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Differential Roles of Mammalian Target of Rapamycin Complexes 1 and 2 in Migration of Prostate Cancer Cells

Smrruthi Vaidegi Venugopal
Clark Atlanta University, smrruthivaidegi.venu@students.cau.edu

ABSTRACT

DEPARTMENT OF BIOLOGICAL SCIENCES

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DIFFERENTIAL ROLES OF MAMMALIAN TARGET OF RAPAMYCIN COMPLEXES 1 AND 2 IN MIGRATION OF PROSTATE CANCER CELLS

Committee Chair: Shafiq A. Khan, Ph.D

Dissertation dated May 2019

In this study, we investigated differential activation and the role of two mTOR complexes in cell migration of prostate cancer cells. Specific knock-down of endogenous RAPTOR and RICTOR by siRNA resulted in decreased cell migration in LNCaP, DU145, and PC3 cells indicating that both mTORC1 and mTORC2 are required for cell migration. EGF treatment induced the activation of both mTORC1 and mTORC2 as determined by complex-specific phosphorylation of mTOR protein. Specific knock-down or inhibition of Rac1 activity in PC3 cells blocked EGF-induced activation of mTORC2, but had no effect on mTORC1 activation. Furthermore, the over-expression of constitutively active Rac1 (Rac1Q61L) resulted in significant increase in cell migration and activation of mTORC2 in PC3 cells, but had no effect on mTORC1 activation. Constitutively active Rac1 (Rac1Q61L) in PC3 cells was localized in the plasma membrane and was found to be in a protein complex which contained mTOR and RICTOR proteins, but not RAPTOR.

In conclusion, we suggested that EGF-induced activation of Rac1 causes the phosphorylation/activation of mTORC2 via RICTOR, specific regulator of mTORC2 activation in numerous cancer cells. The major role played by mTOR in a wide array of cancers has in the recent decades led to the development of numerous mTOR inhibitors. One of the drawback of these first generation mTOR inhibitors are that m TORC1 activity is inhibited but effect on mTORC2 activity require high dosages and prolonged exposure in different cancer cell types including HeLa, PC3, LNCaP, and A549. High dosage of rapamycin and its associated rapalogs required for mTORC2 inhibition is clinically unsuitable. Studies have shown that the dual mTORC1/C2 inhibitors trigger feedback loops causing metastasis and affect the cell viability of normal tissues *in vitro* and *in vivo*. There is a need for specific mTORC1 and mTORC2 inhibitor, which overcome the disadvantages of the previously developed mTOR inhibitors. The Rac1-RICTOR axis suggested in this study could be used as a potential target for the development of mTORC2 inhibitor and lead to a potential therapeutic treatment for aggressive prostate cancer.

DIFFERENTIAL ROLES OF MAMMALIAN TARGET OF RAPAMYCIN COMPLEXES 1 AND 2 IN MIGRATION OF PROSTATE CANCER CELLS

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With God as my witness, I at the outset like to thank my mentor and doctoral advisor Dr. Shafiq A Khan for his unwavering support, advice, encouragement and kindness during my research work at Clark Atlanta University. My sincere thanks also goes to my committee members Dr. Valerie Odero-Marah, Dr. Jenny Yang, Dr. Zhengxin Wang, and Dr. Nathan Bowen for their constructive criticisms, encouragement and affirmation during the last 5 years of this invaluable journey. My special thanks go to Dr. Silvia Caggia, Ms. Ana Cecilia Millena, and Dr. Bethtrice Thompson-Elliott. They were always there to extend a helping hand, a listening ear, and warm hug, when I needed it the most.

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LIST OF ABBREVIATIONS

AKT	Protein kinase B
ANOVA	Analysis of Variance
AR	Androgen Receptor
Bp	base pair
BPH	Benign Prostatic Hyperplasia
BSA	Bovine Serum Albumin
BSA	Bovine Serum Albumin
cDNA	Complementary Deoxyribonucleic Acid
DAPI	4'-6-Diamidino-2-phenylindole
ECL	Enhanced Chemiluminescence
EGF	Epidermal Growth Factor
EMT	Epithelial Mesenchymal Transition
FBS	Fetal Bovine Serum
MET	Mesenchymal Epithelial Transition
mRNA	Messenger Ribonucleic Acid
mTOR	Mammalian Target of Rapamycin
mTORC2	Mammalian Target of Rapamycin Complex 1
mTORC2	Mammalian Target of Rapamycin Complex 2
PBS	Phosphate Buffered Saline

PCR	Polymerase Chain Reaction
PI3K	Phosphatidylinositol-4, 5-bisphosphate 3-kinase
PIN	Prostatic-Intraepithelial Neoplasia
PSA	Prostate Specific Antigen
PVDF	Polyvinylidene Difluoride
Rac1	Ras-Related C3 Botulinum Toxin Substrate 1
RAPTOR	Regulatory Associated Protein of mTOR
RhoA	Ras Homolog Family Member A
RICTOR	Rapamycin-insensitive companion of mTOR
RNA	Ribonucleic Acid
RT-PCR	Reverse Transcription Polymerase Chain
siRNA	small interfering Ribonucleic Acid

CHAPTER I

INTRODUCTION

Metastasis is the process by which cancer cells leave the primary tumor, disseminate and form secondary tumors at anatomically distant sites.[1] Metastasis of epithelial cancer cells involves a complex cascade of molecular processes such as epithelial-to-mesenchymal transformation (EMT), degradation of extracellular matrix, cell migration and invasion of the neighboring tissues, intravasation into blood or lymph vessels, transportation and extravasation, mesenchymal-to- epithelial transformation (MET), and growth of metastatic tumors at distant locations.[1] Among these complex processes, the most critical step in the metastasis of cancer cells is tumor cell motility or cell migration. Cell migration is a multi-step process, which can either be random or directed. Most metastatic cancer cells exhibit directed cell migration, a highly integrated process which is regulated by numerous intracellular proteins.[2] RHO family of GTPases, including RhoA, Rac1, and Cdc42, play a vital role in the regulation of directed cell migration.[3] During cell migration, formation of actin-driven protrusion at the leading edge, called lamellipodia, is regulated by Rac1 activation, whereas focal adhesion and stress fibers formation at the cell body and rear of the cell is regulated by RhoA activation; while filopodia formation, which establishes cell polarity, is regulated by Cdc42.[2]

Several growth factors, chemokines, hormones, and prostaglandins acting through several different types of membrane receptors, have been shown to induce migratory and invasive behavior in cancer cells.[4] Several of these growth factors, including EGF, IGF, bind to their specific receptor tyrosine kinases or G-protein coupled receptors (GPCRs) and signal through their intracellular substrates to induce cell migration in numerous cancer cells including breast, ovarian, and prostate cancer cells.[5-7] The activation of GPCR's or tyrosine kinase receptors by chemokines or growth factors causes the activation of PI3K/AKT/mTOR pathway, which plays a critical role in the induction of cell migration and invasion.[8] Activation of PI3-kinase enzyme leads to the phosphorylation of phosphatidylinositol 4,5-biphosphate (PIP2) to phosphatidylinositol 3,4,4-triphosphate (PIP3), leading to the activation of AKT, which in turn promotes activation of mTOR.[9]

Mammalian target of rapamycin (mTOR) is a highly conserved serine/threonine kinase that exists in two distinct complexes: mTORC1 and mTORC2. The mTORC1 complex is composed of mTOR, RAPTOR (regulatory associated protein of mTOR), PRAS40 (proline-rich Akt substrate), DEPTOR (DEP domain containing mTOR-interacting protein), mLST8 (mammalian lethal with sec-8), and tti1/tel2 (TELO2 interacting protein 1 homolog) proteins.[10-12] mTORC2 complex is composed of mTOR, RICTOR (rapamycin-insensitive companion of mTOR), mSIN1 (mammalian stress-activated map kinase interacting protein 1), PROTOR1/2, DEPTOR, mLST8, and tti1/tel2 proteins.[13-15] Activation of both mTORC1 and mTORC2 complexes are vital for induction of cytoskeletal rearrangement and cell migration in human cancers.[16-18]

Previous studies have shown that in advanced bladder cancer cells, mTORC1 and mTORC2 components regulate cell spreading and migration.[19] In colorectal cancer cells, the knock-down of mTORC1 and mTORC2 component RAPTOR and RICTOR drastically reduced migration and invasion.[20] Studies conducted by Chen *et* al in prostate cancer cells also showed that mTORC1 and mTORC2, acting via Rac1 and RhoA play a role in cell migration and invasion.[21]

Elevated levels of mTOR, p-mTOR, RAPTOR, and RICTOR are observed in both primary and metastatic stages of renal cell carcinoma, gliomas, and prostate cancers.[22-24] In addition, it was observed that these elevated levels are also correlated to poor patient prognosis. Studies have also shown that RAPTOR and RICTOR, components of mTORC1 and mTORC2 are critical for migration in highly invasive and metastatic cancer cells.[19-21] Previous study from our laboratory showed that mTORC1 can be activated by EGF in a PI3K/AKT dependent manner.[4] An independent study also showed that mTOR is differentially phosphorylated at position 2448 and 2481 to regulate the differential activation of mTORC1 and mTORC2, respectively.[25] Another emerging body of evidence showed that Rac1 regulates the activation of mTOR in HeLa and Panc1 cells.[26] However, it is unknown if EGF and Rac1 plays a role in the specific activation of mTORC2 and the possible mechanism utilized by the EGF-Rac1 axis for the activation of mTORC2. In order to address this gap, we hypothesize that EGF can differentially regulate mTOR complexes in a Rac1 dependent manner in prostate cancer cells**.** The following specific aims were proposed to address the hypothesis:

Specific Aim 1: To investigate the potential of EGF to activate mTORC1 and mTORC2 complex in prostate cancer cells.

- Determine if mTORC1 and mTORC2 are essential for cell migration in LNCaP, DU145, and PC3 cells.
- Determine if EGF induced the activation of mTORC1 and mTORC2 in a complex specific manner.

Specific Aim 2: To investigate if Rac1 activates mTOR complexes and the mechanism involved in their activation in prostate cancer cells.

- Determine if either the knockdown or inhibition of Rac1 affects the complex-specific activation of the mTOR complexes in PC3, DU145 and LNCaP cells.
- Determine the effect of stable over-expression of Rac1WT and constitutively active Rac1 (Rac1Q61L) on the Rac1 activation, localization, and migration of PC3 cells.
- Determine if the over-expression of constitutively active Rac1 (Rac1Q61) in PC3 affect the activation of mTOR complexes.
- Determine if active Rac1 promotes activation of the mTOR complexes due to physical interaction with components of mTOR complexes.

CHAPTER II

LITERATURE REVIEW

Prostate Biology and Structure

The prostate is the largest accessory gland in the male reproductive system. It secretes a thin slightly alkaline fluid that forms a portion of the seminal fluid. The prostate gland is composed of glandular and stromal elements and is tightly fused within a pseudo-capsule. The inner layer of the prostate capsule is composed of smooth muscle with an outer layer covering of collagen. The nerve supply to this gland is derived from the prostatic plexus and the arterial supply by the branches of the internal iliac artery.[27]

Prostate Anatomy

The prostate gland is located posterior to the lower portion of the symphysis pubis, anterior to the rectum, and inferior to the urinary bladder between the pelvic diaphragm and the peritoneal cavity. The gland is classically described as "walnut shaped", but is actually conical in shape and surrounds the proximal urethra as it exists from the bladder.[28] The adult prostate and its surrounding structures are shown below in Figure 1.

