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ANDROGEN RECEPTOR AND PIN1 IN PROSTATE CANCER

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Aims of the Thesis

The aim of my thesis is to understand the molecular mechanism involved in Prostate Cancer. Since 20 years, the laboratory of Prof. Antonio Giordano is dedicated to elucidating the molecular basis of different human diseases with particular emphasis in the mechanisms involved on cancer progression and cell cycle control. The discoveries achieved in the last years helped the scientific community to understand the basic mechanism of cell cycle progression and open new ways to cure cancer patients.

In this thesis we demonstrated that the peptidyl prolyl isomerase PIN1 directly interact with the Androgen Receptor and this interaction plays a key role in Prostate Cancer Progression.

INTRODUCTION

Androgen dependent and independent Prostate Cancer

Other than skin cancer, prostate cancer is the most frequently diagnosed malignancy and second leading cause of cancer death amongst men in the United States (1).About 1 man out of 6 will be diagnosed with prostate cancer during his lifetime and 1 man in 36 will die. The androgens testosterone and dihydrotestosterone play an

essential role during the normal physiology of the cells and in the initial steps of tumorigenesis. Androgens bind to a specific androgen receptor (AR), a ligand-dependent transcription factor which controls the expression of a large number of downstream target genes. The androgen receptor (AR) is a critical effector of prostate cancer development and progression and for this reason , AR ablation is the first line of therapeutic intervention in the treatment of disseminated prostate cancers. However, recurrent tumors arise within a median of 2-3 years wherein androgen signaling has been inappropriately restored (2).

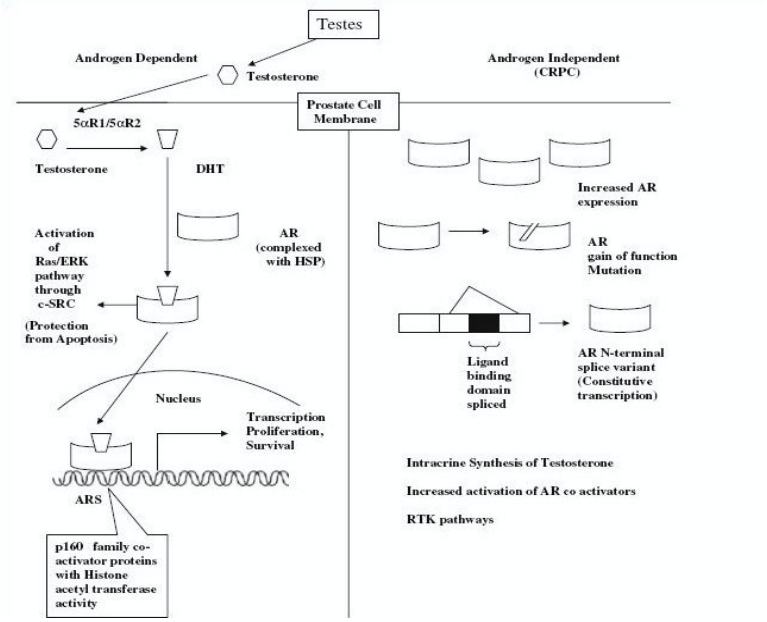

Fig 1: Comparison between androgen dependent and androgen independent way.

A form of more aggressive Prostate Cancer is the androgen independent prostate cancer(AIPC). In this case, there is an AR signaling reactivation and as a consequence, a lethal form of prostate cancer that progress and metastasize. The mechanisms involved in AIPC recurrence are different. One possible mechanism by which a prostate cancer circumvents the effects of androgen ablation therapy is by increasing its sensitivity to very low levels of androgens. There are several potential mechanisms that would allow increased tumor-cell proliferation, despite low circulating androgens in the patient. One mechanism to accomplish this is by increasing the expression of the AR itself. Approximately 30% of tumors that become androgen independent after ablation therapy have amplified the AR gene (3). A second hypersensitive mechanism for tumor progression results in high-level expression of the AR, increased stability, and enhanced nuclear localization of AR in recurrent tumor cells. A third hypersensitive mechanism to circumvent androgen ablation therapy is by increasing the local production of androgens, to compensate for the overall decline in circulating testosterone. Prostate cells could increase the rate of conversion of testosterone to the more potent hormone DHT by increasing 5α-reductase activity.

This would facilitates AR signaling even with significantly lower levels of serum testosterone. Some of these tumors, at least initially, have adapted to the low androgen environment, others acquire mutations that allow them to circumvent the normal growth regulation by androgens. It seems that many cases of AIPC do not develop from a loss of androgen signaling, but rather from the acquisition of genetic changes that lead to aberrant activation of the androgen-signaling axis. These changes are usually missense mutations in the AR gene that decrease the specificity of ligand binding and allow inappropriate activation by various non-androgen steroids and androgen antagonists (4) . In other cases some growth factors such as insulin-like growth-factor-1 (IGF- 1), keratinocyte growth factor (KGF) and epidermal growth factor (EGF), can activate the AR, creating an outlaw receptor, and can therefore induce AR target genes in the absence of androgen (5). These are just some of the mechanisms in which cells can use to escape androgen ablation and it is also possible that a single cancer uses several mechanisms either initially or in a multistep progression to AIPC. The appropriate regulation of androgen activity is necessary for a range of developmental and physiological processes, particularly male sexual development and maturation.

However, excessive production of adrenal androgens can cause premature puberty in young boys and their hypersecretion in females produce a masculine pattern of body hair and cessation of menstruation(6). Their miss-regulation is also implicated in the formation and progression of prostatic adenocarcinoma (7). Therefore, the removal of testicular androgens by castration has long been recognized to result in tumor regression, and surgical or pharmacological androgen ablation remains the predominant form of treatment for advanced prostate cancer. Androgen ablation therapy is often combined with the treatment of nonsteroidal antiandrogens, such as hydroxyflutamide, to block residual androgens action. Androgen Replacement Therapy has been in use for over 60 years to treat, with proven efficacy and safety, on patients with male hypogonadal disorders and/or failure of sexual development. Apart from that, the last decade has witnessed a wider therapeutic role of androgens for nonclassical indications.

Androgen Receptor structure and function.

Androgen receptor (AR), belongs to the family of nuclear receptors. These receptors are a class of ligand-regulated and DNA-sequence specific intracellular receptors that control the activity of genetic networks in response to different signals .

Structural analysis of AR revealed that it contains three major functional domains. The N-terminal activation function (AF)-1 domain (residues 1–555) contains different binding sites for transcriptional regulators including coactivators of the p160 family coactivators, acetyltransferases such as cAMP response element (CREB)-binding protein/p300 acetyltransferases, nuclear receptor corepressor and silencing mediator of retinoid and thyroid hormone receptor . Downstream of the AF-1 lies the DNA-binding domain (DBD) (residues 556–624), which mediates sequence-specific binding to promoters and enhancers of target genes. The hinge region (residues 625–670) links the DBD to the C-terminal domain(8,9). The functional domains of the AR are, conserved with other members of the "classic" receptor subclass.

The C-terminal region of the AR, the hinge region and ligand-binding domain (LBD) is responsible for ligand binding and receptor dimerization. The well conserved DNA binding domain consists of 68 amino acids with two zinc finger structures. In contrast to several other hormone-regulated nuclear receptors, the AR lacks an intrinsic activation function 2 in the LBD domain. The LBD domain, which consists of twelve α-helices, projects away from the hormone-binding pocket in the absence of ligand and undergoes substantial conformational changes in the presence of ligand . The folding of the most C-terminal helix 12 (H12) over the ligand-binding pocket in turn creates new structural surfaces that bind coactivators required for efficient *trans*-activation.

Similar to other members of steroid hormone receptor family such as the estrogen receptor, progesterone receptor, glucocorticoid receptor and mineral-corticoid receptor, the AR becomes activated after ligand binding. Steroid-hormone receptors are trans-acting gene-regulating proteins, involved in the accomplishment of steroid-hormone induced cellular responses.

