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Regulation of the human Androgen Receptor by Corepressors and Signal Transduction in Prostate Cancer

D I S S E R T A T I O N

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Von

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To my Beloved Parents…

ABBREVIATIONS

A	Ampere
Abs.	Absorption
AF	Activation Function
AR	Androgen Receptor
ARE	Androgen Response Element
ARR3	Probasin
as	Antisense
aa	Amino Acid
AI	Androgen-independent
ATCC	American Type Culture Collection
ATP	Adenosine tri phosphate
bp	base pair
BPH	Benign Prostate Hyperplasia
Cas	Casodex
cDNA	Complementary Deoxy ribonucleic acid
ChIP	Chromatin-Immuno precipitation
CoIP	Co-Immuno precipitation
Conc	Concentration
CPA	Cyproterone acetate
CsCl	Cesium Chloride
CSS	Charcoal-stripped serum
CtBP	C-terminus Binding Protein
DBD	DNA Binding Domain
ddH_2O	double distilled water
DHT	Dihydrotestosterone
DMEM	Dulbecco´s Modified Eagles Medium
DMSO	Dimethylsulfoxide
DNA	Deoxy ribonucleic acid
DNase	Deoxy ribonuclease
dNTP	Deoxy ribonucleotide phosphate
DTT	Dithiothreitol
EDTA	Ethylene DiamineTetra Acetate
Epi	microfuge tube
ER	Estrogen Receptor
ERE	Estrogen Response Element
EtBr	Ethidium Bromide
EtOH	Ethanol
FBS	Fetal Bovine Serum
gms	Grams
G-418	Geneticin
Gal_{94}	amino acid 1-94 of Gal 4 activator from yeast
GFP	*Green Fluorescent Protein*
GR	Glucocorticoid Receptor
GRE	Glucocorticoid Response Element
Her2/neu	Human epidermal growth factor receptor-2
Hg	Hygromycin
Hr	Hour
HBD	Hormone Binding Domain

HDAC	Histone deacetylase
HEPES	4-(2-Hydroxyethyl)-1-piperazinethan-sulfonic acid
HSP	Heat Shock Protein
HLH	Helix-loop-Helix
IGF	Insulin-like Growth Factor
IgG	Immunoglobulin G
IP	Immuno Precipitation
Kb	Kilo Base
kDa	Kilo Dalton
l	Liter
L	Leucine
LB	Luria-Bertani
LacZ	β-Galactosidase
LCoR	Ligand-dependent Corepressor
LHRH	Luteinizing Hormone Releasing Hormone
LTR	Long terminal repeat
m	milli, 10^{-3}
M	Molar
MeOH	Methanol
min	Minute
MMTV	Mouse Mammary Tumor Virus
n	nano, 10^{-9}
nARE	negative Androgen Response Element
NCoR	Nuclear Receptor Corepressor
NP-40	Nonidet P40; Ethylenephenyl-Polyethylene glycol
NTD	N-Terminal Domain
OD	Optical Density
OH-F	Hydroxy flutamide
ONPG	o-Nitro-phenol-β-Galacto pyranoside
p	pico, 10^{-12}
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
Pen	Penicillin
PMSF	Phenyl methyl sulfonyl fluoride
Poly-A	poly adenylation signal
PPAR	Peroxisome Proliferators Activated Receptor
PR	Progesterone Receptor
PSA	Prostate specific Antigen
PSCA	Prostate Stem Cell Antigen
R1881	Methyltrienolone
RAR	Retinoic Acid Receptor
RD	Repression Domain
RID	Receptor Interaction Domain
RIP	Receptor Interacting Protein
RLU	Relative Light Units
RNA	Ribonucleic acid
RNAi	RNA interference
RNase	Ribonuclease
rpm	Rotation per minute
RPMI	Roswell Park Memorial Institute
RSV	Raus sarcoma virus

RT	Room temperature
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
s	sense
ss	single stranded
SDS	Sodium Dodecyl Sulfate
sec	second
siRNA	small interfering RNA
SHBG	Steroid hormone binding globulin
SMRT	Silencing Mediator for RAR and TR
SRC1	Steroid Receptor Coactivator 1
Str	Streptomycin
SUMO	Small Ubiquitin-Like Modifier
T3	Thyroid hormone
TAE	Tris Acetate EDTA
TB	Terrific Broth
TBE	Tris Borate EDTA
TE	Tris EDTA
TERT	Telomerase reverse transcriptase
tet	Tetracyclin
TR	Thyroid hormone Receptor
TRIP	Thyroid hormone Receptor Interacting Protein
Triton X-100	Octyl phenol ethylene glycol ether
TSA	Trichostatin A
Tween 20	Polyoxy ethylene sorbitan mono laurate
U	Units
UV	Ultra Violet
V	Volt
VDR	Vitamin D Receptor
VP16	Viral Protein 16 from Herpes simplex virus
wt	Wild-type
(v/v)	Volume by volume
(w/v)	weight by volume
°C	Degree Celsius
α	Antibody
μ	micron, 10^{-6}
φ	hydrophobic amino acid
%	percent

List of Publications

1. Gessner G., Schonherr K., Soom M., Hansel A., **Asim M.**, Baniahmad A., Derst C., Hoshi T., Heinemann S. H.(2005) BKCa channels activating at resting potential without calcium in LNCaP prostate cancer cells. **J Membrane Biol**. Dec; 208(3):229-240.

2. Papaioannou M., Reeb C., **Asim M.**, Dotzlaw H., Baniahmad A. (2005) Co-activator and corepressor interplay on the human androgen receptor. **Andrologia**. Dec; 37(6):211-212.

3. Moehren U, Papaioannou M, Reeb C, Grasseli A, **Asim M**, Farsetti A., Baniahmad A. Wildtype but not the mutant T877A inhibits telomerase TERT subunit gene expression. Communicated

4. Moehren U, Papaioannou M, Reeb C, **Asim M**., Hong W, Baniahmad A. Alien interacts with the human androgen receptor and inhibits prostate cancer cell growth. Communicated

Scientific Conferences

(1) Presented a poster at the German Society of Human Genetics meeting, 2006 entitled "Inhibition of ligand activated androgen receptor by the corepressor LCoR" in Heidelberg.

(2) Abstract entitled "Inhibition of ligand activated androgen receptor by the corepressor LCoR" has been accepted for poster presentation in "Androgens 2006" meeting to be held in September, 2006 in Cambridge, UK.

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1.1 ABSTRACT

The thesis primarily addresses the role of transcriptional corepressor and signal transduction cascades in regulating androgen receptor (AR) activity. AR is a ligand-activated transcription factor and is important for the development of male phenotype. Malfunctioning of AR function has been implicated in the progression of the prostate cancer (CaP). Clinical management of the CaP most often involves the administration of anti-hormones (Cas, CPA) that bind to AR and turn it transcriptionally incompetent and consequently regression of the tumor. Eventually though emerges resistance to therapy 3-4 semesters post-treatment but unfortunately modern medicine doesn't offer any cure at this point. To safeguard the AR function, corepressor molecules negatively modulate AR function on its target genes hence suggested to have protective role against the CaP. Gene regulation by AR and corepressors can be influenced by activated signal transduction machinery often abruptly activated in CaP cells.

The work pursued here in part shows how partial agonist CPA modulates the expression of target genes PSCA and Maspin. It also highlights how interaction of corepressor SMRT is modulated by signal transduction pathways *in vivo*. The work demonstrates the role of Src kinase pathway in regulating AR function in androgen-independent CaP cells. Inhibiting Src by chemical inhibitor PP2, leads to decrease in AR recruitment on target genes *in vivo*. Also it decreases transactivation potential of AR on various target genes. The work also shows that inhibition of Src leads to loss of target gene induction in response to agonist and growth retardation of C4-2, hormone-independently growing cells.

This work also demonstrates the discovery of a new AR corepressor LCoR. Corepressor LCoR potently represses AR transactivation not only in anti-hormone but also in a hormone-dependent manner in CV1 cells. Versatility in repression by LCoR evidenced from the experiments showing potent *in vivo* repression of AR T877A hot spot mutant and another AR mutant which does not interact and thereby not repressed by many other corepressors. This work also shows that for its interaction with AR LCoR uses its C-terminus, which harbours HLH domain that interacts with the DBD of AR *in vivo*. Intriguingly LCoR shows only marginal repression of endogenous AR in CaP cells. This work shows that LCoR is functionally weakened by Src kinase pathway in CaP cells in repressing AR function. Inhibition of Src by Src inhibitor PP2 enhances autonomous silencing function of LCoR. In addition, this inhibition by PP2 enhances its ability to interact and thereby repress AR in CaP. Overexpression of stably-integrated corepressor LCoR in CaP cells leads to compromised growth.

This work demonstrates the importance of molecular *cross talk* between corepressors and signal transduction pathways that functionally modulate the corepressor function and thereby regulate AR transactivation and growth of CaP. It also opens an important avenue in translational cancer research implicating the role of cross talk between signaling cascades and corepressors in CaP.

1.2 ZUSAMMENFASSUNG

In dieser Arbeit wurde die Bedeutung transkriptionaler Corepressoren und der Signaltransduktions-Kaskade bei der Regulation des Androgen-Rezeptors (AR) untersucht. Der AR ist ein durch Liganden aktivierbarer Transkriptionsfaktor und ist für die Entwicklung des männlichen Phänotyps von großer Bedeutung. Dysfunktionen des AR sind mit der Progression von Prostata-Krebs (CaP) assoziiert. Das klinische Management bei der Behandlung von CaP besteht häufig in der Gabe von Anti-Hormonen (Cas, CPA), welche an den AR binden, dessen Transkription unterbinden und daher zu einer Regression des Tumors führen. Nach 1-2 Jahren der Therapie mit Anti-Hormonen kommt es meist zu einer Therapie-Resistenz, die leider zum jetzigen Zeitpunkt keine medizinische Heilung ermöglicht. Corepressoren, die den AR transkriptionell hemmen, scheinen daher eine hemmende Rolle bei der CaP-Proliferation zu haben. In CaP-Zellen kann die Genregulation durch AR und Corepressoren durch eine Aktivierung der Signalkaskade beeinflusst werden.

In dieser Arbeit wurde untersucht, wie der partielle Agonist CPA die Expression der Zielgene PSCA und Maspin reguliert. Es wurde weiterhin analysiert, wie eine Interaktion des Corepressors SMRT durch Signalwege *in vivo* moduliert wird. Die Arbeit zeigt die Bedeutung des Src-Kinase-Signalweges bei der Regulation der AR-Funktion in Androgen-unabhängigen CaP-Zellen. Wird Src durch den chemischen Inhibitor PP2 blockiert, führt dies *in vivo* zu einer verminderten AR-Rekruitierung an Ziel-Genen. Auch das Transaktivierungspotential von AR an verschiedenen Ziel-Genen ist vermindert. In dieser Arbeit wurde weiterhin gezeigt, dass eine Inhibierung von Src zu einem Verlust der Induktion der Zielgene als Antwort auf den Agonisten und zu einem verminderten Wachstum der C4-2-Zellen, einer Hormon-unabhängig wachsende Zelllinie, führt.

Weiterhin wurde in dieser Arbeit LCoR als ein neuer Corepressor für den AR beschrieben. Der Corepressor LCoR unterdrückt die AR-Transaktivierung sowohl durch Anti-Hormone als auch durch Hormone in CV-1-Zellen. Die Vielseitigkeit der Repression von LCoR wurde in Experimenten gezeigt, die eine effektive *in vivo* Hemmung der AR T877A „hot spot"-Mutante und anderer AR-Mutanten zeigen. Diese können mit vielen anderen Corepressoren nicht interagieren und somit nicht reprimiert werden. Das konnte auch nachgewiesen werden für die Interaktion des AR mit dem C-terminalen Ende von LCoR mit einer HLH-Domäne, welche mit der DBD-Domäne des AR *in vivo* reagiert. Interessanterweise zeigte LCoR nur eine geringe Repression des endogenen ARs in CaP-Zellen. In dieser Arbeit wurde auch gezeigt, dass durch den Src-Signalweg in CaP-Zellen LCoR funktionell schwächer wird. Eine Hemmung von Src durch den Src-Inhibitor PP2 führt zu einer Verstärkung der Funktion von LCoR. Darüber hinaus fördert diese Inhibierung durch PP2 eine Interaktion und führt zu einer Unterdrückung der AR-Transaktivierung in CaP-Zellen. Die Überexpression von stabil integrierten LCoR in CaP-Zellen führt zu einer Beeinträchtigung des Wachstums.

Diese Arbeit zeigt die Bedeutung des molekularen „cross talks" zwischen Corepressor und Signaltransduktions-Wegen, welche die Funktion des Corepressors funktionell moduliert, dadurch die AR-Aktivierung und die Proliferation von Prostata-Tumoren reguliert. Für weitere Forschungen könnten möglicherweise die Erkenntnisse bezüglich des „cross talks" zwischen Signalkaskaden und Corepressoren in Hinblick auf die Prostata-Tumoren genutzt werden.

INTRODUCTION

2.1 Androgens and anti-androgens

Androgens mediate a wide range of developmental and physiological responses. Androgens are steroid hormones that induce the differentiation and maturation of male reproductive organs (Cooke et al., 1991). They are major regulators of cell proliferation and cell death in the prostate gland (Isaacs, 1984). Production of androgens occurs in the endocrine glands, primarily in the testes and adrenal gland (Coffey and Isaacs 1981). In prostate, testosterone (a major androgen) is converted to 5 α-dihydrotestosterone (DHT) by 5 α-reductase activity (Russell and Wilson 1994). The androgens have been shown to regulate the expression of many target genes. For example, the expression of prostate specific antigen (PSA) is androgen-regulated (Young et al., 1992, Perry et al., 1996). The connection between androgen action and cell growth control may be explained by the fact that the androgens also regulate several genes involved in cell cycle control, such as cyclin-dependent kinase inhibitors p16 and p21 and cyclin-dependent kinases 2 and 4 (Lu et al., 1997, 1999). Androgens are believed to have, at least, a permissive role in the genesis of CaP as castration of a male before puberty inhibits the growth of the prostate and prevents the initiation of CaP (Isaacs, 1994; Moore 1944).

2.2 The nuclear receptor (NR) superfamily

The mammalian nuclear receptor superfamily comprises more than 45 transcription factors, many of which regulate gene expression in a ligand-dependent fashion (Perissi and Rosenfeld, 2005). The nuclear receptors mediate the actions of lipid-soluble steroid hormones and nonsteroidal lipophilic hormones. There are also several nuclear “orphan receptors” whose regulatory ligands have not yet been identified (Giguere 1999). The ligands of different nuclear receptors are diverse, but the receptors are structurally quite similar to each other (Mangelsdorf et al., 1995). Also a subset of receptors, which includes receptor for thyroid hormone (TR) and Retinoic Acid Receptor (RAR), can actively repress target genes in the absence of ligand, whereas upon binding to cognate ligand almost in all cases the receptors usually become potent transcriptional activators. Conversely, several NRs have been shown to inhibit transcription in a ligand-dependent manner either by

binding to negative response elements or by antagonizing the transcriptional activities of other classes of transcription factors (Weston et al., 2003 and Nettles et al., 2004).

The nuclear receptors are cytosolic or nuclear proteins and the activation of the steroid receptor from an inactive, chaperone-protein bound state requires the binding of the cognate ligand, which induces a conformational change in the receptor structure. This leads to the dissociation of chaperone proteins, receptor dimerization and nuclear shuttling (Moras and Gronemeyer 1998). In the nucleus, a dimerized receptor complex regulates the transcription of the target genes by binding to cognate response element in DNA. There are however some exceptions to this basic model of NR function as in case of Ecdysone receptor, which is predominantly nuclear in nature (Koelle et al., 1991). In addition, nuclear receptors can mediate the so-called non-genomic, DNA binding independent effects of hormones in the cells (Peterziel et al., 1999).

The nuclear receptor family can be divided into subgroups according to the pattern by which they bind to the ligand, to DNA and to each other. Class I, the steroid receptor subfamily, consists of androgen (AR), estrogen (ER), progesterone (PR), glucocorticoid (GR) and mineralocorticoid (MR) receptors. There are receptors that bind diverse products of lipid metabolism such as fatty acids and prostaglandins (peroxisome proliferator activated receptors, PPARs and liver X receptors, LXR) (Aranda and Pascual, 2001 and Gronemeyer et al., 2004).

The modular structure of the NHRs consists of three different functional domains: a variable amino-terminal domain, the central well-conserved DNA binding domain, and the moderately conserved carboxy-terminal ligand binding domain, separated from each other by hinge regions (MacLean et al., 1997).

2.2.1 The Androgen receptor: Genomic effects

The human androgen receptor (AR) gene, located in the chromosome Xq11-12, contains 8

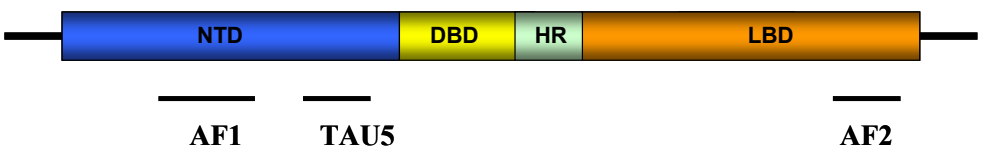


Fig. 2.1 The modular structure of AR. AR is 919 a.a. member of nuclear hormone receptor superfamily containing N-terminus transactivation domain (NTD), a DNA-binding domain (DBD), hinge region (HR) and Ligand-binding domain (LBD). NTD contains a hormone-independent activation function (AF1) and another activation function called, transactivation unit 5 (TAU-5),

and a ligand-dependent activation function (AF2) situated in the LBD. Both of NTD and LBD contribute to transactivation function.

exons (Lubahn et al., 1988). The AR gene is almost universally expressed in different tissues (Faber et al., 1991). AR mediates the biological effects of androgens. Structurally, AR can be subdivided into three well-defined functional domains: the amino terminus transactivation domain (NTD), the DBD, hinge region and the LBD (Fig. 2.1). The transactivation by AR involves the NTD which harbours AF1 and TAU-5 transactivation functions that can act in a ligand-independent manner. AF1 (activation function 1), functions in a ligand-independent manner when artificially separated from the LBD; the AR NTD mutant therefore lose their transactivation potential (Jenster et al., 1995). TAU-5 is activated by PRK1 signal transduction pathway and has been implicated in CaP progression (Metzger et al., 2003). NTD has also been shown to be the target of corepressor-mediated receptor repression (Dotzlaw et al., 2002). The NTD also contains a few homopolymeric amino acid repeats typical for many transcription factors. The most amino-terminal repeat is the polyglutamine (Q) repeat, coded by CAG triplets. Like other genes with the CAG repeats, the repeat length is very polymorphic, ranging from 14 to 35 (Sartor et al., 1999). Lengthening the repeat to 40-62 suggested to be involved in an inherited neuromuscular degenerative disease, Kennedy's disease or spinal and bulbar muscular atrophy, SBMA (La Spada et al., 1991).

Many coactivators such as SRC1 family of coactivators are known to interact with this sequence in an agonist-dependent manner to promote transcription (Ma et al., 1999). The amino acid sequence of DBD is a most highly conserved among members of the nuclear receptor superfamily. It includes zinc fingers, in which four cysteine residues bind one zinc ion in each of the motifs. The zinc fingers have been shown to be fundamental to the binding of the response element in DNA (O'Malley 1990). The first zinc finger harbors the information for the specific recognition of DNA, and the second finger stabilizes the DNA-receptor interaction in contact with the DNA backbone and has been shown to be involved in interaction with other coregulatory molecules (Glass 1994).

The third domain structure in the AR is the carboxy-terminal ligand binding domain. It contains the ligand-dependent transactivation function AF2 which is responsible for optimal transcription activation in response to the ligand. Both the N-terminal transactivation domain and the LBD are responsible for and coordinate the ligand-dependent transactivation function of the NRs (Tora et al., 1989). The N-terminal activation function (AF1) is constitutively active on its own, while the AF2 function in the

LBD is induced upon ligand binding. Because the transactivation function is normally androgen-dependent, the LBD prevents the action of the receptor without the ligand (Kuil and Brinkmann 1996). Deletions in this domain abolish the binding and response of androgens, which results in a constitutively active AR (Jenster et al., 1991). The activities of both activation functions are dependent on the cell type and promoter context (Beato et al., 1995). The N terminus transactivation domain and C terminus LBD of AR are known to interact with each other and this interaction has been shown to contribute to the optimal activation of the AR (He et al., 2000).

Like few other members of NR superfamily, unbound AR is inactive and forms a complex with chaperones/HSPs (Heat shock proteins) in the cytoplasm (Georget et al., 1997; Jenster et al., 1991). The binding of androgens to AR induces a conformational change that leads to dissociation of AR from the HSPs and subsequent receptor dimerisation and translocation into the nucleus, facilitating the ability of AR to bind to its cognate response elements, called AREs (androgen response elements) in the target genes (Claessens et al., 2001; Roche et al., 1992), recruit coregulators to modulate the expression of the target genes (Collingwood et al., 1999; Glass and Rosenfeld, 2000; Xu et al., 1999).

2.2.2 The androgen receptor: Non-genomic effects

The androgens can exert effects, which are considered to be non-genomic (rapid effects) because these effects take place in the presence of transcription inhibitors or they are occurring too fast to involve changes in gene transcription. Until now, the reported non-genomic effects of the steroids at physiological concentrations appear to be receptor-mediated (Heinlein and Chang 2002). The non-genomic actions include stimulation of MAPK (mitogen-activated protein kinase) pathway and induction of cAMP second messenger and PKA (protein kinase A) by androgens. Both of these mechanisms also influence the transcriptional activation of the nuclear AR (Peterziel et al., 1999 and Cato et al., 2002). In addition, the existence of a novel, cell membrane bound androgen receptor has been suggested (Benten et al., 1999) but remains to be identified. One of the effects mediated by this putative receptor is the increase of intracellular calcium levels, which in turn could be able to activate signal transduction cascades such as the PKA, PKC (protein kinase C) and MAPK or to modulate the activity of the transcription factors. Androgen-mediated modulation of the ion channel activity and intracellular calcium levels has been observed in several cell types (Heinlein and Chang 2002) including LNCaP cells (Steinshapir et al., 1991). However, it has not yet been determined whether these non-

genomic effects are mediated through a membrane androgen receptor or by steroid hormone transporter proteins termed as steroid hormone binding globulin or SHBGs.

2.3 Coregulatory proteins of AR

AR activates the expression of the target genes by facilitating transcriptional. AR-mediated transcription requires several auxiliary protein complexes (Hermanson et al., 2002) that can interact sequentially, in combination or in parallel such as coactivators and corepressors. Coactivators are generally recruited to agonist-bound AR e.g., coactivator p300 contains HAT activity and can alter the architecture of chromatin to facilitate transcription. Histone acetylation neutralizes the positive charge of the histone N-terminal tails and weakens the interaction between histones and negatively charged DNA. Thus, the recruitment of chromatin remodeling proteins and acetyltransferases is essential to make the target sequences accessible for the liganded receptor. Conversely, corepressors complexes often contain HDACs, leading to deacetylation of the histone tails, leading to condensed chromatin structure resulting in transcriptional repression (Burke and Baniahmad, 2000 and Hu and Lazar 2000). Recently, another AR coactivator, Lysine-specific demethylase was identified. It interacts with the AR in a ligand-dependent fashion and its knock down in LNCaP cells leads to abrogation of cell proliferation (Metzger et al., 2005).

2.3.1 Corepressors

Nuclear receptor corepressors were originally identified as proteins associated with unliganded type II nuclear receptors which, unlike type I nuclear receptors, can bind to DNA in the absence of a ligand and mediate transcriptional repression (Horlein et al., 1995). Retinoic acid receptor and thyroid hormone receptor are capable of gene repression by interacting with the corepressors and recruiting the HDAC activities, whereas the steroid receptors, including the AR, do not repress transcription in the absence of a ligand (Hu and Lazar 2000). For the ER, the switch from gene activation to gene repression by an antagonist is accomplished by association of the corepressors and HDACs (Shang et al., 2002). Due to the structural and functional similarities between the ER and AR, it has been proposed that the corepression complex may be similarly recruited by antagonist-bound

AR (Shang et al., 2002). Indeed Dotzlaw et al., (2002) have shown the *in vivo* binding of corepressor SMRT to anti-hormone CPA-bound AR.

Different corepressors are known to act by targeting various domains of AR. The corepressor calreticulin for example functions by associating with the DBD of AR and inhibits AR binding to its DNA response elements leading to target gene repression. (Dedhar et al., 1994) interestingly calreticulin has been identified as an androgen-

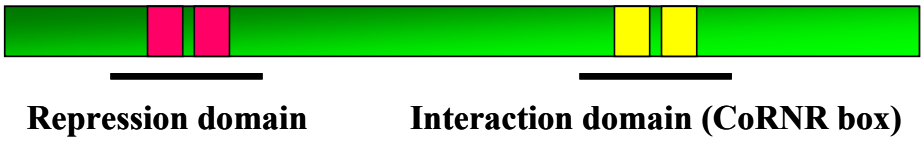


Fig. 2.2 Typical structure of a corepressor. It essentially harbours a CoRNR motif with consensus LXX/HIXXXI/L for its interaction with the nuclear receptor. In addition has repression domain(s) by which it can recruit gene silencing machinery to the receptor thereby inhibiting transcription initiation.

responsive gene in prostate suggesting a feedback control (Zhu et al., 1998). Other kinds of corepressors recruit histone deacetylases (HDACs) to silence gene expression. HDACs play a critical role in altering the acetylation status of core histones leading to deacetylation thereby making chromatin to adopt a condensed conformation inhibitory to transcription by AR as well as by other transcription factors (Grunstein et al., 1997, Xu et al., 1999). Corepressor SMRT (*Silencing Mediator for RAR and TR*), Alien and NCoR (Nuclear receptor corepressor) are the exemplary of those and are known to be recruited to numerous NRs. (Ordertlich et al., 2001, Chen and Evans 1995, Horlein et al., 1995, Dressel et al., 1999). Indeed SMRT and NCoR have been shown to attenuate AR transactivation through competing for interaction between AR and its coactivators. Inhibition of target genes by corepressor Alien has been shown to be Trichostatin A (TSA) (HDAC inhibitor) sensitive and can functionally interact with other molecules of the signaling machinery like mSinA (Moehren et al., 2004). This interaction might have synergistic effect in repressing AR function. This suggests that inhibition of AR activity involves recruitment of corepressors in the form a repression complexes with other transcription inhibitory proteins. Similarly a SMRT (*Silencing Mediator for RAR and TR*)/HDAC complex has been shown to contain *nuclear receptor corepressor*, NCoR (Li et al., 2000). Another class of corepressors interrupt the interaction between AR and its coactivators e.g., Cyclin D1 which suppresses transactivation by AR (Knudsen et al., 1999). The C-terminal domain of cyclin D1 and the hinge region of AR mediate AR-cyclin D1 interaction. Furthermore,

cyclin D1 inhibition of AR may result from its capacity to inhibit the association of coactivator P/CAF and AR (Reutens et al., 2001). Complexity is further added by corepressors that can interrupt the interaction between the NTD and LBD of AR (often called N-C interaction). A key checkpoint Rad family protein, called hRad9 was recently identified as a corepressor for AR in CaP cells. The LBD of AR can interact with the CTD of the hRad9. The FXXLF motif within the CTD of hRad9 interrupts the DHT induced AR N-C terminus interaction. This interaction between AR and Rad9 results in the suppression of AR transactivation (Wang et al., 2004). Another corepressor Alien (Dressel et al., 1999) has been identified previously for TR. Recent data suggest that Alien can be recruited to CPA-bound AR. Stable integration of the corepressor Alien in CaP cells leads to inhibition of LNCaP cell growth in a CPA-dependent manner (Moehren et al., Communicated). The ability of the corepressor to inhibit CaP growth suggests that corepressor inactivation plays an important role in the progression of CaP.

2.3.2 The agonist-dependent corepressors: LCoR

Hormone binding, particularly by NHRs is widely associated with activation of the target gene transcription by the recruitment of coactivators that contain LXXLL motif (called NR box). Corepressors, such as SMRT and NCoR recognize LBDs in a hormone free or, in some cases, antagonist-bound conformation as for AR (Dotzlaw et al., 2002). However, a model of receptor activation where only coactivators are recruited to agonist-bound receptors can not entirely explain the existence of large number of corepressor identified to date. Indeed several NR box-containing corepressor proteins have been identified e.g., TIF1α, (Le Douarin et al., 1995). A similar SET domain containing protein *NR-binding SET-domain-containing protein* (NSD1) contains both NR boxes and CoRNR motifs similar to those of SMRT and NCoR. These motifs control its interaction with multiple NRs in either a ligand-dependent or -independent fashion (Huang et al., 1998). NSD1 contains several distinct coactivation and corepression domains, raising the possibility that their functions may be selectively modulated by secondary signal transduction pathways, thus controlling whether NSD1 acts as a coactivator or a corepressor.

Receptor interacting protein (RIP140) was initially characterized as a coactivator (Cavailles et al., 1995) that interacts through multiple NR boxes with ER. Later work however showed that RIP140 functions as a corepressor that competes with coactivator p160 for binding to agonist-bound LBDs of NHRs, blocking coactivation *in vivo*. (Eng et

al., 1998, Lee et al., 1998, Treuter et al., 1998). RIP140 was reported to inhibit AR-dependent transactivation in agonist-dependent manner (Christian et al., 2006).

Recently also, a ligand-dependent corepressor, LCoR, was identified in a screen for proteins that interacted with the LBD of ERα in an estradiol-dependent manner (Fernandes et al., 2003) LCoR represses ligand-dependent transactivation of PR, GR, VDR and ER. In order to interact with them uses the single NR box having LXXLL motif. Therefore, the LCoR NR box mutant shows loss of interaction with these NHRs (Fernandes et al., 2003) and thereby receptor repression is abolished. LCoR binds to the same coactivator p160

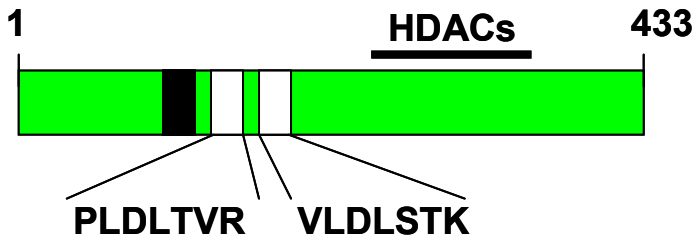


Fig. 2.3 Schematic representation of the primary structure of LCoR. The NR box is represented by black bar. Positions of CtBP binding motifs are indicated by white boxes. HDAC binding domains are over lined. (Fernandes et al., 2003)

binding pocket of NHRs (Fernandes et al., 2003) suggesting that it competes with coactivators for recruitment to selected members of NHRs superfamily. LCoR is described as molecular scaffolds for many transcriptional repressors (White et al., 2004). Corepression of ER and GR can be blocked by HDAC inhibitor TSA, however, that of VDR and PR remained unaffected by TSA treatment suggesting LCoR functions in HDAC-dependent and -independent manner. In fact, LCoR interacts directly with HDACs 3 and 6 but not with 2 and 4 *in vitro* and *in vivo* (Fernandes et al., 2003). Agonist-dependent corepressors have been shown to possess CtBP corepressor binding motifs (Fig. 2.3) and mutation of this binding motif severely attenuates their corepressor function as in case of RIP140. Remarkably the sequence of LCoR revealed also the presence of tandem motifs PLDLTVR and VLDLSTK that are homologous to the consensus P/VLDLS/TXK/R defined as a binding site for CtBPs (Vo et al., 2001) *in vitro* binding and Co-IP studies revealed that the CtBP binding is disrupted only upon mutations in both sites (Fernandes et al., 2003). Immuno-cytochemical studies revealed a substantial overlap of CtBPs and LCoR in discrete nuclear bodies (Fernandes et al., 2003).

The binding of CtBPs may explain the observation that the sensitivity of corepressor LCoR to the HDAC inhibitor is receptor-dependent. While transcriptional repression of ERα and

GR has been shown to be TSA sensitive, the repression of PR and VDR was largely TSA resistant (White et al., 2003). Furthermore, corepression of ERα was partially disrupted by the mutations of the CtBP motifs of the LCoR, corepression of PR function was completely released by the same mutations. This suggests that mode of repression by LCoR is largely dependent on the promoter context same as for CtBP (Chinnadurai 2002).

2.4 Androgen receptor in prostate cancer

Several alterations take place in the AR signaling pathway during the development and progression of prostate cancer. First, the action of the AR in a normal and malignant prostate uses distinct pathways. In a normal prostate gland, androgen-stimulated proliferation of epithelium requires paracrine involvement of stromal cells expressing the AR. In malignant cells the androgen-mediated signaling has been converted to autocrine mode and no interaction with the stroma is needed (Gao et al., 2001). In addition, the emergence of the hormone-refractory tumors during the antihormone-therapy is associated by restoration of the expression of the genes regulated by the AR (Gregory et al., 2001, Kim et al., 2002) such as PSA. It is noteworthy, that hormone-refractory CaP cells though grow in a hormone-dependent manner, they still depend on functional AR signaling for growth and their growth is severely compromised if AR is depleted in cells using RNAi. (Chen et al., 2004 ; Liao et al., 2005; Haag et al., 2005). Many changes that lead to aberrant AR signaling are believed to be caused at least partly by genetic changes in the AR gene.

2.4.1 Germ-line alterations

It has been suggested that a short CAG repeats in the NTD of AR may result in an increased risk of prostate cancer and that the length of the repeat could also be partly responsible for the difference in prostate cancer risk in different racial groups (Edwards et al 1992 and Irvine et al., 1995). For example, Giovannucci et al., (1997) observed that the shorter repeat was associated with an increased risk for metastatic and fatal prostate cancer.

2.4.2 Somatic aberrations of AR in androgen-dependent prostate cancer

Studies have indicated only a few somatic mutations of the AR in untreated prostate cancer (Newmark et al., 1992, Suzuki et al., 1993, Culig et al., 1993, Ruizeweld de Winter et al.,

1994). However, mutations are present in a substantial fraction of prostate cancers. Gaddipati and co-authors (1994) reported that a codon T877A mutation was found in 25% of the patients with untreated metastatic prostate cancer. However, it is now generally accepted that the AR mutations are rare in untreated prostate cancer.

2.4.3 Somatic aberrations of AR gene in hormone-refractory prostate cancer

AR gene amplification

A high-level AR amplification in 30% of the hormone-refractory tumors but not in the specimens taken from the same patients prior to therapy (Visakorpi et al., 1995 and Bubendorf et al., 1999), found the AR gene amplification in 23% of the 54 locally recurrent and 22% of the 62 metastases of hormone-refractory disease. The findings suggest that the amplification of the AR gene may be one of the mechanisms by which the prostate tumors acquire growth advantage in an androgen-depleted environment. The amplification of the AR may sensitize the prostate cancer cells to trace amounts of the androgens (Visakorpi et al., 1995, Culig et al., 1997).

AR gene mutations

One of the best studied mutations in hormone-refractory prostate cancer was the one discovered in the LNCaP prostate cancer cell line (Veldscholte et al., 1992). The LNCaP cell line was originally established from lymph node metastases of a patient treated with hormonal therapy. In the cell line the mutation T877A in the ligand binding domain of the AR enables AR to be activated by other steroid hormones such as estradiol and progesterone, and even by antiandrogen flutamide, suggesting broadened ligand specificity (Culig et al., 1993). It has also been shown that AR point mutations occur spontaneously in transgenic adenocarcinomas of the prostate mouse model (TRAMP), and certain mutations are selected for by the changes (castration) in the androgen environment (Han et al., 2001). The mutated ARs from the flutamide-treated patients were also shown to be stimulated by flutamide. The most frequently found mutation among the flutamide-treated patients was identical to the mutation in the LNCaP (T877A).

In addition to the missense mutations, it has been demonstrated that silent mutations in the AR gene may influence the mRNA stability or transcriptional regulation (Han et al., 2001).

2.5 Cross talk of AR with other signaling pathways

It has been proposed that in the absence of a ligand, the AR activation could take place by cross-talk with various growth factors, protein kinase pathways. CaP progression is often associated with alteration of growth factor or growth factor receptor expression by the tumor (Russell et al., 1998) For example, it has been demonstrated that epidermal growth factor (EGF), epidermal growth factor receptor-2 (ERBB2/Her-2), keratinocyte growth factor (KGF/FGF-7), insulin-like growth factor-1 (IGF-1), protein kinase A (PKA), mitogen-activated protein kinase (MAPK), as well as IL-6 could activate the AR signaling (Culig et al., 1994, Abreu-Martin et al., 1999, Jenster et al., 2000, Craft et al., 1999, Sadar 1999). An additional mechanism underlying ligand-dependent activation of the AR by these alternative pathways may involve phosphorylation of either the AR or its associated proteins (Sadar 1999, Ueda et al., 2002).

It has been shown that the concentration of the IL-6 is elevated in the sera of the patients with metastatic and hormone-refractory prostate cancer (Drachenberg et al., 1999). An IL-6 receptor is also expressed in a normal prostate and in CaP. The IL-6 has been shown to activate AR through the MAPK pathway interacting with the transcriptional coactivator p300 in the prostate cancer cells (Hobisch et al., 1998, Ueda et al., 2002, Debes et al., 2002). It has thus been suggested that a network of protein kinase-coactivator-IL-6 is needed to induce the androgen-independent activation of the AR (Ueda et al., 2002).

The IGF-1 signaling has been implicated in the progression to androgen-independence at least in LNCaP and LAPC-9 xenograft models, in which both the IGF-1 and its receptor IGF-1R are overexpressed (Nickerson et al., 2001). In addition, the inhibition of the IGF-R1 has been shown to result in suppression of tumor growth and invasiveness in rat prostate cancer cells (Putz et al., 1999).

Akt kinase is known to phosphorylate AR and enhances its transactivation potential even at low levels of androgens. Therefore stimuli that increase Akt activity, including Her2 may contribute to progression of CaP. The PI3K pathway is stimulated in LNCaP and PC-3 cells by IL-6 and inhibition of IL-6 induced PI3K activity by wortmanin causes apoptosis in LNCaP cells (Chung et al., 2000) suggesting that this pathway can contribute to the survival and growth of CaP cells.

A recent article suggested forced overexpression of Akt kinase and Raf kinase in androgen-dependently growing CaP cells led to transition to androgen-independent status (Gao et al., 2006).

2.6 AR coregulators in prostate cancer

Fujimoto (2001) reported that the expression of the coactivator SRC1 was higher in refractory CaP. Gregory et al., (2001) found that the expression of both the SRC1 and TIF2 was increased in hormone-refractory prostate cancer. The overexpression of these coactivators affected the ability of low-affinity ligands or minimal concentrations of the adrenal androgens to activate the AR in the castrated environment. Transactivation by AR was further increased by phosphorylation of the p160 coactivators suggesting a link to growth factor signaling pathways. Interestingly, it has also been shown that the ligand-independent activation of the AR does not occur alone through the overexpression of the SRC1; it requires a functional MAPK pathway (Ueda et al., 2002). In the absence of the androgens, the protein-protein interaction of the SRC 1 and AR also seems to require IL-6 (Ueda et al., 2002).

The expression of the CBP has been detected in different prostate specimens, but no alterations in the level of expression have been reported (Comuzzi et al., 2003). It has also been shown that antagonist/agonist balance of antiandrogens flutamide and bicalutamide is influenced by the CBP. Indeed the expression of the CBP increased flutamide-induced AR activity up to 50% of the activation induced by the synthetic androgen R1881. Interestingly, recent work demonstrated that elevated level of corepressor SMRT in CaP cells lead to repression of target genes which are involved in antiproliferative action (Khanim et al., 2004). This suggests that enhanced corepressor expression is not always associated with growth inhibition, rather may promote the growth of CaP cells.

2.7 Specific aim of the present study

Androgen receptor (AR) mediates the biological effects of androgens and is implicated in the progression of the prostate cancer (CaP). AR functions by regulating the expression of target genes, either positively or in a negative manner. However, target gene regulation by other ligands, specifically antagonists has not been well addressed. Regulation of target genes often involves coregulatory proteins. This work attempts to address modulation of

target gene expression by corepressors and therapeutic ligand cyproterone acetate. As many signal transduction pathways are over expressed in CaP, it is possible that they boost the AR function in CaP cells; this possibility has been addressed in the following work. Corepressors repress anti-hormone bound AR function and bring the therapeutic advantage in CaP. It is therefore important to discover new AR corepressors with versatile action, especially those which can repress agonist-bound AR function. Mutants of AR found in CaP cells can be activated by many different non-cognate ligands for the reason that AR can use them as alternate agonists. The decreased corepressor to coactivator ratio observed in androgen-independently growing CaP cells also suggests the link between corepressors and inhibition of growth. Like many other regulatory proteins corepressor function is subject to regulation by post-translational modifications important being phosphorylation as well. Signal transduction pathways may enhance AR function possibly by functionally inactivating the corepressors molecules and this likelihood has also been addressed in the work undertaken.