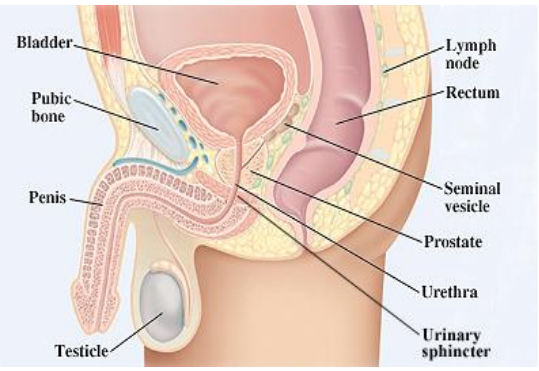

Figure 1. The adult prostate and the surrounding structures.[27]

The prostate is divided into four regions or zones as shown in Figure 2, central zone (CZ), transition zone (TZ), peripheral zone (PZ), and anterior fibromuscular zone. The central zone (CZ) is located at the base of the prostate between the peripheral and the transition zone and comprises of approximately 25% of the glandular tissue. It is cone shaped and surrounds the ejaculatory ducts. The transition zone (TZ) forms only 5% of the glandular tissue and consists of two small lobules of glandular tissue that surrounds the proximal prostatic urethra. The TZ is also the portion of the glandular tissue that is enlarged due to benign prostatic hyperplasia. The peripheral zone (PZ) is the largest of the four zones, comprising approximately 70% of the glandular tissues. In this zone, carcinoma, chronic prostatitis and post inflammatory atrophy are relatively more common than in the other zones. Finally, the anterior fibromuscular stroma, which forms the convexity of the anterior external surface and is devoid of glandular tissue is composed of fibrous and smooth muscle elements.[28-29]

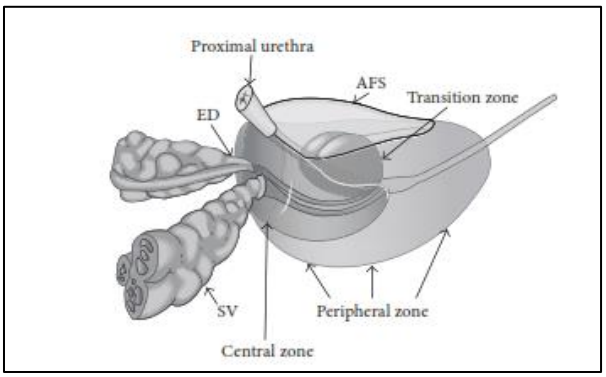

Figure 2. Zonal anatomy of the prostate gland. ED: Ejaculatory ducts; SV: Seminal Vesicles; AFS Anterior Fibromuscular Stroma.[28]

Prostate Growth and Development

The growth and development of the prostate begins in fetal life and is complete at sexual maturity. The prostate develops from the urogenital sinus (UGS) and is found caudal to the neck of the developing bladder. In human males, prostatic morphogenesis begins at 10-12 weeks of gestation.[30] The initial event during prostatic morphogenesis is the out-growth of solid epithelial buds from the urogenital sinus epithelium (UGE) to the urogenital sinus mesenchyme (UGM).[31] The solid epithelial buds grow into the UGM in a precise spatial pattern that establishes the formation of three distinct prostatic lobes: the anterior prostate (AP), dorsolateral prostate (DLP), and ventral prostate (VP).[31] The ductal epithelium of each of the prostate lobe undergoes canalization, which results in the formation of two distinct cell populations: basal epithelial cells and columnar luminal cells. Concurrently, as the ductal epithelium differentiates into basal and luminal cell types, the prostatic mesenchyme/stroma differentiates into a layer of smooth muscle cells that surrounds the prostatic ducts (Figure 3).[32]

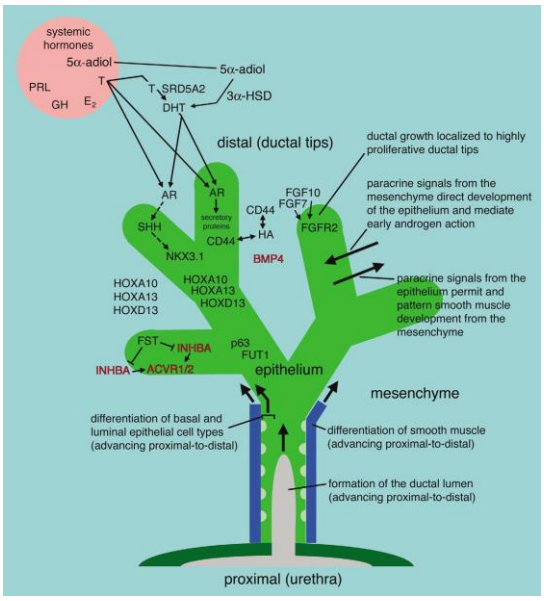

Figure 3. Features of prostatic branching morphogenesis. The diagram shows a generic network of developing prostatic ducts. The developing ductal epithelium is shown in green; the developing prostatic mesenchyme is shown in blue; and the forming ductal lumen is shown in pink.[30]

Histology of the Mature Prostate

Histologically, the mature prostate duct is composed of three cells types (Figure 4). The most abundant are the tall columnar epithelial cells. These cells are androgen-dependent, produce prostatic secretion and are characterized by the expression of androgen receptors and cytokine 8 and 18.[33-34] The second abundant cell type in the prostate duct is basal. These cells form a continuous layer between the luminal cells and the basement membrane and are characterized by the expression of p63 and cytokine 4 and 14.[33] The third prostatic epithelial cell type is neuroendocrine. They are androgen independent and are characterized by the expression of chromogranin A, serotonin, and neuropeptides.[35] Thick layers of stroma surround the epithelial gland and are composed

of mostly smooth muscles and also contain fibroblastic, neuronal, vascular and lymphatic cell types. The stroma is characterized by the expression of smooth muscle alpha-actin and vimentin.[30]

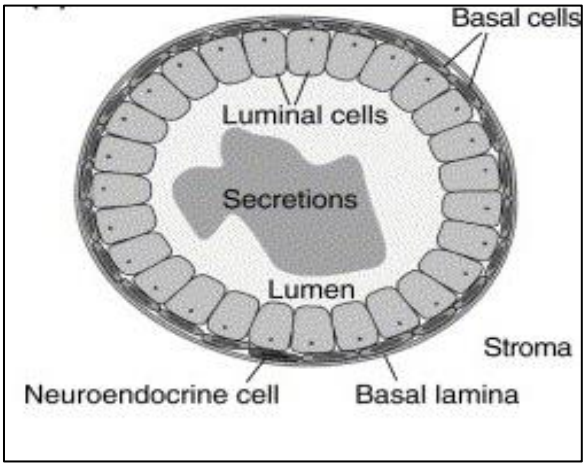

Figure 4. Schematic diagram of the cell types in an adult human prostatic duct.[33]

Mechanism of Prostatic Development

The development of the prostate from UGS is due to the systemic androgen produced by the fetal testis. Cellular response to systemic androgen is mediated by nuclear androgen receptors that are activated by testosterone (T) or dihydrotestosterone (DHT). Studies in mice and humans have shown that sufficient androgen production and activation of androgen receptor are vital for the development of prostate from the urogenital sinus.[36-38] It has also been observed in human and mice studies that, prostate organ growth and morphogenesis is dependent on the ability of circulatory testosterone to activate androgen receptor. Circulatory testosterone activates androgen receptor by directly binding to the receptor and through local conversion of circulating T to the more

potent androgen receptor agonist DHT.[39] In addition, androgen receptors have also been shown to regulate proliferation of prostate epithelial cells by collaborating with growth factors including epidermal growth factor (EGF), fibroblast growth factor 7 (FGF7) and FGF10.[30] Furthermore, around 80-90% of prostate cancer patients are diagnosed with malfunction in androgen signaling.[40]

Prostate Cancer (PCa)

In humans, the prostate is of great pathological significance and is known to be associated with prostatitis, benign prostatic hyperplasia (which are two non-malignant conditions), and adenocarcinoma (most common malignant prostatic disease condition).[27]

Prostate Cancer Incidence and Risk Factors

PCa is the most commonly diagnosed non-cutaneous cancer in males and the second leading cause of cancer related death for men in the United States.[41] The American Cancer Society estimates that there will be approximately 164,690 men diagnosed with PCa and 29,430 will die from PCa in 2018.[42] PCa typically appears in men 65 years or older and is most common in African-American men, followed by Caucasian and Asian men. Studies have shown that, there are several factors that can increase the risk for development of PCa, but the major and most well established risk factors for PCa development are advanced age (65 years or older), race, inherited susceptibility, diet, and environmental factors.[43-44]

Stages of Prostate Cancer

Previous studies have shown that the development of prostatic-intraepithelial neoplasia (PIN) increases the risk for PCa and has been accepted as a precursor to cancer.

PIN lesions are described as either low grade (LGPIN) or high grade (HGPIN). The LGPIN resemble normal prostate with the tufting of luminal epithelial cells, whereas HGPIN are immediate precursors to early invasive carcinoma.[45] It has been shown through histological studies that PCa tissue samples are a combination of normal, pre-neoplastic, and neoplastic foci. A standard pathological grading system, the Gleason grading system, allows researchers to study its molecular pathology. This pathological system is arranged into five groups (1-5).[45-46] To generate a score, the two most prevalent histological patterns are evaluated and rated as Gleason pattern 1-5, with higher numbers representing more aggressive or invasive carcinoma.[47] Based on the Gleason score and the level of prostate-specific antigen (PSA), PCa has been classified into four different stages and they are as follows: Stage I – cancer is confined to the prostate only (one half or less of one lobe), Stage II- cancer is still confined to the prostate, but has spread only throughout the prostate, Stage III- the cancer has spread beyond the outer layer of the prostate and may have spread to the seminal vesicles, and finally Stage IV- cancer has spread beyond the seminal vesicles to nearby tissues or organs including the rectum, bladder or pelvic wall, lymph nodes, and bones (Figure 5).[42]

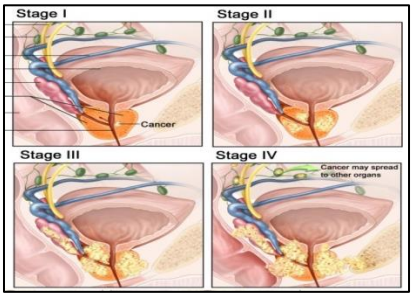

Figure 5. Schematic diagram of the different stages of PCa.[42]

Molecular Mechanisms Involved in Prostate Cancer Initiation and Progression

Multiple molecular events and gene alteration are involved in the initiation and progression of PCa. Studies to-date have implicated several tumor suppressor genes, oncogenes, DNA repair genes, growth factors, and metastasis associated genes in the development of PCa. One of the most common early events involved in PCa is the loss of chromosome 8p, which in turn, leads to the loss of tumor suppressor gene NKX3.1.[48-49] Loss of NKX3.1 has been observed in 20% of PIN, 22% of high grade PCa, 34% of androgen independent PCa, and 78% of PCa metastasis.[50] Studies have shown that the loss of NKX3.1 (known to bind to DNA and cause repression of PSA in normal prostate) causes an increase in the concentration of PSA, which is one of the hallmarks of PCa progression.[51]

The second important molecular event involved in the progression of PCa is the loss of chromosome 10p and the tumor suppressor PTEN (Phosphatase and Tensin deleted on chromosome 10). Loss of chromosome 10p has been observed in 50-80% of prostate tumors and it occurs after the loss of chromosome 8 since it has been mostly observed in carcinomas rather than PINs.[52] Studies have shown that the tumor suppressor gene PTEN in chromosome 10p is drastically reduced in high grade PCa and there exists considerable heterogeneity in PCa that do contain PTEN.[53] The most important consequence of the loss of PTEN is the removal of its inhibitory effect on the activation of PI3K/AKT/mTOR signaling pathway which lead to increased cell proliferation and cell survival in PCa.[54]

A third common event in the progression of PCa is the loss of chromosome 13q, which includes the retinoblastoma tumor suppressor gene (Rb). This deletion of Rb has been observed in at least 50% of prostate tumors.[55-57] Studies have also shown that allelic loss, mutation, or decreased transcription of the Rb gene may also lead to inactivation of the Rb gene in PCa.[57] It has also been observed that expression of wild type Rb in the Rb negative PCa cell line leads to loss of tumorigenicity.[58-59] Thus, inactivation of Rb is crucial for PCa progression.