Upon binding of hormone, the receptor-hormone complex undergoes a conformational change called transformation, which is thought to precede binding of the complex to androgen responsive elements(ARE)in the target cell genome (10). Testosterone — the main circulating androgen — is secreted primarily by the testes, but is also formed by peripheral conversion of adrenal steroids. It circulates in the blood, where it is bound to albumin and sex-hormone-binding globulin (SHBG), with a small fraction dissolved freely in the serum. When free testosterone enters into prostate cells , it is converted to dihydrotestosterone (DHT) by the enzyme 5α-reductase (SRD5A2). DHT is the more active hormone, having fivefold higher affinity for the androgen receptor (AR) than does testosterone(11).

While the subsets of AR target genes that underlie each cellular outcome have yet to be clearly defined, discovery of at least one major AR-dependent target gene, prostate specific antigen (PSA) has had a major impact on disease management (12). PSA is a serine protease in the kallikrein gene family that is secreted into seminal fluid by prostatic epithelial cells and found in the serum.

As it is almost exclusively a product of prostate cells, measurement in blood has proved to be exceptionally useful as tumor marker. Specifically, serum PSA is monitored clinically to detect early stage disease, track tumor burden, monitor the efficacy of therapeutic intervention, and detect the emergence of recurrent tumors post-therapy (13,14). PSA is generally considered to be the most sensitive biochemical marker available for monitoring the presence of prostatic disease, particularly prostate cancer, and response to therapy (15,16).

AR partners.

The binding of androgens to AR induces dissociation of the AR from the HSPs proteins (Heat-Shock-Proteins) and subsequent receptor dimerization and translocation into the nucleus , facilitating the ability of AR to bind its cognate response elements, and recruit coregulators to promote the expression of target genes. The transcriptional activity of AR is greatly modulated by coregulatory proteins. Coactivators such as ARA70 (Androgen Coactivators 70Kd) and ARA55 stabilize the process of ligand binding to AR. The ability of AR to be translocated into the nucleus is regulated by several coregulators such as the F-Actin binding protein Filamin.

Inside the nucleus AR interacts with DNA by targeting specific nucleotide palindromic sequences termed Androgen Response Element (17). A number of coregulators themselves perform enzymatic activities such as phosphorylation or acetylation, modifying either the chromatin surrounding the promoter of the target gene or other coregulators. Among coactivators, the acetyltranferase , CBP (CREB Binding Protein) , the closely related p300 and other nuclear receptor coactivators p/CAF(p300/CBP Associated Factor), SRC1(Steroid Receptor Coactivator-1), and SRC3 (18).

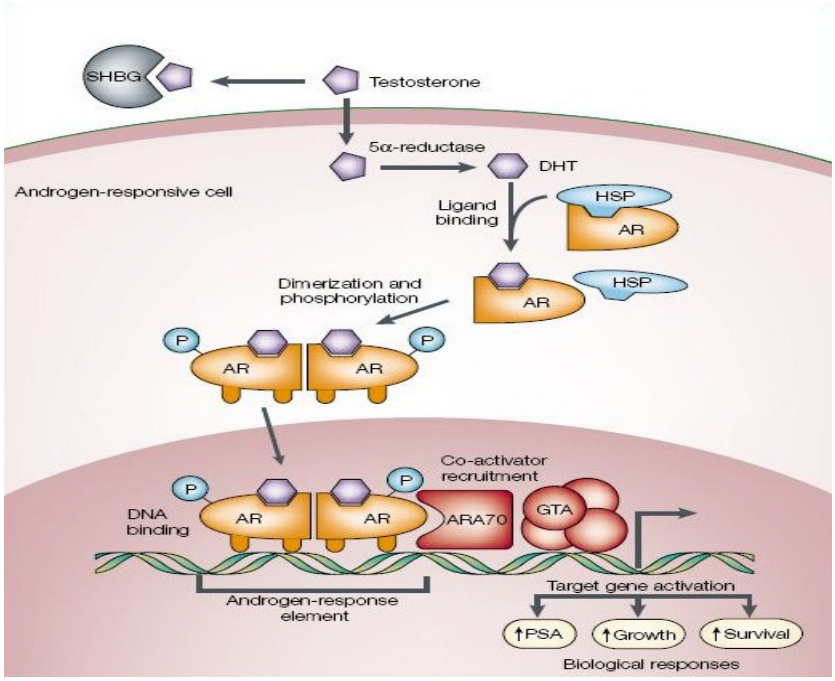

Fig2. **Androgen action.** Testosterone circulates in the blood bound to albumin (not shown) and sex-hormone-binding globulin (SHBG), and exchanges with free testosterone. Free testosterone enters prostate cells and is converted to dihydrotestosterone (DHT) by the enzyme 5α-reductase. Binding of DHT to the androgen receptor (AR) induces dissociation from heat-shock proteins (HSPs) and receptor phosphorylation. The AR dimerizes and can bind to androgen-response elements in the promoter regions of target genes. Co-activators (such as ARA70) and corepressors (not shown) also bind the AR complex, facilitating or preventing, respectively, its interaction with the general transcription apparatus (GTA). Activation (or repression) of target genes leads to biological responses including growth, survival and the production of prostate-specific antigen (PSA).

PIAS (Protein Inhibitor of Activated Signal Transducer and Activator of Transcription STAT) family of proteins and ANPK (Androgen Receptor-Interacting Nuclear Kinase) play major function. Transcriptional activation by AR ultimately requires the recruitment of RNA Pol II(RNA polymerase II) to the promoter of target genes. RNA Pol II recruitment is mediated through the assembly of GTFs (General Transcription Factor)to form the pre-initiation complex, the first step of which is the binding of TBP (TATA box-Binding Protein)near the transcriptional start site . TBP is part of multiprotein complex, the first step of which is the binding of TBP near the transcriptional start site. TBP is part of multiprotein complex which includes TFIID (Transcriptional Factor –IID) that induces DNA bending , bringing sequences upstream of the TATA element in closer proximity, and presumably enabling interaction between GTFs and steroid receptor-coregulators complexes. TFIIB binds directly to TBP and recruits the TFIIF-RNA Pol II complex. TFIIF interacts with TFIIB and RNA Pol II and has a role in transcription initiation and elongation.

The ATPase/ Kinase TFIIE and helicase TFIIH are than recruited to RNA Pol II to facilitate DNA strand separation before transcription initiation. TFIIE and TFIIF are acetylated by p300 and p/CAF. Ubiquitin ligase activity has been identified for two AR coactivators, ARA54 and E6-AP.The coactivators with ubiquitin ligase activity contribute to nuclear receptor transactivation through targeting the degradation of corepressor. AR can also interact with a number of transcription factor including Activator Protein-1,SMAD3(Sma and Mad Related Family),NF-KappaB (Nuclear Factor-KappaB), SRY (Sex-determining Region-Y), and the Ets family of transcription factors.

Transcriptional corepression of androgen-bound AR can be attributed to three corepressors: cyclin D1, calreticulin and HBO1.Cyclin-D1 inhibits AR transactivation through a mechanism independent of its function in cell cycle regulation (7).

The calcium –binding protein calreticulinis localized to the endoplasmic reticulum and in the nucleus and has also been characterized as corepressor of AR.

The AR corepressor HBO1 is a member of MYST protein family that is characterized by a homologous zinc finger and carries an acetyltransferase domain. Although AR is normally thought to function as a homodimer, it has been found to heterodimerize with other nuclear receptors including the ER(Estrogen Receptor), GR (Glucocorticoid Receptor) and TR4(Testicular Orphan Receptor 4) and in each case result in a decrease in AR transcriptional activity. In addition to the transcriptional or genomic mode of action by steroids, androgens, can also exert rapid, nongenomic effect. Nongenomic steroid activity typically involves the rapid induction of conventional second messenger signal transduction cascades. Nongenomic action of androgens can occur through multiple receptors. Androgens also stimulate an elevation in intracellular Ca2+ through GPCR(G-Protein Coupled Receptor) by activating an influx through nonvoltage-gated Ca2+ channels.The elevation of intracellular calcium activates signal transduction cascades, including PKA(Protein Kinase-A),PKC(Protein Kinase-C), and MAPKs(Mitogen Activated Protein Kinase), that can modulate the activity of the ARs and other transcription factors.

