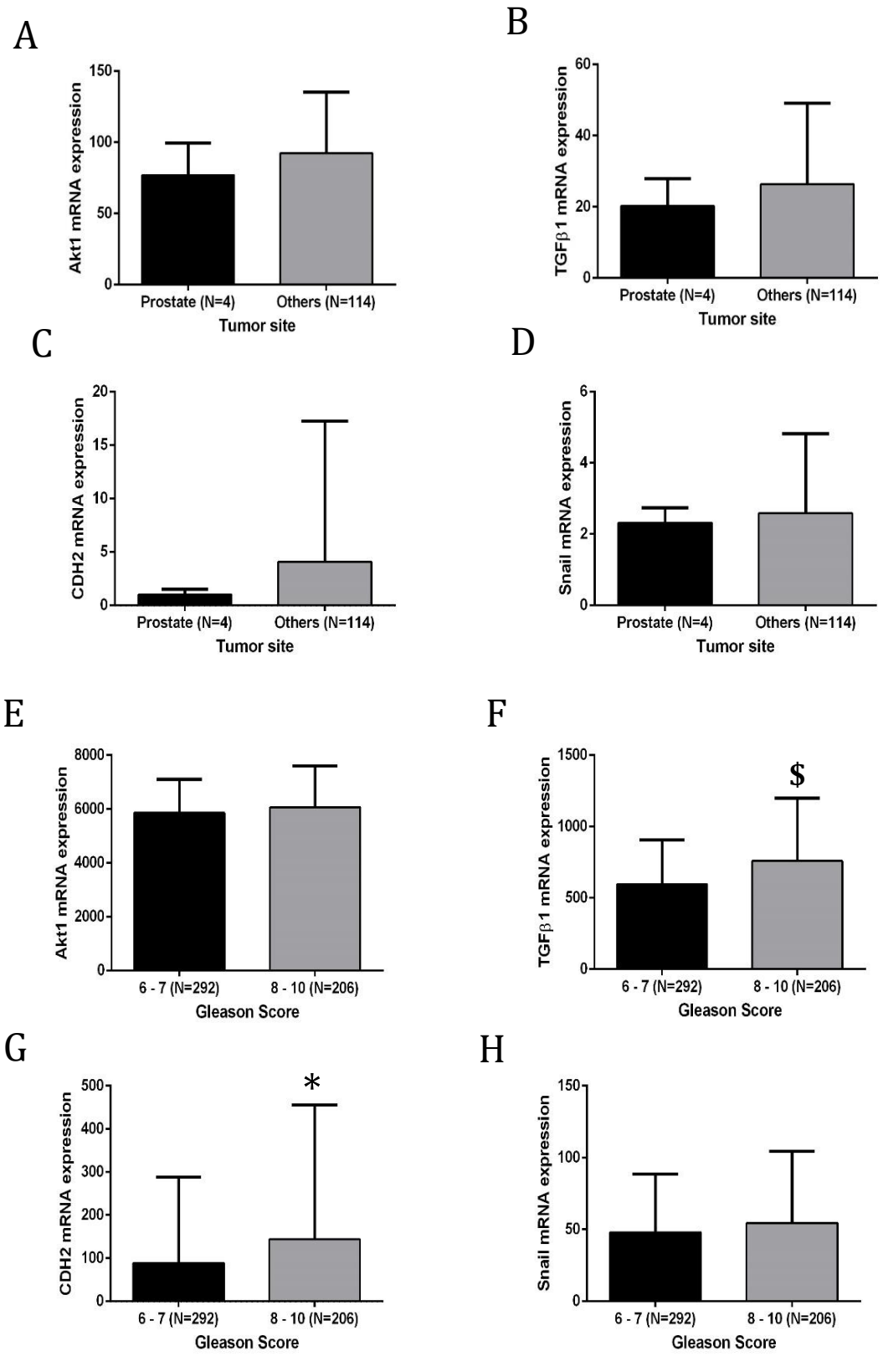
A
Akt1 mRNA expression
150
100
50
0
Prostate (N=4)
Others (N=114)
Tumor site
B
TGFβ1 mRNA expression
60
40
20
0
Prostate (N=4)
Others (N=114)
Tumor site
C
CDH2 mRNA expression
20
15
10
5
0
Prostate (N=4)
Others (N=114)
Tumor site
D
Snail mRNA expression
6
4
2
0
Prostate (N=4)
Others (N=114)
Tumor site
E
Akt1 mRNA expression
8000
6000
4000
2000
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6 - 7 (N=292)
8 - 10 (N=206)
Gleason Score
F
TGFβ1 mRNA expression
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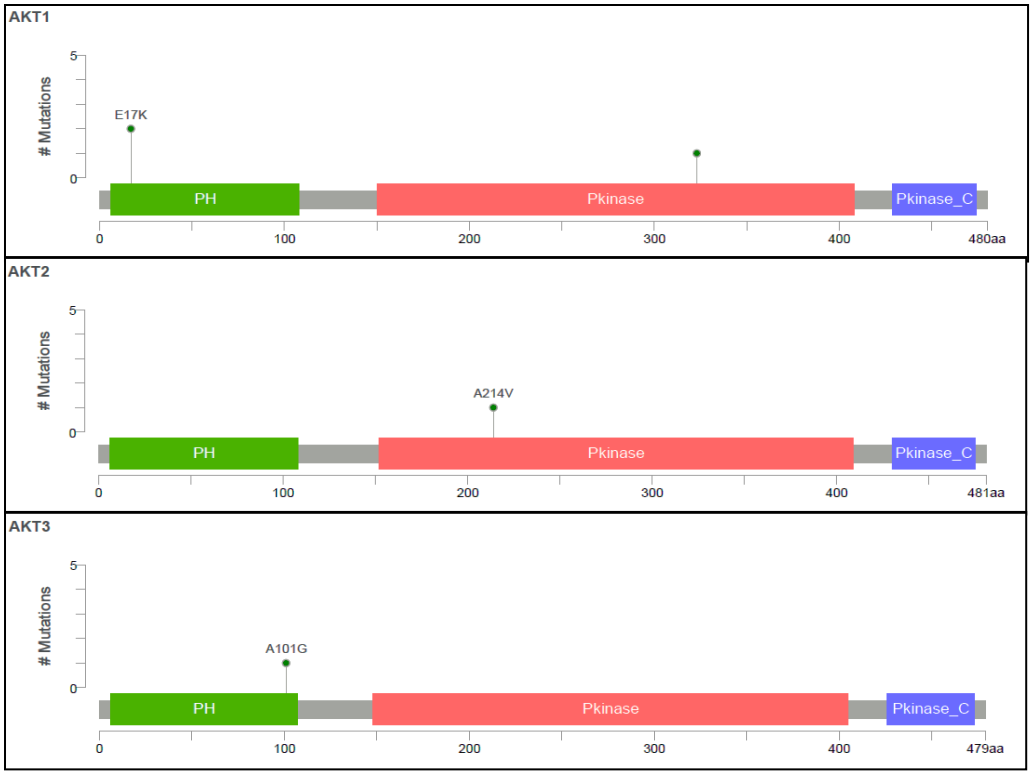
6 - 7 (N=292)
8 - 10 (N=206)
Gleason Score

FIGURE 9: EMT is positively correlated to higher Gleason score and PCa metastasis with no change in the Akt1 expression. (A-D) Castration-resistant PCa from patients in the MSKCC/UMICH study (Robinson D et al, Cell, 2015) stratified based on the anatomic site [prostate and others (bone, muscles, neck, chest wall, thoracic epidural, liver, bladder, penis, pelvis, lymph node, perirectal, retroperitoneum and soft tissue) group] showed no change in Akt1 mRNA level with a trend toward elevation of TGFβ1, CDH2 (N-cadherin) and Snail in the others compared to the prostate group. **(E-H)** Primary prostate adenocarcinoma from patients in the cancer genome atlas study (TCGA, Cell, 2015) stratified based on Gleason score [higher (8-10) and lower (6-7) group] showed no change in Akt1 mRNA level with a significant increase in TGFβ1 and CDH2 (N-cadherin) and a trend toward elevation of Snail in the higher compared to the lower Gleason score group. $^{*}P < 0.05$; $^{\$}P < 0.01$; Unpaired Student t-test for two groups analysis (GraphPad Prism 6.01). Data are presented as means ± SD.



SUPPLEMENTAL FIGURE 1: Mutation in the Akt isoforms based on the data collected from the cBioportal database. This Figure shows the mutations detected in the Akt1, Akt2 and Akt3 isoforms in PCa patient samples based on the 6 genomic analysis studies available in the cBioportal database.

CHAPTER 4

Nodal pathway activation due to Akt1 suppression is a molecular switch for prostate cancer cell epithelial-to-mesenchymal transition and metastasis

Alwhaibi, Abdulrahman et al, **Manuscript accepted by the Journal *Biochemical Pharmacology,* June 2019. Reprinted here with permission of publisher.**

Abstract

Several studies have unraveled the negative role of Akt1 in advanced cancers, including metastatic prostate cancer (mPCa). Hence, understanding the consequences of targeting Akt1 in the mPCa and identifying its downstream novel targets is essential. We studied how Akt1 deletion in PC3 and DU145 cells activates the Nodal pathway and promotes PCa epithelial-to-mesenchymal transition (EMT) and metastasis. Here we show that Akt1 loss increases Nodal expression in PCa cells accompanied by activation of FoxO1/3a, and EMT markers Snail and N-cadherin as well as loss of epithelial marker E-cadherin. Treatment of PCa cells with FoxO inhibitor AS1842856 abrogated the Nodal expression in Akt1 deleted PCa cells. Akt1 deficient PCa cells exhibited enhanced cell migration and invasion *in vitro* and lung metastasis *in vivo*, which were attenuated by treatment with Nodal pathway inhibitor SB505124. Interestingly, Nodal mRNA analysis from two genomic studies in cBioportal showed a positive correlation between Nodal expression and Gleason score indicating the positive role of Nodal in human mPCa. Collectively, our data demonstrate Akt1-FoxO-Nodal pathway as an important mediator of PCa metastasis and present Nodal as a potential target to treat mPCa patients.

Keywords: Akt1; FoxO; Metastasis; Nodal; Prostate cancer

4.1. Introduction

Despite the early diagnosis and significant advances in treatments, prostate cancer (PCa) still ranks the second and third on cancer-related mortalities in men in the United States and Europe, respectively [1, 2]. Recent statistics reveal that the higher mortality of PCa is mainly due to its metastasis to the bone, lungs, and brain [3, 4]. Whereas the 5-year survival rate of non-metastatic PCa patients has always been >99 %, the 5-year survival rate of metastatic PCa (mPCa) patients has been further declined to ≤ 30% [1]. Uncertainty in the molecular mechanisms mediating cancer cell dissemination to distant organs is a major roadblock in the effective management of mPCa [4]. In-depth molecular characterization and identification of novel, druggable targets will pave the way for future therapies in mPCa.

Several investigators over the past 2 decades have demonstrated the integral role of Akt (Protein kinase B) in multiple cellular processes such as, survival, proliferation, growth, invasion, and migration, that are implicated in tumorigenesis and cancer malignancy [5-7]. As a result, many Akt targeting drugs have been developed, tested and a few of these have entered the clinical trials [8]. In prostate cancer, we have demonstrated that Akt is necessary for cancer cell motility [9], survival [10-12], proliferation [11, 13], invasion [9, 14], transendothelial migration [14]. Several preclinical studies have also demonstrated an isoform-specific effect of Akt on cell migration and invasion, which are essential steps in the metastasis. Whereas Akt2 promoted the invasive phenotype of breast and ovarian cancer cells [15, 16], Akt1 was intriguingly found to abrogate cell migration and invasion by inhibiting epithelial-to-mesenchymal transition (EMT) in breast cancer [15, 17-20]. Recently, several reports in various cancer types have emerged explaining an unexpected, counteractive role of Akt1 in the advanced cancers [19, 21-24],

including our findings in PCa [25-27]. Despite the controversial role of Akt1 in the advanced PCa, knowing how Akt1 orchestrates this process is crucial to optimize the current therapies and pave the way for potential new therapies for patients with mPCa. One of the genes that was significantly elevated in our previous gene arrays from the mice experiments on PCa is 'Nodal' [25]. Hence we hypothesized that Nodal pathway activation downstream of Akt1 suppression is involved in the promotion of PCa cell EMT and metastasis.

Nodal, a secreted protein belonging of TGFβ superfamily, is known to be expressed during embryogenesis, where it plays a vital role in inhibiting embryonic cell differentiation and maintains human stem cell pluripotency [28, 29]. In concert with another related signaling partner Lefty, Nodal regulates physiological cell migration to determine anterior-posterior and left-right axises asymmetry during vertebral development [30]. Although Nodal was thought to be absent in adulthood, it is normally expressed in the mammary glands, endometrium and placenta, and a specific population of pancreas and liver cells [31]. Nodal transmits signals by binding to a heterodimeric receptor complex of Activin-like kinase type II receptors (ActRIIA/ActRIIB) and Activin-like kinase type I receptors (ALK4/ALK7) [32]. Activation of ALK4 or ALK7 by type II receptors and a co-receptor Cripto-1 results in the phosphorylation of Smad2/3, which further interacts with Smad4 to enter the nucleus and regulate the expression of the target genes [33]. The activity of Nodal pathway is kept in check by the endogenously-secreted, extracellularly-acting inhibitors Lefty A, Lefty B and Cerberus, which are transcribed in response to Nodal signaling, thus providing a negative feedback mechanism [31, 32]. Lefy A and B block this pathway by binding to Nodal and/or Cripto-1, whereas Cerberus exerts its effect by binding to free Nodal [32].

In this study, we investigated the molecular switch that induces EMT and metastasis in PCa cells following Akt1 suppression. Based on our results, although Akt1 inhibition activates caspases to induce apoptosis in a selected population of the androgen-independent PCa cells, activation of the FoxO3a-Nodal pathway induced downstream of Akt1 suppression in apoptosis resistant population of PCa cells, plays a significant role in promoting PCa cell EMT *in vitro* and lung metastasis *in vivo*. Our results demonstrate that the Nodal-induced cell migration and invasion are mediated through the canonical Smad2/3-dependent pathway and SB505124, a specific antagonist of Nodal receptors ALK4/ALK7, abrogated Nodal-induced Smad2 phosphorylation, PCa cell migration, invasion and metastasis to the lungs. The data from 'the cancer genome atlas (TCGA)' study also indicated that the expression levels of Nodal in PCa patients is directly proportional to their Gleason score. Taken together, these results clearly demonstrate that the activation of Nodal pathway downstream of Akt1 suppression promotes PCa cell EMT in vitro and metastasis *in vivo* and therefore targeting Nodal pathway using SB505124 could serve as a potential therapeutic strategy for the treatment of mPCa.

4.2. Materials and Methods

- **Cell culture, gene silencing by shRNA, antibodies and other reagents**

Human PC3 and DU145 cell lines were purchased from ATCC (Manassas, VA), cultured in DMEM high glucose medium (Hyclone, Logan, UT) with 10% Fetal bovine serum (FBS, Atlanta Biologicals, Atlanta, GA), 100 U/ml penicillin, and 100 mg/ml streptomycin in a humidified incubator at 37°C and 5% CO2. Cells were routinely passaged and when they were 80-90% confluent, transfection was carried out using SMARTvector 2.0 Lentivirus ShControl (non-targeting) and ShAkt1 (ACGCTTAACCTTTCCGCTG) from Dharmacon (Lafayette, CO), followed by selection with puromycin (0.6 ng/ml, Sigma Millipore, St. Louis, MO). Primary

antibodies against Akt1 (Cat #2938), p^{Ser473}Akt (Cat #4060), p^{Thr308}Akt (Cat #2965), pFoxO1/3a (Cat #9464), pFoxO3a (Cat #9465), FoxO3a (Cat #2497), FoxO1 (Cat #2880), pSmad2/3 (Cat #8828), Smad2/3 (Cat #8685), Snail (Cat #3879), E-cadherin (Cat #3195), and N-cadherin (Cat #4061) were purchased from Cell Signaling (Danvers, MA). Nodal antibodies (Cat # SC-28913 and SC-373910) were purchased from Santa Cruz Biotechnology. β-actin antibody (Cat #A5441) was purchased from Sigma (St. Louis, MO). Triciribine (TCBN) and SB505124 were purchased from Selleckchem (Houston, TX), and FoxO1/3a inhibitor (AS1842856) was purchased from Calbiochem (San Diego, CA). All other reagents were purchased from Fisher Scientific.

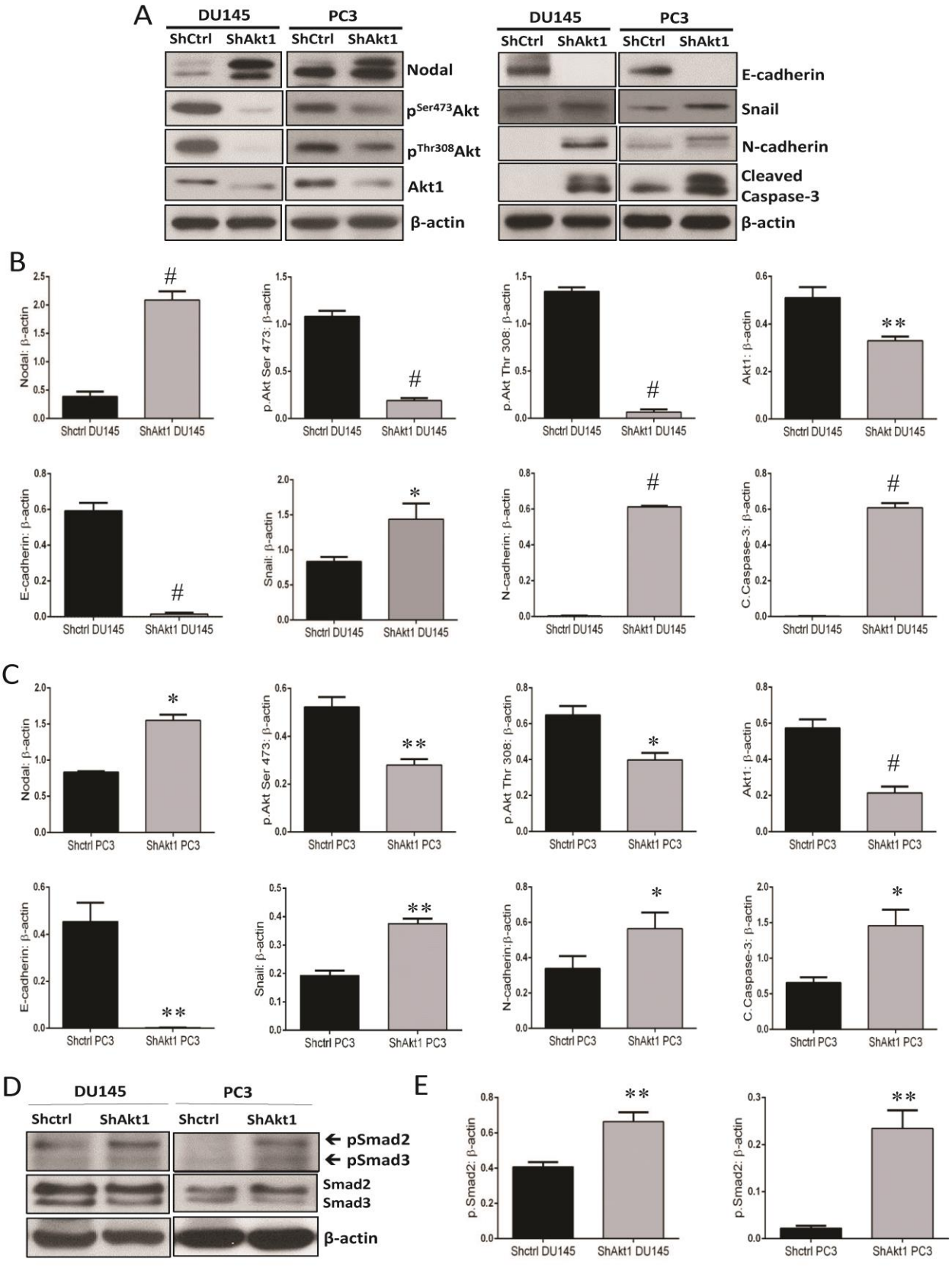
- **Western blot**

Western blotting was performed as published previously [25]. Briefly, the cell lysates were prepared using 1X RIPA lysis buffer (Millipore, Temecula, CA) supplemented with protease and phosphatase inhibitor tablets (Roche Applied Science, Indianapolis, IN). Protein concentration was measured by the DC protein assay (Bio-Rad Laboratories, Hercules, CA) and approximately 30-60 µg of cell lysates in Laemmli buffer were used. Densitometry was performed using NIH ImageJ software.

- **Migration assay**

Migration assay was performed as explained previously [9]. Cells were plated on 6-well plates until 60-70% confluence. Scratches were made in the cell monolayers using 1ml pipette tip followed by a one-time wash with 1X PBS. Cells were incubated with SB505124 (20 µM) or 0.5 % DMSO in DMEM containing 5% FBS for 24 hours. Images of scratches were taken immediately after scratching (0 h) and 24 h after treatment. The rate of migration was measured as a percentage of scratch filling using the equation ([1-T24/T0] X 100), where T24 is the area at the end point (24 hours) and T0 is the area measured immediately after making the scratches.

Figure 1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ShCtrl
ShAkt1
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p^Ser473Akt
p^Thr308Akt
Akt1
β-actin
E-cadherin
Snail
N-cadherin
Cleaved Caspase-3
B
Nodal: β-actin
p.Akt Ser 473: β-actin
p.Akt Thr 308: β-actin
Akt1: β-actin
E-cadherin: β-actin
Snail: β-actin
N-cadherin: β-actin
C.Caspase-3: β-actin
Shctrl DU145
ShAkt1 DU145
C
Shctrl PC3
ShAkt1 PC3
D
Shctrl
ShAkt1
← pSmad2
← pSmad3
Smad2
Smad3
β-actin
E
p.Smad2: β-actin

FIGURE 1: Akt1 silencing promotes Nodal expression and EMT in PC3 and DU145 PCa cells. (A) Representative Western blot images of cell lysates obtained from control (ShControl) and Akt1-silenced (ShAkt1) DU145 and PC3 cells showing changes in the expression of Nodal, EMT markers (N-cadherin and Snail), apoptotic marker cleaved caspase-3, epithelial marker (E-cadherin), phosphorylated Akt and Akt1. **(B-C)** Bar graphs showing band densitometry analysis of Western blot images from cell lysates obtained from control (ShControl) and Akt1-silenced (ShAkt1) DU145 and PC3 cells showing significant increase in the expression of Nodal, EMT markers (N-cadherin and Snail) and apoptotic marker cleaved caspase-3 associated with decreased expression of epithelial marker (E-cadherin), phosphorylated Akt and Akt1 normalized to loading control β-actin, respectively (n=3). **(D)** Representative Western blot images of cell lysates obtained from control (ShControl) and Akt1-silenced (ShAkt1) DU145 and PC3 cells showing changes in the expression of phosphorylated and total expression of Smad2/3. **(E)** Bar graphs showing band densitometry analysis of Western blot images from cell lysates obtained from control (ShControl) and Akt1-silenced (ShAkt1) DU145 and PC3 cells showing a significant increase in the expression of phosphorylated Smad2 normalized to loading control β-actin, respectively, with no changes in the expression of total Smad2 (n=3). Data are presented as mean $\pm$ SEM. *$p<0.05$; **$p<0.01$; #$p<0.001$.

- **Matrigel® invasion assay**

Twenty-four Transwell® permeable plate supports with 8.0 µm polycarbonate membrane and Matrigel® were purchased from Corning Life Sciences (Tewksbury, MA). A concentration of 5mg/ml of Matrigel was used for coating supports. Matrigel® invasion assay was performed as published previously [34]. Briefly, cells were seeded in 6 well plates, washed one time with 1X PBS and treated with either SB505124 (20 µM) or 0.5 % DMSO (diluted with 0.9% saline) for 24 hours. The medium was aspirated, and cells were washed once with 1X PBS, detached using sterile 20 mM EDTA in PBS and washed once with 0.9% saline. Cells were re-suspended in serum-free medium with/without treatment based on the final treatment. Using Countess automated cell counter (Invitrogen), 100,000 cells were seeded on to the Matrigel® in the upper chamber of the transwell plates filled with 100 µl of serum-free medium. Cells that invaded the matrigel and reached the bottom layers of the supports after 24 hours incubation were fixed using 3.7 % paraformaldehyde then stained with 0.5% crystal violet solution. Three bright field images of each insert were taken using an inverted microscope and three blinded reviewers counted the invaded cells. The average number of invaded cells from every three images was calculated and considered for the analysis.

- ***In vivo* nude mouse xenograft model**

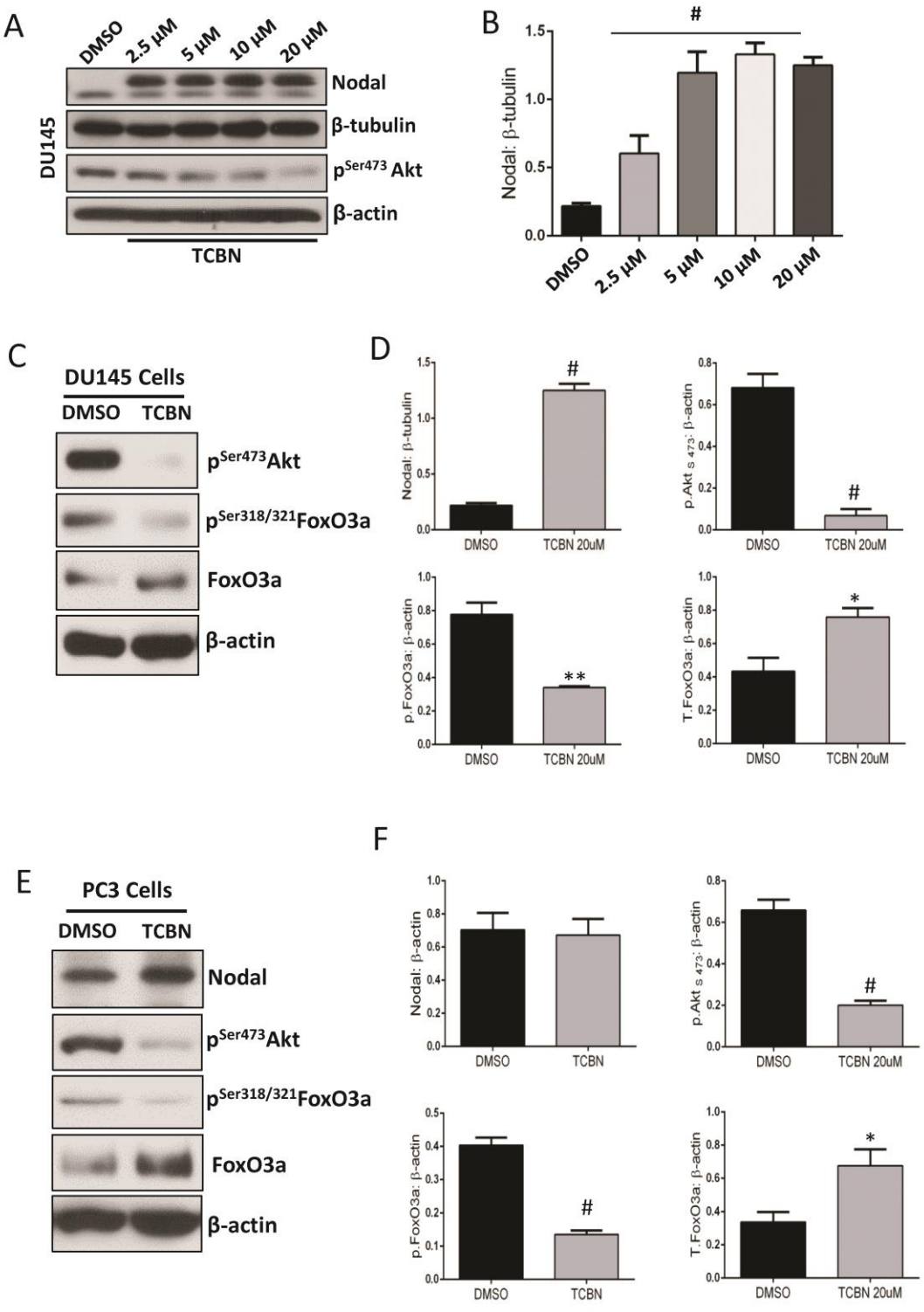
All animal procedures were performed according to the protocol approved by the Institutional Animal Care and Use Committee at the Charlie Norwood Veterans Affairs Medical Center, Augusta, Georgia (protocol 15-08-083). Tumor xenografts were implanted as explained previously [27]. ShAkt1 and ShCtrl DU145 cells were grown to 60-70% confluence in T75 flasks. Cells were washed once with 1X PBS, detached using trypsin and re-suspended in 0.9% saline. A volume of 150-200 µl cell suspension containing $1x10^6$ cells was injected through the

tail vein into 8-week-old nude mice (athymic nude mice; Harlan, Indianapolis, IN). Animals in each group were injected (i.p.) with either 10mg/kg SB505124 dissolved in 75% DMSO (diluted with 0.9% saline) daily for 15 days or with a vehicle only (n=8 in each group). Mice weight was monitored every 3 days up to day 16. On day 16, mice were euthanized and one lobe of the right lungs (post-caval lobe) was collected and snap-frozen for H&E staining while the rest were stained with 15% India ink through intratracheal injection (5 ml). Stained lungs were carefully resected and rinsed in Fekete's solution (300 ml 70% ethanol, 30 ml 37% formaldehyde, 5 ml glacial acetic acid), then placed in fresh Fekete's solution overnight. Lung tissue would remain stained with India ink whereas lung tumor nodules would become unstained and can be visualized by the naked eye. The number of lung nodules was counted by three blinded reviewers and the lung colonization in all groups and the average of their scores were considered for the analysis.

- **The cancer genome atlas (TCGA) study analysis**

The data on the expression of Nodal and corresponding clinical information for 498 prostate cancer patients from the TCGA database [35] were downloaded from cBioPortal (https://www.cbioportal.org/). According to the Gleason Score, patients were stratified into 3 cohorts, Gleason Score of 6-7 (N=292), 8 (N=64) and 9-10 (N=142). Changes in the Nodal mRNA expression was compared to the Gleason score. The same approach was performed on another study on the Prostate Adenocarcinoma [36] with 150 prostate cancer patients. Based on the Gleason Score known for 139 patients from this study, patients were stratified into 3 cohorts, Gleason Score of 6-7 (N=117), Gleason Score of 8 (N=11) and Gleason Score of 9 (N=11), and similar analysis on their mRNA expression of Nodal was conducted. Furthermore, the

Figure 2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5 μM
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20 μM
DU145
Nodal
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p^Ser473 Akt
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Nodal: β-tubulin
DMSO
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10 μM
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TCBN
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p^Ser318/321FoxO3a
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p.FoxO3a: β-actin
T.FoxO3a: β-actin
DMSO
TCBN 20uM
E
PC3 Cells
DMSO
TCBN
Nodal
p^Ser473Akt
p^Ser318/321FoxO3a
FoxO3a
β-actin
F
Nodal: β-actin
p.Akt S 473: β-actin
p.FoxO3a: β-actin
T.FoxO3a: β-actin
DMSO
TCBN
TCBN 20uM

FIGURE 2: Pharmacological inhibition of Akt1 activated FoxO1/3a and promotes Nodal expression in PCa cells. (A-B) Representative Western blot images and respective bar graph of band densitometry analysis on the effect of Akt inhibitor TCBN treatment (72 hours) on Nodal expression in DU145 cells (n=3). **(C-D)** Representative Western blot images and respective bar graph of band densitometry analysis on the effect of Akt inhibitor TCBN treatment (72 hours) on phosphorylation and total expression of FoxO3a in DU145 cells (n=3). **(E-F)** Representative Western blot images and respective bar graph of band densitometry analysis on the effect of Akt inhibitor TCBN treatment (72 hours) on phosphorylation and total expression of FoxO3a as well as Nodal in PC3 cells (n=3). Data are presented as mean $\pm$ SEM. **p<0.05; **p<0.01; #p<0.001*.

association between Nodal mRNA expression and the clinical characteristics of patients in both studies was investigated.

4.3. Statistical analysis

All the data are presented as the mean ± standard error of the mean (SEM) to determine significant differences between treatments and control values. We have used One-way ANOVA for groups of 3 or more and Student's two-tailed t-test for studies including 2 independent groups. Statistical analysis was performed using GraphPad Prism version 6.01 software and results are considered significant when $p\text{-}value < 0.05$.

4.4. Results

- **The nodal expression is increased in Akt1 knockdown androgen-insensitive human PCa cells associated with increased EMT markers and enhanced Smad2/3 pathway**

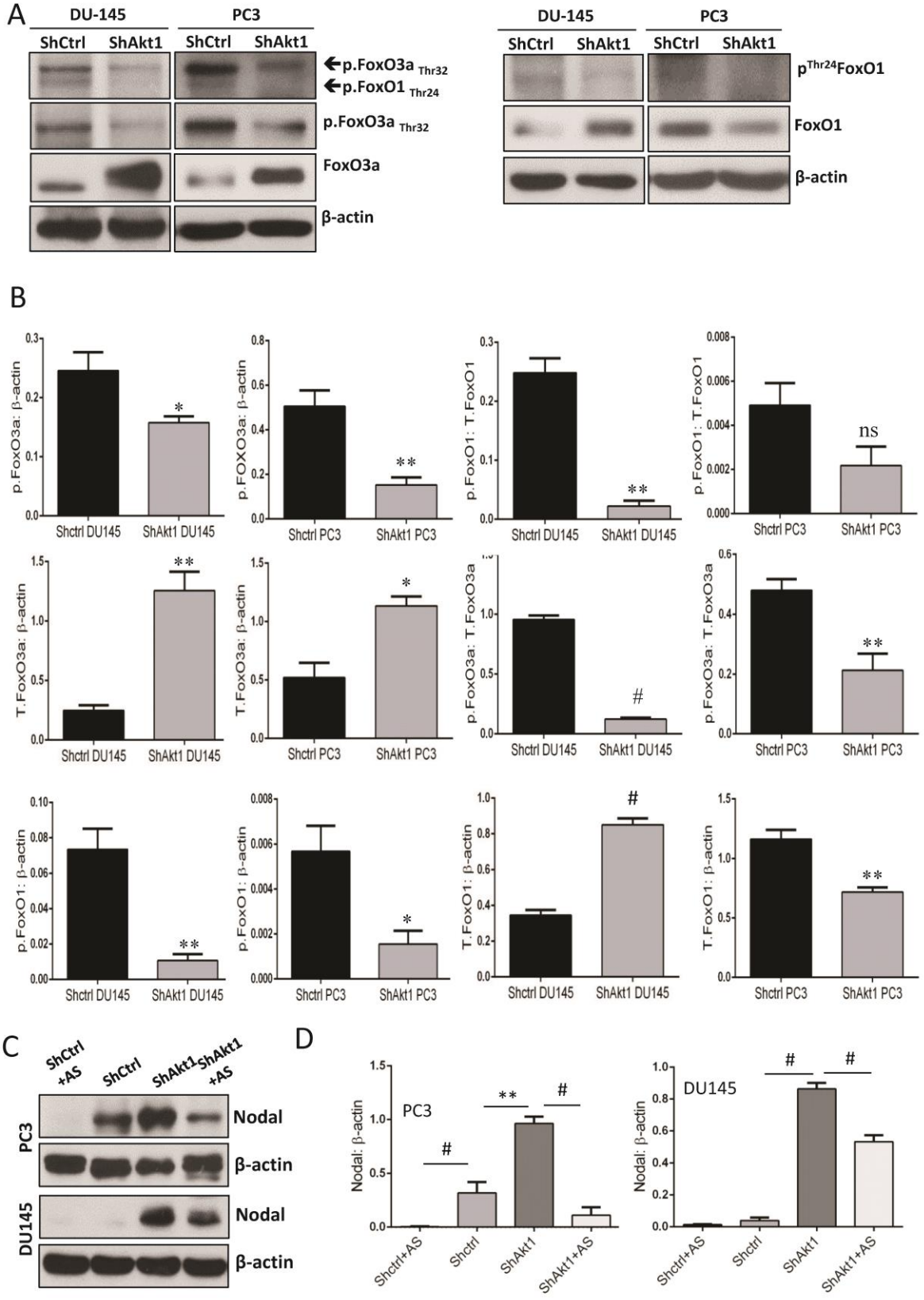
In a recent study, we showed that the pharmacological inhibition of Akt1 using TCBN in the tumor-bearing TRansgenic Adenocarcinoma of the Mouse Prostate (TRAMP) model promoted PCa EMT and metastasis [25, 26]. A gene array analysis of the prostate tumors from these mice revealed an increased expression of several EMT markers, including Nodal when compared to the control prostates [25]. To investigate this using human PCa cell lines, we silenced the expression of Akt1 gene using ShRNAs in PC3 and DU145 cells. Akt1 gene silencing resulted in a significant increase in the Nodal protein expression in both DU145 and PC3 cells (Figure 1A-C). This was accompanied by a complete loss of epithelial marker E-cadherin and increased expression of EMT markers Snail and N-cadherin (Figure 1A-C). Since Akt1 is essential for cell survival, its loss was associated with apoptosis indicated by enhanced cleaved caspase-3 expression (Figure 1A-C). We next determined if increased Nodal expression in ShAkt1 PC3 and DU145 cells results in the activation of the canonical pathway. Our analysis

showed a significant increase in the phosphorylated Smad2 correlating to the Nodal expression levels in ShAkt1 DU145 and PC3 cells with no net changes in the total Smad2/3 expression (Figure 1D-E), thus suggesting that Nodal expression as a result of Akt1 suppression promotes EMT in PCa cells through the canonical Smad2/3 pathway.