3. Materials

3.1 General instruments	Suppliers
Agarose gel electrophoretic unit	Stratagene and Harnishmacher
Analytical balance (s)	Sartorius
Autoclave	Tecnomara/KSG
Bacterial incubator	Stemmert
Balance	Sartorius
Blower	Rothenberger
Cell cryo cooler	Nalgene
Centrifuge	Heraeus biofuge
CO_2 incubator	NUNC
Computer	Intel Pentium IV
Cover slip	Mencel
Deep freezer (-80 °C)	SEPATECH, Heraeus
Dryer	Heraeus
Freezer (-20 °C)	Liebherr
Gel documentation system	Biometra
Ice machine	Scotsman/Ziegra
Incubator	Heraeus
Incubator/Shaker	GFL
Laminar hood	Heraeus, laminair
Luminometer	SIRIUS *Berthold Detection Systems*
Magnetic Stirrer	Janke & Kunkel
Microliter pipettes	Gilson, Eppendorf
Microscope	Axiovert 135, Zeiss
Microwave	Privileg
MilliQ machine	USF, Seral/ Mi
Multipipette	Gilson
Neubauer chamber	Assistent
P^H Meter	WTW pH96
Pipetteboy	Braun/IBS Integra
Quartz cuvette	Hellma
Real time PCR machine	Roche, Corbett research
Refrigerator (4 °C)	Liebherr, Foran
Rocker	Bochem
Shaker	Heidolph
Sonifier	250 Branson
Spectrophotometer	Eppendorf, Lab biochrom
Thermal-Cycler	Eppendorf, PeqLab
Timer	Roth
UV-Trans-illuminator	Bachofer (366 nm), UVP (254nm)
Vacuum pump	IBS Integra
Vortex	Janke & Kunkel, Scientific instruments
Washer	Newamatic

3.2 Consumables

Cell culture dishes	Greiner, NUNC
Cryovials	Nalgene
Falcon 15 ml and 50 ml	Greiner, TPP
Filter tips	Greiner
Latex gloves	Braun
Microfuge tube (1.5 ml and 2.0)	Eppendorf
Nitrile gloves	Roth
Paper towel	Kimberly Clark
Parafilm	parafilm
Pasteur pipette	Assistant
PCR tubes	Biozym
Petridishes	Greiner
Plastic cuvette	Sarstedt
Quick-Seals™	Beckman
Reagent bottles	Schott
Sterile filter (0.2 µm, 0.8 µm)	Millipore, Schleicher & Schüll, Sartorius
Tissue culture bottles	Greiner
Tissue culture dishes	Greiner, NUNC

3.3 Chemicals

Acetic acid	Roth
Active charcoal	Merck
Adenosine tri-phosphate (ATP)	Roche
Agar	Gibco, Invitrogen
Agarose	Roth
Ampicillin-Sodium salt	Merck, Serva
Apo transferrin	Sigma
Bacto tryptone	Applichem
Biotin	Sigma
Boric acid	Merck
Bromophenol blue	Serva, Merck
Calcium chloride	Roth
Calf thymus DNA	Sigma
Cesium chloride	Roth
Chloramphenicol	Roth
Chloroform	Merck
Coenzyme A	PJK GmbH
Di potassium hydrogen phosphate	Merck
Di Sodium hydrogen phosphate	Merck
Dimethylsulfoxide (DMSO)	Serva
Dithiothreitol (DTT)	Sigma
D-Luciferin	PJK GmbH
DMEM cell culture media	Invitrogen
dNTPs (dATP, dCTP, dGTP, dTTP)	MBI
EDTA-sodium salt	Roth
Ethanol	Roth
Ethidium bromide solution (10 mg/ml)	Roth

F12 cell culture media	Sigma
Fetal bovine serum (FBS)	Invitrogen
Ficoll 400	Sigma
Formaldehyde	Roth
Glucose	Roth
Glycerin	Roth
Glycine	Roth
HEPES (n-2-Hydroxyethylpiperazine)	Roth
Hydrochloric acid	Roth
Insulin	Sigma
Isoamyl alcohol	Roth
Isopropanol	Roth
Lithium chloride	Merck
Magnesium acetate	Merck
Magnesium sulfate	Merck
Manganese chloride	Merck
Methanol	Merck
Nonidet® P-40 (NP-40) substitute	Fluka
Oligonucleotide	Eurogentec
Penicillin/Streptomycin	Invitrogen
Phenol	Roth
Phenyl methyl sulfonyl fluoride (PMSF)	Sigma
Potassium acetate	Merck
Potassium chloride	Merck, Roth
Potassium dihydrogen phosphate	Roth
Protein-A-Sepharose	Amersham
RPMI cell culture media	Invitrogen
SDS ultrapure	Serva
Sodium acetate	Roth
Sodium chloride	Merck, Roth
Sodium deoxycholate	Roth
Sodium dihydrogen phosphate	Roth
Sodium bicarbonate	Merck, Roth
Sodium hydroxide pellets	Roth
Sodium pyruvate	Invitrogen
TRI®-Reagent	Peqlab
Tris-Base	Roth
Triton X-100	Sigma
Trypsin	Gibco/Invitrogen
Tween-20	Roth
Xylene cyanol	Sigma
Yeast extract	Difco, Oxoid
β-Mercapto ethanol	Merck, Fluca
3-(n-Morpholino) o-Nitrophenol-β-Galactopyranoside	Roche

3.4 Enzymes, hormones and Signaling inhibitors

ENZYMES

Alkaline Phosphatase (CIAP)	MBI
Klenow Enzyme	MBI
Lysozyme	Amersham, Serva
Proteinase K	Sigma
Restriction endonucleases	MBI Fermentas, Boehringer Mannheim
Ribonuclease (RNase A)	Sigma
Trypsin	Merck
T_4-DNA-Ligase	MBI
T_4-DNA-Polymerase	MBI
Taq DNA Polymerase	Invitrogen

HORMONES

Casodex (Cas)	Zeneca
Cyproterone acetate (CPA)	Sigma
Dihydrotestosterone	Sigma
Geneticin (G-418)	Invitrogen
Hydroxy flutamide (OH-F)	LKT Laboratories Inc, Schering AG
Hygromycin	Sigma
Methyltrienolone (R1881)	Perkin Elmer
Tri-iodo thyronine (T_3)	Sigma

INHIBITORS

AG1517	Alexis
HA1077	Calbiochem
PP2	Calbiochem
U0126	Calbiochem
LY294002	Calbiochem

3.5 Commercial kits and ready-to-use materials

Complete Mini, Protease inhibitor	Roche (Cat # 11 836 152 001)
DOTAP transfection reagent	Carl Roth (L787.3)
Maxiprep, midiprep kit	Marligen (11452-026 and 11451-028)
QIAquick PCR purification Kit	Qiagen (28104)
PCR taq Polymerase Kit	Invitrogen (18038-042)
Salmon Sperm DNA/Protein-A-Agarose	Upstate (Cat # 16157)
Superscript™ One-Step RT-PCR	Invitrogen (12574-030)

3.6 Antibodies employed in ChIP

α-AR (PG21)	Upstate (4 µl per IP)

α-SMRT (PA1-842)	Alexis (4 µl per IP)
α-Alien (PepAK2)	Rabbit polyclonal (home-made, 4 µl per IP)
α-c-myc (9E10, sc-40)	Santa Cruz (5 µl per IP)
α-IgG Rabbit (sc-2027)	Santa Cruz (2 µl per IP)
Goat α-Rabbit IgG (sc-3836)	Santa Cruz (2 µl per IP)

3.7 Oligonucleotides

Sequencing Oligos

Gal_{94}-Primer:	5‘-CCT CGA GAA GAC CTT GAC CTT GAC ATG-3‘
T_7:	5’TAA TAC GAC TCA CTA TAG GG-3´
AR-sense:	5´CAG GAA AGC GAC TTC ACC-3´
rev. primer:	5´AGG CGA TTA AGT TGG GTA3´
AR-67:	5´TTC CAG AAT CTG TTC CAG AGC3´

Oligos employed in ChIP

PSA ARE I sense:	5´TCT GCC TTT GTC CCC TAG AT3´
PSA ARE I antisense.	5´AAC CTT CAT TCC CCA GGA CT3´
PSA ARE III sense:	5´GAG GTT CAT GTT CAC ATT AGT ACA C3´
PSA ARE III antisense:	5´ATT CTG GGTT TGG CAG TGG AGT GC3´
-2.0 kb PSA sense:	5’AGC ATC AGC CTT ATC TCC A3’
-2.0 PSA antisense:	5’ACT CCA ATC TGA TCC TCC A3’
-7.5 kb PSA sense:	5’AGT GAT TCT CCT GCC TCA3’
-7.5 kb PSA antisense:	5’AGC ATG TAG GCT CTG GAA3’
PSCA enhancer sense:	5’GAA CTT TCC CTC TGG ACA C3’
PSCA enhancer antisense:	5’GTG AGG TCA GAA CCC AAC3’
-2.0 kb PSCA sense:	5’TAC CCA GGG CCA TAT CTC3’
-2.0 kb PSCA antisense:	5’GAC CAA GGC TTT ATC ATC AG3’
Maspin promoter sense:	5’CGG CAC TCC TCT CCT AC3’
Maspin promoter antisense:	5’TCA ACC TCC CCA AAT GC3’

-2.0 kb maspin sense: 5'TAC TTG GGA GGC TGA GAC3'

-2.0 kb maspin antisense: 5'GAT CAG GCT TCT AAA GAG AC3'

Real time PCR primers

PSA sense: 5′ACT GCA TCA GGA ACA AAA GCG TGA3′

PSA antisense: 5′CGC ACA CAC GTC ATT GGA AAT AAC3′

PSCA sense: 5′TGC AGG TGGAGA ACT GCA3′

PSCA antisense: 5′TCT GTG AGG AGT GGC ACA3′

Maspin sense: 5′GCT AAA GGT GAC ACT GCA A3′

Maspin antisense: 5′TTT GGT CTG GTCGTT CAC A3′

GAPDH sense: 5′CGG AGT CAA CGG ATT TGG TCG TAT3′

GAPDH antisense: 5′AGC CTT CTC CAT GGT GGT GAA GAC3′

β-Actin sense: 5′ACA GAG CCT CGC CTT TGC CGA3′

β-Actin antisense: 5′CAC GAT GGA GGG GAA GAC G3′

3.8 DNA standard markers

λ DNA EcoRI/HindIII (MBI)

Size (bp)
21227
5148
4973
4268
3530
2027
1904
1584
1375
947
831
564

pUC19 DNA MspI (MBI)

Size (bp)
501
489
404
331
242
190
147
111
110
67
34

3.9 Bacterial strains and Eukaryotic cell lines

Bacterial strains

***Escherichia coli* DH 5α** (Described by Hanahan, 1983)

Genotype of the strain F*'/end*A1 *hsd*R17 ($r_{K-}m_{K+}$) *sup*E44 *thi*-1 *rec*A1 *gyr*A (Nal$_r$) *rel*A1 Δ(*lacZYA-arg*F)$_{U169}$ (φ*80lacZ*Δ*M15*).

***Escherichia coli* HB101** (Described by Boyer and Roulland-Dussoix, 1969)

Genotype of the strain F-*(gpt-proA) 62 leuB6 supE44 ara-14 galK2 lacY1* Δ*(mcrC-mrr) rpsL20 (Str$_r$) xyl-5 mtl-1 recA13*.

Cell Lines

CV1 (ATCC CCL-70)

This cell line was raised from the kidney epithelial cells of African green monkey (*Ceropithecus aethiopis*). The cells grow in an anchorage-dependent manner forming monolayer, show contact inhibition, and exhibit morphology similar to fibroblasts. They were grown in DMEM containing 10% FBS, 1% pen/strep solution and 1 mM HEPES pH 7.5 in the humidified incubator at 5% CO_2. Their utility in NHR research lies in the fact that they lack in endogenous NHR expression, ideal to find out the functions of not only ectopically expressed AR.

LNCaP (ATCC CRL1740)

The cell line was derived from a lymph node metastasis biopsy conducted on a CaP patient. They grow in an androgen-dependent manner and express endogenous mutant form of AR. Cells do not show contact inhibition, and therefore tend to form foci. Cells are grown in RPMI (Roswell Park Memorial Institute) media containing 10% FBS, 1% pen/strep., 1% Sodium pyruvate solution and 1 mM HEPES pH 7.5 in the humidified incubator at 5% CO_2 (Procured from Dr. J. Klug, Giessen).

C4-2

This cell line was derived from parent cell line LNCaP after their painstaking long-term

culture without androgens and therefore they show an androgen-independent phenotype. They grow faster than the parent cell line and do not show growth inhibition by antagonist Cas. They do not show contact inhibition and can form foci. These cells require a growth factor enriched media called "T-media" which is modified from DMEM and contains in addition 10% FBS, 20% F-12 media, 5 μg/ml insulin, 13.6 pg/ml T3, 5 μg/ml apotransferrin, 0.25 μg/ml biotin, 25 μg/ml adenine and 1% pen/strep, 1 mM HEPES pH 7.5. Cells are grown at 37 °C in the humidified incubator at 5% CO_2. (Cells were kindly by Dr. G. Thalmann, Switzerland)

PC3-wt AR

PC3-wt AR cells were derived from PC3 CaP cells. Unlike PC3 cells, these cells express functional AR which has been stably integrated. They are grown in DMEM (Dulbecco's Modified Eagle's Media) containing 10% FBS, 1% pen/strep solution and 1 mM HEPES pH 7.5 in the humidified incubator at 5% CO_2. (Procured from Andrew Cato)

3.10 Plasmids

The following plasmids were used for the work undertaken

Name of the plasmid	**Source/Reference**
Reporter plasmids	
1. pARR3-Luc	R. J. Matusik, Nashville, TN
2. pCMV-LacZ	U. Deutsch, Bad Nauheim
3. pMMTV-Luc	Gast et al., 1998
4. pPSA-Luc	Cleutjens et al., 1997
5. p$(UAS)_4$-TATA-Luc	Frauke Goeman, AG Baniahmad
6. pPSCA-2.7-3.0	Robert Reiter, US
7. pPSCA-TATA	Robert Reiter, US
Control Plasmids	
1. pSG5	Stratagene
2. pcDNA3	Invitrogen
3. pABΔgal	Baniahmad et al., (1990)
4. pCMX-VP16	Ronald Evans

Eukaryotic expression plasmids

1. pSG5-AR	Gast et al., 1998
2. pSG5-hAR-T877A	M. Asim, AG Baniahmad
3. pSG5-hAR-K385R/K518R	M. Asim, AG Baniahmad
4. pVP16-cSMRT	K. Busch, M. Muller
5. pVP16-cLCoR	M. Patz, AG Baniahmad
6. pSG5-LCoR	John White, Canada
7. pSG5-LCoR-mut	John White, Canada
8. pCMX-VP16-Alienα	Udo Moehren, AG Baniahamd
9. pABgal$_{94}$-LCoR	M. Asim, AG Baniahmad
10. pCS2+MT	M. Schulz (Rupp *et al.*, 1994)
11. pCS2+MT-LCoR	M. Asim, AG Baniahmad
12. pAB-VP-ARDBD	M. Asim, AG Baniahmad
13. p5HB-AR-K385E/K518E	J.A. Iniguez-Lluhi
14. pABΔgal-Trip15/Alien	U. Dressel, AG Baniahmad
15. pSG5-AR-ΔHBD	H. Dotzlaw, AG Baniahmad
16. pSG5-AR-ΔNT	Dotzlaw et al., 2002
17. pSG5-AR-Δ39−171	Dotzlaw et al., 2002
18. pSG5-AR-Δ39−328	Dotzlaw et al., 2002
19. pSG5-AR-Δ1−171	Dotzlaw et al., 2002
20. pSG5-AR-Δ1−328	Dotzlaw et al., 2002
21. pSG5-AR-1-328	Dotzlaw et al., 2002
22. pSG5-AR-1-505	Dotzlaw et al., 2002
18. pETE-Hyg	Protopopov, 2002
19. pABgal$_{94}$-Alien	Dressel et al., 1999

4 Methods

4.1 Working with DNA

Storage

All experiments were carried out in sterile environment. Solutions were prepared using double distilled milli Q water as stocks. Solutions were further diluted according to the requirement by making working dilutions. It is recommended to store all Plasmid DNA at –20 °C in TE buffer (Ausubel et al., 1989). The DNA was dissolved in either TE buffer or preferably in ddH_2O.

TE Buffer	
Tris-HCl (P^H 7.5)	10 mM
EDTA (P^H 8.0)	1 mM
Filter sterilised	

4.1.1 Minipreparation of plasmid DNA

TELT method

TELT method was first described by Holmes and Quigley in 1981. Modified by Wilmzig in 1985. The advantage of this method lies in the instant recovery of the plasmid DNA. This method is particularly useful in analytical preparation of the plasmid DNA e.g., for purpose of restriction digestion etc. in generation of plasmid vectors.

LB Medium	
Bacto tryptone	10 g/l
Yeast Extract	5 g/l
NaCl	5 g/l
Autoclaved	

TELT Buffer	
Tris-HCl (P^H 7.5)	50 mM
EDTA	62.5 mM
Triton X-100	0.4 % (v/v)
LiCl	2.5 M
Stored at 4 °C.	

In this method, 1.5 ml of overnight LB-grown bacterial culture was used for the isolation of plasmid DNA. Culture was transferred to a 1.5 ml reaction tube and centrifuged for 3 minutes at 4000 rpm, in Hettich microlitre lab centrifuge. The supernatant was vacuum discarded and the pellet was resuspended in 200 µl of TELT buffer by vortexing and 20 µl

of lysozyme solution (10 mg/ml in ddH_2O). The probes were quickly incubated at 100 °C for 1 min for cell lysis, making solution viscous. Probes were taken out and vigorously shaken manually and then incubated on ice for 10 min. Then probes were centrifuged at 13.000 for 10 min and the pellet was discarded. Supernatant was added with 2 volumes of absolute ethanol and the probes were vortexed followed by incubation at RT for 15 min to precipitate DNA. Again, the probes were centrifuged at 13.000 rpm for 20 min Supernatant was vacuum discarded and the plasmid DNA pellet was twice washed with 1 vol. of 70% ethanol. Ethanol free DNA pellet was dissolved in 30 µl of TE buffer and left at RT for at least 45 min.

In order to carry out restriction analysis, 5 µl of this DNA solution is sufficient. This method is fast but leaves at the end much RNA intact forming smear on gel. Thus, to avoid RNA smear, probes should be mix with gel loading buffer containing RNase A (concentration 0.5 mg/ml) for 10 min before loading to the gel. This remains the method of choice for HB101 strain of E. coli.

Alkaline Lysis method

Devised by Birnboim and Doly (1979) this method of plasmid DNA extraction was started with 1.5 ml of overnight grown bacterial culture in LB under selective antibiotics. Cells were pelleted by centrifuging the culture at 13000 rpm for 1 min in Hettlich table top centrifuge.

Sol I	
Tris-HCl (P^H 8.0)	25 mM
EDTA	10 mM
Glucose	50 mM
Autoclaved, refrigerated	

Sol II	
NaOH	200 mM
SDS	1 % (w/v)
Fresh prepared	

Sol III	
CH_3COOK	3 M
CH_3COOH	115 ml/l
Refrigerated	

The cell pellet was resuspended by vortexing in 100 µl of sol I. This was followed by incubation with 200 µl of sol II at RT, followed by immediately inverting the 1.5 ml reaction tube 3 times to uniformly mix the solution. This solution, not only lyses the cells but leaves genomic DNA entangled in a dense mass which can later on be removed by centrifugation, leaving plasmid DNA in solution. The lysis reaction was then stopped by

adding 150 µl of sol III, and after vortexing, the probes were kept at -20 °C for 5 min and were again spun at 13.000 rpm for 5 min to sediment the heavier mass of genome DNA and residual proteins. The supernatant was again transferred to a fresh 1.5 ml reaction tube and spun once more at 13.000 rpm for 5 min and again transferred into a fresh 1.5 ml reaction tube. Plasmid DNA was precipitated by adding 1 ml of absolute ethanol and vortexing briefly. The probes were incubated at RT for 5 min and centrifuged at 13.000 rpm for 5 min Supernatant was discarded and plasmid DNA was washed with 70% ethanol. The probes were again centrifuged at 13.000 rpm, and air-dried for a short time after discarding the supernatant; the probes were dissolved in 30 µl of TE buffer.

4.1.2 Maxipreparation of plasmid DNA

CsCl method

This method, modification of alkaline lysis method described by Holmes and Quigley (1981), is particularly useful for making large amount of ultra pure plasmid DNA (1-10 mg) free of genomic DNA as well as RNA. Use of CsCl gets rid of RNA impurities by creating a density gradient (Radloff et al., 1967).

Sol I	
Tris-HCl (P^H 8.0)	25 mM
EDTA	10 mM
Glucose	50 mM
Autoclaved	
Added 4 mg/ml lysozyme just before use	

Sol II	
NaOH	200 mM
SDS	1 % (w/v)
Fresh prepared	

Sol III	
CH_3COOK	3 M
CH_3COOH	115 ml/l

TB Medium	
Bacto tryptone	12 g/l
Yeast Extract	24 g/l
Glycerine	4 ml/l
Autoclaved	

10x Phosphate Buffer	
KH_2PO_4	170 mM
K_2HPO_4	720 mM
Autoclaved	
1/10 vol to be added to TB media	

To start with 3 ml of overnight LB grown bacterial “pre culture” was added to the 400 ml of TB (360 ml TB media + 40 ml phosphate buffer) media containing appropriate antibiotics. The culture was grown for 8-9 hrs in the incubator shaker at 37 °C with vigorous shaking after which, growth of the bacteria was stopped by adding 2 ml of

chloramphenicol (34 mg/ml in ethanol), which arrests the bacterial growth by stopping synthesis of new proteins so the energy can more efficiently be used for the amplification of plasmid DNA and therefore, this step is often referred as “plasmid amplification”. Overnight incubation with chloramphenicol was followed by the harvesting of bacterial culture by centrifuging at 4000 rpm for 10 min at 4 °C (Beckmann JA10 rotor). The pellet was resuspended in 10 ml of sol I containing 4 mg/ml lysozyme avoiding bubbles to make a homogenous paste and then allowed to stand at RT for 5 min Lysis follows by the addition of 20 ml of freshly prepared sol II and then inverting the tube 5 times and let it incubated on ice for 10 min That caused cell lysis and the reaction was then stopped by adding 15 ml of sol III which was mixed well by inverting and kept on ice for 10 min. The probes were centrifuged for 15 min at 6000 rpm at 4 °C (Beckmann JA10. The supernatant from each probe was divided into 2 falcons of 50 ml each by filtering through muslin cloth to avoid impurities. Now the DNA was precipitated by adding 0.6 volume of isopropanol which selectively precipitates DNA, mixed by vortexing and centrifuged at 5800 rpm for 15 min at RT (Heraeus minicentrifuge). The DNA pellet obtained was dissolved in 1.5 ml of TE buffer in water bath at 37 °C. After proper dissolution the samples were re united and 4.5 gms of CsCl was added and then dissolved by again keeping in water bath at 37 °C. To each sample, 0.5 ml of ethidium bromide (10 mg/ml) solution was added followed by centrifugation for 10 min, 6000 rpm at RT in Heraeus centrifuged. The supernatant was then collected in Beckman-*Quick-Seal* ^{TM} ultracentrifuge tube was filled to the graduated point using 50% w/v of CsCl solution. The properly weighed tubes were sealed by the thermal sealer and were centrifuged at 55.000 rpm overnight at 20 °C using Beckman XL70 ultracentrifuge with rotor VTi 90. The upper plasmid DNA band, and lower RNA band and the impurities on the wall of the tube were visible. The tube was carefully punctured from the side wall using a needle and the plasmid DNA band was collected with a syringe slowly to avoid nicks in the DNA. The DNA band was transferred to a new Quick seal tube which was filled with 50% w/v CsCl. After proper sealing the tubes were again centrifuged at 70.000 rpm for 3 1/2 hrs at 20 °C to remove EtBr. Plasmid DNA was taken in a fresh 15 ml falcon and 2 ml of CsCl-saturated isopropanol was vortex-mixed. Out of two visible phases, the upper red phase containing EtBr was discarded, and to the lower phase with plasmid DNA, 2 ml CsCl-saturated isopropanol was mixed and discarded unless the lower phase becomes completely colorless. This step was repeated once again for 2 more times to remove any traces of EtBr left. To the plasmid DNA added 2 volumes of milli Q water followed by addition 0.6 volume of isopropanol, mixed well and incubated

at RT for 10-15 min It was centrifuged for 20 min, RT, 6000 rpm, Heraeus minifuge a 4 °C. The pellet was washed with 5 ml 70% EtOH and dried. Finally, the DNA dissolved in 500-700 µl of TE or milli Q water depending on the rough yield.

Maxiprep protocol (Marligen kit-modified)

A typical yield of 1-4 mg DNA is desired. The culture was started as 3 ml overnight grown preculture to inoculate 400 ml LB media containing suitable antibiotic in appropriate concentration and grown overnight. Cells were centrifuged at 4000 rpm for 10 min at 4 °C the supernatant was discarded and cells were homogenized in 10 ml of solution E1 followed by lysed with 10 ml of E2 at RT for 5 min E2 must be gently mixed by inverting the tube 5 times. 10 ml of neutralization buffer E3 was added to avoid excessive lysis by inverting the tube 5 times. The lysate was centrifuged and the supernatant containing the plasmid DNA was loaded sieving through muslin cloth on the affinity column which was pre equilibrated using 10 ml of E4 equilibration buffer. The column was washed once with 60 ml wash buffer E5 to remove the impurities. The DNA was finally eluted in 15 ml of elution buffer E6. To the eluted solution 10.5 ml of isopropanol was added, mixed by brief vortexing and centrifuged at 4000 rpm for 30 min at 4 °C. The DNA pellet obtained was washed with 5 ml of 70% ethanol and centrifuged again at 4000 rpm for 5 min at 4 °C. The pellet obtained was air-dried and subsequently dissolved in appropriate volume of ddH_2O.

4.1.3 Midiprep protocol (Marligen-kit modified)

The midiprep is usually carried out when only moderate amounts (1-2 µg) of highly pure DNA is required as for sequencing etc.

The culture was started with 3 ml overnight grown preculture to inoculate 100 ml LB media containing suitable antibiotic in appropriate concentration and grown overnight. Cells were centrifuged at 4000 rpm for 10 min at 4 °C. Supernatant was discarded and cells were homogenized in 4 ml of solution E1 followed by lysis with 4 ml of E2 and gently mixed at RT for 5 min 4 ml of neutralization buffer E3 was then gently mixed. The lysate was centrifuged and the supernatant containing the plasmid DNA was filtered through muslin cloth on the affinity column which has been pre equilibrated using 10 ml of E4 equilibration buffer. The column was washed twice with 10 ml wash buffer E5 to remove impurities. The DNA was finally eluted in 5 ml of elution buffer E6. To the eluted solution containing DNA, 3.5 ml of isopropanol was added, mixed by brief vortexing and

centrifuged at 4000 rpm for 30 min at 4 °C. The DNA pellet obtained was washed with 3 ml of 70% ethanol and centrifuged again at 4000 rpm for 5 min at 4 °C. The pellet obtained was air-dried and subsequently dissolved in appropriate volume of ddH_2O. A typical yield of 100-200 µg DNA was achievable.

4.1.4 Determination of DNA concentration

Each DNA molecule contains conjugated double bonds in sugar atoms which allows the absorption of ultra-violet spectrum of light and forms therefore the basis for the spectrophotometric determination of DNA concentrated (Ausubel et al., 1989). Briefly, the DNA was diluted in TE, and measured spectrophotometrically at wave-lengths of 260 nm and 280 nm. The following formula was employed for determining the concentration.

Concentration of dsDNA = OD_{260} x dilution factor x 50 = in µg/ml
Purity of DNA = $OD_{260}/OD_{280} \geq 1.8$

Abs. constant for dsDNA is 50, for ssDNA which absorbs more light compared to same quantity of dsDNA this value is 40. The purity of the DNA sample can be checked by the ratio of abs. values at 260/280 nm. For pure DNA sample the value should stand 1.8. Values less than 1.8 indicate the contamination by proteins.

4.1.5 Gel electrophoresis of DNA

A sea weed product, agarose is a natural carbohydrate polymer which forms a matrix and frequently used to resolve the negatively charged DNA fragments under the electric field based on their size (Southern, 1979) with the smaller fragments running faster than the bigger ones. The degree of resolution depends on the size of the lattice formed by the agarose upon solidification. High concentration agarose forms smaller pores and are therefore resolving fragments with very little difference in size, however low percentage (w/v) of agarose that forms lattice with bigger pores and useful for resolving fragments with significant difference in size. There are two kinds of gels which have here been used.

Analytical agarose gel

Agarose gels with varying percentage of agarose were prepared with 0.8-1.5% of agarose (Johnson and Grossman, 1977; Southern, 1979). The required quantity of agarose powder was dissolved in the 0.5x TBE containing 0.2 µg/ml EtBr, by boiling in a microwave oven. Once cooled to 55 °C the solution was poured in a gel chamber containing appropriate size combs for casting the wells for loading the DNA samples.

10x TBE Stock	
Tris-HCL (P^H 8.3)	0.9 M
Boric Acid	0.9 M
EDTA	20 mM

10x Gel Loading Dye	
Ficoll 400	40 %(w/v)
Bromophenol blue	0.05 %(w/v)
Xylene cyanol	0.05 %(w/v)

Ethidium bromide (EtBr) Stock Solution
EtBr 10 mg/ml in ddH_2O

The DNA to be tested in gel was dissolved in water or TE. To the appropriate DNA volume, 10x gel loading dye was mixed and was loaded in the well along with 0.5 µg DNA standard markers to be run parallely. The electric field of 10 volts/cm was applied to separate the fragments obtained by restriction digestion of a plasmid DNA (for fragments >2kBp 5V/cm). After completion of the run the fragments of the DNA were visualized on the UV trans-illuminator (254 nm) and photographed by Polaroid camera or digital gel documentation system.

Preparatory agarose gel

50x TAE	
Tris	2 M
EDTA	0.5 M
P^H adjusted to 7.7 with acetic acid	

The gel electrophoresis with aim to obtain DNA for further experimentation, especially cloning fragments was carried out in gels made out of another derivative of agarose having low melting point (LMP agarose). For that purpose generally 1% LMP agarose gels are

common in use. The LMP agarose was dissolved in 1x TAE buffer (containing 0.5 µg/ml EtBr) to form a gel (Blin et al., 1975) and fragments were separated for ligation. The electrophoresis was carried out at 4 °C with 4 volts/cm potential difference. After 1 hr running the gel was visualized on a UV trans-illuminator with 365 nm wavelength. The fragment was cut out of the gel, weighed and diluted with 4 volumes of ddH_2O. For dissolution the probe was incubated in water bath at 65 °C for 10 min with mixing in between at RT and was stored for future use at -20 °C.

4.1.6 Cleavage of DNA using restriction enzymes

Restriction enzymes isolated from prokaryotes have become molecular scissors for routine research in molecular biology. Type II restriction enzymes, also called restriction endonucleases are the most common among them. They recognize a specific sequence in the DNA which is most often a palindrome, and break the phosphodiester bond, linking two nucleotide entities together. Depending on the exact site of cleavage they either generate overhangs which being sticky in nature with affinity towards the complementary sequence therefore preferred in cloning. Blunt ends, however are generated when the enzyme cut on both strands at the same site (Ausubel et al., 1989).
The enzyme activity is shown in units (U), with 1U representing the amount required to digest 1 µg of λ-DNA in 1 hr at 37 °C. Generally for analytical digestion of DNA 1 U suffices for 5 µl of miniprep DNA or 1 µg of DNA, however, when the purpose is clone a restriction fragment use of more units can be recommended in order to restrict every single plasmid molecule, which otherwise will hinder the selection of the right clone afterwards.

4.1.7 Dephosphorylation of DNA ends

The restriction fragment to be subcloned into a vector contains a 5′ phosphate group generated as a result of restriction acts as a substrate for ligation also with its own 3′ hydroxyl group, which may severely decrease the efficiency of ligation. Therefore, in order to prevent the self intra-molecular ligation the 5′ phosphate group was enzymatically removed by incubating the DNA fragment with 5 U of calf intestinal alkaline phosphatase (CIAP) at 37 °C for 30 minutes. This decreases the probability of intra molecular ligation and enhances the occurrence of intermolecular ligation between fragment to be cloned and the vector.

4.1.8 Filling of 5´ recessive termini and trimming of 3´ overhangs

In order to generate compatible ends for ligation, the protruding ends at the 5´ were filled by Klenow fragment to generate blunt ends. Klenow uses the 3´ OH group as primer and extends it 5´→3´direection adding nucleotides complementary to the opposite strand (Tabor and Richardson, 1987). This reaction uses dNTPs (dATP, dCTP, dGTP, dTTP; 5 mM solution) as substrate and requires incubation at 37 °C for 30 min. Another situation which prerequisites the presence of blunt end at a 3´ overhang site involves the trimming of protruding ss nucleotides by 3`→5` exonuclease activity of T_4-DNA polymerase.

4.1.9 Ligation

10x Ligase Buffer	
Tris-HCl P^H 7.8	400 mM
$MgCl_2$	100 mM
DTT	100 mM
ATP	5 mM

To carry out sticky-sticky ends ligation between two DNA molecules, a vector to insert ration 1:2 to 1:3 was used however, for blunt-blunt end ligation a higher vector to insert ration should be used. e.g., 1:4-1:5. The ligation was carried out in a total reaction volume of 30 µl with 3 µl T_4-DNA ligase buffer and 3 to 10 units of the ligase depending on the ends of the DNA. Mixture was kept overnight in dark at RT for overnight. Later on 15 µl of the reaction mixture was used to transform competent E. coli cells.

4.1.10 The Polymerase Chain Reaction

The method to amplify a DNA fragment or molecule became automated with the discovery of the bacteria *Thermus aquaticus* which grows in the hot springs of the Yellowstone national park in the US. To amplify the given fragment of DNA, PCR is almost always *in vitro* method of choice. It utilizes two spanning primers for the exponential increase in the DNA amount. The key to the reaction is a thermo stable DNA polymerase which can carry out many cycles of addition of nucleotide to the existing DNA strand at elevated temperatures without being inactivated.

PCR is also used to quantify the amount of DNA precipitated by an antibody directed to a DNA binding protein and hence indicates the strength of *in vivo* recruitment of a protein in question on the DNA fragment amplified.

Component	**Volume [µl]**	**Final Concentration**
10 x Buffer	3.5	1x
10 mM dNTPs	0.7	0.2 mM each
50 mM $MgCl_2$	1.050	1.5 mM
100 mM Fwd primer	0.175	0.5 µM
100 mM Rev. primer	0.175	0.5 µM
Taq-Pol (5 U/µl)	0.175	
H_2O	24.225	
DNA	5.00	
	Σ 35.000	

In order to avoid pipetting errors a master mix containing the above-mentioned components and DNA was prepared and individually added to each of the sample.

PCR employing thermal cycler involves repeated cycles of denaturation, annealing and extension which were programmed as shown below

Initial denaturation	94 °C (hot start PCR)	
Denaturation	94 °C for 45 sec.	30 to 39 cycles
Annealing	depending on the T_m of primer (Generally chosen T_m + 5 to 10 °C)	
Extension	72 °C, Approx. 90 sec. (depending on length of DNA)	
Final extension	72 °C for 10 min	
Cooling	4 °C	

It is noted that the PCR was carried out in “Hot start” mode by initially denaturing the DNA at 94 °C for 3 min. The primary advantage of hot start PCR is that the product obtained are of high specificity as a result of precise annealing of the primers at correct locations. Therefore it avoids amplification of any non-specific DNA fragment.

4.2 Working with RNA

Working with RNA is more demanding than working with DNA, because of the chemical instability of the RNA and the ubiquitous presence of RNases: Unlike DNases, RNases do not need metal ion co-factors and can maintain activity even after prolonged boiling or autoclaving. Therefore special precautions were taken when working with RNA. It is therefore recommended to allocate a special area for RNA work only.

4.2.1 Storage of RNA

RNA was aliquoted in ethanol or isopropanol and stored at -70 °C. Most RNA molecules are relatively stable at this temperature. Ethanol or isopropanol were discarded by centrifugation and the RNA pellet was resuspended in appropriate amounts of RNase-free water. As for DNA, RNA sample should always be kept on ice when preparing an experiment.

4.2.2 Extraction of RNA from eukaryotic cells

The extraction was carried out by the modified method from Chomczynski and Sacchi (1987) for the isolation of highly pure RNA from the eukaryotic cells. Cells were washed first with PBS. 1 ml of Trifast® reagent (a metaphasic solution of phenol and guanidium iso thio cyanate) was added to each of the six well on cell culture plate. The cells were gently resuspended 5-6 times with blue microtip after which it was incubated for 5 min at RT. 0.2 ml of chloroform was then added and the samples were shaken vigorously by hand for 15 sec followed by RT incubation for 3-10 min the samples were centrifuged at 12.000 rpm for 5 min Out of two phases formed, upper aqueous phase was taken into a fresh 1.5 ml reaction tube and vortex-mixed with 0.5 ml isopropanol. Samples were incubated on ice for 10-15 min and centrifuged at 12.000 rpm for 30 min the supernatant was discarded and the pellet was washed with 1 ml of 75% ethanol by vortexing and centrifugation for 8 min at 4 °C. The pellet was air-dried for 20 sec by letting the lid open and then dissolved in appropriate amounts of RNase free water and the concentration of RNA was measured by nanodrop® instrument. Alternatively concentration measurement was done by conventional UV-Vis spectrophotometer with the abs. constant 40 for RNA.

Conc. of RNA = OD_{260} x dilution factor x 40 = µg/ml

4.2.3 cDNA synthesis and Real-Time PCR

The RNA pellet obtained from a single well of the six well plate, was dissolved in ~25 µl of water. 2 µg RNA was dissolved in final 8 µl volume of water and mixed with 4 µl of

Mastermix	
First strand buffer	4 µl
DTT, 0.1 M	2 µl
dNTP 10 mM	1 µl

primer mix (2 µl oligo + 2 µl random) and kept at 70 °C for 15 min before leaving on ice for 1 min followed by addition of 7 µl master mix with gentle mixing and brief centrifugation. Further, incubation first at 25 °C for 10 min and 42 °C for 2 min was completed. To the reaction mixture 1 µl of Superscript II was added and again incubated at 42 °C for 55 min and later cooled to 4 °C. The prepared cDNA was stored at -20 °C. To carry out real time PCR, 8 µl of SYBR green (Invitrogen) was taken in light cycler capillaries and 2 µl of the cDNA was added. The capillaries were covered and briefly centrifuged to bring the liquid to bottom. These capillaries were then transferred to light cycler for real time transcript analysis.

4.3 Working with Bacteria

4.3.1 Bacterial transformation

Transformation of HB101/DH5α Strains

50-100 µl (depending on degree of competence) competent cells were thawn on ice. 0.5 µg of plasmid DNA was carefully mixed by very gentle pipetting. DNA and cells were Incubated on ice for 30 min Heat shock was given at 37 °C water bath for 2 min followed by addition of 1 ml cold LB and cells were incubated at 37 °C for an hr and centrifuged at 3000 rpm for 5 min 1 ml of the supernatant was discarded and rest plated on amp-agar plate and incubated at 37 °C incubator overnight. Bacterial colonies were observed 16 hrs later.