AR also interacts with intracellular tyrosine Kinase c-Src, triggering c-Src activation. One of the targets of c-Src is the adapter protein SHR

(SH2 Containing Protein), an upstream regulator of the MAPK pathway (18). AR phosphorylation by ERK2 is associated with enhanced AR transcriptional activity and an increased ability to recruit the coactivator ARA 70.The SRC family of transcriptional coactivators: SRC1, SRC3, and TIF2(Transcription Intermedary Factor -2) are targets of MAPK phosphoryation that result s in an increased ability of these coactivators to recruit additional coactivators complexes to the DNA-bound receptor. The non genomic rapid stimulation of the second messenger cascades by androgens may ultimately exert biological effects through modulation of the transcriptional activity of AR or other transcription factors. Such modulation may occur through direct phosphorylation of activators or their coregulators.

AR phosphorylation site

It is increasingly clear that the AR is also an integration point for signals coming not only from steroid hormones but from other pathways including kinases.

Post-translational modifications such as acetylation and sumoylation have been shown to influence the transactivation potential of the AR (19,20). However, it is not clear whether phosphorylation has an effect on the properties and activity of the AR. It has been shown that the AR is a phosphoprotein (21,22) and extra phosphorylation of the AR is induced when cells are exposed to androgens, in addition to the so-called basal AR phosphorylation observed in the absence of androgens(23). Phosphorylation occurs predominantly at serine residues, which are mainly located in the N-terminal domain. Androgen receptors (ARs) are phosphorylated at multiple sites in response to ligand binding, but the kinases mediating AR phosphorylation and the importance of these kinases in AR function have not been established. However specific phosphorylation sites have been identified for most members of the steroid receptor family (24,25).

Furthermore, phosphorylation is correlated with the three AR isoforms that appear on an SDS/polyacrylamide gel (26).

Within minutes after the start of *de novo* synthesis, the AR appears as a 110 kDa isoform, whereas the generation of the second (112 kDa) isoform appear within 15 min as shown by radioactive methionine-labelling studies. Only after hormone binding does the third (114 kDa) isoform appear. The AR isoform pattern is correlated with AR phosphorylation as was shown in previous studies by using phosphatases. The dephosphorylation of AR by phosphatases resulted in the loss of one isoform either in the presence or absence of hormone . This effect was also observed when AR phosphosites were mutated . Furthermore, several phosphorylation sites have been identified. The first identified phosphosites, Ser-81, Ser-94 and Ser-650, were found by mutagenesis analyses in combination with SDS/PAGE (27). Ser-308 was the first phosphosite identified by mutagenesis and MS Ser-16, Ser-81, Ser-94, Ser-256, Ser-308, Ser-424 and Ser-650 were all identified and confirmed as phosphosites by mutagenesis, peptide mapping and MS (28).

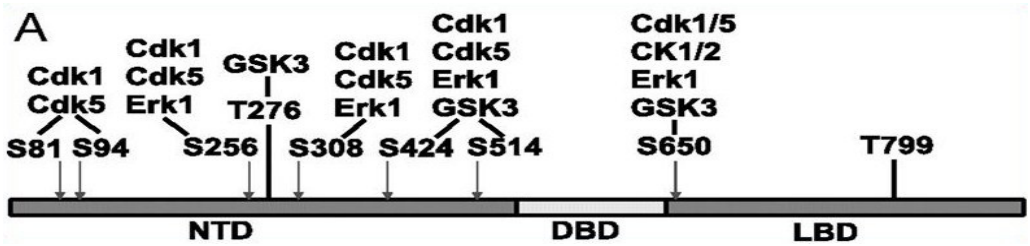

Fig. A) The AR becomes phosphorylated at multiple site after ligand binding but the identity of all the kinases that directly phosphorylate ARs and the functional importance of AR phosphorylation is not clearly esthabilished .

However, cell-free *in vitro* phosphorylation reaction studies on AR mutants also demonstrated Ser-213, Ser-515 and Ser-791 are potential phosphosites. Some data suggest that stress kinase signaling regulates AR Ser 650 phosphorylation and that Ser 650 phosphorylation regulates AR export (27,29).

Recently Narayanan et al. discovered a selective nuclear androgen receptor exporter (SNARE) that functions to exclude AR from the nucleus. SNARE-1 binds wild-type and mutant ARs and efficiently inhibits their transactivation activity and ability to induce PSA gene expression.

SNARE-1 inhibits the androgen-sensitive growth of LNCaP cells and tumor xenografts. Quantitative subcellular localization studies suggest that SNARE-1 inhibits nuclear translocation of AR, but also facilitates export of nuclear AR that has been translocated by an agonist. Mechanistic studies indicate that SNARE-1 rapidly phosphorylates p38 mitogen-activated protein kinase (MAPK) and Ser650 of the AR facilitating the nuclear export of AR. Additionally, SNARE-1 was found to promote ubiquitination of AR in LNCaP cells (30). Transitions into and within the mitotic cell cycle are dictated by the coordinate activation of cyclin-dependent kinase (CDK)/cyclin complexes, wherein cyclin binding induces the catalytic activity of the kinase (31,32). Mitogenic signaling pathways generally induce cell cycle progression through ordered activation of CDK-cyclin complexes, whereas anti-mitogenic signals that result from extracellular events (e.g., nutrient depletion) or intracellular insults (e.g., DNA damage) typically serve to attenuate CDK function. Although the signals that dictate commitment to the cell cycle are often cell type-specific, the core machinery that drives the cell cycle engine is well conserved.

Prior to mitogenic stimulation cells can exit the cell cycle and enter into a resting stage deemed "G0. At this stage, several key gatekeepers of cell cycle transitions are invoked to prevent unscheduled cell cycle progression (33).

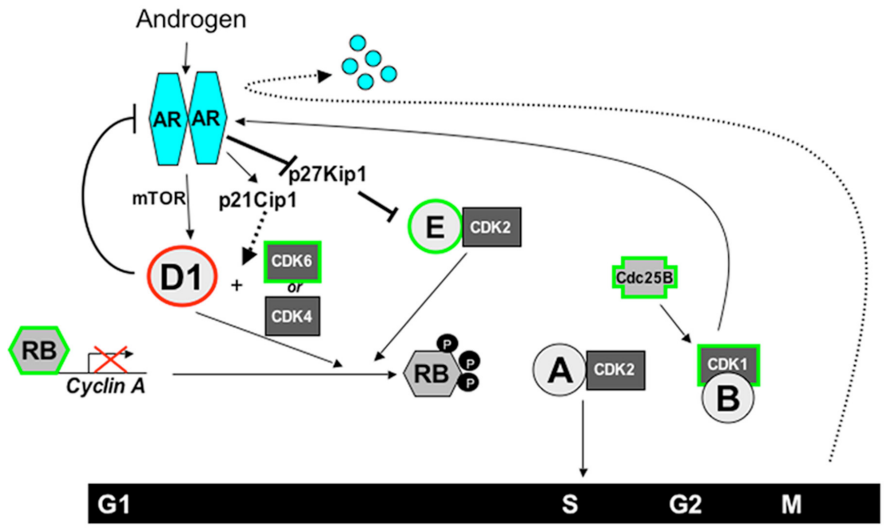

Figure 3:(from Balk et all2008) AR-cell cycle crosstalk**.**

Activated AR stimulates the accumulation of cyclin D1 (D1), through mammalian Target of Rapamycin (mTOR), to activate CDK4 and promote phosphorylation of the retinoblastoma (RB) tumor suppressor. In addition, AR-induced expression of p21Cip1 and degradation of p27Kip1 further enhance cycD1/CDK4 and cycE/CDK2-dependent inactivation of RB and allow expression of E2F target genes like cyclin A (CycA). Cyclin A in turn activates CDK2 to drive G1-S phase transition. Subsequently engaged components of the cell cycle machinery then impinge on AR to regulate the androgen response. Elevated cyclin D1 acts as in a negative feedback loop to attenuate AR activity, thereby modulating androgen action. In G2-phase, CDK1 promotes the phosphorylation and activation of AR. However, AR is degraded in M-phase and is purposed to be a "licensing factor" for DNA replication. Components that suppress AR function are outlined in *red*, whereas positive effectors of AR activity are outlined in *green*.