- **Increased Nodal expression in ShAkt1 PCa cells is reliant on FoxO3a signaling**

It is well established that Akt1 regulates FoxO1/3a transcriptional activity via direct phosphorylation followed by a nuclear exit and subsequent proteasomal degradation [37]. Therefore, it was in our interest to determine whether FoxOs have a role in enhancing Nodal expression in PCa cells. Treatment with Akt inhibitor TCBN significantly increased Nodal expression in DU145 cells in a dose-dependent manner (Figure 2A-B). The increase in Nodal expression in TCBN-treated DU145 and PC3 cells was associated with decreased phosphorylated FoxO3a and increased total FoxO3a, thereby correlating the activity and nuclear presence of FoxO3a (Figure 2C-F) with the increased Nodal expression. Similarly, Akt1 gene silencing in PC3 and DU145 cells also resulted in a significant decrease in the phosphorylation and increase in the total expression of FoxO3a in DU145 cells (Figure 3A-B). Interestingly, treatment with FoxO inhibitor AS1842856 resulted in a significant decrease in the Nodal expression in ShAkt1 as well as ShCtrl PC3 and in ShAkt1 DU145 cells (Figure 3C-D) indicating an important role of FoxO transcription factors in the regulation of Nodal expression in PCa cells.

Figure 3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DU-145
PC3
ShCtrl
ShAkt1
ShCtrl
ShAkt1
pThr24FoxO1
FoxO1
β-actin
B
p.FoxO3a: β-actin
Shctrl DU145
ShAkt1 DU145
p.FOXO3a: β-actin
Shctrl PC3
ShAkt1 PC3
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C
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PC3
Nodal
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Nodal
β-actin
D
PC3
Nodal: β-actin
Shctrl+AS
Shctrl
ShAkt1
ShAkt1+AS
DU145
Nodal: β-actin
Shctrl+AS
Shctrl
ShAkt1
ShAkt1+AS

FIGURE 3: Increased Nodal expression in Akt1-deficient PCa cells is attenuated by treatment with FoxO inhibitor AS1842765. (A-B) Representative Western blot images and bar graph of the band densitometry analysis of ShControl and ShAkt1 DU145 and PC3 cells probed for phosphorylated and total expression of FoxO1 and FoxO3a showing a significant decrease in FoxO phosphorylation in ShAkt1 PCa cells compared to ShControl, respectively (n=6). **(C-D)** Representative Western blot images and bar graph of the band densitometry analysis of ShControl and ShAkt1 DU145 and PC3 cells probed for Nodal expression showing significant increase in Nodal expression in ShAkt1 PCa cells and a significant decrease in Nodal expression in ShAkt1 PCa cells by treatment with FoxO1/3a inhibitor AS1842765 (10 μM; 72 hours; n=3). Data are presented as mean ± SEM. **p<0.05; **p<0.01; #p<0.001*.

- **The Nodal pathway inhibition in vitro blunted the migration and invasion of ShAkt1 PCa cells**

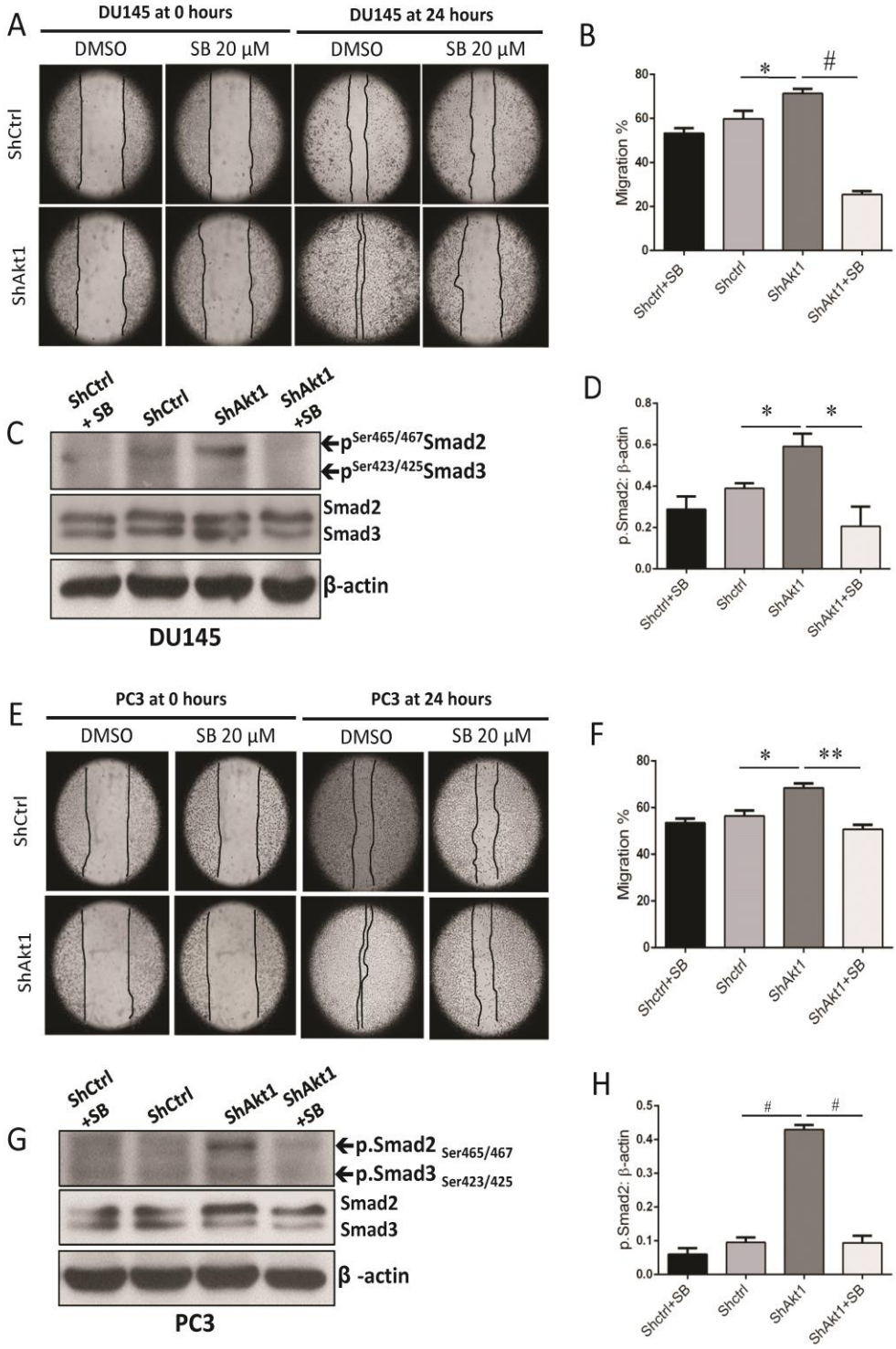
Increased expression of Nodal correlating to the increased expression of EMT markers in ShAkt1 PCa cells suggested a role for the Nodal pathway in the regulation of cell motility and invasion, the features of aggressive, metastatic cancer cells. To test this, we determined the effect of Nodal pathway inhibitor SB505124 on migration and invasion of ShCtrl and ShAkt1 DU145 and PC3 cells. Our analysis indicated a significant increase in the migration rate of ShAkt1 DU145 (Figure 4A-B) and PC3 cells (Figure 4E-F) compared to their respective controls in a cell monolayer scratch assay. This was accompanied by an increase in the phosphorylation levels of Smad2 in ShAkt1 DU145 (Figure 4C-D) and PC3 cells (Figure 4G-H). Increased cell migration of ShAkt1 DU145 and PC3 cells was significantly inhibited by treatment with Nodal pathway inhibitor SB505124 (Figure 4A-B and 4E-F, respectively), which was accompanied by a significant reduction in the expression levels of phosphorylated Smad2 (Figure 4C-D and 4G-H, respectively).

In the next step, we determined whether inhibition of Nodal pathway would affect the rate of invasion of PCa cells. Our analysis indicated a significant increase in the invasion of ShAkt1 DU145 (Figure 5A-B) and PC3 cells (Figure 5C-D) compared to their respective controls in a Matrigel® based Transwell invasion assay. Enhanced invasion of ShAkt1 DU145 and PC3 cells were significantly inhibited by treatment with Nodal pathway inhibitor SB505124 (Figure 5A-B and 5C-D, respectively). Together, these results clearly demonstrated the importance of Nodal signaling in the regulation of PCa cell migration and invasion downstream of Akt1 suppression.

- **The Nodal pathway inhibition in vivo attenuated lung metastasis of ShAkt1 PCa cells**

The efficacy of Nodal pathway inhibitor SB505124 to suppress enhanced migration and invasion of ShAkt1 PCa cells suggested the utility of SB505124 to inhibit PCa metastasis *in vivo*. To test this, we performed lung metastasis assay by administering ShCtrl and ShAkt1 DU145 cells via tail-vein ($1x10^6$ cells suspended in 150-200 µl saline) to athymic nude mice with and without treatment by the Nodal pathway inhibitor SB505124 (10 mg/kg daily and for 15 days) or vehicle (DMSO) via i.p. route. Whereas nodules were not clearly visible in the lungs of ShCtrl DU145 cell administered mice, numerous DU145 cell tumor nodules were visible in the lungs of ShAkt1 DU145 cells administered mice, visualized by India ink staining (Figure 6A). Treatment with SB505124 resulted in a significant reduction in the number of ShAkt1 DU145 tumor nodules in the mouse lungs as compared to the DMSO treated controls (Figure 6A-B). In order to confirm this further, we analyzed the lung sections from a different set of mice for the presence of DU145 cell tumor colonies. Our analysis indicated that the mice administered with ShAkt1 DU145 cells had >5-fold higher number of tumor colonies compared to the mice administered with ShCtrl DU145 cells, and it was significantly inhibited by treatment with SB505124 (Figure 6C-D). This treatment, however, had no effect on the animal weight (Supplemental Figure 1A). Finally, we determined if SB505124 has any direct effect on PCa tumor growth. To do this, we implanted ShAkt1 DU145 cell tumor xenografts in athymic nude mice and treated them with DMSO (vehicle) or SB505124. Our results indicated that the Nodal pathway inhibitor has no significant effect on the growth of ShAkt1 DU145 cell tumor xenografts *in vivo* (Figure 6E-F and Supplemental Figure 1B).

Figure 4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DU145 at 24 hours
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SB 20 μM
DMSO
SB 20 μM
ShCtrl
ShAkt1
B
Migration %
Shctrl+SB
Shctrl
ShAkt1
ShAkt1+SB
C
ShCtrl + SB
ShCtrl
ShAkt1
ShAkt1 +SB
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pSer423/425Smad3
Smad2
Smad3
β-actin
DU145
D
p.Smad2: β-actin
E
PC3 at 0 hours
PC3 at 24 hours
F
G
p.Smad2 Ser465/467
p.Smad3 Ser423/425
H
PC3

FIGURE 4: Nodal pathway inhibition blunts Smad2/3 phosphorylation and abrogates enhanced migration of Akt1-deficient DU145 and PC3 cells. (A) Representative images from the scratch assay (0 and 24 hours) in ShControl and ShAkt1 DU145 cell monolayers showing the migration of PCa cells in the presence and absence of Nodal pathway inhibitor SB505124 after 24 hours. **(B)** Bar graph showing the significant inhibitory effect of Nodal pathway inhibitor SB505124 on ShAkt1 DU145 cell migration compared to ShControl cells (n=3). **(C)** Representative Western blot images of cell lysates obtained from ShControl and ShAkt1 DU145 cells showing changes in the expression of phosphorylated and total expression of Smad2 in the presence and absence of Nodal pathway inhibitor SB505124. **(D)** Bar graphs showing the band densitometry analysis of Western blot images from cell lysates obtained from ShControl and ShAkt1 DU145 cells showing a significant decrease in the expression of phosphorylated Smad2 in ShAkt1 cell lysates by treatment with Nodal pathway inhibitor SB505124 (n=3). **(E)** Representative images from the scratch assay (0 and 24 hours) in ShControl and ShAkt1 PC3 cell monolayers showing the migration of PCa cells in the presence and absence of Nodal pathway inhibitor SB505124 after 24 hours. **(F)** Bar graph showing the significant inhibitory effect of Nodal pathway inhibitor SB505124 on ShAkt1 PC3 cell migration compared to ShControl cells (n=3). **(G)** Representative Western blot images of cell lysates obtained from ShControl and ShAkt1 PC3 cells showing changes in the expression of phosphorylated and total expression of Smad2 in the presence and absence of Nodal pathway inhibitor SB505124. **(H)** Bar graphs showing the band densitometry analysis of Western blot images from cell lysates obtained from ShControl and ShAkt1 PC3 cells showing a significant decrease in the expression of phosphorylated Smad2 in ShAkt1 cell lysates by treatment with Nodal pathway inhibitor SB505124 (n=3). Data are presented as mean $\pm$ SEM. *$p<0.05$; **$p<0.01$; #$p<0.001$.

- **Changes in Nodal expression positively correlates with the Gleason score of human PCa**

Since Nodal expression was significantly increased in PCa cells and its pharmacological suppression attenuated PCa cell migration and invasion *in vitro*, and metastasis *in vivo*, we determined if there is a correlation between increased Nodal expression and the stage of PCa. To do this, we collected the information available from cBioportal on changes in the Nodal expression in PCa patients enrolled in the TCGA studies, based on their Gleason score. The prostate adenocarcinoma patient characteristics of TCGA provisional study (n=498) and the MSKCC genomic analysis of prostatic adenocarcinoma study (n=150) are shown in Supplemental Tables 1 and 2, respectively. When the patients were stratified based on the age, Nodal mean mRNA expression was significantly higher in the group with age >60 years compared to age ≤ 60 (3.99 vs. 2.48, respectively; $p = 0.0376$). Interestingly, when patient data were stratified based on the Gleason Score, Nodal expression was higher in patients with a higher Gleason score (2.6 in Gleason score 6-7 vs. 4.659 in Gleason score 9-10; $p = 0.042$, respectively) (Figure 7A; Supplemental Table 1). Similarly, Nodal expression was significantly upregulated in the higher Gleason Score (9) compared to lower Gleason Score (6-7) group (6.521 vs. 6.15; $p = 0.0025$, respectively) in TCGA MSKCC study (Figure 7B and Supplemental Table 2). These results indicate that the Nodal expression in PCa is directly proportional to the stage of the disease progression.

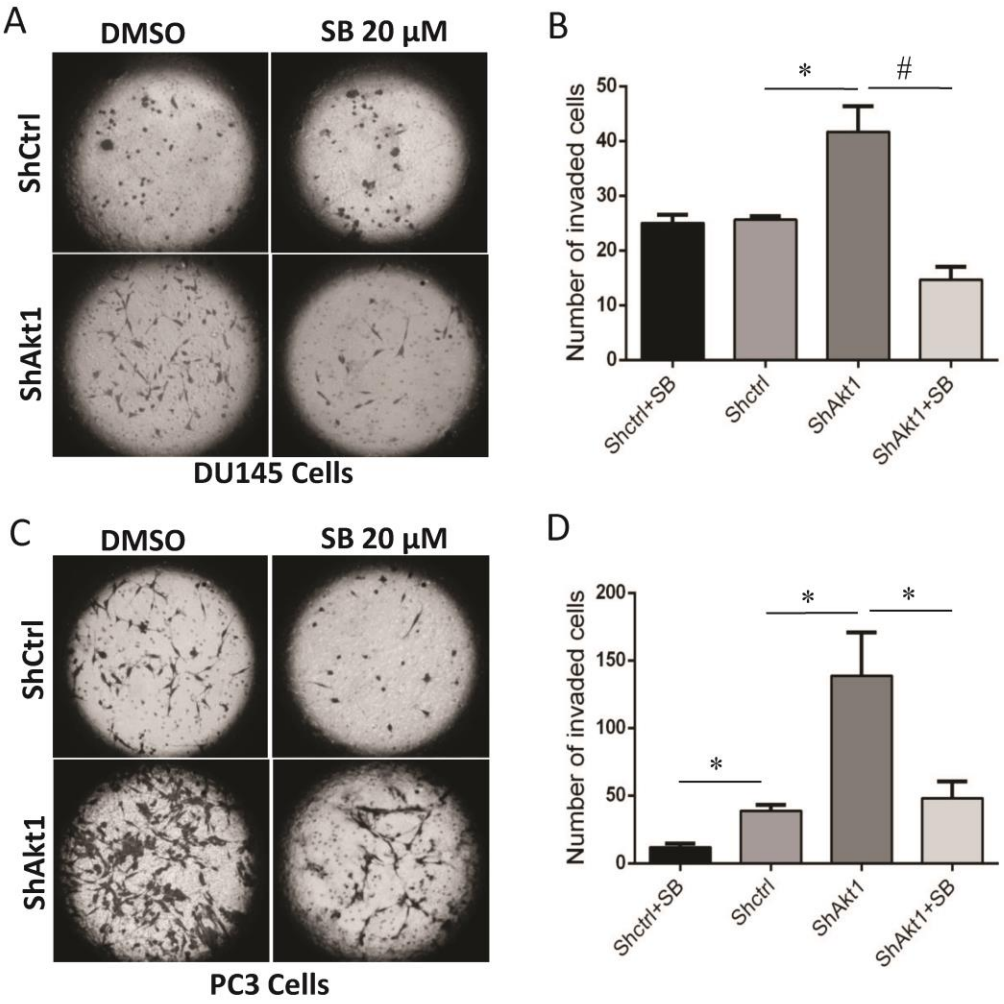
4.5. Discussion

The oncogene Akt, although promotes tumor survival, proliferation, and growth, its suppression has been implicated in promoting tumor cell EMT, motility, and invasion *in vitro* [17, 19, 24, 38, 39] and metastasis *in vivo* in several cancer types such as the breast, prostate,

head and neck, and non-small cell lung cancers [19, 23-25]. Recent studies in our laboratory have identified that Akt suppression in advanced PCa promotes EMT and metastasis [25, 26]. Interestingly, endothelial-specific loss of Akt1 alone promoted PCa metastasis to the mouse lungs [27], indicating that the endothelial cells, by opening its endothelial-barrier [40], also might contribute to the increased PCa metastasis upon pharmacological Akt inhibition. Although promotion of EMT with Akt1 gene deletion has been implicated as a common feature in all these studies, reports on the mechanisms by which Akt1 suppression promotes EMT in different cancer types have been disparate suggesting that our understanding on how Akt1 suppression promotes cancer metastasis is far from complete. From our gene array studies in the TRAMP mouse prostates, we found a significantly higher expression of Nodal with Akt suppression as compared to Akt intact prostates [25]. Based on this, we hypothesized that activation of Nodal pathway as a result of Akt1 inhibition promotes PCa cell EMT and the pharmacological inhibition of this pathway attenuates Akt1 deficient PCa cell metastasis *in vivo*.

In the current study, we report for the first time that Akt1 is directly involved in the regulation of Nodal expression. Genetic deletion of Akt1 or pharmacological suppression of total Akt that promoted PCa cell EMT and metastasis also resulted in increased Nodal expression. This was accompanied by reduced FoxO1/3a phosphorylation, enhanced FoxO3a expression, and their transcriptional activation. The ability of FoxO inhibitors to blunt the effect of Akt1 suppression on Nodal expression indicated that Nodal is under the transcriptional control of FoxO1/3a. The effect of Nodal pathway inhibitor to mitigate the enhanced cell migration and invasion observed in Akt1 silenced PC3 and DU145 cells through the suppression of canonical Smad2/3 pathway indicated the involvement of Nodal in promoting EMT in these cells. The efficacy of Nodal pathway inhibitor to suppress Akt1-deficient DU145 cell lung metastasis in

Figure 5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DMSO
SB 20 µM
ShCtrl
ShAkt1
DU145 Cells
B
Number of invaded cells
Shctrl+SB
Shctrl
ShAkt1
ShAkt1+SB
C
DMSO
SB 20 µM
ShCtrl
ShAkt1
PC3 Cells
D
Number of invaded cells
Shctrl+SB
Shctrl
ShAkt1
ShAkt1+SB

*

FIGURE 5: Nodal pathway inhibition abrogates enhanced invasion of Akt1-deficient DU145 and PC3 cells. (A) Representative images from the Matrigel Boyden chamber invasion (0 and 24 hours) by ShControl and ShAkt1 DU145 cells showing the invasion of PCa cells in the presence and absence of Nodal pathway inhibitor SB505124 after 24 hours. **(B)** Bar graph showing the significant inhibitory effect of Nodal pathway inhibitor SB505124 on ShAkt1 DU145 cell invasion compared to ShControl cells (n=3). **(C)** Representative images from the Matrigel Boyden chamber invasion (0 and 24 hours) by ShControl and ShAkt1 PC3 cells showing the invasion of PCa cells in the presence and absence of Nodal pathway inhibitor SB505124 after 24 hours. **(D)** Bar graph showing the significant inhibitory effect of Nodal pathway inhibitor SB505124 on ShAkt1 PC3 cell invasion compared to ShControl cells (n=3). Data are presented as mean $\pm$ SEM. *$p<0.05$; **$p<0.01$; #$p<0.001$.

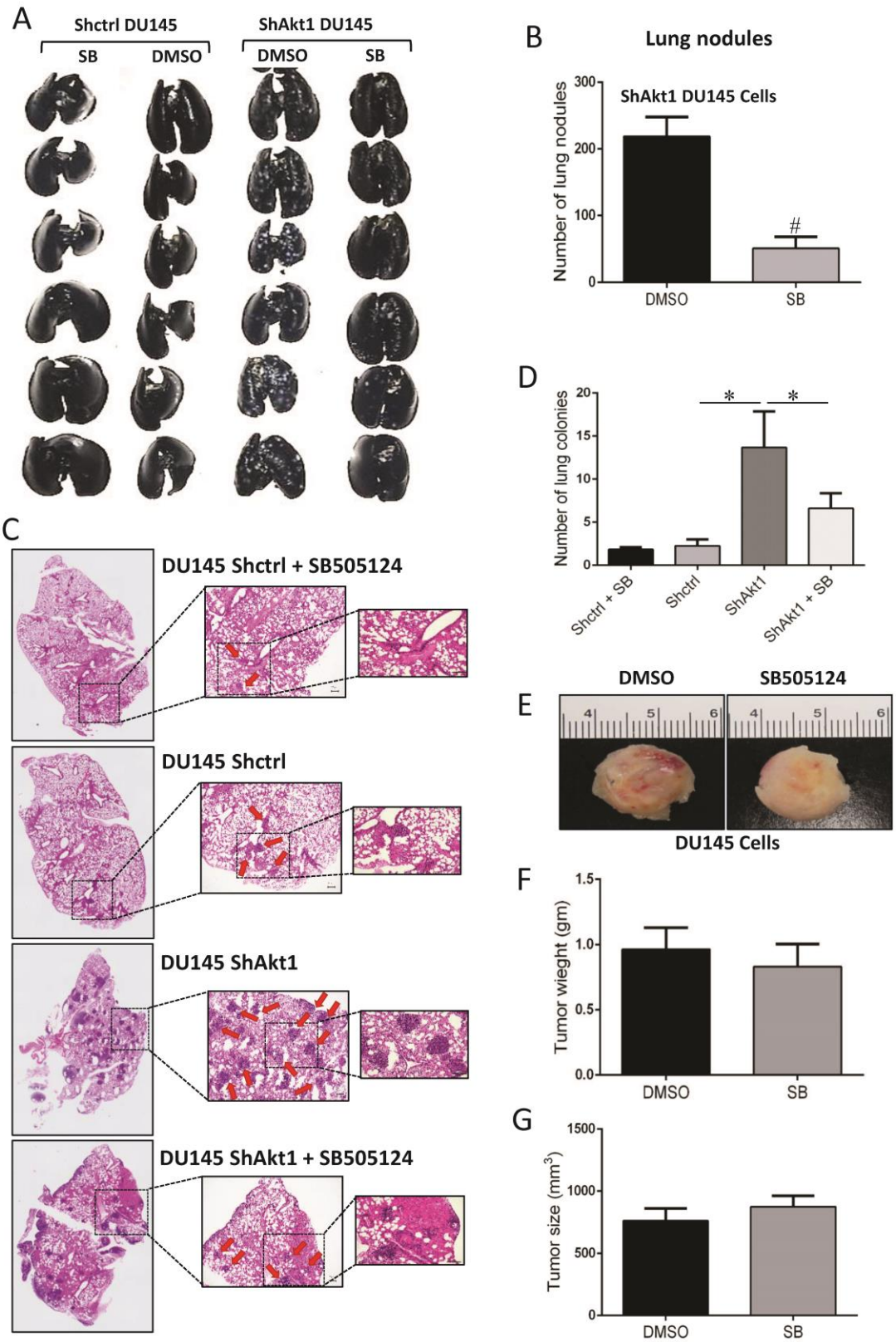
mice with no significant effect on the growth of DU145 cell tumor xenografts demonstrated the specific role of Nodal in promoting PCa metastasis in the advanced stages. The direct relationship between the increased Nodal mRNA expression in PCa patient samples correlating to their Gleason score and age as analyzed from the TCGA database further strengthened our conclusions that Nodal is a specific mediator of PCa metastasis.

The ability of PCa cells to migrate and invade as a result of EMT also determines its metastatic potential [25, 34]. Therefore, it is necessary to confirm a direct link between the observed changes in the Nodal expression in the Akt1-deficient PCa cells and their increased migration and invasion. Whereas the Nodal promoted cell invasion via increased MMP2 expression in glioma [41], it promoted PCa EMT via increased N-cadherin, Vimentin and Snail, and it enhanced MMP2 and CXCR4 expression in pancreatic cancer cells [42]. Nodal also activated cell migration and invasion via increased MMP2 and MMP9 expression in bladder cancer cells [43]. Nodal has also been demonstrated to effect through the Smad2/3-independent mechanisms such as the promotion of angiogenesis via ERK1/2 and HIF1α dependent VEGF signaling in glioma [44] and stimulation of aggressive melanoma by inducing EMT through increased MMP2 and MMP9 expression and Snail stabilization-mediated by GSK-3β pathway [45]. Nodal promotes EMT-like phenotype through p38 MAPK activation in choriocarcinoma and breast cancer cells [46] and confers stemness and malignancy via activating Wnt/β-catenin-Oct4 axis in PCa and lung cancer [47]. The results from our study in PCa demonstrate that Akt1 inhibition in Pca cells enhances Nodal expression through activation of downstream FoxO1/3a transcriptional factors and promotes Pca cell migration, invasion *in vitro* and metastasis *in vivo*. Therefore, suppression of Nodal pathway by SB505124 inhibits the canonical Smad2/3 signaling and thus attenuates the enhanced motility and invasion of Akt1 silenced PCa cells.

Despite the wealth of literature with mechanisms demonstrating Nodal functions, we still do not have a clear understanding of Nodal expression regulation in cancer cells. A recent study linked an elevated expression of Nodal to the promotion of cell motility in breast cancer and invasive ductal carcinoma via MAP kinase-interacting serine/threonine kinase-1 activation [48]. In a distinct scenario, our data are in agreement with the above reports and indicates a reciprocal relationship between Akt1 activity and Nodal expression, where gene silencing or pharmacological inhibition of the former in the highly metastatic PCa cell lines resulted in increased expressionof the latter. In this study, we also provide the novel evidence on the role of Akt1-FoxO signaling in enhancing PCa cell-derived Nodal expression, in turn, promoting EMT and metastasis. In support of this, Fu and Peng reported an interesting observation in ovarian cancer cells, where Nodal pathway arbitrated a negative feedback loop on Akt1 activity thus reducing FoxO3a phosphorylation and enhancing its mRNA as well as protein expression through the canonical Smad pathway [49]. Although there is a potential role for FoxO1 in regulating Nodal expression, the predominance of FoxO3a accompanied with very low levels of FoxO1 in the PCa cells indicated that the majority of the observed effects are regulated by FoxO3a.

Although Nodal has been shown to induce apoptosis in ovarian cancer cells [50], it activated the canonical Smad2/3 pathway to induce vascular mimicry and promoted the expression of EMT markers such as the Snail and Slug in breast cancer cells [51]. Even in PCa, we observed increased cleaved caspase-3 levels in Akt1 silenced PC3 and DU145 cells correlating to the Nodal expression indicating that some level of apoptosis is associated with Nodal expression. A significant increase in the apoptosis of tumor cells was also observed in

Figure 6


A
Shctrl DU145
ShAkt1 DU145
SB
DMSO
DMSO
SB
B
Lung nodules
ShAkt1 DU145 Cells
Number of lung nodules
300
200
100
0
#
DMSO
SB
C
DU145 Shctrl + SB505124
DU145 Shctrl
DU145 ShAkt1
DU145 ShAkt1 + SB505124
D
Number of lung colonies
20
15
10
5
0
*
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Shctrl + SB
Shctrl
ShAkt1
ShAkt1 + SB
E
DMSO
SB505124
DU145 Cells
F
Tumor wieght (gm)
1.5
1.0
0.5
0.0
DMSO
SB
G
Tumor size (mm3)
1500
1000
500
0
DMSO
SB

*

FIGURE 6: Nodal pathway inhibition blunts enhanced mouse lung metastasis of Akt1-deficient DU145 cells. (A-B) Representative images of athymic nude mouse lungs infused with India ink through trachea and bar graph showing a significant increase in the number of PCa tumor nodules upon administration of ShAkt1 DU145 cells compared to ShControl, which was significantly inhibited by treatment with Nodal pathway inhibitor SB505124 (10 mg/kg/day; Day 15; n=6). **(C-D)**. Representative H&E stained lung section images showing a significant increase in lung colonization of ShAkt1 DU145 compared to ShControl DU145 cells, which was significantly attenuated by a 15-day treatment with SB505124 (n=4). **(E-F)** Representative images of ShAkt1 DU145 tumor xenografts implanted (s.c.) in athymic nude mice and a bar graph showing no significant inhibition of tumor growth by Nodal pathway inhibition (Day 15; n=6). Data are presented as mean $\pm$ SEM. **p<0.05; **p<0.01; #p<0.001*.

Akt1 deficient TRAMP mice [25]. Despite this, Nodal expression was directly linked to the promotion of PCa cell EMT, migration, and invasion.

Nodal has been reported for its potential role in several cancers, including PCa [47, 52-54]. Interestingly, Nodal mRNA expression was directly proportional to the age, Gleason score and the metastasis stage of PCa in our analysis of the TCGA study data obtained from cBioportal. Our findings from rodent studies along with the patient data on Nodal expression strongly suggest the potential therapeutic use of Nodal inhibitors to inhibit mPCa. Our results corroborate a previous report linking increased Nodal staining intensity to high-grade prostate tumor biopsies compared to low-grade samples [53]. In summary, whereas the clinical data signify a positive correlation between Nodal expression and the stage of PCa, our overall findings clearly demonstrate the important role of Nodal pathway activation as a consequence of Akt1 suppression leading to the activation of FoxO1/3a in promoting EMT and metastasis in the advanced PCa. Furthermore, our study present FoxO along with Nodal as two therapeutic targets in the treatment of mPCa.

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Authors' contributions: Conception and design: AA and PRS; Data production, analysis, and interpretation: AA, AV, SA, MA, and PRS; Writing the manuscript: AA and PRS. All authors reviewed the manuscript.

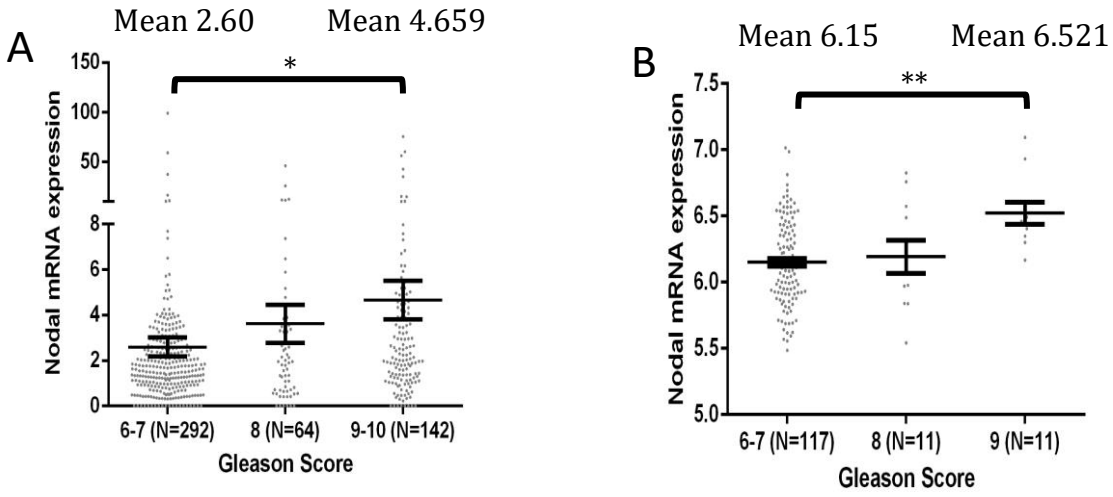
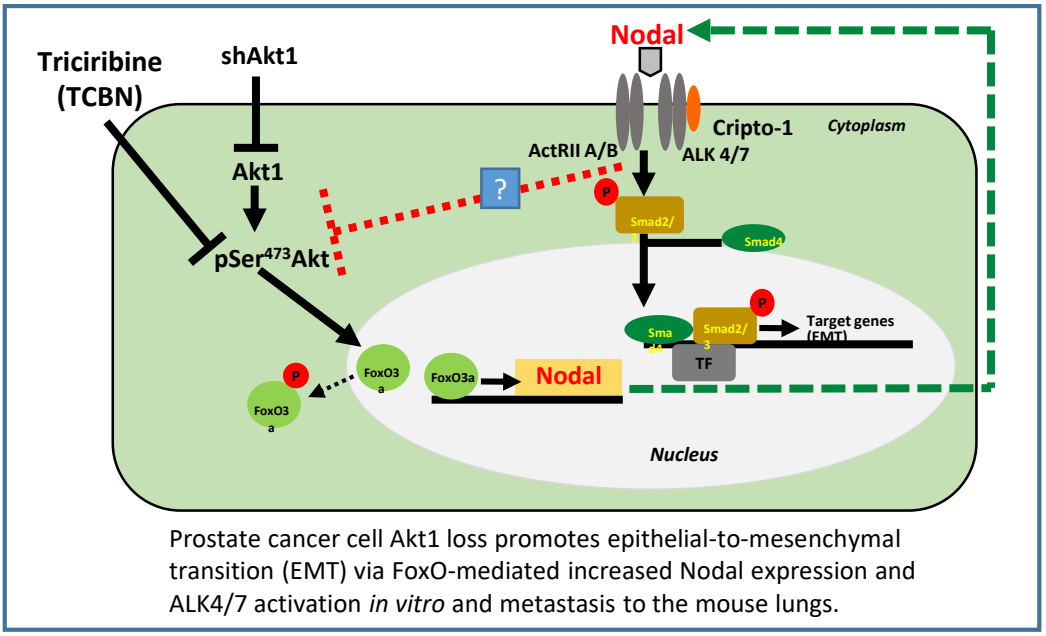


FIGURE 7: Genomic data on Nodal expression in human PCa tissues positively correlates with the Gleason score and cancer metastasis.

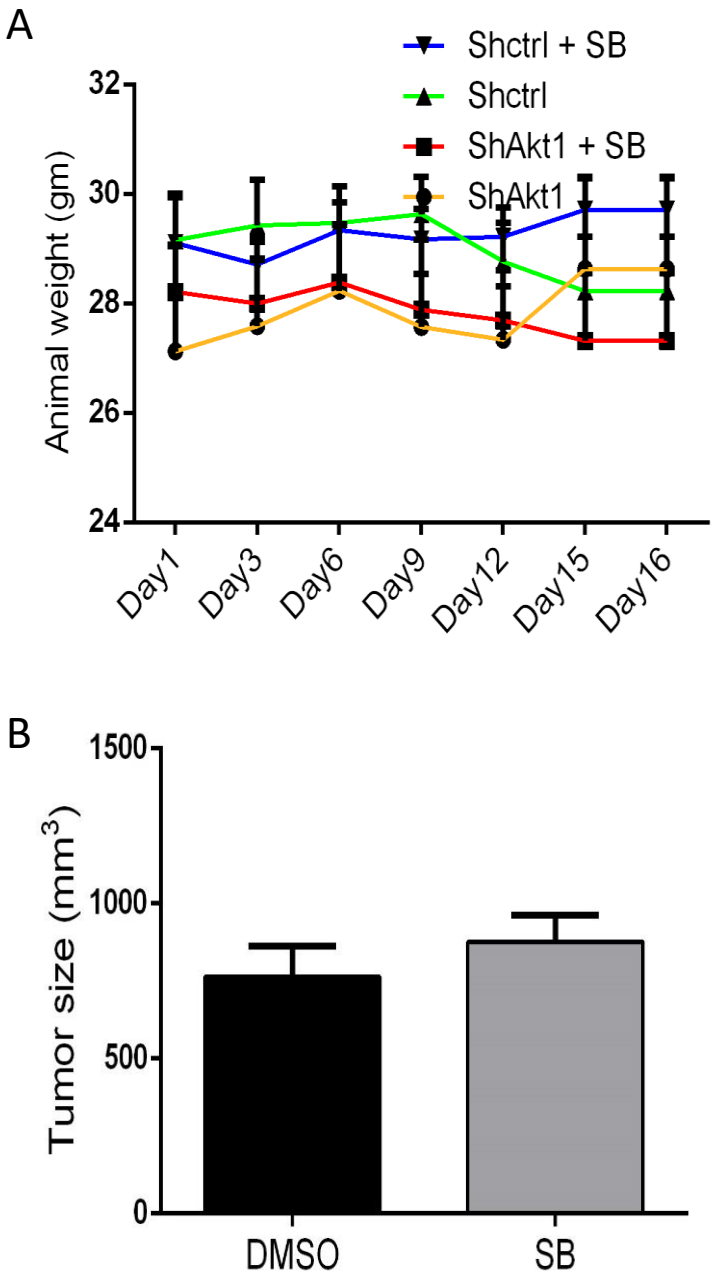
(A). Comparison between prostate cancer tissues with different Gleason scores (known for 498 out of the 501 patients) shows that Nodal expression is significantly upregulated with higher (9-10) compared to lower Gleason score (6-7). **(B)** Comparison between prostate cancer tissues with different Gleason scores (known for only 135 out of the 150 patients) shows that Nodal expression is significantly upregulated with higher (9) compared to lower Gleason score (6-7). Data are presented as mean $\pm$ SEM. *$p<0.05$; **$p<0.01$; #$p<0.001$.