An important late-stage molecular event in the progression of PCa is the loss of chromosome 17, which encodes the well characterized p53 tumor suppressor gene. It is important in normal cells for entry into the synthesis phase of cell cycle and also to promote apoptosis in cells with DNA damage.[60] Mutations in p53 gene are common in human neoplasmas, but there is only a low frequency of mutation of this gene in PCa. Abnormal expression of p53 in PCa has been associated with bone metastasis and androgen independent disease state.[61] In addition, studies have also shown that abnormal p53 expression or loss of p53 in PCa has been correlated with uncontrolled cell growth, high histological grade, late stage, and clinical disease progression in PCa.[62]

Unlike genes discussed previously that have been implicated at specific times in the progression of PCa, androgen receptor (AR), a nuclear hormone receptor family of transcription factors necessary for growth terminal differentiation and functioning of the prostate gland, is important at all stages.[40] AR is the most extensively studied receptor in PCa due to its requirement in both normal prostate and PCa progression.[50] Studies have shown that AR blockade can delay progression of PCa and has been used to-date to treat

patients unable to undergo radical surgery or with cancer that has progressed beyond the prostate.[50] Over the past years, several mechanisms have been identified by which AR promotes prostate tumorigenesis. Some of the most widely studied mechanisms include AR hypersensitivity, constitutive AR activation via cross talk with other signaling pathways, elevated tumoral androgen production, and altered recruitment or expression of AR coregulators.[40] However, previous study has also shown that in most patients with advanced stages of PCa, the tumor progresses in an androgen independent state with cell proliferation occurring independent of androgens. Numerous mechanisms have been postulated for androgen insensitivity in PCa progression and some of the most important mechanisms involve mutation, amplification, deletion of AR gene, and structural changes in the AR protein.[63-65]

Apart from AR, there is another important molecular event that takes place during PCa progression, which is the inappropriate expression of members of the growth factor families such as interluekin-6 (IL-6), epidermal growth factor (EGF), transforming growth factor β (TGF-β), and insulin-like growth factors (IGF).[50] Studies have shown that these growth factors are associated with invasion and metastasis of PCa as they are involved in the activation of cell proliferation and cell survival signaling pathways: Ras-MAPK (mitogen activated protein kinase) and PI3K/AKT/mTOR.[66-69]

As can been observed from the above discussion, there are numerous gene alterations and molecular events that lead to PCa progression. However, most often these events and alterations lead to activation of two major signaling pathways: Ras-MAPK and PI3K/AKT/mTOR, which are essential for progression of PCa. In this discussion, we

will focus our efforts on the PI3K/AKT/mTOR pathway with special focus on mTOR complexes and Rac/RhoGTPase in PCa progression.

PI3K/AKT/mTOR Signaling Pathway in Normal Prostate and Prostate Cancer Progression

The phosphatidylinositol 3 kinase (PI3K) pathway is a complex signaling pathway that responds to the availability of various stimulations such as growth factors, nutrients, and hormones.[5] This pathway has been shown to coordinate a number of direct input signals from growth factors including EGF and TGFβ, and tyrosine kinase receptors including IGF-1R and EGFR.[6]

The central role in the PI3K/AKT/mTOR pathway is played by PI3K heterodimer protein, which belongs to the class IA of phosphoinositol-3-kinase family of proteins. This heterodimer is composed of two subunits, with the regulatory subunit (p85) regulating the activity of the catalytic subunit (p110), in response to upstream signals including receptor binding, activation or localization of enzymes.[70]

Following the allosteric activation of the catalytic site of the PI3K protein (Figure 6), phosphorylates phosphatidylinositol 4, 5-biphosphate (PIP2) phosphorylation leads to the active second messenger phosphatidylinositol 3,4,4-triphosphate (PIP3).[6] As a result, the PIP3 is recruited to the plasma membrane and activates pyruvate dehydrogenase kinase 1 (PDK1). Activation of PDK1 has been known to recruit and activate AKT (serine/threonine family of protein kinase).[71-72] Activated AKT phosphorylates a number of important downstream effectors including tuberous sclerosis complex 2 (TSC2), glycogen synthesis kinase 3 (GSK3), forkhead box O (FOXO) transcription factor, p27,

and BAD (BclxL/Bcl-2 associated death promotor)(pro-apoptotic), and in turn, regulates processes that coordinate cell growth, survival, proliferation, and metabolism in cells.[73] One of the major downstream effector of AKT signaling and also necessary for prostate tumorigenesis is the serine/threonine protein kinase, mammalian target of rapamycin (mTOR). This protein kinase exists as two distinct complexes: mTORC1 and mTORC2.[15] The mTORC1 complex consists of mTOR, RAPTOR (regulatory associated protein of TOR), PRAS40, mLST8, DEPTOR, tti1/tel2 and assembles following the AKT phosphorylation of TSC2, which allows the accumulation of GTP bound Rheb, an mTORC1 activator.[15, 74] The mTORC2 complex consists of mTOR, RICTOR (rapamycin independent component of TOR), DEPTOR, mSin1, mLST8, PROTOR1/2, and tti1/tel2. It is known to be activated by S6K1 (one of the substrates of mTORC1) and ribosome association.[15] It is also known that mTORC1 and mTORC2 are regulated by TSC1/2.[75-76] Each of the components of mTOR complexes, their function and role in PCa will be described in detail in the results section.

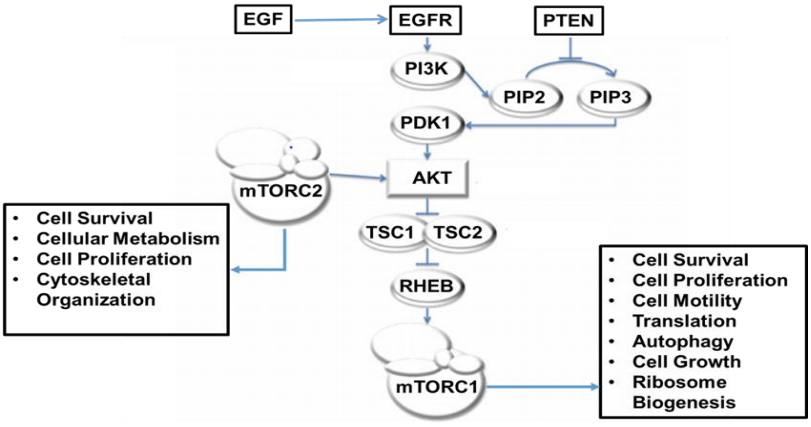

Figure 6. Schematic representation of the PI3K/AKT/mTOR signaling pathway.

The PI3K pathway signaling is controlled endogenously by a number of negative regulators. PTEN protein acts as a pathway repressor by dephosphorylating PIP3 to PIP2.[15] The p85 regulatory subunit of PI3K also functions as a negative regulator by binding, stabilizing, and inhibiting p110's catalytic activity.[15] It has been shown that liver kidney kinase B1 (LKB1) acts as a tumor suppressor by negatively regulating mTOR via the activation of AMPK. In addition, TSC2 also acts as a tumor suppressor by negatively regulating mTOR through small G-protein Rheb.[77-78]

PI3K/AKT/mTOR Signaling in Normal Cellular Processes

Numerous studies have shown that the PI3K/AKT/mTOR pathway is significantly dysregulated in human PCa. To understand the mechanism involved in PI3K pathway dysregulation leading to PCa progression, there exists a need to understand the normal cellular processes controlled by PI3K/AKT/mTOR signaling pathway.[15] Here, we will highlight some of the major cellular processes controlled by PI3K pathway. PI3K protein is a major regulator of metabolism through its role as a critical downstream effector of the insulin receptor. It has been shown in knockout and transgenic mouse models that class IA PI3K is necessary for effective insulin signaling and glucose uptake.[79] Studies have also shown that the loss of PTEN tumor suppressor in embryonic stem cells increase cell proliferation, which is associated with decreased levels of cell cycle inhibitor p27.[53] AKT, another downstream effector of PI3K pathway, plays a critical role in cell survival as it phosphorylates critical proapoptotic targets including BAD, which then triggers release of BAD from its target protein Bcl-2 and promote survival of neurons and other cell types.[80-81] mTOR (downstream effector of AKT activation) coordinates the

maturation of multiple hematopoietic lineages, demonstrating a critical role in cellular differentiation.[82] The downstream targets of mTORC1: 4EBP1 and S6K1 are major regulators of mRNA translation and have been shown to control cell size and proliferation.[83] The various components of the pathway described above and the cellular processes they impact clearly describe the vital role played by PI3K/AKT/mTOR signaling in cellular homeostasis and PCa.

PI3K/AKT/mTOR Signaling Pathway in Prostate Cancer

Dysregulation in the PI3K/AKT/mTOR pathway has been detected in multiple prostatic cancer tissue studies, suggesting that this pathway plays an important role in the development and progression of PCa. Studies have shown that PI3K/AKT/mTOR pathway is upregulated in approximately 30-50% of PCa and aberrant signaling of the molecules of this pathway have also been detected in PCa cell lines and xenografts.[84]

PTEN, one of the most important negative regulators of PI3K/AKT pathway, has been shown to be deleted or mutated in PCa. This, in turn, leads to the expression of inactive PTEN protein and increased activity of the PI3K/AKT pathway. Studies have shown that mutations in the PTEN tumor suppressor are common events in PCa with loss of heterozygosity at the PTEN locus in 60% of PCa samples. It has also been shown that decreased expression of PTEN has been found in 85% of primary PCa compared to normal tissues from the same patient.[52, 85-86] The loss of PTEN is correlated with Gleason score and pathologic stage of primary tumor and increased incidence of lymph node metastasis.[87-89] *In vivo* studies with mice having heterozygous deletion for PTEN developed PIN with 100% incidence. In addition, mice with homozygous deletion for

PTEN die *in utero,* while mice with prostate specific PTEN deletion developed invasive PCa.[90]

In vitro and preclinical studies have shown that the inactivation of the PTEN protein leads to constitutive activation of AKT and mTOR resulting in dysregulation of cell size and cell growth.[90] AKT protein staining intensity and percentage of positively stained cells were greater in PCa than in non-neoplastic cells.[91] Furthermore, phospho-AKT levels were also significantly greater in high-grade prostate tumors *vs* low or intermediate grade tumors.[92] Studies have also shown that, phospho-AKT is significantly increased in castration-resistant PCa when compared to hormone sensitive tissues and has been associated with decreased disease specific survival.[15]

Recent studies have also shown the importance of p110β catalytic subunit of PI3K in PCa. Both p85α and p110β are essential for androgen induced AR transactivation because they are required for cell proliferation and tumor growth. Conditional knockout mice studies have evaluated the effect of p110β deletion in the presence of PTEN deletion.[93] The prostate has normal appearance in the presence of only p110β deletion and development of high grade PIN in presences of PTEN loss alone. However, the ablation of p110β prevented tumorigenesis caused by PTEN loss.[94] Another study has also shown that increased AKT activation related to PTEN loss, was drastically decreased due to the ablation of p110β activity.[84]

Another important molecule that has been shown in studies to be constitutively upregulated due to PTEN loss is mTOR.

Mammalian Target of Rapamycin Complexes (mTORC)

mTOR is a typical serine/threonine protein kinase that belongs to the phosphoinositide 3-kinase (PI3K)-related kinase family and thus, has a high sequence similarity to the PI3K protein and inhibited by PI3K inhibitors. mTOR is a large (289 kDa) serine/threonine protein with several domains (Figure 7). The amino-terminal of mTOR contains tandem HEAT (huntington, elongation factor 3, a subunit of protein phosphatase 2A and TOR1) repeats that form an extended super helical array with surfaces for protein-protein interaction.[95] The FKBP12-rapamycin-binding (FRB), within the central region of mTOR, is the binding domain for the FKBP12-rapamycin complex. The catalytic domain of mTOR is near the carboxyl terminus. This kinase domain resembles a lipid (phosphatidylinositol) kinase domain. The mTOR protein is additionally characterized by FRAP-ATM-TTRAP (FAT) and C-terminal FAT (FATC) domains. The FAT domain is between the HEAT repeats and the FRB domain. The FATC domain is at the extreme carboxyl terminus. The FAT and FATC are always found together, which suggests that they interact.[95]

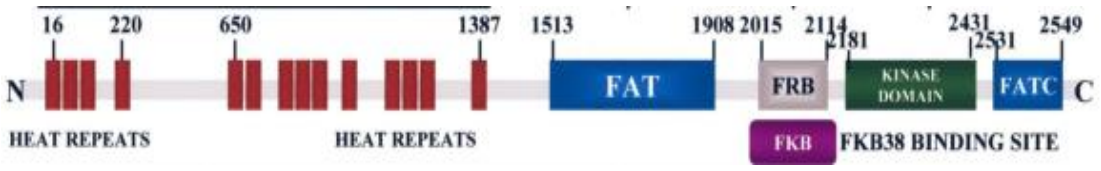

Figure 7. A schematic representation of mTOR.