4.3.2 Preparation of competent cells

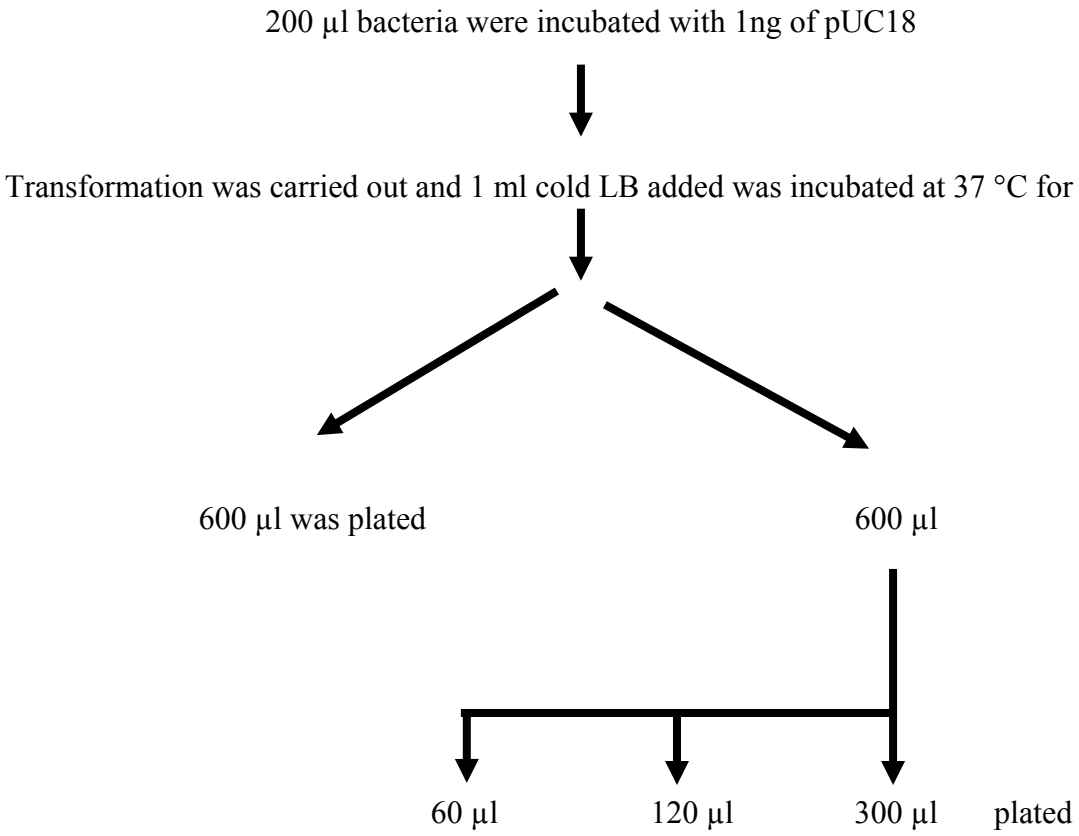
LB Media	
Bacto tryptone	10g/l
Yeast extract	5 g/l
NaCl	10g/l
P^H adjusted to 7.5 and autoclaved	

TB Buffer	
PIPES	10 mM
$CaCl_2$	15 mM
KCl	250 mM
$MnCl_2$	55 mM
P^H adjusted to 6.7 with KOH	

This is modified method from Inoue et al., 1990. In 25 ml LB culture (containing 50 µg/ml streptomycin) a single colony from agar plate was inoculated and incubated at 37 °C with shaking till the OD_{600} reaches 0.07 to 0.1. (This usually takes overnight). Next day 0.5 ml culture was inoculated in 400 ml LB media and grown at 18 °C till OD_{600} reached 0.6. The culture was equally divided into two centrifuge tubes and incubated on ice for 10 min. The culture was then centrifuged at 4800 rpm for 10 min at 4 °C. The pellet was resuspended in 40 ml TB buffer followed by incubation on ice for 10 min. The culture was centrifuged at 3200 rpm for 10 min at 4 °C. The pellet was homogeneously resuspended in 10 ml of DMSO mix (9.3 ml cold TB + 0.7 ml DMSO, RT) and kept at 4 °C for 10 min and finally divided into 0.2 ml aliquots and immediately transferred to liquid nitrogen. The can be stored for long term at -80 °C.

4.3.3 Determining degree of competence

The high degree of competence is an essential prerequisite to find the correct clone out of a ligation mixture. Good competent cells have degree of competence between $1x10^7$-$1x\ 10^8$. Colony number counted next day. The degree of competence can be calculated as follows 10^7 degree is considered good and denotes the amount of colony to be formed for each µg of the pUC18 or pBluescript DNA transformed. The degree of competence was checked with the help of following flow-chart. 60 µl has 5 pg of DNA transformed and should in produce 100 colonies to achieve 10^7 degree of competence.



4.3.4 Preparation of glycerol stock

Bacterial cells were stored for long-term use at -80 °C by preserving in glycerol which was used as a cryoprotectant. (Ausubel *et al.,* 1989)

Agar Plates	
Bacto tryptone	10 g/l
Yeast extract	5 g/l
NaCl	10 g/l
Agar	15 g/l
P^H adjusted to 7.5 and autoclaved	

Glycerol Stock	
Bacterial suspension	10%
Glycerol	40%
LB media	60%
Stored at -80 °C	

For instant availability, bacteria were preserved at 4 °C in agar in Petri plate containing appropriate concentration of antibiotics.

4.4 Culture and transfection of mammalian cells

Cell growth conditions were already standardized. All cell culture work was performed in cell culture facility.

The eukaryotic cells used in the study grow in an anchorage-dependent manner however CaP cell lines do not exhibit contact inhibition and therefore can grow into clustered if left unpassaged. Cells were generally maintained in 145 diameter maxi dishes with 15 ml of appropriate media. Depending on their growth behaviour the cells were splitted one to three times per week. In order to split the cells, the media was first aspirated off and then cells were washed with 1x PBS. The PBS was also aspirated off and 2 ml trypsin (invitrogen) was added to cells from the side of the dish. The cells were incubated for 2-4 min at 37 °C dependent on the strength of adherence of cells. The cells were mixed with 10 of culture media and gently mixed and divided in a new dish, high or low depending on their use. The cells were transferred to 5% CO_2 incubator for growth.

Cell line	**Passage**
CV1	thrice weekly
PC3-wtAR	Once weekly
C4-2	twice weekly
LNCaP	Once weekly

4.4.1 Preparation of T-media

Ingredients	**quantity**	**Final concentration**
DMEM (1g/l glc)	400 ml	80%
F-12	100 ml	20%
Supplements		
Insulin	1 ml of 2.5 mg/ml	5 μg/ml
T_3	1 ml of 6.825 ng/ml	13.6 pg/ml
Apo-transferrin	1 ml of 2.5 mg/ml	5 μg/ml
Biotin	1 ml of 0.125 mg/ml	0.25 μg/ml
Adenine	1 ml of 12.5 mg/ml	25 μg/ml
Penicillin/ streptomycin	5 ml	100 U/l; 100 μg/ml
FBS	50 ml	10%
HEPES, pH 7.5	12.5 ml of 1 M	1 mM

4.4.2 Colony formation assay

The assay carries a goal to test the effect of overexpression of the protein in question on the abilities of cells to form colonies in a cell culture dish. The assay was carried out in 10 cm cell culture dish. 0.5 Million C4-2 cells were grown for at least 48 hrs before transfection. The cells were transfected using calcium phosphate method with a total of 10 μg of DNA expressing protein of interest along with the expression vector providing the ability to

transfected cells to grow in antibiotic hygromycin thereby selecting transfected cells. Non-transfected cells eventually die in a couple of weeks. The transfected cells were washed after 24 hrs and the media was changed. 24 hours later, cells were treated with hygromycin, hormones and inhibitors. The cells were washed with 1x PBS and the media was changed twice a week. After death of all non-transfected cells (2 weeks), the transformed clones overexpressing the protein started growing to form colonies. They were further allowed to grow for 6-8 weeks. The number of colonies obtained was counted and the cells were trypsinised and frozen at that point for future usage.

4.4.3 Preparation of hormone-depleted serum

For LNCaP and C4-2 cells
500 ml thawn FBS was mixed with 25 gms of active charcoal and mixed on a magnetic stirrer for 1 hr at RT. The charcoal was pelleted by centrifuging at 7500 rpm for 15-30 min and repeated in a new tube. The FBS was filtered with 0.45 μ filter to avoid charcoal particles followed by sterile filtering using 0.22 μ filter and stored at -20 °C.
For CV1 the FBS was first heat inactivated by incubating at 56 °C for 30 min.

4.4.4 Calcium phosphate-based transfection method

The calcium phosphate protocol (Wigler et al., 1978) was used to analyse both transient and stable expression of genes. In this protocol a calcium phosphate/DNA precipitate was formed which adheres to the surface of adherent cells and was taken up by the cells via a poor defined endocytosis.

A total of 216 μl of DNA-transfection mix per well of a six well plate

DNA-transfection mix:

Scheme for transfection for each of Six well		
H_2O/DNA	183.6 μl	Make the Total DNA concentration 5.4 μg using Calf thymus DNA
10xHEBS	21.6 μl	Vortex mix
2 M $CaCl_2$	10.8 μl	Vortexed for 10 seconds, 10-20 min RT, Vortex and add to cells

Calcium Chloride Solution
$CaCl_2$ 2 M
Filter sterilized and stored at -20 °C.

Calculated amount of water was taken and 1 to 5.4 μg of plasmid DNA was dissolved in water and added 21.6 μl of 10x HEBS. Vortexed and added 10.8 μl of 2M $CaCl_2$ and incubated for 15 min at RT for crystal formation. The mix was directly pipetted. The media was changed after 16 hrs and cells were incubated for another 24 to 72 hrs depending on the requirement of the experiment.

4.4.5 DOTAP Transfection protocol

Use of HEPES buffer free media is recommended

Cells were seeded out in normal media in one well of a six-well plate (with antibiotics and serum) and allowed to reach 60 to 70% level of confluence. 2.5 μg of DNA was diluted and a media free of antibiotics and serum to a final concentration of 0.1 μg/μl.
Pipetted 16 μl DOTAP in a fresh sterile polystyrene tube and adjusted the volume to 50 μl by the addition of media free of antibiotics and serum. DNA solution was mixed gently with the DOTAP mix and incubated at RT for 10-15 min. The solution was carefully transferred to cells grown in a media containing serum free of antibiotics.

4.4.6 Determining the transfection efficiency

In any kind of transfection the goal is to achieve the maximal possible uptake of DNA by as many cells as possible. It is required to test especially the effect of ectopically expressed protein on the test system. Many methods are in use. The method used during the work was tested for their transfection efficiency. Transfection efficiency is routinely tested by employing an expression plasmid coding for peGFP (enhanced green fluorescent protein) inside the cells. 16 hrs after Transfection with the methods to be evaluated cells were washed with PBS and media was changed. 72 hrs later cells were visualised under the phase contrast microscope to visualize the cells giving peGFP signal in the form of emitting green fluorescent light. For C4-2 cells, it turns out that at least 4-5 times better

transfection efficiency (~18-20%) can be achieved with calcium phosphate method compared to DOTAP Transfection protocol. It is also observed that the kind of serum, normal or depleted, had no effect on the transfection efficiency.

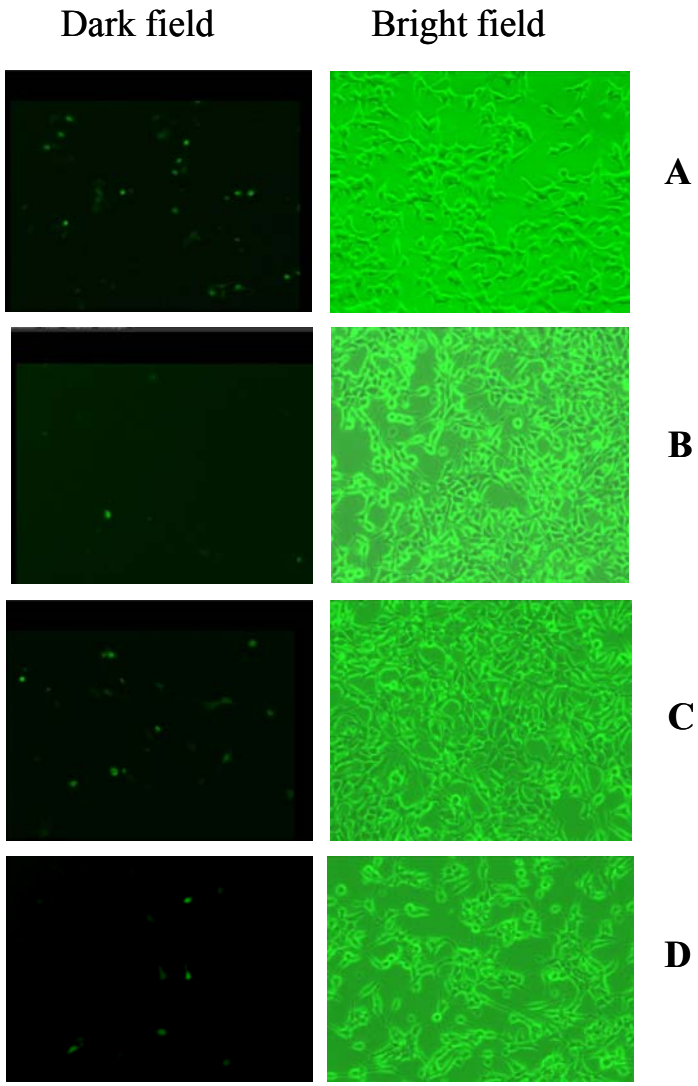


Fig. 4.1 Determination of transfection efficiency using GFP. Photomicrographs of C4-2 cells transfected with eGFP. The microscopic images show the relative efficiency of the two commonly used Transfection methods for C4-2 cells. 2 µg of peGFP plasmid for each well of a six-well plate was transfected. On right side cells showing green emission represent those which were transfected. Panel A and B represent cells transfected using media containing normal serum with Calcium phosphate and DOTAP method respectively. Panel C and D represent the cells transfected in media containing stripped serum using calcium phosphate method and DOTAP method respectively.

4.4.7 Cryopreservation of mammalian cells

Cryopreservation Media	
FBS	40%
DMSO	15%
Media	45%

Cells were trypsinised and 10 ml normal media added. Cells were then transferred into a fresh 15 ml falcon and centrifuged to spin down. After centrifugation, the media was aspirated off and the cell pellet was resuspended in 1 ml of normal media by gentle shaking. Then, 1 ml of the cryo media was added and mixed by inverting. Out of this 2 ml, 1.8 ml media containing cells were transferred to the cryovial which was then kept in a small cooler used to gradually cool down the cells to avoid cold damage. The cooler was transferred at least for a couple of day to the –80 °C after which the cryovials were transferred to liquid nitrogen tank.

4.4.8 Reviving mammalian cells

The cells stored in cryovial were taken out in a 15 ml falcon and 10 ml media was added. After gentle mixing cells were briefly centrifuged, supernatant media was discarded and the cell pellet was resuspended in 10 ml of fresh media and transferred to a maxi cell culture dish in total 15 ml volume and transferred to incubator.

4.4.9 Cell counting using Neubauer chamber

Using a Pasteur pipette a small fraction of cells was transferred to the Neubauer chamber and cells were cover slipped and counted at least in three 16X squares and the average was taken. The average was divided by 16 and multiplied by the constant $2.5 \times 10^{5.}$ This gave the number of cells in each ml of the trypsinised cells.

4.5 Firefly luciferase assay

The firefly luciferase assay is most often used these days in nuclear hormone research to check the strength of transcription from a given receptor in the presence of hormone/inhibitor. The luciferase cDNA fused to a hormone responsive promoter is

Lysis Buffer	
Tris-Acetate P^H 7.8	50 mM
MgAc	10 mM
EDTA	0.1 mM
Triton X-100	1 %
DTT	4 mM
PMSF	0.2 mM
DTT and PMSF freshly added to the lysis buffer	

Luciferase Substrate Solution	
Tris-Acetate P^H 7.8	50 mM
Luciferin	360 µM
ATP	1 mM
Coenzyme A	400 µM

expressed on the activation of receptor and forms the luciferase enzyme which can be measured using a Luminometer and the appropriate substrate. The amount of enzyme formed represents the activity of the receptor *in vivo*. The reaction requires Luciferin, ATP, coenzyme A, Mg^{++} and oxygen. Light emitted by the reaction of enzyme on the substrate Luciferin is measured by luminometry.

Briefly, the transfected cells were lysed using 400 µl of the lysis buffer for 15 min at RT and the probes were stored on ice until all samples were collected. 50 µl of sample was then read by Luminometer which pumps in 100 µl of the luciferase substrate buffer and measures the luciferase units which can be represented in the form of the graph.

4.6 β-Galactosidase assay

ONPG Substrate solution
4 mg/ml ONPG in 0.1 M potassium phosphate with pH 7.0

Z Buffer	
Na_2HPO_4	60 mM
NaH_2PO_4	40 mM
KCl	10 mM
$MgSO_4$	1 mM
P^H calibration 7.0 + 2.7 ml β-Mercapto ethanol per Liter	

1 M potassium phosphate solution pH 7.0	
1M K_2HPO_4	6.15 ml
1M KH_2PO_4	3.85 ml
Filled up to 100 ml with Milli Q.	

The reporter plasmid pCMV-LacZ contains a gene that codes for the enzyme β-galactosidase. The CMV promoter is presumed to be unaffected by nuclear hormone receptor mediated transcriptional activation and hence transfected as an internal control in order to normalize Luciferase values. The method uses a substrate *o*-Nitro-phenol-β-Galacto pyranoside (ONPG) which is converted into yellow coloured o-nitro phenol which can be photometrically measured at 420 nm.

In a fresh 1.5 ml reaction tube 600 µl of Z-buffer was taken and mixed with 100 µl of cell lysate. To the mix ONPG solution was added. The mix was shaken vigourously and incubated at 37 °C in a water bath till yellow. The absorbance was taken at 420 nm. The measurement of product formed was done according to the following formula.

Units

$$\text{LacZ units} = \frac{OD_{420} \times 1000}{\text{time (in min)}}$$

4.7 Chromatin immuno precipitation

4.7.1 Protein cross linking and chromatin preparation

Cells (LNCaP and C4-2) were grown to 95% confluence, taking into account that before harvesting they were grown in hormone-depleted media for at least 48 hrs if the experiment was done for hormone studies. Cells were treated with the hormone for the required period of time. It is prudent to change the media a couple of hours before treating with hormone as it may make the cells relatively more synchronised. If the experiment requires the treatment with inhibitor it was done at least 24 hours before harvesting.

ChIP Dilution Buffer	
Triton X-100	1% (v/v)
EDTA	2 mM
NaCl	150 mM
Tris/HCl, P^H 8.0	20 mM

SDS Lysis Buffer	
SDS	1.0% (w/v)
EDTA	10 mM
Tris/Cl P^H 8.0	50 mM

In order to cross link proteins with the chromatin, 37% formaldehyde was directly given to the media so that the end concentration in the media becomes 1%. The cells were then incubated with formaldehyde for exactly 10 min at 37 °C incubator. To stop over-cross linking the cells were treated with 2 M glycine (end concentration 125 mM) for 5 min at RT which was given directly into the media. The media was aspirated off and the cells were washed twice with cold PBS. This was followed by addition of 1.5 ml cold PBS containing protease inhibitor cocktail PIC (Complete mini, Roche) and the cells were scraped off into a fresh 15 ml falcon and were spun down at 4000 rpm for 5 min at 4 °C. The supernatant was discarded and the cells at this point were frozen at -80 °C. The pellet

(from two maxi cell culture dishes) was resuspended in 2 ml of cold SDS-lysis buffer containing protease inhibitor cocktail. Cells were then sonicated to isolate and shear chromatin to fragments of desired size. For generating ~500 bp fragments (for LNCaP and C4-2) cells were sonicated 10-12 times with 10 sec pulse and 10% output (Branson sonifier 250). Sonication of each sample was performed at an interval of 1-2 min in order to avoid froth formation.

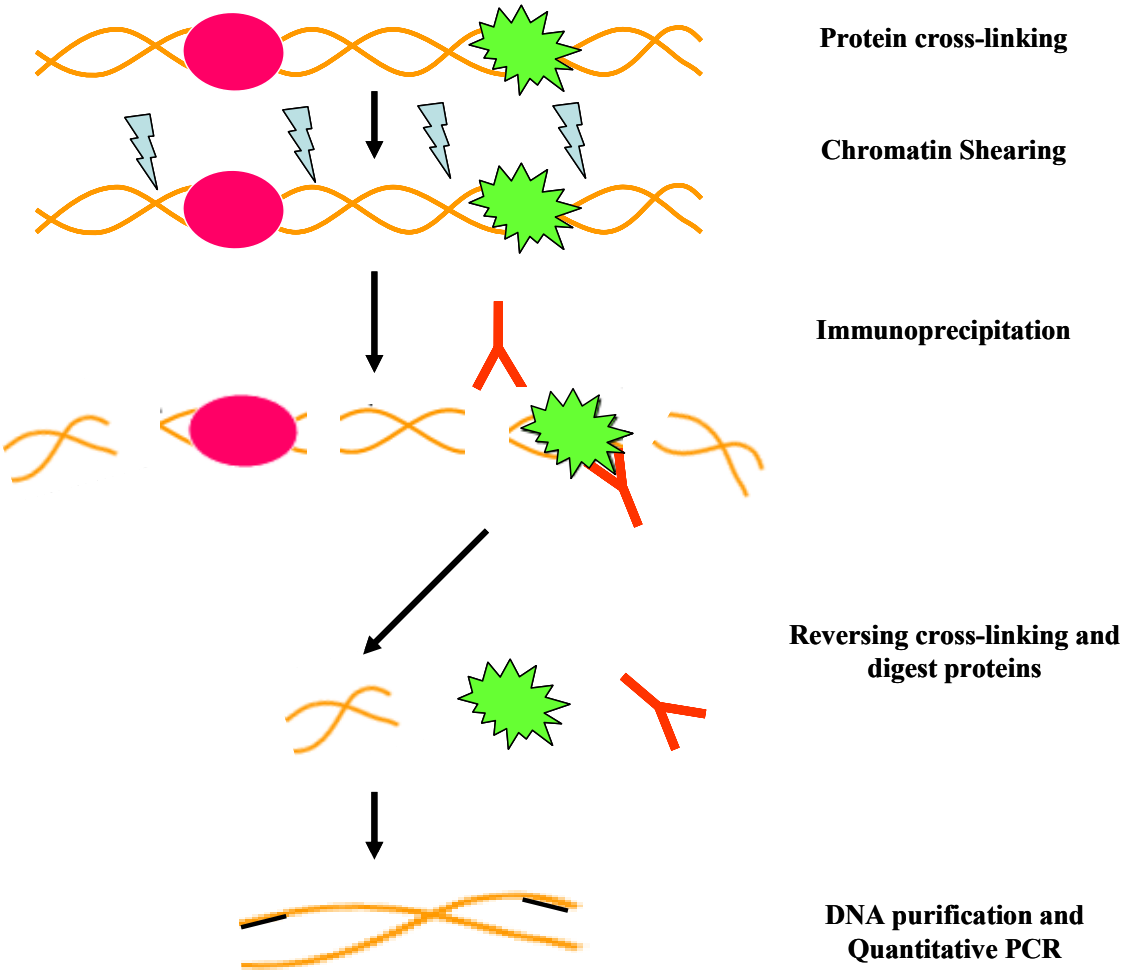


Fig. 4.2 Schematic representation of Chromatin immuno precipitation (ChIP) assay. Chromatin-associated proteins are cross linked. Cells are harvested and chromatin prepared into appropriate sized fragments. Chromatin is used for immuno precipitation with specific antibodies and complex isolated following which the cross linked proteins are separated from the chromatin and subsequently digested. The chromatin is purified and used for the detection of DNA elements using known primer sequences.

The sonicated cells were centrifuged at 5800 rpm at 4 °C for 10 min. The soluble chromatin remained in the supernatant while the cell debris settled down in the form of pellet. The supernatant was then diluted to 10 ml using ice cold ChIP dilution buffer containing protease inhibitor cocktail. The diluted chromatin was incubated with 40 µl of

protein A sepharose/salmon sperm DNA for 1 hr on a rocking platform at 4 °C. This step is called preclearing the chromatin by allowing all non specific proteins to bind to beads prior to incubation with specific antibodies and thereby lowering the possibility of false-positive results. Out of this 10 ml small portion (200 µl) was taken as input control.

4.7.2 Immuno precipitation (IP)

Low Salt Buffer	
SDS	0.1% (w/v)
Triton X-100	1% (v/v)
EDTA	2 mM
Tris/HCl, P^H 8.0	20 mM
NaCl	150 mM

High Salt Buffer	
SDS	0.1% (w/v)
Triton X-100	1% (v/v)
EDTA	2 mM
Tris/HCl, P^H 8.0	2 mM
NaCl	500 mM

LiCl Buffer	
LiCl	0.25 M
NP-40	1% (v/v)
Deoxycholate	1% (w/v)
EDTA	1 mM
10 mM Tris/HCl, P^H 8.0	

From the above step the beads were spun down by brief centrifugation at 4 °C. The cleared supernatant was used for immuno precipitation with specific antibodies. 1.5 ml of the diluted chromatin was incubated overnight (at least 6 hours) at 4 °C on a rocking platform to allow antibodies to bind to specific proteins (immuno precipitation). After incubation 35 µl of sepharose beads were added and the incubation continued further for 3 1/2 hours as before. The samples were centrifuged at 2000 rpm for 2 min at 4 °C and the supernatant discarded. The beads were sequentially washed with the following buffers with each washing followed by 10 min incubation on a rocking platform for 10 min and subsequent centrifugation at 2000 rpm for 2 min at 4 °C.

> 1x 1ml of low salt wash buffer
> 1x 1ml of high salt wash buffer
> 1x 1ml of LiCl wash buffer
> 2x 1ml of TE buffer

Elution Buffer	
SDS	1% (w/v)
$NaHCO_3$	0.1 M
Freshly prepared	

To the beads left after the last wash, 100 µl of freshly prepared elution buffer was added. The 1.5 ml reaction tubes were kept for 15 min on a fixed vortex machine at RT. The

samples were again spun at 2000 rpm for 2 min at 4 °C and the supernatant was collected in a fresh 1.5 ml reaction tube. The above elution was repeated with 100 µl of elution buffer. The elute was combined together and to each 200 µl sample, in order to reverse-cross linking, 8 µl of 5 M NaCl was added, mixed by vortexing and kept overnight (To 50 µl input, 150 µl elution buffer was added mixed and reversed the cross linking with NaCl). After overnight incubation each sample was treated with 4 µl 500 mM EDTA + 8 µl 1M Tris-Cl pH 6.5 + 1 µl of 10 mg/ml Proteinase K + 1 µl of 10 mg/ml RNaseA. The samples were further incubated for 3 hrs at 55 °C. The DNA was purified using QIAquick PCR purification kit to perform qPCR.

4.7.3 Chromatin purification for PCR

All centrifugation steps were performed at 13.000 rpm at RT. 200 µl combined elute was mixed with 1 ml of PB and mixed by vortexing. The column was placed on the provided 2 ml tube. Half of the elute (600 µl) was pipetted to the column and spun for 30-60 sec. The supernatant was discarded and the rest 600 µl was again pipetted to the previously used column and centrifuged once again. The column was washed with 0.75 ml of wash buffer. After discarding the wash buffer column was again centrifuged for additional 1 min the column was kept on a fresh 1.5 ml reaction tube and 50 µl of EB buffer was poured in the centre of the column matrix and allowed incubation for 1 min The reaction tubes were centrifuged and the elute containing DNA can be used for performing PCR.

4.7.4 Design and Standardization of ChIP PCR

In order to get the end result of a ChIP experiment PCR was subsequently performed. The primers spanning the response elements were selected based on the known complementary sequence in the DNA. The primers were generated by using a high output Genefisher program available as ftp and online operated at bibiserv.techfak.uni-bielefeld.de/**genefisher.**
The primers employed in ChIP study were all designed in a way that they lead to the generation of approx. 500 bp PCR products which could be run in 1% agarose gel and visualized on UV-trans illuminator. The PCR was standardized primarily by manipulating the annealing temperature while keeping the extension temp. and denaturation temp. same. The key to obtaining single and specific product lies in the Tm of the primers and the annealing temp. was always chosen to be 5 to 10 °C higher than the Tm. This worked

nicely in fulfilling the objective. Additionally, PCR could be standardized by employing Gradient PCR, where simultaneous amplification of the same PCR reaction mixture took place at different annealing temperatures. This program is available on the Peqlab thermal cycler available in the laboratory. All ChIP experiments therein have been reproduced.

5. Results

The research work pursued here provides an insight into how target genes expression is regulated by androgen receptor (AR). The work shows how interaction of corepressor SMRT with AR is decreased in cell type specific manner. The work also shows how Src and related kinases potentially enhance AR function on its target genes and can positively stimulate growth of prostate cancer (CaP). Further this works identifies a new corepressor LCoR for AR. It demonstrates the role of LCoR in repressing AR function and CaP growth. The work sheds light on how LCoR function is attenuated by signal transduction pathway which results in enhanced AR activity.

5.1 Plasmid construction

For the work undertaken many plasmids were constructed. Briefly the cDNA/part of cDNA was taken out from the parent vector and cloned into another acceptor vector in correct orientation to the desired promoter that eventually drives the expression of cDNA. cDNA fragments were cloned into a polylinker site of the expression vector that contains a series of restriction sites for cloning different cDNA with choice of various reading frame, an essential prerequisite for the production of a functional proteins.

All plasmids were sequenced to confirm their correctness. The following description shows how the cDNA from donor DNA was cloned into the polylinker site of the acceptor plasmid. It shows the resistance that they carry for a particular antibiotic, helping in the identification of bacteria carrying them thereby maintaining the purity in subsequent usages.

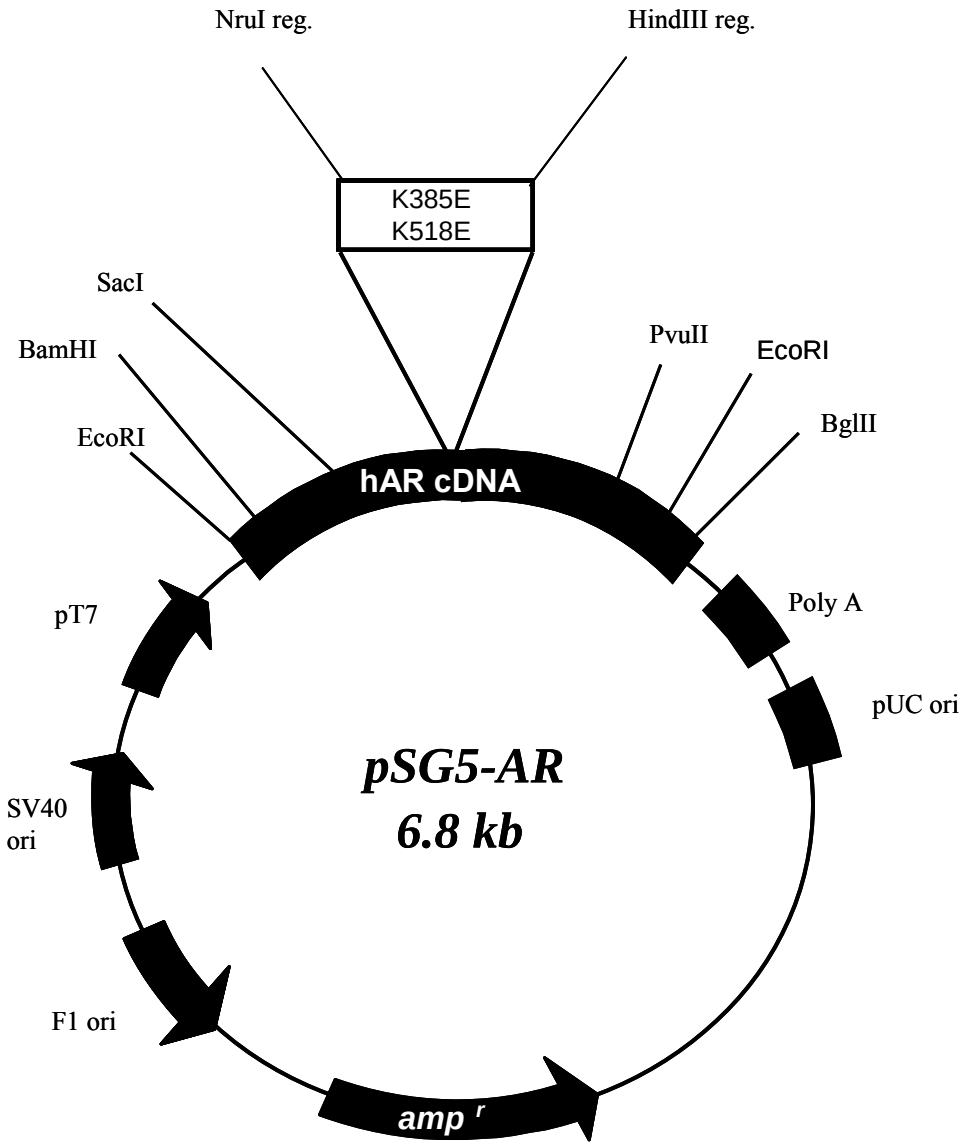
Clone Chart: pSG5-hARK385E, K518E

Cloning strategy:

Nru1 + Hind III (0.6 kb fragment) from p5HbAR K385E, K518E into Nru1 + Hind III/ CIAP treated pSG5

Selection:

Ampicillin



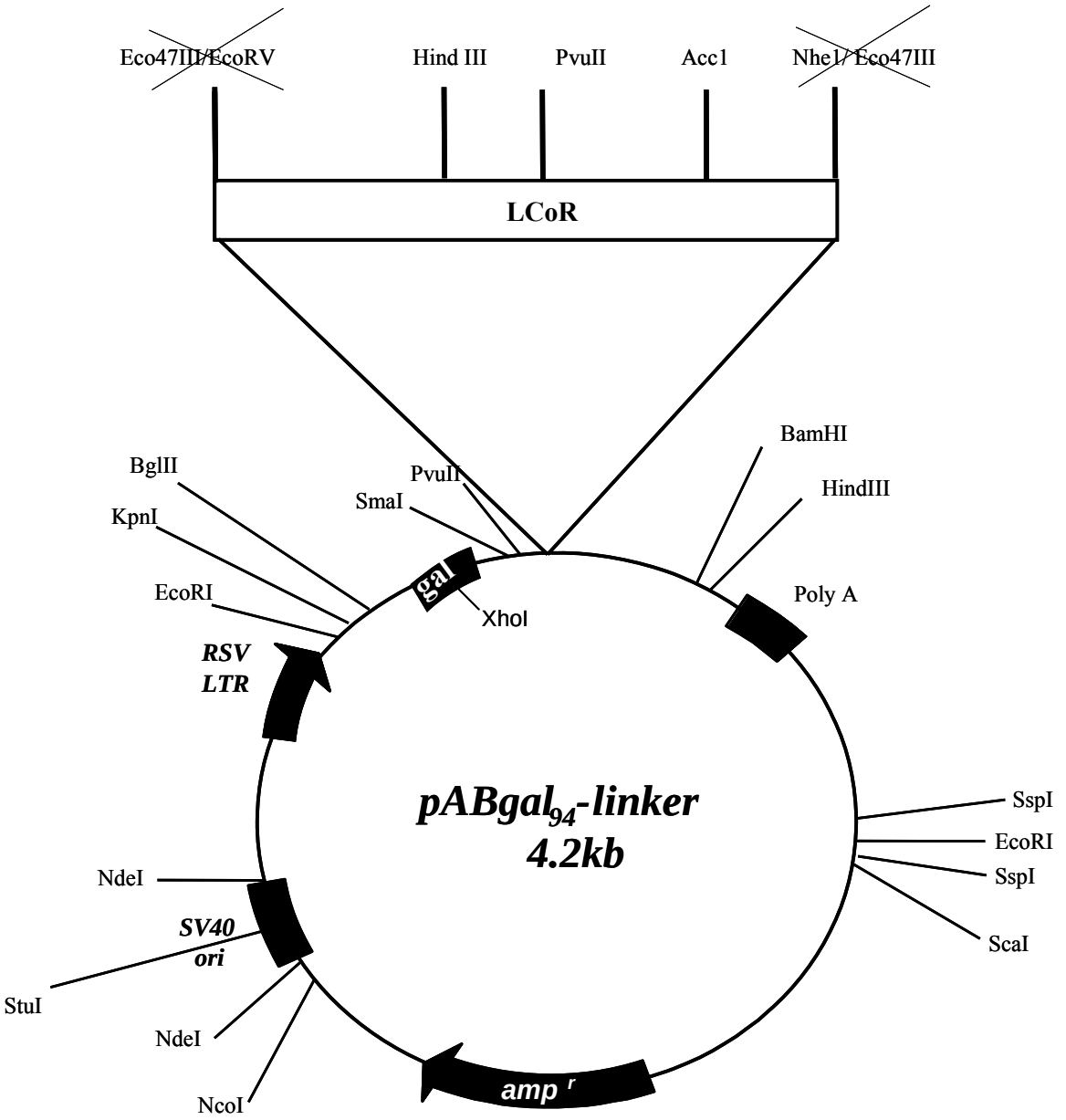
Clone Chart: pAB-gal$_{94}$ LCoR

Cloning strategy:

Nhel, Klenow, EcoRV fragment (1,3 kb) from pCMV-VP-LCoR into pAB gal$_{94}$-linker Eco 47III, CIAP treated

Selection:

Ampicillin



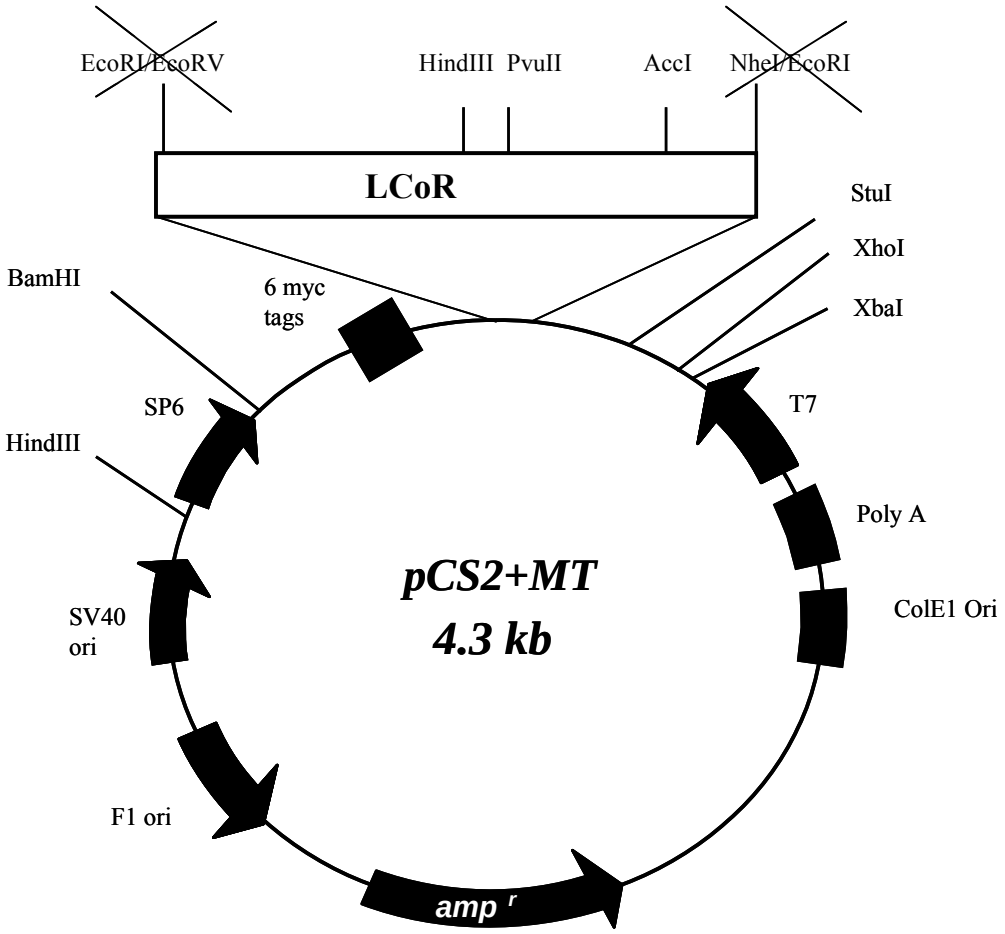
Clone Chart: pCS2-MT-LCoR

Cloning strategy:

NheI, Klenow, EcoRV fragment (1.3 kb) from pCMV-VP-LCoR into EcoRI, Klenow, and CIAP treated pCS2-MT

Selection:

Ampicillin



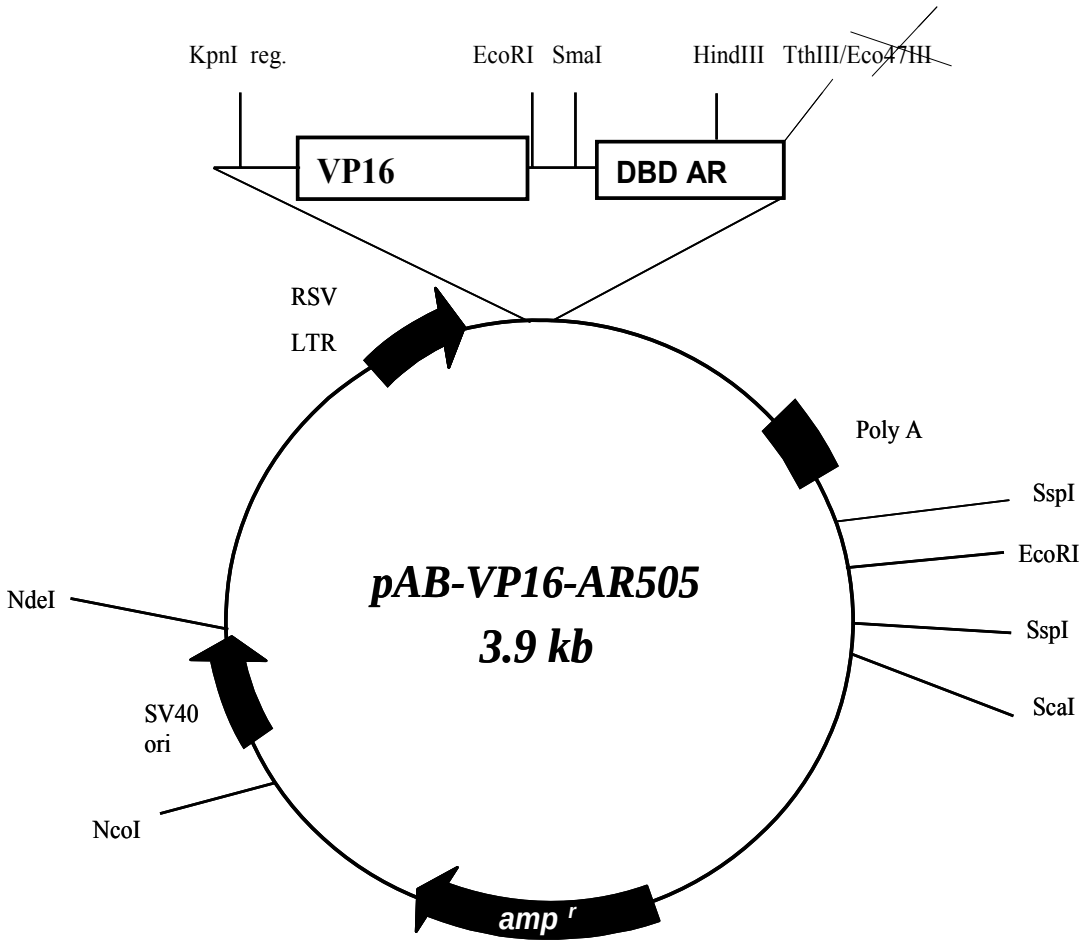
Clone Chart: pAB-VP-DBD

Cloning strategy:

TthI, Klenow, KpnI fragment from pABgal$_{94}$-linker
Into KpnI + Eco47 III digested pAB-VP-AB-505

Selection:

Ampicillin



5.2 Regulation of target gene expression in prostate cancer cells

AR mediates the biological effects of androgens. Activated AR translocates to the nucleus and bind to the cis- regulatory consensus elements for the binding of AR (called Androgen response elements, AREs). Binding to AR attract coactivators and/or corepressors in order to build transcription permissive or repressive complexes resulting in the modulation of target gene expression. The following section addresses the direct regulation of androgen target gene by AR and its corepressors.