*

Working Hypothesis



Prostate cancer cell Akt1 loss promotes epithelial-to-mesenchymal transition (EMT) via FoxO-mediated increased Nodal expression and ALK4/7 activation *in vitro* and metastasis to the mouse lungs.



Supplemental Figure 1: (A) Graph showing no significant effect of treatment with Nodal pathway inhibitor on the weight of DU145 cell administered athymic nude mice (n=6). **(B)** Bar Graph showing no significant effect of treatment with Nodal pathway inhibitor on the size/volume of the DU145 tumor xenografts in athymic nude mice (n=6).

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A

Characteristics	No. of patients (N=498)	%
Age [Range]		
≤ 60 [41-60 yrs]	223	44.78
> 60 [61-78 yrs]	275	55.22
Gleason score		
6	45	9.04
7	247	49.59
8	64	12.85
9	138	27.71
10	4	0.80
T stage [pT]		
T1	177	35.54
T2	174	34.94
T3	53	10.64
T4	2	0.40
Unknown	1	0.20
NA	91	18.27
N stage [pN]		
N0	345	69.28
N1	80	16.06
NA	73	14.66
M stage [pM]		
M0	456	91.57
M1	3	0.60
NA	39	7.83
Survival status		
Alive	488	97.99
Dead	10	2.01

B

Characteristics (Number of patients)	Mean Nodal mRNA expression	p value
Age [Range]		
≤ 60 [41-60 yrs]	2.48	0.0376
> 60 [61-78 yrs]	3.99	
Gleason score		
6-7 (292)	2.60	0.042 (GS
8 (64)	3.62	6-7 vs. GS
9-10 (142)	4.66	9-10)
T stage [pT]		
T1-2 (351)	3.19	0.5167
T3-4 (55)	2.56	
N stage [pN]		
N0 (345)	3.06	0.3711
N1 (80)	3.89	
M stage [pM]		
M0 (456)	3.326	0.2296
M1 (3)	9.072	
Survival status		
Alive (488)	3.325	0.8937
Dead (10)	2.980	
Overall survival Median		
≤ 30.49 mths (249)	3.398	0.8253
> 30.49 mths (249)	3.238	

Supplemental Table 1: (A) Table showing the clinical characteristics of 498 patients of the TCGA study [Prostate adenocarcinoma (TCGA, Provisional)] extracted from cBioportal. **(B)** Table showing the associations between the mRNA expression levels of Nodal and clinical characteristics indicating that Nodal expression is significantly upregulated with higher age (>60 years) and Gleason score (9-10) compared to lower age (≤ 60 years) and Gleason score (6-7), respectively.

A

Characteristics	No. of patients (N=150)	%
Age [years]	Unknown	
Gleason score		
6	41	27.33
7	76	50.67
8	11	7.33
9	11	7.33
10	0	0
NA	11	7.33
T stage [pT]		
T1	80	53.33
T2	58	38.67
T3	6	4
T4	1	0.67
NA	5	3.33
N stage [pN]	Unknown	
M stage [pM]	Unknown	
Survival status	Unknown	

B

Characteristics (Number of patients)	Mean Nodal mRNA expression	P value
Gleason score		
6-7 (117)	6.15	0.0062 (GS 6-7 vs. GS 9)
8 (11)	6.19	
9-10 (11)	6.52	
T stage [pT]		
T1-2 (138)	6.184	0.5592
T3-4 (7)	6.104	
Disease free Survival Median		
≤ 45.37 mths (70)	6.22	0.2557
> 45.37 mths (70)	6.15	
Overall survival	Unknown	

Supplemental Table 2: (A) Table showing the available clinical characteristics of 150 patients of the TCGA study [Prostate adenocarcinoma (MSKCC, cancer cell 2010)] extracted from cBioportal. **(B)** Table showing the associations between the mRNA expression levels of Nodal and clinical characteristics indicating that Nodal expression is significantly upregulated with higher (9) compared to lower Gleason score (6-7).

CHAPTER 5

Modulation in the microRNA repertoire is responsible for the stage-specific effects of Akt suppression on murine neuroendocrine prostate cancer

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Abstract

Recent studies indicate a stage-specific, differential role for the oncogene Akt on various cancers. In prostate cancer (PCa), suppression of Akt activity in the advanced stages promoted transforming growth factor-β (TGFβ) pathway-mediated epithelial-to-mesenchymal transition (EMT) and metastasis to the lungs. In the current study, we performed Affymetrix analysis to compare the expression profile of microRNAs in the mouse prostate tissues collected at the prostatic inter-epithelial neoplasia (PIN) stage from Transgenic adenocarcinoma of the mouse *(TRAMP)/Akt1*$^{+/+}$ versus *TRAMP/Akt1*$^{-/-}$ mice, and at the advanced stage from *TRAMP/Akt1*$^{+/+}$ mice treated with triciribine (Akt inhibitor) versus DMSO-treated control. Our analysis demonstrates that in the early stage, Akt1 in the *TRAMP* prostate tumors express a set of miRNAs responsible for regulating cancer cell survival, proliferation, and tumor growth, whereas, in the advanced stages, a different set of miRNAs that promote EMT and cancer metastasis is expressed. Our study has identified novel Akt-regulated signature microRNAs in the early and advanced PCa and demonstrates their differential effects on PCa growth and metastasis.

Keywords: Biochemistry; Bioinformatics; Cancer research

5.1.Introduction

Metastatic prostate cancer (PCa) is the leading cause of cancer-related deaths in men in the US and the Europe [1]. Although slow-growing cancer, PCa that has metastasized to the bone, lungs, and brain are difficult to treat [2]. Uncertainties in the molecular mechanisms leading to the switch from early to advanced PCa is the underlying reason for the unreliable screening measures and ineffective treatments that are currently used in the management of PCa [3]. Recent studies from our laboratory have indicated that transforming growth factor-β (TGFβ)-induced epithelial-to-mesenchymal transition (EMT) plays an important role in this process [4]. TGFβ, that plays a tumor suppressor role in the early stages switches to a metastasis promoter in the advanced stages [4-6]. However, the mechanisms that regulate this switch are not clearly understood.

Recently we showed that Akt1, the predominant Akt isoform in the PCa cells [7] and vascular cells [8-10] plays a dual, reciprocal role in tumor growth and metastasis [11]. Similar results have also been reported in four other types of cancer such as the breast [12 13], liver [14], non-small cell lung [15] and head and neck [16]. Furthermore, a very recent study from our lab has indicated that the specific loss of Akt1 in endothelial cells promotes prostate cancer metastasis [17]. These studies have identified Akt1 to promote tumor growth but suppress cancer metastasis. The above studies also have identified a reciprocal link between Akt1 and TGFβ pathways in promoting cancer cell EMT and metastasis. Until today, the molecular mechanisms connecting these two pathways in the regulation of EMT and metastasis have not been identified.

Micro-RNAs are novel players in the modulation of cellular signaling in various physiological and pathological processes [18]. There are several microRNAs that have been

identified to regulate the tumor progression, EMT, and metastasis in PCa [19]. Interestingly, one of the studies linking Akt1 suppression to EMT in breast cancer demonstrated the involvement of microRNAs, mir200 cluster in particular in the process [12]. However, such a link between Akt1 activity, microRNAs expression regulation, tumor growth, EMT, and metastasis has not been shown in other cancer types.

In the current study, we performed microRNA array on an Affymetrix platform to identify the signature microRNAs followed by bioinformatics analysis to identify the potential microRNA regulated pathways in the early prostatic inter-epithelial neoplasia (PIN) [20] stage and the advanced stage (31 week old mice) TRansgenic Adenocarcinoma of the Mouse Prostate *(TRAMP)* PCa tissues in the presence and absence of Akt1 gene in the early stage (12-week old mice; PIN stage) and between DMSO and triciribine (Akt inhibitor) treatment in the advanced stage. Our results indicate different signatures of the microRNA by Akt1 in the PIN and advanced PCa, with a clear role of Akt1-regulated microRNAs in the regulation of cell survival and proliferation in the early stages and EMT and metastasis in the advanced stages.

5.2. Materials and Methods

- ***Generation and genotyping of TRAMP/Akt1$^{+/-}$ and TRAMP/Akt1$^{-/-}$ Mice***

Akt1$^{-/-}$mice (C57BL/6 background) were generated and maintained as reported previously [8]. In order to generate *TRAMP/Akt1*$^{-/-}$ transgenic mice, C57BL/6 *Akt1*$^{+/-}$ male was crossed with *TRAMP* (C57BL/6 background) female mice (Jackson, Bar Harbor, ME). All experiments were carried out in accordance with guidelines set by Augusta VA Medical Center. DNA was extracted from the tails of 10- to 21-day old litters (Qiagen, Valencia, CA). *TRAMP* transgene (600bp) was detected by PCR (forward: 5'-GCGCTGCTGACTTTCTAAACATAAG-3' and reverse: 5'-GAGCTCACGTTAAGTTTTGATGTGT-3') with an annealing temperature of 55℃.

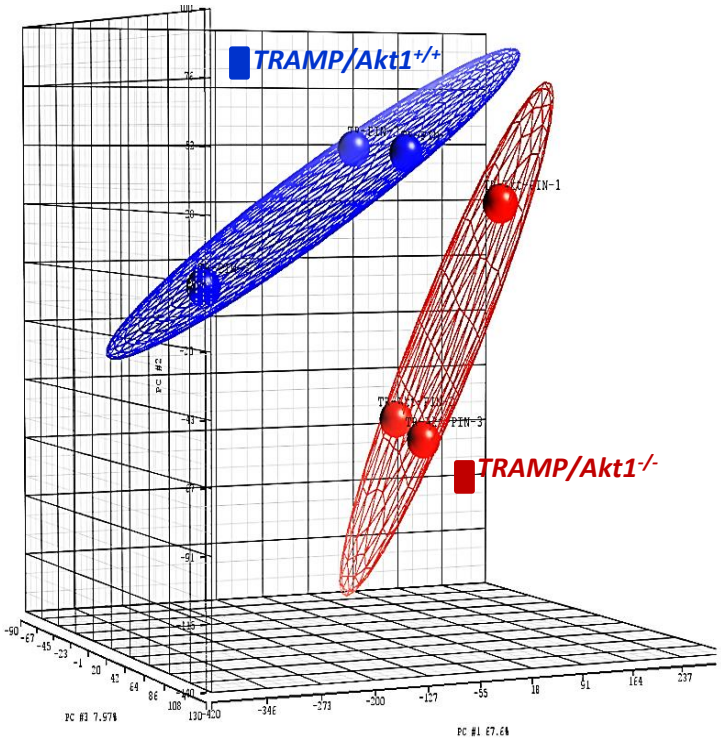
The internal positive control (forward: 5'-CTAGGCCACAGAATTGAAAGATCT-3' and reverse: 5'-GTAGGTGGAAATTCAGCATCATCC-3') produced a 324bp fragment. Primers to confirm Akt1 gene knockout (forward: 5'-TCCAGGACCAGGGGAGGATGTTTCTACTG-3' and reverse: 5'-ACGACATGGTGCAGCAATGGCCAGCG-3') yielded a 600bp band. Primers for *Neo* gene (forward: 5'-TGAGACGTGCTACTTCCATTTGTCACGTCC-3' and reverse: 5'-ACAGGCCGCTACTATGCCATGAAGATCCTC-3') generated a 1200bp fragment [11]. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals. All tests were performed with the approval of the Charlie Norwood VAMC Institutional Animal Care and Use Committee (approval #15-08-083).

- ***TRAMP prostate miRNA isolation and microarray profiling***

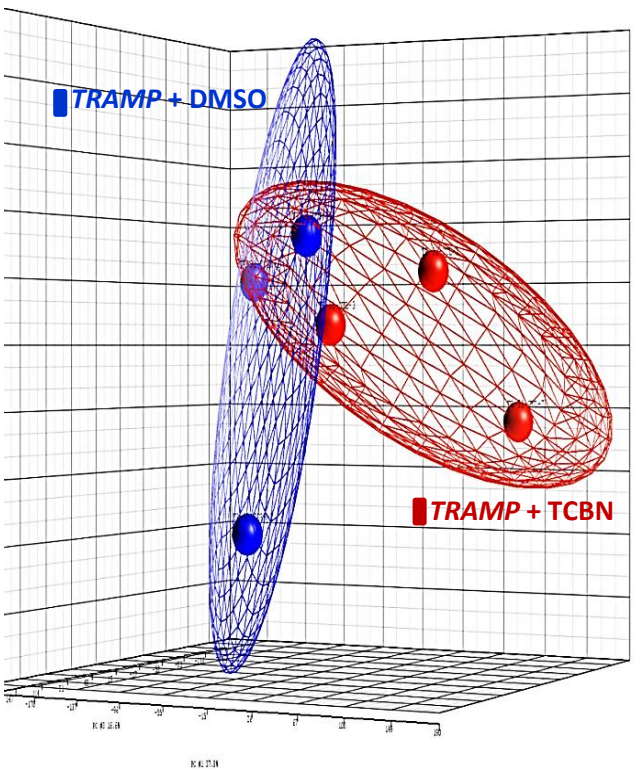
We subjected the prostate tissues collected from *TRAMP/Akt1*$^{+/+}$ and *TRAMP/Akt1*$^{-/-}$ mice at 12 weeks (PIN stage) age for Affymetrix® technology-based microRNA array analysis. To determine the specific effect of pharmacological suppression of Akt in advanced PCa, we subjected the prostate tissues collected from *TRAMP/Akt1*$^{+/+}$ mice treated with DMSO (control) or triciribine (Selleckchem, Houston, TX) for 5 weeks starting from week 26 and collecting at 31 weeks for the microRNA array analysis. miRNAs were isolated from mouse prostates using Qiagen miRNeasy Kit according to manufacturer's protocol. The concentration of miRNA was determined using a NanoDrop spectrophotometer (Thermo Scientific) and the quality of miRNA was analyzed using an Agilent 2100 Bioanalyzer. Microarrays were performed on miRNA using an Affymetrix GeneChip® miRNA 4.0 Array at the Integrated Genomics Core, Augusta University, GA. The miRNA profiles for the early stage prostate tumors with or without the Akt1 gene and the advanced prostate tumors with DMSO (control) or triciribine treatment were determined and analyzed.

FIGURE 1

A



B



C

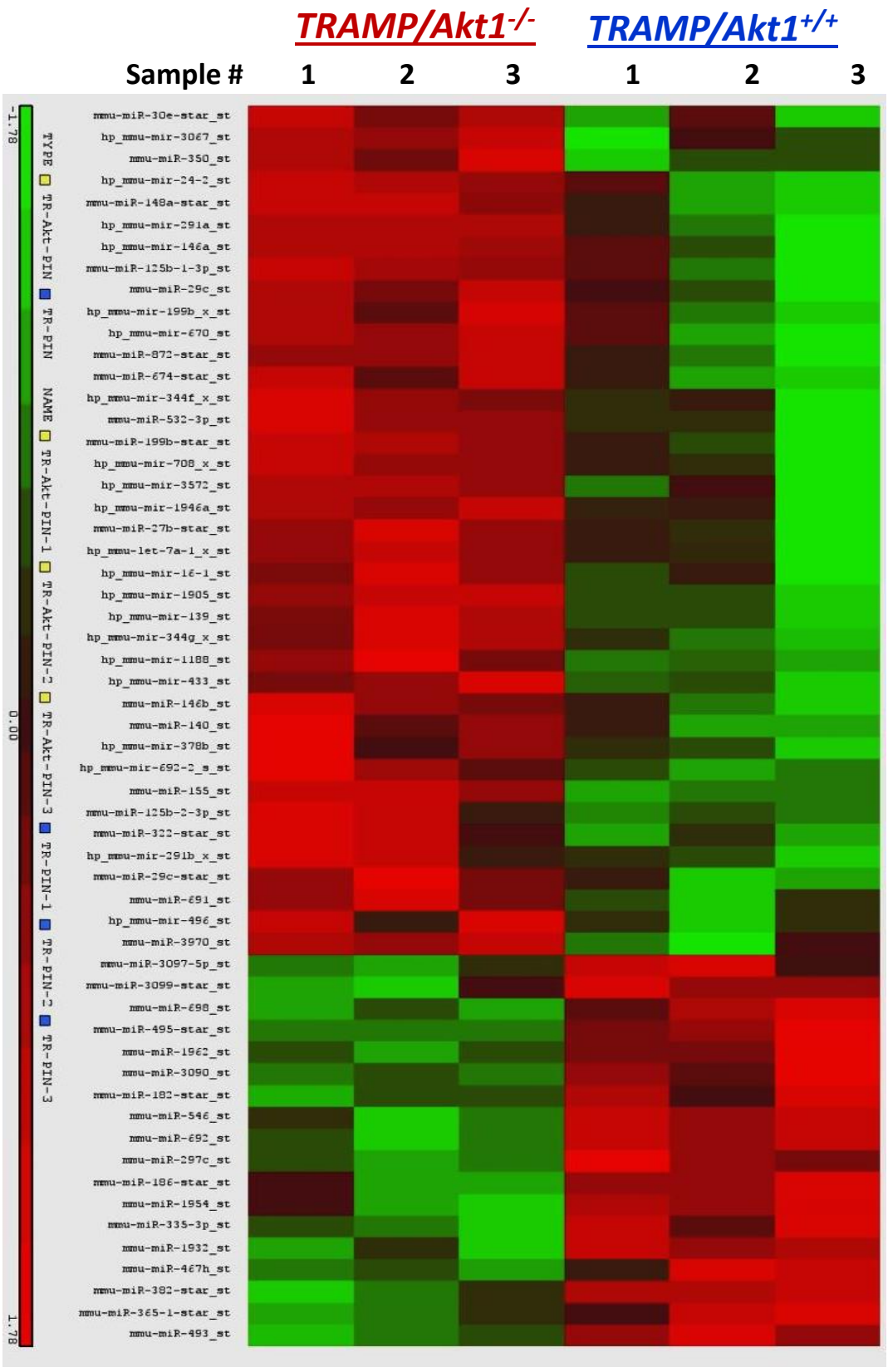
Pathway Name	Enrichment Score	Enrichment p-value	% genes in pathway that are present	TR-Akt1-PIN vs TR-PIN score	# genes in list, in pathway
Pathways in cancer	22.5899	1.55E-10	8.33333	1.8637	125
Wnt signaling pathway	19.6736	2.86E-09	10.1382	1.78544	66
Focal adhesion	13.7705	1.05E-06	8.32396	1.67597	74
Adherens and Tight junction	9.90508	4.99E-05	9.56284	1.76326	71
Matrix-receptor interactions	9.31652	8.99E-05	9.51009	1.61127	65
Prostate cancer	7.61258	0.000494196	8.26087	1.88998	38
Regulation of actin cytoskeleton	7.49147	0.000557822	7.05128	1.8564	66
Transcriptional regulation in cancer	7.33192	0.000654315	7.16846	1.86271	60
VEGF signaling pathway	1.68937	0.184636	5.76923	1.75209	21

D

Pathway Name	Enrichment Score	Enrichment p-value	% genes in pathway that are present	TR-DMSO vs TR-TCBN score	# genes in list, in pathway
Pathways in cancer	18.416	1.00E-08	14.1333	1.79551	212
MAPK signaling pathway	16.1029	1.02E-07	14.1956	1.80586	180
Wnt signaling pathway	19.0138	5.53E-09	16.8971	1.79399	110
Regulation of actin cytoskeleton	8.82737	0.000147	13.3547	1.81522	125
Adherens and Tight junction	8.9183	0.000134	15.847	1.79523	139
TGF-beta signaling	9.37164	8.51E-05	15.9574	1.86195	60
ECM-receptor interaction	6.30004	0.001836	14.6974	1.72916	51
Hedgehog signaling pathway	8.08318	0.000309	17.6471	1.86199	36
Notch signaling pathway	6.52075	0.001473	16.6667	1.80608	33

Figure 1: Akt-regulated microRNAs differentially regulate PCa pathways in the early and advanced stages. (A) Principle component analysis (PCA) mapping of *TRAMP/Akt1*$^{+/+}$ and *TRAMP/Akt1*$^{-/-}$ profiling. *TRAMP/Akt1*$^{+/+}$ group (indicated by red color) was clustered distinctly from *TRAMP/Akt1*$^{-/-}$ group (indicated by blue color). **(B)** Principle component analysis (PCA) mapping of 31 weeks old, 5 weeks treated *TRAMP*+DMSO and *TRAMP*+Triciribine prostate tissue profiling. *TRAMP*+DMSO group (indicated by blue color) was clustered distinctly from *TRAMP*+Triciribine group (indicated by red color). **(C)** Table showing pathways affected by the microRNA expression in *TRAMP/Akt1*$^{-/-}$ compared to *TRAMP/Akt1*$^{+/+}$ mouse prostates as determined by the KEGG pathway analysis. **(D)** Table showing pathways affected by the microRNA expression in *TRAMP*+Triciribine compared to *TRAMP*+DMSO mouse prostates as determined by the KEGG pathway analysis.

FIGURE 2

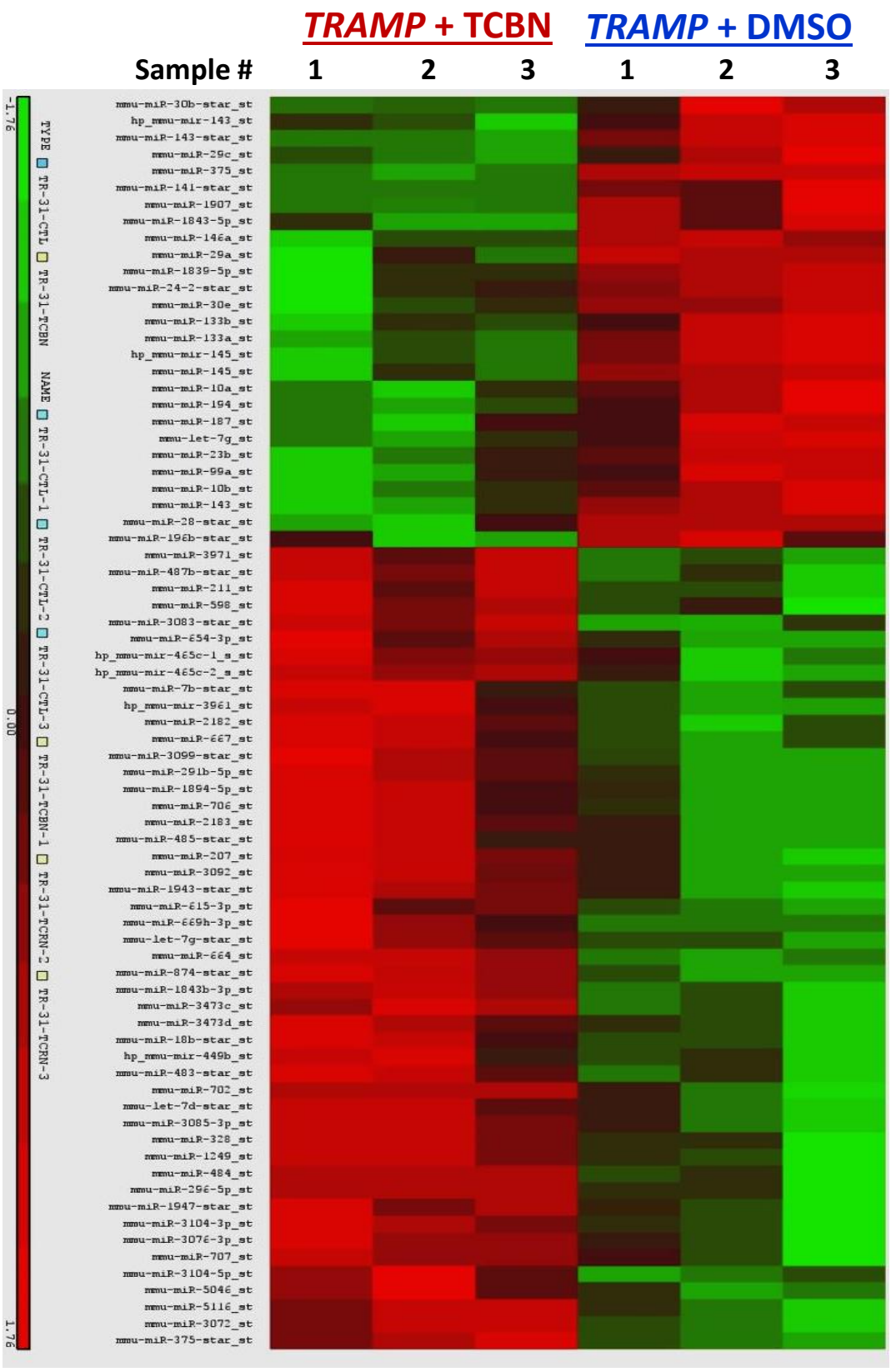


12 Weeks (PIN Stage)

Figure 2: Akt suppression in the early and advanced stages of PCa modulates a different set of microRNAs. Alteration in the miRNAs in *TRAMP/Akt1*$^{-/-}$ mouse prostates compared to *TRAMP/Akt1*$^{+/+}$ shown in a Heat-map (n=3).

Figure 3: Akt suppression in the early and advanced stages of PCa modulates a different set of microRNAs. Alteration in the miRNAs in *TRAMP*+Triciribine mouse prostates compared to *TRAMP*+DMSO shown in a Heat-map (n=3).

FIGURE 3



31 Weeks (Metastasis Stage)

- ***Normalization and pathway analysis of microRNA array***

The miRNA expression was normalized to the average of the house keeping genes (snoRNA251, snoRNA202, snoRNA142, and U6) provided in the miRNA PCR arrays. The miRNA profile of *TRAMP/Akt1*$^{-/-}$ was normalized to *TRAMP/Akt1*$^{+/+}$ (early stage), while the miRNA profile of triciribine treated advanced tumor-bearing *TRAMP/Akt1*$^{+/+}$ was normalized with the respective DMSO treated controls (late stage). T-tests were used to calculate the p-value to determine the significant difference in miRNA expression between the groups. The p-value cutoff of 0.05 and the miRNAs with a fold change above 1.5 were considered differentially expressed for further analyses. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) signaling pathway analyses were performed using DIANA-miRPath version 3.0 (http://diana.imis.athena-innovation.gr/DianaTools/index.php) on differentially expressed microRNAs target genes [21]. Analysis of EMT genes regulated by microRNAs was determined using the epithelial-to-mesenchymal transition gene database (dbEMT; http://dbemt.bioinfo-minzhao.org/). Principal component analysis (PCA) was performed between control and test *TRAMP* tumors both in the early and advanced stages.

- ***Ingenuity pathway analysis***

Ingenuity Pathway Analysis (IPA, Qiagen Bioinformatics) is a software that transforms a list of molecules into a set of relevant networks associated with pathology based on extensive records maintained in the Ingenuity Pathways Knowledge Base [22]. Highly interconnected networks are predicted to represent a significant biological function [23]. IPA was used to connect 132 genome-wide association study (GWAS)- implicated cancer genes along with microRNA and various cancer pathways [24 25]. Significantly changed miRNAs associated with Akt1 inhibition from the two experimental sets were uploaded in IPA and core analyzed. Genes that are differentially

regulated by miRNAs, as well as miRNAs, were mapped to molecular pathways, canonical pathways, and biological functions that are predominantly associated with cancer. All genes that were directly affected by the pathway in cancer are shown.

5.3. Data and Statistical Analysis

All the studies performed using the KEGG, Ingenuity, and miR-Path databases were performed in an unbiased manner without focusing on any specific targets or signaling pathways. dbEMT database analysis was performed specifically to look into the known and potential genes/targets regulated by each or combination of the most up- or down-regulated miroRNAs as obtained from the KEGG and miR-Path analysis on EMT and cancer metastasis. All the data are presented as mean ± SD and were calculated from multiple independent experiments performed in quadruplicates. For normalized data analysis, data was confirmed that normality assumption was satisfied and analyzed using paired sample t-test (dependent t-test) and/or further confirmed with non-parametric test Wilcoxon signed rank test. For all other analyses, Student's two-tailed t-test or ANOVA test were used to determine significant differences between treatment and control values using the GraphPad Prism 4.03 software and SPSS 17.0 software. Data with $P<0.05$ were considered significant.

5.4. Results

- ***Akt1 gene deletion in the early (PIN) and pharmacological suppression in the advanced (metastasis) PCa in TRAMP prostate reveal expression changes in microRNAs involved in different signaling pathways***

Principle component analysis (PCA) mapping of *TRAMP/Akt1*$^{+/+}$ and *TRAMP/Akt1*$^{-/-}$ showed that *TRAMP/Akt1*$^{+/+}$ group was clustered distinctly from *TRAMP/Akt1*$^{-/-}$ group (Figure 1A).

KEGG pathway based all microRNA target prediction analysis indicated changes in the expression of several genes involved in the regulation of cancer growth, Wnt signaling pathway, focal adhesion, extracellular matrix interactions and cell-cell junctions etc. (Figure 1C). As supported by the literature, these results indicated that Akt1 predominantly regulates cancer pathways, Wnt Signaling pathways, Focal adhesions, junctional proteins, extracellular matrix interactions, actin cytoskeleton and VEGF signaling pathway in the promotion of tumor growth in the early stages and that the absence of Akt1 gene suppresses these effects.

Principle component analysis (PCA) mapping of *TRAMP/Akt1*$^{+/+}$ + DMSO and *TRAMP/Akt1*$^{+/+}$ + triciribine in the advanced stages showed that *TRAMP/Akt1*$^{+/+}$ + DMSO group was clustered distinctly from *TRAMP/Akt1*$^{+/+}$ + triciribine group (Figure 1B). KEGG pathway based all microRNA target prediction analysis of the *TRAMP/Akt1*$^{+/+}$ + DMSO and *TRAMP/Akt1*$^{+/+}$ + triciribine treated advanced stage prostate cancer tissues indicated changes in the expression of several genes predominantly involved in the regulation of the Cancer pathways, Wnt signaling pathway and cytoskeletal remodeling, similar to what was observed in the early stages. Interestingly, Akt suppression by triciribine in the late stages also promoted EMT-regulating pathways such as the MAP kinase signaling, TGFβ pathway, Notch and Hedgehog signaling etc. (Figure 1D). A highly diverse group of microRNA repertoire was observed in these mouse prostate samples (*TRAMP/Akt1*$^{-/-}$ compared to *TRAMP/Akt1*$^{+/+}$ prostates versus *TRAMP + DMSO* compared to *TRAMP + triciribine*) at two different stages of the disease (Figures 2 and 3, respectively) suggesting an important role of microRNAs in stage-specific effects of Akt suppression on PCa.

- ***Akt1 deletion in TRAMP mice alters expression changes in selective microRNAs that regulate cell survival and proliferation in early PCa***

FIGURE 4

A

TRAMP/Akt1$^{-/-}$ vs. *TRAMP/Akt1*$^{+/+}$ (12 wks)		
miRNA with seed sequence	***Fold change***	***p-value***
miR-155-5p (miRNAs w/seed UAAUGCU)	↑12.527	0.0004517
miR-199a-5p (and other miRNAs w/seed CCAGUGU)	↑7.318	0.0228678
miR-29b-3p (and other miRNAs w/seed AGCACCA)	↑5.217	0.0431547
miR-30a-3p (and other miRNAs w/seed UUUCAGU)	↑4.902	0.0451509
miR-125b-1-3p (miRNAs w/seed CGGGUUA)	↑3.915	0.0475152
miR-146a-5p (and other miRNAs w/seed GAGAACU)	↑3.408	0.0207138
miR-322-3p (miRNAs w/seed AACAUGA)	↑2.887	0.0331661
miR-125b-2-3p (miRNAs w/seed CAAGUCA)	↑2.567	0.0331728
miR-485-5p (and other miRNAs w/seed GAGGCUG)	↓-2.720	0.0227016
miR-493-3p (miRNAs w/seed GAAGGUC)	↓-2.364	0.0090527
miR-467e-5p (and other miRNAs w/seed UAAGUGU)	↓-2.230	0.0288641
miR-3099-5p (miRNAs w/seed CAGCUUC)	↓-2.078	0.0273878
miR-365-1-5p (and other miRNAs w/seed GGGACUU)	↓-1.983	0.0291202
miR-3090-3p (and other miRNAs w/seed CCCAGGU)	↓-1.549	0.0328666

B

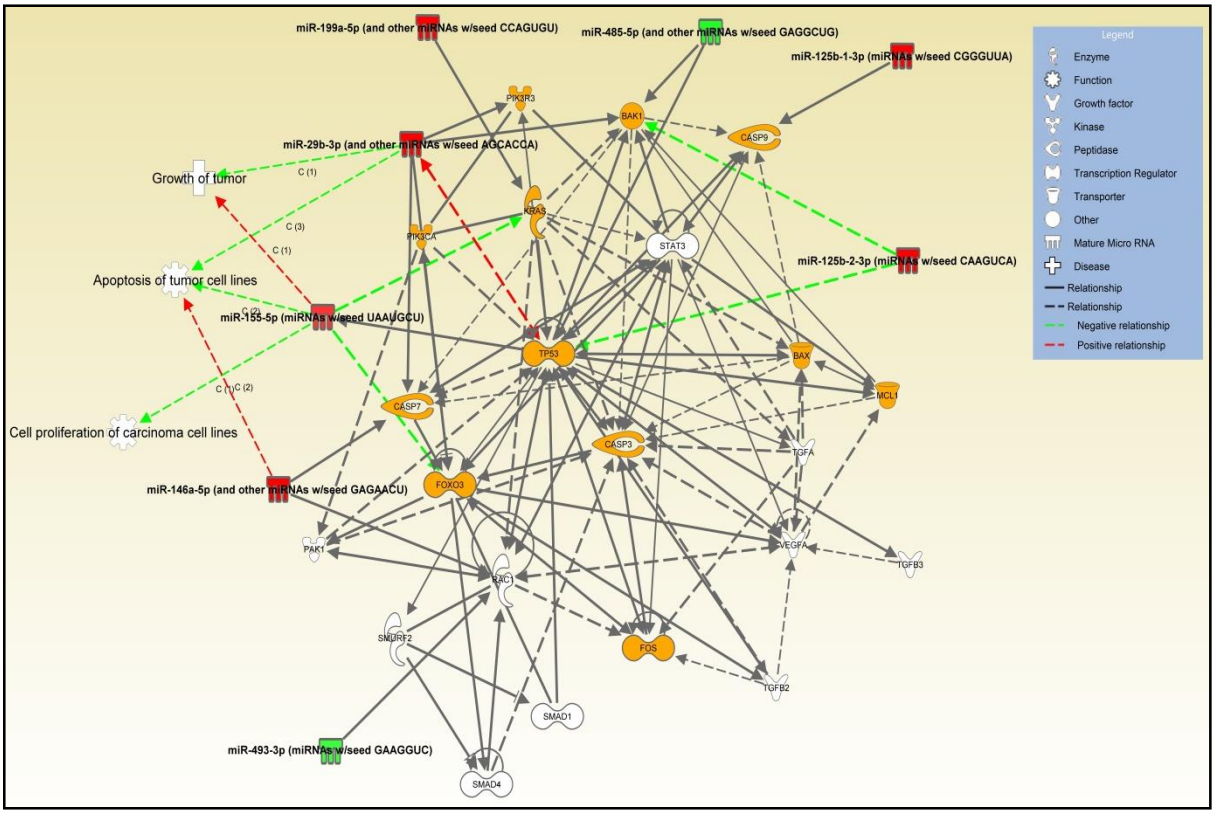


Figure 4: MicroRNA expression changes in *TRAMP/Akt1*$^{-/-}$ mouse prostates compared to *TRAMP/Akt1*$^{+/+}$ show the integral role of Akt1 in cell survival and proliferation. **(A)** Selected miRNAs differentially regulated in *TRAMP/Akt1*$^{-/-}$ mouse prostates compared to *TRAMP/Akt1*$^{+/+}$. **(B)** Signaling network analysis using Ingenuity Pathway Analysis software involving microRNAs identified from the study indicating the integral role of Akt1-regulated microRNAs in cell survival, proliferation and growth in the early stage PCa.