Mechanistic investigations have showed that AR plays a master regulation of G1-S phase progression, able to induce signals that promote G1 cyclin-dependent-Kinase activity and finally, phosphorylation /inactivation of the receptor of the retinoblastoma tumor suppressor protein (34).

Like PR, the majority of identified phosphorylation sites for AR are located in the N-terminal domain. Although many of the NTD sites have been tested in vivo, assigning function to them has been difficult. Studies of transcriptional activation by AR on several androgen response elements containing promoters carried out in various cell lines has provided little information on the function of NTD site-specific phosphorylation, Many phosphorylation mutants exhibited no aberrant activation characteristics when compared with wild type.(35,36,37,38). However, greater differences in activity can be detected when specific signaling pathways are activated or inhibited. For example, overexpression of cyclin D3/CDK11p58 inhibits AR activity by phosphorylating Ser308A mutant partially elevates hormone dependent activity and prevents cyclin D3/ CDK11p58-mediated repression of AR transcriptional activity (39).

Several studies have suggested that AKT-mediated phosphorylation of Ser213 and Ser791 (numbered based on an AR length of 919 amino acids) reduces AR activity (40). Mutation of the AR Ser213 to alanine caused resistance to AKT-mediated suppression of activity in DU145 cells(41) . Palazzolo et al. found that substituting alanines for both of the sites also prevented AKT-mediated inhibition of AR transcriptional activity. Surprisingly, substitution of aspartic acids at either site blocked hormone binding and, therefore, ligand-dependent AR protein stabilization, ligand-mediated translocation, and AR transcriptional activity(42).

The remaining sites in the AR NTD domain also have been shown to have important functional roles. When a fragment of the androgen receptor (amino acids 507–660) is expressed, Ser515 and Ser578 are phosphorylated in response to EGF treatment(43).

The AR Ser515Ala mutation exhibited a more severe phenotype than the Ser578Ala and the double mutant displayed little to no activity; furthermore, EGF treatment had no effect on the activity of this mutant.

A Ser578Ala substitution results in increased nuclear localization of AR in the absence of ligand but eliminates AR transcriptional response to EGF. AR Ser578Ala also exhibits increased binding to Ku-70/80 regulatory subunits of DNA dependent protein kinase in addition to nuclear retention of the AR in association with hyperphosphorylation at Ser 515(44).

Finally, the Ser515Ala mutant is not phosphorylated on Ser650, which is located in the hinge region of AR (45).

One possible explanation for this effect is that phosphorylation at Ser515 mediates a conformational change of the AR thus making the Ser650 phosphosite either more available to phosphatases or less accessible to kinases. Attempting to assign function to the AR Ser650 phosphorylation site itself has produced conflicting reports. Early studies have suggested that blocking phosphorylation of this site resulted in 30% reduced activity with an MMTV-Luc reporter in CV1 cells . This was in direct contrast to later reports examining the function of Ser650 phosphorylation on AR activity in various cell lines using various reporters in which no phenotype was detected .

However, careful examination of these studies revealed that the original observation by Zhuo et al. was evident at only high concentrations of receptor . Wong et al. also found that at high concentrations of receptor the Ser650Ala mutant is less active than wild type . AR Ser650 phosphorylation also plays an important role in nuclear export of AR in response to stress kinase signaling (29).

A recent report has shown that protein phosphatase 1 (PP1) inhibition increases phosphorylation at AR Ser650 which causes a marked increase in nuclear export of AR which is not observed for the Ser650Ala mutant(46) .

This study suggests that PP1 plays a critical role in regulating AR protein stability and nuclear localization through dephosphorylation of AR at Ser650. In addition to Ser–Pro motifs, several tyrosine phosphorylation sites are present in the NTD of AR. A number of candidate sites have been identified in AR isolated from cells overexpressing Src. Based on the overall level of tyrosine phosphorylation in AR, substituting Phe for Tyr534 reduced the Tyr phosphorylation most substantially suggesting that this is a major site under these conditions(47).

The Tyr534Phe mutant also exhibited reduced activity and DNA binding at low doses of ligand and defective nuclear translocation in response to various stimuli . Finally, Tyr534Phe mutant expression caused growth inhibition in both cell lines and tumor xenografts containing the Tyr534Phe mutant grew more slowly than tumors expressing WT AR in castrated mice, thereby demonstrating a role for Tyr534 phosphorylation in prostate cancer cell growth under androgen-depleted conditions. Two additional tyrosine phosphorylation sites have been identified in cells treated with heregulin or transfected with constitutively active Ack (Cdc42 associated kinase).

Mutation of these sites, Tyr267 and Tyr363 to phenylalanine (Tyr267Phe and Tyr363Phe, respectively), reduced Ackinduced reporter activation and recruitment to the enhancer, thus demonstrating the importance of these sites in AR basal and ligand-dependent activity as well as in potentiation of AR activity by kinase signaling. In addition, substituting Phe at both of these sites also reduced tumor growth of Ack-driven tumor xenografts in castrated nude mice.

Recently has been demonstrated that Androgen receptor (AR) interacts with β-catenin and can suppress its coactivation of T cell factor 4 (Tcf4) in prostate cancer (PCa) cells. Pin1 is a peptidyl-prolyl *cis*/*trans* isomerase that stabilizes β-catenin by inhibiting its binding to the adenomatous polyposis coli gene product and subsequent glycogen synthase kinase 3β (GSK-3β)-dependent degradation. Higher Pin1 expression in primary PCa is correlated with disease recurrence, and this study found that Pin1 expression was markedly increased in metastatic PCa.(48).

Pin1

Phosphorylation of proteins on serine/threonine residues preceding proline (pSer/Thr-Pro) is a key regulatory mechanism for the control of cell proliferation and transformation (49). The pSer/Thr-Pro moiety in proteins exists in two distinct *cis*- and *trans*-conformations, whose conversion is catalyzed specifically by Pin1, which specifically acts only on phosphorylated Ser/Thr-Pro bonds (50,51).

The significance of this phosphorylation in cell cycle control was obscured until the discovery of the PIN1 protein (protein interacting with NIMA (never in mitosis A)-1) . Therefore Pin1 plays an important role in cell cycle regulation. Functionally, Pin1 catalyzes the conversion of *cis*- and *trans*-conformations of target proteins after phosphorylation, thereby having profound effects on their catalytic activity, dephosphorylation, protein-protein interactions, and subcellular localization. Pin1 is essential for mitotic progression and is required for the DNA replication checkpoint (52).

A comparison of steroid hormone receptor sequences indicates that Ser-Pro motifs predominate in the NH2-terminal hyper-variable regions and hinge regions, occur rarely in the hormone-binding domains, and are not detected within the zinc finger DNA-binding

domains (53) . Pin1 is normally expressed at very low levels in most normal tissues, although significant levels of Pin1 are often found in cell types that normally undergo active cell division. Pin1 is overexpressed in some human malignancies and that its expression closely correlates with the level of cyclin D1 in human breast cancer (54).