5.2.1 *In vivo* recruitment of AR and its corepressors on positively-regulated target genes in CaP cells

AR-mediated transactivation involves its recruitment of AR to the androgen response element (AREs) in the regulatory region of target genes. PSA gene expression is known to be positively stimulated by AR and AR binding has previously been shown to this element (Young et al., 1992). Prostate stem cell antigen (PSCA) gene was shown to be positively regulated by androgens and an ARE has been found in its enhancer region (Jain et al., 2002). PSCA is a cell surface marker and is overexpressed in CaP (Reiter et al., 1998). Anti-PSCA monoclonal antibodies inhibit tumour growth and metastasis formation and prolong the survival of mice bearing human prostate cancer xenografts (Saffran et al., 2001 and Watabe et al., 2002). Therefore, PSCA is emerging as an important diagnostic marker and therapeutic target in CaP. However, direct *in vivo* involvement of AR and its corepressors in regulating PSCA expression has not been addressed.

Target gene regulation by AR often involves coactivators and corepressors. To test the possibility of direct regulation of PSCA expression by AR and its corepressors *in vivo*, ChIP analyses were performed. LNCaP and C4-2 cells were grown in androgen-deprived media and treated with R1881 (10^{-8} M), CPA (10^{-7} M) and Cas (10^{-7} M) for 1 hr. Afterwards, chromatin was prepared and immuno precipitated with antibodies against AR, corepressor SMRT and Alien. Subsequently, PCR was performed with specific primers designed based on the known DNA sequence spanning putative AREs to detect the binding of AR, SMRT and Alien under various AR ligands (Fig. 5.1). Under positive control conditions upon no hormone treatment (referred as control) in LNCaP cells, on PSA enhancer element, binding of AR and both of its tested corepressors, Alien and SMRT was observed. Upon treatment with agonist R1881, the recruitment of AR was enhanced while

that of corepressor SMRT was lost. It also resulted in strong recruitment of corepressor Alien. Treatment of LNCaP cells with partial agonist CPA also induced enhanced AR recruitment on this element. In addition, CPA treatment also led to strong recruitment of corepressor SMRT and Alien in line with a previous report (Dotzlaw et al., 2002). Cas treatment however led to loss of AR recruitment on PSA enhancer in concordance with recent data suggesting that Cas treatment of androgen-independently growing cells lead to association of AR to nuclear matrix (Whitaker et al., 2004) and that Cas treatment

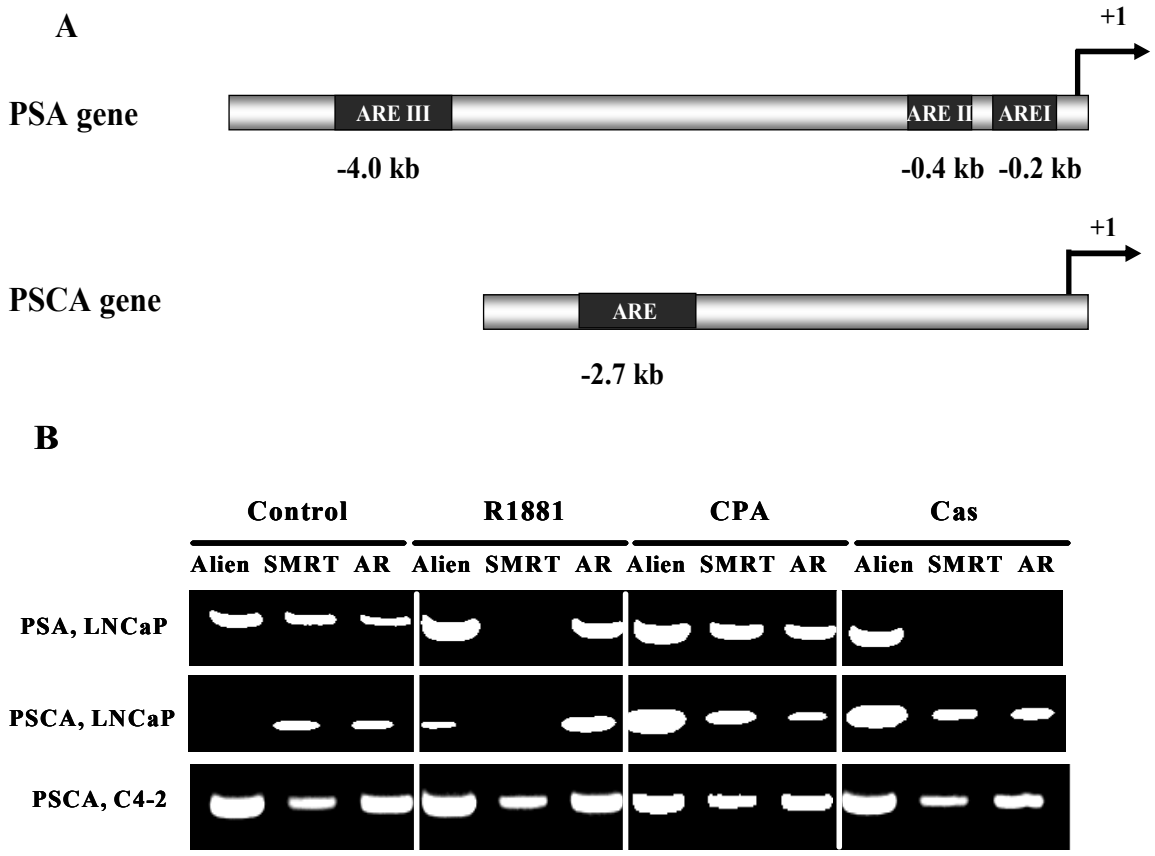


Fig. 5.1 AR and corepressor SMRT and Alien are recruited to the regulatory elements in the target genes. A. Schematic representation of the regulatory regions in the androgen target genes. PSA promoter contains two separate AREs situated app. 0.2 and 0.4 kb upstream of the transcription start site. PSA enhancer situated -4.0 kb upstream also contains a functional AREs. PSCA enhancer contains an ARE situated 2.7 kb upstream of the transcription start site. Presence of an ARE situated 0.3 kb upstream in the maspin promoter. B. 0.75 Mi LNCaP cells were seeded out in CSS containing RPMI 1640 medium and C4-2 cells were seeded in CSS containing T-media containing normal serum. Cells were grown for 4 days after which the fresh CSS T-media was given. Cells were further grown for 48 hrs and were treated with ligands R1881 (10^{-8} M), CPA (10^{-7} M) and Cas (10^{-7} M) for 1 hr. after which ChIP procedure was followed. The immuno precipitated DNA fragments were purified and subjected to amplification by specific primers spanning the AREs in the corresponding response element. The figure shows the result of a ChIP experiment.

interferes with the recruitment of AR to its target sites (Farla et al., 2005). Similar results from ChIP experiment on PSCA were obtained (Fig. 5.1). On PSCA enhancer AR binding

was enhanced in response to R1881 and that of SMRT was decreased. Under conditions of CPA treatment, Alien was strongly recruited, while the binding of AR and SMRT remained unchanged compared to no hormone conditions. These results indicate that both PSA and PSCA are target genes for direct AR action and their expression might be modulated by the corepressors recruited to AR under the influence of various ligands. However, AR recruited is to PSCA enhancer in presence of Cas. This is in contrast to loss of AR binding observed under Cas treatment on PSA enhancer raising the possibility that antagonistic action of ligands on AR action may well vary in part by the differences in the response elements. ChIP experiment performed in C4-2 cells on PSCA enhancer element revealed that AR recruitment was marginally enhanced by R1881 and remained unchanged in presence of CPA or Cas. Binding of corepressor SMRT was only marginally enhanced in response to CPA, however remained unchanged by R1881 or Cas.

5.2.2 *In vivo* recruitment of AR and its corepressors on maspin promoter element

The Maspin is a member of serine protease family and a tumour suppressor implicated in inhibition of tumour progression and invasion (Cher et al., 2003). Maspin expression is known to be negatively modulated by androgens, in addition, maspin, a tumor suppressor was identified to be expressed in prostate and is negatively regulated by androgens (Zhang et al., 1997). *In vitro* binding assays and transfection experiments revealed the binding of AR to these AREs elements. Here, the recruitment of AR and corepressors and their molecular actions are poorly understood. ChIP data show the recruitment of corepressors SMRT and Alien on key target genes (previous section). The possibility of direct binding of AR and its corepressors was also explored on maspin promoter element. ChIP experiments were performed in LNCaP and C4-2 cells under similar conditions as mentioned in the previous section. Maspin promoter is known to contain an ARE which has been shown to interact with AR *in vitro*. Results indicate that under conditions without any hormone (referred as control), both AR and Alien were recruited (Fig. 5.2). Treatment of LNCaP cells with R1881 enhanced the binding of corepressor Alien and led to the abolishment of AR binding. Upon CPA treatment binding of Alien and AR is decreased however, the recruitment of SMRT is seen. Interestingly, under conditions of Cas treatment, the recruitment of AR was decreased while that of Alien remained unchanged. Similarly, the ChIP experiment was performed in C4-2 cells and the immuno precipitated DNA was subject to amplification with primers spanning maspin putative AREs. In C4-2

cells under conditions without any hormone treatment recruitment of corepressor AR and AR was observed, in addition binding of SMRT is also observed. R1881 treatment led to a marginal decrease in binding of AR and both of the corepressors tested, Alien and SMRT. Upon treatment of C4-2 cells with CPA, surprisingly, the binding of AR and corepressors SMRT and AR was completely abolished. Cas induced loss of AR and SMRT recruitment, parallely decreasing the recruitment of Alien.

Results obtained in the absence/presence of R1881 are in contrast to those obtained for PSA and PSCA. On positively regulated PSA and PSCA, R1881 promoted the recruitment of AR (Fig. 5.1), contrarily, R1881 induced loss of AR recruitment on maspin promoter in LNCaP cells and significantly decreased AR recruitment in C4-2 cells. This indicates that a differential recruitment of hormone-bound AR depends in part on the nature of response

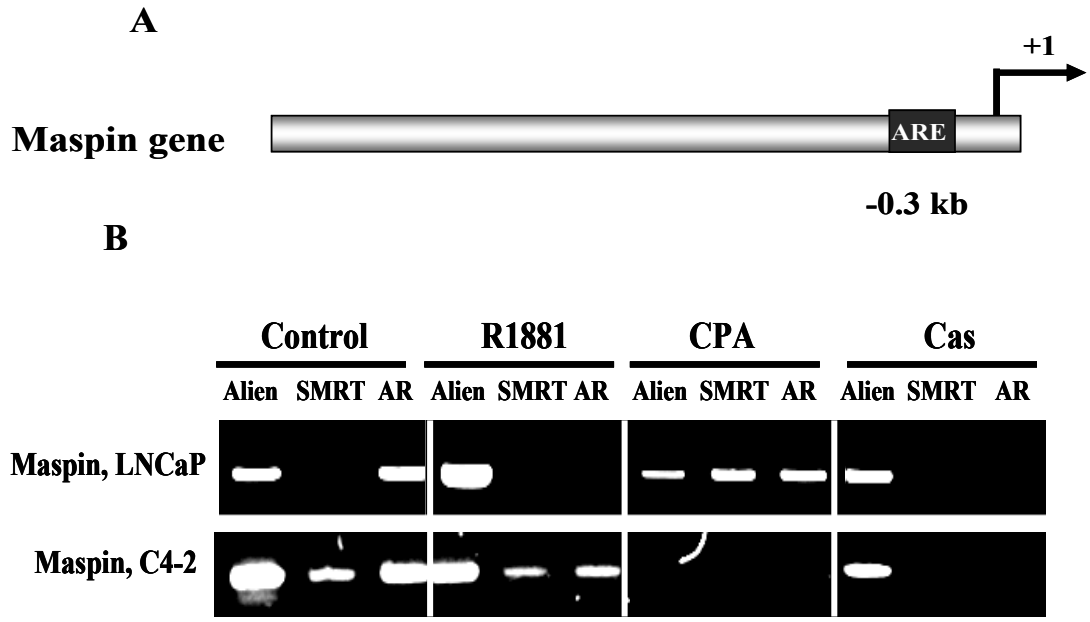


Fig. 5.2 AR and corepressors are differentially recruited to maspin promoter in response to various AR ligands. (A) Graphical representation of maspin promoter element and the position of AREs. (B) 0.75 Mi LNCaP/C4-2 cells were seeded out in RPMI1640/T-media containing normal serum respectively. Cells were grown for 4 days after which the fresh CSS containing RPMI/T-media was given. Cells were further grown for 48 hrs and were afterwards treated with ligands R1881 (10^{-8} M), CPA (10^{-7} M) and Cas (10^{-7} M) for 1 hr. after which ChIP procedure was followed. The immuno precipitated DNA fragments were purified and subjected to amplification by specific primers spanning the AREs in the corresponding response element. The figure shows the result of a ChIP experiment.

element. Similar decrease or loss of AR recruitment was observed in response to partial agonist CPA in LNCaP and C4-2 cells respectively. This suggests that agonist/partial agonist -bound AR confirmation is unstable on the maspin response element in contrast to

strong AR recruitment by R1881 on a positive regulatory element. However, Cas treatment led to loss of AR recruitment on maspin promoter.

5.2.3 Differential effect of CPA on PSCA and maspin expression in C4-2 cells

Previous section focused on determining regulation of AR and its tested corepressors SMRT and Alien recruitment under the influence of various ligands. To determine the influence of CPA treatment on the expression of PSCA and maspin genes, real time PCR was performed. Effect of partial antagonist CPA was tested on the expression of PSCA and maspin in mRNA level in androgen-dependently growing C4-2 cells. C4-2 cells growing in 6-well culture plates were treated for 48 hrs with CPA (10^{-7} M). RNA was isolated using Trifast reagent, reverse transcribed to cDNA and was subjected to amplification by light cycler using primers specific for PSCA and maspin mRNA and results were normalised using GAPDH. Treatment of C4-2 cells with partial antagonist CPA decreased expression

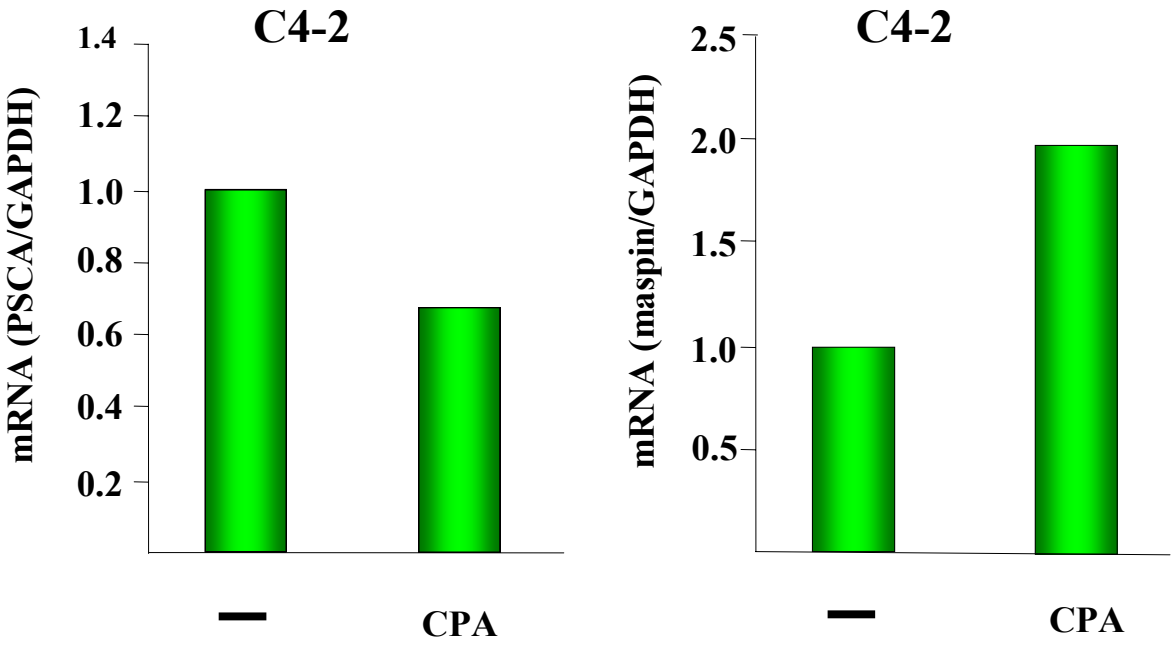


Fig. 5.3 Contrasting effect of partial antagonist CPA on the expression of PSCA and maspin mRNA in CaP cells. 200.000 C4-2 cells/well of the six well tissue culture dish were seeded out in CSS containing T-media. 24 hrs later cells were treated with CPA (10^{-7} M) for 48 hrs. Afterwards total cellular RNA was isolated, reverse transcribed to cDNA and was subjected to amplification by light cycler using specific primers and internal control primers against GAPDH. The graph represents the GAPDH normalised values of the PSCA and maspin transcripts.

of PSCA (Fig. 5.3) gene, which is positively regulated by androgens advocating that CPA shows only marginal inhibitory effect in repressing AR transactivation of target genes. The same RNA preparations were used for real time PCR using maspin primers. Contrasting to results obtained for PSCA, CPA led to enhanced expression of maspin transcripts further highlighting the contrasting regulation of PSCA and maspin genes by AR in C4-2 cells. ChIP data (fig 5.2) in fact reveals the loss of CPA-bound AR binding to maspin element in C4-2 cells.

Add together, the results from ChIP and real time PCR experiments demonstrate that PSCA and maspin are oppositely regulated by AR signaling. It suggests that AR recruitment on maspin promoter in the absence of hormone repress maspin expression. However, loss of AR recruitment from this element leads to its enhanced expression.

5.3 Src kinase inhibition decreases AR transactivation and the growth of androgen-independently growing cells

Kinase cascades are known to be overexpressed in different kinds of cancer. e.g., in breast cancer cells, Src kinase leads to failure of antagonistic action of Tamoxifen (Shah et al., 2005). Failure of CaP anti-hormone therapy may also involve action by Src. In fact, Src is over expressed in androgen-independently growing CaP cells and correlate with the increased metastatic potential of cancer cells (Slack et al., 2001). This part focused, therefore to determine the possible role of Src in modulating AR function and the growth of CaP cells.

5.3.1 Inhibition of Src by PP2 decreases AR transactivation in C4-2 cells

The ability of Src kinase to modulate AR transactivation function was tested using luciferase reporter assays in androgen-independently growing C4-2 cells *in vivo*. Cells were transfected with MMTV-Luc and PSA-Luc androgen responsive reporters and pCMX-LacZ which was used as an internal control. AR-mediated transactivation was repressed by treatment of cells with Src inhibitor PP2 (Fig. 5.4). To test whether attenuation of AR transactivation by PP2 on target genes expression is a promoter-specific phenomenon or AR function is in general reduced; two different AR target elements, MMTV and PSA, were used. Indeed AR transactivation potential was reduced to almost 50% on both MMTV and PSA reporter elements. This suggests that in androgen-

independently growing cells AR function on its target genes is still important. To test, whether PP2-mediated decrease in target gene expression is due to decrease in AR transactivation of its target genes or the decrease in target gene expression in general

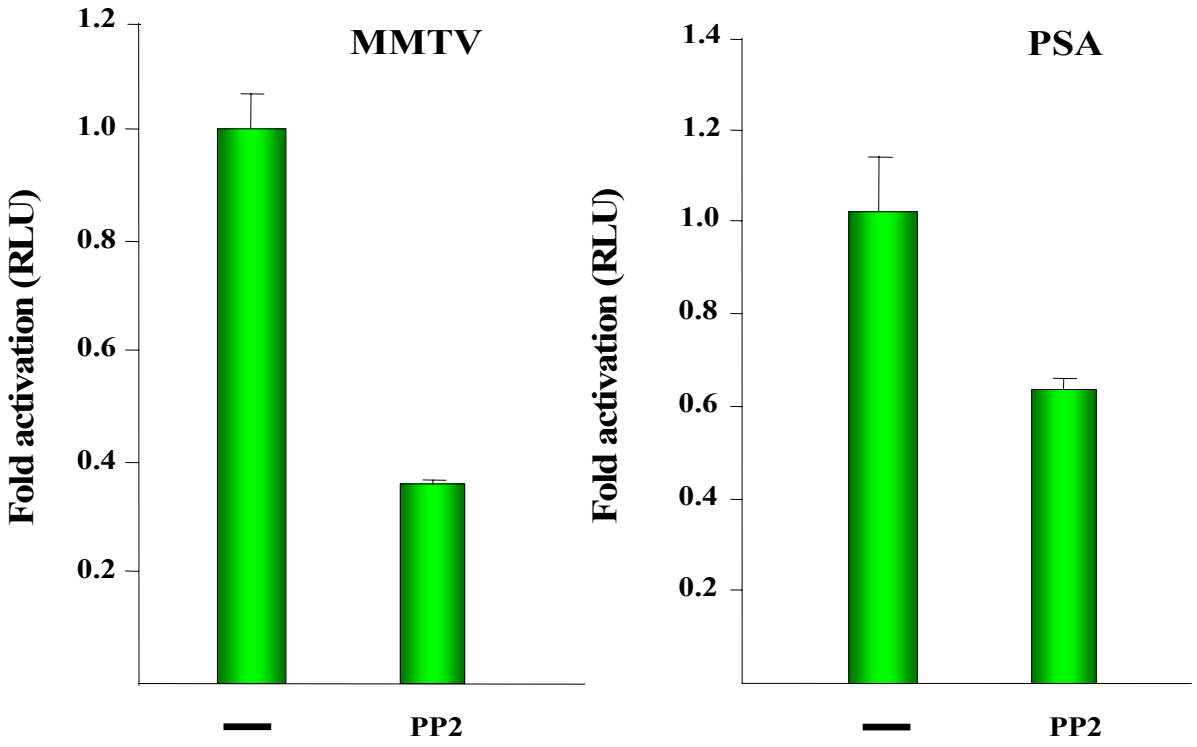


Fig. 5.4 Inhibition of Src kinase by chemical inhibitor PP2 leads to decrease in AR transactivation of target genes in C4-2 cells. 300.000 C4-2 cells were seeded out in normal serum containing T-media. 24 hrs later cells were transfected with 1.0 µg each of MMTV-luc and PSA-luc reporter plasmids along with 0.2 µg of pCMV-LacZ which was used as an internal control to normalize transfection efficiency. After washing, cells were treated with 1 µM PP2 for 72 hrs. Cells were lysed and luciferase values were measured. Values obtained without the inhibitor in each case were set as 1. The graph represents the activation of reporter expression as LacZ normalized relative luciferase units.

irrespective of the promoter, the effect of PP2 was tested on the expression of another control gene driven by CMV enhancer. The C4-2 cells were transfected with pCMV-LacZ reporter plasmid and treated with PP2 similar to the above experiment. In fact, the expression of this gene remained unaffected by PP2 treatment (Fig. 5.5) suggesting that the PP2 specifically decreases AR transactivation on its target genes. The enhanced transactivation by AR observed in C4-2 cells is in part boosted by overexpression of Src kinase as evident from inhibitor experiment and which may decrease the response to anti-hormone therapy. This may act as a potential growth-inhibition escaping mechanism

adopted by CaP cells for sustained growth. In fact AR activity has been shown to be subject to regulation by many signaling kinases including Her2 (Craft et al., 1999).

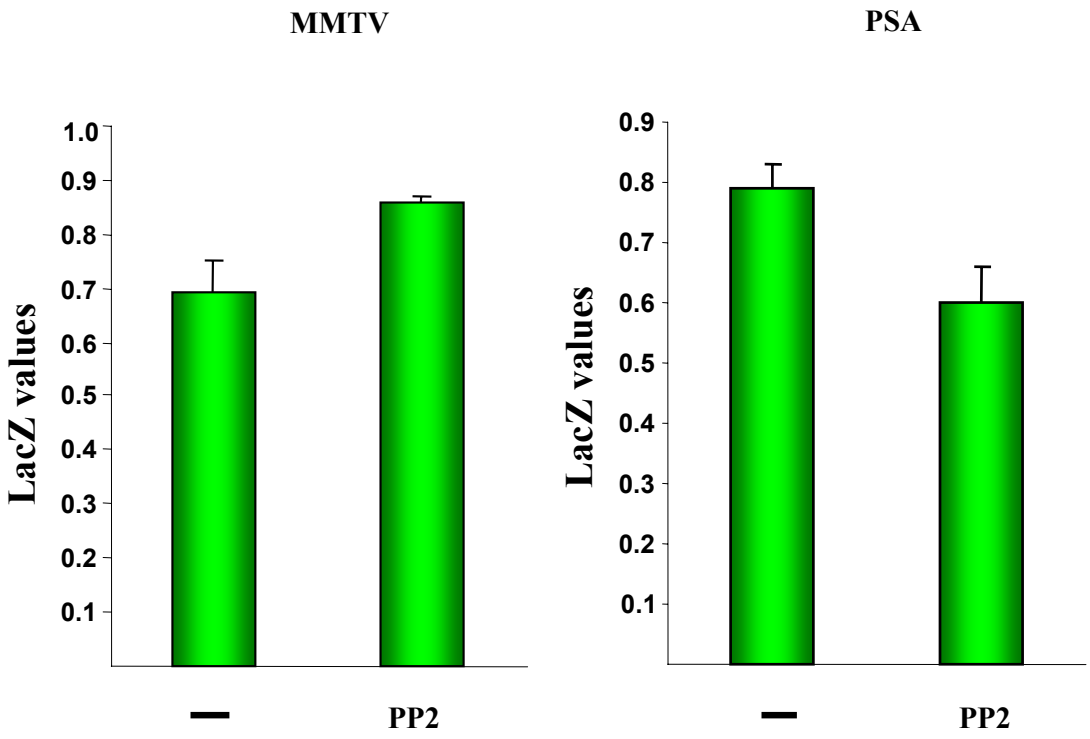


Fig. 5.5 PP2 does not affect expression of β-galactosidase in C4-2 cells. 300.000 C4-2 cells were seeded out in normal serum containing T-media. 24 hrs later cells were transfected with 0.2 µg of pCMV-LacZ which was used as an internal control to normalize transfection efficiency. After washing, cells were treated with 1 µM PP2 for 72 hrs. Cells were lysed and β-galactosidase values were measured and graph plotted.

5.3.2 Src inhibition leads to decreased recruitment of AR on target genes *in vivo*

To determine the effect of Src inhibition on AR recruitment to its target genes, chromatin immuno precipitation (ChIP) assay was performed. C4-2 cells were treated for 48 hrs with Src inhibitor PP2 (1 µM), following which cells were treated with R1881 (10^{-8} M) for 1 hr. The nucleo protein complexes were immuno precipitated with anti-AR and anti-IgG antibodies and purified DNA fragments were subsequently amplified by PCR with primers

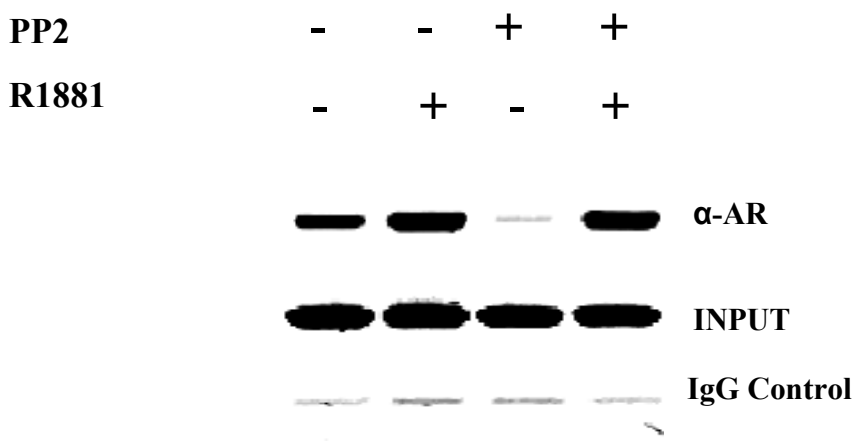


Fig. 5.6 Src inhibition leads to decreased AR recruitment on its target genes. 0.75 Mi C4-2 cells were seeded out in T-media containing normal serum. Cells were grown for 4 days after which they were grown for 48 hrs in T-media containing CSS and were simultaneously treated with 1μM PP2 for 48 hrs. Before lysis, cells were treated with ligands R1881 (10^{-8} M) for 1 hr. after which ChIP procedure was followed. Prior to immuno precipitation, part of chromatin was saved that functions as input here. Rest was immuno precipitated with anti-AR antibody or normal rabbit IgG that acts as negative control for the experiment. Fragments were purified and subjected to amplification by specific primers spanning the enhancer region of PSA containing ARE. After PCR, amplified products were run on 1% agarose gel, visualised by UV light and pictured by the gel documentation system, the figure shows the result of a ChIP experiment.

for PSA enhancer. In concordance to the recruitment of AR observed on PSCA element in the absence of R1881 (section 5.2.1, fig. 5.1) AR recruitment was also observed on androgen-responsive PSA enhancer in the absence of R1881 (Fig. 5.6). Treatment of C4-2 cells with R1881 led to increase in AR recruitment. Intriguingly, C4-2 cells pre-treated with Src inhibitor exhibited drastic loss of AR recruitment on PSA enhancer in the absence of R1881. However, AR was strongly recruited upon treatment of PP2-pre-treated cells with R1881. These experiments indicate that recruitment of AR to its target genes is indeed subject to regulation by PP2-mediated Src inhibition. This also suggests that decrease in reporter gene expression by chemical blockade of Src (sec. 5.3.1) may potentially involve decreased recruitment of AR in the presence of PP2. However, PP2-mediated decrease in AR recruitment was overcome by R1881 to similar level as seen in the absence of PP2.

5.3.3 Effect of PP2-mediated Src inhibition on *in vivo* target gene expression

In order to test the effect of Src blockade on target gene expression, real time RT-PCR experiment was performed. Briefly, C4-2 cells were treated with PP2 (1 μM) for 48 hrs. The following day cells were treated with R1881 (10^{-10} M). 24 hrs later cells were harvested and RNA isolation was performed. The prepared RNA was subjected to reverse-transcription to synthesize cDNA, which was used for performing real time amplification with primers specific for PSA gene. Values were normalized to actin control.

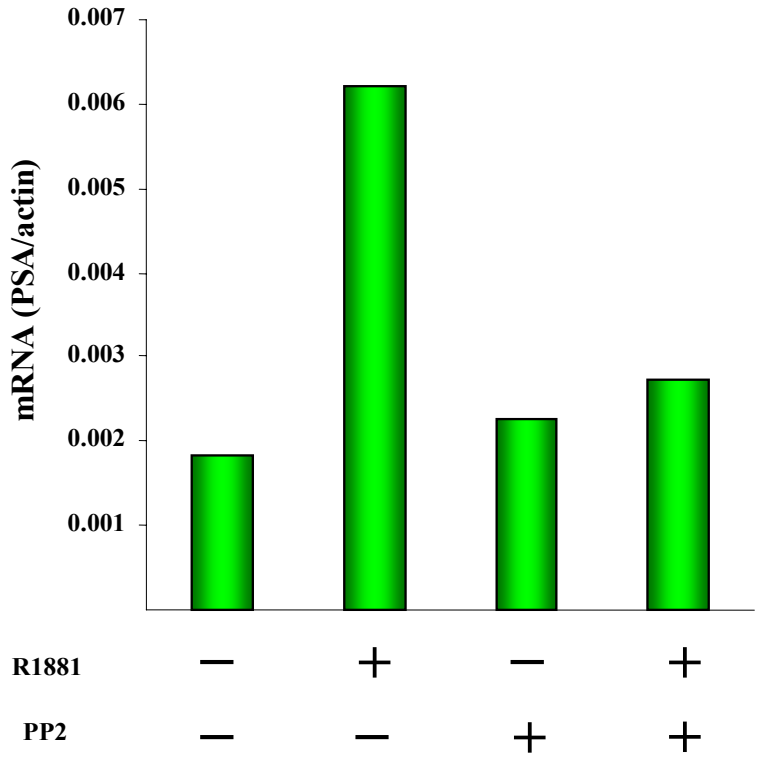


Fig. 5.7 Src kinase inhibition by PP2 leads to loss of agonist induced PSA mRNA induction in C4-2 cells. 200.000 C4-2 cells/well were seeded out in CSS containing T-media in six well tissue culture dishes. 24 hrs later cells were treated with R1881 (10^{-10} M) for 48 hrs. Afterwards total cellular RNA was isolated, reverse transcribed to cDNA and was subjected to amplification by light cycler using specific primers and control primers against Actin. The graph represents the actin normalised values of the PSA transcripts.

It is evident from this experiment that C4-2 cells show a basal leaky expression of PSA gene transcripts in the absence of agonist R1881 (Fig. 5.7) which may be explained largely by the agonist-independent recruitment of AR on PSA enhancer. In fact PSA gene is marginally expressed in C4-2 cells even in the absence of agonist and the expression has been shown to go up upon agonist treatment (Jia and Coetzee, 2005). Treatment with R1881 led to further 3-fold induction of PSA gene transcripts, in agreement of enhanced

AR recruitment under that condition (Fig. 5.6). Interestingly, R1881-mediated induction of PSA mRNA expression, however, was lost in C4-2 cells pre-treated with PP2. The real time experiments are not in total agreement to ChIP experiment.

Taken together, results from transient transfection, real time and ChIP experiments suggest that a basal level of PSA transcripts are expressed in C4-2 cells in the absence of agonist. Agonist R1881 leads to enhanced AR transactivation function on target genes reflected in the induction of PSA gene transcription. PP2-mediated blockade of Src suggests that induction of PSA transcription by AR in response to agonist is significantly contributed by Src signal transduction pathway, blocking which leads to decrease in AR target gene expression (Fig. 5.4) and induction (Fig. 5.7) as well. Treatment of C4-2 cells growing in androgen-depleted T-media with Src kinase inhibitor PP2 alone however, leads to loss of AR recruitment, surprisingly no further decrease in PSA gene transcript was observed. This suggests that the C4-2 cells express basal level of PSA mRNA independent of AR transactivation function. However, induction of PSA expression by AR requires not only enhanced AR occupancy but Src-mediated signaling plays an important role in AR transactivation leading to enhanced PSA expression.

The hAR is synthesized as a single 110 kDa protein, which becomes rapidly phosphorylated to a 112 kDa protein (Brinkmann et al., 1992). AR is a phosphoprotein and is heavily phosphorylated not only by signal transduction machinery-mediated cross-talk independent of agonist, but AR is phosphorylated in response to agonist that leads to conformational changes and receptor activation. Indeed, AR is known to interact with Src kinase in response to agonistic ligand (Kousteni et al., 2001) it is possible that certain phosphorylation sites in AR are phosphorylated by a subset of kinases while others are subject to phosphorylation by other signal kinase stimuli. Phosphorylation of various domains may have different functional consequences on receptor activation. In fact Src kinase is known to be overexpressed in CaP and enhances the AR function at very low concentration of agonist R1881 (Castoria et al., 2003) suggesting that it enhances the transactivation function of AR and may also cooperate in its ability to bind to target genes.

5.3.4 Src kinase blockade by PP2 decreases in C4-2 cell growth

The functional consequence of PP2-mediated decrease in AR transactivation and loss of induction of target gene expression in response to agonist was tested in relation to cellular growth. C4-2 cells were grown in T-media containing 10% normal serum (serum which

has not been depleted of androgens) and were treated with PP2 (1 μM) with media change twice a week for a period of 6 weeks and thereafter the effect of PP2 on cellular growth was tested. Treatment of C4-2 cells leads to reduced growth of cells in response to PP2

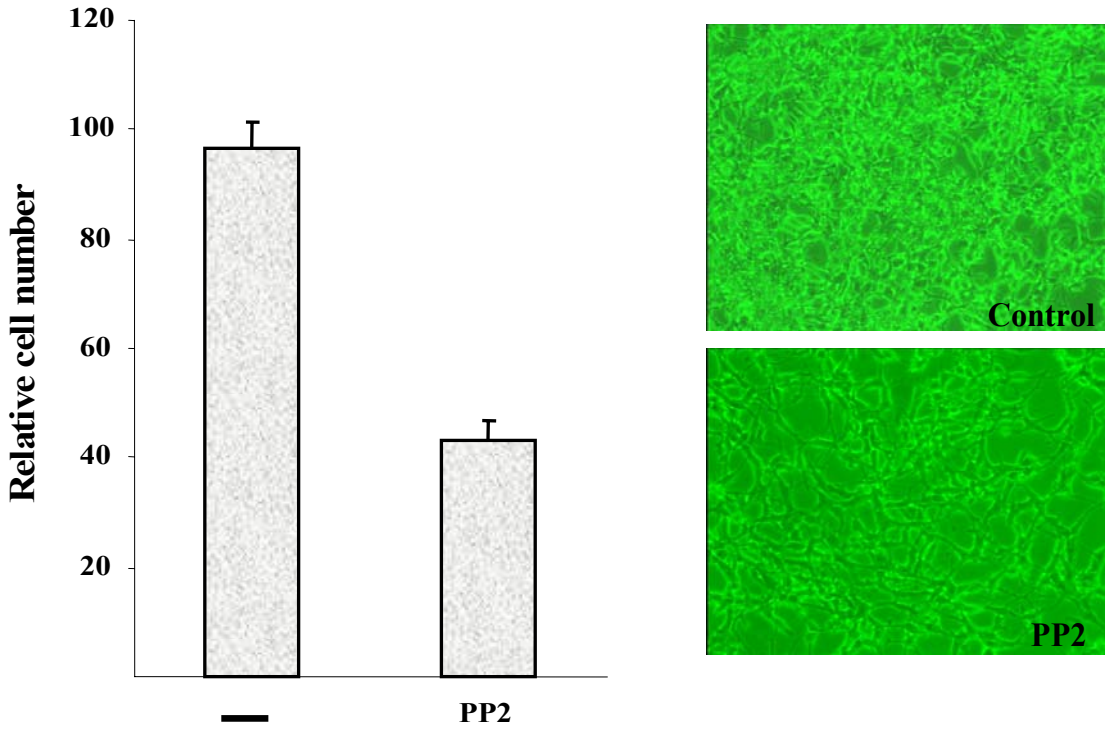


Fig. 5.8 Src kinase inhibition leads to decrease in growth of C4-2 cells. 200.000 C4-2 cells were cultured in 10 cm tissue culture dishes in T-media containing 10% normal serum for a period of 6 weeks. Cells were given fresh media twice a week along with 1 μM PP2. After 6 weeks cells were counted and pictures using light microscope assisted camera. The graph shows relative cell number (each triplicate).

(Fig. 5.8). While untreated cells grow to form a homogeneous monolayer with small intercellular spaces, Src inhibitor PP2 treated cells show retarded growth and an explicit decrease in cell number.

Taken together these results indicate that androgen-independently growing cells do depend on AR-mediated signaling for growth. This also employs that enhanced AR transactivation observed in androgen-independently growing C4-2 cells is in part contributed by over expression of Src signal transduction pathway. This also suggests that CaP cells achieve androgen-independent growth in part by overexpressing Src kinase. In essence, the results implicate the role of Src kinase in enhancing AR transactivation on its target genes. This induction is blocked by PP2-mediated Src blockade, in part by decreased AR transactivation on its target genes thereby allowing decrease in cellular growth.