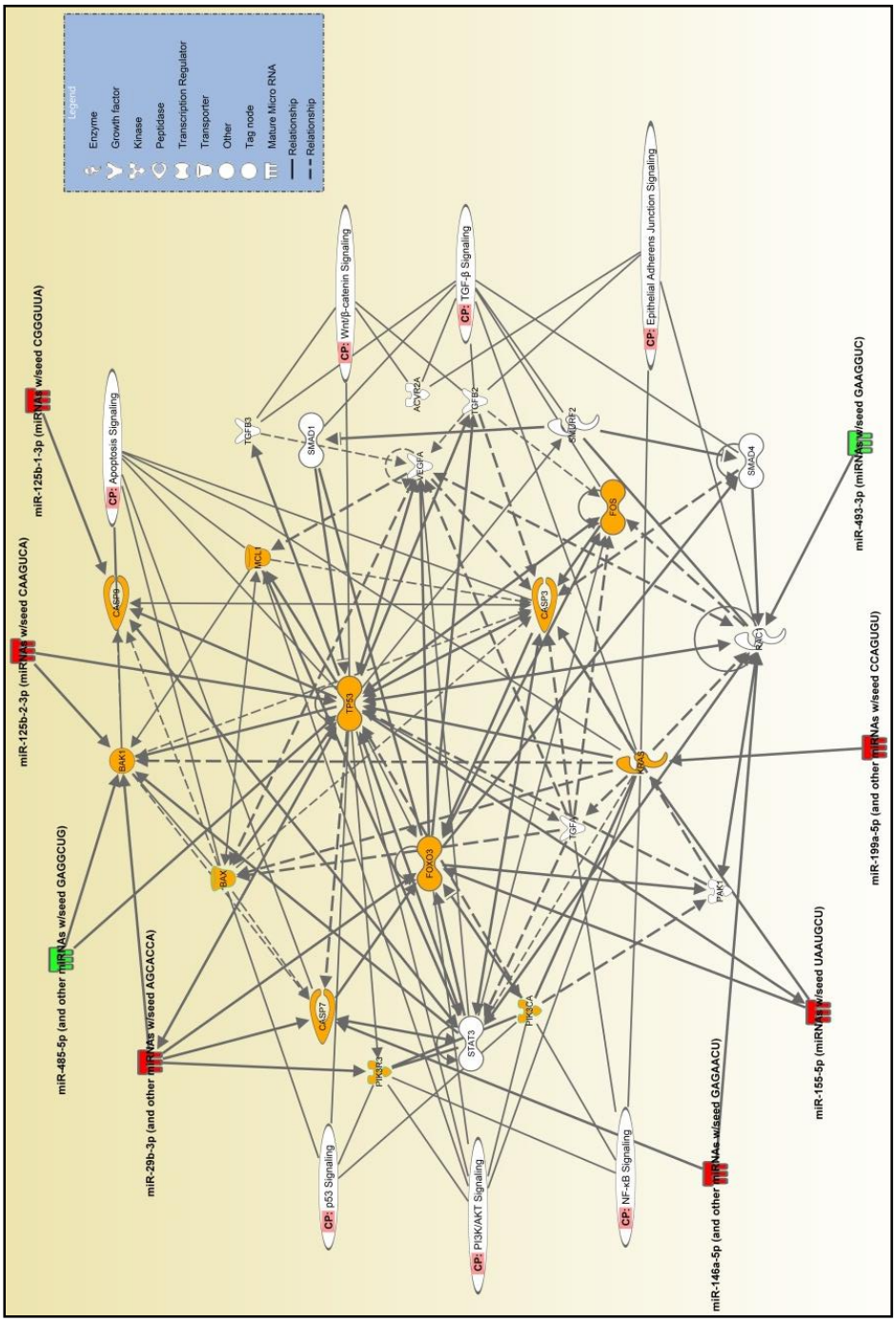


Figure 5: Signaling network analysis using Ingenuity Pathway Analysis software involving microRNAs identified from the study indicating the integral role of Akt1-regulated microRNAs in cell survival, proliferation and growth in the early stage PCa.

There were significant changes in the repertoire of microRNA expression in *TRAMP/Akt1*$^{-/-}$ compared to *TRAMP/Akt1*$^{+/+}$ prostates (Figures 2 and 4A). While ~5-13-fold increase in miR-155-5p, miR199a-5p, and miR-29b-3p was observed in *TRAMP/Akt1*$^{-/-}$ compared to *TRAMP/Akt1*$^{+/+}$ prostates, this was also associated with a 2-3-fold decrease in miR-485-5p and miR-493-3p (Figure 4A). Based on the Ingenuity Pathway Analysis® system that converts a list of microRNAs and/or genes of interest in particular disease pathology into a set of functional networks based on the reported biological interactions, we identified that the net effect of Akt1 gene deletion in TRAMP prostate at early cancer stage such as PIN stage will be suppression of proliferation and promotion of apoptosis (Figures 4B and 5), thus inhibiting oncogenic transformation and tumor growth. All the microRNAs that were modulated by Akt1 gene deletion in the PIN stage *TRAMP* prostate were previously characterized for their target genes and cellular function in various cancers. The gene targets of the upregulated microRNAs in the PIN-stage *TRAMP* prostates, such as the mir155-5p, mir29b-3p, mir199a-5p, mir125b-1-3p, mir674-3p and mir29b-3p because of Akt1 gene knockdown, as identified by the Gene ontology and KEGG pathway (DIANA-miRPath database) analyses has informed about the integral role of these microRNAs in the promotion of cell survival and/or proliferation (Figure 6A; Supplemental Table 1). Similarly, GO and KEGG analysis on the target genes of downregulated miRNAs such as mir485-5p, mir3097-5p, mir460e-5p, mir3090-3p, mir365-1-5p and mir3099-5p identified their role in promoting cellular arrest and apoptosis (Figure 6B; Supplemental Table 2), suggesting that Akt inhibition in the early stages of PCa has a tumor suppressive effect.

- ***Pharmacological inhibition of Akt in the advanced PCa-bearing TRAMP mice alters expression changes in selective microRNAs that regulate EMT and metastasis***

We observed significant changes in the repertoire of microRNA expression in triciribine treated compared to DMSO treated control prostates, which are entirely different from the early stage tumors (Figures 3 and 7A). While ~5-fold increase in miR-669h-3p, miR3104-3p and miR-598-3p were observed in triciribine treated compared to DMSO treated control prostates, more changes were observed in the downregulated microRNAs resulted in ~7-17-fold decrease in miR-375-3p, let-7a-5p, miR-10a-5p and miR-143-3p (Figure 7A). Based on the Ingenuity Pathway Analysis®, we identified that the net effect of Akt activity suppression using triciribine in *TRAMP* prostate in the advanced stages will be the promotion of cellular migration, invasion, malignancy and differentiation to mesenchymal type as demonstrated by changes in the expression of smooth muscle cell actin-α and TGFβ signaling (Figures 7B and 8), thus promoting metastatic ability. Analysis based on KEGG pathway analysis and dbEMT database analysis also indicated that the changes in these microRNAs with Akt suppression in advanced PCa will promote EMT and metastasis.

Although several microRNAs that were modulated by Akt suppression in the advanced stage *TRAMP* prostate were previously characterized for their target genes and cellular function in various cancers, information regarding some of the highly downregulated microRNAs such as mir375-3p was not available in these databases or in the literature. The gene targets of the up-regulated microRNAs such as the mir669h-3p, mir5046, mir3092-3p, mir328-3p, mir296-5p and mir674-5p because of Akt suppression by triciribine treatment in the advanced PCa tissues as identified by the dbEMT database analyses has informed about the integral role of these microRNAs in the promotion of EMT and metastasis (Figure 9A and Supplemental Table 3). Similarly, the gene targets of the down-regulated microRNAs such as the mir145a-5p, mir30c-5p, mir10a-5p, mir143-5p, let7a-5p and mir133a-5p because of Akt activity suppression by

triciribine treatment in the advanced PCa tissues as identified by the dbEMT database has informed about the integral role of these microRNAs in the suppression of EMT and metastasis (Figure 9B and Supplemental Table 4). Overall, the results suggest that Akt inhibition in the advanced stages of PCa would promote metastasis. Complete lists of microRNAs identified in the Affymetrix microarrays comparing *TRAMP/Akt1*$^{-/-}$to *TRAMP/Akt1*$^{+/+}$ prostates versus *TRAMP* + *DMSO* to *TRAMP* + Triciribine treated prostates are provided in Supplemental Tables 5 and 6, respectively.

5.5. Discussion

Our study has demonstrated for the first time that Akt(1) suppression during the early and advanced stages of PCa induces stage-specific changes in the repertoire of microRNAs involved in the differential regulation of oncogenic transformation, tumor growth, and metastasis. Mechanistically this involves microRNA-mediated regulation of genes involved in cell survival and proliferation in the early stages and deregulation of TGFβ, MAP kinase, Notch and Hedgehog signaling in the later stages.

Akt has been indisputably regarded as a pro-tumorigenic kinase in various cancers [26 27]. Several studies from our laboratory have indicated that Akt is indispensable for the survival, motility, and proliferation of PCa cells *in vitro* and tumor growth *in vivo* [7 28-32]. Intriguingly, recent studies from various laboratories in different cancer types such as the breast [12 13], liver [14], non-small cell lung [15] head and neck [16], have reported a different, paradoxical effect of Akt suppression on cancer metastasis. Our most recent study in PCa has clearly demonstrated that although Akt1 gene deletion in *TRAMP* mice prevents oncogenic transformation and tumor growth in the prostate, the pharmacological suppression of Akt kinase activity in *TRAMP* mice

FIGURE 6

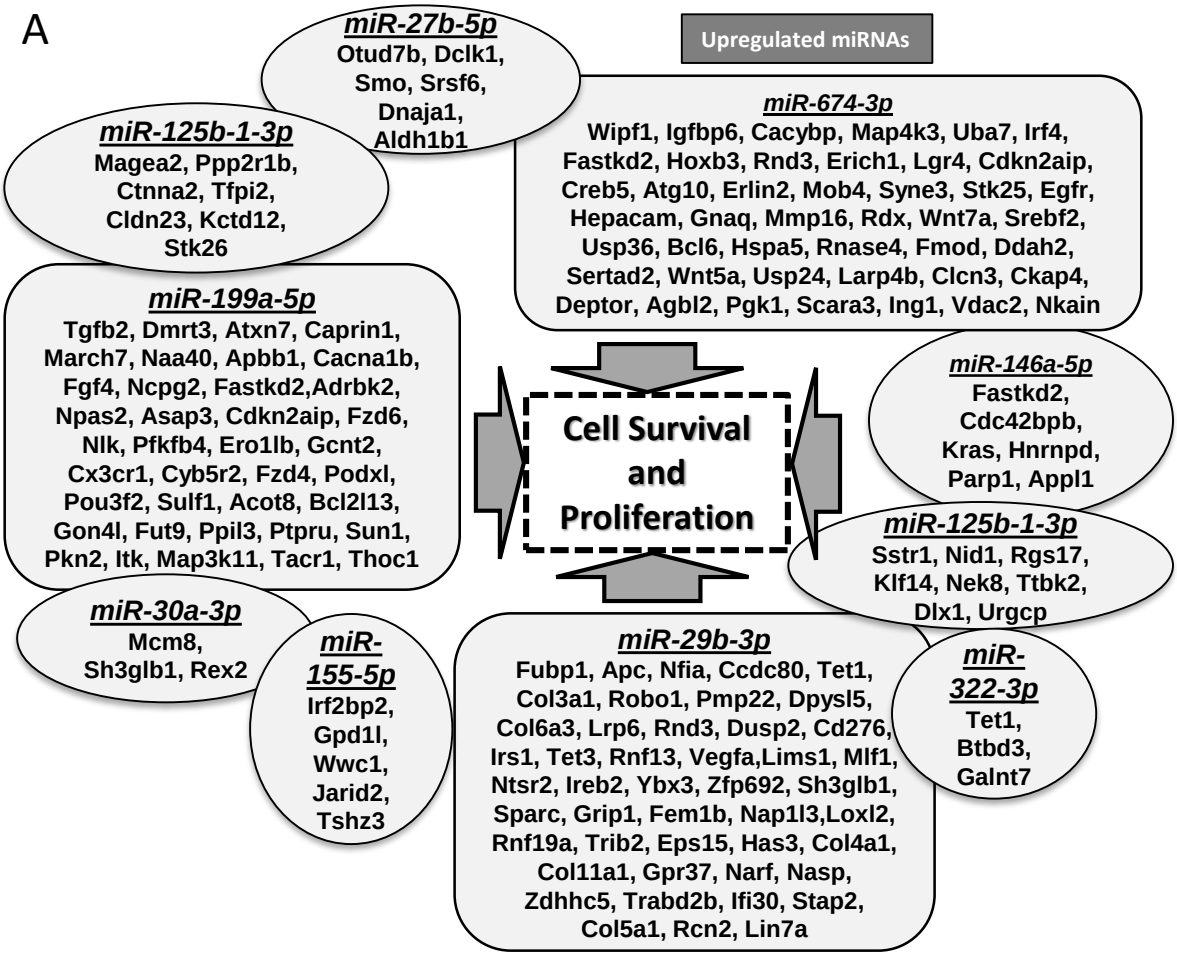

A
Upregulated miRNAs
miR-27b-5p
Otud7b, Dclk1, Smo, Srsf6, Dnaja1, Aldh1b1
miR-125b-1-3p
Magea2, Ppp2r1b, Ctnna2, Tfpi2, Cldn23, Kctd12, Stk26
miR-674-3p
Wipf1, Igfbp6, Cacybp, Map4k3, Uba7, Irf4, Fastkd2, Hoxb3, Rnd3, Erich1, Lgr4, Cdkn2aip, Creb5, Atg10, Erlin2, Mob4, Syne3, Stk25, Egfr, Hepacam, Gnaq, Mmp16, Rdx, Wnt7a, Srebf2, Usp36, Bcl6, Hspa5, Rnase4, Fmod, Ddah2, Sertad2, Wnt5a, Usp24, Larp4b, Clcn3, Ckap4, Deptor, Agbl2, Pgk1, Scara3, Ing1, Vdac2, Nkain
miR-199a-5p
Tgfb2, Dmrt3, Atxn7, Caprin1, March7, Naa40, Apbb1, Cacna1b, Fgf4, Ncpg2, Fastkd2,Adrbk2, Npas2, Asap3, Cdkn2aip, Fzd6, Nlk, Pfkfb4, Ero1lb, Gcnt2, Cx3cr1, Cyb5r2, Fzd4, Podxl, Pou3f2, Sulf1, Acot8, Bcl2l13, Gon4l, Fut9, Ppil3, Ptpru, Sun1, Pkn2, Itk, Map3k11, Tacr1, Thoc1
Cell Survival and Proliferation
miR-146a-5p
Fastkd2, Cdc42bpb, Kras, Hnrnpd, Parp1, Appl1
miR-125b-1-3p
Sstr1, Nid1, Rgs17, Klf14, Nek8, Ttbk2, Dlx1, Urgcp
miR-30a-3p
Mcm8, Sh3glb1, Rex2
miR-155-5p
Irf2bp2, Gpd1l, Wwc1, Jarid2, Tshz3
miR-29b-3p
Fubp1, Apc, Nfia, Ccdc80, Tet1, Col3a1, Robo1, Pmp22, Dpysl5, Col6a3, Lrp6, Rnd3, Dusp2, Cd276, Irs1, Tet3, Rnf13, Vegfa,Lims1, Mlf1, Ntsr2, Ireb2, Ybx3, Zfp692, Sh3glb1, Sparc, Grip1, Fem1b, Nap1l3,Loxl2, Rnf19a, Trib2, Eps15, Has3, Col4a1, Col11a1, Gpr37, Narf, Nasp, Zdhhc5, Trabd2b, Ifi30, Stap2, Col5a1, Rcn2, Lin7a
miR-322-3p
Tet1, Btbd3, Galnt7
B
miR-485-5p
Dlec1, Mcl1, Arhgef11,Vat1
miR-3097-5p
Fgf13, Gas7
miR-3099-5p
Snd1, Cse1l, Mcl1
Cell Survival and Proliferation
miR-467e-5p
Nxph1, Bag2, RFwd2, Cdcc8, Nampt, Chek1, Cysltr1, Dimt1
miR-365-1-5p
Ank2, Ptgs2, Rad1, Wnt9b
miR-3090-3p
Ank2, Ptgs2, Rad1, Kras, Grhl2, Wnt5b, Rasal2, Mgat3, Mutyh, Swap70, Epn2, Hmgn1, Bloc1s2
Downregulated miRNAs

Figure 6: KEGG and Gene Ontology (mirPath) analysis indicate modulation cell survival and proliferation by Akt-regulated microRNAs in the early PCa. **(A)** Diagram showing highly upregulated miRNAs in *TRAMP/Akt1*$^{-/-}$ mouse prostates compared to *TRAMP/Akt1*$^{+/+}$, and their predicted and known targets indicating their predominant involvement in the cell survival and proliferation in the early PCa. **(B)** Diagram showing highly down-regulated miRNAs in *TRAMP/Akt1*$^{-/-}$ mouse prostates compared to *TRAMP/Akt1*$^{+/+}$, and their predicted and known targets indicating their predominant involvement in the cell survival and proliferation in the early PCa.

-bearing advanced PCa using triciribine-augmented metastasis to distant tissues such as the lungs, liver, and kidney [11]. One of the key mechanisms by which Akt suppression leading to increased metastasis in PCa [11] and breast cancer [12 13] has been identified to be the deregulation of various genes involved in the TGFβ-mediated EMT of cancer cells.

How Akt(1) suppression leads to deregulation of the TGFβ pathway to promote EMT and cancer metastasis is not clearly understood and literature in this area is scarce. One of the first studies reporting a connection between Akt1 suppression, TGFβ expression and EMT in breast cancer indicated the down-regulation of mir200 clusters such as mir200a, mir200b, and mir200c, which subsequently led to reduced expression of E-Cadherin and increased expression of vimentin and EMT transcription factor Zeb1 [12]. Although the involvement of microRNAs was not investigated, a causal relationship between Akt1 suppression and promotion of EMT via increased transcription factor Twist1 expression was also reported by another group in breast cancer cells [13]. Although increased invasion as a result of Akt suppression has also been reported by other laboratories in NSCLC [15], liver [14] and head and neck [16] cancer, the involvement of microRNAs and TGFβ pathway in the process have not been investigated. Similarly, our recent study in PCa demonstrated changes in the expression of a plethora of genes involved in the TGFβ and EMT pathways. Results reported in the current study is the second in any cancers, after breast cancer [13] and is the first report in PCa that demonstrate the involvement of stage-specific expression of various microRNAs linking Akt1 activity suppression, activation of TGFβ pathway and EMT.

Unlike breast cancer cells, analysis of *TRAMP* PCa tissues did not reveal a difference in the expression of mir200 family with Akt1 activity suppression in either of the early or advanced stages, indicating that different sets of microRNAs are involved in various cancers. In the early

FIGURE 7

A

TRAMP + TCBN vs. *TRAMP* + DMSO (26-31 week)		
miRNA with seed sequence	**Fold change**	**P-value**
miR-669h-3p (and other miRNAs w/seed AUGCAUA)	↑5.541	0.0394994
miR-3104-3p (miRNAs w/seed CGCUCUG)	↑5.203	0.0196368
miR-598-3p (miRNAs w/seed ACGUCAU)	↑4.778	0.0292323
miR-674-5p (and other miRNAs w/seed CACUGAG)*	↑4.763	0.0339797
miR-291a-5p (and other miRNAs w/seed AUCAAAG)	↑4.388	0.0173373
miR-296-5p (miRNAs w/seed GGGCCCC)	↑4.359	0.026896
miR-485-3p (miRNAs w/seed GUCAUAC)	↑4.295	0.0459664
miR-3092-3p (miRNAs w/seed AAUGGGG)	↑4.101	0.0162596
miR-375-3p (and other miRNAs w/seed UUGUUCG)	↓-16.424	0.0001088
let-7a-5p (and other miRNAs w/seed GAGGUAG)	↓-11.697	0.0330701
miR-10a-5p (and other miRNAs w/seed ACCCUGU)*	↓-8.522	0.0302061
miR-143-3p (and other miRNAs w/seed GAGAUGA)	↓-6.860	0.0067206
miR-30c-5p (and other miRNAs w/seed GUAAACA)	↓-5.428	0.0180734
miR-133a-3p (and other miRNAs w/seed UUGGUCC)	↓-5.304	0.0344731
miR-145a-5p (and other miRNAs w/seed UCCAGUU)	↓-4.781	0.0064273
miR-100-5p (and other miRNAs w/seed ACCCGUA)	↓-4.675	0.0365975
miR-24-1-5p (and other miRNAs w/seed UGCCUAC)	↓-3.937	0.0418752

B

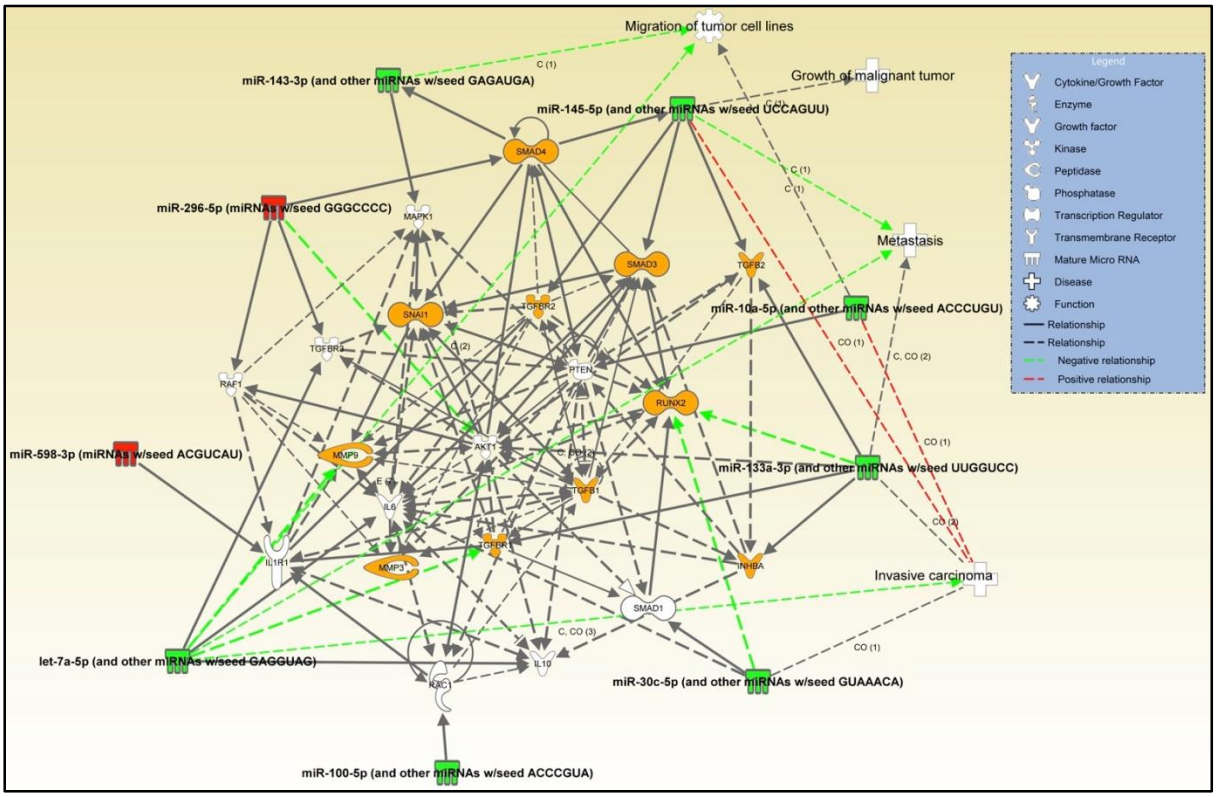


Figure 7: MicroRNA expression changes in *TRAMP/*Triciribine mouse prostates compared to *TRAMP/*DMSO show promotion of EMT with Akt suppression. (A) Selected miRNAs differentially regulated in Triciribine treated *TRAMP*+ mouse prostates compared to DMSO treated control *TRAMP*+. **(B)** Signaling network analysis using Ingenuity Pathway Analysis software involving microRNAs identified from the study indicating the integral role of Akt-regulated microRNAs in EMT and PCa metastasis in the advanced stages.

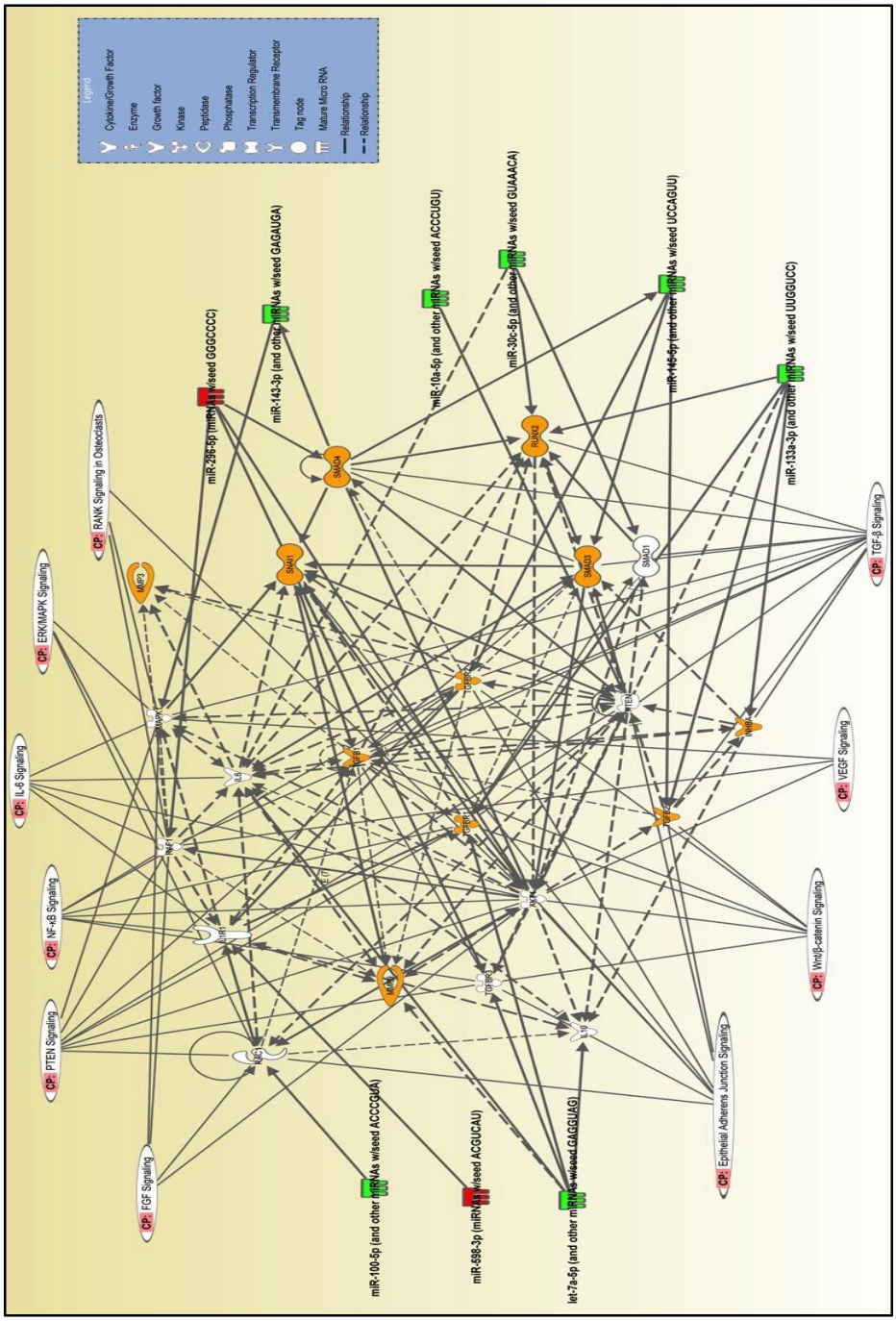


Figure 8: Signaling network analysis using Ingenuity Pathway Analysis software involving microRNAs identified from the study indicating the integral role of Akt-regulated microRNAs in EMT and PCa metastasis in the advanced stages.

-(PIN) stages, Akt1 gene deficiency in the *TRAMP* prostate resulted in significant increase in mir155-5p, mir199a-5p, mir29b-3p and mir30a-3p as well as a decrease in the expression of mir485-5p, mir493-3p and mir467e-5p, all of which that have been demonstrated to regulate the cell survival and proliferation in the early stages of cancer as analyzed by the KEGG, GO and IPA databases. Among these, mir155-5p has been shown to induce gastric cancer cell apoptosis [33] and promote autophagy in cervical cancer cells [34]. On the other end, in hepatocellular carcinoma [35] and colorectal cancer [36] mir155-5p has demonstrated its ability to resist apoptosis and promotes cellular proliferation, respectively. Intriguingly, although mir199a-5p was found to suppress tumor growth from colorectal cancer cells [37], papillary thyroid carcinoma [38], triple-negative breast cancer [39] and proliferation of esophageal cancer cells [40], its down-regulation was shown to promote prostate adenocarcinoma progression [41]. Furthermore, mir29b-3p has been shown to act as a tumor suppressor in glioblastoma where it can inhibit cell growth and induce apoptosis *in vitro* [42]. In addition, a reciprocal correlation was found between miR-30a-3p expression and esophageal cancer cells proliferation [43]. This clearly underlines the cell type-specific effect of miRNAs despite the nature of the disease. Although miR-485-5p has been shown to suppress breast cancer and hepatocellular carcinoma progression [44] [45], the proliferation of NSCLC [46], its reduced expression was associated with poor gastric cancer prognosis [47]. Such a complexity indicate that the stage-specific effects of Akt on PCa growth and metastasis is orchestrated by several but not a single miRNA. In general, the microRNAs detected in the early PCa stage with Akt1 gene deletion were not involved in the regulation of TGFβ pathway, MAP Kinase pathway or EMT indicating that the effect of Akt1 suppression on these events is limited to the advanced stages.

In the advanced stages, Akt1 inhibition by triciribine treatment for 6 weeks resulted in the increased expression of mir669h-3p and mir3104-3p as well as decreased expression of mir375-3p, le7a-5p, mir10a-5p and mir143-3p all of which are the signature microRNAs in the

modulation of TGFβ and EMT pathway as analyzed using the KEGG, GO, IPA and dbEMT databases. In spite of the significant upregulation of miR-375 in the serum of castration-resistant PCa patients [48], we observed a significant reduction of miR-375-3p with tricirbine treatment in the advanced tumor-bearing TRAMP mice. Interestingly, during their investigation for the miRNA-Runx1/2 signaling network in the regulation of PCa progression in TRAMP mice and by looking at the temporal miRNAs expression in TRMAP's tumors, Farina *et al* have also noticed a significant reduction in miR-375-3p expression as the tumor develops in these mice compared to wild-type controls [49]. Although its expression was measured up to 21-week-old mice, the expression of Runx1/2, which are targets for miR-375-3p, was elevated in 33week-old TRAMPs indicating the potential reduction of miR-375-3p during that stage. However, since we had TRAMP+DMSO as our control, treatment with TCBN was the only reason responsible for the further reduction in this miRNA, assuming its low level in the control animals. Another study reported that loss of let-7a expression in human PCa specimens was correlated to higher Gleason score and more importantly to higher EZH2 expression [50], which is known to regulate molecular features of cancer stem cells (CSC), thus EMT [51]. The suppressive activity of miR-143-3p on ovarian cancer progression was reported through downregulation of TGFβ activated kinase-1 (TAK1) [52]. Interestingly, we observed a significant reduction in miR-143-3p with TCBN treatment, which is potentially involved in augmenting TGFβ-induced PCa metastasis upon Akt inhibition in the advanced stage PCa. Currently, there is no information related to the role of miR-669h-3p and miR-3104-3p in cancer, which represents novel topics for further investigation. Our analysis thus demonstrates a significant role of Akt-regulated microRNAs in the stage-specific regulation of PCa.

FIGURE 9

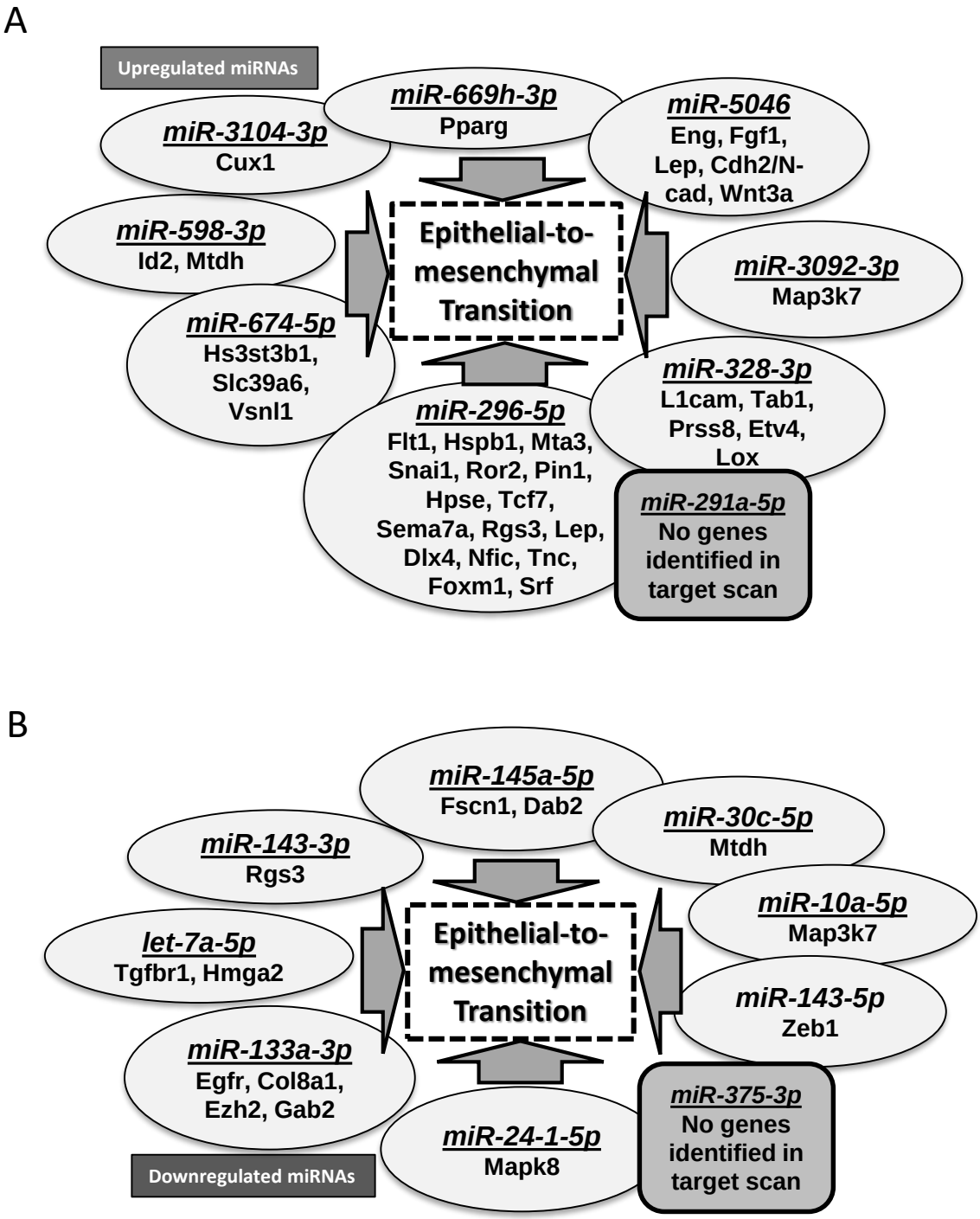

A
Upregulated miRNAs
miR-669h-3p
Pparg
miR-3104-3p
Cux1
miR-5046
Eng, Fgf1, Lep, Cdh2/N-cad, Wnt3a
miR-598-3p
Id2, Mtdh
Epithelial-to-mesenchymal Transition
miR-3092-3p
Map3k7
miR-674-5p
Hs3st3b1, Slc39a6, Vsnl1
miR-328-3p
L1cam, Tab1, Prss8, Etv4, Lox
miR-296-5p
Flt1, Hspb1, Mta3, Snai1, Ror2, Pin1, Hpse, Tcf7, Sema7a, Rgs3, Lep, Dlx4, Nfic, Tnc, Foxm1, Srf
miR-291a-5p
No genes identified in target scan
B
miR-145a-5p
Fscn1, Dab2
miR-30c-5p
Mtdh
miR-143-3p
Rgs3
miR-10a-5p
Map3k7
let-7a-5p
Tgfbr1, Hmga2
Epithelial-to-mesenchymal Transition
miR-143-5p
Zeb1
miR-133a-3p
Egfr, Col8a1, Ezh2, Gab2
miR-375-3p
No genes identified in target scan
miR-24-1-5p
Mapk8
Downregulated miRNAs

Figure 9: KEGG and Gene Ontology (mirPath) analysis indicate modulation epithelial-to-mesenchymal transition and metastasis by Akt-regulated microRNAs in the advanced PCa. (A) Diagram showing highly upregulated miRNAs in Triciribine-treated *TRAMP+* mouse prostates compared to DMSO treated control *TRAMP+* prostates, and their predicted and known targets indicating their predominant involvement in the regulation of EMT in the advanced PCa. **(B)** Diagram showing highly downregulated miRNAs in Triciribine-treated *TRAMP+* mouse prostates compared to DMSO treated control *TRAMP+* prostates, and their predicted and known targets indicating their predominant involvement in the regulation of EMT in the advanced PCa.