Mammalian Target of Rapamycin (mTOR) due to its large size and multiple domains is found in two structurally and functionally distinct multiprotein complexes:

mTORC1 and mTORC2. The mTORC1 complex is composed of mTOR, RAPTOR, PRAS40, DEPTOR, mLST8, tti1/tel2.[10-12] The mTORC2 complex is composed of mTOR, RICTOR, mSIN1, PROTOR1/2, DEPTOR, mLST8 and tti1/tel2.[13-15] Studies have shown that each of the components has distinct molecular functions and interaction sites on the mTOR protein. The molecular functions of each of these components with respect to their complexes are as follows. In mTORC1 complex, RAPTOR acts as a scaffold protein, regulating the assembly, localization, and substrate binding ability of mTORC1; PRAS40 and DEPTOR act as negative regulators of mTORC1 activity and tti1/tel2 act as scaffold proteins regulating the assembly and stability of mTORC1 (Figure 8).[96] In mTORC2 complex, RICTOR acts as the scaffolding protein, regulating the assembly, localization and substrate binding of mTORC2; mSIN1 also acts as a scaffold protein regulating the assembly of mTORC2 and its interaction with mTORC2 downstream substrate: SGK1; PROTOR1/2 is required to increase mTORC2-mediated activation of SGK1; DEPTOR in the mTORC2 also acts as a negative regulator; mLST8 is essential for mTORC2 activity and tti1/tel2 acts as scaffolding proteins regulating the assembly and stability of mTORC2 (Figure 8).[96]

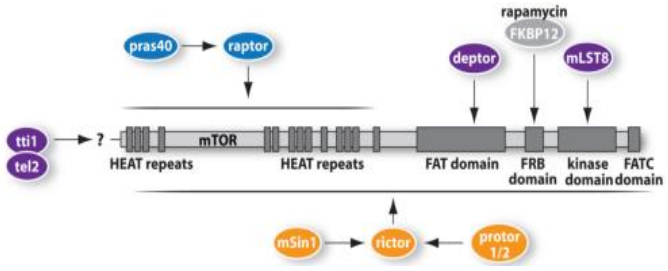

Figure 8. A schematic representation of the binding sites of the different components of mTORC1 and mTORC2 to the mTOR protein.

Function of mTORC1 and mTORC2 in Normal Cellular Processes

Normal cellular process controlled by mTORC1 and mTORC2 are shown in Figure 9

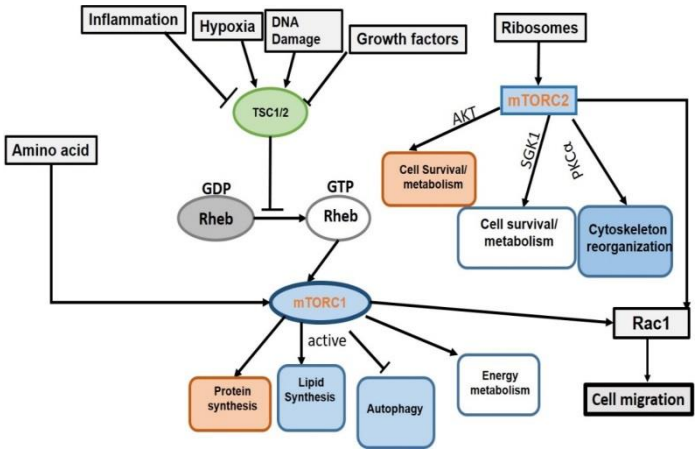

Figure 9. Schematic representation of the functions of mTORC1 and mTORC2 in normal cellular processes.

mTORC1 Activation and Subsequent Cellular Functions

mTORC1 is the best characterized of the two mTOR complexes and is activated by numerous upstream signals. Studies have shown that mTORC1 is activated by growth factors, stress, energy status, oxygen, and amino acids. This, in turn, controls major cellular processes including protein and lipid synthesis, cell motility, cell survival, cell size, and autophagy. One of the key proteins involved in transmitting upstream signals are TSC1 and TSC2, which function as GTPase-activating proteins (GAP) for Rheb (Ras homolog enriched in brain) GTPase. The GTP-bound Rheb then directly interacts with mTORC1 and stimulates its kinase activity[96]. In addition, studies have also shown that TCS1/2 act as negative regulators of mTORC1 by converting Rheb into its inactive GDP-bound form.[97] Numerous studies have shown how upstream signals such as growth factors, energy levels, oxygen levels, DNA damage, and amino acids, activate mTORC1

in a TSC1/2 dependent and independent manner. TSC1/2 transmits upstream growth factor signals, through PI3K and Ras pathway effectors: protein kinase B (Akt/PKB) and extracellular-signal-regulated kinase1/2 (ERK1/2), respectively. These effectors then directly phosphorylate TSC1/2 to its inactive state and cause mTORC1 activation.[98-100] Studies have also shown that AKT activates mTORC1 in a TSC1/2 independent manner by phosphorylating and causing the dissociation of PRAS40 (inhibitor of mTORC1 activation) from the RAPTOR component of mTORC1.[101-102] It has also been shown that pro-inflammatory cytokines including tumor necrosis factor-α (TNFα) using IκB kinase β, phosphorylates TSC1, causing inhibition of TSC1/2 and activation of mTORC1.[103] In addition, it has been shown that TSC1/2 transmits low energy levels, oxygen levels, and DNA damage signals to inactivate mTORC1. In response to hypoxia or low energy levels, adenosine monophosphate activated protein kinase (AMPK) phosphorylates TSC1 and causes inactivation of mTORC1 by increasing the GAP activity of TSC1/2 towards Rheb[104]. Studies have also shown that mTORC1 can be activated in a TSC1/2 independent manner using amino acids including leucine and arginine. It has been shown that upon amino acid sensing, the amino acids promote loading of the Rag family proteins RagA/B with GTP.[105-106] This, in turn, enables the interaction with RAPTOR component of mTORC1 and translocation of mTORC1 from the cytoplasmic to the lysosomal surface, where the Rag GTPase docks on Ragulators (multiple subunit complexes).[107] This Rag-Ragulator mechanism sensitive to amino acids then enables the GTP-loaded Rheb to activate mTORC1.

Activation of mTORC1 leads to a number of cellular processes (Figure 9). Major cellular process controlled by mTORC1 is the synthesis of proteins. It controls protein synthesis by directly phosphorylating the translational regulators eukaryotic translation initiation factor 4E (eIF4E) binding protein 1 (4E-BP1) and S6 kinase 1 (S6K1). The phosphorylation of E4-BP1 is required for initiation of cap-dependent translation by preventing the binding of 4E-BP1 to eIF4E.The phosphorylation of S6K1 causes its activation, which leads to increase in mRNA biogenesis, translational initiation, and elongation.[108] Another important cellular process that is controlled by mTORC1 is the synthesis of lipids (which required by proliferating cells to generate cell membrane). In response to insulin or sterol depletion, activated mTORC1 activates sterol regulatory element binding protein1/2 (SREBP1/2) and translocate it from the endoplasmic reticulum to the nucleus. This translocation activates the transcription of numerous genes involved in fatty acid and cholesterol synthesis.[109-111] In addition, studies have also shown that mTORC1 phosphorylates lipin-1 preventing its entry into the nucleus and suppressing SREBP1/2 functions.[112]

The third cellular process controlled by mTORC1 is the positive regulation of cellular metabolism and ATP production. Studies have shown that mTORC1 controls cellular metabolism by increasing the glycolytic flux by activating transcription and translation of hypoxia inducible factor 1α, a positive regulator of glycolytic genes.[109, 113-115] Studies have also shown that mTORC1 controls ATP production by increasing mitochondrial content and the expression of genes involved in oxidative metabolism. This increase is mediated by the nuclear association between PPAR-γ coactivator 1α and

transcription factor Ying-Yang 1, which positively regulates mitochondrial biogenesis and oxidative function.[116]

The fourth cellular process controlled by mTORC1 is the promotion of cellular growth by negatively regulating autophagy (central degradation process required to recycle damaged organelles and also for organismal and cellular adaptation to nutrient deprivation).[117] Studies have shown that mTORC1 directly phosphorylates and suppresses ULK1/Atg13/fip200 (umc-15-like kinase 1/mammalian autophagy-related gene 13/focal adhesion kinase family-interacting protein), a kinase complex required for the initiation of autophagy. In addition, activation of mTORC1 is also known to regulate another suppressor of autophagy called death associated protein 1 (DAP1).[118-119]

mTORC2 Activation and Subsequent Cellular Functions

mTORC2 is poorly characterized of the two mTOR complexes. mTORC2 is activated by growth factors including insulin and EGF that stimulate the PI3K pathway.[120] Recently, it has been shown that ribosome association is needed for mTORC2 activation and mTORC2 binds ribosomes in a PI3K-dependent manner.[120] Like mTORC1, mTORC2 also controls several cellular processes such as metabolism, cell survival, apoptosis, cell growth, proliferation by directly causing phosphorylation of AKT, serum and glucocorticoid-induced protein kinase 1 (SGK1) and protein kinase C-α (PKC-α).[96] Studies have shown that activated mTORC2 directly phosphorylates AKT at serine 473 hydrophobic motif for maximal activation of AKT. This maximal activation of AKT, in turn, regulates metabolism, survival, apoptosis, growth, and proliferation through phosphorylation of several downstream effectors of AKT.[121] It has also been

shown through several studies that mTORC2 directly activates SGK1, a kinase controlling ion transport and cell growth.[122] PKC-α is the third substrate activated by mTORC2. This activation has been shown to regulate cell shape in cell type specific manner by affecting actin cytoskeleton.[123]

Role of mTORC1 and mTORC2 in Cancer

Several studies have shown the importance of mTOR signaling in cancer pathogenesis. Many PI3K signaling pathway components including TSC1/2, PTEN and AKT (upstream effectors of mTORC1 and mTORC2) have been known to be mutated, deleted or overexpressed in human cancers. This, in turn, resulted in the over-activation of mTORC1 and mTORC2. [84, 124] Studies have also shown that loss of p53 (common event in cancer) also promotes activation of mTORC1.[83] This oncogenic activation of mTOR complexes induces processes required for cancer cell growth, survival, and proliferation.

A growing body of evidence has shown that the dysregulation of protein synthesis, which is controlled by mTORC1 at the level of 4E-BP1/eIF4E, plays a critical role in tumor formation. Studies have also shown that loss of 4EBP1 and subsequent activation of cap-dependent translation promotes cell cycle progression and cell proliferation.[125] In addition, it has also been shown that 4E-BP1/eIF4E mediate the effects of oncogenic AKT signaling on mRNA translation, cell growth, and tumor progression.[126]

A hallmark of proliferating cancer cells is the increase in *de novo* lipid synthesis, which is controlled by mTORC1 activation.[127] Studies have shown that over-activation

mTORC1 (observed in colon, bladder, Breast and PCa, in part due to upstream PI3K components) promotes the activation and translocation of SREBP1 to the nucleus. [109] This, in turn, drives the expression of components of the oxidative branch of the pentose phosphate pathway, which control the production of reducing equivalents and ribose-5-phosphate needed for lipogenesis and nucleotide biosynthesis, respectively.[109]

Studies have shown that over-activation of mTORC1 in cancer cells strongly inhibits autophagy. *In vivo* and *in vitro* studies have shown that deficiency in essential components of autophagy promotes the accumulation of protein aggregates, damaged mitochondria, and reactive oxygen species, which promotes DNA damage and tumorigenesis.[128]

An emerging body of evidence has also suggested the role of mTORC2 in cancer. In glioma studies, the overexpression of mTORC2 subunit of RICTOR has been observed. It has also been observed that the forced overexpression of mTORC2 endows cancer cells with increased proliferative and invasive capability.[129-130] *In vivo* studies in mice have shown that, the development of PCa by loss of PTEN requires mTORC2 function. These results thus support the important role of mTORC2 in promoting tumorigenesis.[131]

mTORC1 and mTORC2 have been recently implicated in regulation of RhoGTPase and thus affect cancer progression. Before we discuss the role of Rac1, mTORC1, and mTORC2 in cancer progression, we will discuss in detail what is Rac1, how Rac1 is activated, what is its function in normal cellular processes, and finally its role in cancer cell progression.