5.4 Molecular characterization of interaction of corepressor SMRT with endogenous AR in LNCaP and C4-2 cells: the role of signaling cascades

CaP cells exhibit strong AR transactivation and enhanced cellular growth, suggesting decreased/lost influence of corepressors in inhibiting AR function. AR activity is known to be regulated by corepressor SMRT function (Dotzlaw et al., 2002, Liao et al., 2003). Indeed tyrosine kinase signaling is known to negatively regulate the interaction between SMRT and TR (Hong and Privalsky, 2000). Similarly PKA signaling which is known to activate AR, inhibits the binding of SMRT to AR (Dotzlaw et al., 2002). Reports also suggest that SMRT function is potently inhibited by MAPK cascade that operated downstream of the growth factor receptor. Activation of MAPK leads to SMRT

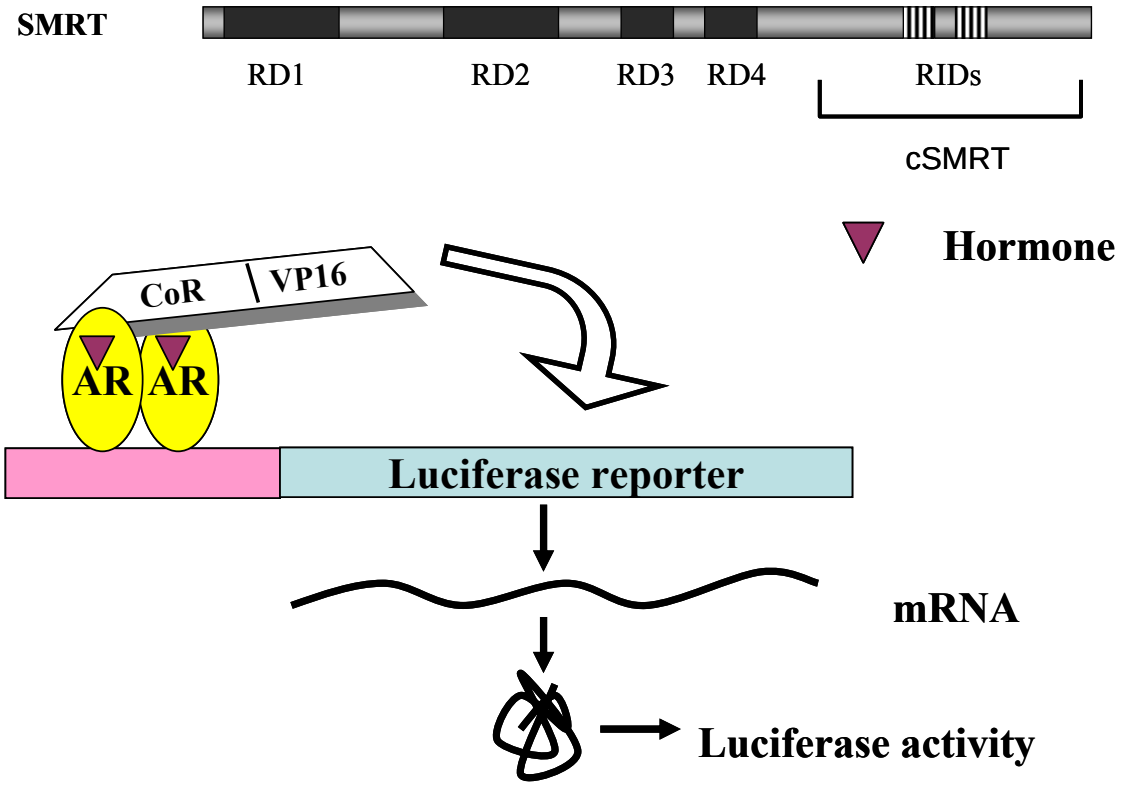


Fig 5.9 Graphical representation of mammalian-one-hybrid to detect interaction of corepressor SMRT with AR *in vivo*. Upper panel shows the graphical representation of corepressor SMRT, which contains four repression domains (RDs) and two receptor interaction domains (RIDs). Lower panel shows the methodology of Mammalian-one-hybrid. C-terminus 422 aa of corepressor SMRT were expressed as a fusion protein along with VP16 transactivator. The ligand-regulated interaction of SMRT with AR brings VP16 into the close proximity of promoter allowing a strong activation of the reporter luciferase gene and the product formed can directly be measured using Luminometer.

phosphorylation and its nuclear import (Hong and Privalsky, 2000) indicating that SMRT-mediated AR repression is influenced by signal transduction. To test the influence of signaling cascades in regulating AR and SMRT interaction, mammalian-one-hybrid assay was performed. The assay utilized a hybrid protein, which retains the receptor interaction domains of SMRT (Fig 5.9) deleting the repression domain, fused to a potent viral transactivator protein VP16. The system works on a simple principle- interaction of corepressor SMRT with AR, brings VP16 into the close proximity of the target gene promoter allowing expression of the luciferase gene (fig 5.9) and the strength of interaction can be quantitatively measured by the amount of luciferase units obtained under a given set of conditions.

LNCaP cells were cotransfected with VP16 empty vector or VP16-cSMRT along with MMTV-Luc reporter. pCMV-LacZ was used as internal control (Fig. 5.10). In LNCaP cells the binding of AR with VP16-cSMRT fusion protein was not observed *in vivo* in CPA-dependent manner (Fig. 5.10 A). It is possible that abruptly activated signal transduction machinery may influence this interaction. Experiment indicate that indeed interaction was enhanced significantly on co-treatment of cells with PRK-1 inhibitor HA1077 (Fig. 5.10 B), or MAPK inhibitor U0126 (Fig. 5.10 C) and Src kinase inhibitor PP2 (Fig. 5.10 E) in a CPA-dependent fashion. In contrast, the treatment with tyrosine-kinase inhibitor AG1517, did not lead to enhancement of interaction of SMRT with endogenous AR in LNCaP cells (Fig. 5.10 D). Rather AG1517 treated LNCaP cells showed a general decreased in reporter activation and induction. These results indicate that abrupt activation of specific signal transduction cascades in CaP cells may function in multiple ways to allow the growth of CaP cells. Loss of interaction of corepressor SMRT with AR by signaling cascades identified by using inhibitors may allow target gene expression and growth of cells leading to failure of CPA-based anti-hormone therapy.

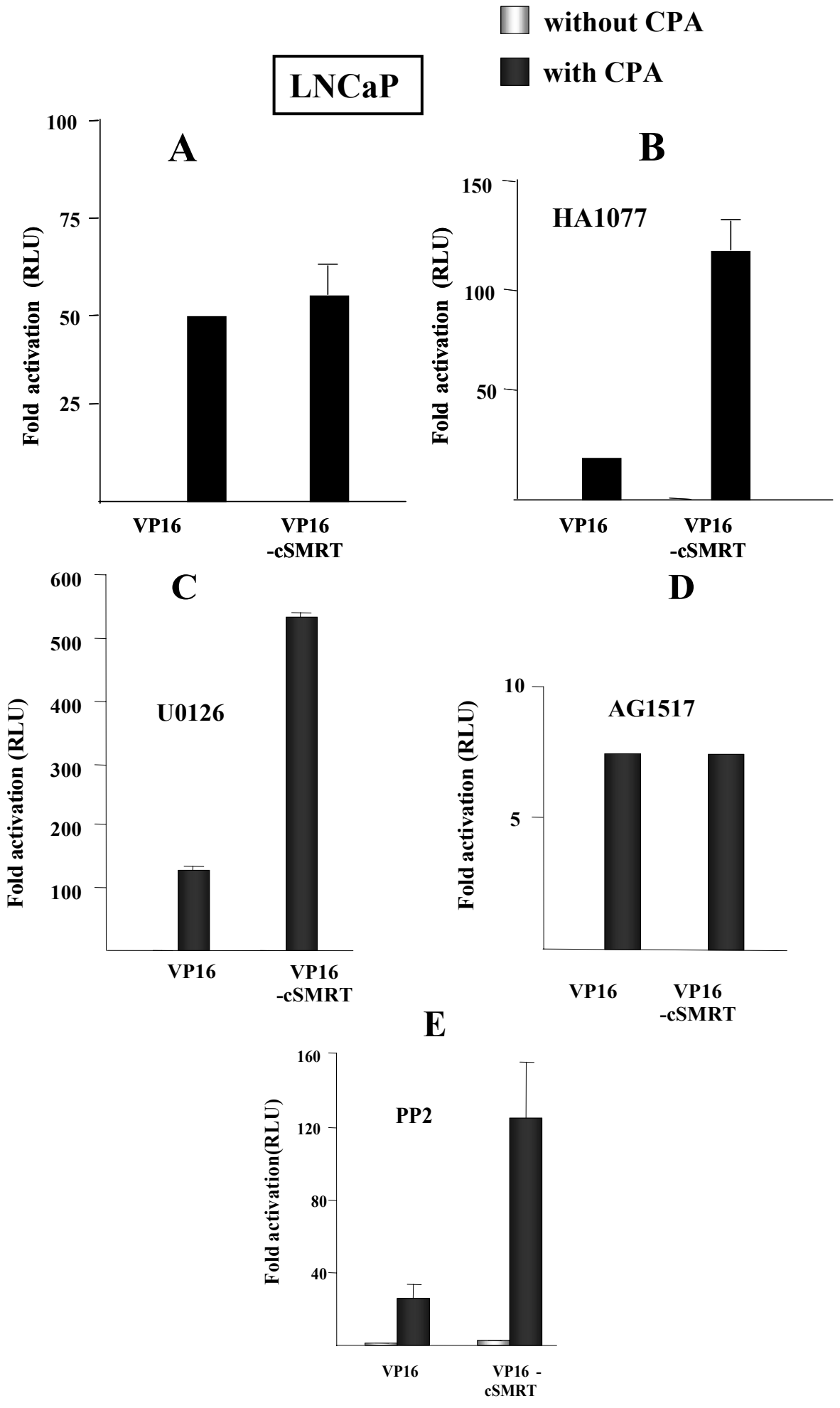
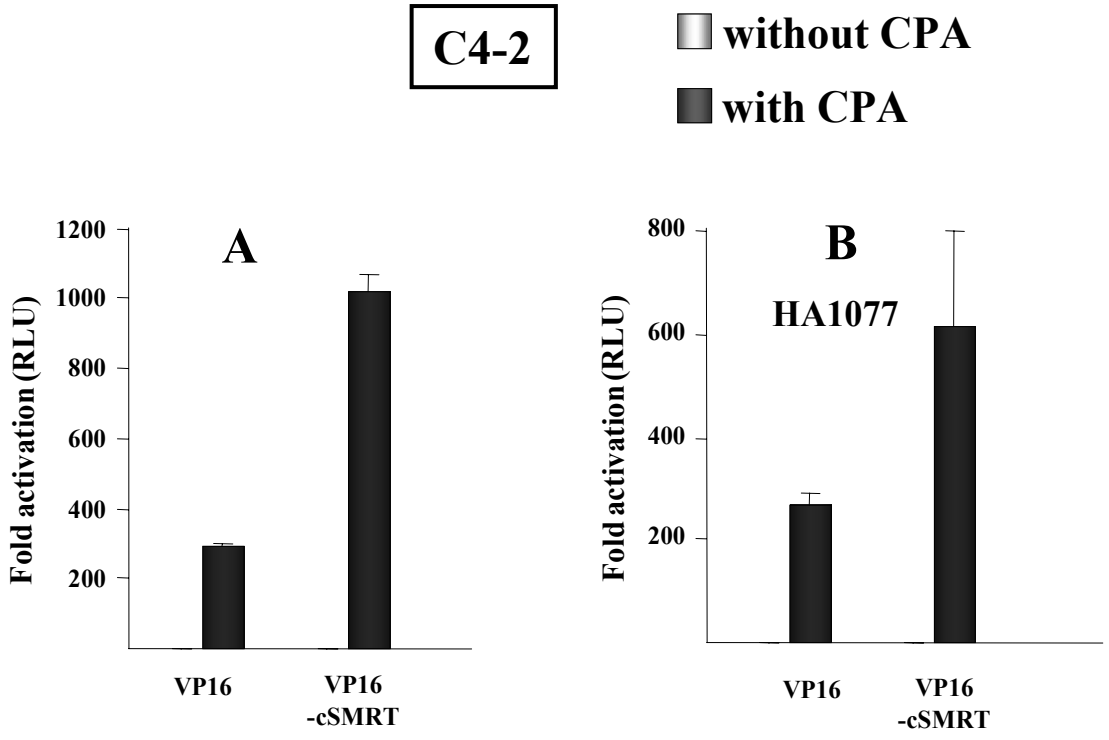

without CPA
with CPA
LNCaP
A
B
HA1077
C
U0126
D
AG1517
E
PP2
Fold activation (RLU)
Fold activation(RLU)
VP16
VP16 -cSMRT

Fig. 5.10 Interaction of corepressor SMRT with AR in LNCaP cells is differentially modulated by signal transduction pathways. 300.000 LNCaP cells were seeded out in androgen-deprived T-media and were transfected with MMTV-luc (1 μg), 0.22 μg of empty vector VP16 or VP16-cSMRT. pCMX-LacZ (0.4 μg) was used as internal control. 16 hrs after transfection media was changed with androgen-deprived fresh RPMI 1640 media and were treated with CPA (10^{-7} M) alone (A), or co treated with PRK inhibitor HA1077 1 μM, (B), MAPK inhibitor U0126 1 μM (C), tyrosine kinase inhibitor AG1517 1 μM (D) and Src inhibitor PP2 also 1 μM (E) for a period of 72 hrs. Cells were lysed and luciferase values were measured. Experiment was conducted in triplets and values obtained for VP16 empty vector in the absence of any inhibitor were arbitrarily set as 1. The graph represents the activation of reporter expression as LacZ normalised relative luciferase units.

On the other hand, Experimental results indicate 3-fold increase in SMRT interaction with AR in C4-2 cells in a CPA-dependent manner over values obtained for empty vector VP16, in the presence of CPA (fig 5.11 A) raising the possibility that signal transduction pathways may act differentially to regulate interaction of corepressor SMRT interaction in cell-type specific manner. It is notable that two different tyrosine kinase inhibitors PP2 and AG1517 were used. AG1517 inhibits the membrane tyrosine kinase and therefore blocks many essential growth factor signals and inhibits the growth not only of the LNCaP cells but also the reporter expression as evident from Fig. 5.11 D. PP2 in contrast being more specific and inhibits non-receptor cytosolic tyrosine kinases such as focal adhesion kinase (FAK).



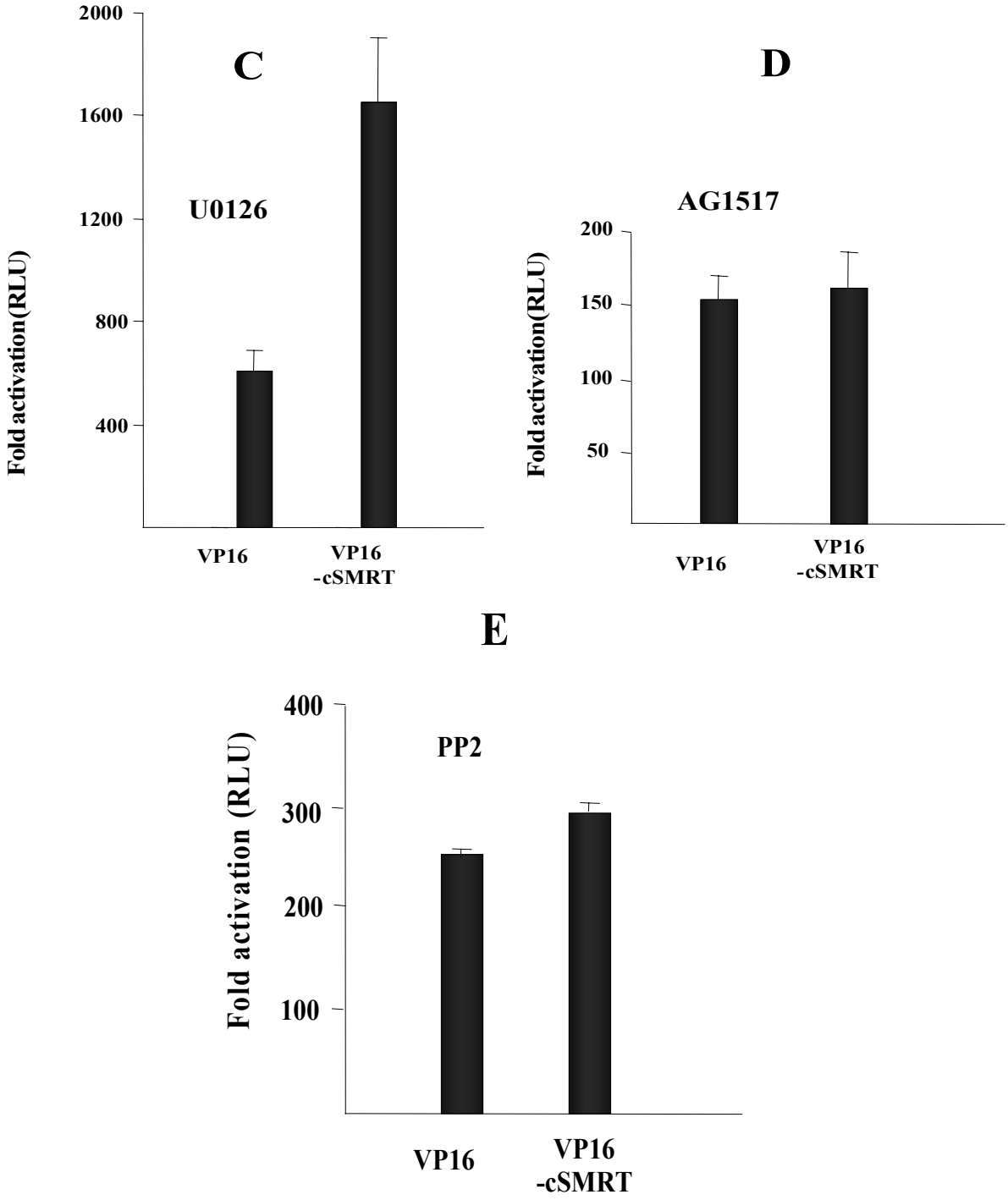


Fig. 5.11 *in vivo* interaction of corepressor SMRT with endogenous AR is regulated by signal transduction pathway in a CPA-dependent manner in C4-2 cells. 200.000 C4-2 cells were seeded out in androgen-deprived T-media and were transfected with MMTV-luc (1 µg), 0.22 µg of empty vector VP16 or VP16-cSMRT. pCMX-LacZ (0.4 µg) was used as a internal control. 16 hrs after transfection media was changed with androgen-deprived fresh RPMI 1640 media and were treated with CPA (10^{-7} M) alone (A), or cotreated with PRK inhibitor HA1077 1 µM, (B), MAPK inhibitor U0126 1 µM (C), tyrosine kinase inhibitor AG1517 1 µM (D) and Src inhibitor PP2 also 1 µM (E) for a period of 72 hrs. Cells were lysed and luciferase values were measured. Experiment was conducted in triplets and values obtained for VP16 empty vector in the absence of any inhibitor were arbitrarily set as 1. The graph represents the activation of reporter expression as LacZ normalized relative luciferase units.

Intriguingly, PRK1 inhibitor HA1077 did not alter interaction of SMRT with AR *in vivo* (Fig. 5.11 B) However, the same inhibitor promoted interaction of SMRT with AR in LNCaP cells (fig 5.10 B). Similarly MAPK inhibitor U0126 did not modulate interaction of SMRT with AR in C4-2 cells (Fig. 5.11 C). Interestingly receptor tyrosine-kinase inhibitor AG1517 and non-receptor tyrosine kinase inhibitor PP2, both led to loss of SMRT interaction in C4-2 cells (Fig. 5.11 D & E) presumably by functionally inactivating SMRT interaction with AR through a phosphorylation-mediated mechanism. This further emphasizes the differential behaviour of signaling kinase in regulating corepressor interaction with AR in cell type-specific manner.

5.5 Characterisation of LCoR as a transcriptional corepressor for AR

5.5.1 LCoR represses wt AR in a ligand-dependent manner

LCoR was identified as an interaction partner in a screen for protein that interacted with the LBD of ERα (Fernandes et al., 2003). Transient transfection experiments were performed to test the ability of LCoR to modulate AR function. Report suggests that AR bound to various ligands shows different functionality in terms of its interaction with corepressor molecules (Dotzlaw et al., 2002) by allowing a slightly different conformation of AR LBD. Whether wt-LCoR is able to repress wt-AR transactivation function under the influence of various ligands was therefore tested. Activated AR by synthetic agonist R1881, partial antagonist CPA and natural hormone DHT as drastically repressed upon cotransfection of LCoR suggesting that LCoR can act as AR corepressor not only in an agonist-dependent manner (Fig. 5.12) but also in a partial agonist CPA-dependent manner. Cas and OH-F which act as pure antagonist for wt-AR did not allow higher transactivation by wt-AR and therefore LCoR-mediated transcriptional corepression on wt-AR can not be envisaged.

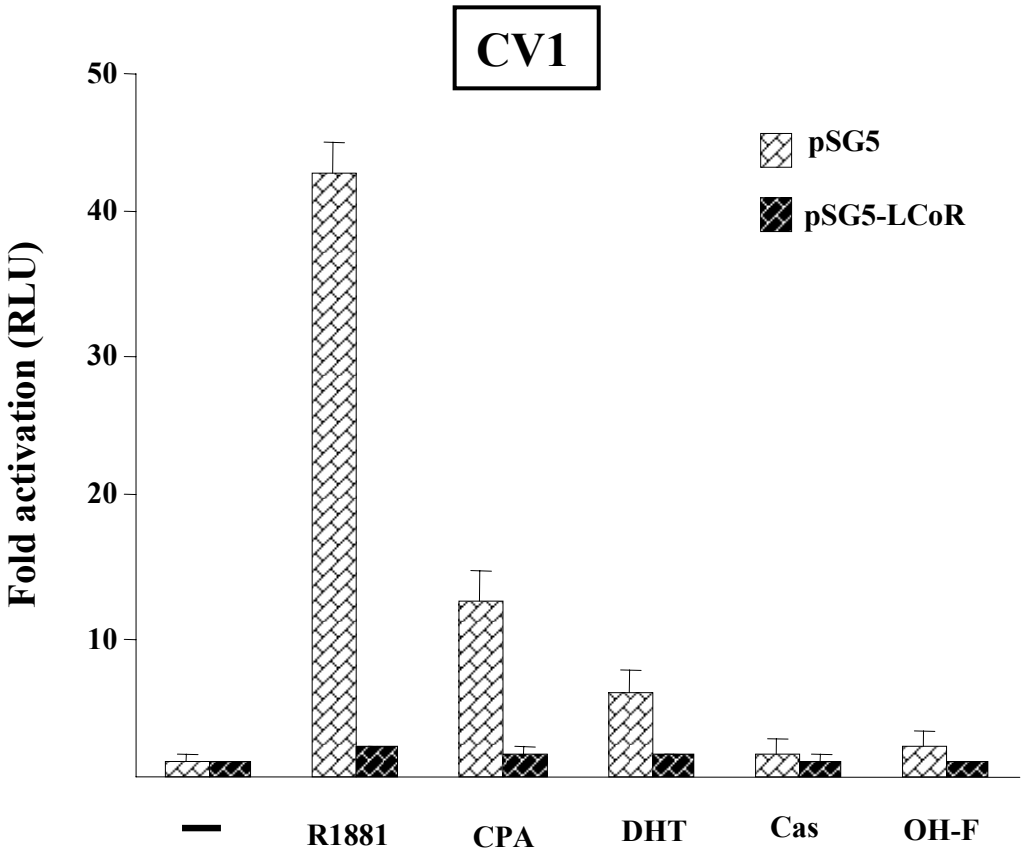
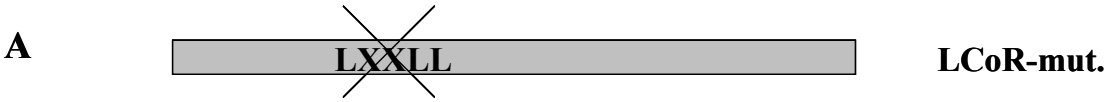


Fig. 5.12 wt-LCoR represses wt-AR in a ligand-dependent fashion. 90.000 CV1 were seeded out in androgen deprived DMEM media and cells were transfected with pMMTV-Luc (1 µg), hAR (0.2 µg) and 1 µg each of pSG5 (control) and pSG5-LCoR. 0.4 µg pCMV-LacZ was used as an internal control. 16 hrs post-transfection, cells were treated with R1881 (10^{-10} M), CPA (10^{-7} M), DHT (10^{-7} M), Cas (10^{-7} M) or OH-F (10^{-7} M) for 72 hrs following which cells were lysed, luciferase measurement was done and graph was plotted arbitrarily setting control in the absence of hormone as 1. The experiments were conducted in triplets and the graph represents fold reporter activation as LacZ normalised relative luciferase units.

LCoR has been shown to repress GR, PR and ER by interacting with their LBDs involving its NR box and a mutant form of LCoR where its NR box with sequence motif LXXLL was exchanged with LSKAA (called LCoR-mut, fig. 5.12 A) led to loss of



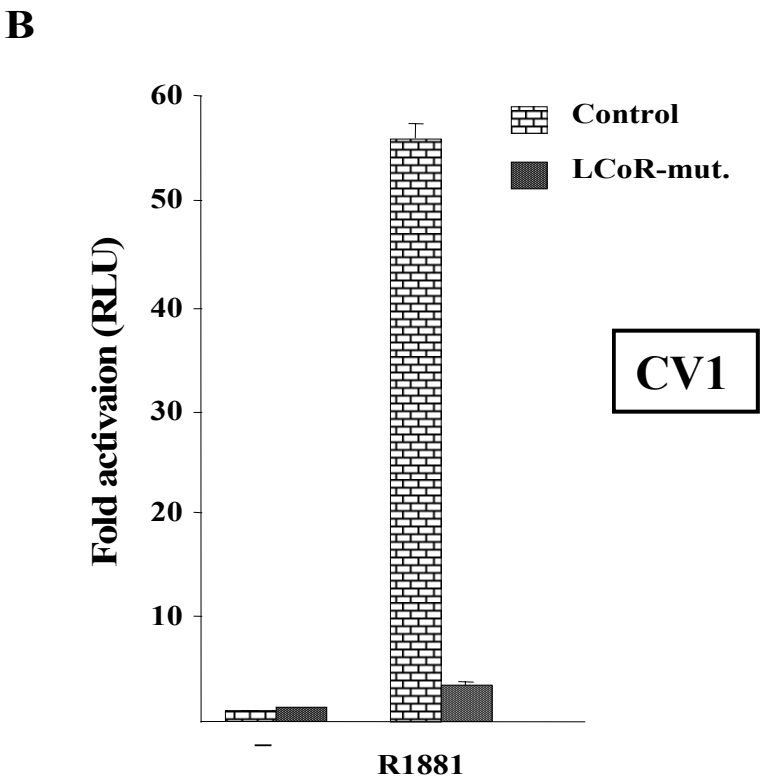


Fig. 5.13 LCoR-mut represses wt-AR in a ligand-dependent fashion. (A) Graphical representation of LCoR NR box and LCoR-mut. (B) 90.000 CV1 were seeded out in androgen deprived DMEM media and cells were transfected with pMMTV-Luc (1 µg), hAR (0.2 µg) and 1 µg each of pSG5 (control) and pSG5-LCoR. 0.4 µg pCMV-LacZ was used as an internal control. 16 hrs post-transfection, cells were treated with R1881 (10^{-10} M) for 72 hrs following which cells were lysed, luciferase measurement was done and graph was plotted arbitrarily setting control in the absence of hormone as 1. The experiments were conducted in triplets and the graph represents fold reporter activation as LacZ normalised relative luciferase units.

repression of these receptors (Fernandes et al., 2003) therefore LCoR-mut. was also tested for its ability to repress AR function. In fact, coexpression of LCoR-mut. leads to a robust decrease in AR transactivation in agonist-dependent manner (Fig. 5.13 B).

To determine the minimal concentration of the corepressor with apparently no or very little corepressor function transient transfection was performed. Intriguingly however, cotransfection of as little LCoR plasmid as 250 ng was sufficient to sharply decrease AR transactivation to more than ~50 fold on MMTV promoter in an agonist-dependent fashion (fig 5.14). This suggests that LCoR poses a “super repression” on agonist-activated AR function.

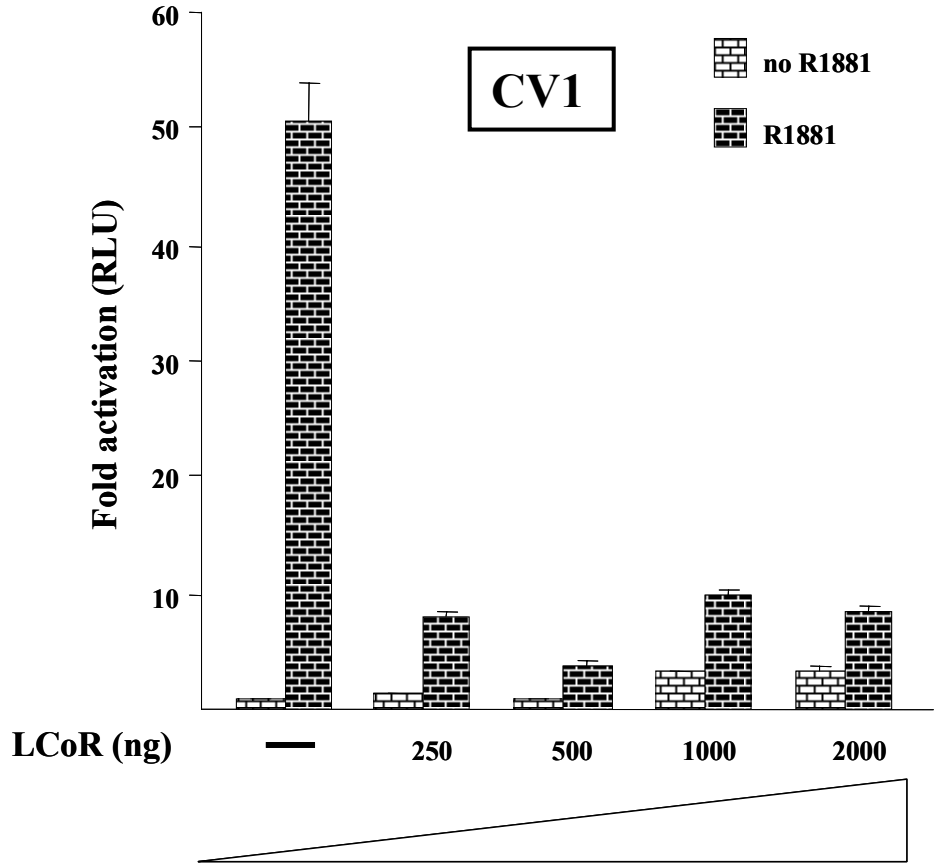


Fig. 5.14 wt-LCoR acts as a transcriptional corepressor for AR. 100.000 CV1 cells seeded out in hormone deprived DMEM media and were transfected with pMMTV-Luc (1 µg), wt-AR (0.2 µg) and varying amount of LCoR shown. 16 hrs post-transfection, media was exchanged with the fresh media and cells were treated with synthetic agonist R1881 (10^{-10} M) for 72 hrs. Cells were harvested, luciferase measurement was done and graph was plotted arbitrarily setting values obtained for pSG5 in the absence of hormone as 1. The experiment was conducted in triplets and the graph represents fold reporter activation.

The agonist-independent enhancement of basal luc values for 1 and 2 µg of LCoR suggests that higher amounts of this corepressor though not able to interact and repress AR, can nevertheless, squelch some transcription repressor proteins from repressing basal AR transactivation in the absence of ligand leading to the higher basal values in the absence of ligand.

5.5.2 LCoR also acts as a transcriptional corepressor for mutant ARs in CV1 cells

Because many CaP cells, including LNCaP and C4-2, harbour a mutant form of AR-T877A bearing mutation in the LBD and has been shown recently to positively stimulate

the growth of CaP cells (Sun et al., 2006). It is possible that in addition to broadened ligand spectrum this mutant may not be repressed by corepressors hence promoting cell growth. Therefore transient transfection experiments were carried out to determine whether wt-LCoR is able to repress T877A mutant of AR *in vivo*. Similar results were obtained as for

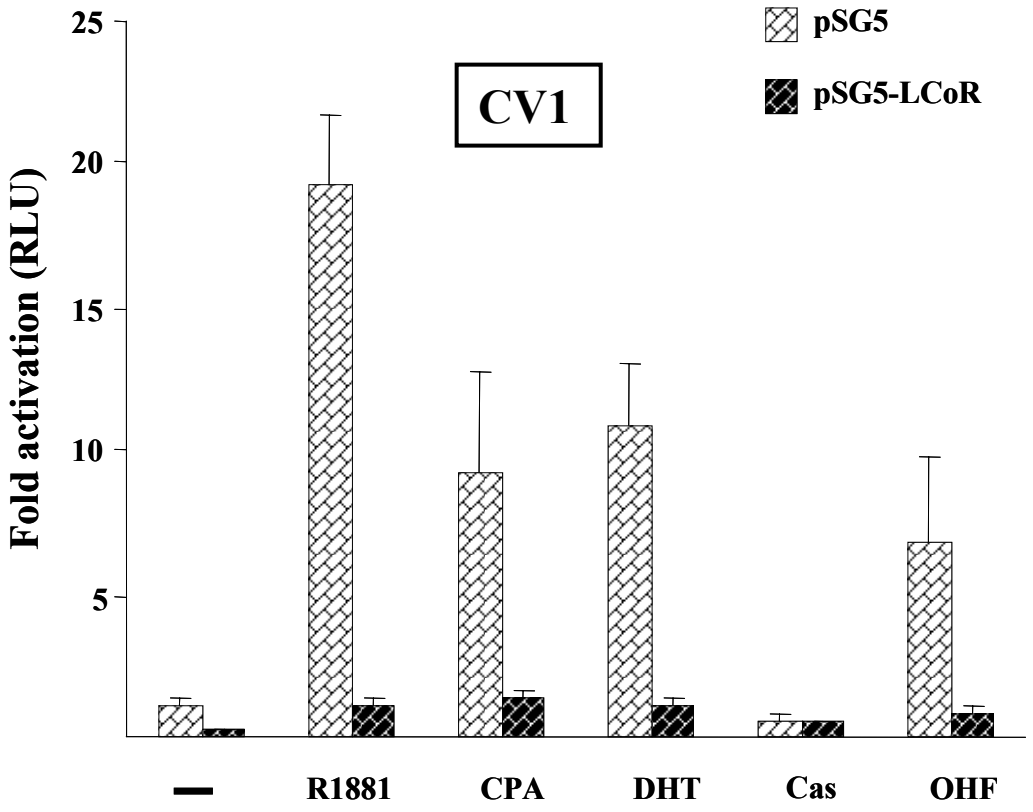


Fig. 5.15 LCoR-wt represses ligand-activated T877A mutant of AR in a ligand-dependent fashion. 90.000 CV1 were seeded out in androgen deprived DMEM media and cells were transfected with pMMTV-Luc (1 µg), hAR (0.2 µg) and 1 µg each of pSG5 (control) and pSG5-LCoR. 0.4 µg pCMV-LacZ was used as an internal control. 16 hrs post-transfection, cells were treated with R1881 (10^{-10} M), CPA (10^{-7} M), DHT (10^{-7} M), Cas (10^{-7} M) or OH-F (10^{-7} M) for 72 hrs following which cells were lysed, luciferase measurement was done and graph was plotted arbitrarily setting avg. values obtained for pSG5 in the absence of hormone as 1. The experiments were conducted in triplets and the graph represents fold reporter activation as LacZ normalised relative luciferase units.

wt-AR suggesting that T877A mutant activated by agonists R1881 and DHT and partial agonist CPA can be repressed by wt-LCoR in ligand-dependent fashion. In contrast, this mutant activated by OH-F, which acts as a partial agonist for T877A mutant can also be repressed by LCoR (Fig. 5.15). These data suggest that LCoR-mediated repression may be independent of the AR conformation attained in presence of various ligands and therefore allows repression by LCoR in presence of various AR ligands in CV1 cells. Agonist-bound AR is known in general, to bind coactivators which eventually lead to gene expression. Agonist-dependent repression of LCoR suggests that LCoR may be a part of negative

feedback loop in AR transactivation presumably by competing with coactivators and thereby fine-regulation the AR transactivation.

Next, a double point mutant of AR, which shows very strong transactivation in response to ligand (called AR SUMO mutant, K385E, K518E) (Poukka et al., 2000) which does not interact with and thereby not repressed by corepressor SMRT and Alien (Dotzlaw et al., 2002), was tested for LCoR mediated repression. wt-LCoR represses AR SUMO mutant-

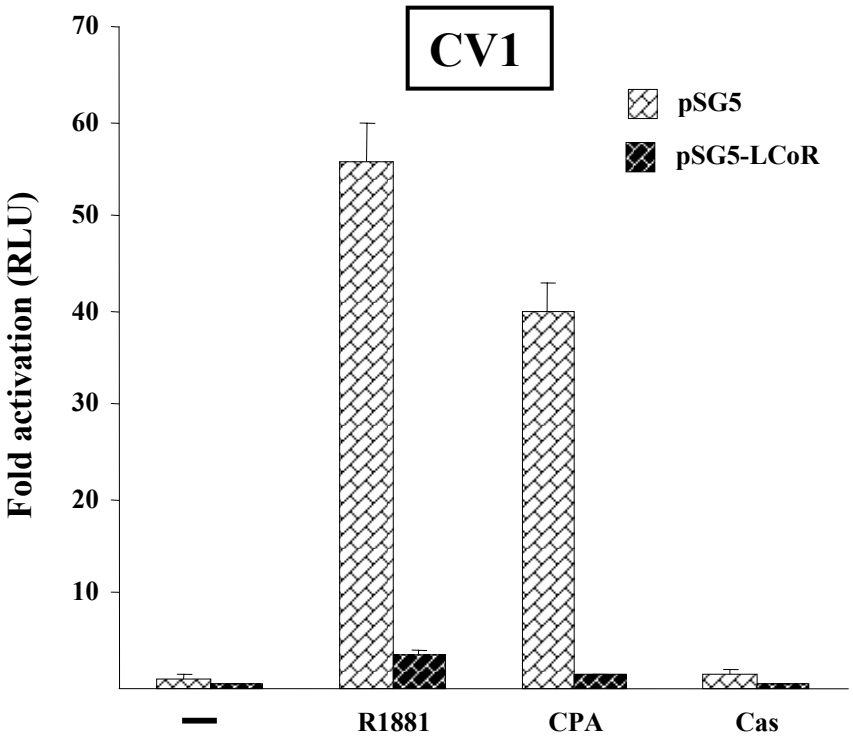


Fig. 5.16 wt-LCoR represses AR-Sumo mutant in a ligand-dependent fashion. 100.000 CV1 were seeded out in androgen deprived DMEM media and cells were transfected with pMMTV-Luc (1 µg), hAR (0.2 µg) and 1 µg each of pSG5 (empty vector control) and pSG5-LCoR. 16 hrs post-transfection, cells were treated with R1881 (10^{-10} M), CPA (10^{-7} M), DHT (10^{-7} M), Cas (10^{-7} M) or OH-F (10^{-7} M) for 72 hrs following which cells were lysed, luciferase measurement was done and graph was plotted arbitrarily setting avg. values obtained for empty vector control in the absence of hormone as 1. The experiments was conducted in triplets and the graph represents fold reporter activation.

mediated transactivation *in vivo* (Fig. 5.16), raising the possibility that LCoR can indeed interact with the AR mutant in a ligand-dependent fashion and that this interaction is not dependent on point mutation in AR NTD or LCoR can potentially target another domain of AR for its interaction. This indicates that LCoR differs from other corepressors in that it is able to repress AR function in a hormone-dependent fashion, may also target an entirely different domain of AR for mediating its repression.

5.5.3 LCoR interacts with AR in an agonist-dependent manner *in vivo*

To demonstrate that repression of AR by LCoR involves its direct *in vivo* interaction with agonist-bound AR on its target elements, modified mammalian-one-hybrid assay was performed. Our unpublished results (Michaela Patz, Diploma thesis) suggested that the CPA-induced binding of LCoR with AR does not require its NR box; rather AR interacts via C-terminus of LCoR, which harbours an HLH interaction motif.