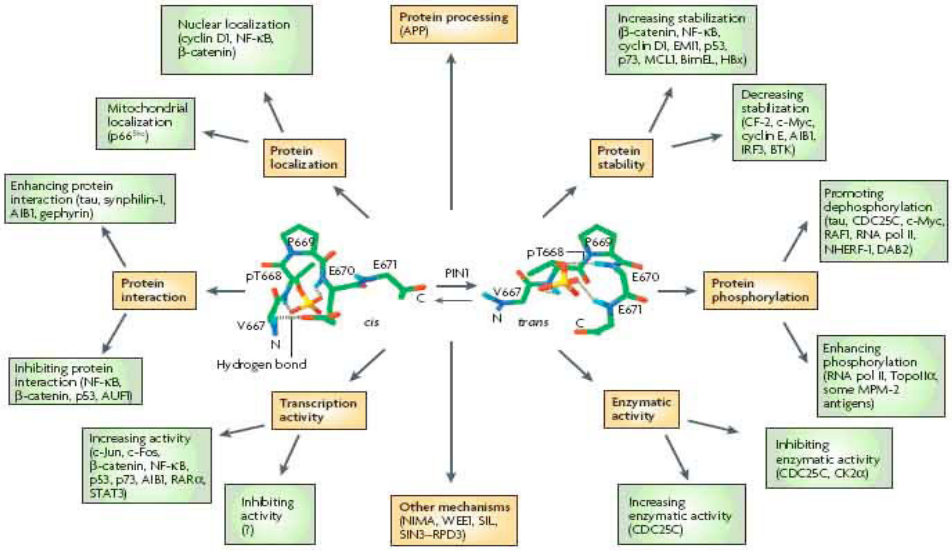

Fig 4: The different roles of PIN1 in cellular physiology

PIN1 is a member of the evolutionarily conserved peptidylprolyl isomerase (PPIase) family of proteins. PIN1 has a two-domain structure that consists of an N-terminal WW domain (amino acids 1–39) and the Cterminal PPIase domain (amino acids 45–163). The WW domain binds only to specific pSer/Thr-Pro-motifs and the PPIase isomerase domain catalyzes the conformational switch from cis to trans of target proteins. This fact is especially important because Pro-directed kinases and phosphatases are conformation-specific and act only on the trans isoform (55,56). For this reason, PIN1 is important for many physiological activities of the cell (Fig. 4).

In cell cycle control, PIN1 was originally identified and defined as a protein important in mitosis (57).

Depletion of PIN1 in yeast and human cells induces mitotic arrest and its over-expression blocks the cells in the G2 phase of the cell cycle (51). Since the discovery of PIN1, a plethora of protein targets have been discovered, many of which are involved in the G0 and G1/S control (58). PIN1 controls Cyclin D1 mRNA levels and it is involved in regulation of CyclinD1, c-MYC and Cyclin E protein stability. PIN1 -/- MEF showed proliferative defects in cell cycle entry after serum deprivation. In addition, PIN1 is a target of E2F transcription factors and its mRNA and protein levels fluctuate during cell cycle

(59). Even in cancer pathology, PIN1 over-expression was found in 38 different tumour types out of 60, including most common human cancers such as prostate, cervical, brain, ovary, lung, breast, liver cancer, and melanoma . Pin1 overexpression has been observed in a subset of primary prostate cancers, and its expression correlates with increased risk of recurrence after radical prostatectomy (60). The relationship between PIN1 overexpression and Prostate Cancer progression has been correlate with the ability that PIN1 has to antagonize β-catenin /Tcf4 binding and transcriptional activity. Abrogation of this interaction enhance β-catenin /Tcf4 signling and contribute to aggressive biological behavior in Prostate Cancer. Pin1 stabilizes β-catenin by inhibiting its binding to the adenomatous polyposis coli gene product and subsequent glycogen synthase kinase 3β (GSK-3β)-dependent degradation. Increased expression of Pin1 in transfected LNCaP PCa cells strongly accelerated tumor growth in vivo in immunodeficient mice (48). However, the functional effects of Pin1 overexpression on β-catenin nuclear signaling in PCa cells (and in particular in PTEN-deficient cells), and how it contributes to more aggressive biological behavior have not been determined.

Moreover, PIN1 expression is an excellent prognostic marker in prostate cancer (61). It is now clear that Pin1plays a catalytic role in oncogenesis in solid cancers. Some groups have already started to study the role of PIN1 in Prostate Cancer. PIN 1 has also been suggested as new marker in PCa (61). RYO et al. have already suggested PIN1 like a good target for patients with prostate cancer with different kind of experiments. In those studies a retrovirus-mediated RNA interference targeting Pin1was expressed in PC3 and LNCaP cells, and cell growth and several transformed properties were investigated.

As result the stable expression of Pin1-specific small interfering RNA constructs in PC3 and LNCaP cells significantly reduced cellular proliferation, colony formation, migration, and invasion but strongly enhanced the apoptotic response induced by serum depletion or treatment with anticancer agents. Furthermore, Pin1depletion significantly suppressed tumorigenic potential in athymicmice, resulting in the inhibition of both tumor growth and angiogeneisis.

These results strongly suggest that Pin1plays an important role not only in tumorigenesis but also in the maintenance of the transformed phenotype in prostate cancer cells.

Hence, Pin1 may serve as a promising therapeutic target, particularly for recurrent prostate tumors. Published data establish Pin1 as an independent prognostic marker in PCa patients after radical prostatectomy (61).

Here we demonstrate that PIN1 form a protein complex with Androgen Receptor and we will suggest the role that this interaction plays in Prostate Cancers. This interaction could represent the starting point for the development of new drugs in patients with diagnosed prostate cancer.

Results

PIN1 directly interact with AR

In the last decade many evidences have suggested a relationship between PIN1 and Androgen Receptor Activity.(48,61). Higher Pin1 expression in primary Prostate Cancer cells is correlated with disease recurrence, and Pin1 expression was found markedly increased in metastatic Prostate Cancer. Ayala et al. have shown that Pin1 expression levels were tightly correlated with both a higher probability and a shorter period of tumor recurrence following radical

prostatectomy by a comprehensive immunohistochemical analysis. PIN1 can control AR activity through an indirect mechanism mediated its ability to inhibit β-catenin degradation. From our perspective, since AR contains many pSer/Pro, a direct mechanism could be hypothesized. First of all we used a specific prostate cancer cell line (LNCaP) and then we performed a GST-pull down experiment using GST alone and GST-PIN1 to determinateif PIN1 and AR could interact (FIG5).

In a GST gene fusion system, the GST sequence is incorporated into an expression vector alongside the gene sequence encoding the protein of interest. Induction of protein expression from the vector's promoter results in expression of a fusion protein. This GST-fusion protein can then be purified from cells via its high affinity for glutathione. It is fused to the N-terminus of a protein. Agarose beads can be coated with glutathione, and such glutathione-Agarose beads bind GST-proteins. These beads are then washed, to remove contaminating bacterial proteins.

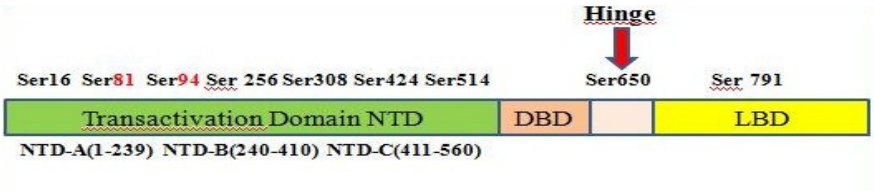

Fig:5a Possible site of ineraction between Pin1 and AR

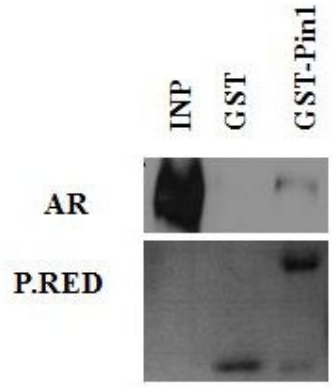

Fig5b: **In vitro and in vivo interaction between PIN1 and AR** a) Potential PIN1 binding site targets in AR protein. b) GST-PIN1 interaction with AR. A specific band was detected in the GST-PIN1 lane and no band is detected in GST control lane.