Rac1 GTPase- Member of the Rho/RAC GTPase Family

The Rho/Rac GTPases are a family of small G-proteins widely implicated in normal physiology and disease. They play an important role in cytoskeleton rearrangement and are key regulators of cellular adhesion, migration, proliferation, survival differentiation, and malignant transformation.[132] The members of this family of proteins in humans are divided into six classes: Rho (RhoA, RhoB and RhoC), Rac (Rac1, Rac2, Rac3 and RhoG), Cdc42 (Cdc42, Tc10, TCL, Chp/Wrch-2 and Wrch-1), RhoBTB, Rnd, and RhoT.[133] The most well characterized and widely studied members of the Rho/Rac GTPase family are RhoA, Rac1 and Cdc42 but in this discussion we will focus our attention only on Rac1.

The Rac1 gene is located on chromosome 7 (7q22) and is ubiquitously expressed.[134] Like other members of its family, Rac1 functions as a molecular switch that cycle between an inactive state that binds GDP and an active state that binds GTP. GTP is hydrolyzed to GDP through their intrinsic GTPase activity to render the Rac1 protein inactive.[135] The switch between GDP and GTP is primarily regulated by two types of proteins: GEFs (Guanine Nucleotide Exchange Factor) that facilitate GTP loading and thereby activate the Rac1 and GAPs (GTPase Activating Proteins) that stimulate the hydrolysis of GTP by enhancing the intrinsic GTPase activity of Rac1.[136] Studies have shown that Rac1 has its own specific GEFs that cause activation of Rac1 and these will be discussed in the following paragraph.

Regulation of Rac1 by GEFs and GAPs

As mentioned above, Rac1 cycles between an inactive state that binds GDP and active state that binds to GTP. It has been known that the guanine nucleotides have picomolar affinity for Rac1 and as a result their dissociation rate from Rac1 protein is very slow. In order, to produce fast responses such as actin cytoskeleton rearrangement, GEFs accelerate the GDP/GTP exchange rate by several orders of magnitude.[137] The mechanism by which GEFs weaken the binding of nucleotide has been recently investigated. According to this model, the bound nucleotide in Rac1 is sandwiched between two loops called switch 1 and 2. These regions together with the phosphate-binding loop interact with the phosphate and a coordinating magnesium ion in the Rac1 specific GEFs.[137-138]

Studies have shown that Rac1 is activated by a variety of stimuli including growth factors (EGF) and G-protein coupled receptor ligands (CXCL12/SDF-1α).[136] The aforementioned stimuli activate two very specific GEFs: Tiam1 and p-REX1. p-REX1 (specific Rac1-GEF) is directly activated by free $G_{\beta\gamma}$ subunit released when CXCL12 binds the GPCR receptor.[139] The other Rac1 specific GEF –Tiam1 is activated by activation of the RTK pathway, activated by growth factors.[140-141] Recent studies have also shown that both p-REX1 and Tiam1 are overexpressed in metastatic PCa cells, which lead to over activation of Rac1 protein.[142]

Function of Rac1 in Cellular Processes

The main role of Rac1 is regulation of cytoskeleton reorganization as it promotes actin assembles required for lamellipodia and membrane ruffle formation.[143] The

regulation of cytoskeleton dynamics by Rac1 is essential in normal cells for maintenance of morphology, polarity, adhesion, and migration.[133] The control of cytoskeleton reorganization via Rac1 involves two mechanisms (Figure 10).

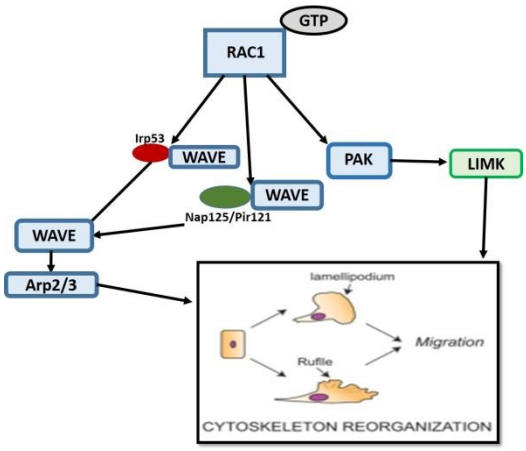

Figure 10. Schematic representation of the functions of Rac1 in normal cellular processes.

The first mechanism is through activation of Arp2/3, which has a prominent role in actin polymerization through WAVE/Scar indirect activation. WAVE can be activated by disassemble of Rac-Nap1-PIR121 or through IRSP53 activation.[144] The second mechanism is through Pak (p21-activated kinase). The Rac1-GTP binds to the N-terminal GTPase binding domain of group I Pak (Pak 1, 2 and 3), which causes release of auto-inhibition and enhanced kinase activity.[24]

Studies have shown that Rac1 is involved in other cellular functions such as cell cycle regulation, endocytosis, phagocytosis, and integrin-mediated adhesion. Rac1 regulates transcription via JNK/c-Jun, is involved in p38 activation by stress stimuli, activates the MKK3/6-p38MAPK pathway, and mediates invasion by H-Ras in mammary

epithelial cells. It has been also shown that Rac1 cooperates with Raf to activate the Erk pathway.[145]

Role of Rac1 and mTOR Complexes (mTORC1 and mTORC2) in Cancer Progression

The above discussion details the critical role of mTOR complexes and Rac1 for the normal function of cells. Recent studies have shown that the levels of mTOR and cytoplasmic p-mTOR were greater in PCa tissues *vs* normal prostatic epithelium, with mTOR levels in cancer cells twice that of benign tissues.[145] p-mTOR was detected at low levels in the cytoplasm and at moderate to high levels along the membrane in normal prostatic epithelium, while in cancer cells strong immunoreactivity of p-mTOR was detected both at membrane and cytoplasmic levels (60). Comparison between the levels of signaling molecules downstream of mTOR, including 4E-BP1 and S6, also showed higher levels in PCa vs. normal cells.[145] Another independent study has observed that the expression of Rac1-GTP is drastically increased in HG-PIN and prostate carcinoma compared to benign secretory epithelium.

Recent studies by Gupta *et al* has shown that, mTORC2 component RICTOR regulates Rac1 and causes cell spreading and migration in advanced bladder cancer cells.[19] Another independent study in colorectal cancer cells showed that mTORC1 and mTORC2 regulate Rac1 and cause migration and invasion in these cells.[20] Studies conducted by Chen *et al* in PCa cell lines, DU145 and 22RV1 have also shown that mTORC1 and mTORC2 regulate Rac1 and cause migration and invasion in these cells.[21] Another study conducted by Saci *et al.*, in HeLa and Panc1 cells showed that Rac1

activated mTORC1 and mTORC2. In addition, it was also shown that Rac1 directly interacts with mTOR protein in both HeLa and Panc1 cells.[26]

From all of the above discussions and literature presented, we see that there exists a gap in our understanding of the Rac1-mTOR complexes (mTORC1 and mTORC2) axis and it implications leading to PCa progression. Thus, in order to understand the role played by Rac1-mTOR axis in PCa progression, there exists a need to determine if Rac1 activates mTOR complexes and the mechanism by which Rac1 activates the complexes in PCa cells. This investigation will not only help us understand the progression of PCa but also shed light on a new signaling mechanisms that can be potentially exploited for treatment of PCa.

CHAPTER III

MATERIALS AND METHODS

Cell Culture, Chemicals and Reagents

LNCaP, DU145, and PC3 cells were obtained from American Type Culture Collection (ATCC) (Rockville, MD). LNCaP cells were maintained in RPMI, supplemented with 5% FBS and DU145 and PC3 cells were maintained in MEM, supplemented with 5% FBS in 5% CO_2 environment at 37°C, as previously described[146-147]. Recombinant human Epidermal Growth Factor (EGF) was purchased from R&D systems (Minneapolis, MN). The antibodies against His-tag, p-mTOR (Ser-2448), p-mTOR (Ser-2481), and mTOR were purchased from Cell Signaling Technology (Danvers, MA). Anti-Rac1 antibody was purchased from BD Biosciences (New Jersey, NY). Protein A/G agarose beads, RAPTOR, RICTOR, and Rac1 siRNA oligos were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-α-Tubulin antibody was purchased from Sigma–Aldrich (St Louis, MO). The anti-rabbit and anti-mouse immunoglobulins coupled with horseradish peroxidase (IgG-HRP) were obtained from Promega (Madison, WI). Rac1 inhibitor (NSC23677) was obtained from Torcis Biosciences (Bristol, UK). Fluorescent stain for cell nuclei: DAPI (4', 6-diamidino-2-phenylindole) and for actin: Rhodamine-Phallodin was purchased from Roche Diagnostics (Indianapolis, IN) and Cytoskeleton Inc. (Denver, MO), respectively.

siRNA Transfection

To knockdown endogenous RAPTOR, RICTOR, or Rac1 expression, DU145 and PC3 cells were plated in 6-well plates at a density of $1.5x10^5$ cells per well in antibiotic-free growth medium, supplemented with 5% fetal bovine serum (FBS) overnight. DU145 and PC3 cells were transfected with control, RAPTOR, RICTOR, or Rac1 siRNA according to the manufacturer's instructions (Santa Cruz Biotechnology). In brief, cells were washed once with antibiotic free-growth medium, followed by the addition of the siRNA duplex supplemented with antibiotic-free growth medium with 5% FBS. After 24 hrs, the transfection media were replaced with fresh media and the cells were incubated for an additional 24 hrs. These cells were used for western blot analysis, migration, and Rac1 activation assays.

Migration Assay

In vitro cell migration assays were performed as described previously.[148] The outside and inside of the trans-well insert membranes were coated with 50 µl of rat tail collagen (50 µg/ml). The cells were harvested and re-suspended in MEM supplemented with 0.2% BSA (30,000 cells per 100 µl). Epidermal growth factor (EGF, 10 ng/ml) and MEM with 0.2% BSA were used as chemoattractant and control treatments, respectively. Aliquots of 100 µl of cell suspensions were loaded into trans-well inserts, in a 24- well plate and incubated at 37°C for 5 hrs. Non-migrating cells were removed with cotton swabs and fixed using 3.7% paraformaldehyde (pH 7.5) for 20 min at room temperature. Migrated cells were stained with 3 ng/ml of DAPI, according to the manufacturer's instructions and visualized using an Axiovert 200M Carl-Zeiss microscope (Gottingen,

Germany). The results were expressed as a migration index defined as: the average number of cells per field for test substance/the average number of cells per field for the control.

Western Blot Analysis

Cell lysates were collected and western blots were carried out as described previously.[148] In brief, individual samples (30–40 μg proteins) were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis in 7.5 or 10% gels and transferred to polyvinylidene difluoride membranes (Millipore). After blocking with 5% fat-free milk in TBST (Tris-buffered saline containing 0.01 %Tween-20) for 1 hr at room temperature, the membranes were incubated with appropriate dilutions of specific primary antibodies (1:1000 dilution for anti-p-mTOR(Ser-2448), anti-p-mTOR(Ser-2481), anti-mTOR, and anti-His-tag antibodies, 1:500 for anti-Rac1, 1:3000 for anti-α-Tubulin) overnight at 4°C. After washing, the blots were incubated with anti-rabbit or anti-mouse IgG-HRPs for 1 hr. The blots were developed in enhanced chemiluminescence mixture (Thermo Fisher Scientific, Rockford, IL). The density of specific protein bands were determined by ImageJ analysis software and normalized using α-Tubulin as the loading control.