In conclusion, our study provides the necessary clues that the expression of different sets of microRNAs during the early and the advanced stages of PCa plays a major role in the differential regulation of many signaling pathways such as the Akt and TGFβ pathways and that the microRNAs are also responsible for linking these pathways together. Our results will lay the foundation for many future discoveries that may lead to the development of various tools in the management of PCa by identifying the key microRNAs involved in the regulation of different signaling pathways, determining changes in the microRNA expression in cancer biopsies and/or body fluids as a biomarker for staging and for future therapies. A major limitation of our study is that the data is specific to a murine model of PCa and hence have limited clinical relevance. Nevertheless, cellular studies involving human PCa cell lines in our laboratory have yielded similar effects of Akt suppression on EMT and metastasis [11]. However, because of the significant differences between the murine and the human microRNAs involved in various pathologies, more studies on the specific microRNAs involved in human PCa and their specific effects on cell signaling pathways, EMT and metastasis are warranted. This will be the focus of future research in our laboratory.

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Supplemental TABLE 1. Genes and cellular pathways regulated by the upregulated microRNAs in *TRAMP/Akt1*$^{-/-}$ compared to *TRAMP/Akt1*$^{+/+}$ prostate

Genes	Gene associated pathways and cellular processes
Irf2bp2	Transcription
Gpd1l	Glycerophospholipid biosynthesis; Metabolism of fatty acid, triacylglycerol and ketone bodies; Metabolism of lipids and lipoproteins
Wwc1	Hippo signaling; Endoderm differentiation
Jarid2	Pluripotency of stem cells; Epigenetic regulation of gene expression
Tshz3	Transcription
Tgfb2	FoxO signaling pathway; MAPK signaling pathway; Cytokine-cytokine receptor interaction; MicroRNAs in cancer; Pathways in cancer; Extracellular matrix organization; Endocytosis
Atxn7	Chromatin modifying enzymes; Post-translational protein modification; Deubiquitination; Metabolism of proteins
Caprin1	Regulation of translation; Dendrite morphogenesis
March7	T-cell proliferation; Protein ubiquitination
Naa40 (Patt1)	N-terminal protein amino acid acetylation; Lipid metabolism
Apbb1	DNA double strand break response; DNA repair
Cacna1b	MAPK signaling pathways; Calcium signaling pathway
Fgf4	MAPK signaling pathway; RAF/MAP kinase cascade; Rap1 signaling pathway; Focal adhesion-PI3K-Akt-mTOR-signaling pathway; Signaling by VEGF; Signaling to ERKs; Signaling to RAS; Interleukin-2 signaling; Interleukin-3, 5 and GM-CSF signaling; Insulin receptor signaling cascade; Embryonic stem cells (ESC) pluripotency pathways; Regulation of actin cytoskeleton; VEGFR2 mediated cell proliferation
Ncapg2	Mesodermal commitment pathway ; Cell cycle; Condensation of prophase chromosomes; Endoderm differentiation
Fastkd2	Protein phosphorylation; Cellular respiration; Mitochondrial large ribosomal subunit assembly
Adrbk2 (Grk3)	Chemokine signaling pathway; Hedgehog signaling pathway; Thromboxane A2 receptor signaling; Signaling by GPCR; Clathrin-mediated endocytosis; Membrane trafficking
Npas2	Metabolism of fatty acid, triacylglycerol and ketone bodies; Metabolism of lipids and lipoproteins; Regulation of lipid metabolism by peroxisome proliferator-activated receptor alpha (PPARα)
Asap3	Endocytosis; Fc gamma R-mediated phagocytosis
Cdkn2aip	Regulation of signal transduction; Protein stability and cell growth
Fzd6	MTOR signaling pathway; β-catenin independent Wnt signaling; Wnt signaling in cancer; Proteoglycans in cancer; ESC pluripotency pathways
Nlk	FoxO signaling pathway; IL-6 signaling pathway; MAPK signaling pathway; Presenilin action in Notch and Wnt signaling; β-catenin independent Wnt signaling; Noncanonical Wnt signaling pathway; Adherens junction; Ectoderm differentiation
Pfkfb4	AMPK signaling pathway; Glycolysis and metabolism of carbohydrates
Ero1lb	Protein processing in endoplasmic reticulum
Gcnt2	Glycosphingolipid biosynthesis; Terminal O-glycans residues modification; Metabolic pathways
Cx3cr1	Chemokine signaling pathway; Signaling by GPCR; Cytokine-cytokine receptor interaction

TABLE 1. Continued

Genes	Gene associated pathways and cellular processes
Cyb5r2	Amino sugar and nucleotide sugar metabolism; Metabolism and oxidation by cytochrome P450
Fzd4	β-catenin independent Wnt signaling; Hippo signaling ; Wnt signaling pathway; MTOR signaling; Signaling by GPCR; Clathrin-mediated endocytosis; Ectoderm differentiation; Membrane trafficking; Mesodermal commitment pathway; Proteoglycans in cancer; Pluripotency
Podxl	Ectoderm differentiation
Pou3f2	Mecp2 and associated Rett syndrome
Sulf1	Wnt signaling pathway; BMP signaling pathway; Endothelial growth factor receptor signaling pathway; Fibroblast growth factor receptor signaling pathway; Cell apoptosis; Angiogenesis; Endothelial cell proliferation; Cell migration; Prostatic bud formation; Bone development
Acot8	Synthesis of bile acids and bile salts; Metabolism of fatty acid, triacylglycerol, and ketone bodies; Metabolism of lipids and lipoproteins; Alpha-linolenic acid (ALA) metabolism; Peroxisomal lipid metabolism
Bcl2l13	Apoptosis
Gon4l	Transcription
Fut9	Glycosphingolipid biosynthesis; Mannose type O-glycan biosynthesis; Metabolic pathways
Ppil3 (CypJ)	Regulation of mRNA splicing and protein folding
Ptpru	Canonical Wnt signaling pathway; Erythropoietin receptor signaling; Transmembrane receptor protein tyrosine phosphatase signaling pathway; Signaling by stem cell factor-KIT; Cell adhesion; Cell proliferation; Cell migration; Protein dephosphorylation
Sun1	Cell cycle; Cytoskeletal anchoring at nuclear membrane and nucleokinesis involved in cell motility in cerebral cortex radial glia guided migration
Pkn2	PI3K-Akt signaling pathway; Signaling by Rho GTPases
Itk	Chemokine signaling pathway, T-cell receptor signaling pathway; Leukocyte transendothelial migration; Protein phosphorylation; Cell proliferation
Map3k 11	FCERI mediated MAPK activation; Gastrin-CREB signaling pathway via PKC and MAPK; Interleukin-2, 3, 5 and GM-CSF signaling; Oncogenic MAPK signaling; RAC1 signaling; Signaling by PDGF; Signaling by RAS; Angiopoietin like protein 8 regulatory pathway; VEGFR2 mediated cell proliferation; Protein phosphorylation; Cell proliferation; Cell apoptosis
Tacr1	Gastrin-CREB signaling pathway via PKC and MAPK; Signaling by GPCR; Clathrin-mediated endocytosis; Membrane trafficking; Epithelial cell proliferation and migration; Vascular permeability
Thoc1	Gene expression; Cleavage of growing transcript in the termination region; mRNA 3'-end processing
Fubp1	Transcription; Gene expression
Apc	Wnt signaling pathways; Hippo signaling pathway; β-catenin phosphorylation cascade; Pathways and microRNAs in cancer; Apoptosis; Deubiquitination; ESC pluripotency pathways; Metabolism of proteins; Post-translational protein modification; Regulation of actin cytoskeleton
Nfia	Transcription
Ccdc80	Regulation of extracellular matrix organization and cell-substrate adhesion
Tet1	Endoderm differentiation; Epigenetic regulation of gene expression; Mesodermal commitment pathway
Col3a1	TGFβ signaling pathway; Collagen biosynthesis and modifying enzymes; Endothelins; Extracellular matrix organization; Focal adhesion; Senescence and autophagy in cancer; miRNA targets in ECM and membrane receptors

TABLE 1. Continued

Genes	Gene associated pathways and cellular processes
Robo1	Netrin-1 signaling; Activation of Rac; Regulation of epithelial cell migration and cell-cell adhesion
Pmp22	α6β1 and α6β4 Integrin signaling; Neural crest differentiation; Regulation of cell proliferation and cell death
Dpysl5	Regulation of signal transduction
Col6a3	PI3K-Akt signaling pathway; Signaling by PDGF; miRNA targets in ECM and membrane receptors; Assembly of collagen fibrils and other multimeric structures; Collagen biosynthesis and modifying enzymes; ECM-receptor interaction; Focal adhesion
Lrp6	Canonical Wnt signaling pathway in cancer; Presenilin action in Notch and Wnt signaling; MTOR signaling pathway; Wnt/β-catenin signaling pathway in leukemia; Regulation of FZD by ubiquitination
Rnd3	Rho protein signal transduction; Actin cytoskeleton organization; Cell adhesion and migration
Dusp2	FCERI mediated MAPK activation; Gastrin-CREB signaling pathway via PKC and MAPK; Interleukin-2, 3, 5 and GM-CSF signaling; RAF/MAP kinase cascade; Signaling by EGFR; PDGF signaling; RAS signaling; Cytokine signaling in immune system; VEGFR2 mediated cell proliferation; Protein dephosphorylation; Endoderm differentiation
Cd276 (B7-H3)	Cell adhesion molecules; Regulation of cell proliferation; Immune response; Cytokine production
Irs1	AGE/RAGE pathway; AMPK signaling pathway; Adipocytokine signaling pathway; α6 β4 signaling pathway; DAP12 signaling; EPO receptor signaling; Factors and pathways affecting insulin-like growth factor (IGF1)-Akt signaling; Fc epsilon receptor (FCERI) signaling; FoxO signaling pathway; Gastrin-CREB signaling pathway via PKC and MAPK; Growth hormone receptor signaling; IGF1 pathway; IL-4 signaling pathway; Insulin signaling pathway; Integrins in angiogenesis; Interleukin-2, 3, 5 and GM-CSF signaling; Leptin signaling pathway; MAPK family signaling cascades; Oncostatin M signaling pathway; PI3K-Akt signaling in cancer; Prolactin signaling pathway; RAF/MAP kinase cascade; Signaling pathways in glioblastoma; Signaling by EGFR; Signaling by GPCR; Signaling by PDGF; Signaling by VEGF; Signaling to ERKs; Signaling to RAS; MTOR signaling pathway; MicroRNAs in cancer
Tet3	Epigenetic regulation of gene expression; DNA demethylation; Protein O-linked glycosylation; Oxidation-reduction process; MECP2 and Associated Rett Syndrome
Rnf13	Integral component of membrane
Vegfa	PI3K-Akt signaling pathway; HIF-1 signaling pathway; Rap1 signaling; Oncostatin M signaling pathway; Signaling by VEGF; Interleukin-4 and 13 signaling; Integrins in angiogenesis; miR-148a/miR-31/FIH1/HIF1α-Notch signaling in glioblastoma; Pathways in cancer; MicroRNAs in cancer; Proteoglycans in cancer; Focal adhesion; Epithelial cell differentiation; Angiogenesis; Endothelial cell chemotaxis; Cytokine-cytokine receptor interaction; Differentiation pathway
Lims1 (PINCH)	NIK/NF-kappa B signaling; TNF-mediated signaling pathway; Cell junction organization; Cell-cell communication; Focal adhesion assembly; Cell-extracellular matrix interactions; Cell-substrate adhesion; Regulation of cytoskeletal remodeling and cell spreading by IPP complex components; GTPase activity
Mlf1	Transcriptional misregulation in cancer; Transcription; Cell cycle arrest
Ntsr2	Signaling by GPCR; Gastrin-CREB signaling pathway via PKC and MAPK
Ireb2 (Irp2)	Validated targets of C-MYC transcriptional activation; Metabolic process; Iron ion transport
Ybx3	Apoptosis; Tight junction; Spermatogenesis; Organ growth; Regulation of necroptotic process
Zfp692	Transcription
Sh3glb1 (Bif1)	Senescence and autophagy in cancer; Endocytosis; Regulation of cytokinesis; Regulation of protein stability
Sparc	Senescence and autophagy in cancer; Extracellular matrix organization; Cellular response to growth factor stimulus; CM proteoglycans; Vesicle-mediated transport; Bone development; Response to cytokine

TABLE 1. Continued

Genes	Gene associated pathways and cellular processes
Grip1 (SRC-2)	Intracellular signal transduction; Androgen receptor signaling pathway; Brain-Derived Neurotrophic Factor (BDNF) signaling pathway; Glutamate binding; Trafficking of AMPA receptors; Transcription
Fem1b	Apoptosis; Branching involved in prostate gland morphogenesis; Epithelial cell maturation involved in prostate gland development; Post-translational protein modification
Nap1l3	Nucleosome assembly
Loxl2	Canonical and Non-Canonical TGFβ signaling; Assembly of collagen fibrils and other multimeric structures; Collagen formation; Extracellular matrix organization; Cell adhesion; Endothelial cell migration; Endothelial cell proliferation; Epithelial to mesenchymal transition; Angiogenesis
Rnf19a	Microtubule cytoskeleton organization; Protein polyubiquitination
Trib2	MAP kinase activity; Cell differentiation; Protein kinase activity; Proteasomal ubiquitin-dependent protein catabolic process
Eps15	EGF/EGFR signaling pathway; Notch signaling pathway; Signaling events mediated by Hepatocyte Growth Factor Receptor (c-Met); Clathrin-mediated endocytosis; Membrane trafficking; Cell proliferation
Has3	Glycosaminoglycan metabolism; Hyaluronan metabolism; Metabolism of carbohydrates; Cell adhesion; Extracellular matrix assembly
Col4a1	PI3K-Akt signaling pathway; Signaling by PDGF; Epithelial cell differentiation; Protein digestion and absorption; Collagen formation; Assembly of collagen fibrils and other multimeric structures; Extracellular matrix organization; ECM-receptor interaction; Focal adhesion; Vesicle-mediated transport; miRNA targets in ECM and membrane receptors
Col11a1	Collagen formation; Assembly of collagen fibrils and other multimeric structures; Extracellular matrix organization; ECM-receptor interaction; Focal adhesion; Endodermal cell differentiation; Proteoglycan metabolic process
Gpr37	Adenylate cyclase-inhibiting G-protein coupled receptor signaling pathway; Signaling by GPCR; MAPK cascade; Dopamine metabolic process
Narf	Oxidation-reduction process
Nasp	DNA replication; Cell proliferation; Protein transport; Male gonad development; Response to testosterone
Zdhhc5	Protein palmitoylation
Trabd2b	Wnt signaling pathway; Protein oligomerization; Protein oxidation; Proteolysis
Ifi30	Cytokine signaling in immune system; Interferon signaling; Oxidation-reduction process
Stap2	Signaling by PTK6; Tyrosine phosphorylation of STAT protein
Col5a1	Collagen formation; Assembly of collagen fibrils and other multimeric structures; Extracellular matrix organization; Focal adhesion; Protein digestion and absorption; miRNA targets in ECM and membrane receptors; Cell adhesion; Cell migration; Blood vessel development; Integrin biosynthetic process
Rcn2	Protein binding
Lin7a	Exocytosis; Epithelial cell apical/basal polarity; Protein transport; Protein-containing complex assembly
Mcm8	Activation of ATR (ATM- and rad3-related kinase) in response to replication stress; Cell cycle; DNA replication
Rex2	Ribosome biogenesis in eukaryotes
Sstr1	Somatostatin signaling pathway; Neuropeptide signaling pathway; Signaling by GPCR; cAMP signaling pathway; Regulation of cell proliferation
Nid1	Degradation of the extracellular matrix; Extracellular matrix organization; Laminin interactions; Cell-matrix adhesion

TABLE 1. Continued

Genes	Gene associated pathways and cellular processes
Rgs17	Signaling by GPCR; Regulation of GTPase activity
Klf14	Transcription by RNA polymerase II
Nek8	Hippo signaling; Protein phosphorylation
Ttbk2	Anchoring of the basal body to the plasma membrane; Cell migration and shape; Organelle biogenesis and maintenance
Dlx1	Notch signaling pathway; BMP signaling pathway; TGFβ signaling; Cell differentiation; Transcription by RNA polymerase II
Urgcp	Cell cycle
Cdc42bpb	Actin cytoskeleton reorganization; Cell migration; Cell polarity; Intracellular signal transduction; Protein phosphorylation
Kras	PI3K-AKT-mTOR signaling pathway; Rac1/Pak1/p38/MMP-2 pathway; Oncogenic MAPK signaling pathway; TNFα signaling pathway; Interleukin-2, 3, 5 and GM-CSF signaling; Tie2 signaling; VEGF signaling pathway; FoxO signaling pathway; BDNF-TrkB signaling; Apelin signaling pathway; Chemokine signaling pathway; ErbB signaling pathway; Gastrin-CREB signaling pathway via PKC and MAPK; Signaling by EGFR in cancer; Signaling by FGFR; Signaling by GPCR; Signaling to ERKs; Signaling to RAS; RAF activation; Oxytocin signaling pathway; Prolactin signaling pathway; GnRH signaling pathway; Estrogen signaling pathway; FRS-mediated FGFR1 signaling; Fc epsilon receptor (FCERI) signaling; Gap junction; Choline metabolism in cancer; Apoptosis; B cell receptor signaling pathway; Acute myeloid leukemia; Central carbon metabolism in cancer;
Hnrnpd	mRNA processing; mRNA splicing; Transcription
Parp1	NF-kappa B signaling pathway; Signaling by TGFβ receptor complex; Apoptosis; Base excision repair; Downregulation of SMAD2/3:SMAD4 transcriptional activity; Fas Ligand (FasL) pathway and Stress induction of Heat Shock Proteins (HSP) regulation; Generic transcription pathway; Metabolism of proteins
Appl1	Follicle Stimulating Hormone (FSH) signaling pathway; Caspase activation via extrinsic apoptotic signaling pathway; Coregulation of androgen receptor activity; Pathways in cancer; Apoptosis
Btbd3	Regulation of proteolysis; Response to stress; Dendrite morphogenesis
Galnt7	Metabolism of proteins; O-glycan biosynthesis; Post-translational protein modification; Carbohydrate metabolic process
Magea2	Signal transduction by p53 class mediator; Transcription; Regulation of protein catabolic process (acetylation and sumoylation); Cellular senescence
Ppp2r1b	AMPK signaling pathway; Activated TLR4 signaling; βcatenin phosphorylation cascade; DAP12 signaling; Fc epsilon receptor (FCERI) signaling; Hippo signaling pathway; FCERI mediated MAPK activation; Gastrin-CREB signaling pathway via PKC and MAPK; IRS-mediated signaling; Interleukin receptor SHC signaling; Interleukin-2, 3, 5 and GM-CSF signaling; MAPK family signaling cascades; PI3K-Akt signaling pathway; RAF activation; RET signaling; Signaling by EGFR; Signaling by GPCR; Signaling by Interleukins; Signaling by Rho GTPases; Signaling by VEGF; Signaling by wnt in cancer; Signaling to ERKs; Signaling to RAS; TGFβ signaling pathway; Regulation of TP53 Activity; Cell cycle; Glucose metabolism; Glycogen metabolism; Tight junction; Toll-like receptors cascades; VEGFR2 mediated cell proliferation;
Ctnna2	Hippo signaling pathway; Pathways in cancer; Adherens junction; Cell differentiation; Bacterial invasion of epithelial cells; Leukocyte transendothelial migration;
Tfpi2	Regulation of endopeptidase activity and extracellular matrix structural
2610018G03Rik (Stk26)	MAPK cascade; LKB1 signaling; Protein phosphorylation; Cell migration; Apoptosis; Apoptotic cleavage of cellular proteins
Cldn23	Cell adhesion molecules (CAMs); Cell-Cell communication; Leukocyte transendothelial migration; Tight junction
Kctd12	Component of cell junction; Regulates RNA and protein binding

TABLE 2. Genes and cellular pathways regulated by the downregulated microRNAs in *TRAMP/Akt1*$^{-/-}$ compared to *TRAMP/Akt1*$^{+/+}$ prostate

Genes	Gene associated pathways and cellular processes
Mcl1	PI3K signaling and apoptosis
Dlec1	Pathways in lung cancer and prostate cancer
Arhgef11 (RhoGEF)	Pathways in cancer
Snd1	MAPK signaling and viral carcinogenesis
Cse1l	P53 pathway; Ras-induced cancer; Apoptosis
Ank2	Metabolism of proteins; Post-translational protein modification; Membrane trafficking in cancer
Ptgs2	VEGF signaling, TNF signaling; NFKB signaling; Interleukin-4/10/13/17 signaling pathways; Cytokine signaling in immune system; Lipid and lipoproteins metabolism
Rad1	Cellular senescence; Regulation of cell cycle and cell division; DNA repair
Wnt9b and Wnt5b	MTOR and Wnt signaling pathways; Signaling regulating proteoglycan in cancer and basal cell carcinoma,; Signaling regulating pluripotency of stem cells
Kras	PI3K-AKT-mTOR signaling; Ras signaling; MAPK signaling, FoxO signaling; EGFR and FGFR signaling; Rap1 signaling; Signaling regulating autophagy and apoptosis; BDNF-TrkB signaling; Chemokines signaling pathways; Gap junction; Viral carcinogenesis; Proteoglycans in cancer
Grhl2	Regulation of tumor metastasis via RhoG; Cell junction protein expression
Rasal2	Ras signaling, MAPK1/MAPK3 signaling; IRS-mediated signaling; VEGFA-VEGFR2 signaling; Signaling to p38 MAPK via RIT and RIN
Mgat3	Metabolic signaling; Metabolism of proteins; Expansion and stabilization of the E-cadherin adherens junction; Post-translational protein modification and N-Glycan biosynthesis
Swap70	Oncogene; Regulation of actin rearrangement
Epn2	Clathrin-mediated endocytosis; Membrane trafficking and ubiquitin-binding adaptor proteins
Hmgn1	P38 MAPK signaling pathway; MAPK-Erk Pathway; DNA Repair
Fgf13	PI3K-Akt signaling; Rap1 signaling; NRF2 pathway; MAPK signaling; Ras signaling; ERK pathway; TGFβ pathway; Regulation of actin cytoskeleton; Proliferation and differentiation of skeletal muscle; Ribosomal biogenesis and neuronal polarization and migration
Gas7	Regulation of N-WASP/FAK/F-actin and hnRNP U/β-TrCP/β-catenin pathways in lung cancer; Regulation of neuronal cell morphology via microtubule and actin filament assembly
Mutyh	Base excision repair; Oxidative damaged gene repair
Vat1	Immune system and neutrophil degranulation
Bloc1s2	Apoptosis
Bag2	Cellular responses to stress; Protein processing in endoplasmic reticulum; Regulation of HSF1-mediated heat shock response
RFwd2	P53 signaling pathway; P53-Dependent G1/S DNA damage checkpoint; Autodegradation of the E3 ubiquitin ligase COP1
Ccdc8	Microtubule cytoskeleton organization; Regulation of phosphatase activity; Post-translational protein modification
Nampt	NOD-like receptor signaling pathway; Adipogenesis; Metabolism of Nicotinate; Metabolism of water-soluble vitamins and cofactors
Chek1	ATM signaling pathway; ATR signaling; DNA repair; Regulation of TP53 activity through phosphorylation; Regulation of p53 signaling pathway and retinoblastoma (RB) in cancer
Cysltr1	GPCR downstream signaling; Endothelins; Leukotriene receptors; Gastrin-CREB signaling pathway via PKC and MAPK
Dimt1	rRNA modification in the nucleus and cytosol; Gene expression

TABLE 3. Genes involved in the EMT pathways regulated by the upregulated microRNAs in Triciribine-treated advanced *TRAMP* prostates compared to DMSO-treated *TRAMP* prostates

Genes regulated by miRNAs	EMT genes
PPARG	TGFβ1, N-cadherin, Vimentin, Fibronectin, MMPs
CUX1	TGFB1 migratory effect
ID2	α-SMA
MTDH	N-cadherin, Vimentin, Snail, Fibronectin
HS3ST3B1	Snail
Slc39a6	Snail
Vsnl1	Snail
Flt1	N-cadherin, Vimentin
Hspb1	Snail, Vimentin
Mta3	Snail
Snai1	Snail
Ror2	Snail
Pin1	Snail
Hpse	α-SMA, Vimentin, Fibronectin, MMP9
Tcf7	MMP7
Sema7a	TGFβ1-induced EMT
Rgs3	Snail
Lep	N-cadherin, Vimentin, Snail, Fibronectin, Slug
Dlx4	Twist, Snail
Nfic	Vimentin
Tnc	α-SMA, Fibronectin
Foxm1	Snail, Vimentin, ZEB1, ZEB2
Srf	RhoA

TABLE 4. Genes involved in the EMT pathways regulated by the downregulated microRNAs in Triciribine-treated advanced *TRAMP* prostates compared to DMSO-treated *TRAMP* prostates

Genes regulated by miRNAs	EMT genes
TGFBR1	Slug
HMGA2	N-cadherin, Vimentin, Snail, Slug, Twist
RGS3	Snail
FSCN1	Vimentin, Snail
DAB2	N-cadherin
MTDH	N-cadherin, Vimentin, Snail, Fibronectin
MAP3K7/TAK1	N-cadherin, Vimentin, Fibronectin
MAPK8	HMGA2 and FSP-1
EGFR	Twist-1, N-cadherin, Vimentin, Fibronectin, MMP9
COL8A1	Vimentin, FSP-1 and MMP2
EZH2	Fibronectin, N-cadherin, Vimentin, Slug
GAB2	ZEB1
HS3ST3B1	Snail

CHAPTER 6

miR-199a-5p and let-7a-5p regulated by Akt1 modulate prostate cancer epithelial-to-mesenchymal transition via transforming growth factor-β type-I receptor

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Abstract

Although Akt1 suppression in the advanced prostate cancer (PCa) promotes metastasis, how Akt1 orchestrates this is far from clear. The focus of this study was to identify the miRNAs responsible for Akt1-mediated PCa epithelial-to-mesenchymal transition (EMT). NanoString® based miRNA and mRNA profiling of PC3 and DU145 cells and their analysis by mirPath, dbEMT and the Ingenuity databases were carried out. cBioportal genomic database and RT-PCR analysis of PCa patient tissues were performed to determine a causal link between miR-199a-5p, let-7a-5p and TGFβ-RI expression with respect to the Gleason score. Locked nucleic acid (LNA) miR-199a-5p inhibitor and/or let-7a-5p mimic were used to test their effects on TGFβ-RI expression, EMT, motility, and invasion of PC3 and DU145 cells. Akt1 loss in PC3 and DU145 cells predominantly induced changes in the miRNA and mRNA profiles that regulated EMT. These include increased miR-199a-5p and decreased let-7a-5p expression associated with increased TGFβ-RI expression. LNA-miR-199a-5p inhibitor or let-7a-5p mimic effects confirmed their EMT inducing and inhibiting effects, respectively, correlating to their anticipated effects on cell motility, invasion, and TGFβ-RI expression. A correlation between increased miR-199a-5p and TGFβ-RI expression with reduced let-7a-5p was observed in high Gleason score PCa tissues and cBioportal database. Akt1-mediated miR-199a-5p and let-7a-5p expression changes modulated TGFβ-RI expression and EMT in PCa, highlighting the potential benefits of miR-199a-5p and let-7a-5p in therapy and/or early screening of mPCa.

Keywords: Akt1; miR-199a-5p; let-7a-5p; TGFβ; prostate cancer

6.1.Introduction

Mortality in prostate cancer (PCa), the second leading cause of cancer-related deaths among men in the United States [1], is primarily due to its metastasis [2, 3]. Mechanistically how metastatic PCa (mPCa) emerges from the primary tumors is a current gap in our knowledge base, in turn, a major roadblock in its therapeutic development and the primary reason for high mortality. Digital rectal examination and serum prostate-specific antigen, two gold standard screening methods used for PCa detection and follow up, however, have very low sensitivity and high non-specificity [4]. Therefore, there is an urgent need for more accurate diagnostic and predictive tools as well as improved therapies for mPCa.

The transforming growth factor-β (TGFβ) signaling has long been demonstrated to be a growth suppressor in the cells of epithelial origin, which is the major source of solid tumors [4, 5]. A tumor suppressor in the early stages, TGFβ signaling switches into a promoter of cancer cell invasion and metastasis [6, 7]. It is an adaptive response by the advanced stage cancer cells to differentiate into a different cell type in order to evade the growth suppressive effects of TGFβ [5, 8]. Our laboratory has demonstrated both the growth suppressive [9] and epithelial-to-mesenchymal transition (EMT) inducing effects of TGFβ in PCa [10]. EMT, the process of cancer cell differentiation to a mesenchymal phenotype, is a pre-requisite for cancer cell invasion [11] and dissemination [12].

Interestingly, we noticed an amazing paradoxical correlation between TGFβ and Akt pathway in the regulation of early and late stage PCa. Being a growth suppressor, Akt inhibition in TGFβ-stimulated PCa cells [7, 13] and endothelial cells [14] did not surprise us. However, the

finding that suppression of Akt1 is a crucial step in the acquisition of PCa EMT and promotion of metastasis was remarkable [15]. Similar effects were observed in Akt1 deficient endothelial cells promoting endothelial-to-mesenchymal transition [16, 17] and promoting PCa metastasis [18]. Furthermore, human PCa tissues with high Gleason score showed reduced phosphorylated Akt1 expression/activity and enhanced TGFβ level compared to lower Gleason score tissues [*Alwhaibi et al, Oncoscience, 2019, In Press*]. Interestingly, Akt1 gene deletion or pharmacological Akt suppression using triciribine or MK2206 activated various signaling molecules that belong to the TGFβ superfamily [15, 19, 20] indicating the existence of an Akt1-TGFβ signaling axis in PCa metastasis. A paradoxical effect of Akt1 suppression on metastasis promotion has also been reported in other types of cancers [21] but with limited molecular insights.

MicroRNAs (miRNAs or miRs) are small non-coding RNAs that post-transcriptionally regulate gene expression by binding to the 3' untranslated regions (3' UTR) of the target mRNAs [22]. Several recent studies have implicated the importance of miRNAs in the regulation of TGFβ signaling [23, 24], EMT and cancer metastasis [25], including PCa [20, 26]. Studies also support the use of miRNAs as prognostic or predictive biomarkers of PCa [PMID: 17616669 [27]. However, having discovered over 2000 miRNAs in humans that regulate one-third of the total gene pool in the genome [28], our understanding of the miRNAs regulating cancer metastasis is still in the primitive stage. Akt1 has been demonstrated to regulate miR-200a-5p in TGFβ-induced EMT in human breast cancer cells [29]. Apart from this, Akt substrate FoxO is a master regulator of miRNAs in cancer cells [30]. Hence it is important to profile the Akt1 regulated miRNAs in mPCa and study their involvement in the modulation of TGFβ pathway.

In the current study, on a NanoString® platform, we profiled Akt1 regulated miRNAs in human PC3 and DU145 mPCa cells and identified miR-199a-5p and let-7a-5p as key Akt1 regulated miRNAs in the activation of TGFβ pathway and promotion of EMT. Bioinformatics analysis, data mining from the cBioportal database, and RT-PCR analysis of PCa biopsies confirmed increased miR-199a-5p and decreased let-7a-5p expression in high Gleason score compared to low Gleason score PCa tissues associated with increased TGFβ type-I receptor (TGFβ-RI) expression. Treatment with miR-199a-5p inhibitor and/or let-7a-5p mimic diminished TGFβ-RI expression and EMT marker expression in PC3 and DU145 cells, in turn, inhibiting their migration and invasion. Our study supports the use of miR-199a-5p and let-7a-5p for early detection and treatment of mPCa.

6.2. METHODS AND MATERIALS

- ***Cell culture, shRNA gene silencing, antibodies, and other reagents***

Androgen-independent human PCa (PC3 and DU145) cells were purchased from ATCC (Manassas, VA), grown and maintained in DMEM high glucose medium (Hyclone, Logan, UT) with 10% FBS (Atlanta Biologicals, GA), 100 U/ml penicillin, and 100 mg/ml streptomycin in a humidified incubator at 37 °C and 5% CO_2. Gene silencing was performed using SMARTvector 2.0 Lentivirus ShControl (non-targeting) and ShAkt1 (5′-3′: ACGCTTAACCTTTCCGCTG) from Dharmacon (Lafayette, CO), followed by selection with 0.6 ng/ml puromycin (Sigma, St. Louis, MO). Primary antibodies against TGFβ-RI (Cat# 31013) was purchased from Abcam (Cambridge, MA). Antibodies for Snail (Cat #3879), E-cadherin (Cat #3195), N-cadherin (Cat #4061), and cleaved caspase-3 (Cat#9664) were purchased from Cell Signaling (Danvers, MA).

- ***NanoString® based miRNA and mRNA profiling***

ShControl and shAkt1 cells were used for the miRNA and mRNA analysis. Briefly, cells were plated in 6-well plates until 60-70 % confluence, washed 2 times with 1X PBS, lysed for RNA isolation using RNeasy Plus Mini Kit (Qiagen, Cat# 73414) according to the manufacturer's instructions. The purity and concentration of RNA were determined using a NanoDrop (Thermo Scientific) and the quality of miRNA was analyzed using an Agilent 2100 Bioanalyzer. nCounter gene fusion panel (NanoString Technologies®, Inc.) was used to determine the copy numbers of miRNA and mRNA targets in each sample according to the manufacturer's instructions. Changes in the miRNA and mRNA targets were normalized to a set of housekeeping genes in the designed panels. After normalization, positive ratio values indicated higher gene expression in ShAkt1 compared to Shctrl cells, while negative values indicated higher genes expression in Shctrl compared to ShAkt1 group. All ratios were presented with P values. Only significantly changed miRNA and mRNA were considered for further analysis.

- ***In vivo miRNA isolation and quantitative RT-PCR***

miRNAs were extracted from tumor nodules isolated from the lungs of nude mice that were injected with either shctrl or shAkt1 DU145 cells and sacrificed after 16 days from the injection. miRNAs extraction was carried out using miRNeasy Mini Kit (Cat# 1038703) and RNeasy MiniElute Cleanup Kit (Cat#74204) purchased from Qiagen and according to the manufacturer's protocols. Nanodrop analyzer (Thermo Scientific, Inc.) was used to test RNA samples purity and concentration. cDNA synthesis was performed using the miScript II RT Kit (Cat#218161, Qiagen). miScript SYBR® Green PCR Kit (Cat#218075, Qiagen) was used for real-time qRT- PCR. The primers for *hsa*-miR-199a-5p is (5′-3′: CCCAGUGUUCAGACUACCUGUUC) and the primer for *hsa*-let-7a-5p (5′-3′:

UGAGGUAGUAGGUUGUAUAGUU) was purchased from Qiagen. RNU6 (RNA, U6 small nuclear 2) and SNORD61 (small nucleolar RNA, C/D box) were used as normalization reference genes to analyze the expression of miRs.

- ***miRNA transfection***

Locked Nucleic Acid (LNA) technology developed, highly stable and efficient human miR-199a-5p inhibitor (5′-3′: CCCAGUGUUCAGACUACCUGUUC; Cat# YI04101096), let-7a-5p mimic (5′-3′: UGAGGUAGUAGGUUGUAUAGUU; Cat# YM00470408), and their negative controls (Cat# YI00199006 and Cat# YM00479902, respectively) were purchased from Qiagen. miRNA transfection was carried out using lipofectamine 2000 (Invitrogen, USA) in Opti-MEM (Thermo Scientific). Cells were incubated in 6-well plates until 60-70% confluent. A final concentration of 25 nM miR-199a-5p inhibitor and 10 nM let-7a-5p mimic were transfected into the target cells and further incubated for a total of 72 hours.

- ***Western blotting***

Western blotting was performed as published previously [18]. Briefly, the whole cell lysates were prepared using 1X RIPA lysis buffer (Millipore, Temecula, CA) supplemented with protease and phosphatase inhibitor tablets (Roche Applied Science, Indianapolis, IN). Protein concentration was measured by the DC protein assay (Bio-Rad Laboratories, Hercules, CA) and approximately 30ug to 40 µg of cell lysates mixed with 2X Laemmle's buffer were used. Densitometry was performed using NIH ImageJ software.

- ***mirPath v.3 and dbEMT analysis***

KEGG (Kyoto Encyclopedia of Genes and Genomes) analysis on mirPath v.3 databases (Diana.imis.athena-innovation.g) was performed to predict all targets genes or signaling pathways regulated by miRNAs. dbEMT (Epithelial-Mesenchymal Transition (EMT) gene

database) analysis was performed specifically to look into the role of known or potential genes/targets, that are regulated by each or combination of the profiled microRNAs, obtained from the KEGG and miR-Path on EMT and cancer progression/metastasis (dbemt.bioinfo-minzhao.org).