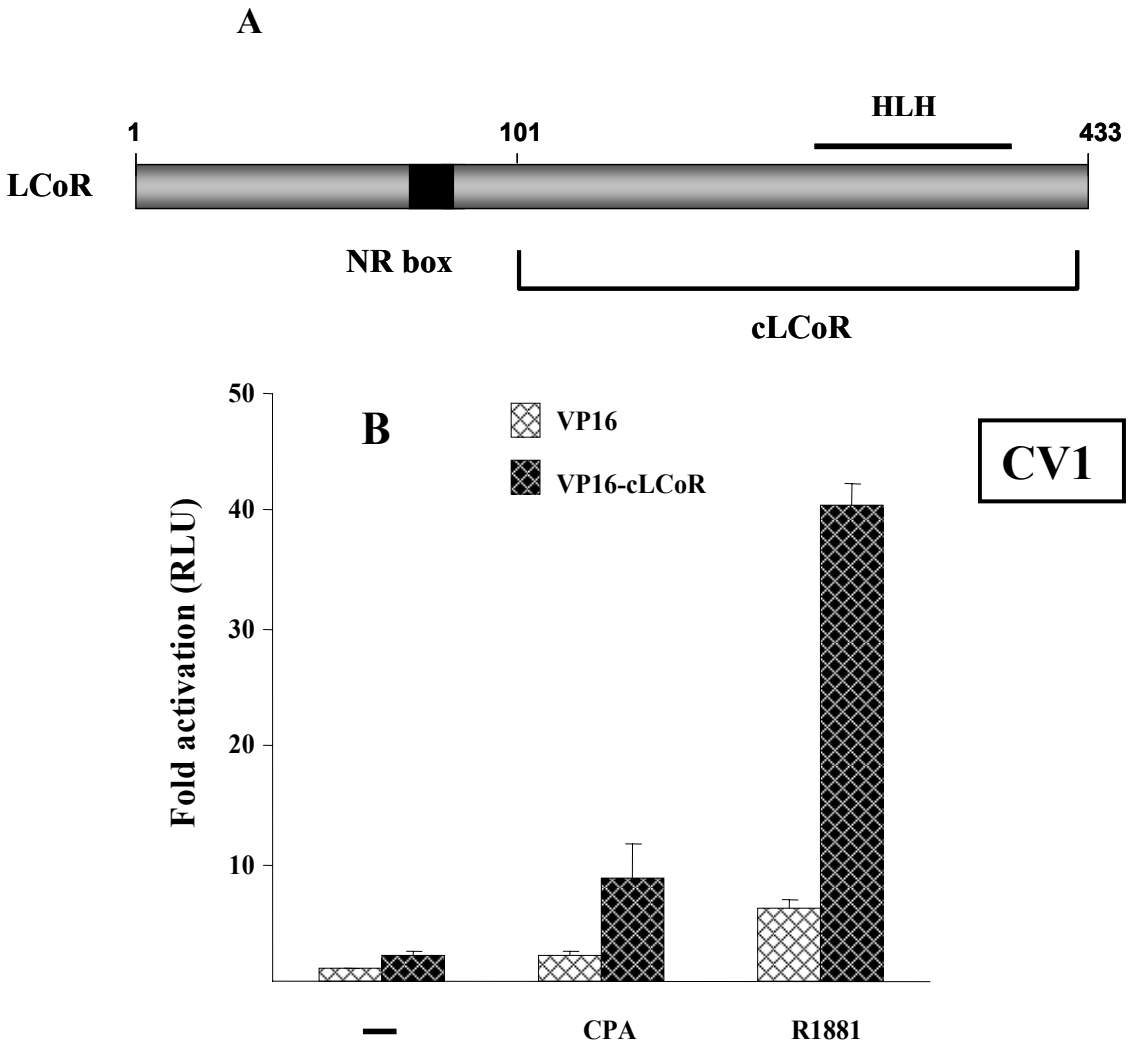


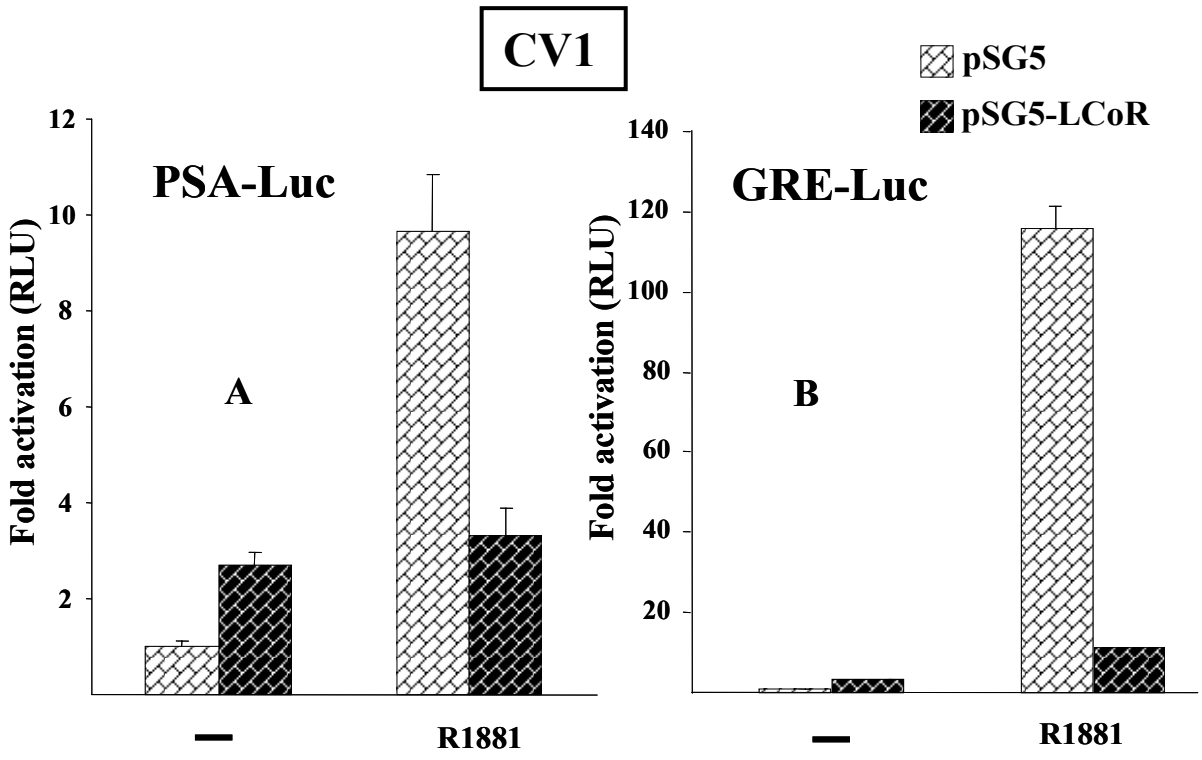
Fig. 5.17 LCoR interacts with AR in partial agonist CPA- and agonist R1881-depdendent manner. (A) LCoR is a 433 aa residue protein. It has a single NR box for interaction with selected members of nuclear hormone receptor superfamily. A centrally situated region is responsible for its interaction with HDACs, and another CtBP binding motif is also present. The C-terminal part of protein harbours a HLH interaction motif. VP16-cLCoR was constructed by cloning last 332 C-terminus aa into pCMX-VP16 empty vector. (B) 100.000 CV1 cells were seeded out in androgen-deprived DMEM were transfected with pMMTV-Luc (1 µg), pCMX-VP16-cLCoR (2 µg) and hAR (50 ng). 16 hrs post-transfection, fresh media was given and cells were treated with CPA (10^{-7} M) or R1881 (10^{-10} M) for 72 hrs. Cells were harvested and luciferase measurements were done and graph was plotted arbitrarily setting avg. values obtained for empty vector control VP16 in the

absence of hormone as 1. The experiments was conducted in triplets and the graph represents fold reporter activation.

Previous report (Fernandes et al., 2003) suggested that LCoR functions as a corepressor in agonist-dependent manner for ER, PR etc. It was found (in previous section) that LCoR acts as a corepressor for R1881-bound AR as well, therefore the mammalian-one-hybrid was aimed at finding whether VP16-cLCoR is able to interact with R1881-bound wt-AR was performed. AR shows 7-fold interaction with cLCoR over empty vector VP16 in a R1881-depdendent fashion (Fig. 5.17). VP16-cLCoR also interacts with CPA-bound AR, used here as a positive control. Taken together, these results indicate, that in order to repress AR, LCoR directly interacts *in vivo* with AR in a ligand-dependent manner, an essential prerequisite for a protein to fulfill the criteria to function as a corepressor.

5.5.4 Response element specificity of LCoR-mediated AR repression

PSA and probasin genes are known to be activated by functional AR signaling (Zhang et al., 2000 and Cleutjens et al., 1997). Therefore it was important to test the effect of LCoR mediated AR repression in context of natural chromatin of AR target genes.



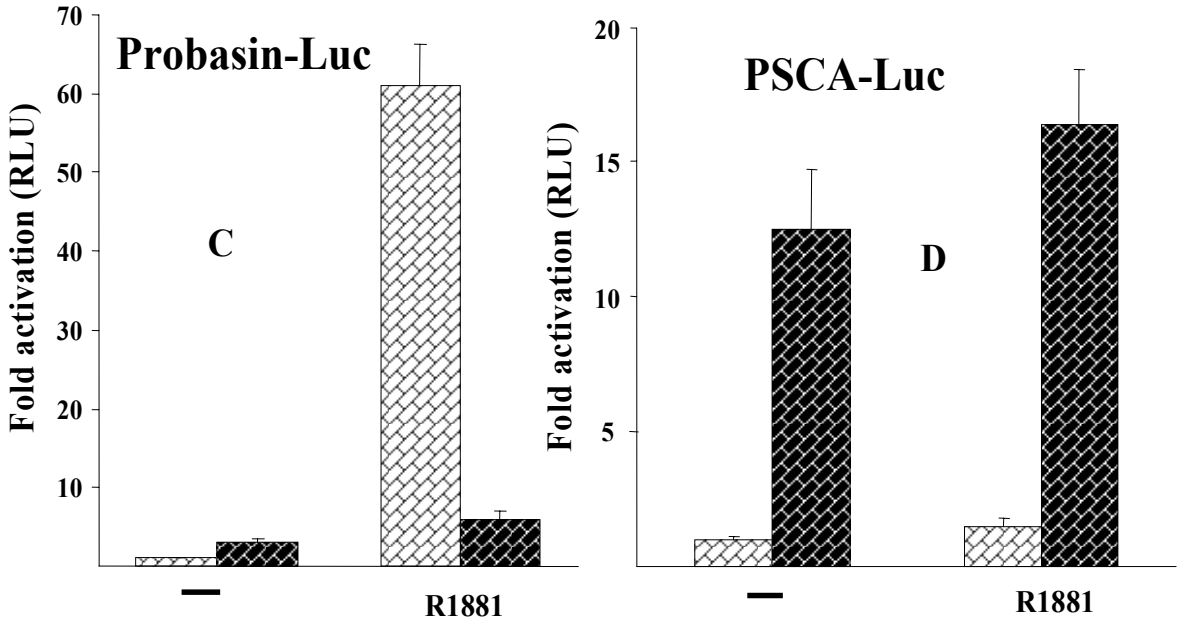


Fig. 5.18 Response element specificity of LCoR mediated repression of AR. 100.000 CV1 cells were seeded out in androgen-deprived DMEM media and cells were transfected with reporter plasmids (1 µg each) of PSA-Luc, Probasin-Luc, GRE-tk2-Luc and PSCA (2,7-3.0)- Luc along with 0.2 µg of hAR and 1 µg of pSG5 or pSG5-LCoR. 16 hrs post transfection, media was changed and cells were treated with 10^{-10} M of R1881 for 72 hrs. Cells were lysed and luciferase units were measured. (A) LCoR shows agonist-dependent repression of PSA promoter. (B) GRE-tk2-Luc, an artificial response element which is responsive to members of nuclear hormone receptor superfamily is also repressed in agonist-dependent fashion when LCoR is expressed. (C) androgen-responsive mouse probasin promoter is also repressed by LCoR in agonist-dependent fashion. (D) PSCA promoter activation by LCoR. Graph was plotted arbitrarily setting values in the absence of hormone as 1 for each reporter. The experiments was conducted in triplets and the graph represents fold reporter activation.

PSA gene codes for prostate specific antigen in humans and is overexpressed in conditions like benign prostate hyperplasia and CaP and therefore is widely used not only for the diagnosis of the disease but also to monitor the response of anti-hormone therapy which decreases PSA expression in the initial phase of the treatment.

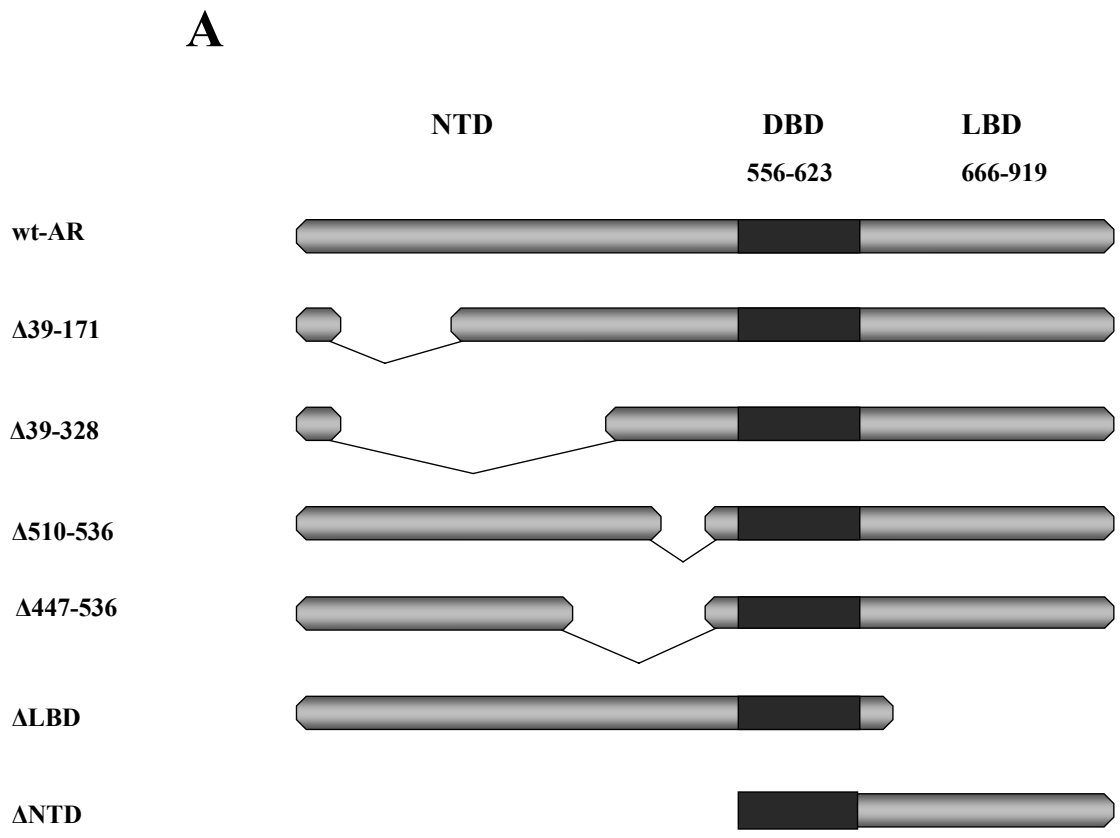
AR transactivation potential on natural promoters PSA and probasin is significantly repressed by the coexpression of LCoR in a hormone-dependent manner (fig 5.18 A & C) which highlights the physiological relevance of LCoR to repress AR transactivation, which may hence play a protective role against the disease by repressing AR mediated gene activation and thereby the growth of CaP. GRE-tk2-Luc, an artificial promoter which is responsive to androgens in addition to glucocorticoids is also repressed by LCoR in a ligand-dependent fashion (Fig. 5.18 B). Interestingly another AR responsive gene *Prostate stem cell antigen* (PSCA) which is known to be activated by androgens (Jain et al., 2002) shows enhanced transcription by LCoR independent of hormone (Fig. 5.18 D), suggesting

that there exists promoter specificity in repression of AR by LCoR. These results are contrary to the corepression effect on LCoR seen on other elements and on other members of NHR (Fernandes et al., 2003). It is known that androgen receptor-mediated expression of target gene often involves other -cis elements in addition to AREs as Sp1 and therefore it is possible that the minimal PSCA promoter containing 300 bp upstream of transcription start site including ARE is not in itself sufficient to be inducible by AR and therefore AR may be recruited to this minimal element without exhibiting a strong induction by hormone. Indeed transient transfections carried out in CV1 cells with this minimal promoter element barely show hormone induction by DHT and only ~2 fold hormone induction by synthetic agonist R1881. Presumably therefore, cotransfection of LCoR may squelch transcription inhibitory molecules, which are recruited to a feeble functioning AR allowing AR to show a little transactivity on this minimal element without hormone suggesting that in this particular context LCoR-mediated effect is only an artifact.

5.5.5 LCoR-mediated repression does not involves C or N terminus of AR

Corepressors are regulatory proteins that, though by themselves cannot bind to target DNA directly, rather can influence the function of NHRs by interacting with various AR domains (Baniahmad 2005). e.g., corepressor SMRT targets NTD of CPA-bound AR (Dotzlaw et al., 2002), however, other set of corepressors interact with the LBD of AR e.g. hRad9 etc. (Wang et al., 2004). In order to interact with NHRs, SMRT and NCoR use a “CoRNR”motif with consensus LXXI/LXXXI/L. This is similar in sequence with “NR” box of coactivator proteins (Hu and Lazar, 1999). Binding of antagonist exposes a hydrophobic cleft in the helix-12 situated in the LBD, which allows the binding of corepressors carrying such kind of hydrophobic motif. LCoR also has a motif similar to LXXLL motif of coactivator proteins and indeed known to interact with many members of NHR superfamily (Fernandes et al., 2003) via their LBD. Therefore, the binding with NHRs is disrupted by mutations in this motif called LSKAA resulting in loss of repression (Fernandes et al., 2003). Results suggest that LCoR with mutant LXXLL motif is able to repress AR transactivation to the same degree as observed by the wt-LCoR in a hormone-dependent manner (Fig. 5.13 B). Various deletion mutants of LCoR were therefore tested for their ability to interact with AR (Michaela Patz, Diploma thesis). These experiments suggested that C-terminus aa 101-end, which excludes LXXLL motif is involved in its interaction with AR in receptor-negative CV1 cells. In fact the C-terminus of LCoR contains a protein-protein interaction HLH (Helix-loop-helix) motif suggesting that LCoR

may use HLH motif to interact with AR. In order to map down the domain of AR that facilitates its interaction with LCoR various AR deletion mutants lacking one or few functions/domains were used. Various truncations of the N-terminus as well as NTD-deleted AR mutants were used to map down part of AR protein that interacts with LCoR in a hormone-dependent manner. These truncations included Δ39-171 that deletes part of AF1 ligand-independent transactivation function, Δ39-328 which excludes the N-terminal part lacking part of AF1 and TAU5 activation functions. Two other NTD deletions, Δ510-536 and Δ 447-536 were also included. In addition, a C-terminal deletion which deletes LBD of AR was individually transfected along with reporter and AR expression vector.



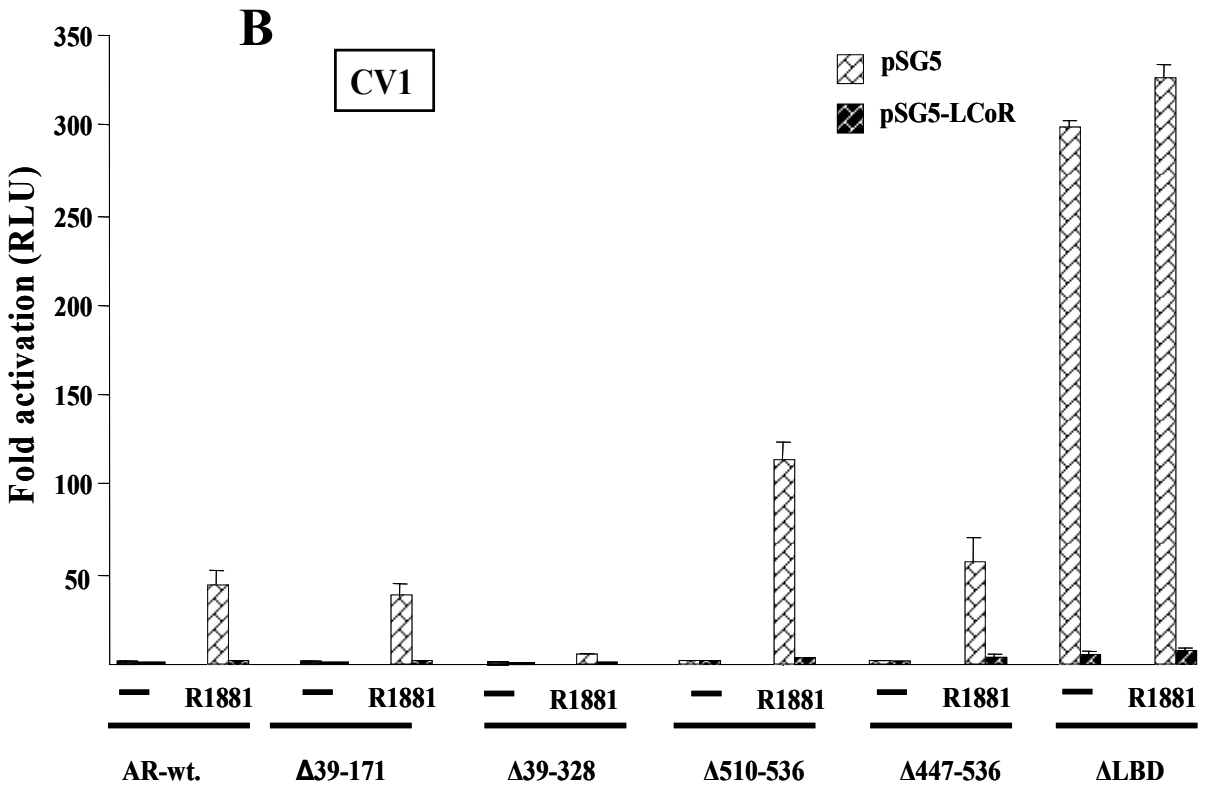


Fig. 5.19 LCoR does not interact with LBD or NTD of the AR in CV1 cells. (A) Various AR mutants used to map down interaction domain with LCoR are shown. (B) Cells were transfected with pMMTV-Luc (1 µg) along with 1 µg each of pSG5 and pSG5-LCoR.wt. In addition 0.2 µg of various AR mutant expression plasmids that lead to truncated AR proteins were also transfected. 16 hrs post-transfection cells were treated with 10^{-10} M R1881 for 72 hrs. Afterwards, cells were harvested and luciferase activity was measured. Graph was plotted against the arbitrarily set value of 1 for control pSG5 obtained in the absence of ligand. "Δ" indicates the part of the AR proteins deducted from the functional protein.

As evident from the result, f.l. wt-LCoR was able to repress various AR N-terminus truncations (Fig. 5.19 B). These truncations included part of AF1 function and TAU-5 function, which play decisive role in transactivation imparted by NTD. Similarly, AR truncation devoid of the complete LBD was also repressed significantly by coexpression of f.l. wt-LCoR. Results indicate that various N- and C-terminal deletions were repressed by wt-LCoR. It raises the possibility that LCoR may have two different interaction motifs for interaction with AR. (1) By its LXXLL motif it can bind to the LBD of AR as it binds to ER, PR, VDR and GR (Fernandes et al., 2003) and (2) and by the C-terminus which harbours HLH motif, it may interact with some other domain of the AR. The first possibility was ruled out previously (fig 5.13 B) whereby LCoR-mut was able to repress wt-AR in a ligand-dependent manner. In order to rule out the possibility of bifacial interaction, similar experiments were performed LCoR mutant where LXXLL motif was replaced by LSKAA.

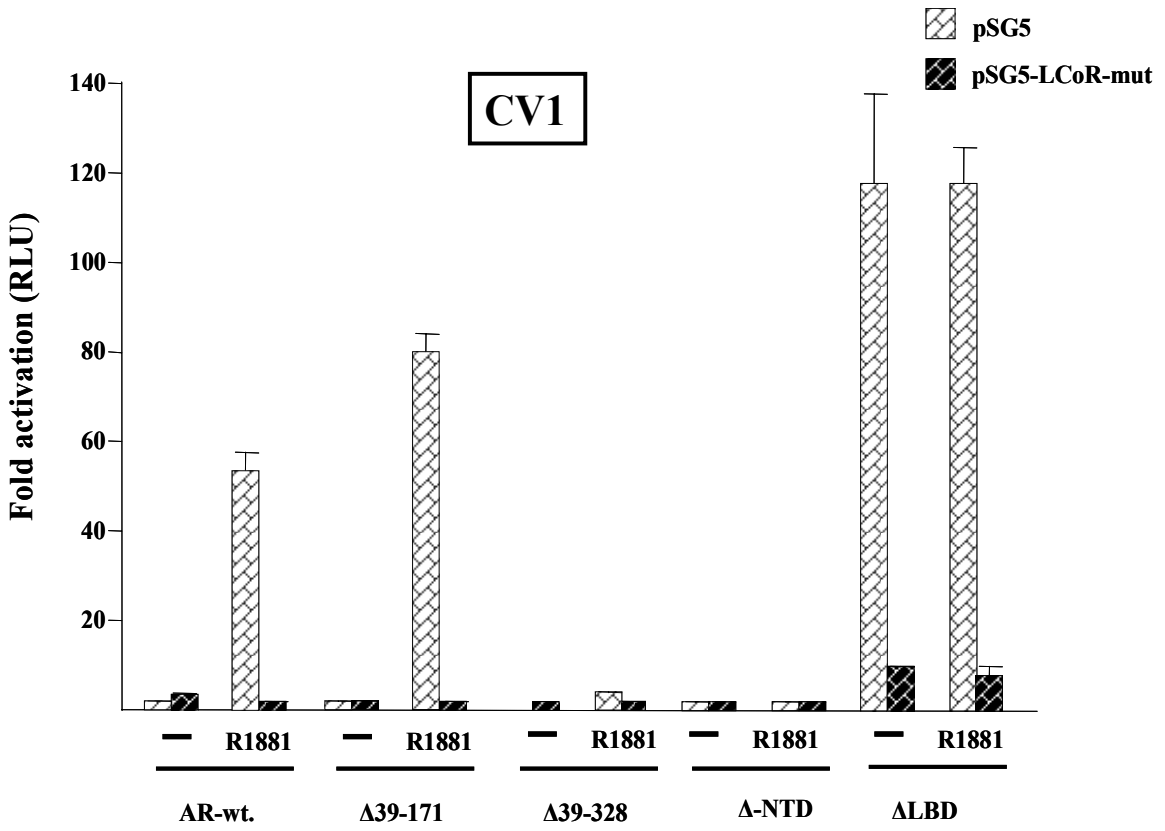


Fig 5.20 LCoR-mut does not interact with LBD or NTD of the AR in CV1 cells. 100.000 CV1 cells were seeded out in androgen-deprived DMEM media and were transfected with pMMTV-Luc (1 µg) along with 1 µg each of pSG5 and pSG5-LCoR.wt. In addition 0.2 µg of various AR mutants expression plasmids that lead to truncated AR proteins were also transfected. 16 hrs post-transfection cells were treated with 10^{-10} M R1881 for 72 hrs. Afterwards, cells were harvested and luciferase activity was measured. Graph was plotted against the arbitrarily set value of 1 for control pSG5 obtained in the absence of ligand. "Δ" indicates the part of the AR proteins deducted from the functional protein.

Surprisingly, various AR mutants were robustly repressed by LCoR-mut. as well (5.20), excluding the possibility of a bifacial mode of LCoR interaction with AR. Had there been a bifacial interaction of LCoR with AR, i.e., 1) Interaction with AR-LBD through its NR box, and 2) interaction with other domain(s) through its HLH motif, LCoR would have been unable to repress one of the mutant used in the above experiment. This means that interaction of LCoR with AR does not involve its "NR" box. These results indicate that a different domain of AR, (not LBD) possibly the DBD may be involved in interaction with both wt and mutant LCoR.

5.5.6 Mammalian-one-hybrid experiments demonstrate wt- and mutant LCoR target the DBD of AR in a hormone-dependent fashion

Above experiments, ruled out the possibility of the involvement of AR NTD or LBD as a target for LCoR interaction and pointed out towards the DBD of AR to be a potential target for interaction and repression by LCoR. Indeed, some coactivators have been shown to target the DBD of the AR in order to activate its function e.g., Ubc9 has been shown to activate AR function by targeting part of the DBD (Poukka et al., 1999). It is therefore, possible that corepressors can target also the same domain to modulate AR transactivation.
To test this possibility, first another mutant of AR, whereby the entire NTD was replaced by VP16- transactivation domain was tested for LCoR-mediated repression. This mutant was referred to as VP16-DBD-LBD. Cotransfection of this mutant together with LCoR wild type led to the repression of this AR construct in a hormone-dependent manner (Fig. 5.21 B), suggesting that NTD of AR does not play a role in LCoR-mediated repression. As LBD mutant of AR was robustly repressed both by LCoR-wt and LCoR-mut., this advocated the potential involvement of the DBD of AR in interaction with LCoR.
Therefore, to demonstrate the absolute involvement of the DBD in interacting with corepressor LCoR, a mutant was generated from the parent vector VP-AR-505 (referred in the text as VP16-DBD-LBD) by deleting the LBD, which led to the generation of VP16-AR-DBD, whereby the DBD of AR is expressed as a fusion protein with VP16 transactivation domain. This mutant was tested for LCoR-mediated repression in mammalian-one-hybrid experiments performed in CV1 cells. Briefly, cells were transfected either with VP16 empty vector or VP16-AR-DBD, along with reporter plasmid and with or without LCoR-wt or LCoR-mut. As evident, VP16 alone or in combination with LCoR-wt or LCoR-mut was incapable to drive the expression of reporter containing AREs (Fig. 5.22). Interestingly transfection of VP16-AR-DBD led to very robust enhancement of reporter activity attributed to the binding through DBD and transactivation mediated by VP16 transactivation domain. Cotransfection of LCoR-wt or LCoR-mut led to a dramatic decrease in reporter activity. Because of the lack of LBD any hormone induction by R1881 was not seen.

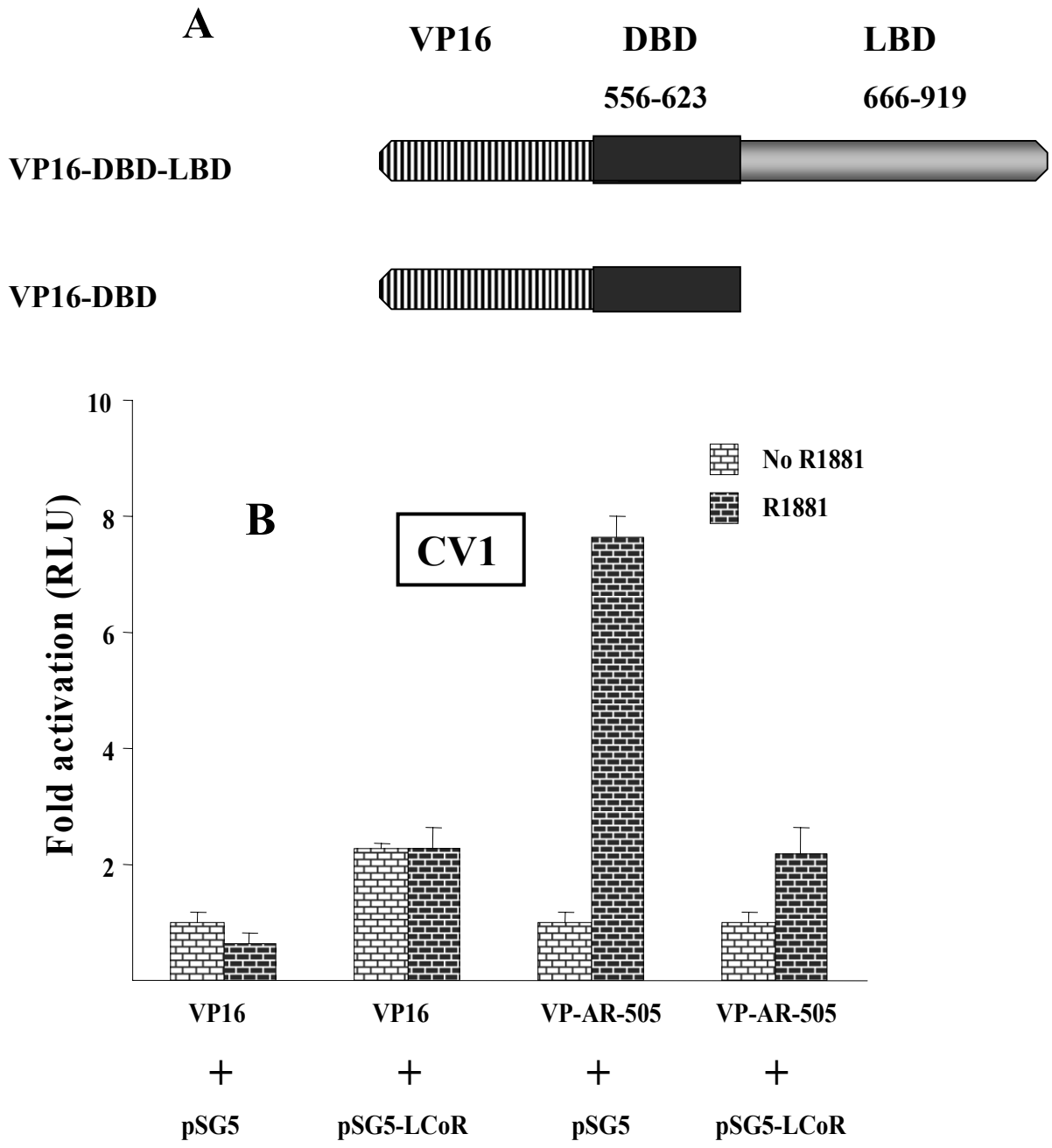


Fig. 5.21 LCoR represses the VP16-DBD-LBD mutant of AR in CV1 cells. (A) Graphical representation of VP16-AR fusion proteins. (B) 100.000 CV1 cells were seeded out in androgen-depleted DMEM and were transfected with pMMTV (1.0 µg), VP16 or VP-AR-505 (0.1 µg) and 1 µg each of either pSG5 or pSG5-LCoR. 16 hrs post transfection media was changed with fresh media and cells were incubated with R1881 (10^{-10} M) for 72 hrs. Afterwards, cells were harvested and luciferase activity was measured. Graph was plotted against the arbitrarily set value of 1 for control obtained in the absence of ligand.

This data clearly indicate that LCoR indeed targets the DBD of AR in order to interact and repress its transactivation function. Research demonstrate that many coregulatory proteins despite having motif similar to "NR" or "CoRNR" motif may not always bind to the AF2 hydrophobic cleft exposed in response to hormone binding such as coactivator SRC-1

which also interacts also in addition to the CAG repeats situated at the NTD of AR (Ma et al., 1999). In addition corepressor SMRT, having a CoRNR motif does target the NTD of the AR (Dotzlaw et al., 2002).

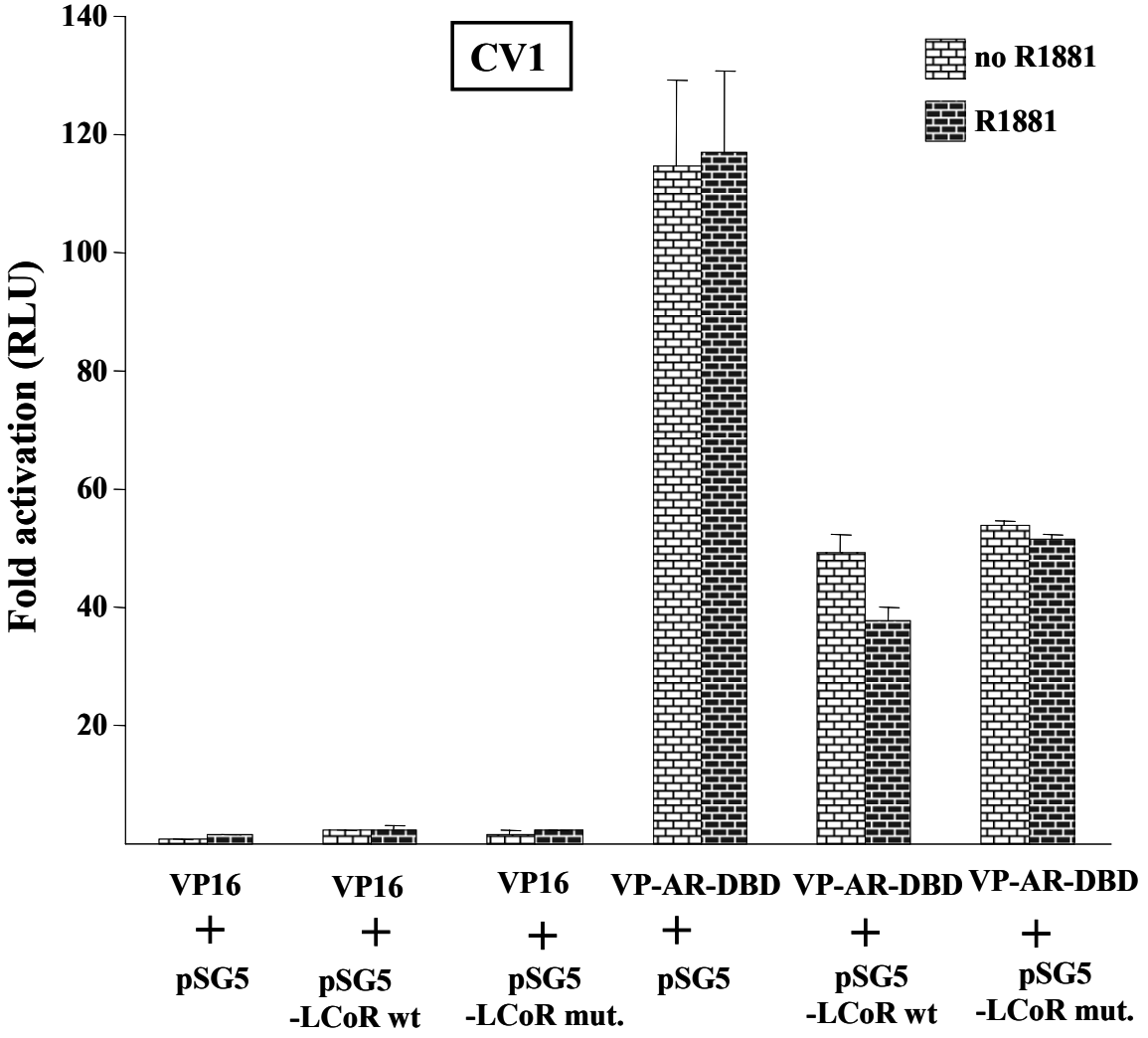


Fig. 5.22 Both wt-LCoR and LCoR-mut., target DBD of AR in CV1 cells. 100.000 CV1 cells were seeded out in androgen-depleted DMEM and were transfected with pMMTV (1.0 µg), VP16 or VP-AR-DBD (0.1 µg) and 1 µg each of either pSG5 or pSG5-LCoR. 16 hrs post transfection media was changed with fresh media and cells were incubated with R1881 (10^{-10} M) for 72 hrs. Afterwards, cells were harvested and luciferase activity was measured. Graph was plotted against the arbitrarily set value of 1 for cotransfected empty vector pSG5+VP16 obtained in the absence of ligand.

These experiments suggest a novel mode of AR repression by corepressor LCoR which differs from other members of NHR superfamily where LCoR targets the LBDs. This indicates that LCoR is equipped with multiple mode of interaction that varies in receptor context further adding intricacy to its repression function. These data also demonstrate,

however, that the repression of VP16-DBD alone by LCoR is not as potent as seen for wt- or other point mutant forms of AR. Individual corepressors are known to form complexes with each other e.g., Alien a corepressor for TR functionally interacts and forms complex with another corepressor Sin3A (Moehren et el., 2004). In the similar way, SMRT and NCoR are known to form complexes with each other, it is possible that LCoR can form silencing complex with other corepressors recruited to either NTD of LBD that synergises in repressing AR transactivation. It is also possible that VP16 acts as a much stronger transactivator compared to the NTD of AR and therefore cannot be completely repressed by LCoR hence not allowing it to completely repress the activated DBD-VP16.