Overexpression of Wild Type Rac1 (Rac1WT) and Active Rac1 Mutant (Rac1Q61L) in PC3 Cells

Bacterial Stab containing pcDNA3-EGFP-Empty Vector (EV) (plasmid#13031), pcDNA3-EGFP-Rac1WT (plasmid#12980), and pcDNA3-EGFP-Rac1Q61L (plasmid#12981) (constitutively active Rac1) plasmids were purchased from Addgene. Plasmids were isolated and purified, according to the manufacturer's protocol, using

ZYMOPURE™ plasmid Maxiprep kit (Zymo Research). Purified plasmids (2 µg) were transfected into PC3 cells, using Lipofectamine 3000 transfection reagent for 24 hrs. Cells were sorted based on EGFP expression using BD Jazz Cell Sorter (BD Bioscience, New Jersey, NY). The enriched populations were grown in MEM, supplemented 10% FBS with different concentrations of the selective antibiotic G418 (400 µg/ml for PC3-EV, and PC3-Rac1WT, and 800 µg/ml for PC3-Rac1Q61L), to prevent the growth of non-EGFP expressing cells.

Immunoprecipitation

PC3-EV, PC3-Rac1WT, and PC3-Rac1Q61L cells were incubated with or without EGF (10 ng/ml, 3 min) and cell lysates were prepared using 1X cell lysis buffer (Cell Signaling Technology, Beverly, MA). Total cell lysates containing 1000-1100 µg of total proteins were incubated with 1:50 dilution of anti-His-tag antibody for 24 hrs under gentle rotation at 4°C, followed by incubation with 100 µl of protein A/G agarose beads (0.5 ml of agarose in 2.0 ml of PBS with 0.02% sodium azide) overnight at 4°C under, gentle rotation. Immune-complexes were washed 3 times with 1X cell lysis buffer to remove non-specific proteins adhered on the agarose beads. The resulting immune-complexes were eluted using 2X Laemmeli's buffer at 60°C for 10 min. Resulting eluates were analyzed by western blot analysis with anti-mTOR, anti-RAPTOR, or anti-RICTOR antibodies.

Rac1 Activation Assay

PC3-EV, PC3-Rac1WT, and PC3-Rac1Q61L cells were plated at a density of $1.5x10^5$ cells per well. Cells were serum-starved for 2 hrs. The cells were then pre-

incubated with or without Rac1 inhibitor NSC23677 (10 μM) for 30 min, followed by treatment with EGF (10 ng/ml, 3 min). Rac1 activity was measured in the cell lysate proteins (0.1-0.2 mg/ml) with GLISA Rac1-activation assay (colorimetric format, Cytoskeleton Inc., Denver, MO) according to manufacturer's protocol.

Fluorescence Analysis and Actin Staining

PC3-EV, PC3-Rac1WT, and PC3-Rac1Q61L cells were plated on glass cover slips placed in 6-well plates, at a density of $1.5x10^5$ cells per well for 48 hrs. Cells were serum-starved for 2 hrs and then treated with EGF (10 ng/ml, 3 min). Cells were fixed with 3.7% paraformaldehyde, washed with 1X PBS, and permeabilized with 0.1% of Triton-X. Cells were incubated with Rhodamine-phalloidin for 30 min to detect F-actin and DAPI for 10 min to detect the nuclei and mounted using Vectashield mounting medium (Vector Laboratories, Burlingame, CA). EGFP-fluorescence was used for Rac1 localization in PC3-Rac1WT and PC3-Rac1Q61L cells.

Statistical Analysis

All experiments were repeated at least three times using different cell preparations. The results are presented as means ± SEM of three independent experiments and images from a single representative experiment are presented. Analysis of variance and Duncan's modified multiple range tests were employed to assess the significance of differences among various treatment groups ($p< 0.01$).

CHAPTER IV

RESULTS

mTORC1 and mTORC2 are Essential for Migration in DU145 and PC3 Prostate Cancer

We and others have shown that PI3K/AKT/mTOR is essential for cell migration and invasion.[4, 149-150] To understand the relative contribution of mTORC1 and mTORC2 in cell migration, we knocked down the expression of RAPTOR and RICTOR (main players in mTORC1 and mTORC2, respectively) by over 80% using siRNA in PC3 and DU145 cells and then performed cell migration in the presence or absence of EGF. EGF induced a significant increase in cell migration in cells transfected with control siRNA whereas it failed to cause any increase in cell migration in RAPTOR and RICTOR deficient cells (Figure 11A, 11B and 11C). These results demonstrated that both mTORC1 and mTORC2 complexes are required for cell migration in PC3 and DU145 cells.

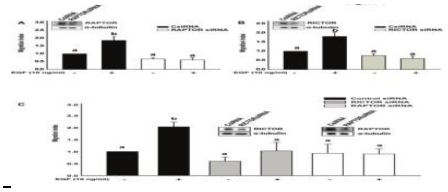

Figure 11. RAPTOR and RICTOR are essential for cell migration in PC3 and DU145 cells.

EGF Induces the Activation of both mTORC1 and mTORC2 in PC3, DU145 and LNCaP Cells

Previous studies have shown that mTOR protein is phosphorylated at serine 2448 (Ser-2448) or serine 2481 (Ser-2481) depending upon its association with active mTORC1 or mTORC2, respectively.[25] To investigate if EGF activates both mTORC1 and mTORC2 in PC3, DU145 and LNCaP cells, we treated PC3, DU145 and LNCaP cells with EGF (10 ng/ml, 3min) and determined the phosphorylation of mTOR protein at respective phosphorylation sites. EGF treatment induced phosphorylation of mTOR protein at both Ser-2448 and Ser-2481 (Figure 12A) in PC3, DU145 and LNCaP cells Thus, indicating that EGF induces activation of both mTORC1 and mTORC2 in PC3, DU145 and LNCaP cells.

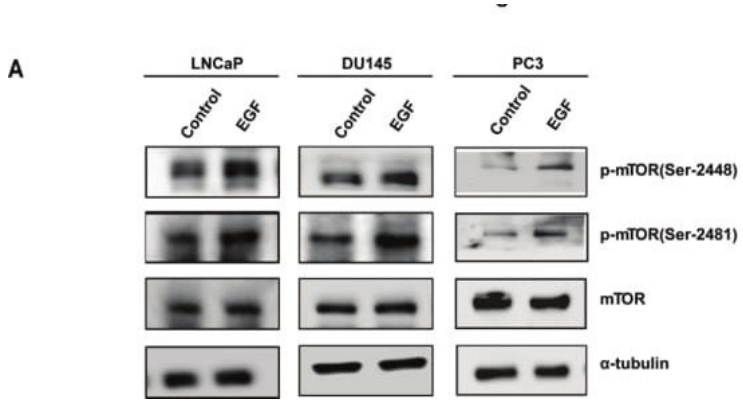

Figure 12. EGF induces the activation of mTORC1 and mTORC2 in PC3, DU145 and LNCaP cells.

Role of Rac1 in Activation of mTOR Complexes (mTORC1 and mTORC2) in PC3, DU145 and LNCaP Cells

Studies have demonstrated that Rac1 has the capability to regulate mTORC1 and mTORC2 activity and affect cell size in HeLa cells.[26] Therefore, we determined a possible role of Rac1 in the activation of both mTOR complexes in PC3, DU145 and LNCaP cells. We knocked down Rac1 by over 80% using Rac1 siRNA in PC3 cells and investigated the effects of EGF on phosphorylation of mTOR in control and Rac1 deficient cells. As shown in Figure 13A, EGF-induced activation of mTORC1 was unaffected however; the activation of mTORC2 was significantly reduced in Rac1 deficient cells.

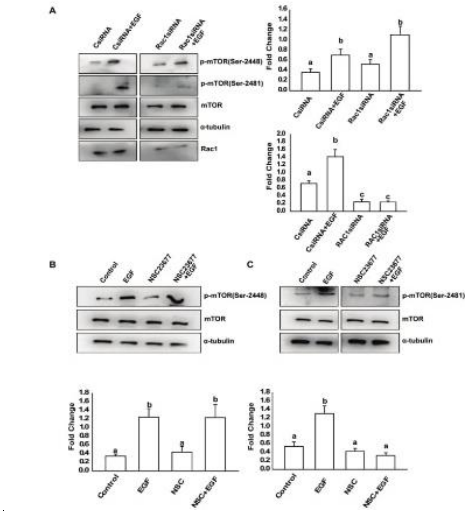

Figure 13. Activation of mTORC2 was decreased in the absences of total and active Rac1 in PC3 cells.

In parallel experiments, inhibition of Rac1 by a specific inhibitor (NSC23677) blocked EGF-induced activation of mTORC2, but had no effects on activation of mTORC1 by EGF (Figure 13B, 13C, 14A and 14B) in PC3, DU145 and LNCaP cells. These results suggest that EGF induced activation of mTORC1 is upstream and mTORC2 is downstream of activated Rac1 in PC3, DU145 and LNCaP cells.

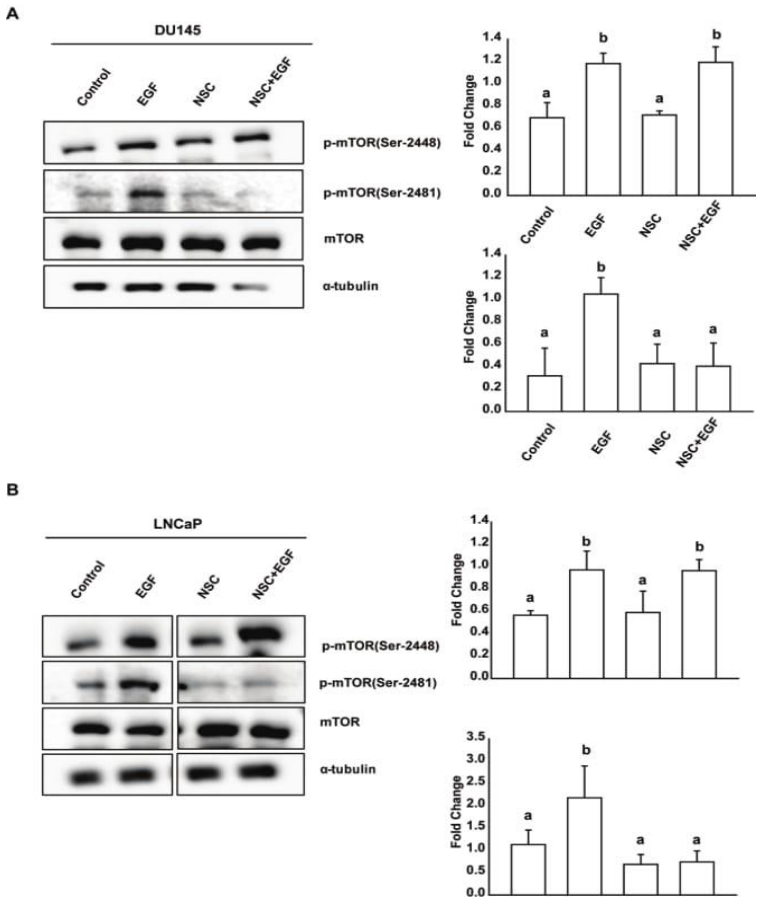

Figure 14. Activation of mTORC2 was decreased in the absences of total and active Rac1 in DU145 and LNCaP cells.

Overexpression of Rac1 Increased Migration and Affected Rac1 Localization in PC3

PC3 cells were transfected with EGFP-EV, EGFP-Rac1WT, or EGFP-Rac1Q61L (constitutively active Rac1) plasmids to establish stably over-expressing empty vector (PC3-EV), wild type Rac1 (PC3-Rac1WT) or constitutively active Rac1 (PC3-Rac1Q61L) cells. Over-expression of Rac1 in these cells was confirmed by qRT-PCR and western blot analysis (Appendix A: Figure 1A and 1B).