- ***Migration assay***

The migration assay was performed as previously published [31]. Cells were grown on 6-well plates until reaching 70-80% confluence. Scratch was made in the cell monolayer using 1ml pipette tip followed by a one-time wash with 1X PBS. Cells were incubated after miRNA transfection in serum-free DMEM high glucose medium for 24 hours then replaced with full medium thereafter. Images of scratches were taken immediately after scratching (0 hours) and 48 hours after transfection. The rate of migration was measured as a percentage of scratch filling using the equation ([1-T48/T0] X 100), where T48 is the area at the end point (48 hours) and T0 is the area measured immediately after making the scratches.

- ***Matrigel® invasion assay***

Matrigel invasion assay was performed as previously reported from our laboratory [10]. Briefly, 24-well Transwell® permeable plate supports with 8.0 μm polycarbonate membrane and Matrigel® were purchased from Corning Life Sciences (Tewksbury, MA). A concentration of 5mg/ml of Matrigel was used for coating supports. Briefly, 24 hours after miRNA transfection process, explained previously in the migration assay, the medium was aspirated, and cells were washed once with 1X PBS, detached using sterile 20 mM EDTA in PBS and washed once with PBS. Cells were re-suspended in serum-free medium. Using Countess automated cell counter (Invitrogen), 100,000 cells were seeded on to the Matrigel® in the upper chamber of the transwell plates filled with 100 µl of serum-free medium. Cells that invaded the matrigel and

reached the bottom layers of the supports after 48 hours incubation were fixed using 3.7 % paraformaldehyde then stained with 0.5% crystal violet solution. Three bright field images of each insert were taken using an inverted microscope and three blinded reviewers counted the invaded cells. The average number of invaded cells from every three images was calculated and considered for the analysis.

- ***Ingenuity® pathway analysis***

Ingenuity Pathway Analysis (IPA, Qiagen Bioinformatics) is a software that transforms a list of molecules into a set of relevant networks associated with pathology based on extensive records maintained in the Ingenuity Pathways Knowledge Base [20]. Highly interconnected networks are predicted to represent significant biological functions. IPA was used to map the significantly changed/shared miRNAs associated with akt1 inhibition in both cell lines to genome-wide association study (GWAS)-implicated cancer genes along with molecular canonical/non-canonical pathways and biological functions observed with various cancer. All genes that were directly affected by the upregulated or downregulated miRNAs were subjected for further analysis.

- ***De-paraffinization of FFPE PCa tissues and extraction of miRNAs***

PCa tissues with different Gleason scores were obtained from Augusta University pathology department. The use of blinded, archived PCa tissues in research was exempted by Augusta University Institutional Review Board. The tissues consisted of Gleason score 6 (3+3) (N=#), Gleason score 7 (3+4) (N=#), Gleason score 7 (4+3) (N=#), Gleason score 8 (4+4) (N=#), Gleason score 9 (4+5) (N=#), Gleason score 9 (5+4) (N=#) and Gleason score 10 (5+5) (N=#). 5μm-thick sections (7-10) were made from each specimen block, graded by a pathologist, and transferred to 1.5 mL tube each. Before RNA extraction, FFPE tumor tissues were de-

paraffinized with xylene and rehydrated with 100% ethanol, followed by incubation with Proteinase-K at 56 °C overnight (Qiagen, Hilden, Germany) for tissue digestion. miRNAs were extracted using a miRNeasy FFPE Kit from Qiagen (Cat# 217504), reported as the best kit for this task [32], according to the manufacturer's protocol. The purity and concentration of miRNA samples were determined using a NanoDrop (Thermo Scientific). Total RNA was stored at − 80 °C until used. cDNA synthesis and real-time qRT- PCR for miR-199a-5p and let-7a-5p were performed as explained previously.

- ***cBioportal genomic data analysis of MSKCC study***

The data on the expression (log2 normalized) of miR-199a-5p and let-7a-5p as well as the corresponding clinical information published by the MSKCC team [33] was obtained from cBioPortal (https://www.cbioportal.org/). miR-199a-5p and let-7a-5p were analyzed in the dataset. To investigate the correlation of miRNAs expression with Gleason scores, patients with Gleason score 6 or 7 (n=87) were grouped together and compared to those with Gleason score 8 or 9 (n=17). The student t-test was used for the analysis of differential expression of miR-199a-5p and let-7a-5p between the two cohorts. \

6.3. Statistical analysis

All the data are presented as the mean ± standard deviation (SD) to determine significant differences between treatments and control values. We have used One-way ANOVA for groups of 3 or more and Student's two-tailed t-test for studies including 2 independent groups. Statistical analysis was performed using GraphPad Prism version 6.01 software and results are considered significant when p-value < 0.05.

6.4. RESULTS

- ***Akt1 inhibition changes the microRNA expression profile in PC3 and DU145 cells***

Expression profiling of 827 miRNAs in Akt1-silenced PC3 and DU145 human PCa cells was carried out using Nanostring® technology to identify the actual change in miRNA copy numbers with Akt1 gene ablation (Figure 1). Out of the mapped miRNAs, 222 and 125 miRNAs changed their expression significantly ($P<0.05$) in DU145 and PC3 cells, respectively, from which a total of 61 miRNAs showed matched expression. Among these, 35 mRNAs were downregulated and 26 upregulated in both cell types with Akt1 gene silencing (Figure 2). Matched miRNAs were further analyzed for their potential targets using DIANA-mirPath v 3.0 software and for their potential involvement in various cell processes promoting cancer metastasis as well as regulation of EMT gene expression based on the published studies using the db-EMT database, respectively (Figure 3A-D), and confirmed based on our Nanostring® mRNA analysis (Figure 3E). Bioinformatics analysis using Ingenuity® was performed on the matched upregulated and downregulated miRNAs to identify the mRNAs that are potentially involved in the regulation of cancer cell EMT (Supplemental Figure 1). Out of the 35 downregulated miRNAs, five miRNAs were linked to the genes regulating the TGFβ pathway. Among these, only let-7a-5p showed a negative correlation with the expression of TGFβ1 and TGFβR1 as well as the cancer cell migration and metastasis. Three out of the 26 upregulated miRNAs were linked to the TGFβ pathway. From these, only miR-199a-5p showed a positive correlation with TGFβR1, TGFβR2, proangiogenic gene expression and invasive carcinoma. Overall, from the two aggressive Akt1-silenced PCa cell lines, miR-199a-5p upregulation and let-7a-5p downregulation were found to be potentially involved in the regulation of TGFβ pathway, in turn, promoting PCa cell EMT, thus warranting further investigation (Figure 1).

FIGURE 1

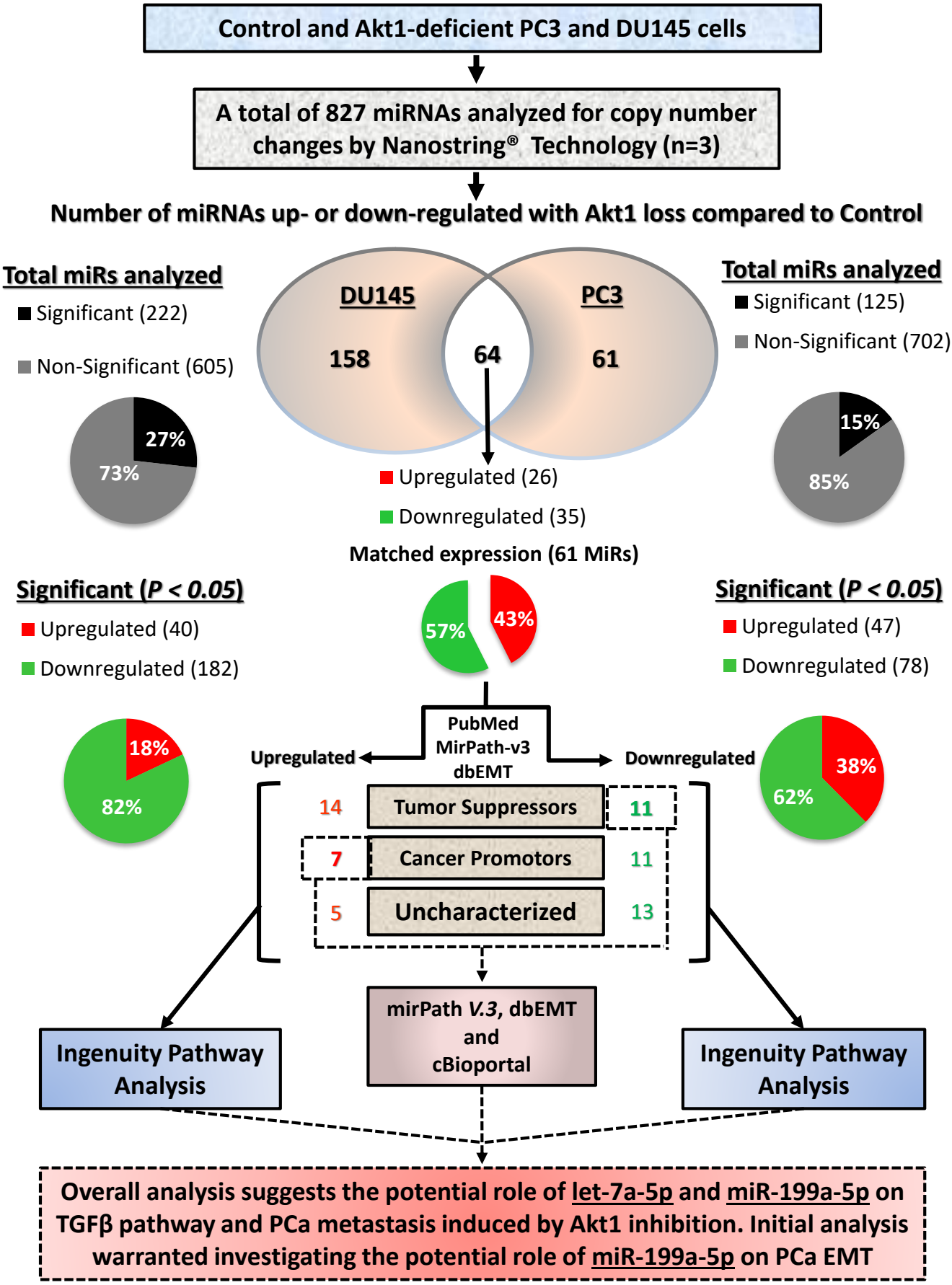

Control and Akt1-deficient PC3 and DU145 cells
A total of 827 miRNAs analyzed for copy number changes by Nanostring® Technology (n=3)
Number of miRNAs up- or down-regulated with Akt1 loss compared to Control
Total miRs analyzed
Significant (222)
Non-Significant (605)
27%
73%
DU145
158
64
PC3
61
Upregulated (26)
Downregulated (35)
Total miRs analyzed
Significant (125)
Non-Significant (702)
15%
85%
Matched expression (61 MiRs)
Significant (P < 0.05)
Upregulated (40)
Downregulated (182)
18%
82%
57%
43%
PubMed
MirPath-v3
dbEMT
Upregulated
Downregulated
14
Tumor Suppressors
11
7
Cancer Promotors
11
5
Uncharacterized
13
Significant (P < 0.05)
Upregulated (47)
Downregulated (78)
38%
62%
Ingenuity Pathway Analysis
mirPath V.3, dbEMT and cBioportal
Ingenuity Pathway Analysis
Overall analysis suggests the potential role of let-7a-5p and miR-199a-5p on TGFβ pathway and PCa metastasis induced by Akt1 inhibition. Initial analysis warranted investigating the potential role of miR-199a-5p on PCa EMT

Figure 1: Flow chart describing the profiling of Akt1-regulated miRNAs in human PC3 and DU145 PCa cells. Among the 827 miRNAs analyzed using the NanoString® technology, 222 miRNAs in DU145 cells and 125 miRNAs in PC3 cells were found to be modulated by Akt1 gene silencing. Out of the 61 matched miRNAs from DU145 and PC3 cells with Akt1 loss (26 up- and 35 down-regulated), there were 7 up-regulated cancer promoters and 11 down-regulated cancer suppressors, which were subjected for bioinformatics analysis using the Ingenuity®, mirPath *V.3*, TargetScan, dbEMT, and cBioportal databases to identify miR-199a-5p and let-7a-5p as the key Akt1-regulated miRNAs modulating PCa cell EMT.

FIGURE 2

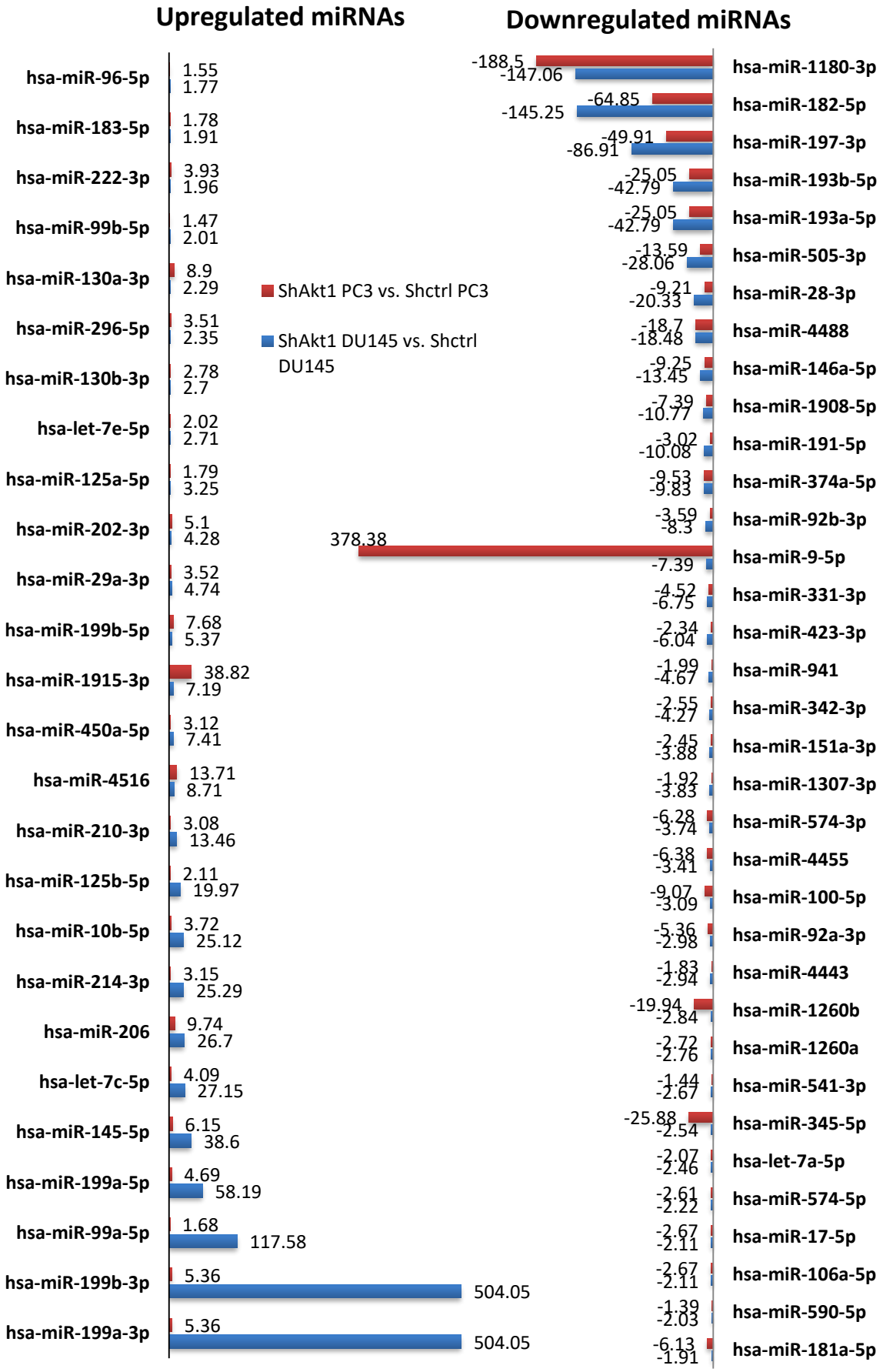

Upregulated miRNAs
Downregulated miRNAs
ShAkt1 PC3 vs. Shctrl PC3
ShAkt1 DU145 vs. Shctrl DU145
hsa-miR-96-5p 1.55 1.77
hsa-miR-183-5p 1.78 1.91
hsa-miR-222-3p 3.93 1.96
hsa-miR-99b-5p 1.47 2.01
hsa-miR-130a-3p 8.9 2.29
hsa-miR-296-5p 3.51 2.35
hsa-miR-130b-3p 2.78 2.7
hsa-let-7e-5p 2.02 2.71
hsa-miR-125a-5p 1.79 3.25
hsa-miR-202-3p 5.1 4.28
hsa-miR-29a-3p 3.52 4.74
hsa-miR-199b-5p 7.68 5.37
hsa-miR-1915-3p 38.82 7.19
hsa-miR-450a-5p 3.12 7.41
hsa-miR-4516 13.71 8.71
hsa-miR-210-3p 3.08 13.46
hsa-miR-125b-5p 2.11 19.97
hsa-miR-10b-5p 3.72 25.12
hsa-miR-214-3p 3.15 25.29
hsa-miR-206 9.74 26.7
hsa-let-7c-5p 4.09 27.15
hsa-miR-145-5p 6.15 38.6
hsa-miR-199a-5p 4.69 58.19
hsa-miR-99a-5p 1.68 117.58
hsa-miR-199b-3p 5.36 504.05
hsa-miR-199a-3p 5.36 504.05
-188.5 -147.06 hsa-miR-1180-3p
-64.85 -145.25 hsa-miR-182-5p
-49.91 -86.91 hsa-miR-197-3p
-25.05 -42.79 hsa-miR-193b-5p
-25.05 -42.79 hsa-miR-193a-5p
-13.59 -28.06 hsa-miR-505-3p
-9.21 -20.33 hsa-miR-28-3p
-18.7 -18.48 hsa-miR-4488
-9.25 -13.45 hsa-miR-146a-5p
-7.39 -10.77 hsa-miR-1908-5p
-3.02 -10.08 hsa-miR-191-5p
-9.53 -9.83 hsa-miR-374a-5p
-3.59 -8.3 hsa-miR-92b-3p
378.38 -7.39 hsa-miR-9-5p
-4.52 -6.75 hsa-miR-331-3p
-2.34 -6.04 hsa-miR-423-3p
-1.99 -4.67 hsa-miR-941
-2.55 -4.27 hsa-miR-342-3p
-2.45 -3.88 hsa-miR-151a-3p
-1.92 -3.83 hsa-miR-1307-3p
-6.28 -3.74 hsa-miR-574-3p
-6.38 -3.41 hsa-miR-4455
-9.07 -3.09 hsa-miR-100-5p
-5.36 -2.98 hsa-miR-92a-3p
-1.83 -2.94 hsa-miR-4443
-19.94 -2.84 hsa-miR-1260b
-2.72 -2.76 hsa-miR-1260a
-1.44 -2.67 hsa-miR-541-3p
-25.88 -2.54 hsa-miR-345-5p
-2.07 -2.46 hsa-let-7a-5p
-2.61 -2.22 hsa-miR-574-5p
-2.67 -2.11 hsa-miR-17-5p
-2.67 -2.11 hsa-miR-106a-5p
-1.39 -2.03 hsa-miR-590-5p
-6.13 -1.91 hsa-miR-181a-5p

Figure 2: Bar graphs showing fold changes in the matched miRNAs in DU145 and PC3 cells with Akt1 gene silencing. The graphs show up- and down-regulated miRNAs in Akt1 silenced DU145 and PC3 cells compared to respective Akt1 intact controls as analyzed by NanoString®.

Only changes in the miRNA that are statistically significant ($P < 0.05$) are shown (n=3). Data are presented as mean ± SD.

- ***Human PCa tissues with high Gleason score express higher miR-199a-5p and lower let-7a-5p compared to the low Gleason score tissues***

In order to validate the clinical relevance of our findings, miR-199a-5p, and let-7a-5p expression was determined in the published MSKCC study dataset of human PCa specimens with two different cohorts of high and low Gleason scores. Clinicopathological characteristics of 113 patients with miRNAs are summarized in Figure 4B. A total of 104 patients were classified into two groups on their Gleason score to low (6-7; n=87) and high (8-10; n=17). The expression of miR-199a-5p was significantly elevated and let-7a-5p significantly reduced in the high compared to the low Gleason score group (Figure 4B and D, respectively). These results suggested that the elevation of miR-199a-5p and inhibition of let-7a-5p expression could be linked to their higher tumor grade and, potentially, tumor cell EMT and metastasis. Further analysis of mRNAs based on the median miR-199a-5p and let-7a-5p expression confirmed the significant positive correlation between miR-199a-5p and TGFβRI expression (Figure 5A-B) with a trend toward the elevation of TGFβ1 expression with higher miR-199a-5p levels (Figure 5C). Surprisingly, however, increased let-7a-5p expression was associated with a significant increase in TGFβRI with a trend toward inhibition of TGFβ1 expression (Figure 5D-F).

- ***Transfections with miR-199a-5p inhibitor and/or let-7a-5p mimic impairs PC3 and DU145 cell invasion***

In order to confirm that let-7a-5p and miR-199a-5p expressions were modulated in Akt1 deficient PC3 and DU145 cells, we collected control and Akt1-deficient DU145 tumor colonies from the mouse lungs previously administered with these cells via tail-vein. Analysis of these colonies revealed decreased let-7a-5p and increased miR-199a-5p with Akt1 gene deletion

FIGURE 3

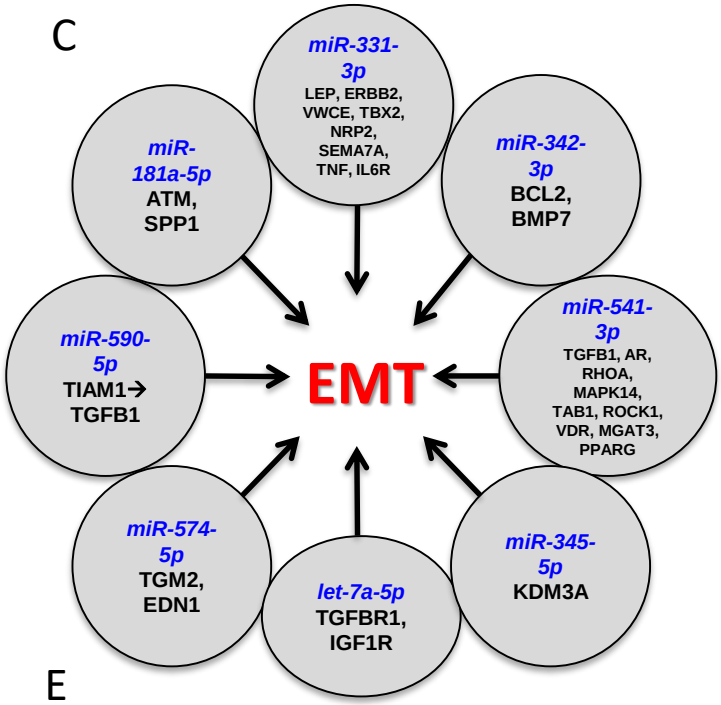
A

miRNAs	EMT genes
miR-181a-5p	α-SMA, TGFβ, p.Smad2/3
miR-590-5p	VIM
miR-574-5p	ZEB1, VIM, N-cad, Snail, TGFβ1, p.Smad3, α-SMA
let-7a-5p	Slug, Runx2, VIM, ZEB1, Twist, Snail
miR-345-5p	N-cad, Twist1
miR-541-3p	p.Smad2/3, α-SMA, VIM, Snail, Slug, α-SMA, Fibronectin, MMP9, ZEB1
miR-342-3p	MMP9, N-cadherin, VIM, α-SMA
miR-331-3p	VIM, N-cadherin, Fibronectin, TGFβR1, TGFβ1, p.Smad2/3, VIM, α-SMA, Snail, Twist1, STAT3
miR-106a-5p	VIM, Fibronectin, α-SMA, N-cadherin

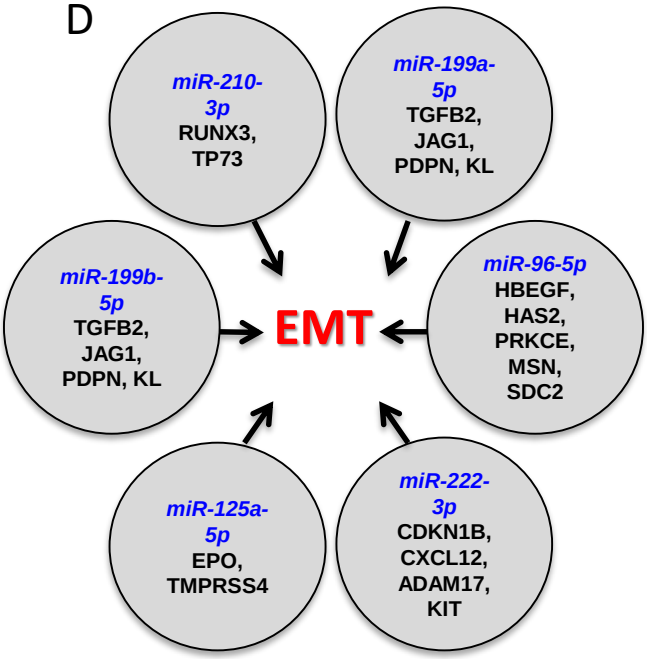
B

miRNAs	EMT genes
miR-199b-5p miR-199a-5p	TGFB2, α-SMA, Snail, Vim, RHOA, Slug, Twist, N-cad, MMP7, MMP9
miR-125a-5p	ZEB2, Integrin-α5
miR-222-3p	TGFβ1
miR-96-5p	Slug, TGFβ2

C



D



E

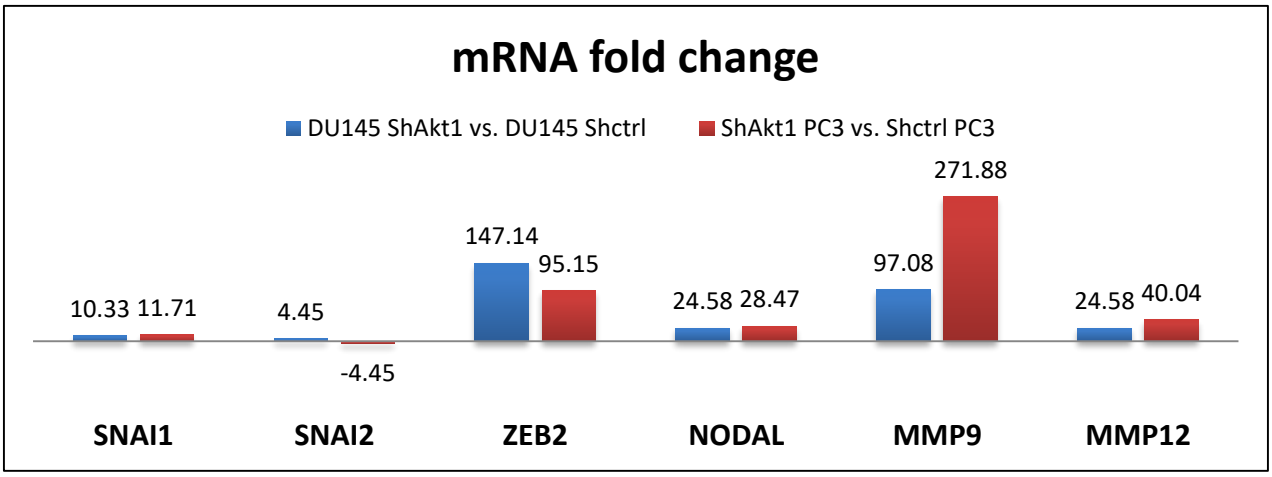
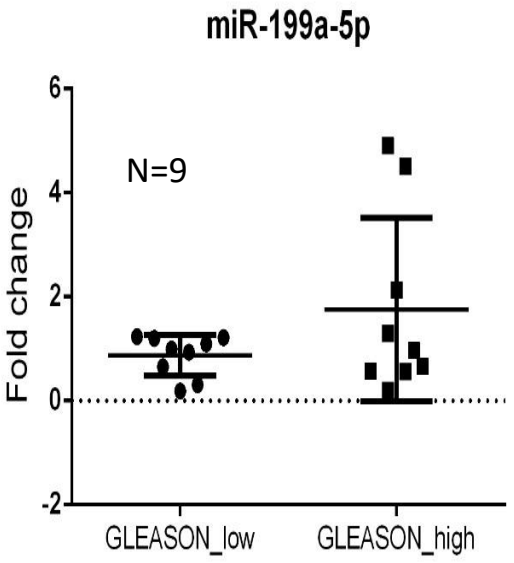


Figure 3: KEGG and Gene Ontology (mirPath) analysis indicate modulations in EMT and metastasis regulating miRNAs regulated by Akt1 in PC3 and DU145 PCa cells. (A and C) Chart showing highly upregulated miRNAs in *Akt1*$^{-/-}$ PC3 and DU145 cells compared to *Akt1* intact controls, and their predicted and known targets indicating their predominant involvement in the regulation of EMT and metastasis. **(B and D)** Diagram showing highly down-regulated miRNAs in *Akt1*$^{-/-}$ PC3 and DU145 cells compared to *Akt1* intact controls, and their predicted and known targets indicating their predominant involvement in the regulation of EMT and metastasis. **(E)** Bar graph showing highly upregulated EMT regulating mRNAs in *Akt1*$^{-/-}$ PC3 and DU145 cells compared to *Akt1* intact controls identified by NanoString® mRNA analysis.

Only changes in the miRNA that are statistically significant ($P < 0.05$) are shown (n=3). Data are presented as mean ± SD.

FIGURE 4

A



B

Characteristics	No. of patients (N=113)	%
Age [years]	Unknown	
Gleason score		
6	32	
7	55	
8	9	
9	10	
10	0	
NA	7	
T stage [pT]		
T1	65	
T2	40	
T3	3	
T4	1	
NA	4	
N stage [pN]	Unknown	
M stage [pM]	Unknown	
Survival status	Unknown	

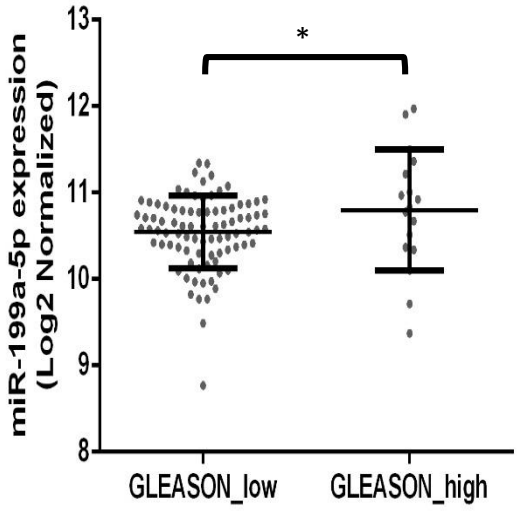
C

Characteristics (Number of patients)	Mean miR-199a-5p expression (Log2 Normalized)	P value
Gleason score		
Low; 6-7 (87)	10.54	0.0465
High; 8-9 (17)	10.80	

D

Characteristics (Number of patients)	Mean let-7a-5p expression (Log2 Normalized)	P value
Gleason score		
Low; 6-7 (87)	13.78	0.0371
High; 8-9 (17)	13.35	

E



F

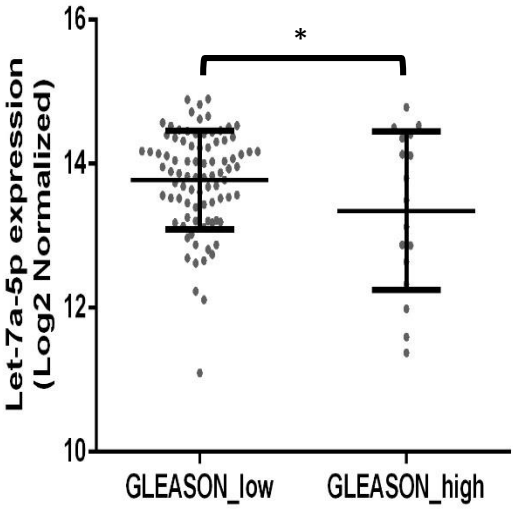


Figure 4: miR-199a-5p and let-7a-5p expression changes correlate with PCa Gleason score. (A) The table shows the characteristics of 113 (those with miR-199a-5p and let-7a-5p expression determined) out of 217 patients with prostatic adenocarcinoma of the TCGA study [Prostate adenocarcinoma (MSKCC, Cancer Cell 2010)]. **(B-C)** Data showing the mean normalized Log2 miR-199a-5p and let-7a-5p expression changes, respectively, in the high and low Gleason score cohorts. 104 out of 113 patients were included in the analysis (seven were excluded with unknown Gleason Scores). **(D-E)** Comparison between the two cohorts showing that miR-199a-5p expression is significantly up-regulated in the higher (8-9) compared to lower Gleason score (6-7) samples (N=17 and 87, respectively) indicating the EMT and metastasis-promoting the role of miR-199a-5p. In contrast, let-7a-5p expression was significantly down-regulated in the higher (8-9) compared to lower (6-7) Gleason score cohort (N=17 and 87, respectively) indicating its potential anti-metastatic role in PCa.

* ($P < 0.05$). Data are presented as mean ± SD.

(Figure 6A). Based on our TargetScan 7.2 software analysis, we identified that let-7a-5p has a direct target site on TGFβRI mRNA (Supplemental Figure 2). Transfection of DU145 cells with miR-199a-5p inhibitor (25 nM) and/or let-7a-5p mimic (10 nM) was performed to determine their effect on TGFβRI expression. Western blotting results showed that overexpression of let-7a-5p mimic or miR-199a-5p inhibitor in DU145 cells results in reduced expression of TGFβRI, compared non-target miRNA control (Figure 6B). Overexpression of let-7a-5p mimic or miR-199a-5p inhibitor in DU145 cells also resulted in impaired invasion in a Matrigel® based assay system (Figure 6C and D), with no significant effect on cell viability assessed by an MTT assay (Figure 6E). Similar to invasion, overexpression of let-7a-5p mimic or miR-199a-5p inhibitor in PC3 and DU145 cells resulted in impaired migration in a scratch assay (Figure 7A-D). There were no significant differences between single transfections with let-7a-5p mimic or miR-199a-5p inhibitor in PCa cells compared to combined transfections on TGFβRI expression, invasion and migration.

6.5. DISCUSSION

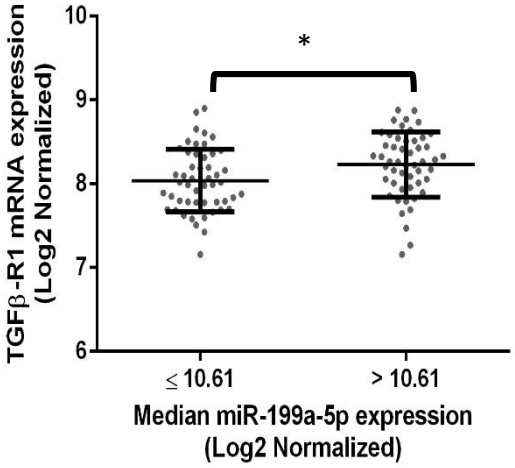
Although unexpected, a flurry of recent reports from various laboratories has demonstrated that Akt1 suppression in the advanced cancers worsens the condition by augmenting the process of EMT, in turn, promoting metastasis [21]. Interestingly, in spite of different molecular mechanisms reported by various laboratories for this unforeseen role of Akt1 in the advanced cancers, it does get reconciled at one single point, which is EMT [21]. miRNAs are one of the first set of molecules proposed to promote EMT in the absence of Akt1 expression in a breast cancer study [29]. Unfortunately, further research on the Akt1-miRNA axis in any other cancers was not performed since its first report in 2009. We recently demonstrated the role

FIGURE 5

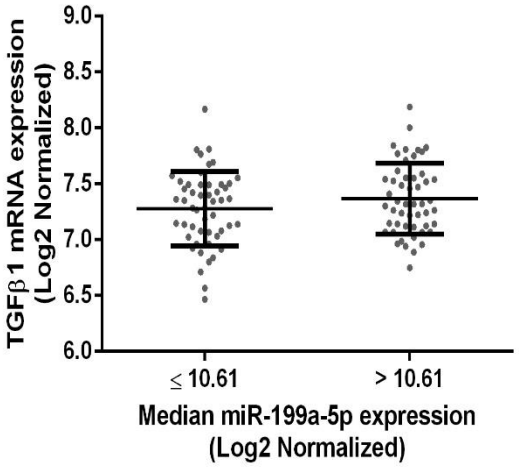
A

Median miR-199a-5p expression (Log2 Normalized)	Number of patients	Mean TGFβ-R1 mRNA expression (Log2 Normalized)	P value	Mean TGFβ1 mRNA expression (Log2 Normalized)	P value
≤ 10.611	51	8.037	0.0123	7.278	0.1699
> 10.611	51	8.229		7.367	

B



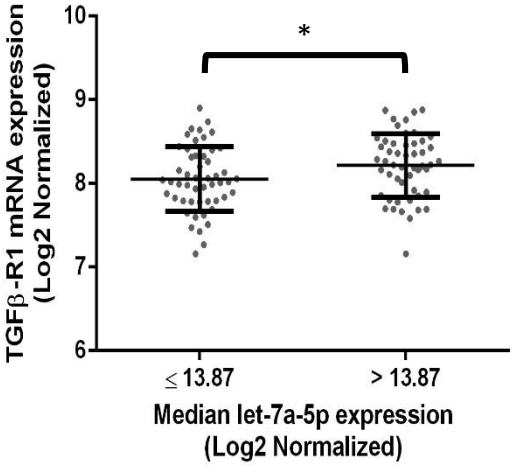
C



D

Median Let-7a-5p expression (Log2 Normalized)	Number of patients	Mean TGFβ-R1 expression (Log2 Normalized)	P value	Mean TGFβ1 expression (Log2 Normalized)	P value
≤ 13.872	51	8.052	0.0352	7.373	0.1220
> 13.872	51	8.215		7.273	

E



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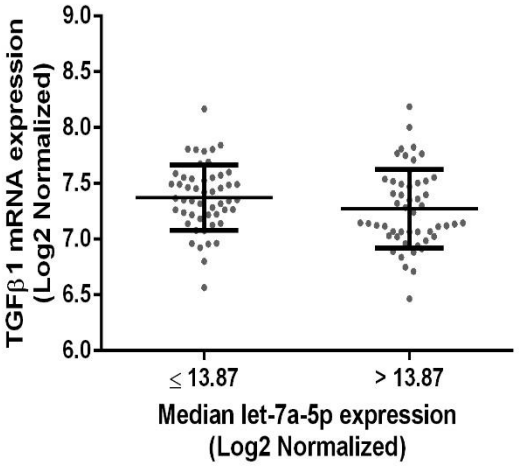


Figure 5: High miR-199a-5p and low let-7a-5p expression correlate with increased TGFβ1 and TGFβ-RI expression. (A) Data showing the mean TGFβ-RI and TGFβ1 expression from the TCGA study based on the median miR-199a-5p expression. **(B-C)** Comparison based on the median miR-199a-5p expression of 102 patients out of the 113 total patients (11 were excluded; 7 with unknown Gleason score, 2 outliers and 2 with unknown TGFβ-RI and TGFβ1 expression) showing a correlation between higher miR-199a-5p expression and higher TGFβ-RI expression associated with a positive trend towards increased TGFβ1 (Log2 normalized) expression. **(D)** Data showing the mean TGFβ-RI and TGFβ1 expressions from the TCGA study based on the median let-7a-5p expression. **(E-F)** A comparison between high and low let-7a-5p expression based on the median value for 102 patients showed lower let-7a-5p expression significantly associated with lower TGFβ-RI expression and a positive trend towards increased TGFβ1 expression.