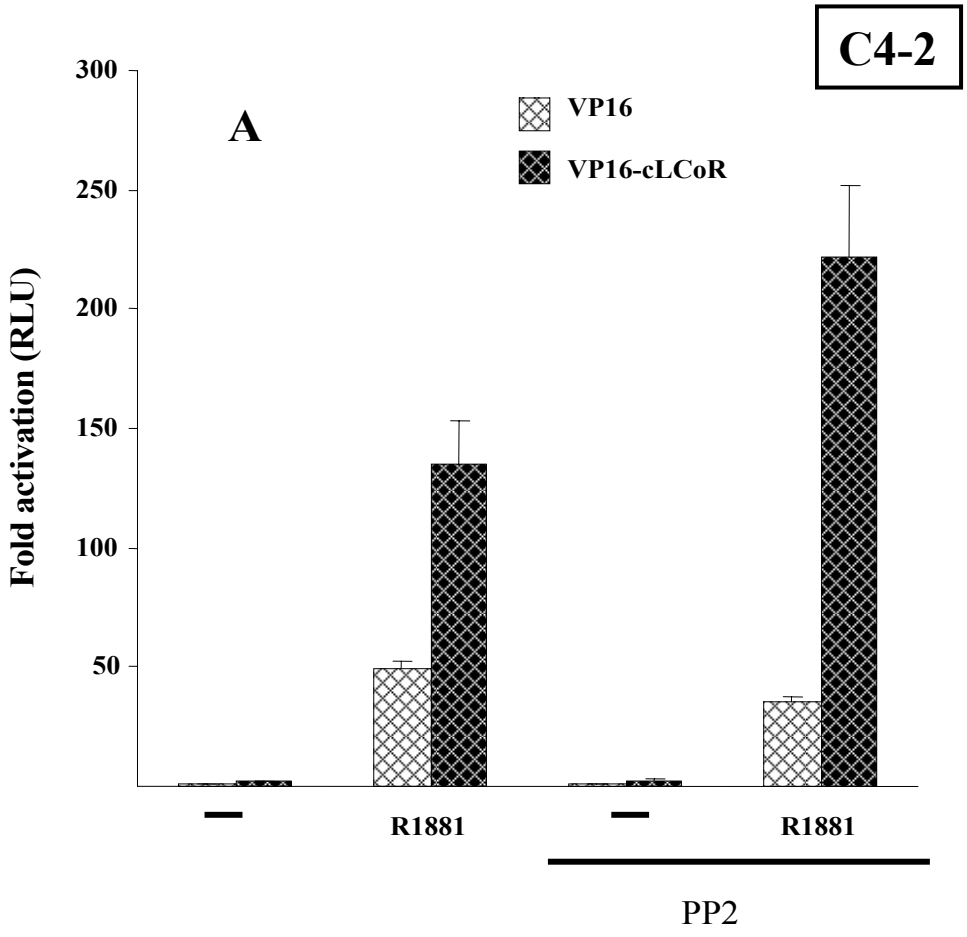
5.6 Functional attenuation of LCoR in CaP cells

Many coregulators are post-translationally modified that lead to modulation of their normal physiological function. LCoR has been shown to be expressed in CaP cells (Fernandes et al., 2003). However, CaP have been reported to show overexpression of AR signaling as reflected in PSA expression, a marker for CaP progression (Chen et al., 2004, Gregory et al., 2001, Jenster 2000, Viscorpi et al., 1995). The question arises how the presence of such a potent ligand-dependent corepressor in CaP is tolerated by CaP cells. It is possible as discussed in section 5.4 that LCoR is functionally attenuated by CaP cells using one or more strategies, similar to SMRT. That prompted to the investigation whether ectopically expressed LCoR is able to interact and thereby repress endogenous AR function in androgen-independently growing C4-2 cells.

5.6.1 Cell type-specific regulation of LCoR interaction with AR is modulated by Src signal transduction pathway

Results depicted in the previous section demonstrate that LCoR shows a robust repression of AR by interacting with it in a ligand-dependent manner in CV1 cells. The ability of LCoR to interact with endogenous AR in C4-2 cells was tested by performing mammalian-one-hybrid assay. C4-2 cells were transfected with VP16 empty vector or with VP16-cLCoR and MMTV-luc reporter. Intriguingly, these results demonstrate that the binding of LCoR to endogenous AR was dramatically reduced in C4-2 cells (Fig. 5.23 A) compared to the strong LCoR binding observed in CV1 cells (Fig. 5.17), a possible strategy adopted by cells to escape LCoR-mediated AR repression.

Many signal transduction pathways are overexpressed in androgen-independently growing cells which may potentially decrease this interaction in order to overcome AR repression. In C4-2 cells, introduction of dominant-negative Ras restores sensitivity to Casodex (Bakin et al., 2003). Similarly, treatment of C4-2 cells with Her2 tyrosine kinase inhibitor, AG825 leads to apoptosis in C4-2 cells but the same amount was not able to induce apoptosis in LNCaP cells indicating that overexpression of Her2 may provide androgen-independence to C4-2 cells. Therefore a battery of chemical inhibitors of specific signal transduction pathways was employed to test the effect of blockade of specific signaling pathway on interaction of AR with LCoR in C4-2 cells. As evident from the experiment blocking Src kinase signaling using its chemical inhibitor PP2 leads to enhanced interaction of LCoR with AR in C4-2 cells (Fig. 5.22 A). These results demonstrate that Src signaling functionally interferes with the binding of LCoR to AR *in vivo*. To test whether this



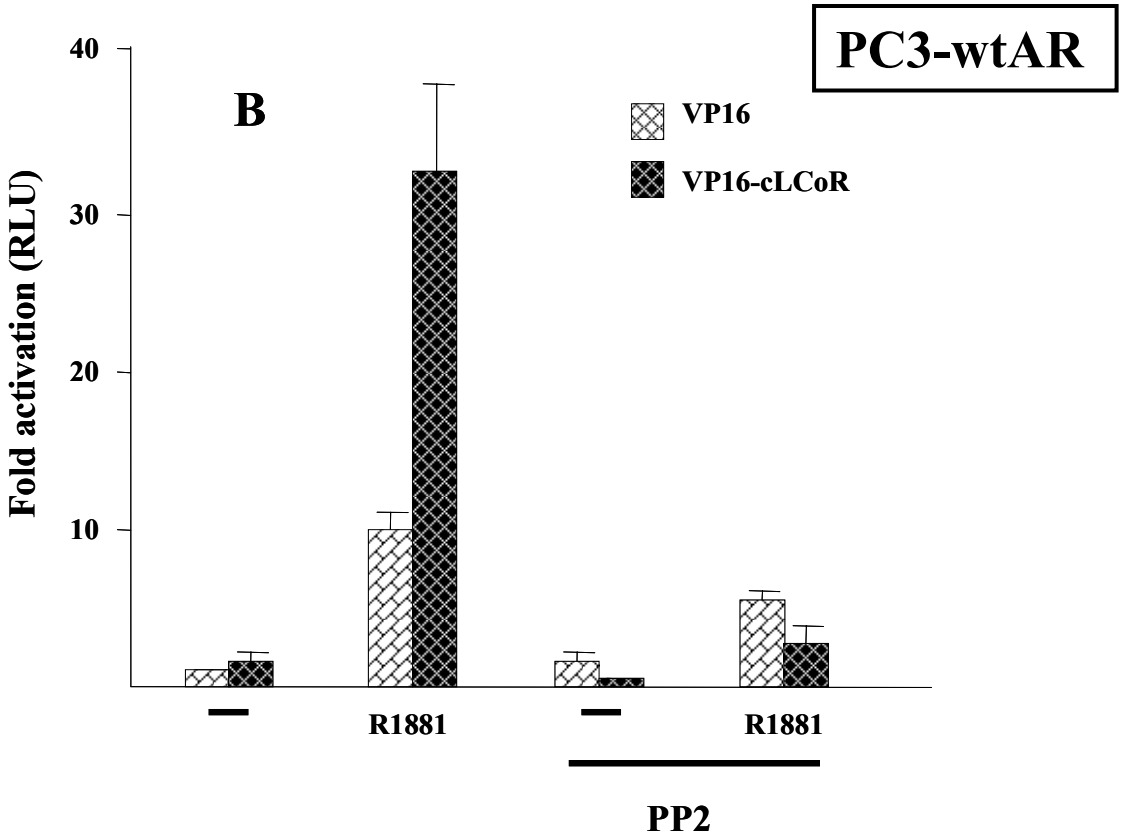


Fig. 5.23 Src kinase regulates interaction of LCoR with AR in CaP cells in a cell type-specific manner. (A) 300.000 C4-2 cells (B) 100.000 PC3-wt AR cells were seeded out in androgen-depleted T media and DMEM media respectively. Following day, cells were transfected with MMTV-Luc (1 µg), VP16 or VP16-cLCoR (1 µg for C4-2, 2 µg for PC3-wt AR) along with 0.4 µg of pCMV-LacZ which was used as an internal control. 16 hrs post transfection cells fresh media was given and cells were treated with R1881 (10^{-10} M) and Src inhibitor PP2 (1 µM) for 72 hrs. Afterwards, cells were harvested and luciferase measurements were done and graph was plotted arbitrarily setting each empty vector control VP16 in the absence of hormone as 1. The experiments were conducted in triplets and the graph represents fold reporter activation as LacZ normalised relative luciferase units.

Src-mediated decrease in LCoR interaction is a common strategy adopted by androgen-independently growing cells or is specifically harnessed by C4-2 cells, similar mammalian-one-hybrid assay was performed in PC3-wtAR CaP cells that grow in an androgen-independent manner. In PC3-wtAR cells, LCoR shows interaction with AR in a ligand-dependent manner (Fig. 5.23 B), similar to that seen in C4-2 cells, suggesting a marginal interaction in both cell lines. In contrast to enhanced interaction of LCoR observed with AR in C4-2 cells by Src blockade, treatment of transfected PC3-AR cells with PP2 led to loss of LCoR interaction with AR. These data indicate that decreased LCoR interaction with AR in C4-2 cells by Src kinase may be a cell type-specific phenomenon specifically

adopted by C4-2 cells, to escape LCoR-mediated repression, to allow loss of regulation of AR transactivation and thereby growth in an androgen-dependent fashion.

5.6.2 Regulation of LCoR autonomous silencing function in a cell-type specific manner

Many repressor proteins act as autonomous silencers to suppress transcription. LCoR has been shown to function as an autonomous transcription corepressor (Fernandes et al., 2003). It is possible that the repression function can be regulated in cell type-specific manner, a full length LCoR was fused to Gal-DBD to test its repressional effect on Gal-responsive promoter element (called p$(UAS)_4$-TATA-Luc). In addition other corepressors namely NCoR and Alien were also tested. Experiments were carried out in CV1 and in C4-2 cells. Results obtained from CV1 cells indicate that corepressor Alien shows 3-fold repression over control Gal empty vector however; corepressor NCoR and LCoR shows a potent repression function on the gal-responsive promoter (Fig. 5.24 A). This strong repression by LCoR explains largely its strong repressional effect seen on AR (Fig. 5.14) in a ligand-dependent manner in CV1 cells. Intriguingly however, similar experiment in C4-2 cells showed that LCoR repression function is severely compromised (Fig. 5.24 B). The observed decrease in LCoR repression potential may involve signal transduction cascades which may phosphorylate LCoR and decrease its repression function that may still allow LCoR to maintain its interacting function with AR but can compromise its silencing function. Similar decrease was observed in the repression potential of another corepressor NCoR in C4-2 cells. However, no change was observed in Alien repression potential in both cell lines. These results indicate a novel mechanism that CaP can utilise to weaken specific corepressors at multiple levels. In addition to decreasing the interaction of corepressor with AR, the repression potential can also be attenuated to minimise the effect of corepressor on the AR.

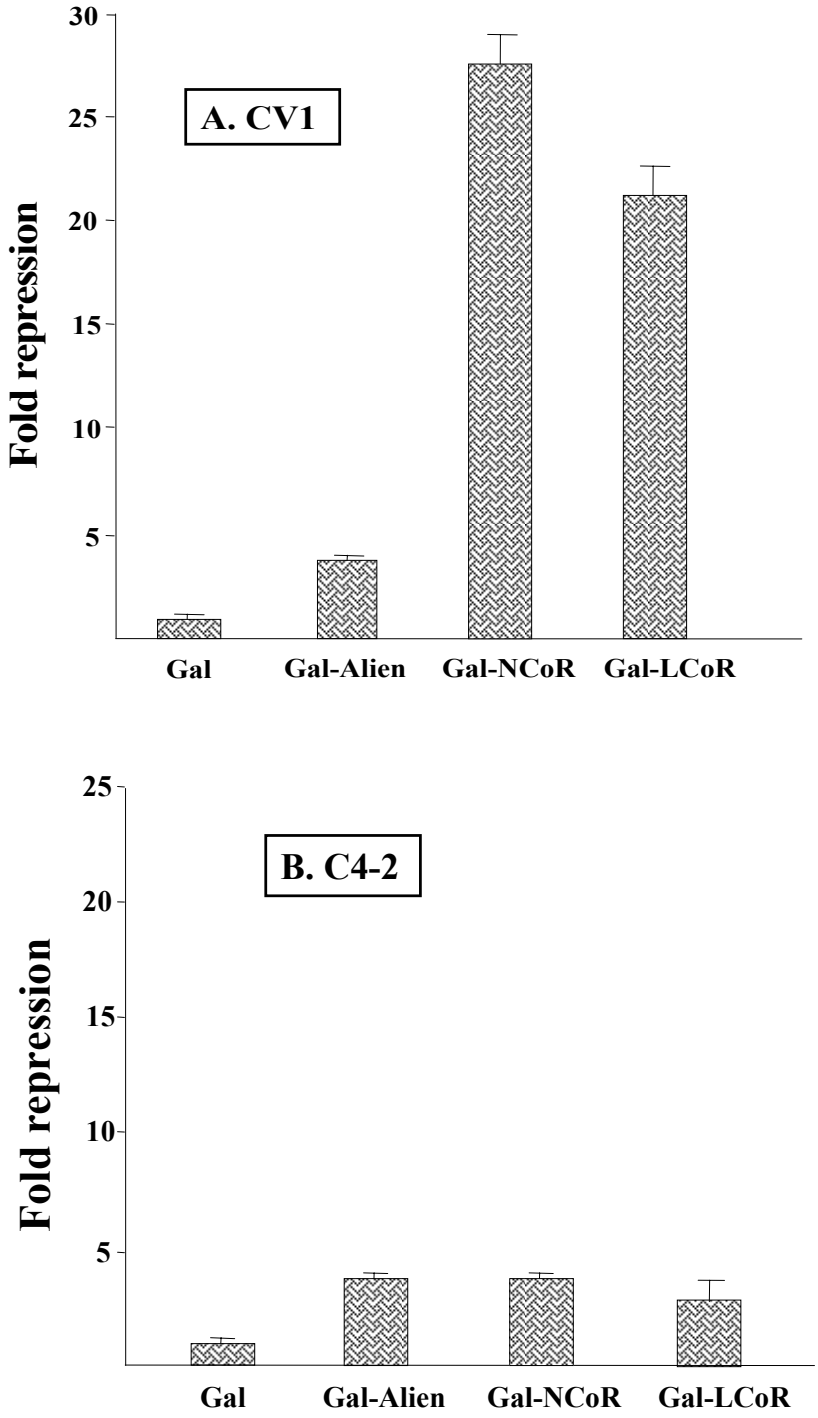


Fig. 5.24 Autonomous silencing function of LCoR is severely retarded in CaP cells compared to CV1 cells. (A) 100.000 CV1 cells (B) 300.000 C4-2 cells were seeded out in androgen-depleted DMEM and T media respectively. Following day, cells were transfected with $p(UAS)_4TATA$-Luc (1 µg) reporter containing Gal binding sites along with 1 µg each of Gal-Alien, Gal-NCoR and Gal-LCoR plasmids. 16 hrs post transfection fresh media was given and cells were treated with R1881 (10^{-10} M) and Src inhibitor PP2 (1 µM) for 72 hrs. Afterwards, cells were harvested and luciferase measurements were done and graph was plotted with respect to values obtained for empty gal. The experiments was conducted in triplets and the graph represents fold reporter repression over gal control.

It is possible that all corepressors in general show reduced repression function in CaP cells due to activated signaling kinases and that it is not LCoR specific phenomenon, to rule out this possibility, another corepressor "Alien" which is acts as a corepressor for selected members of NHR superfamily and it has been shown that Alien is a phosphorylation target by PKA pathway (Dotzlaw et al., 2002) therefore, was included to test whether its repression potential is also regulated by Src kinase. As is evident from the experiment, LCoR shows a dramatic decrease in its repression potential in C4-2 cells compared to CV1 cells (Fig. 5.24 B), however the repression shown by Alien remains unchanged indicating that autonomous repression potential of LCoR is specifically decreased by Src kinase and the cancer cell uses specific signaling machinery in order to functionally inactivate its repression function thereby providing a growth stimuli to cells.

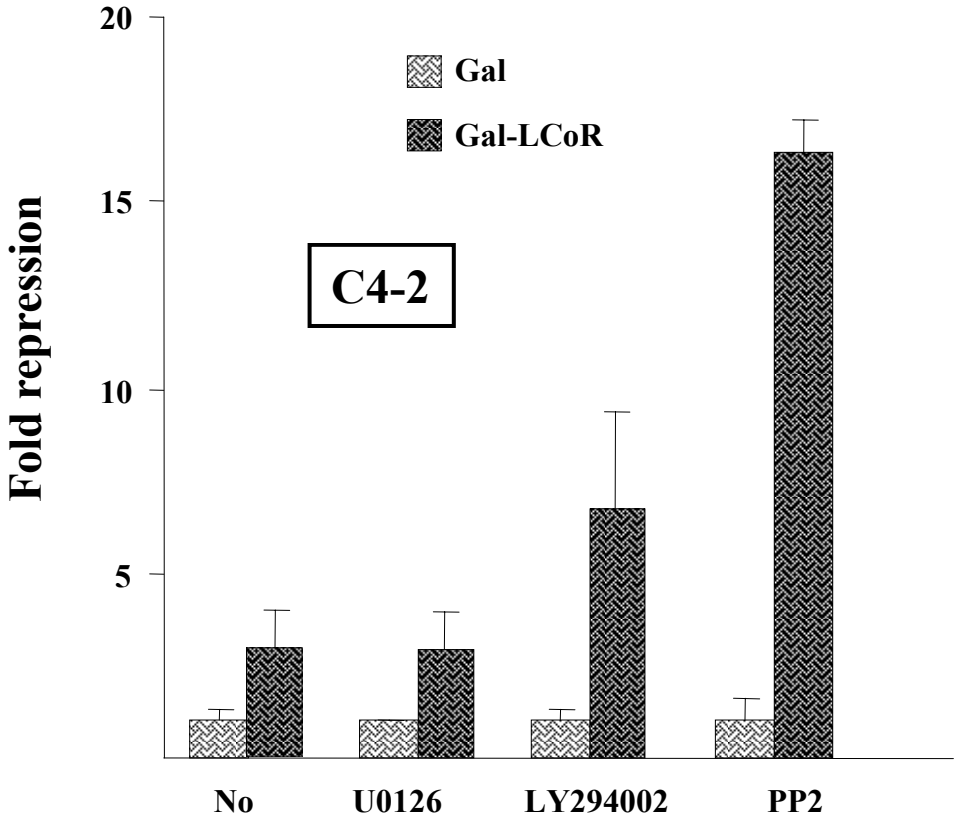


Fig. 5.25 Src kinase signal transduction pathway decreases LCoR repression function in CaP cells. 300.000 C4-2 cells were seeded out in androgen-depleted T media. Following day, cells were transfected with $p(UAS)_4TATA$-Luc (1 µg) reporter containing Gal binding sites along with 1 µg Gal-LCoR plasmid. 16 hrs post transfection fresh media was given and cells were treated with R1881 (10^{-10} M) and MAPK inhibitor U0126 (1 µM), LY294002 (1 µM) and Src inhibitor PP2 (1 µM) for 72 hrs. Afterwards, cells were harvested and luciferase measurements were done and graph was plotted with respect to values obtained for each empty gal. The experiments was conducted in triplets and the graph represents fold reporter repression over gal empty vector control.

Therefore, the role of signal transduction pathways was tested to explain weakened repression potential of LCoR in C4-2 cells. C4-2 cells were transfected with Gal-DBD fused LCoR and Gal responsive luciferase reporter. A battery of signal transduction inhibitors was tested for its effect on LCoR repression function. Gal-LCoR showed feeble repression of Gal-responsive luciferase promoter (Fig. 5.25). Inhibition of MAPK using inhibitor U0126, did not alter its repressional potential. In addition, blocking PI-3K kinase by LY294002 led to a marginal increase in LCoR repression. Interestingly however, blocking Src kinase using specific chemical inhibitor PP2 led to a sharp increase in the autonomous repression potential of LCoR.

Taken together, results obtained by these experiments clearly indicate that activating signaling kinases can indeed influence corepressor LCoR function and that this functional inactivation by signaling kinases may act at multiple levels. In case of LCoR, Src kinase not only decreases its interaction with AR, as evident from mammalian-one-hybrid experiments (Fig. 5.23 A), but also attenuates its autonomous repression function (Fig. 5.24 B), which may work in synergy to functionally attenuate LCoR from acting as a potent corepressor in CaP cells. Src-kinase mediated signaling may potentially play a role by post-translationally modifying LCoR by means of phosphorylation of its tyrosine residues which may play an important role in LCoR repression function. Indeed sequence

Tyrosine predictions

Name	Position	Context	Score	Prediction
S1	12	FAAEYTSKN	0.818	*Y*
S2	200	AKPHYEFNL	0.955	*Y*
S3	273	TGDQYSYSS	0.603	*Y*
S4	325	SGQPYPTSD	0.526	*Y*
S5	384	STLEYKVKE	0.589	*Y*

Fig. 5.26 LCoR sequence analysis. Predicted tyrosine residue(s) in its aa sequence, which are potential phosphorylation targets by tyrosine kinases. The score against each sequence represents the probability of phosphorylation with the "1" theoretical value obtained for an experimental phosphorylation. Y is a one word abbreviation for tyrosine (Source: Netphos 2.0 Bioinformatics server, Technical University of Denmark).

analysis of LCoR suggests many potential residues which can be phosphorylated by tyrosine kinases and might lead to its functional inactivation (Fig. 5.26). However a function point mutation-based analysis is lacking.

5.6.3 Decreased AR repression in CaP cells: Implications of signal transduction

AR signaling promotes the growth of normal prostate and progression of CaP and the therapy carries therefore the goal to repress AR function. Corepressors also repress AR function and therefore have been suggested to protective effect against CaP. In fact corepressors are known to be functionally recruited to antihormone CPA-bound AR to repress its function (Dotzlaw et al., 2002). In this direction, the ability of LCoR was tested to repress endogenous AR in androgen-independently growing C4-2 cells. Briefly, C4-2 cells were co transfected with AR responsive reporter plasmid along with LCoR expression vector. In fact, LCoR-mediated AR repression is dramatically reduced in C4-2 cells (fig 5.27). This was a puzzling phenomenon observed because the same T877A mutant of AR is able to be strongly repressed in CV1 cells (Fig. 5.15). This decreased effect of LCoR can however be explicitly explained on the basis of (1) decreased *in vivo* interaction of LCoR with AR (2) decreased autonomous repression potential of LCoR in C4-2 cells by Src signal transduction pathway. Therefore experiment was conducted to test the effect of Src inhibition by PP2 on LCoR-mediated AR repression in C4-2 cells. The repression of AR by LCoR was significantly enhanced in a ligand-dependent fashion upon cotreatment of cells with Src kinase inhibitor PP2 (Fig. 5.27). These results indicate that indeed, C4-2 cells, by over expressing Src kinase specifically inhibit LCoR to function as AR corepressor.

These experiments suggested that the repression potential of LCoR is significantly compromised in CaP cells, presumably as a part of sustained growth promoting strategy adapted by CaP cells.

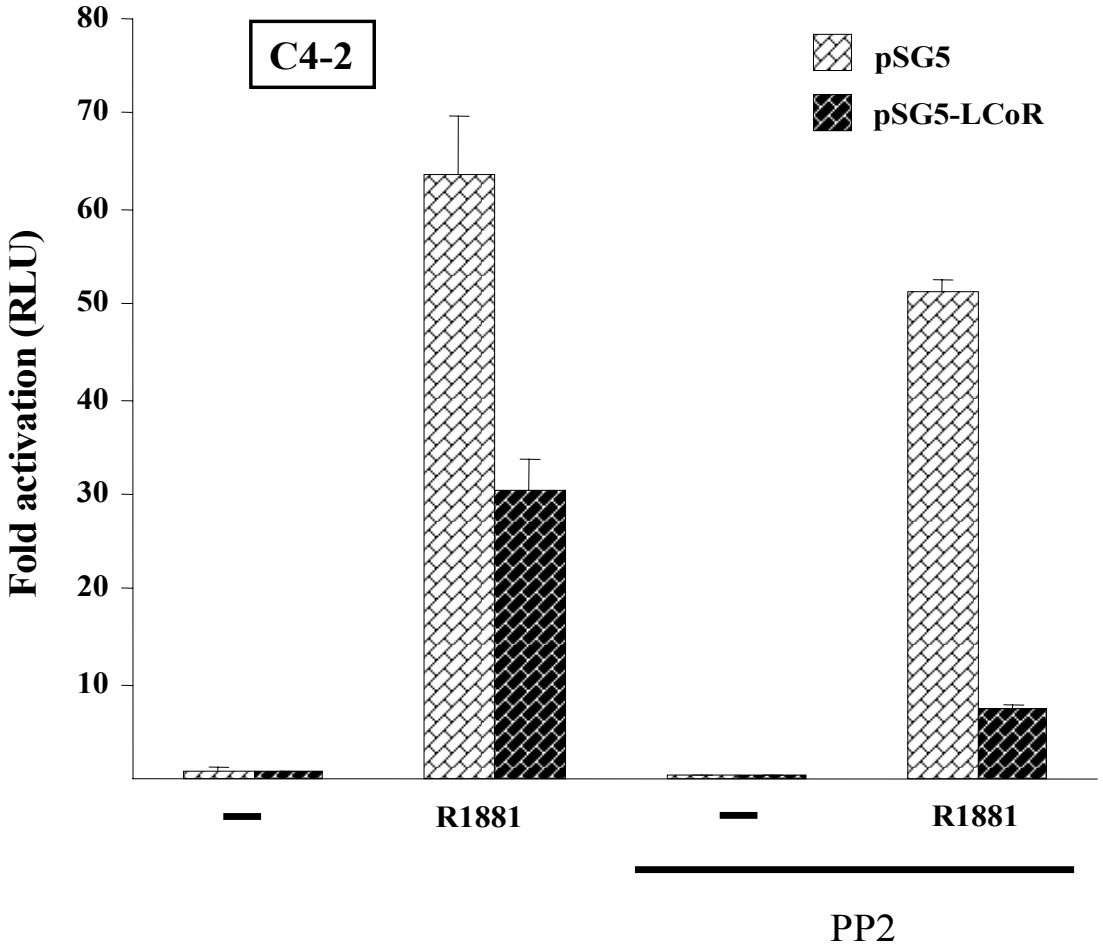


Fig. 5.27 LCoR-mediated AR repression is attenuated and PP2-mediated blockade of Src kinase strengthen LCoR-mediated AR repression in C4-2 cells. 300.000 C4-2 cells were seeded out in androgen-depleted T media. Following day, cells were transfected with MMTV-Luc (1 µg) reporter plasmid, pSG5 or pSG5-LCoR (1 µg) and pCMX-LacZ (0.4 µg; internal control). 16 hrs post transfection fresh media was given and cells were treated with R1881 (10^{-10} M) and Src inhibitor PP2 (1 µM) for 72 hrs. Afterwards, cells were harvested and luciferase measurements were done. The experiments were conducted in triplets and the graph represents fold reporter activation as LacZ normalised relative luciferase units.

5.7 Modulation of CaP cell growth by LCoR

5.7.1 LCoR inhibits growth of androgen-independently growing C4-2 cells and promotes neuroendocrine differentiation

Prostate cancer is an endocrine cancer that involves anomalies in AR function. Many CaP grow hormone-independently and regress upon anti-hormone therapy that antagonises the receptor function. In many therapy resistance cancers, however, the tumour becomes

androgen-independent and therefore leads to therapy failure, in some other cases of CaP, upon long-term Cas treatment, the AR pathway is bypassed (Hobisch et al., 2006) means

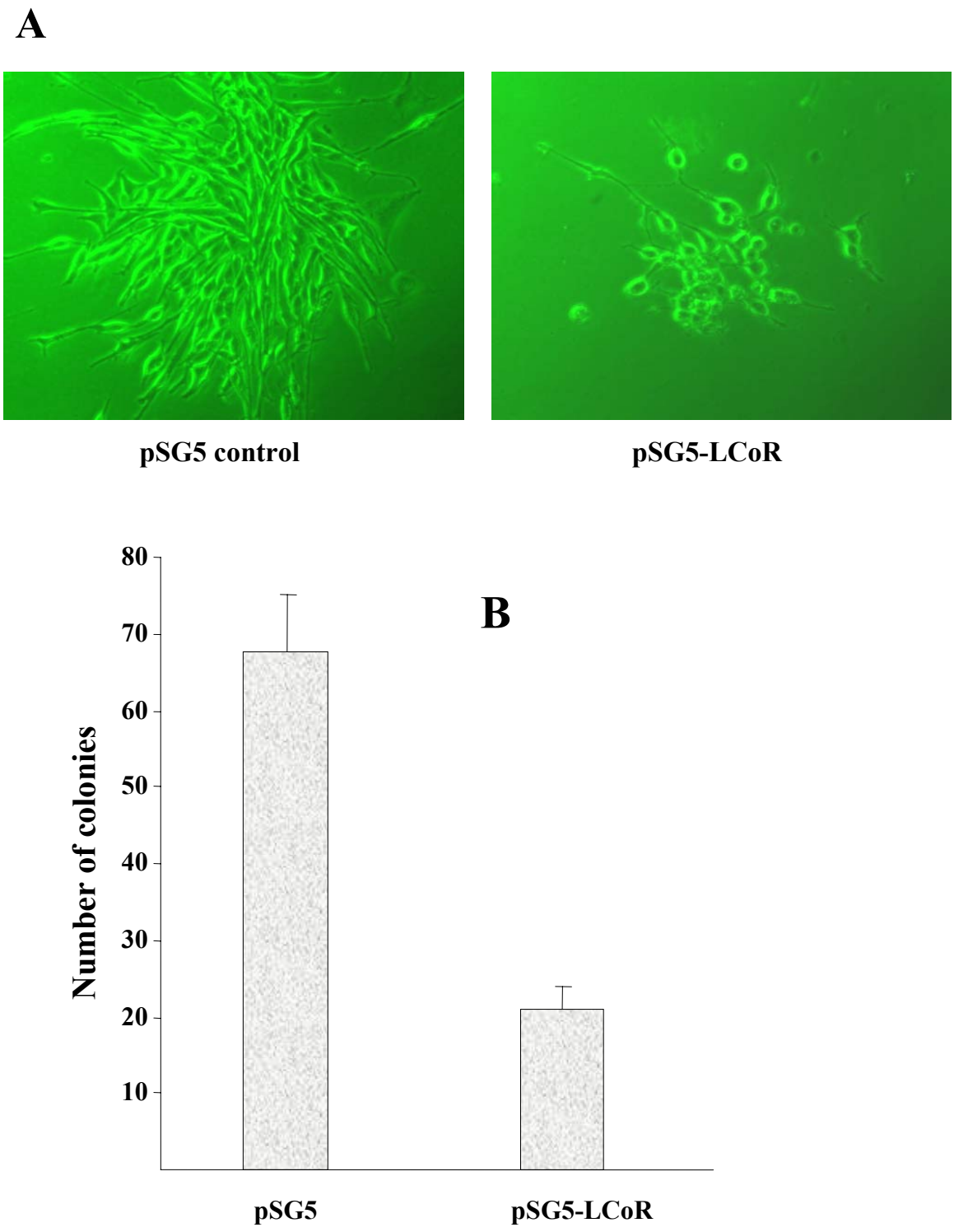


Fig. 5.28 LCoR represses cell growth and induces neuroendocrine transdifferentiation in androgen-independently growing C4-2 cells. (A) Light microscope photographs of C4-2 cells grown in T-medium containing normal serum. C4-2 cells were seeded out at the density of 200.000 cells per 10 cm tissue culture dish. Cells were transfected with pSG5 empty vector or pSG5-LCoR along with pETE vector in 5:1 molar ratio employing calcium phosphate transfection protocol. Cells were selected with 200 µg/ml hygromycin over a period of 6 weeks with continuous fresh

media change twice a week. (B) C4-2 cells were numbered for colonies formed. Average of the triplicate was taken and the graph was plotted.

that AR becomes promiscuitely/constitutively active. As previously described, both kinds of tumours though depend on functional AR signaling and regress upon AR depletion. It is known that AR, in addition to promoting growth of CaP cells, also suppresses neuro-endocrine differentiation of CaP AR function cells (Wright et al., 2003) and AR knock down using siRNA leads to a neuro-endocrine phenotype that manifest itself by inducing dendrite-like cellular processes. Corepressor which represses may thereby also lead to neuro-endocrine differentiation in addition to decreased CaP growth. Therefore the ability of LCoR to repress C4-2 cellular growth was also tested. Clones of C4-2 cells stably overexpressing LCoR were generated. Cells with control vector or LCoR expression vector, in combination with pETE (which provides resistance to hygromycin, Protopopov et al., 2002) were cotransfected and selected over a period of 6 weeks on hygromycin (200 µg/ml) in normal serum-containing T-media. LCoR overexpression in C4-2 cells led to inhibition of growth (Fig. 5.28). Cells overexpressing LCoR produce less colonies compared to those transfected with empty vector pSG5, indicating the potential growth inhibitory effect of the corepressor on cellular growth. The morphological analysis of cells suggest a differentiation pattern similar to that observed in neuro-endocrine differentiation, which further indicates the repression of AR by LCoR. This experiment indicates that corepressor function is important for inhibition of AR and thereby the growth of CaP cells.

5.7.2 Combinatorial effect of agonist R1881 and Src inhibition on CaP growth

Overexpression of LCoR in androgen-independently growing C4-2 cells leads to a decrease in colony number (fig. 5.28). Src inhibition by a specific chemical inhibitor leads to enhanced *in vivo* binding of LCoR with AR and also strengthens its autonomous repression potential thereby allowing AR to be more robustly repressed in C4-2 cells (sec. 5.6). To test the effect of enhanced AR repression by LCoR under the influence of Src inhibition, another colony formation assay was performed in androgen-depleted T-media. Briefly, 500.000 C4-2 cells were seeded out in 10 cm tissue culture dishes and were cotransfected with or without pSG5-LCoR together with PETE. Cells were selected over a period of 8 weeks on hygromycin (200 µg/ml) with regular exchange of fresh media twice weekly. Cells growing under no hormone “control” conditions show decreased growth and smaller colonies (Fig. 5.29). Notably, cells transfected with LCoR showed less cells per

colony compared to the cells transfected with empty vector control. However, R1881 treatment led to a growth acceleration and formation of cellular foci in both, cells treated

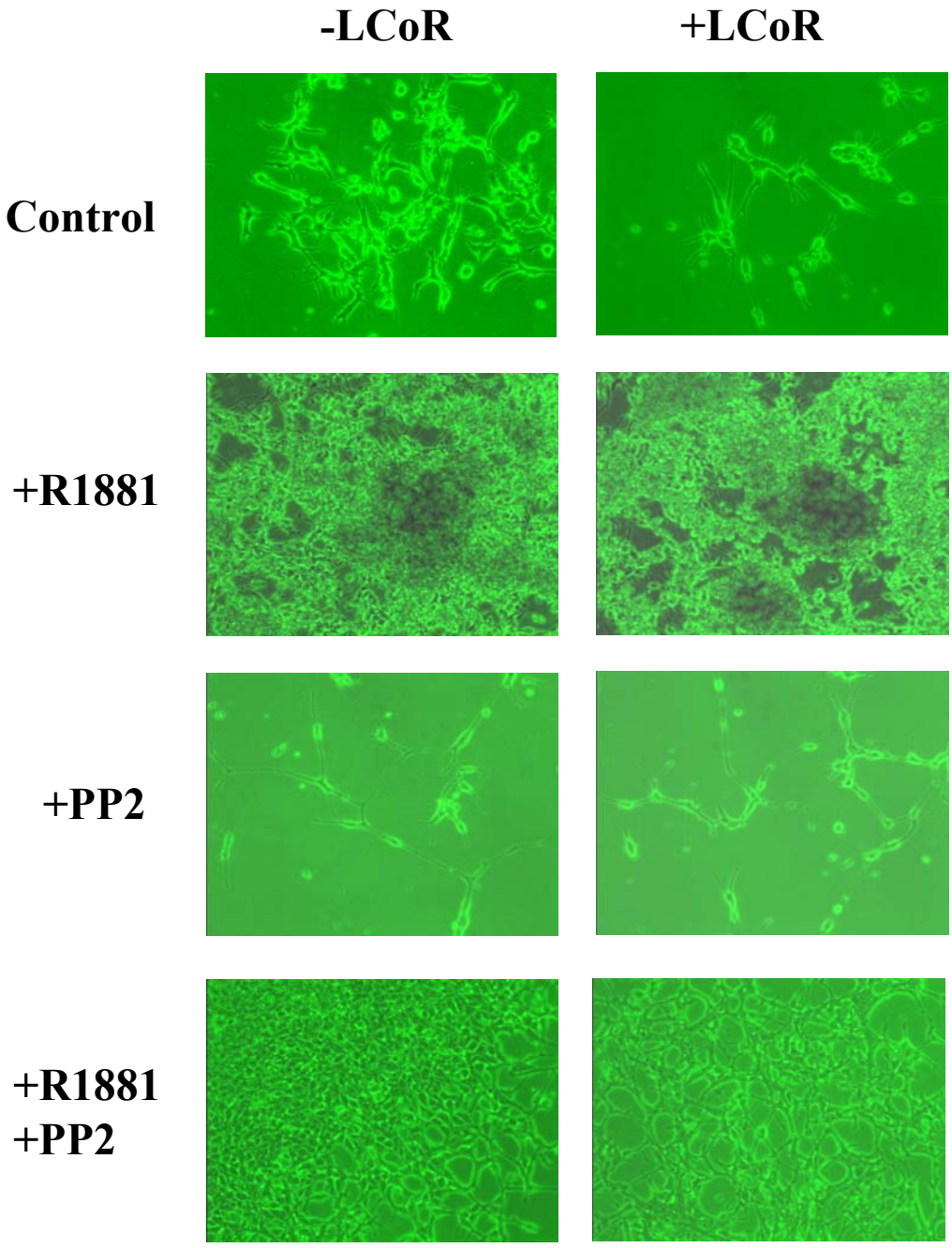


Fig. 5.29 Cotreatment of LCoR expressing C4-2 cells with PP2 and R1881 represses growth. Photomicrographs of C4-2 cells stably expressing empty vector control or LCoR. 500.000 C4-2 cells were grown in T-medium containing androgen-depleted serum. Cells were transfected with pSG5 empty vector or pSG5-LCoR along with pETE vector in 5:1 molar ratio employing calcium phosphate transfection protocol. Cells were selected with 200 µg/ml hygromycin over a period of 8 weeks with continuous fresh media change twice a week.

with empty vector control and in LCoR-transfected cells (Fig. 5.29). Interestingly no decrease either in cell number or in colony number was observed (Fig. 5.30), suggesting that LCoR may require some of the components such as growth factors present in the normal serum containing media to allow long-term repressional effect on cellular growth.

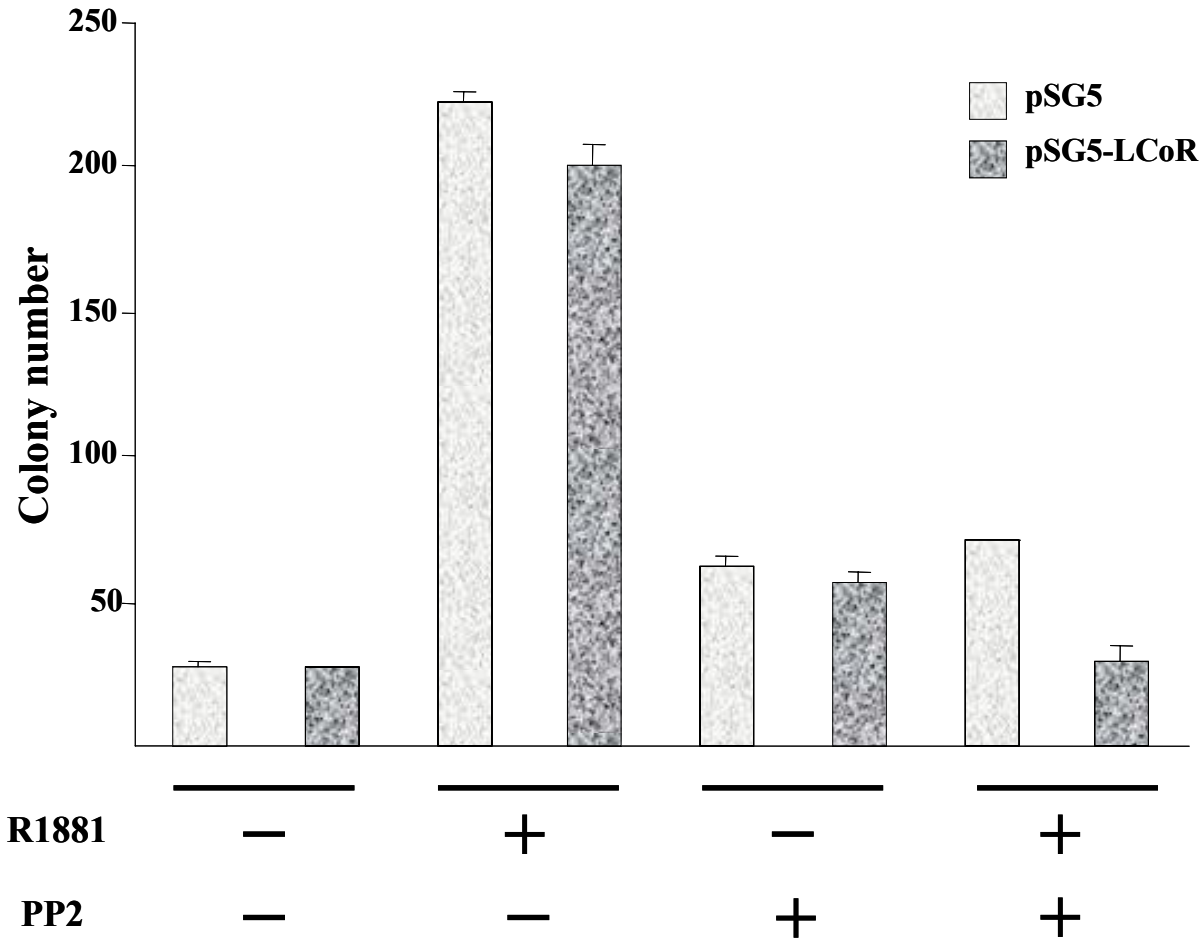


Fig. 5.30 PP2-mediated blockade of Src kinase enhances growth inhibitory effect of LCoR in C4-2 cells. 500.000 C4-2 cells stably expressing empty vector control or LCoR, were grown in T-medium containing androgen-depleted serum. Cells were transfected with pSG5 empty vector or pSG5-LCoR along with pETE vector in 5:1 molar ratio employing calcium phosphate transfection protocol. Cells were selected with 200 µg/ml hygromycin over a period of 8 weeks with continuous fresh media change twice a week. C4-2 cells were numbered for the colonies formed. Average of duplicate was taken and the graph was plotted.