Rac1 activation assays confirmed the activation levels of Rac1 in these cell lines (Figure 15A). Basal Rac1 activity was unaffected in cells over-expressing empty vector (PC3-EV) and wild-type Rac1 (PC3-Rac1WT), however there was a 2-fold increase in Rac1 activity in cells over-expressing constitutively active Rac1 (PC3-Rac1Q61L). EGF treatments caused an increase in Rac1 activity in PC3-EV and PC3-Rac1WT cells, whereas Rac1 activity was unaffected in cells over-expressing constitutively active Rac1. EGF induced Rac1 activity was more than 2-fold higher in PC3-Rac1WT cells compared to PC3-EV cells. For migration analysis, there was no significant difference in cell migration between PC3-EV and PC3-Rac1WT cells; however, PC3 cells over-expressing constitutively active Rac1 (PC3-Rac1Q61L) exhibited 2-fold increase in migratory ability (Figure 15B). Treatment with EGF caused significant increase in cell migration in PC3-EV and PC3-Rac1WT cells; EGF effects on cell migration in PC3-Rac1WT were 2-fold higher than those in PC3-EV cells. EGF treatment did not cause any additional increase in cells over-expressing constitutively active Rac1. We also performed fluorescence analysis to determine the localization of Rac1 in cells over-expressing wild type (PC3-Rac1WT) or constitutively active Rac1 (PC3-Rac1Q61L) (Figure 15C). Under basal

conditions, Rac1 was localized in the cytoplasm - in a diffused manner in PC3-Rac1WT cells, whereas it was localized at the plasma membrane in the PC3-Rac1Q61L cells. After stimulation with EGF, Rac1 was localized at the plasma membrane in PC3-Rac1WT cells as well. These results show that increased Rac1 activity leads to an increase in cell migration, which involves localization of Rac1 at the membrane in migrating cells.

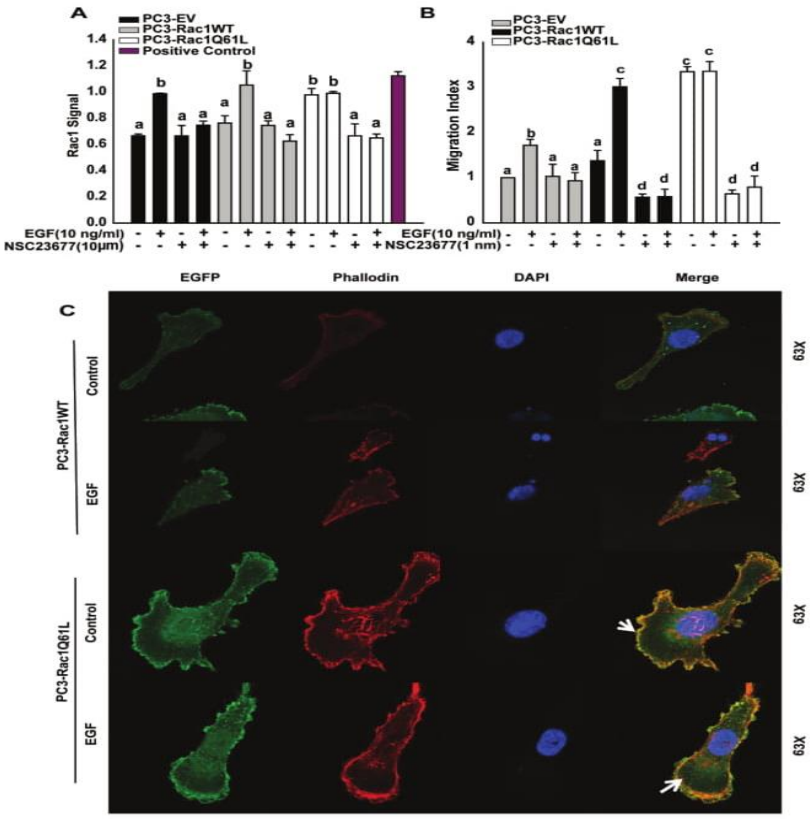

Figure 15. Wild type Rac1 (PC3-Rac1WT) and constitutively active Rac1 (PC3-Rac1Q61L) cells induced increased activation of Rac1, cell migration, and induced constant localization of Rac1 at the leading edge of these cells.

Active Rac1 Promotes Activation of mTORC2 via mTORC2 Component RICTOR

Activation of Rac1 leads to phosphorylation and activation of mTORC2 complex suggesting that Rac1 may physically interact with components of mTORC2 complex. To investigate this possibility, we determined the activation of mTORC1 and mTORC2 in above cell lines. As shown in Figure. 16A, mTORC1 complex was not activated in unstimulated cell lines while mTORC2 complex was active in PC3 cells overexpressing constitutively active Rac1. On the other hand, EGF treatment induced activation of mTORC1 and mTORC2 complexes in all cell lines in PC3 cells transfected with either EV or Rac1WT. Additionally, EGF caused activation of mTORC1, but had no additional effect on the activation of mTORC2 in PC3 cell over-expressing constitutively active Rac1. Immunoprecipitation of His-tagged Rac1 from PC3 cells over-expressing wild type or constitutively active protein followed by western blot analysis of mTOR, RICTOR, and RAPTOR proteins is shown in Figure 16B. Both mTOR and RICTOR were associated with Rac1 in EGF treated PC3-Rac1WT cells and in PC3 cell over-expressing constitutively active Rac1, whereas RAPTOR was not detected. Our data suggest that the activation of Rac1 was necessary to activate mTORC2 and was dependent on the proximity of Rac1 to RICTOR.

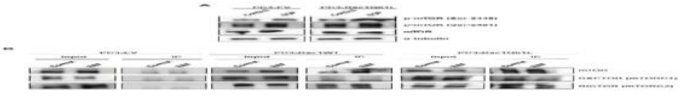

Figure 16. Rac1 forms a complex with mTORC2 through RICTOR in wild type Rac1 (PC3-Rac1WT) and constitutively active Rac1 (PC3-Rac1Q61L) cells.

CHAPTER V

DISCUSSION

In this study, we demonstrated that mTORC1 and mTORC2 complexes were activated by EGF signaling and are required for cell migration. mTORC1 was activated directly by EGF while the activation of mTORC2 complex was indirectly dependent on the activation of Rac1 protein. This data suggested that mTORC1 acts upstream and mTORC2 acts downstream of activated Rac1 to induce cell migration in PCa cells.

PI3K/AKT/mTOR axis is one of the major signaling pathways involved in cancer progression and metastasis.[151-152] Over-expression of growth factor (EGF, TGFβ, IGF), mutations in the PI3K protein as well as deletion or mutation of the tumor suppressor PTEN, causes up-regulation of AKT and mTOR in numerous cancer types.[5] We have previously shown that TGFβ and EGF induced activation and phosphorylation of AKT, p70S6K, and S6K (latter of the two protein are downstream substrates of mTORC1 activation) in PC3 cells.[4] In addition, we have also shown that knockdown of the PTEN levels induced the upregulation of p-AKT and increased migratory behavior in DU145 cells.[153] Dysregulated activation of AKT and mTOR via afore mentioned factors, has been shown to be essential for cell migration in several cancers such as gastric, breast, colon, prostate, and hepatocellular carcinoma.[150, 154-155] We previously reported that rapamycin caused a decrease in migration in PC3 and DU145 cells.[4]

Studies in bladder, breast, prostate, and colon cancer indicated that knockdown of RAPTOR and RICTOR (regulatory components of mTORC1 and mTORC2 respectively), caused decrease in cell migration.[19-21] In the current study, we also demonstrated that PC3 and DU145 cells exhibited a decrease in cell migration as a result of RAPTOR and RICTOR knockdown. These results suggest that the activation of PI3K, AKT, mTORC1, and mTORC2 are critical for migration in cancer cells.

mTOR, a 289 kDa serine/threonine kinase, acts as a master controller of numerous cellular functions including migration, invasion, and cytoskeletal rearrangement in normal and cancer cells.[156] The mTOR kinase nucleates into two protein complexes termed as mTORC1 and mTORC2.[157] The major regulator of mTORC1 kinase activity is regulatory associated protein of mTOR (RAPTOR).[158-159] Mechanism of regulation of mTORC1 requires two major cellular inputs: amino acids and growth factors. Amino acids accumulate at the Lysosomal lumen and initiate the interaction between v-ATP and Ragulators (Gunine exchange factors specific for Rag-GTPases).[160] The Ragulators through their guanine nucleotide exchange factor activity causes Rag-GTPase to recruit and activate mTORC1 at the lysosomal surface.[105-106] Another mode of mTORC1 regulation is through growth factors such as Insulin or insulin like growth factors. These growth factors activate receptor tyrosine kinases (RTKs), which promotes PIP3 production through phosphoinositide-3-kinase (PI3K).This production initiates AKT activation, which lead to the activation of mTORC1 by two mechanisms.[160] First, active AKT reduces the interaction of proline-rich AKT substrate 40 kDa (PRAS40) with mTORC1. Secondly, active AKT phosphorylates and inactivates

TSC1/2, which stimulates mTORC1 kinase activity via Rheb.[160] A previous study showed that in HEK293 and 293T cells, serum or IL3 acting via p-AKT induced mTORC1 kinase activity by phosphorylating mTOR at Ser-2448.[161] A similar induction of mTORC1 activity by phosphorylation at Ser-2448 has been demonstrated in breast and pancreatic cancer cells.[161] We also demonstrate that the stimulation by EGF in PC3, DU145 and LNCaP cells causes the phosphorylation of mTORC1 at Ser-2448. Less is known about mTORC2, compared to mTORC1. mTORC2 is insensitive to amino acids, but responds to growth factors through a poorly understood mechanism. Soliman *et al.* showed that the inhibition of PI3K by wortmannin decreased mTORC2 associated mTOR Ser-2481 phosphorylation.[162] Similarly Copp *et al.* demonstrated that insulin promoted the RICTOR associated phosphorylation of mTOR at Ser-2481, which is associated with mTORC2 kinase activity.[25] Our results confirmed those of Copp *et al.*, showing that EGF was also able to induce phosphorylation of mTORC2 associated mTOR Ser-2481 in PC3, DU145 and LNCaP cells. We also investigated the potential upstream regulators of mTORC2 phosphorylation after EGF stimulation. A study conducted by Sac *et al* in HeLa cells, showed that Rac1 regulates the activity of mTORC1 and mTORC2 under a serum stimulated condition.[26] We have shown that in Rac1 depleted PC3 cells, mTORC1 associated mTOR Ser-2448 phosphorylation was unaffected whereas mTORC2 associated mTOR Ser-2481 phosphorylation was drastically reduced under EGF stimulated condition. In a parallel experiment, we observed that in the absence of active Rac1, the phosphorylation of mTORC1 associated mTOR Ser-2448 was unaffected whereas mTORC2 associated mTOR Ser-2481 phosphorylation was decreased under

EGF stimulated conditions. These results indicate that Rac1 plays a role in the regulation of mTORC2 associated mTOR Ser-2481 phosphorylation.

Rac1 is a member of the Rho/Rac GTPase family and cycles between GTP (guanine tri-phosphate) active and GDP (guanine di-phosphate) inactive states.[137] Rac1 has been implicated in numerous cellular processes including cell adhesion, proliferation, differentiation, and cell migration.[132] Studies have shown that the over-expression of Rac1 in PCa patient tissue samples is associated with poor patient prognosis.[145] We investigated the effect of Rac1 over-expression on Rac1 activity, its localization and cell migration (Figure 17).

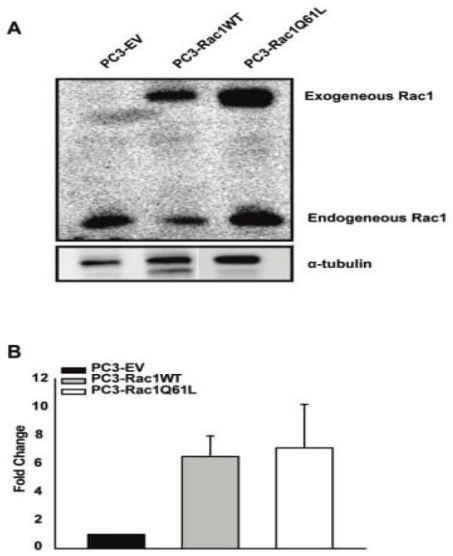

Figure 17. Over-expression of EGFP-Rac1WT or EGFP-Rac1Q61L (Rac1 constitutively active mutant) plasmids in PC3 cells were confirmed using western blot analysis and qRT PCR.