* ($P < 0.05$). Data are presented as mean ± SD.

FIGURE 6

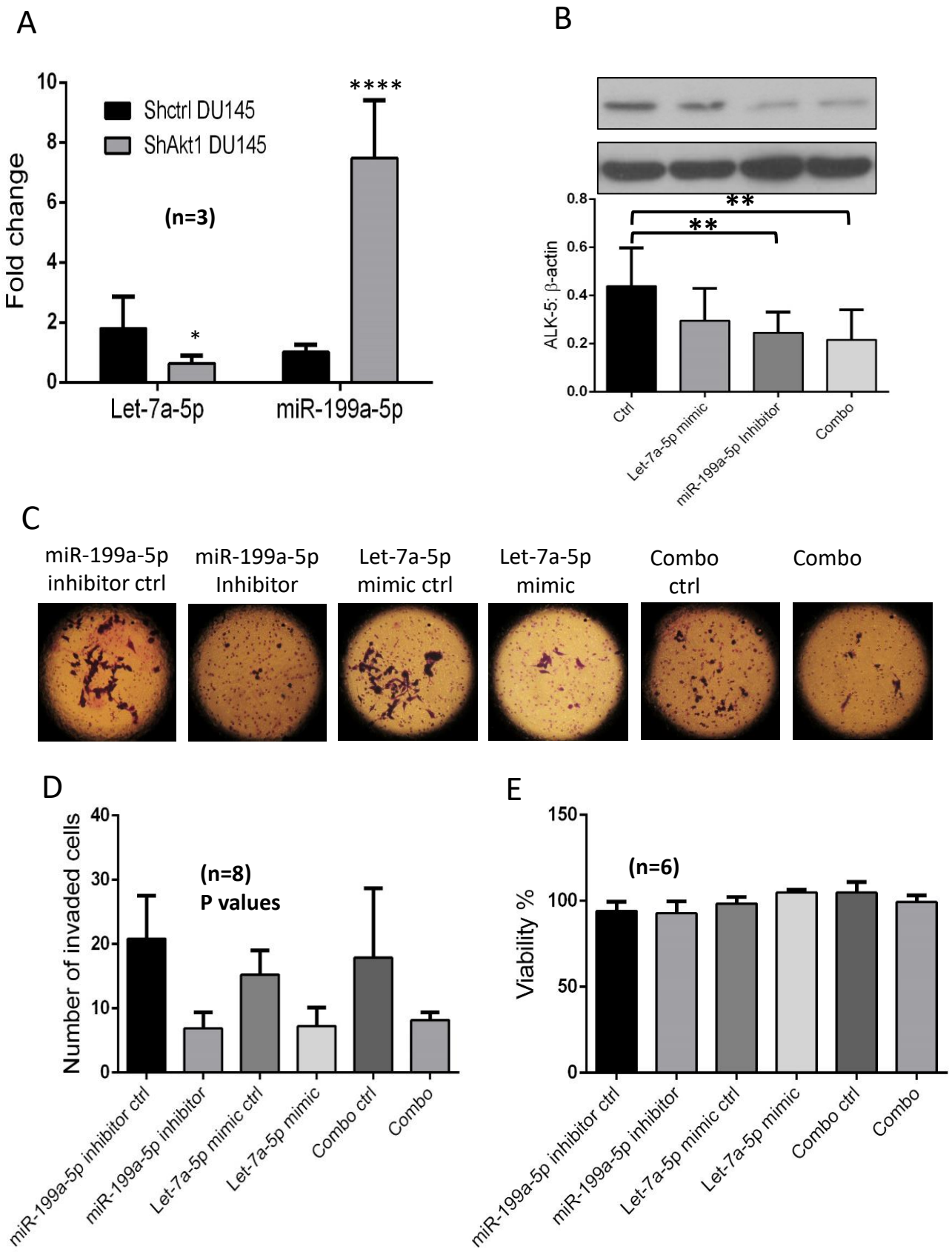

A
Fold change
Shctrl DU145
ShAkt1 DU145
(n=3)
****
*
Let-7a-5p
miR-199a-5p
B
ALK-5: β-actin
**
**
Ctrl
Let-7a-5p mimic
miR-199a-5p Inhibitor
Combo
C
miR-199a-5p inhibitor ctrl
miR-199a-5p Inhibitor
Let-7a-5p mimic ctrl
Let-7a-5p mimic
Combo ctrl
Combo
D
Number of invaded cells
(n=8)
P values
miR-199a-5p inhibitor ctrl
miR-199a-5p inhibitor
Let-7a-5p mimic ctrl
Let-7a-5p mimic
Combo ctrl
Combo
E
Viability %
(n=6)
miR-199a-5p inhibitor ctrl
miR-199a-5p inhibitor
Let-7a-5p mimic ctrl
Let-7a-5p mimic
Combo ctrl
Combo

Figure 6: Inhibition of miR-199a-5p and activation of let-7a-5p inhibited TGFβRI expression, EMT and cell invasion. (A) Quantitative real-time PCR data for miR-199a-5p and let-7a-5p in DU145 colony collected from the mouse lungs previously administered (via tail-vein) with ShControl and ShAkt1 DU145 cells shpwing increased miR-199a-5p and decreased let-7a-5p expression (n=5). **(B)** The use of miR-199a-5p inhibitor (25nM) and/or let-7a-5p mimic (10nM) in DU145 cells inhibited TGFβRI (ALK5) (n=3). **(C)** miR-199a-5p inhibition and/or let-7a-5p activation in DU145 cells resulted in impaired cell migration (n=3). **(C)** Neither miR-199a-5p inhibition and/or let-7a-5p activation in DU145 cells had any significant effect on cell viability (n=3).

* ($P < 0.05$); ** ($P < 0.01$); *** ($P < 0.001$); **** ($P < 0.0001$); unpaired Student t-test for two groups analysis (GraphPad Prism 6.01). Data are presented as mean ± SD.

of Akt-miRNA axis in the potential regulation of EMT in a TRAMP mouse model of PCa [20]. Whereas miR200a was the predominant Akt1-regulated miRNA identified in breast cancer cells, this, however, was unchanged in the Akt-suppressed TRAMP prostates suggesting that the Akt1-regulated miRNAs in different cancers types may vary based on its origin and/or specific mutations that they may hold. Furthermore, there are differences between human and mouse miRNAs in their nomenclature as well as their function and molecular regulation. Hence, it is important to profile the Akt1-regulated miRNAs in advanced cancers and study their molecular and functional implications in advanced cancers for translational purposes.

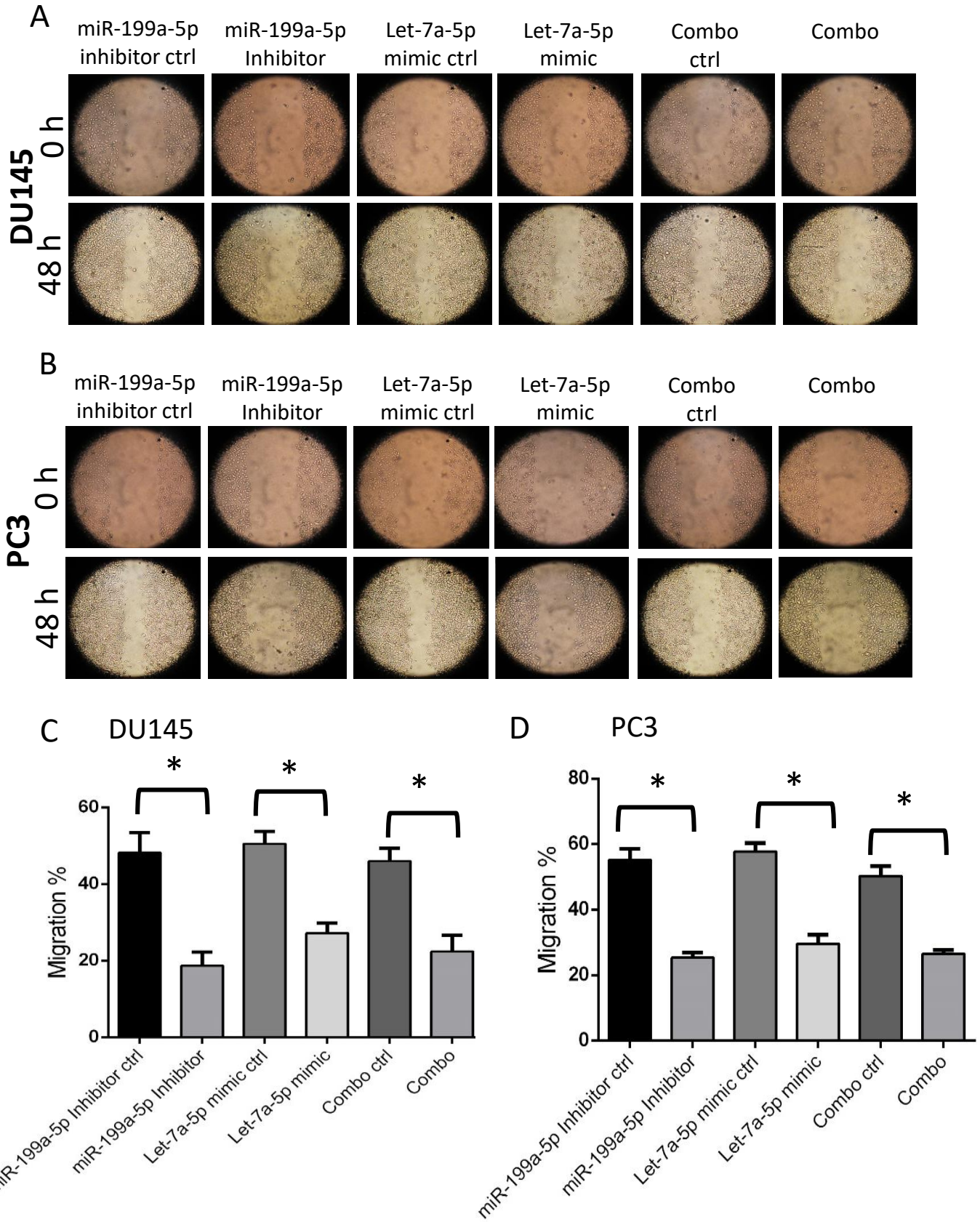
In the current study, we used Akt1 intact and Akt1 gene silenced human PC3 and DU145 metastatic PCa cells to profile their miRNA and mRNA expression in the presence and absence of the Akt1 gene. Our analysis indicated that the absence of Akt1 induced changes in the expression of several microRNAs and mRNAs in both cell types that were collectively involved in the regulation of EMT. This indicated that the miRNAs serve as key molecules in the promotion of EMT in the advanced PCa when Akt1 is inhibited. This was further supported by the bioinformatics analyses of Akt1-regulated miRNAs that were common to both the PC3 and DU145 cells. Ingenuity pathway analysis predicted increased miR-199a-5p and/or reduced Let-7a-5p as a result of Akt1 suppression in PC3 and DU145 cells responsible for the promotion of EMT, both of which reciprocally regulated the TGFβ signaling pathway. The TargetScan analysis identified a direct target of Let-7a-5p on TGFβRI mRNA, suggesting that a reduced Let-7a-5p would promote EMT by activating the TGFβ signaling pathway. Interestingly, we did not detect a direct target for miR-199a-5p on any of the TGFβ pathway molecule suggesting that the effect of miR-199a-5p on PCa cell EMT could be due to the secondary effects. The genomic analysis of PCa patient samples using the data available from the cBioportal database indicated

increased miR-199a-5p and reduced Let-7a-5p in high Gleason score compared to the low Gleason score PCa tissues, which was corroborated in our Real-Time PCR study involving PCa patient tissues. Transfections with either miR-199a-5p inhibitor or Let-7a-5p mimic resulted in impaired motility and invasion of PCa cells. Collectively, our study revealed that miR-199a-5p and Let-7a-5p regulated by Akt1 in the advanced PCa modulates the TGFβ signaling pathway, in turn, regulating EMT.

Literature indicates the important role of miRNAs in PCa [34]. Previous reports from other laboratories on the role of Let-7a-5p in cancer metastasis is highly in agreement with our findings in this study. Let-7a-5p, in combination with another long-coding RNA has shown to develop doxorubicin resistance in DU145 PCa cells [35]. Down-regulation of Let-7a-5p expression has been reported to predict lymph node metastasis and its prognosis in colorectal cancer [36]. Concomitant downregulation of let-7a-5p and let-7f-5p miRNAs have been reported in the plasma and stool samples collected from early-stage colorectal carcinoma [34, 37]. In the current study, we show for the first time that the under-expression of let-7a-5p leading to EMT in the advanced PCa is regulated by Akt1. Furthermore, our study has identified TGFβRI as the primary target of let-7a-5p in PCa cells, where overexpression with let-7a-5p mimic was observed to inhibit TGFβRI expression, PCa cell migration, and invasion. TGFβRI as the direct target of let-7a-5p was also confirmed by TargetScan software analysis. Overall, this finding is a very important step forward in our knowledge on how Akt1 downregulation in the advanced PCa suppresses let-7a-5p expression, in turn, promoting PCa cell EMT *via* increased TGFβRI expression.

Unlike let-7a-5p, the literature on the potential role of miR-199a-5p in cancer is conflicting and their potential role in the regulation of EMT in cancer is not very clear. A recent

FIGURE 7


A
miR-199a-5p inhibitor ctrl
miR-199a-5p Inhibitor
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DU145
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Combo ctrl
Combo
PC3
0 h
48 h
C
DU145
Migration %
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20
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60
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miR-199a-5p Inhibitor ctrl
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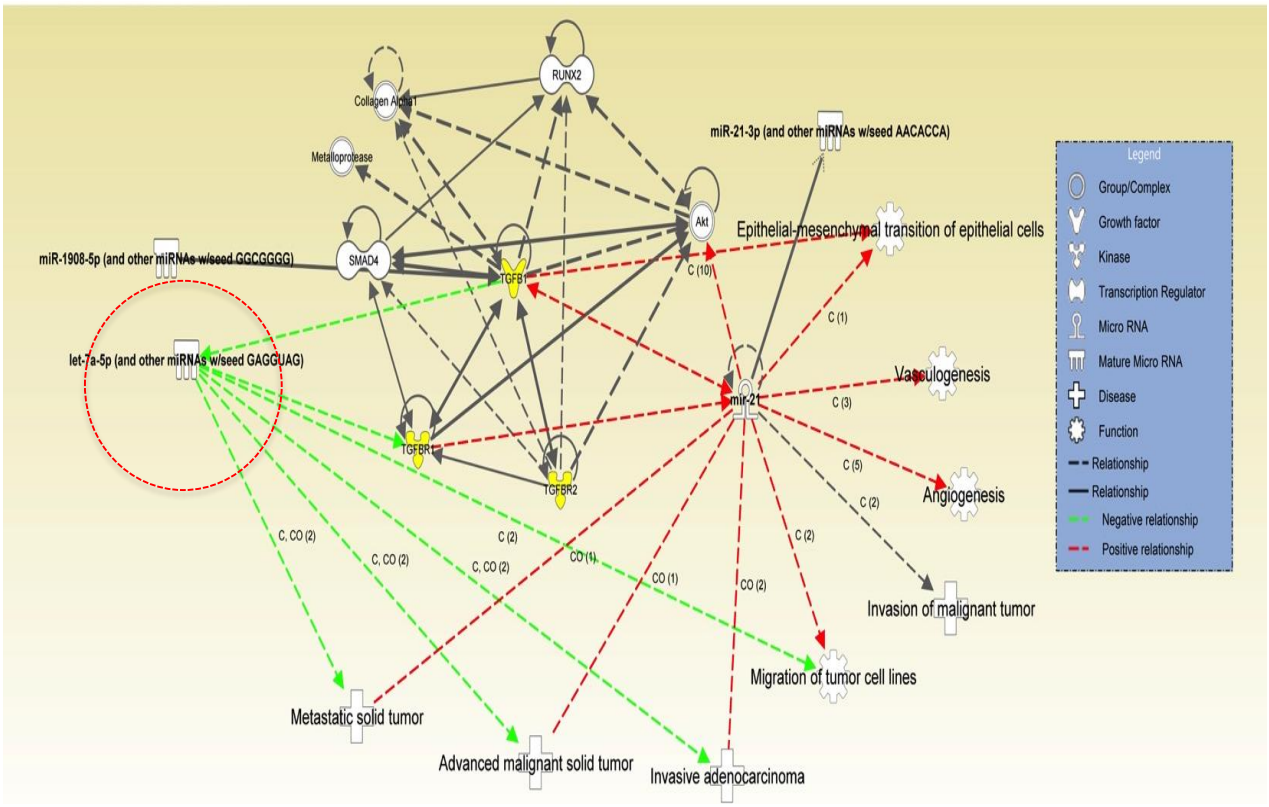
Figure 7: miR-199a-5p inhibition and/or let-7a-5p activation impairs DU145 and PC3 cell motility. (A-B) Representative images from the scratch assay (0 and 48hrs) for DU145 and PC3 cells show the regulatory role of miR-199a-5p and let-7a-5p on cell migration (n=3) **(C-D).** 25nM of miR-199a-5p inhibitor and/or 10nM of let-7a-5p mimic significantly inhibited the motility of both DU145 and PC3 cells (n=3)**].**

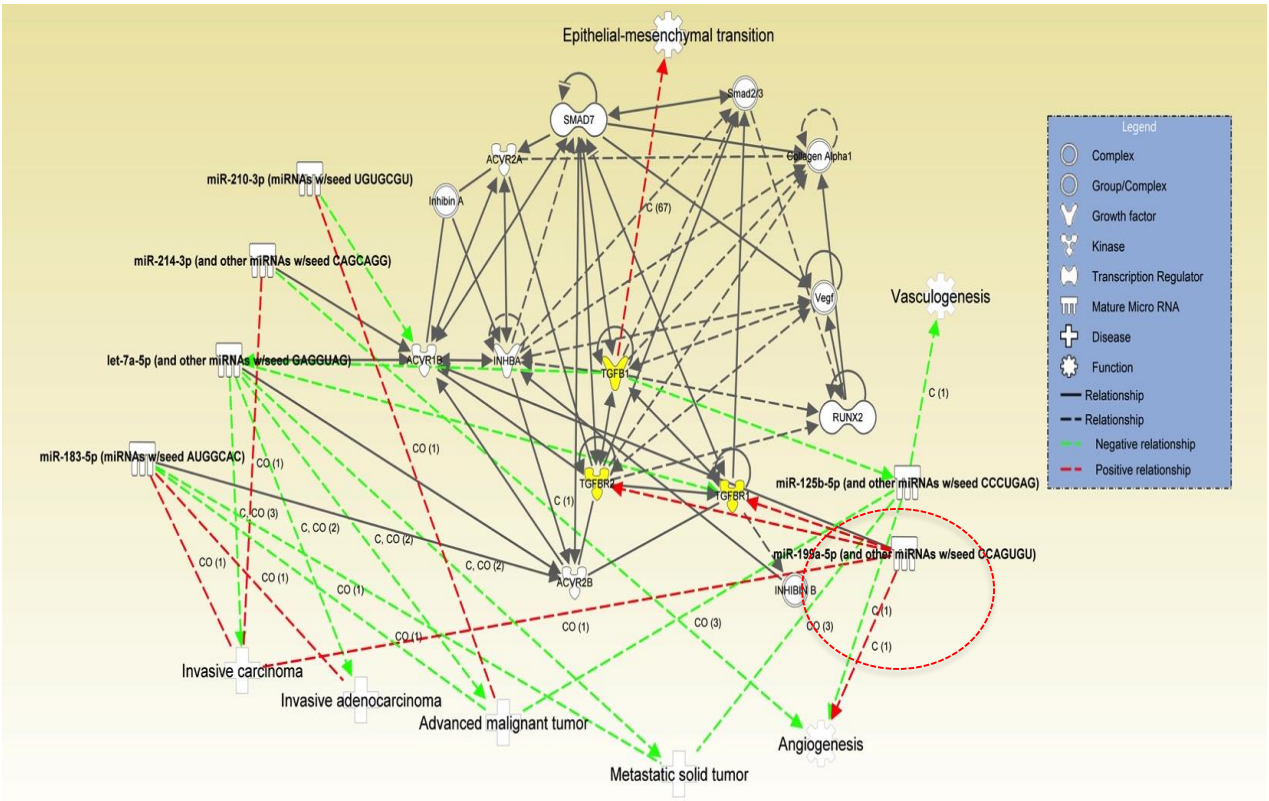
*P < 0.001. Data are presented as mean ± SD.

SUPPLEMENTAL FIGURE 1

A



B

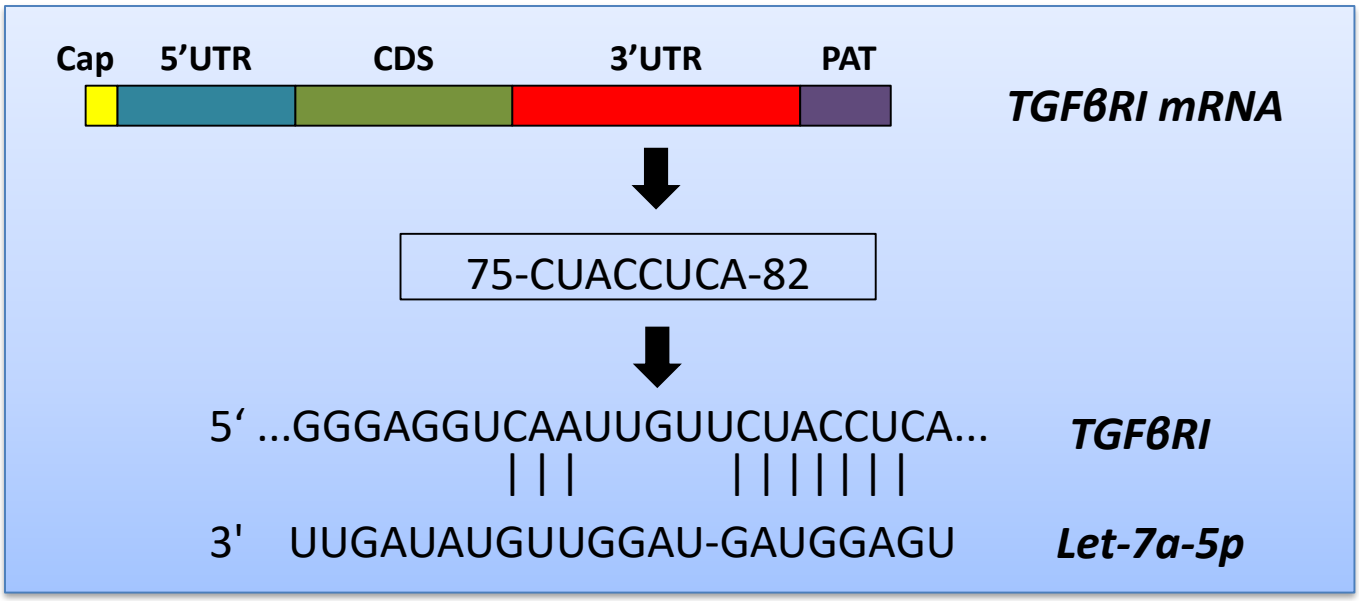


Supplemental Figure 1: Ingenuity pathway analysis of upregulated and downregulated miRNAs. (A) Ingenuity pathway analysis for all downregulated miRNAs mapped to PI3Kinase-Akt, TGFβ and Wnt signaling pathways show that out of the 35 miRNAs, 5 miRNAs only were linked to TGFβ pathway among which Let-7a-5p only showed a negative relationship to TGFβ1 and TGFβRI expression, cancer invasion and metastasis. **(B)** Ingenuity pathway analysis for all upregulated miRNAs mapped to PI3Kinase-Akt, TGFβ and Wnt signaling pathways show that out of the 26 miRNAs, 3 miRNAs only were linked to TGFβ pathway among which miR-199a-5p only showed a positive relationship to TGFβR1/R2 expression, cancer invasion, and metastasis.

study has shown reduced expression of miR-199a-5p in PCa tissues compared to the normal prostate and BPH tissues [38]. This, in fact, is highly in agreement with our findings that mir-199a-5p expression correlates with Akt1 activity. It has been demonstrated that Akt1 activity is higher in the primary prostate tumors compared to normal prostate tissues [15, 39]. Hence, a high Akt1 activity correlating to the reduced miR-199a-5p expression in a primary PCa tissue is not surprising. This might also be a cancer cell adaptation to keep the TGFβ pathway in check, which otherwise functions as a tumor suppressor in the early cancers [5]. However, when the Akt1 activity declines as the cancers progress to the advanced stages, an increase in the miR-199a-5p expression is also expected, which is what we see in Akt1 deficient PC3 and DU145 cells. cBioportal analysis and the RT-PCR analysis of patient samples show a positive correlation between miR-199a-5p and TGFβRI expression. The TGFβRI expression was also reduced when PC3 or DU145 cells were transfected with miR-199a-5p inhibitor. However, a direct target for miR-199a-5p on TGFβRI was not identified in TargetScan software analysis. This suggests that the effect of the miR-199a-5p inhibitor on TGFβRI expression could be secondary. Nevertheless, our study shows for the first time that Akt1 suppression in the advanced PCa increases miR-199a-5p expression, in turn, promoting TGFβRI expression in PC3 or DU145 cells in the regulation of cell motility and invasion.

In conclusion, our data showed that miR-199a-5p elevation and let-7a-5p inhibition resulting from Akt1 suppression in the advanced PCa activates the TGFβ pathway through increased TGFβRI expression, thus promoting PCa cell EMT. The elevated miR-199a-5p and reduced let-7a-5p expression in the high Gleason score human PCa tissues and the ability of miR-199a-5p inhibitor or let-7a-5p mimic to inhibit PCa cell migration and invasion demonstrates its potential therapeutic benefits and utility as biomarkers for mPCa.

SUPPLEMENTAL FIGURE 2



Supplemental Figure 2: Targetscan® predicted TGFβ-RI a target of let-7a-5p, but not miR-199a-5p. TGFβ-RI is a target of let-7a-5p based on Targetscan and downregulation of let-7a-5p accompanied by Akt1 inhibition is expected to increase TGFβ-RI expression. TGFβR1 does not indicate a target site for miR-199a-5p, indicating that miR-199a-5p might inhibit a gene that negatively regulates TGFβR1

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Chapter 7

Integrated summary

Metastatic prostate cancer (mPCa) is the second largest cancer-related killer in men in the US and other Western countries. Treatments for mPCa is limited due to our incomplete understanding of the molecular mechanisms leading to the disease progression and lack of reliable early screening methods, in turn, resulting in high mortality. Investigations directed towards understanding the molecular mechanisms contributing to the mPCa development is hence a high-impact area of research for the National Cancer Institute, the Department of Defense, American Cancer Society and the Veterans Affairs. One of the pre-requisites in attaining a more aggressive, motile and invasive phenotype in cancer cells is a phenomenon called epithelial-to-mesenchymal transition (EMT), which is the trans-differentiation of epithelial origin cancer cells into mesenchymal cells conferring it a less proliferative but high migratory, and invasive properties.

In our laboratory, we have demonstrated two key signaling pathways that are crucial in the induction of EMT and metastasis progression, which are transforming growth factor-β (TGFβ) and Akt pathways. Akt, also known as protein kinase-B is a serine-threonine kinase that can phosphorylate a plethora of substrates to activate or inhibit their signal transducing properties. These substrates include, but are not limited to, several other kinases, adaptor proteins, cytoskeletal proteins, phosphatases, growth factor receptors, and transcription factors, etc. Among the three

Akt isoforms (Akt1, Akt2, and Akt3), Akt1 is the most predominant and ubiquitously expressed isoform in the tumor and stromal cells whose contributions to EMT leading to mPCa is currently being investigated.

The main goal of this Ph.D. dissertation is to identify the signaling partners of Akt1 in the modulation of PCa cell EMT *in vitro* and metastasis *in vivo*. To dissect this, we first determined the activity levels of Akt in low Gleason score compared to high Gleason score human PCa biopsies as analyzed from the data available in the cBioportal repository, using archived patient samples available in the Pathology Department of Augusta University and through human PCa (PC3 and DU145) cells and murine PCa tissues. Second, we determined to see if there is a correlation between Akt1 activity and TGFβ pathway activation in high low Gleason score compared to high Gleason score human PCa, once again through a cBioportal and pathological analysis of human and murine PCa tissues. Third, we investigated the role of Nodal pathway identified through a gene array in PCa EMT as a result of Akt1 activity suppression or genetic knockdown. Forth, we profiled the mRNAs and micro-RNAs (miRNAs) regulated by Akt1 in the early and advanced stage murine and human PCa using the advanced NanoString® technology, and unveiled their physiological and pathological significance in mPCa through bio-informatics analysis using Ingenuity pathway analysis, mirPath, KEGG analysis, dbEMT database, cBioportal, TargetScan etc. Fifth, we identified two key miRNAs regulated by Akt1 in the promotion of mPCa and characterized the effects of their expression modulation on PCa cell EMT, motility, and invasion through the upregulation of TGFβ pathway.

In the 1st phase of this thesis research (Aim 1), we used a neuroendocrine TRansgenic Adenocarcinoma of the Mouse Prostate (TRAMP) model to demonstrate that the genetic ablation or pharmacological inhibition of Akt1 in mice blunts oncogenic transformation and prostate cancer (PCa) growth. Surprisingly, triciribine (TCBN)-mediated Akt inhibition in 25-week old, tumor-bearing TRAMP mice and Akt1 gene silencing in aggressive PCa cells EMT and promoted metastasis to the lungs. Mechanistically, Akt1 suppression leads to increased expression of EMT markers such as Snail1 and N-cadherin and decreased expression of epithelial marker E-cadherin in TRAMP prostate, and in PC3 and DU145 cells. Akt1 is essential for the oncogenic transformation and tumor growth in various cancers. Following this, we performed genome atlas analysis of Akt pathway molecules in human prostatic adenocarcinoma, castration-resistant PCa, neuroendocrine PCa and metastatic PCa for mutations, genetic alterations, mRNA and protein expressions and activating phosphorylations from the cBioportal data repository. Results from the protein data analysis from the cBioportal were compared to the results of our data on human PCa tissue analysis and the cellular effects of Akt1 suppression using MK-2206 on PCa cell aggressiveness. Our study indicated the possibility of a dual role for Akt1 in PCa and warranted a large-scale analysis of the early and advanced stage PCa clinical samples for further clarity.

In the next step, we focused on understanding the consequences of targeting Akt1 in the mPCa. To do this, we studied how Akt1 gene ablation in human metastatic PC3 and DU145 PCa cells activated the Nodal pathway (one of the members of the TGFβ superfamily) to promote PCa EMT and metastasis in a preclinical model. Our results showed that Akt1 loss increased Nodal expression in both PCa cells, which was accompanied by the activation of FoxO1/3a, increased

expression of EMT markers Snail and N-cadherin, and the loss of epithelial marker E-cadherin. Treatment with pan FoxO inhibitor AS1842856 abrogated the Nodal expression in Akt1 deleted PCa cells. Akt1-deficient PCa cells exhibited enhanced cell migration and invasion *in vitro*, and lung metastasis *in vivo*. Enhanced PCa cell invasion *in vitro* and lung metastasis *in vivo* in Akt1-deficient human metastatic DU145 cells were attenuated by concomitant treatment with Nodal pathway inhibitor SB505124. Nodal mRNA analysis from two genomic studies in cBioportal indicated a positive correlation between Nodal expression and high vs. low Gleason score indicating the positive role of Nodal in promoting human mPCa progression. In summary, these results demonstrated an important role for Akt1-FoxO3a-Nodal pathway in promoting PCa EMT and metastasis, and present FoxO and Nodal as two potential druggable targets to treat mPCa patients.

In the second phase of the thesis dissertation, we focused on profiling the Akt1-regulated miRNAs involved in PCa EMT and in identifying suitable candidates for targeting, and for early screening. Although our Phase-I studies demonstrated that Akt1 suppression in the advanced PCa promotes metastasis involving TGFβ and Nodal pathways, there were still several unanswered questions. The focus of this phase of the thesis was to identify the miRNAs responsible for Akt1-mediated PCa EMT, and how targeting these miRNAs can modulate PCa cell EMT and invasion. In the first step, we performed Affymetrix analysis to compare the expression profile of miRNAs in the mouse prostate tissues collected at the prostatic inter-epithelial neoplasia (PIN) stage from *TRAMP/Akt1*$^{+/+}$ versus *TRAMP/Akt1*$^{-/-}$ mice, and at the advanced stage from *TRAMP/Akt1*$^{+/+}$ mice treated with triciribine (Akt inhibitor) versus DMSO-treated control. Our study indicated that in

the early stages of PCa, Akt1 in the TRAMP prostate tumors regulated the expression of a set of miRNAs responsible for promoting cancer cell survival, proliferation, and tumor growth, whereas, in the advanced stages, a different set of miRNAs that promote EMT and cancer metastasis were expressed. In the second step, we first performed a NanoString® based miRNA and mRNA profiling of human PC3 and DU145 cells and the identified miRNAs were analyzed using the bioinformatics tools such as the MirPath, dbEMT and the Ingenuity® pathway analysis. cBioportal genomic database and RT-PCR analysis of PCa patient tissues were performed to complement the bioinformatics study findings, and to determine a causal link between miR-199a-5p, let-7a-5p and TGFβ-RI expression with respect to the Gleason score of the human PCa tissues. Locked nucleic acid (LNA) technology-based miR-199a-5p inhibitor and/or let-7a-5p mimic (that had higher stability and transfection potential compared to conventional miRNA expression modulators) were used to test their effects on TGFβ-RI expression, EMT, motility, and invasion of PC3 and DU145 cells. Akt1 loss in PC3 and DU145 cells predominantly induced changes in the miRNA and mRNA profiles that regulated EMT. These included increased miR-199a-5p and decreased let-7a-5p expression accompanied by increased expression of TGFβ-RI (activin-linked kinase-5; ALK5). LNA-miR-199a-5p inhibitor or LNA-let-7a-5p mimic effected their EMT inducing and inhibiting effects on PC3 and DU145 cells, respectively. This was further correlated to their effects on cell motility, invasion, and TGFβ-RI expression. A correlation between increased miR-199a-5p and TGFβ-RI expression with reduced let-7a-5p was observed in high Gleason score PCa tissues and cBioportal database. Akt1-mediated miR-199a-5p and let-7a-5p expression changes modulated

TGFβ-RI expression and EMT in PCa, highlighting the potential benefits of miR-199a-5p and let-7a-5p in therapy and/or early screening of mPCa.