LNCaP cells were collected and lysated .(See material and method for detail) Total protein extract was incubated O.N. with GST- PIN1. The interaction was detected by western blot using Androgen Receptor specific antibody. The figure shows that Pin1 and AR interact. To assess the in vivo interaction between PIN1 and AR, LNCaP cells were immunoprecipitated with anti-PIN1 antibody and analyzed by western blot with anti-AR antibody. We utilized LNCaP cells that overexpressed Pin1 or without Pin1 as negative control.

Co-immunoprecipitation is a purification procedure to determine if two different proteins interact. An antibody specific to the protein of interest is added to a cell lysate. Then the antibody-protein complex is pelleted usually using protein-A/G agarose, which binds most antibodies. If there are any proteins that bind to the first protein, they will also be pelleted. Identification of proteins in the pellet can be determined by western blot.

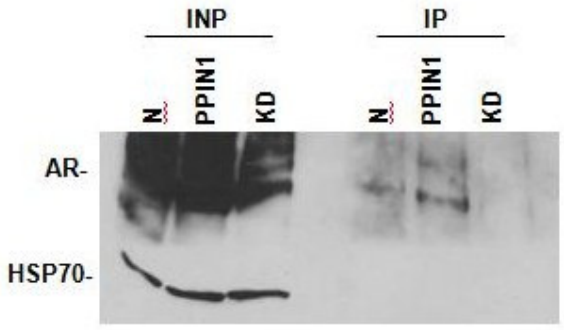

FIG:6 PIN1 interacts with AR *in vivo*. Cells were immunoprecipitated with anti-PIN1 antibody, analyzed by western blot with anti-AR antibody. The membrane was normalized with HSP-70 antibody.

In the following experiments we will determinate witch part of the AR receptor was involved in the interaction with PIN1. We splitted the AR in two different segments: the first one containing the amino-terminal domain NTD and the DNA binding domain DBD and the second matching the LDB domain. These fragments were obtained by PCR amplification using as template the androgen receptor full length and cloned in the PCDNA6HisMyc mammalian expression vector.

The NTD-DBD domain corresponds from the a.a 1 to a.a. 625. The LBD domains contains the a.a. 626-919. The domains were than transfected in the HEK293 cell lines, after tranfection total lysate protein were pulled-down ON with GST or GST-PIN1 and analyzed by immunobt with AR antibody. The analysis showed that NTD domain interacts with PIN1. HSP70 antibody was used to normalize samples. (Fig7)

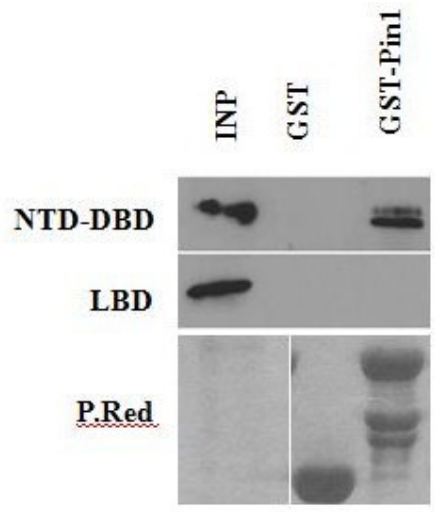

Fig 7:Pin1 interacts with AR-NTD-DBD fragment. Total protein lysate was pull down with GST-Pin1. Note no band is detected in GST control line .

The NTD domain contains six different potential Pin1 binding sites. To narrowed the region of interaction on AR protein the amino-terminal domain was split in three fragments NTD-A, NTD-B, NTD-C consisting rispectively of aminoacids 1-239, 240-410, 411-560.

The fragments obtained by PCR were transfected in HEK293 cells.(for transfection see material and methods below).The GST-pull-down experiment limit the area of interaction on the first 239 aa of AR. (Fig 8)

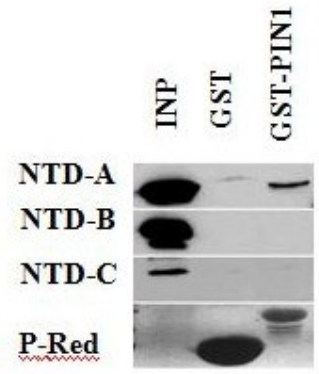

Fig. 8 The amino terminal domain NTD of AR was splitted in three fragments and pulled down with GST-Pin1. GST alone was used as negative control. Pin1 interacts with NTD-A corresponding of the first 239 a.a. of AR

It is widely accepted that PIN1 interacts with phosphorylated Ser/Thr motif followed by Proline. To identify the motif that is recognized by PIN1 we first identify the Ser/Thr motifs that are present on the first 239 a.a. of the receptor. We found that 2 of those Ser81 and Ser94 are potential binding site of Pin1. To explore witch Ser/Pro motif is bound by PIN1 isomerase we expressed wild-type S81A and S94A mutant AR in 293FT cells.

The obtained mutant proteins were transfected in 293FT cells and subsequently pulled down with GST tagged PIN1. The immunoblotting assay showed a markedly decrease of interaction when Ser 81 is substituted with Ala in contrast with the S94A mutant that doesn't shows any decrease of interaction compared with AR wild tipe.(Fig9)

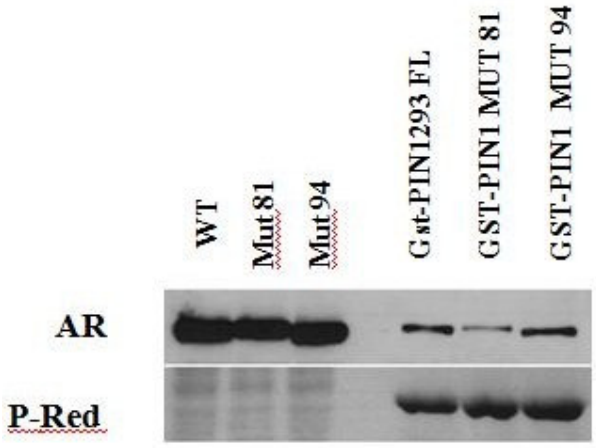

Fig 9: The figure shows a markedly decrease of interaction when Ser 81 is substituted with Ala in contrast with the S94A mutant that doesn't shows any decrease of interaction compared with AR wild type.

This finding open a new scenario about the role of AR SER81in prostate control. The role of Ser81 has been widely studied, recently according to our data Gioeli et al. discovered that LHS cells stably expressing wild-type and S81A mutant AR showed differences in the regulation of endogenous AR target genes, suggesting that S81 phosphorylation regulates promoter selectivity. Is well known that PIN1 recognized pSER/THR our next goal was to identify witch kinases is responsible for binding between the two proteins.

CDK1, CDK3 and recently CDK9(63,64) are until now considering the 3 kinases involved in the phosphorylation of SER81. To examine in detail the kinase responsible of the interaction between PIN1 and the receptor we conducted some experiments using the dominant negative of all the kinases that seems to be involved in Ser81 phosphorylation. Different papers report that the CDK1, CDK3, CDK5 are involved in Ser81 phosphorylation but the exact role that these phosporylation plays in AR activity are non well knowed yet. Recently Gioeli et.al found an association between (CDK)9 and AR. CDK9 phosphorylates the AR on Ser81 *in vitro*. Phosphorylation is specific to AR Ser81 because CDK9 did not phosphorylate the AR on other serine phosphorylation sites. Overexpression of CDK9 with its cognate cyclin, Cyclin T, increased Ser81 phosphorylation levels in cells. Small interfering RNA knockdown of CDK9 protein levels decreased hormone-induced S81 phosphorylation.