Firstly, to investigate the effect of over-expressing Rac1 on the Rac1 activity of PC3 cells, GLISA assay was performed. Basal Rac1 activity was unaffected in cells over-

expressing empty vector (PC3-EV) and wild-type Rac1 (PC3-Rac1WT) but there was a 2-fold higher Rac1 activity in cells over-expressing constitutively active Rac1 (PC3-Rac1Q61L). EGF treatment caused an increase in Rac1 activity in PC3-EV and PC3-Rac1WT cells, whereas Rac1 activity was unaffected in cells over-expressing constitutively active Rac1. EGF induced increase in Rac1 activity was more than 2-fold higher in PC3-Rac1WT cells compared to PC3-EV cells. These results are in agreement with studies in breast cancer, urothelial carcinoma and squamous cancer of head and neck, which showed that more invasive and metastatic stages of cancer are correlated to higher Rac1 activity.[163-165] Next, we determined cell migration in all three cell lines in response to EGF. In untreated cells, there was no difference in cell migration in PC3-EV and PC3-Rac1WT cells but basal cell migration was 2-fold higher in PC3-Rac1Q61L cells. Treatment with EGF caused significant increase in cell migration in PC3-EV and PC3-WT cells; EGF effects on cell migration in PC3-Rac1WT were 2-fold higher than those in PC3-EV cells. EGF treatment did not cause any additional increase in cells over-expressing constitutively active Rac1. Therefore, indicating that higher Rac1 activity resulted in higher migration in PC3-Rac1WT and constitutively active Rac1 cells. This result concurred with PCa patient tissue studies, which indicated that drastically higher active Rac1 was associated with aggressive PCa stages.[145] Finally, we investigated the effect of Rac1 overexpression on Rac1 localization, under treated and untreated conditions. Under basal conditions, Rac1 was localized in the cytoplasm in a diffused manner in PC3-Rac1WT cells, whereas it was localized at the membrane in constitutively active Rac1 cells. In EGF treated cells, Rac1 was localized at the membrane of PC3-

Rac1WT cells as well. These finding confirm that Rac1 needs to be bound to GTP, in order to be trans-localized to the leading edge of migrating cells.[166] Thus, higher Rac1 activity in Rac1WT and constitutively active Rac1 cells caused Rac1 to localize at the leading edge, which in turn caused an increase in cell migration.

Next, to investigate whether activation of mTORC2 downstream of active Rac1 was caused by the physical interaction of Rac1 with components of mTORC2 proteins, we first determined the activation of both mTOR complexes in empty vector and constitutively active Rac1 cells. As shown, mTORC1 complex was not activated in unstimulated cell lines while mTORC2 complex was active in PC3 cells overexpressing constitutively active Rac1. On the other hand, EGF treatment induced activation of mTORC1 and mTORC2 complexes in all cell lines in PC3 cells transfected with EV or Rac1WT. On the other hand, EGF caused activation of mTORC1 but had no additional effect on activation of mTORC2 in PC3 cell over-expressing constitutively active Rac1. This finding concurs with the role of Rac1 in regulation of mTORC2 by anchoring it to the membrane of migrating cells.[26] Next we immunoprecipitated Rac1 with His-tag antibody (located at the C-terminal of EGFP) from all three over-expressed cell lines, with and without EGF and then western blotted for mTOR, RICTOR, or RAPTOR protein. In control cells, mTOR and RICTOR were present in Rac1WT and constitutively active Rac1 cells but the presences of mTOR and RICTOR was increased in constitutively active Rac1 cells. In case of EGF treated cells, presence of mTOR and RICTOR was increased in Rac1WT cells, whereas presence of mTOR and RICTOR was unaffected in constitutively active Rac1 cells. Finally, with or without EGF, RAPTOR

protein was absent in PC3-Rac1WT and constitutively active Rac1 cells. This result indicates that activated Rac1 promotes mTORC2 activation due to proximity of Rac1 to RICTOR. This result also concurs with Copp et al study, demonstrating that RICTOR promotes mTORC2 activation by phosphorylating mTORC2 associated mTOR Ser-2481.[31] A review by Lam et al indicates that Rac1 has a hypervariable region at the C-terminal, which is essential for interaction and phosphorylation of its downstream substrates.[173] The direct interaction between Rac1 and RICTOR at this hypervariable region is elusive.

CHAPTER VI

MAJOR FINDINGS AND SIGNIFICANCE

On the basis of our study, we conclude that Rac1 plays a vital role in the regulation of mTORC2 and may provide a potential approach towards development of mTORC2 specific inhibitor. We also showed that EGF induced mTORC1 and mTORC2 specific phosphorylation, which are indicators of their kinase activity. In this study, it was also observed the active Rac1 is not required for mTORC1 activation, but is critical for mTORC2 activation in LNCaP, DU145, and PC3 cells. Our investigation also showed that stable over-expression of Rac1WT and constitutively active Rac1 in PC3 increased Rac1 activity and associated cell migration. Additionally, we also observed the constant localization of Rac1 at the leading edge of constitutively active Rac1 over-expressed cells. Finally, the study also showed that Rac1 forms a complex with RICTOR associated mTORC2 in the presences of EGF and affects phosphorylation of mTORC2 associated mTOR Ser-2481 in PC3 cells. Although we were able to address Rac1 requirement for mTORC2 activation, Rac1 and RICTOR interaction towards PCa cell migration is still incomplete, therefore further studies need to be completed to address this aspect of our findings. Based on our current finding, we have developed a working model for mTORC2 activation as shown in Figure 18. We hypothesize that the stimulation by EGF induces the activation of mTORC1 by phosphorylating mTOR at Ser-2448.

This, in turn caused the activation of Rac1, which upon close proximity with RICTOR affects the activation of mTORC2.

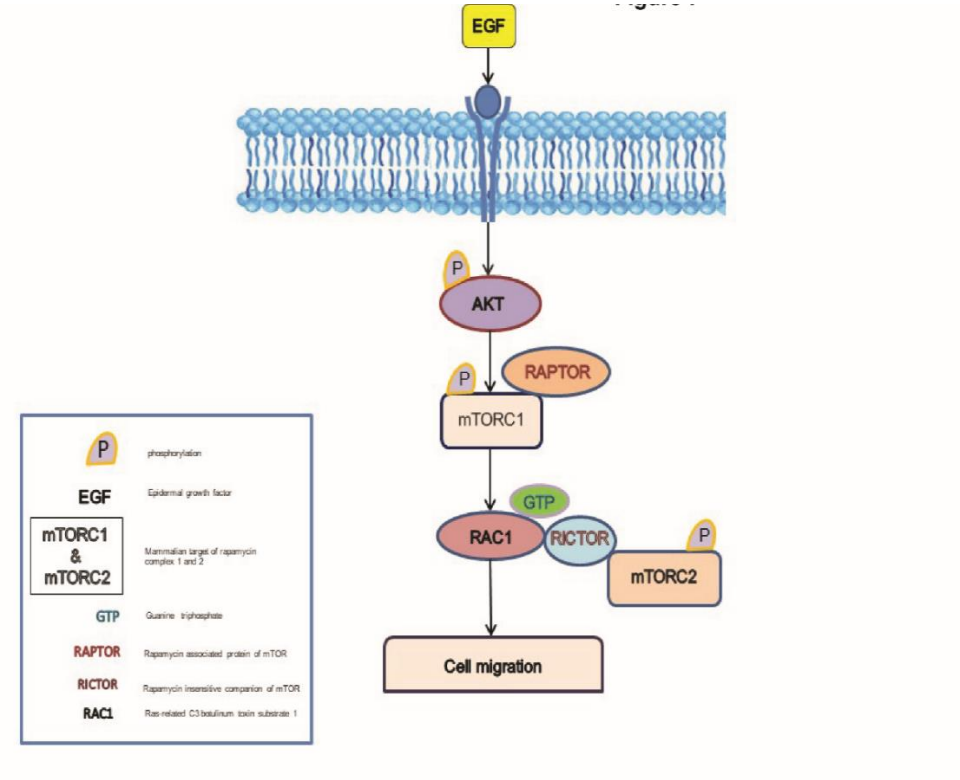

Figure 18. Working model of mTORC2 activation by active RAC1 using PI3K/AKT signaling pathway.

In addition to the current study, stably over-expressed constitutively active Rac1 cells were utilized in another study. This study focused on the importance of small G-protein Giα2 in migration of prostate cancer cells. In constitutively active Rac1 cells, knockdown of Giα2 drastically affected the migration of the cells. In addition, the polarity of the constitutively active Rac1 cells were also affected in Giα depleted condition.[148] Thus, the stably over-expressing cell line was utilized in two separate and distinct studies.

Based on our finding from both studies, we conclude that active Rac1 is downstream of mTORC1 and upstream of mTORC2 in prostate cancer cells. In addition, we also showed that activation of Rac1 by EGF regulates activity of mTORC2 via RICTOR in prostate cancer cells. With respect to the Giα2 studies, we observed that mechanism utilized by active Rac1 is independent from the mechanism used by Giα2 to regulate polarity of prostate cancer cells. Thus Rac1 has a role to play in both regulation of protein function and polarity of migrating prostate cancer cell. Based on the overexpression and activation of Rac1 in numerous cancers.[145,167] small molecular inhibitor such as ZINC08010136, and ZINC07949036 have been developed.[168] Most of these inhibitors target the Rac1-GEF axis to inhibit migration and invasion in breast, prostate both *in vitro* and *in vivo*.[168] One of the drawbacks of these inhibitors is that they affect the proliferation of normal tissues. Recently, another Rac1 inhibitor known as ZINC69391 was developed, which inhibits Rac1 activation caused by EGF.[168] It has been shown, both *in vivo* and *in vitro* studies, to inhibit invasion and migration in highly metastatic breast cancer cells.[168] The combination treatment with ZINC69391 and a specific small peptide that blocks the interaction of Rac1 and RICTOR can lead to the inhibition of both the feedback loop associated with mTORC2 activation and migration induced by the activation of Rac1 in metastatic prostate cancer patients.

APPENDIX A

SUPPLEMENTARY MATERIALS AND METHODS

Quantitative Real-Time PCR (qPCR) Analysis

Total RNAs were isolated from PC3-EGFP-EV, PC3-EGFP-Rac1WT and PC3-EGFP-Rac1Q61L cells using Trizol reagent (Invitrogen, Carlsbad, CA) as previously described.[169] The concentration and purity of all RNA samples were measured using Nanodrop 2000c Spectrophotometer (Thermo Fisher Scientific, Waltham, MA). The optical density readings were taken at 260 nm. Total RNA (2 µg) was reverse transcribed into cDNA as previously described.[169] Quantitative Real Time PCR (qRT-PCR) was performed using GoTaq Master Mix (Promega, Madison, WI) on BioRad CFX Connect Real time PCR System (BioRad, Hercules, CA) as previously described.[170] qRT-PCR was carried out in triplicate and the results were calculated using the ΔΔCT method whereby each sample (gene of interest) is normalized to its corresponding GAPDH values and the average of each individual ΔCt values was used for comparison between groups.[171] Data from qPCR were normalized to GAPDH expression level, which was used as the internal control. The Rac1 human gene-specific primer pair was designed with Beacon-Designer 5.0 as described previously.[172] The following primers were used: Rac1 forward: 5'-TTA CGC CCC CTA TCC TAT CC-3', Rac1 reverse: 5'- AAC GAG GGG CTG AGA CAT TT -3', GAPDH forward: 5'- GAA GGT GAA GGT CGG AGT C -3' and GAPDH reverse: 5'- GAA GAT GGT GAT GGG ATT TC -3'.

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