Translational impact

In summary, this thesis provides novel insights into the role of Akt1-mediated Nodal pathway, modulation of FoxO and β-catenin transcription factors, and several miRNAs in murine and human mPCa disease progression. Our findings are clinically relevant as the pharmacological inhibition of FoxO (AS1842856), β-catenin (ICG-001), Nodal (SB505124), and ALK5 (SM431542), as well as the miRNAs such as miR-199a-5p and let-7a-5p will potentially be useful in targeting mPCa. Analysis of expression changes in miR-199a-5p and let-7a-5p may also be utilized as early screening markers for mPCa.

Appendix

Face pages of accepted/published articles

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Genetic deletion and pharmacological inhibition of Akt1 isoform attenuates bladder cancer cell proliferation, motility and invasion

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Invasion

ABSTRACT

Isoform specific expression, intracellular localization and function of Akt in bladder cancer are not known. In the current study, we identified Akt1, followed by Akt2 and Akt3 as the predominant Akt isoform in human T24 and UM-UC-3 metastatic bladder cancer cells. Whereas Akt1 is localized at the membrane, cytoplasm and nucleus, Akt2 is solely cytoplasmic and Akt3 is mostly localized in the nucleus in T24 cells. ShRNA-mediated Akt1 knockdown resulted in impaired T24 cell survival, proliferation, colony formation, migration and microinvasion. Whereas pharmacological inhibition of Akt1 resulted in impaired T24 and UM-UC-3 cell motility, viability and proliferation, effect of pharmacological inhibition by Akt2 inhibitor was limited to proliferation in T24, but not UM-UC-3 cells. Our data provide important clues on the therapeutic benefits of targeting Akt1 for bladder cancer therapy.

1. Introduction

Bladder cancer is the fourth most common cancer among men in the Western countries (Siegel et al., 2014). Lack of tools for the early detection and therapeutic strategies for metastatic bladder cancer are the major problems. Candidate genes regulating bladder cancer are still under investigation. Recent studies indicate that de-regulation of catalytic domain of PI3-Kinase (p110α), phosphatase and tensin homolog (PTEN), Akt, p53, and fibroblast growth factor receptor (FGFR) can lead to the development and growth of bladder cancer (Aveyard et al., 1999; Cairns et al., 1998; Cappellen et al., 1997; Junker et al., 2008; Knowles et al., 2009; Lopez-Knowles et al., 2006). High-grade non-invasive and invasive bladder cancers are linked with loss of PTEN and p53 activity (Ching and Hansel, 2010; Puzio-Kuter et al., 2009). Mutations or loss of PTEN has been linked to many cancers (Cairns et al., 1997; Coleman et al., 2014; Huang et al., 2014; Kim et al., 1998; Li et al., 2014; Risinger et al., 1997; Sakurada et al., 1997), including bladder cancer (Aveyard et al., 1999; Cordon-Cardo, 2008; Kanda et al., 2009) thus generating research interest in PTEN/Akt pathway in bladder cancer (Saal et al., 2007).

Akt (protein kinase B) is a serine–threonine kinase that exists in three different isoforms namely Akt1, Akt2 and Akt3 (Somanath et al., 2006). Although Akt has been demonstrated in bladder cancer (Mundhenk et al., 2011), the predominant isoform expressed in bladder cancer and its effect on oncogenic response is not yet clear. Mutation in Akt1 E17K has been reported in 2/44 (4.8%) bladder cancer cell lines and 5/184 (2.7%) bladder tumors (Askham et al., 2010). In addition, significantly higher levels of Serine-473 phosphorylated (active) Akt have been reported in primary bladder carcinoma (Harris et al., 2008; Qian et al., 2009; Wu et al., 2004). However, until today, whether Akt1 is the predominant isoform in bladder cancer cells and whether its activation is necessary for bladder cancer progression is not clear. Here, we examined isoform specific expression and subcellular localization of Akt isoforms in metastatic human T24 bladder cancer cells.

2. Material and methods

2.1. Cell Lines, reagents and antibodies

Human T24 (bladder carcinoma) cells, UM-UC-3 (bladder carcinoma) and human dermal micro-vascular endothelial cells

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Original Article

Suppression of Akt1-β-catenin pathway in advanced prostate cancer promotes TGFβ1-mediated epithelial to mesenchymal transition and metastasis

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ABSTRACT

Akt1 is essential for the oncogenic transformation and tumor growth in various cancers. However, the precise role of Akt1 in advanced cancers is conflicting. Using a neuroendocrine TRansgenic Adenocarcinoma of the Mouse Prostate (*TRAMP*) model, we first show that the genetic ablation or pharmacological inhibition of Akt1 in mice blunts oncogenic transformation and prostate cancer (PCa) growth. Intriguingly, triciribine (TCBN)-mediated Akt inhibition in 25-week old, tumor-bearing *TRAMP* mice and Akt1 gene silencing in aggressive PCa cells enhanced epithelial to mesenchymal transition (EMT) and promoted metastasis to the lungs. Mechanistically, Akt1 suppression leads to increased expression of EMT markers such as Snail1 and N-cadherin and decreased expression of epithelial marker E-cadherin in *TRAMP* prostate, and in PC3 and DU145 cells. Next, we identified that Akt1 knockdown in PCa cells results in increased production of TGFβ1 and its receptor TGFβ RII, associated with a decreased expression of β-catenin. Furthermore, treatment of PCa cells with ICG001 that blocks nuclear translocation of β-catenin promoted EMT and N-cadherin expression. Together, our study demonstrates a novel role of the Akt1-β-catenin-TGFβ1 pathway in advanced PCa.

Introduction

Despite the early screening methods and hormone ablation therapies, prostate cancer (PCa) still remains the second leading cause of cancer-related mortality [1] in men in the western countries due to a higher incidence of metastasis [2]. PCa develops from a prostatic intraepithelial neoplasia (PIN) that eventually progresses towards invasive carcinoma [3]. The underlying mechanisms that determine the switch from normal to neoplastic and further to motile, invasive and metastatic cancer cells remain unclear.

Transforming growth factor β (TGFβ) isoforms [4] are among the best-characterized stimuli for phenotypic switching of a variety of cells such as myofibroblast differentiation [5–7], endothelial-to-mesenchymal transition (EndMT) [8] and epithelial-to-mesenchymal transition (EMT) [9]. Although a tumor suppressor early on [10,11], prolonged stimulation by TGFβ induces EMT, shuns the tumor suppressive role, and promotes cancer invasion and metastasis [12,13]. Although TGFβ1 induces apoptosis in prostate and bladder cancer cells via activation of P38 MAP kinase and JNK pathways [14], it induces PCa cell EMT via activation of Rac1 and P21 activated kinase-1 pathway [15,16]. Thus, TGFβ1 is one of the first candidates known to engage in a dual, reciprocal role in the early and advanced cancers.

Akt (protein kinase B), a 3-gene family of serine-threonine kinase [17,18] promotes oncogenic transformation [19] and tumor growth [20], including PCa [21–24]. Akt has also been implicated in the regulation of AR signaling to promote prostate tumor growth, where in $PTEN^{+/-}$ mice, AR was observed to be present both in the cytoplasm and the nucleus, in $PTEN^{+/-}/Akt1^{-/-}$ mice, AR was only localized in the nucleus [25]. However, the precise role of Akt in advanced cancer is not clear. We have reported that $Akt1^{-/-}$ mice

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Lack of adequate pneumococcal vaccination response in chronic lymphocytic leukaemia patients receiving ibrutinib

Chronic lymphocytic leukaemia (CLL) is characterized by profound dysfunction in innate and adaptive immunity and, subsequently, infections are commonly incurred by patients. Historically, successful vaccination in CLL patients has been a challenge in the era of anti-CD20$^+$ based therapies (Sinisalo *et al*, 2007). With an emerging armamentarium of novel targeted CLL therapy, additional data is needed in vaccine efficacy. Ibrutinib, an irreversible inhibitor of Bruton tyrosine kinase (BTK), has been associated with the development of pneumonia in 4–18% of patients (Byrd *et al*, 2014; Farooqui *et al*, 2015). BTK is essential for B cell function and development as well as Toll-like receptors, which are involved in innate and adaptive immunity (Brunner *et al*, 2005). To our knowledge, this is the first report evaluating the efficacy of pneumococcal vaccination in CLL patients receiving ibrutinib.

SLy2 (Src homology domain 3 lymphocyte protein) is a haematopoietic adapter protein containing SH3 and SAM1 domain (HACS1 or referred to as SAMSN1) with immunomodulatory properties (Claudio *et al*, 2001). SAMSN1 has been correlated with decreased immunity to pneumococcal vaccination. Murine models with overexpression of SAMSN1, have demonstrated decreases in interleukin 5 receptor α (IL5Rα) expression and thus decreases in B-1 cells and IgM concentrations. The ability of B-1 cells to create IgM antibodies is critical to generation of immunity following pneumococcal vaccination. Given the potential role of SAMSN1 on pneumococcal vaccination response, this investigation assessed SAMSN1 expression in both ibrutinib and CLL control patients.

This prospective, single-centre, non-blinded study evaluated the effectiveness of immunization with Pneumococcal Conjugate Vaccine 13 (PCV13) in 2 study cohorts (ibrutinib or control). All study participants gave written consent for participation. Eligible patients had histologically confirmed CLL with an Eastern Cooperative Oncology Group performance score ≤2 and having received no active treatment for CLL (control cohort) or actively receiving ibrutinib 420 mg once daily (active treatment cohort). Patients were ineligible if they had they received any pneumococcal vaccination in the last 2 years, treatment with anti-CD20 monoclonal antibodies within the last 6 months, or intravenous immunoglobulin replacement within 6 of study enrolment.

At Day 0 of study enrolment, both cohorts received a single dose (0·5 ml) of PCV13. Serum pneumococcal antibody assessment was performed on study subjects before vaccination (Day 0, vaccination day) and 30 days after vaccination administration. IgG antibodies specific for 13 pneumococcal serotypes were measured by microsphere photometry. CD19$^+$ cells were isolated using Dynabeads® CD19 pan B [Invitrogen (Life Technologies) Carlsbad, CA, USA by standard procedure. Western blot analysis was performed to identify BTK (Cell Signaling, St. Louis, MO, USA) and SAMSN1 (Abcam, Cambridge, MA, USA) expression at Day 0 and Day 30. Adequate vaccination response was defined as ≥2-fold increase in post-vaccination serotype-specific IgG over baseline levels of 3 serotypes (Hartkamp *et al*, 2001; Pasiarski *et al*, 2014). The primary outcome analysis compared changes in PCV serotype changes between ibrutinib and CLL control group using Student's two-tailed *t*-tests. A significance level of 0·05 was considered significant for the primary outcomes. Bonferroni adjustment was applied to secondary outcomes to correct for multiple hypothesis testing; the resulting significance level was 0·05/12 = 0·004.

See Table I for specific study patient demographics. All of the CLL control patients (4/4) generated a ≥2-fold increase in ≥3 of pneumococcal serotypes, whereas (0/4) of ibrutinib patients generated an adequate immune response to PCV13 (P = 0·029; *post-hoc* Fisher exact). The mean post-immunization antibody difference was −0·05 μg/ml for ibrutinib patients compared to 18·2 μg/ml in control patients. Specifically, 5 serotypes 1 (P = 0·03), 3 (P = 0·03), 5 (P = 0·01), 6B (P = 0·009) and 18C (P = 0·03, T-test) were increased at Day 30 in CLL controls compared to ibrutinib patients. However, after Bonferroni adjustment changes these were not statistically significant. Ibrutinib patients had significantly higher SAMSN1 expression at baseline compared to controls and this was significantly reduced following vaccination with PCV13 at Day 30 evaluation (Fig 1).

Historically, the rate of pneumococcal vaccination response in CLL patients has been reported as 22–71% (Hartkamp *et al*, 2001; Sinisalo *et al*, 2007; Pasiarski *et al*, 2014). Pasiarski *et al* (2014) demonstrated that 58% of CLL patients positively responded compared to healthy controls and that the frequency of plasmablasts was significantly lower at 1 week post-vaccination. Notably, our study identified no increase when evaluating specific pneumococcal antibody titres in our ibrutinib-treated patients. Limitations in this investigation include small study size and differences in disease stage and, subsequently, more prior therapies. Since ibrutinib emerged as a front-line treatment option for CLL, clinicians now face the

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ARTICLE

Translational Therapeutics

Endothelial Akt1 loss promotes prostate cancer metastasis via β-catenin-regulated tight-junction protein turnover

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BACKGROUND: Cancer research, in general, is focused on targeting tumour cells to limit tumour growth. These studies, however, do not account for the specific effects of chemotherapy on tumour endothelium, in turn, affecting metastasis.
METHODS: We determined how endothelial deletion of Akt1 promotes prostate cancer cell invasion in vitro and metastasis to the lungs in vivo in endothelial-specific Akt1 knockdown mice.
RESULTS: Here we show that metastatic human PC3 and DU145 prostate cancer cells invade through Akt1-deficient human lung endothelial cell (HLEC) monolayer with higher efficiency compared to control HLEC. Although the endothelial Akt1 loss in mice had no significant effect on RM1 tumour xenograft growth in vivo, it promoted metastasis to the lungs compared to the wild-type mice. Mechanistically, Akt1-deficient endothelial cells exhibited increased phosphorylation and nuclear translocation of phosphorylated β-catenin, and reduced expression of tight-junction proteins claudin-5, ZO-1 and ZO-2. Pharmacological inhibition of β-catenin nuclear translocation using compounds ICG001 and IWR-1 restored HLEC tight-junction integrity and inhibited prostate cancer cell transendothelial migration in vitro and lung metastasis in vivo.
CONCLUSIONS: Here we show for the first time that endothelial-specific loss of Akt1 promotes cancer metastasis in vivo involving β-catenin pathway.

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INTRODUCTION

Currently, research in the development of cancer therapy more focused on the pathways promoting tumour cell growth and invasion. Studies that address the specific role of a pathway in stromal cells and how drugs affect stroma when used for cancer therapy are fewer. Among the cells in the tumour microenvironment, tumour endothelium plays a significant role not only in tumour angiogenesis, perfusion and metastasis[1–3] but also as the first line of defense in a patient's fight against cancer cell metastasis to other vital organs. Hence, it is important to determine the specific role of a pathway and the effect of a drug on tumour vasculature alone so as to improve the efficacy and minimise the side effects of cancer chemotherapy.

Preclinical and clinical research evidence has revealed the integral role of phosphatase and tensin homologue (PTEN)-Akt pathway in multiple cancers,[4] including prostate cancer.[5] A number of studies from our laboratory have indicated that pharmacological and genetic inhibition of Akt, particularly Akt1, inhibits prostate and bladder cancer cell function in vitro and tumour xenograft growth in vivo.[6–8] We previously reported that, drugs such as statins and angiotensin receptor blocker candesartan, that have the ability to normalise Akt1 activity in prostate cancer by inhibiting hyperactive Akt1 in prostate cancer cells,[9–11] and activating Akt1 from its basal state in endothelial cells, led to the inhibition of prostate cancer cell transendothelial migration in vitro.[12] We have also reported that Akt1 gene knockout in mice promoted tumour vascular permeability and angiogenesis in a murine B16F10 melanoma model.[13] Most recently, we demonstrated that endothelial-specific knockdown of Akt1 results in increased vascular permeability via FoxO- and β-catenin-mediated suppression of endothelial tight-junction claudin expression, mainly claudin-5.[14] Since many inhibitors of Akt are in different phases of clinical trials for various types of cancers, it is important to understand the effect of Akt1 suppression in endothelial cells of tumour vasculature, and its consequences on tumour growth and metastasis.

In the current study, we investigated the effects of endothelial-specific knockdown of Akt1, a major endothelial isoform of Akt[13] on prostate cancer cell invasion in vitro and metastasis in vivo using murine lung colonisation model of in vivo metastasis. Our analysis revealed that Akt1 deficiency in human lung microvascular endothelial cells (HLECs) enhances the ability of human metastatic PC3 and DU145 prostate cancer cells to migrate across the endothelial monolayer in vitro, and murine RM1 prostate cancer cell metastasis to the lungs in vivo, with no changes in the growth of RM1 tumour xenografts in vivo. The akt1 loss in HLECs resulted in increased translocation of phosphorylated β-catenin from the endothelial-barrier junctions to the cytosol and the nucleus, in turn, suppressing the transcription of endothelial tight-junction proteins such as claudin-5, ZO-1 and ZO-2.

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Modulation in the microRNA repertoire is responsible for the stage-specific effects of Akt suppression on murine neuroendocrine prostate cancer

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Abstract

Recent studies indicate a stage-specific, differential role for the oncogene Akt on various cancers. In prostate cancer (PCa), suppression of Akt activity in the advanced stages promoted transforming growth factor-β (TGFβ) pathway-mediated epithelial-to-mesenchymal transition (EMT) and metastasis to the lungs. In the current study, we performed Affymetrix analysis to compare the expression profile of microRNAs in the mouse prostate tissues collected at the prostatic inter-epithelial neoplasia (PIN) stage from Transgenic adenocarcinoma of the mouse *(TRAMP)/Akt1*$^{+/+}$ versus *TRAMP/Akt1*$^{-/-}$ mice, and at the advanced stage from *TRAMP/Akt1*$^{+/+}$ mice treated with triciribine (Akt inhibitor) versus DMSO-treated control. Our analysis demonstrates that in the early stage,

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Genome atlas analysis based profiling of Akt pathway genes in the early and advanced human prostate cancer

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ABSTRACT

Recent studies conducted in the mouse and cellular models suggest a stage-specific, differential effect of Akt activity modulation on tumor growth and metastasis in various cancers. In prostate cancer (PCa), although the deletion of Akt1 gene in a neuroendocrine model of TRansgenic Adenocarcinoma of the Mouse Prostate (TRAMP) blunted oncogenic transformation and tumor growth, Akt1 suppression in the advanced PCa resulted in the activation of transforming growth factor-β pathway and enhanced metastasis to the lungs. Such a dual role for the Akt isoforms and its signaling partners has not been investigated in human PCa. In the current study, we performed genomic database analysis of Akt isoforms and associated pathway molecules in human prostate adenocarcinoma, castration-resistant PCa, neuroendocrine PCa and metastatic PCa for mutations, genetic alterations, mRNA and protein expressions and activating phosphorylations from cBioportal. Results from the protein data analysis from the cBioportal were compared to the results of our data on human PCa tissue analysis and the cellular effects of Akt1 suppression using MK-2206 on PCa cell aggressiveness. Our study indicates the existence of a dual role for Akt1 in PCa and warrants a large-scale analysis of the early and advanced stage PCa clinical samples for further clarity.

INTRODUCTION

Metastatic prostate cancer (PCa) is the leading cause of cancer-related deaths in men in the US and Europe [1]. Although slow-growing cancer, PCa that has metastasized to the bone, lungs, and brain becomes difficult to treat [2]. Uncertainties in the molecular mechanisms leading to the switch from early to advanced PCa are the underlying reason for the unreliable screening measures and ineffective treatments in the management of early and metastatic PCa [3].

Phosphoinositide-3-Kinase (PI3K)/Akt pathway has a well-established role in the regulation of cellular processes essential for cell survival such as metabolism, proliferation, growth, anti-apoptosis and cytoskeletal reorganization [4]. Aberrant activation of the PI3K/Akt pathway has been recognized as an essential step towards the initiation and progression of many cancers [5]. Activation of this pathway is driven by genetic mutation or activity deregulation of the upstream components such as receptor tyrosine kinases (RTKs) [6], non-RTKs such as Src family kinases [7] or modulation of the downstream components including PTEN inactivation or deletion, PI3K constitutive activation or amplification, Akt hyperactivation and other genetic changes in signaling molecules involved

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Endothelial stromelysin1 regulation by the forkhead box-O transcription factors is crucial in the exudative phase of acute lung injury

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ABSTRACT

Enhanced vascular permeability is associated with inflammation and edema in alveoli during the exudative phase of acute respiratory distress syndrome (ARDS). Mechanisms leading to the endothelial contribution on the early exudative stage of ARDS are not precise. We hypothesized that modulation of endothelial stromelysin1 expression and activity by Akt1-forkhead box-O transcription factors 1/3a (FoxO1/3a) pathway could play a significant role in regulating pulmonary edema during the initial stages of acute lung injury (ALI). We utilized lipopolysaccharide (LPS)-induced mouse ALI model *in vivo* and endothelial barrier resistance measurements *in vitro* to determine the specific role of the endothelial Akt1-FoxO1/3a-stromelysin1 pathway in ALI. LPS treatment of human pulmonary endothelial cells resulted in increased stromelysin1 and reduced tight junction claudin5 involving FoxO1/3a, associated with decreased trans-endothelial barrier resistance as determined by electric cell-substrate impedance sensing technology. *In vivo*, LPS-induced lung edema was significantly higher in endothelial Akt1 knockdown (*EC-Akt1*$^{-/-}$) compared to wild-type mice, which was reversed upon treatment with FoxO inhibitor (AS1842856), stromelysin1 inhibitor (UK356618) or with shRNA-mediated FoxO1/3a depletion in the mouse lungs. Overall, our study provides the hope that targeting FoxO and styromelysin1 could be beneficial in the treatment of ALI.

1. Introduction

Disruption of the alveolar-capillary unit symbolizes the exudative phase of acute respiratory distress syndrome (ARDS) [1–3]. ARDS is an important cause of acute respiratory failure that is often associated with multiple organ failure and high mortality among ICU patients. ARDS incidence ranges from 10 to 86 cases per 100,000, with the highest rates reported in Australia and the United States [2]. Histologically, the slightly thicker part of blood-air barrier is also composed of the extracellular matrix (ECM) [4]. Endothelial injury and consequent vascular permeability ensuing influx of protein-rich fluid into the alveolar air spaces is a well-established pathological event occurring in the acute/exudative stage of ARDS [5]. Therefore, targeting disruption of the capillary endothelial barrier could provide a potential therapy for ARDS.

Capillary vascular permeability regulated by paracellular pathways plays a prominent role in lung edema [6,7]. This is largely regulated by adherens junction (AJ) proteins such as VE-cadherin and tight junction (TJ) proteins such as claudins [8,9]. Pulmonary endothelium expresses higher levels of claudin5 than the alveolar epithelium and a decrease in claudin5 expression is associated with aberrant vascular permeability and severe ALI in patients with pneumonia [9,10]. Although vascular

Abbreviations: ARDS, acute respiratory distress syndrome; ALI, acute lung injury; LPS, lipopolysaccharide; FoxO, forkhead box-O; MMP, matrix metalloprotease; ECM, extracellular matrix; AJ, adherens junction; TJ, tight junction; ZO-1, zonna occluden 1; EC, endothelial cell; BALF, broncho-alveolar lavage fluid; HMEC, human microvascular endothelial cell; HPAEC, human pulmonary artery endothelial cell; WT, wild-type; DAPI, 4′,6-diamidino-2-phenylindole; FRET, fluorescence resonance energy transfer; MPO, myeloperoxidase; ELISA, enzyme-linked immunosorbent assay; IPA, ingenuity pathway analysis; GWAS, genome-wide association studies; ANOVA, analysis of variance

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ARTICLE

Modulation of the p75 neurotrophin receptor using LM11A-31 prevents diabetes-induced retinal vascular permeability in mice via inhibition of inflammation and the RhoA kinase pathway

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Abstract

Aims/hypothesis Breakdown of the inner blood–retinal barrier (BRB) is an early event in the pathogenesis of diabetic macular oedema, that eventually leads to vision loss. We have previously shown that diabetes causes an imbalance of nerve growth factor (NGF) isoforms resulting in accumulation of its precursor proNGF and upregulation of the p75 neurotrophin receptor ($p75^{NTR}$), with consequent increases in the activation of Ras homologue gene family, member A (RhoA). We also showed that genetic deletion of $p75^{NTR}$ in diabetes preserved the BRB and prevented inflammatory mediators in retinas. This study aims to examine the therapeutic potential of LM11A-31, a small-molecule $p75^{NTR}$ modulator and proNGF antagonist, in preventing diabetes-induced BRB breakdown. The study also examined the role of $p75^{NTR}$/RhoA downstream signalling in mediating cell permeability.

Methods Male C57BL/6 J mice were rendered diabetic using streptozotocin injection. After 2 weeks of diabetes, mice received oral gavage of LM11A-31 (50 mg kg^{-1} day^{-1}) or saline (NaCl 154 mmol/l) for an additional 4 weeks. BRB breakdown was assessed by extravasation of BSA–AlexaFluor-488. Direct effects of proNGF were examined in human retinal endothelial (HRE) cells in the presence or absence of LM11A-31 or the Rho kinase inhibitor Y-27632.

Results Diabetes triggered BRB breakdown and caused significant increases in circulatory and retinal TNF-α and IL-1β levels. These effects coincided with significant decreases in retinal NGF and increases in vascular endothelial growth factor and proNGF expression, as well as activation of RhoA. Interventional modulation of $p75^{NTR}$ activity through treatment of mouse models of diabetes with LM11A-31 significantly mitigated proNGF accumulation and preserved BRB integrity. In HRE cells, treatment with mutant proNGF (10 ng/ml) triggered increased cell permeability with marked reduction of expression of tight junction proteins, zona occludens-1 (ZO-1) and claudin-5, compared with control, independent of inflammatory mediators or cell death. Modulating $p75^{NTR}$ significantly inhibited proNGF-mediated RhoA activation, occludin phosphorylation (at serine 490) and cell permeability. ProNGF induced redistribution of ZO-1 in the cell wall and formation of F-actin stress fibres; these effects were mitigated by LM11A-31.

Conclusions/interpretation Targeting $p75^{NTR}$ signalling using LM11A-31, an orally bioavailable receptor modulator, may offer an effective, safe and non-invasive therapeutic strategy for treating macular oedema, a major cause of blindness in diabetes.

Sally L. Elshaer and Abdulrahman Alwhaibi contributed equally to this work.

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RESEARCH ARTICLE | *Translational Physiology*

Angiotensin II type 2 receptor stimulation with compound 21 improves neurological function after stroke in female rats: a pilot study

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[1]*Program in Clinical and Experimental Therapeutics, Charlie Norwood Veterans Affairs Medical Center and University of Georgia, College of Pharmacy, Augusta, Georgia;* [2]*Department of Physiology, Augusta University, Augusta, Georgia; and* [3]*Department of Neurology, Augusta University, Augusta, Georgia*

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Eldahshan W, Ishrat T, Pillai B, Sayed MA, Alwhaibi A, Fouda AY, Ergul A, Fagan SC. Angiotensin II type 2 receptor stimulation with compound 21 improves neurological function after stroke in female rats: a pilot study. *Am J Physiol Heart Circ Physiol* 316: H1192–H1201, 2019. First published March 1, 2019; doi:10.1152/ajpheart.00446.2018.—The angiotensin II type 2 receptor (AT_2R) agonist, compound 21 (C21), has been shown to be neurovascularly protective after ischemic stroke in male rats. In the current study, we aim to study the impact of C21 treatment on female rats. Young female Wistar rats were subjected to different durations of middle cerebral artery occlusion (MCAO) (3 h, 2 h, and 1 h) using a silicone-coated monofilament, treated at reperfusion with 0.03 mg/kg ip of C21 and followed up for different times (1, 3, and 14 days) after stroke. Behavioral tests were performed (Bederson, paw grasp, beam walk, and rotarod), and animals were euthanized for infarct size analysis and Western blot analysis. In vitro, primary male and female brain microvascular endothelial cells (ECs) were grown in culture, and the expression of the AT_2R was compared between males and females. At 1 day, C21 treatment resulted in an improvement in Bederson scores. However, at 3 days and 14 days, the impact of C21 on stroke outcomes was less robust. In vitro, the expression of the AT_2R was significantly higher in female ECs compared with male ECs. In conclusion, C21 improves Bederson scores after stroke in female rats when administered early at reperfusion. The ability of C21 to exert its neuroprotective effects might be affected by fluctuating levels of female hormones.

NEW & NOTEWORTHY The present study shows the neuroprotective impact of C21 on ischemic stroke in female rats and how the protective effects of C21 can be influenced by the hormonal status of female rodents.

AT_2R; brain microvascular endothelial cells; compound 21; ischemic stroke; female rodents; peroxisome proliferator-activated receptor-γ

INTRODUCTION

Over a lifetime, women suffer from more strokes, and older women are more likely to die from strokes than men (38, 48). In the United States, although stroke has declined to be the fifth leading cause of death overall, it is still the third leading cause of death in women according to the American Heart Association. In addition, women tend to suffer from a greater disease burden and worse functional outcomes after stroke compared with men, since they have longer life expectancy and are diagnosed with stroke at a later age (38, 48). Because of that, there has been a recent emphasis on increasing the representation of females in preclinical research, to better understand the targets and outcome differences seen in humans (33).

The only Food and Drug Administration-approved biologic treatment for ischemic stroke is tissue plasminogen activator (tPA). The administration of tPA follows strict criteria and should be generally administered within 4.5 h of the appearance of stroke symptoms (22, 42). In addition to intravenous thrombolysis with tPA, reperfusion therapy can be carried out using mechanical thrombectomy, which must be performed within 6 h (46). More recently, the DAWN and DEFUSE-3 clinical trials extended the time window of mechanical thrombectomy with stent retriever up to 24 h in a specific patient population with mismatch between the severity of clinical deficit and infarct volume (3, 44). These studies provide ample opportunities for research into agents that can be administered in combination with reperfusion, to further improve stroke outcomes.

One of the drug targets that has recently been studied in preclinical models of ischemic stroke is the angiotensin II type 2 receptor (AT_2R). We, and others, have shown that stimulation of the AT_2R with a nonpeptide, small-molecule, compound 21 (C21), is neurovascularly protective and restorative, and it improves post-stroke cognitive impairment in male rats (1, 4, 26). The mechanism includes reduction of neuroinflammation and promotion of angiogenesis (4, 14), but the molecular mechanisms remain unclear. Recently, cross-talk has been demonstrated between the AT_2R and the transcription factor peroxisome proliferator-activated receptor-γ (PPARγ). The activation of PPARγ in stroke has been shown to be neuroprotective (45) and promotes neuronal survival (35). In addition, the deletion of PPARγ in neurons increases the susceptibility of neurons to ischemia in vitro (60). The mechanism of PPARγ protection involves preservation of mitochondrial function and protection against oxidative stress and apoptotic cell death (16). Interestingly, C21 has been shown to have a preventive effect on stroke-induced brain injury in male mice, partly due to activation of PPARγ (52). Although C21 has been extensively studied and characterized in male rodents, no studies exist to date on the impact of C21 on young female rodents after stroke. Because studies of the AT_2R in other vascular beds have revealed significantly altered expression in females compared with males, and the effect is estrogen-

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Nodal pathway activation due to Akt1 suppression is a molecular switch for prostate cancer cell epithelial-to-mesenchymal transition and metastasis

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ABSTRACT

Several studies have unraveled the negative role of Akt1 in advanced cancers, including metastatic prostate cancer (mPCa). Hence, understanding the consequences of targeting Akt1 in the mPCa and identifying its downstream novel targets is essential. We studied how Akt1 deletion in PC3 and DU145 cells activates the Nodal pathway and promotes PCa epithelial-to-mesenchymal transition (EMT) and metastasis. Here we show that Akt1 loss increases Nodal expression in PCa cells accompanied by activation of FoxO1/3a, and EMT markers Snail and N-cadherin as well as loss of epithelial marker E-cadherin. Treatment with FoxO inhibitor AS1842856 abrogated the Nodal expression in Akt1 deleted PCa cells. Akt1 deficient PCa cells exhibited enhanced cell migration and invasion *in vitro* and lung metastasis *in vivo*, which were attenuated by treatment with Nodal pathway inhibitor SB505124. Interestingly, Nodal mRNA analysis from two genomic studies in cBioportal showed a positive correlation between Nodal expression and Gleason score indicating the positive role of Nodal in human mPCa. Collectively, our data demonstrate Akt1-FoxO3a-Nodal pathway as an important mediator of PCa metastasis and present Nodal as a potential target to treat mPCa patients.

1. Introduction

Despite the early diagnosis and significant advances in treatments, prostate cancer (PCa) still ranks the second and third on cancer-related mortalities in men in the United States and Europe, respectively [1,2]. Recent statistics reveal that the higher mortality from PCa is mainly due to its metastasis to the bone, lungs, and brain [3,4]. Whereas the 5-year survival rate of non-metastatic PCa patients has always been > 99%, the 5-year survival rate of metastatic PCa (mPCa) patients has been further declined to ≤30% [1]. Uncertainty in the molecular mechanisms mediating cancer cell dissemination to distant organs is a major roadblock in the effective management of mPCa [4]. In-depth molecular characterization and identification of novel, druggable targets will pave the way for future therapies for mPCa.

Several investigators over the past 2 decades have demonstrated the integral role of Akt (Protein kinase B) in multiple cellular processes such as, survival, proliferation, growth, invasion, and migration, that are implicated in tumorigenesis and cancer malignancy [5–7]. As a result, many Akt targeting drugs have been developed, tested and a few of these have entered the clinical trials [8]. In prostate cancer, we have demonstrated that Akt is necessary for cancer cell motility [9], survival [10–12], proliferation [11,13], invasion [9,14], transendothelial migration [14]. Several preclinical studies have also demonstrated an isoform-specific effect of Akt on cell migration and invasion, which are essential steps in the metastasis. Whereas Akt2 promoted the invasive phenotype of breast and ovarian cancer cells [15,16], Akt1 was intriguingly found to abrogate cell migration and invasion by inhibiting epithelial-to-mesenchymal transition (EMT) in breast cancer [15,17–20]. Recently, several reports in various cancer types have emerged explaining an unexpected, counteractive role of Akt1 in the advanced cancers [19,21–24], including our findings in PCa [25–27]. Despite the controversial role of Akt1 in the advanced PCa [28], knowing how Akt1 orchestrates this process is crucial to optimize the current therapies and pave the way for potential new therapies for patients with mPCa. One of the genes that was significantly elevated in our previous gene arrays from the mice experiments on PCa is 'Nodal' [25]. Hence we hypothesized that Nodal pathway activation downstream of Akt1 suppression is involved in the promotion of PCa cell EMT and metastasis.

Nodal, a secreted protein belonging of TGFβ superfamily, is known

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Review

The unconventional role of Akt1 in the advanced cancers and in diabetes-promoted carcinogenesis

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ABSTRACT

Decades of research have elucidated the critical role of Akt isoforms in cancer as pro-tumorigenic and metastatic regulators through their specific effects on the cancer cells, tumor endothelial cells and the stromal cells. The pro-cancerous role of Akt isoforms through enhanced cell proliferation and suppression of apoptosis in cancer cells and the cells in the tumor microenvironment is considered a dogma. Intriguingly, studies also indicate that the Akt pathway is essential to protect the endothelial-barrier and prevent aberrant vascular permeability, which is also integral to tumor perfusion and metastasis. To complicate this further, a flurry of recent reports strongly indicates the metastasis suppressive role of Akt, Akt1 in particular in various cancer types. These reports emanated from different laboratories have elegantly demonstrated the paradoxical effect of Akt1 on cancer cell epithelial-to-mesenchymal transition, invasion, tumor endothelial-barrier disruption, and cancer metastasis. Here, we emphasize on the specific role of Akt1 in mediating tumor cell-vasculature reciprocity during the advanced stages of cancers and discuss how Akt1 differentially regulates cancer metastasis through mechanisms distinct from its pro-tumorigenic effects. Since Akt is integral for insulin signaling, endothelial function, and metabolic regulation, we also attempt to shed some light on the specific effects of diabetes in modulating Akt pathway in the promotion of tumor growth and metastasis.

1. Introduction

In the advanced stages, cancer cells become highly invasive and eventually spread to distant organs, resisting treatments and risking the patients' lives [1,2]. Once tumor cells acquire the ability to invade the surrounding tissues, the process of metastasis is instigated, and the cells enter the circulation through the lymphatic or vascular networks [3]. Loss of cell-cell adhesion and acquisition of the migratory features allow malignant tumor cells to dissociate from the primary tumor, break the cell-matrix interactions and disintegrate the extracellular matrix (ECM) network that enables their invasion to the surrounding areas [4]. Upon reaching a congenial microenvironment, these cells settle and adhere to a new location, start to colonize and profusely proliferate to generate the life-threatening secondary tumors [1,5]. Akin to the primary tumors, secondary tumors must also re-initiate angiogenesis in order for their growth to exceed 1–2 mm^3 in size. Without angiogenesis, these metastasized tumors are deprived of oxygen and nutrients delivered through diffusion and thereby fail to develop further [2]. Indeed, these events demonstrate the importance of vascular networks in cancer metastasis. Thus, cooperation between the tumor and vascular compartments ensures the overall growth of tumors, their trans-endothelial migration, invasion as well as metastasis and colonization in distant organs [6,7].

Although the cross-talk between the tumor and vascular compartments is crucial in the regulation of tumor growth and metastasis, less attention has been given to the mechanisms by which tumor and vascular cells reciprocate with each other within the tumor microenvironment [8]. In a recent review, we outlined the importance of Src family of kinases (SFKs) in the regulation of tumor vascular permeability, endothelial-barrier regulation, tumor growth and metastasis [9]. In addition to SFKs, another important molecule that mediates such a cross-talk is protein kinase B (PKB or Akt), a serine-threonine kinase that exists in three isoforms namely Akt1, Akt2 and Akt3 [10]. Akts are known to elicit isoform-, cell- and context-specific effects [11–14]. In this review, we will shed the light on the molecular aspect of Akt1 to understand how it orchestrates the interaction between the tumor cells and the vascular compartment in the advanced stages of cancer. Here, we also present a molecular comparison between the specific effects of

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