Additionally, treatment of LNCaP cells with the CDK9 inhibitors, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole and Flavopiridol, reduced

Ser81 phosphorylation further, suggesting that CDK9 regulates Ser81 phosphorylation. Pharmacological inhibition of CDK9 also resulted in decreased AR transcription in LNCaP cells. Collectively these results suggest that CDK9 phosphorylation of AR Ser81 is an important step in regulating AR transcriptional activity and prostate cancer cell growth. We conducted the experiment using the dominant negative of CdK1, CdK2, CdK3, CdK5, CdK9 and preliminary results shown that CDK9 could be involved in PIN1 and AR interaction (data not shown). Further, to investigate the involvement of S81 in AR-dependent transcriptional activation of AR, a luciferase system assay was used. 293FT cells were transfected with AR WT, AR S81 and probasin promoter cloned upstream of the firefly luciferase gene (*luc*) with high levels of luciferase expression that can be measured with a luminometer. Transfected cells were treated with DHT or the ethanol vehicle for 4 or 24 hours. Transcriptional activity was measured in Relative Light Unit/second (RLU/s) and normalized to Renilla activity. We observed a reduction in ARS81 trascriptional activity compare to AR WT. The difference is statistically significant. These experiments show that phosphorylation of S81 is required for transcriptional activation of AR target gene promoters (Fig 10).

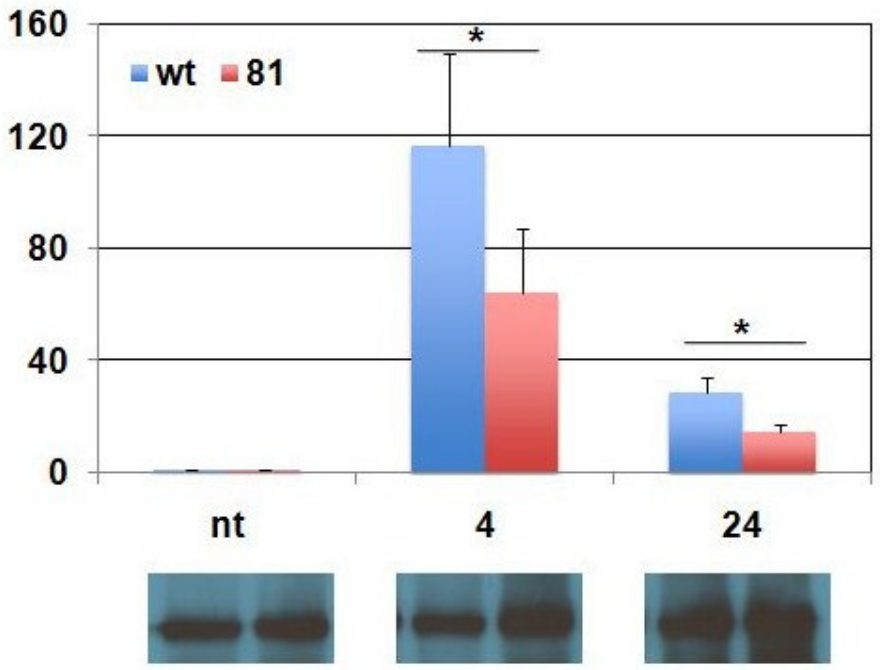

Fig 10: The figure shows that phosphorylation of S81 is required for transcriptional activation of AR target gene promoters.

Since Pin1 controls the phosphorylation of target proteins and AR activity is regulated by phosphorylation, we investigated if it is the case for AR protein. As read out of the system, we used S650 phospho-antibody the only commercial available antibody. In fig. 11 we showed that after DHT treatment the AR S81 is less phosphorylated on Ser 650 than AR wt. This result suggests that Pin1 can control AR phosphorylation after ligand stimulation(Fig 11).

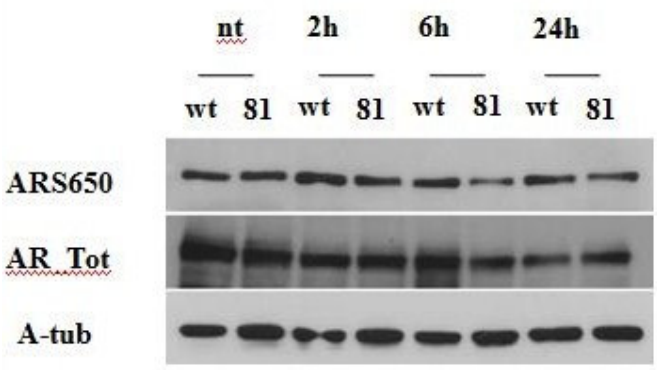

Fig 11: The figure shows S650 phosphorilation after DHT treatment. Cells were grow three days in media without hormones and than a concentration 10-8 of DHT was added. Time course shows that transfected in 293ft cells with mutate Ser81there is a sensitive decrease of Ser650 phosphorylation.

Discussion

The fundamental clinical problem for disseminated prostate cancer is the transition of androgen dependent desease to androgen independent desease after androgen ablation therapy. Collectively, data suggest that although advanced prostate cancer may be functionally independent of physiologic levels of androgen, it is not independent of the AR. For these reasons even though androgen ablation is a most commonly prescribed treatment in the first stage of pathology it is not curative. Development of new strategies for treatment of prostate cancer is limited partly by lack of full understanding of the mechanism by which androgen regulates prostate cancer cell proliferation and which molecular mechanisms are involved in AR function in the absence of hormones. In this cascades of events Pin1 has been showed to play an important role in oncogenesis, and its expression increase dramatically in metastatic prostate cancer; for these reason Pin1 is a potent predictor of recurrence for patients with prostate cancer.

Recently, it has been reported that Pin1 is overexpressed in human prostate cancer cell lines and prostate cancer tissues, and its expression closely correlates with the level of cyclin D1 in tumors. Even if it is widely diffused in the scientific community that there is a link between Pin1 expression and prostate cancer progression, since now nobody knows exactly how the functions of AR and Pin1 are correlated. To address this question in this thesis we provide evidence that AR directly interact with Pin1 and we clarify the role that this interaction plays in prostate cancer progression. We found that Pin1 bounds directly to AR and this interaction lead to structural modification that increases AR activity on target genes.

Pin1 bounds to pSer81 of AR , after binding there is an increase of receptor transcriptional activity and also we found that Ser81 phosphorylation control even the phosphorylation on other part of the receptor. In detail we found that the phosphorylation on Ser81 lead to increase phosphorylation of S650. These finding suggest that AR transcriptional activity may also be modulated directly or indirectly by serine/threonine kinase and phosphatases in absence of hormone in AIPC. Early studies have suggested that by blocking phosphorylation on this site resulted in 30% reduced activity with an MMTV-Luc reporter in CV1 cells (62). It is well known that one major regulatory

mechanism in cell proliferation and transformation is phosphorylation of proteins on serine or threonine residues preceding proline (pSer/Thr- Pro) by various prodirected protein kinases, such as MAP kinases, cyclin-dependent kinases, JNK, and GSK3β. Interestingly, the pSer/Thr-Pro motifs in proteins exist in two completely distinct *cis* and *trans* conformations, whose conversion is normally restrained by phosphorylation, but catalyzed specifically by the essential prolyl isomerase Pin1. By isomerizing pSer81, Pin1 induce conformational changes in androgen receptor. This, phosphorylation- dependent prolyl isomerization is a critical mechanism in phosphorylation signaling. Diverse agonists including activators of protein kinase A (forskolin) and protein kinase C [phorbol-12-myristate-13-acetate (PMA)] increased Ser 650 phosphorylation. Ser 650 phosphorylation occurs by both hormone-dependent and hormone- independent mechanisms (androgen, protein kinase A, EGF, and protein kinase C) suggest that modification of this site might be used to regulate steroid receptor function in response to a variety of physiological stimuli. Gioeli et al. have investigated which signal transduction pathways regulate Ser 650 phosphorylation and determined the effect of these signaling pathways on AR function.

We have found that Ser81 phosphorylation enhance phosphorylation on Ser650 and this phosphorylation improved the transcription of the specific prostate gene probasin. Our future plan is to analyze the interaction between PIN1 and AR with a biochemical structural approach. We plan to model the interaction between PIN1 and AR by a computational approach and to design new specific ligands that inhibit this interaction. Moreover, to gain insight in *in vivo* model, we will analyze the PIN1 KO mice to discover if the AR and AR signal pathway is altered.

In conclusion our results give new insights in the molecular mechanism of AR function and open new ways to design a pharmacological approach to cure prostate cancer.

Materials and Methods

Cells culture conditions

LNcaP prostate cancer cell lines were purchased from American Type Culture Collection (ATCC, Rochville, MD, USA), 293FT were from Invitrogen (Invitrogen Corp, Carlsbad, CA, USA). Cells were grown at 37 ^{o}C, in a 5% CO_2/95% atmosphere. Cell media in this study included growth medium (RPMI or DMEM with 2 mmol/L l-glutamine, 0.1 mmol/L nonessential amino acids, 50 units/mL penicillin, 50µm/mL streptomycin, 6 ng/mL insulin and 10% FBS) and hormone-free medium (phenol red–free DMEM with 2 mmol/L L-glutamine, 0.1 mmol/L nonessential amino acids, 50 units/mL penicillin, 50 µg/mL streptomycin, and 3% charcoal-stripped FBS).

Reagents

Antibodies were purchased from: Pin1 (600-401-A20), 6XHis (600-401-382) from Rockland Immunochemicals, Gilbertsville, PA, USA; AR (sc-7305), HSP70 (SC-24) from Santa Cruz Biotechnology, Santa Cruz, CA, USA; ARpS650 from Signalway (11120-1); α-tubulin (T-6074) from Sigma Inc., St Louis, MO, USA

Plasmids

shRNA plasmids Pin1 (SHCLNG-NM_006221) were from Sigma Inc., St Louis, MO, USA. Scrambled shRNA (17920), psPAX2 packaging plasmid (12260), pMDG.2 envelop plasmid (12259) and PwPI (12254) were from Addgene Inc, Cambridge, MA, USA. For overexpression experiments, the IMAGE: 3941595 clone was utilized to amplify the Pin1 human gene with the oligonucleotide primers PIN1-BamHIF GCGGATCCGCGGCAGGAGGGAAGATGG at the 5' end and PIN1-EcoRIR GCGAATTCCTGGGCTCCCCACCCTCAC at the 3' with BamHI and EcoRI adaptor sequences, respectively. The plasmid was sequenze verified.

GST pull-down experiment: the PCR generated Pin1 (PIN1-BamHIF and PIN1-EcoRIR) were ligated in the pGEX-2T plasmid for the prokaryotic expression vector (Stratagene Inc., La Jolla CA, USA). The AR sub-domains were amplified from the clone BC132975 with the following primers: ARNTD-DBD:

ARNTD-BamHIF ATGGATCCAGGATGGAAGTGCAGTTAGGGC

ARDBD-ECORIR ATGAATTCCAGCTTCCGGGCTCCCAG

ARLBD:

ARLBD-BamHIF

ATGGATCCACCATGGGACACGACAACAACCAGC

ARLBD-XHOIR ATCTCGAGAAGC TTCACTGGGTGTGG

ARNTDA:

ARNTD-BamHIF

ATGGATCCAGGATGGAAGTGCAGTTAGGGC

ARNTDA-R ATT GAA TTC CTT ACA CAA CTC CTT GGC GTT G

ARNTDB:

ARNTDB-F ATT GGA TCC ACC ATG GCA GTG TCG GTG TCC ATG GGC

ARNTDB-R ATT GAA TTC CGC CAG GTC CCC ATA GCG G

ARNTDC:

ARNTDC-F ATT GGA TCC ACC ATG AGC CTG CAT GGC GCG GGT G

ARNTD-ECORIR

ATGAATTCGGTCTGGGGTGGAAAGTACC

After *BamHI/EcoRI or XhoI* double digestion, fragments were ligated in pcDNA6 His/Myc vector. ARS81 e ARS94 was generated by site-directed mutagenesis with the QuickChange mutagenesis kit (Stratagene, La Jolla, Calif.) with the following primers:

ARS81A-f GCAGCAAGAGACT**GCC**CCCAGGCAGCAG

ARS81A-r CTGCTGCCTGGGGGCAGTCTCTTGCTGC5

ARS94A-f GTGAGGATGGT**GCT**CCCCAAGCCCATCG

ARS94A-r CGATGGGCTTGGGGAGCACCATCCTCAC

Luciferase experiments: the Probasin luciferase was purchased from Addgene (p159 pPR-luc: 8392).

Lentiviral production

To generate knock down cells, lentiviral particles were produced as described (http://www.broadinstitute.org/genome_bio/trc/publicProtocols.html). Briefly, $1x10^6$ 293FT cells (Invitrogen Corp, Carlsbad, CA, USA) were transfected with 2.25 µg of PAX2 packaging plasmid, 0.75 µg of PMD2G envelop plasmid and 3µg of pLKO.1 hairpin vector utilizing

30 µl of Fugene HD (Roche Applied Science, Indianapolis, IN, USA) in 10 cm plate. Polyclonal populations of Pin1 kd and scrambled cells were generated by infection with 1 MOI (multiplicity of infectious) of shRNA lentiviral particles. $2.5x10^5$ cells were plated in a multi 6 wells plate. The day after, the cells were transduced with 1 MOI of lentiviral particle in 10% FBS MEM medium. After 3 days post-infection, the cells were selected with 2 µg/ml of puromycin (Sigma-Aldrich, St Louis, MO, USA) for 1 week

Pull-down analyses

GST and GST-Pin1 proteins were produced in BL21 bacteria cells. Cells were grown to mid log phase and then induced to express protein by adding 0.25mM of isopropyl-1-thio-b-D-galactopyranoside (IPTG, Roche Applied Science, Indianapolis, IN, USA). The cultures were shaken for 4 h; bacteria were then pelleted and resuspended in NENT buffer (20mM Tris (pH 8), 100mM NaCl, 1mM ethylenediaminetetraacetic acid (EDTA), 0.5% NP-40). Cell suspensions were sonicated and pelleted so that the supernatant could be collected.

The remaining bacteria were then resuspended in NENT buffer plus 2% of N-lauryl-sarcosine, then pelleted and finally, the supernatants were collected again. The combined supernatants were incubated with glutathione agarose beads (Sigma Inc., St Louis, MO, USA) overnight at 4 ^{o}C. The agarose was then pelleted and washed three times in NENT buffer. The GST protein was analyzed by electrophoresis gel and blue coomassie staining. 1mg of protein was pulled-down with 10 ug of GST or GST-Pin1.

Co-immunoprecipitation assay

Sub-confluent LNcaP cells were harvested and proteins were prepared as follows: the cell pellet was resuspended in lysis buffer (20 mM Tris HCl pH 8, 137 mM NaCl, 10% glycerol, 1% NP40, 2 mM EDTA). 1mg of proteins was immunoprecipitated, utilizing 4 μg of PIN1, AR antibody or mouse IgG overnight at 4°C Extracts were incubated with antibodies and protein A/G beads (Pierce) for 3 h at 4°C. Immunopellets were washed extensively and subjected to SDS-PAGE followed by immunoblot analyses to detect Pin1 or AR proteins.

Luciferase Assay

The luciferase assay was purchased from Promega (Wisconsin, USA). 293FT cell were seeded in 96 well multiplate at $2x10^4$ cells/well and growth in hormone-free medium for three days. Transfections were performed with Fugene HD (Roche Applied Science, Indianapolis, IN, USA) in accordance with the manufacturer's protocol. Cells were treated with DHT for 4 or 24h then lysed with 5x buffer from Luciferase Assay System kit (Promega, Wisconsin, USA). The luminescence of each sample was measured in a single tube luminometer (Berthold Technologies, GmbH & CO, Germany). Each transfection was performed three times in order to overcome the variability inherent in transfections.

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