Treatment of cotransfected cells with PP2 however led to a drastic decrease in cell number, intriguingly there were more colonies compared to no hormone control conditions (Fig. 5.29 & 5.30). This data is in line with the results showing the growth inhibitory effect of Src blockade on cellular growth (sec. 5.3 fig. 5.8). Co treatment of cells with PP2 and R1881 resulted in decrease both in cellular growth and colony number (Fig. 5.29 & 5.30). Data suggest that C4-2 cells stably transfected with LCoR show growth retardation in

response to long-term cotreatment with agonist R1881 and Src inhibitor PP2. This implies that LCoR function to repress CaP cellular growth indeed is subject to attenuation by Src signal transduction pathway and that androgen-independently growing C4-2 cells depend on AR activity for survival and growth.

6. Discussion

6.1 Modulation and functional consequence of AR recruitment on PSCA and PSA target genes

AR is the biological mediator of androgen action. Recruitment of AR on target genes results in either transcriptional activation or transcriptional repression. This involves the tight regulation of AR function by coactivators and corepressors. Corepressors are recruited to anti-hormone CPA-bound AR and lead to the repression of target gene expression (Dotzlaw et al., 2002). AR mediated activation of target genes is generally seen as a growth promotion strategy by CaP cells. Recently PSCA was identified as a cell surface marker which is overexpressed in CaP. PSCA is an androgen-regulated gene and transient transfections involving PSCA-Luc have demonstrated strong induction of reporter in a ligand-dependent manner in non-CaP cells. To test whether AR ligands regulate physical interaction of AR and corepressors Alien and SMRT on PSCA target gene in CaP cells ChIP experiment was performed. For the first time these results indicate that, ligands differentially regulate the recruitment of AR on PSCA enhancer containing an ARE *in vivo*. In LNCaP cells, AR is recruited to PSCA enhancer in response to agonistic ligand R1881 (Fig. 5.1), similar effect were seen with the positive control PSA suggesting that PSCA acts also as a model gene to test direct AR action as PSA. Agonist R1881 also leads to loss of corepressor SMRT recruitment on both the enhancers. Treatment of LNCaP cells with partial agonistic ligand CPA leads to robust recruitment of corepressor Alien and regain of SMRT recruitment on both PSA and PSCA enhancer in line with previous report suggesting enhanced binding of SMRT and Alien to CPA-bound AR in mammalian-one-hybrid assay (Dotzlaw et al., 2002). Casodex leads to a steep decrease in AR recruitment to PSA enhancer in line with a recent report suggesting that Cas interferes with the stable DNA binding of AR (Farla et al., 2005). However, this can not be a general effect of Cas on AR as the recruitment of AR is seen on PSCA enhanced under similar conditions in LNCaP cells. It is possible that Cas-mediated prevention of AR from stably recruiting to DNA elements may in part dictated by response element thereby adding further intricacy to the regulation of target gene expression. ChIP experiment in C4-2 cells indicates a robust recruitment of AR even in the absence of agonist suggesting AR activation by autocrine growth loops and possibly by signal transduction cascades. No changes either in the recruitment of corepressor Alien or SMRT were observed under all experimental

conditions except a decrease in AR recruitment in the presence of Cas. This suggest that AR may be constitutively active on its target genes allowing them to grow in an androgen-independent manner, however, transient transfections revealed that indeed AR transactivation function is modulated by various ligands suggesting that in C4-2 cells the activity of AR might be regulated by some other unknown corepressors which might be differentially recruited to regulate AR function in C4-2 cells. In fact, treatment of C4-2 cells with CPA leads to a decrease in PSCA mRNA (sec. 5.2.3 fig. 5.3) emphasizing the regulation of constitutively recruited AR by ligands which may potentially function by to date unidentified corepressors.

6.2 negative regulation of maspin by AR in CaP cells

Maspin is a member of serine protease family and is a tumour suppressor and inhibits tumour progression (Cher et al., 2003). Interestingly the expression of maspin is known to be down-regulated by androgens (Zhang et al., 1997), however evidence for a molecular mechanism of direct regulation of maspin by AR transactivation function are lacking. Till date there are no reports on how this negative regulation works for the AR target gene exist. For the first time, ChIP in LNCaP and C4-2 CaP cells show recruitment of AR and corepressor Alien and SMRT on maspin promoter (sec. 5.2.2) raising a possibility that its expression may be subject to direct regulation by AR and the tested corepressors SMRT and Alien. Under conditions of no hormone treatment (referred as control), AR is recruited to maspin possibly repressing its expression in order to promote cell invasion. Corepressor Alien seems to play an important role in the AR recruitment mediated repression of maspin, which is co-recruited with SMRT and also alone on maspin under conditions of no hormone in C4-2 and LNCaP cells respectively. Interestingly, the binding of AR is decreased or completely lost in presence of agonist R1881 or partial agonist CPA in both cell lines. This suggests that the response elements itself in part governs the recruitment of AR, by allowing unliganded or non-cognate ligand bound confirmation to be stable to DNA (as seen without hormone), while, preventing the stable DNA binding of agonist R1881 or CPA bound AR further indicating the role of corepressor Alien in allowing maspin gene to remain under repressed state in CaP cells. CPA treatment in addition to decreasing AR recruitment on maspin, also lead to a sharp decrease in Alien recruitment suggesting that the DNA-AR-CPA conformation may be inhibitory to Alien recruitment. To test the functional implications of decreased AR and Alien binding on maspin

promoter, real time experiments were performed, which indicated enhanced maspin mRNA expression by CPA treatment (sec. 5.2.3 fig. 5.3). Taken together, these results suggest that (1) Negative-regulation of target gene expression is a phenomenon not restricted to TR and RAR only (2) AR recruitment on the negative response element is dictated in part by the nature of the cis-DNA element possibly by allowing different AR conformations than observed on a positive element to stably bind to it (3) negative regulation might function by corepressors differentially recruited in response to various ligands. This also suggests that on a negative response element CPA- bound AR does not allow robust binding of Alien.

6.3 Cell type-specific regulation of corepressor SMRT interaction with AR in CaP cells

Eukaryotic transcription factors can exert both positive and negative effects on gene expression. Activity of corepressors is also subject to regulation in turn by signal transduction cascades, allowing an indirect effect on the activity of NRs. CaP cells, like other cancer cells device many cellular alterations that combinely, or synergistically result in their faster growth and escape from therapeutic intervention. As evident, AR repression by corepressors hold a great promise, which have been shown to therapeutic anti-hormone bound AR leading to target gene repression. It is possible that in order to overcome effects of anti-hormone therapy, CaP may specifically inactivate corepressor SMRT which interacts with antihormone bound AR (Dotzlaw et al., 2002). In other cells types corepressor SMRT and CtBP have been shown to be functionally inactivated by cellular signaling pathways (Hong and Privalsky 1998, 2000, Barnes et al., 2003). To test whether signaling kinases can also affect the ability to corepressor SMRT in repressing AR function, mammalian-one-hybrid assays have been performed to test the modulation of interaction of endogenous AR in LNCaP and C4-2 cells *in vivo*. On testing, a wide spectrum of inhibitors found that the binding of SMRT is regulated by MAPK pathway and its specific blockade by its inhibitor U0126 leads to enhanced interaction of SMRT with AR mutant found in LNCaP cells (Fig. 5.10 C). This data is in line with a previous report showing that tyrosine kinases negatively regulate SMRT interaction with TR, another member of NR superfamily (Hong et al., 1998). In addition, protein kinase C-related kinase (PRK1) is known to be overexpressed in CaP cells, shown to enhance AR transactivation function by potentiating TAU-5 function and have been implicated in CaP progression

(Metzger et al., 2003). In line with this blocking PRK1 by HA1077, its specific inhibitor, leads to a significant increase in SMRT AR interaction in LNCaP (Fig. 5.10 B) but not in C4-2 cells (Fig. 5.11 B) suggesting that AR SMRT interaction in C4-2 cells is not regulated by PRK1 signaling and may potentially involve other signaling cascades which might impart androgen-independently growing properties to these cells (Unni et al., 2004). This also suggests that PRK1-mediated enhancement of AR transactivation (Metzger et al., 2003) may in part involve loss of physical interaction and potentially its functional inactivation to repress AR function *in vivo*. In contrast, AR transactivation in C4-2 remains unaffected by blockade of PRK1 (Fig. 5.11 B) further highlight the molecular differences between the signaling pathways in regulating interaction of SMRT with AR within the two cell lines.

This suggest that androgen-dependently and androgen-independently growing cells may specifically use different subset of signal transduction pathways to regulate SMRT function rather than the notion that increasing number of signal transduction pathways are overexpressed with the transition of CaP to an androgen-independent state.

Interestingly, SMRT binds to AR *in vivo* in C4-2 cells without blocking any specific signal transduction pathway (Fig. 5.11 A) , however this binding is not observed in LNCaP cells (Fig. 5.10 A) indicating that SMRT is more functionally active in C4-2 cells compared to LNCaP cells. Indeed enhanced levels of SMRT in CaP cells have been correlated with repression of target genes associated with antiproliferative action (Khanim et al., 2004) and may thereby provide a growth promoting strategy to androgen-independently growing C4-2 cells. To test the implication of enhanced SMRT interaction in LNCaP cells by blocking MAPK, Src and PRK-1, functional experiments, with f.l. SMRT on AR function are sought. Our preliminary results with ectopic expression of SMRT in LNCaP cells and cotreatment with U0126 MAPK inhibitor indicate a more robust effect of corepressor SMRT in repressing AR function in LNCaP cells (Michael Eisold, Diploma thesis). Results obtained from stably integrated SMRT from colony formation experiments done to test the effect of SMRT and MAPK blockade on the growth of LNCaP cells also indicate a dramatic decrease in colony number in U0126 and CPA-dependent manner.

The influence of tyrosine kinase blockade on SMRT and AR interaction was also tested in LNCaP and C4-2 cells. In LNCaP cells, SMRT binding was not influenced by tyrosine kinase inhibitor AG1517 (Fig. 5.10 D), however similar experiment in C4-2 cells led to the loss of SMRT interaction with AR (Fig. 5.11 D). It implies that signaling kinases do not always lead to loss of corepressor function rather in some cases they may rather potentiate

their effect. In C4-2 cells, SMRT interaction is decreased with AR by blocking receptor tyrosine kinases. Results from mammalian-one hybrid experiment suggest that the binding of SMRT to AR is subject to modulation by other kinases as well. Using PP2 the chemical inhibitor of Src kinase pathway, the interaction is enhanced in a CPA-dependent manner *in vivo*, suggesting that Src kinase pathway may negatively influence binding of corepressor SMRT with AR and may potentially be involved thereby in activating AR function. Because Src kinase also acts through MAPK as well, it is possible that the U0126-mediated enhancement of SMRT interaction with AR observed by blocking MAPK may be a result of blockade of Src kinase situated upstream of MAPK. Results from mammalian-one-hybrid performed in C4-2 cells suggest that signaling pathways that lead to decreased SMRT binding in LNCaP cells are ineffective in regulating this interaction suggesting this interaction to be a cell type specific phenomenon.

6.4 Src kinase provides a growth promotion strategy to androgen-independently growing cells

As discussed in section 2.3, AR activation in addition to mutations, is attributed to signal transduction pathways. Signal transduction pathways are over expressed in many types of cancers and lead to therapy failure including for breast carcinoma. Src, a non-receptor tyrosine kinase is activated in breast carcinoma and leads to the failure of antagonist Tamoxifen-based therapy by allowing it to function as a potent agonist (Shah et al., 2005). The role of Src kinase in regulating androgen-independent growth was tested in C4-2 cells. Src kinase inhibitor PP2 leads to the decreased AR transactivation on two different response elements (Sec. 5.3.1 fig. 5.4), however the transcription from a control gene pCMV-LacZ remains unaffected (Fig. 5.5). It is notable that the AR transactivation seen in fig. 5.4 is contributed by androgens and growth factors. ChIP experiments done to correlate decreased reporter activity to AR transactivation and recruitment indicated a basal AR recruitment in the absence of hormone (Fig. 5.6). The recruitment is further enhanced when cells were treated with agonist R1881. Part of the result is in concordance with section 5.2.1 that AR is recruited to PSA enhancer in the absence of agonistic ligand which is attributed to its cross-talk and possible activation by other non-cognate ligands as a result of broadened specificity. Src blockade, however dramatically reduced the recruitment of AR in the absence of R1881, which strengthens the idea of agonist-independent recruitment of AR to PSA enhancer, is indeed a result of its cross talk with

signal transduction pathways. Surprisingly, R1881 overcomes the effect of decreased AR recruitment by PP2.

The real-time experiment performed to test and to correlate the transient transfections and ChIP (Sec. 5.3.3 fig. 5.7). Taken together, these results indicate that AR is recruited to a certain degree independent of androgens to its target genes. C4-2 cells also express PSA at a basal level, which could be attributed to the androgen-independent marginal recruitment of AR allowing the expression of PSA, in addition, one report suggest that PSA basal expression in androgen-independently growing cells does not requires AR binding (Jia et al., 2005). However, induction of PSA mRNA by R1881 does require enhanced AR binding (fig. 5.7). This induction of AR-mediated PSA expression may require Src kinase signaling blocking which, leads to decreased target gene expression as evident from reporter assay (Fig. 5.4) and from real time PSA expression analysis. Long-term PP2 treatment leads to growth inhibition of C4-2 cells in androgen-containing growth factor rich T-media, suggesting that PP2-mediated Src blockade not only affects induction of target gene expression, rather leads to decreased growth of C4-2 cells presumably by interfering with the expression of target genes which play important function in cell cycle.

6.5 LCoR interacts with the DBD of AR in a hormone-dependent fashion

LCoR was initially identified in screen for proteins that interacted with the LBD of ERα in a hormone-dependent manner (Fernandes et al., 2003). The C-terminus of LCoR, harbouring an HLH interaction motif and devoiding of a NR box exhibited a CPA-dependent interaction with AR (Michaela Patz, Diploma thesis). As for other members of NHR superfamily, NR box interacts with the helix-12 in the LBD in a ligand-dependent manner; it raised the possibility that LBD may not be involved in interaction with LCoR. Indeed, these speculations turned out to be correct when various N-terminal and LBD-mutants of AR were robustly repressed by LCoR w.t. or in the similar way by LCoR-NR box mutant (sec. 5.5.5 fig. 5.19 & 5.20). Another hybrid mutant of AR, where NTD was replaced by VP16 transactivation domain was used to find out if N-terminus is not targeted by LCoR (Fig. 5.21 B). This data suggested that LCoR does not target the NTD. Later experiment with VP16-DBD confirmed that LCoR targets the DBD of AR. (Sec. 5.5.5 fig. 5.22). This data suggest that LCoR specifically targets the DBD of AR. It raises two possibilities explaining the repression of AR transactivation. Either LCoR binding to DBD may interfere with the recognition of AREs by the DBD thereby preventing stable binding

of AR to cognate response elements. Another possibility is that DBD-bound LCoR may recruit HDAC or CtBP silencing machinery to DBD thereby repressing AR mediated transactivation. Solution to this enigma comes from the mammalian-one-hybrid studies. The results obtained from these studies clearly indicate that the binding of VP16-cLCoR with AR can be enhanced several folds in a CPA or R1881-dependent manner (Sec. 5.5.3 fig. 5.17). Had there been a displacement of AR through its DBD by LCoR, this enhanced interaction could not be realised. In fact the second Zn finger present in the DBD of AR which has been shown to assist interaction with coregulatory proteins (O' Malley, 1990) is highly conserved among different members of NHR superfamily. Agonist-dependent repression of LCoR suggests that LCoR may be a part of negative feedback loop in AR transactivation presumably by (co)competing with coactivators and thereby fine-regulating the AR transactivation.

6.6 Functional attenuation of LCoR in androgen-independent prostate cancer

The question arises why potent corepressors which are expressed in CaP allow AR to remain superactive in CaP cells. It is possible that ratio of coactivators to corepressors, which has been shown to be high in CaP cells (Liu et al., 2004) gives competitive advantage to coactivators to bind to AR in CaP cells. This increase in ratio of coactivators to corepression may arise from (1) the overexpression of coactivators or decreased expression of corepressors or (2) functional attenuation of corepressors by signal transduction pathways which are known to be activated in CaP cells.

Also there are many signaling pathways known to be hyperactivated in CaP cells (sec 2.4) and as coregulatory proteins are phosphorylation target by many of these abruptly activated pathways it is possible that LCoR may be functionally attenuated by phosphorylation and may therefore not be able to repress AR function in CaP cells as strongly as seen in CV1 cells (Fig. 5.14). Therefore, a battery of specific chemical inhibitors was screened for identification of possible involvement of specific signaling cascades, which may phosphorylate and thereby attenuate LCoR repression function. The results of signaling inhibition experiments to test LCoR ability to repress AR indicate the involvement of a specific tyrosine kinase called Src. Indeed a member of Src family, called FAK (focal adhesion kinase) is known to be overexpressed in CaP cells (Slack et al., 2001,) and its expression is continued during the progression of CaP (Rovin et al., 2002). Utilising

specific inhibitor called PP2 for blocking this pathway; it is possible to enhance the repression potential of LCoR and thereby enhancing its repressive effect on AR.

6.7 Model

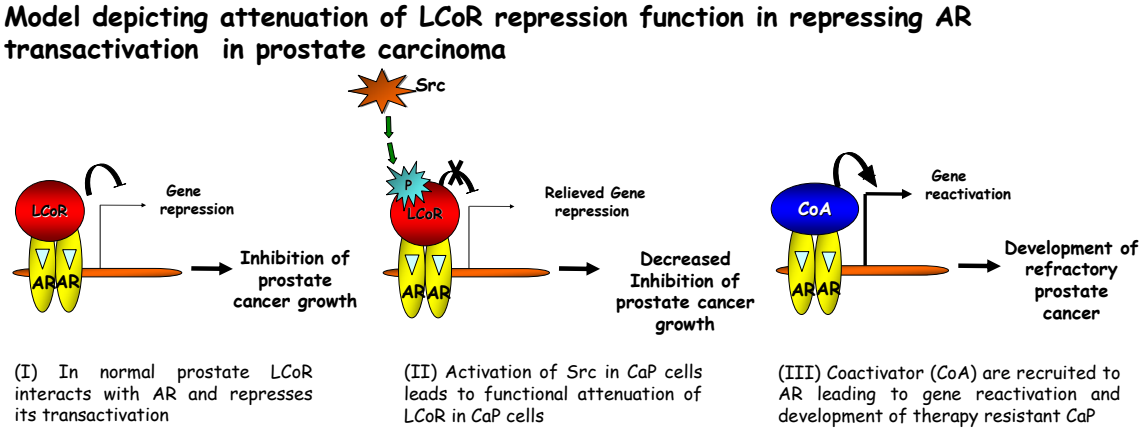


Fig. 6.1 LCoR is expressed in normal prostate and represses target gene transcription in a hormone-dependent manner. Over expression of Src kinase leads to functional attenuation of LCoR to interact with and to repress AR function and thereby giving a competitive advantage to coactivators to enhance AR function and the growth of CaP cells.

7. Outlook

A diverse array of AR target genes has been identified. The molecular mechanism for the upregulation of target gene expression has been very well explored. However, target genes such as maspin, downregulate in response to androgens. The mechanism to explain negative regulation by androgens in only poorly understood and the involvement of AR and its corepressors is not clear. Important goal here would be to find out how in presence of various agonists, the expression of maspin varies and how exactly AR is involved in repressing maspin expression. TR is known to recruit corepressors in the absence of cognate ligand and repress target gene function. Similarly, in context of maspin the role of AR needs to be explored. Another possibility is the recruitment of corepressors to AR on target genes, which may also account for target gene repression. AR knock down would be important here to test direct regulation by AR.

Corepressor LCoR represses growth of androgen-independently growing C4-2 cells and LCoR-mediated AR repression can be boosted by blocking Src kinase signaling by chemical inhibitor PP2. The question is how the potentiation of LCoR function modulates

the expression of endogenous target genes in C4-2 and LNCaP cells. To test this, real-time PCR experiments can be carried out to ascertain (1) the influence of LCoR on target gene expression (2) Influence of Src blockade upon target gene expression. Also, a ChIP experiment would be important to test the whether LCoR is ligand-dependently recruited on AR at target genes. In addition, to test the combinatorial effect of LCoR and Src blockade, colony formation experiments with C4-2 cells are required. Because, Src and LCoR individually repress the growth of C4-2 cells, an enhanced combinatorial effect is envisaged. In addition Xenografts of C4-2 cells, stably overexpressing LCoR can be grown in nude mice to test the influence of corepressor LCoR on the growth of cancer *in vivo*. LCoR sequence analyses show many potential tyrosine and ser/thr kinase phosphorylation sites suggesting that LCoR may be phosphorylated by other signaling cascades as well in addition to Src. Blockade of Src leads to decrease in AR transactivation and decreased growth of C4-2 cells, suggesting that Src may contribute to hormone-independent AR activation in C4-2 cells. Here a CaP cell line engineered to over express Src will be generated and their growth sensitivity will be tested for various ligands, in addition, xenografts will be generated in nude mice to test their growth behavior and sensitivity to regression upon anti-hormone administration.

At this point importance of individual corepressors in regulating AR function is largely lacking. Further work in this direction should elaborate the role of AR corepressors specifically SMRT, NCoR, Alien and LCoR in regulating AR function and thereby cellular growth in normal prostate cells and CaP cells. RNAi-mediated individual corepressor knock down will reveal the functional significance of each of these corepressors in regulating the expression of both positively and negatively regulated genes and in turn regulating CaP growth.

As has been emphasized through out the study, activation of signal transduction pathway plays an important role in inactivating corepressor. However at this point cell type over expression of specific signaling molecules remains largely unexplored. In this direction, differential kinome expression in normal and malignant prostate cells can be ascertained by protein-ChIP based analyses as well as by DNA microarray to detect difference in kinome expression. This would be instrumental in developing effective and more precisely tailored drugs based on the signal transduction inhibitors, against CaP. These findings will also be important in finding out new signal transduction pathways that lead to corepressor inactivation.

Also there is a great thrust in finding new target genes which play important role in CaP progression and its metastasis. In the last decade, over 2 dozens of androgen-regulated genes have been found, employing reporter assays and electrophoretic mobility shift assays. Regulation of many of those however, does not entirely correlate with the aggressive behaviour of the disease and failure of anti-hormone therapy. In order to find out new androgen target genes ChIP on Chip assay can be performed to find out new target genes of androgens. In addition microarray-based studies could be proven effective in finding target genes which are negatively modulated by androgens.

8. REFERENCES

Abreu-Martin M. T., Chari A., Palladino A. A., Craft N. A. and Sawyers C. L. (1999): Mitogen activated protein kinase kinase kinase 1 activates androgen receptor-dependent transcription and apoptosis in prostate cancer. Mol Cell Biol. 19:5143-5154.

Alen, P., Claessens, F., Verhoeven, G., Rombauts, W. and Peeters, B. (1999): The androgen receptor amino-terminal domain plays a key role in p160 coactivator-stimulated gene transcription. Mol Cell Biol. 19, 6085-6097.

Arai Y., Tatsuhiro Y. and Osamu Y. (1990): Prognostic significance of prostate specific antigen in endocrine treatment for prostate cancer. J Urol. 144: 1415-1419.

Aranda A. and Pascual A. (2001): Nuclear hormone receptors and gene expression. Physiol Rev. Jul;81(3):1269-1304.

Ausubel F. M., Brent R., Kingston R. E., Moore D. D., Seidman I. G., Smith J. A. and Struhl K. (1989): Current protocols in molecular biology. Greene Publishing associates and Wiley-Interscience.

Bakin R. E., Gioeli D., Bissonette E. A. and Weber M. J. (2003): Attenuation of Ras signaling restores androgen sensitivity to hormone-refractory C4-2 prostate cancer cells. Cancer Res. Apr 15;63(8):1975-1980.

Baniahmad A. (2005): Nuclear hormone receptor corepressors. J Steroid Biochem Mol Biol. 93: 89-97.

Barnes C. J., Vadlamudi R. K., Mishra S. K. Jacobson R. H., Li F. and Kumar R. (2003): Functional inactivation of a transcriptional corepressor by a signaling kinase. Nat Struc. Biol. 10(8):622-628

Barradell L. B. and Faulds D. (1994): Cyproterone. A review of its pharmacology and therapeutic efficacy in prostate cancer. Drugs Aging 5(1): 59-80.

Beato M., Heerrlich P. and Chambon P. (1995): Steroid hormone receptors: many actors in search of a plot. Cell 83:851-857.

Benten W. P., Lieberherr M., Stamm O., Wrehlke C., Guo Z. and Wunderlich F. (1999): Testosterone signaling through internalizable surface receptors in androgen receptor-free macrophages. Mol Biol Cell Oct;10(10):3113-3123.

Berrevoets C. A., Umar A., Trapman J. and Brinkmann A. O. (2004): Differential modulation of androgen receptor transcriptional activity by the nuclear receptor corepressor (N-CoR). Biochem J Pt.3:731-738.

Birnboim H. C. and Doly J. (1979): A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. 7: 1513-1523.

Blin N., von Gabain A. and Bujard H. (1975): Isolation of large molecular weight DNA from agarose gels for further digestion by restriction enzymes. FEBS Lett. 53, 84-86.

Boyer H. W. and Roulland-Dussoix D. (1969): A complementation analysis of the restriction and modification of DNA in Escherichia coli. J Mol Biol. May 14;41(3):459-472.

Brinkmann A. O., Jenster G., Kuiper G. G., Ris C., van Laar J. H., van der Korput J. A., Degenhart H. J., Trifiro M. A., Pinsky L. and Romalo G. (1992): The human androgen receptor: structure/function relationship in normal and pathological situations. J Steroid Biochem Mol Biol. Mar;41(3-8):361-368.

Bubendorf L., Kononen J., Koivisto P., Schraml P., Moch H., Gasser T. C., Willi N., Mihatsch M. J., Sauter G. and Kallioniemi O. P. (1999): Survey of gene amplifications during prostate cancer progression by high-throughout fluorescence in situ hybridization on tissue microarrays. Cancer Res. Feb 15;59(4):803-806.

Burfeind P., Chernicky C. L., Rininsland F. and Ilan J. (1996): Antisense RNA to the type I insulin-like growth factor receptor suppresses tumor growth and prevents invasion by rat prostate cancer cells in vivo. Proc Natl Acad Sci USA 93:7263-7268.

Burke L. J. and Baniahmad A. (2000): Corepressors 2000. FASEB J. 14, 1876-1888.

Castoria G., Lombardi M. and Barone M. V. (2003): Androgen-stimulated DNA synthesis and cytoskeletal changes in fibroblasts by a nontranscriptional receptor action. J Cell Biol. 161:547–556.

Cato A. C., Nestl A., and Mink S. (2002): Rapid actions of steroid receptors in cellular signaling pathways. Sci STKE. Jun 25;2002(138):RE9.

Cavailles V., Dauvois S., L'Horset F., Lopez G., Hoare S., Kushner P. J. and Parker M. G. (1995): Nuclear factor RIP140 modulates transcriptional activation by the estrogen receptor. EMBO J. Aug 1;14(15):3741-3751.

Chen C. D., Welsbie D. S., Tran C., Baek S. H., Chen R., Vessella R., Rosenfeld M. G. and Sawyers C. L. (2004): Molecular determinants of resistance to antiandrogen therapy. Nat Med. Jan;10(1):33-39.

Chen J. D. and Evans R. M. (1995): A transcriptional corepressor that interacts with nuclear hormone receptors. Nature 377: 454-457.

Cher M. L., Biliran H. R. Jr., Bhagat S., Meng Y., Che M., Lockett J., Abrams J., Fridman R., Zachareas M., and Sheng S. (2003): Maspin expression inhibits osteolysis, tumor growth, and angiogenesis in a model of prostate cancer bone metastasis. Proc Natl Acad Sci USA Jun 24;100(13):7847-7852.

Chinnadurai G. (2002): CtBP, an unconventional transcriptional corepressor in development and oncogenesis. Mol Cell Feb;9(2):213-224.

Chomczynski P. and Sacchi N. (1987): Single-step method of RNA isolation by acid guanidinium thiocyanatephenol-chloroform extraction. Anal Biochem. 162: 156-159.

Christian M., White R. and Parker M. G. (2006): Metabolic regulation by the nuclear receptor corepressor RIP140. Trends Endocrinol Metab. Aug;17(6):243-250.

Chung T. D., Yu J. J., Kong T. A., Spiotto M. T., and Lin J. M. (2000): Interleukin-6 activates phosphatidylinositol-3 kinase, which inhibits apoptosis in human prostate cancer cell lines. Prostate. Jan;42(1):1-7.

Claessens F., Verrijdt G., Schoenmakers E., Haelens A., Peeters B., Verhoeven G., and Rombauts W. (2001): Selective DNA binding by the androgen receptor as a mechanism for hormone-specific gene regulation. J Steroid Biochem Mol Biol. 76, 23-30.

Cleutjens K. B., van der Korput H. A., van Eekelen C. C., van Rooij H. C., Faber P. W. and Trapman J. (1997): An androgen response element in a far upstream enhancer region is essential for high, androgen-regulated activity of the prostate-specific antigen promoter. Mol Endocrinol. 11: 148-161.

Coffey D. S. and lsaacs J. T. (1981): Prostate tumor biology and cell kinetics--theory. Urology 17[Suppl 3]: 40-53.

Collingwood T. N., Urnov F. D. and Wolffe A. P. (1999): Nuclear receptors: coactivators, corepressors and chromatin remodeling in the control of transcription. J Mol Endocrinol. 23: 255-275.

Comuzzi B., Lambrinidis L., Rogatsch H., Godoy-Tundidor S., Knezevic N., Krhen I., Marekovic Z., Bartsch G., Klocker H., Hobisch A. and Culig Z. (2003): The transcriptional coactivator cAMP response element-binding protein-binding protein is expressed in prostate cancer and enhances androgen- and anti-androgen-induced androgen receptor function. Am J Pathol. 162: 233-241.

Cooke P. S., Young P. and Cunha G. R. (1991): Androgen receptor expression in developing male reproductive organs. Endocrinology 128(6): 2867-2873.

Craft N., Chhor C., Tran C., Belldegrun A., DeKernion J., Witte O. Said J., Reiter R. E. and Sawyers C. L. (1999): Evidence for Clonal Outgrowth of Androgen-independent Prostate Cancer Cells from Androgen-dependent Tumors through a Two-Step Process. Cancer Res. 59: 5030-5036.

Craft N., Shostak Y., Carey M. and Sawyers C. L. (1999): A mechanism for hormone-independent prostate cancer through modulation of androgen receptor signaling by the HER2/neu tyrosine kinase. Nat Med. 5: 280-285.

Culig Z., Hobisch A., Cronauer M. V., Cato A. C., Hittmair A., Radmayr C., Eberle J., Bartsch G. and Klocker H. (1993): Mutant androgen receptor detected in an advanced-stage prostatic carcinoma is activated by adrenal androgens and progesterone. Mol Endocrinol. 7:1541-1550.

Culig Z., Hobisch A., Cronauer M. V., Radmayr C., Trapman J., Hittmair A., Bartsch G. and Klocker H. (1994): Androgen receptor activation in prostatic tumor cell lines by insulin-like growth factor-I, keratinocyte growth factor, and epidermal growth factor. Cancer Res. 54: 5474-5478.

Culig Z., Hobisch A., Hittmair H., Peterziel C., Radmayr C., Bartsch G., Cato A. C. B. and Klocker H. (1997): Hyperactive androgen receptor in prostate cancer: what does it mean for new therapy concepts? Histol Histopathol. 12:781-786.

de Voogt H. J. (1992): The position of cyproterone acetate (CPA), a steroidal anti-androgen, in the treatment of prostate cancer. Prostate Suppl. 4: 91-95.

Debes J. D., Schmidt U., Huang H. and Tindall D. J. (2002): P300 mediates androgen-independent transactivation of the androgen receptor by interleukin 6. Cancer Res. 62:5632-5636.

Dedhar S., Rennie P. S., Shago M., Hagesteijn C. Y., Yang H., Filmus J., Hawley R. G., Bruchovsky N., Cheng H., Matusik R. J. and Giguere V. (1994): Inhibition of nuclear hormone receptor activity by calreticulin. Nature 367 (6462):480-483.

Dotzlaw H., Moehren U., Mink S., Cato A. C., Iniguez Lluhi J. A. and Baniahmad A. (2002): The amino terminus of the human AR is target for corepressor action and antihormone agonism. Mol Endocrinol. 16: 661-673.

Dotzlaw H., Papaioannou M., Moehren U., Claessens F., and Baniahmad A. (2003): Agonist-antagonist induced coactivator and corepressor interplay on the human androgen receptor. Mol Cell Endocrinol. 213: 79-85.

Drachenberg D. E., Elgamal A. A., Rowbotham R., Peterson M. and Murphy G. P. (1999): Circulating levels of interleukin-6 in patients with hormone-refractory prostate cancer. Prostate 41:127-133.

Dressel U., Thormeyer D., Altincicek B., Paululat A., Eggert M., Schneider S., Tenbaum S. P., Renkawitz R. and Baniahmad A. (1999): Alien, a highly conserved protein with characteristics of a corepressor for members of the nuclear hormone receptor superfamily. Mol Cell Biol. 19: 3383-3394.

Edwards S. M., Badzioch M. D., Minter R., Hamoudi R., Collins N., Ardern-Jones A., Dowe A., Osborne S., Kelly J., Shearer R., Easton D. F., Saunders G. F., Dearnaley D. P. and Eeles R. A. (1999): Androgen receptor polymorphisms: association with prostate cancer risk, relapse and overall survival. Int J Cancer 84(5): 458-465.

Eng F. C., Barsalou A., Akutsu N., Mercier I., Zechel C., Mader S., and White J. H. (1998): Different classes of coactivators recognize distinct but overlapping binding sites on the estrogen receptor ligand binding domain.J Biol Chem. Oct 23;273(43):28371-28377.

Faber P. W., van Rooij H. C., van der Korput H. A., Baarends W. M., Brinkmann A. O., Grootegoed J. A. and Trapman J. (1991): Characterization of the human androgen receptor transcription unit. J Biol Chem. 266: 10743-10749.

Farla P., Hersmus R., Trapman J., and Houtsmuller A. B. (2005): Antiandrogens prevent stable DNA-binding of the androgen receptor. J Cell Sci. Sep 15;118(Pt 18):4187-4198.

Fernandes I. and White J. H. (2003): Agonist-bound nuclear receptors: not just targets of coactivators. J Mol Endocrinol. Aug;31(1):1-7.

Fernandes I., Bastien Y., Wai T., Nygard K., Lin R., Cormier O., Lee H. S., Eng F., Bertos N. R., Pelletier N., Mader S., Han V. K., Yang X. J. and White, J. H. (2003): Ligand-dependent nuclear receptor corepressor LCoR functions by histone deacetylase-dependent and -independent mechanisms. Mol Cell 11: 139-150.

Fujimoto N., Mizokami A., Harada S. and Matsumoto T. (2001): Different expression of androgen receptor coactivators in human prostate. Urology 58: 289-294.

Gao J., Arnold J. T. and Isaacs J. T. (2001): Conversion from a paracrine to an autocrine mechanism of androgen-stimulated growth during malignant transformation of prostatic epithelial cells. Cancer Res. Jul 1;61(13):5038-5044.

Gao H., Ouyang X., Banach-Pertosky W. A., Gerald W., Shen M. M. and Abate-Shen C. (2006): Combinatorial activities of Akt and B-Raf/Erk signaling in a mouse model of androgen-independent prostate cancer. Proc Natl Acad Sci USA Sep 26;103(39):14477-14482.

Gaddipati J. P., McLeod D. G., Heidenberg H. B., Sesterhenn I.A., Finger M.J., Moul J. W. and Srivastava S. (1994): Frequent detection of codon 877 mutation in the androgen receptor gene in advanced prostate cancers. Cancer Res. 54:2861-2864.

Georget V., Lobaccaro J. M., Terouanne B., Mangeat P., Nicolas J. C. and Sultan C. (1997): Trafficking of the androgen receptor in living cells with fused green fluorescent protein-androgen receptor. Mol Cell Endocrinol. 129: 17-26.

Giguere V. (1999): Orphan nuclear receptors: from gene to function. Endocr Rev. Oct;20(5):689-725.

Giovannucci E., Stampfer M. J., Krithivas K., Brown M., Dahl D., Brufsky A., Talcott J., Hennekens C. H. and Kantoff P. W. (1997): The CAG repeat within the androgen receptor gene and its relationship to prostate cancer. Proc Natl Acad Sci USA 94: 3320-3323.

Glass C. K. (1994): Differential recognition of target genes by nuclear receptor monomers, dimers, and heterodimers. Endocr Rev. 15: 391-407.

Glass, C. K., and Rosenfeld, M. G. (2000): The coregulator exchange in transcriptional functions of nuclear receptors. Genes Dev 14, 121-141.

Gregory C. W., He B., Johnson R. T., Harris Ford O., Mohler J. M., French F. S. and Wilson E. M. (2001): A mechanism for androgen receptor-mediated prostate cancer recurrence after androgen deprivation therapy. Cancer Res. 61: 4315-4319.

Gronemeyer H., Gustafsson J. A., and Laudet V. (2004): Principles for modulation of the nuclear receptor superfamily. Nat Rev Drug Discov. Nov;3(11):950-64.

Grunstein M. Histone acetylation in chromatin structure and transcription. (1997): Nature 389(6649):349-352.

Haag P., Bektic J., Bartsch G., Klocker H., and Eder I. E. (2005): Androgen receptor down regulation by small interference RNA induces cell growth inhibition in androgen sensitive as well as in androgen independent prostate cancer cells. J Steroid Biochem Mol Biol. Aug;96(3-4):251-258.

Han G., Foster B. A., Mistry S., Buchanan G., Harris J. M., Tilley W. D. and Greenberg N. M. (2001): Hormone status selects for spontaneous somatic androgen receptor variants that demonstrate specific ligand and cofactor dependent activities in autochthonous prostate cancer. J Biol Chem. 276: 11204-11213.

Hanahan D. (1983): Studies on transformation of Escherichia coli with plasmids. J Mol Biol. Jun 5;166(4):557-80.

He B., Kemppainen J. A. and Wilson E. M. (2000): FXXLF and WXXLF sequences mediate the NH2-terminal interaction with the ligand binding domain of the androgen receptor. J Biol Chem. 275: 22986-22994.

Heinlein C. A. and Chang C. (2002): Androgen receptor (AR) coregulators: An overview. Endocr Rev. 23: 175-200.

Heinlein C. A. and Chang C. (2004): Androgen receptor in prostate cancer. Endocrine Rev. 25(2):276-308.

Hermanson O., Glass C. K., and Rosenfeld M. G. (2002): Nuclear receptor coregulators: multiple modes of action. Trends Endocrinol Metab. 13: 55-60.

Hobisch A., Eder I. E., Putz T., Horninger W., Bartsch G., Klocker H. and Culig Z. (1998): Interleukin-6 regulates prostate-specific protein expression in prostate carcinoma cells by activation of the androgen receptor. Cancer Res. 5(8):4640-4645.

Hobisch A., Fritzer A., Comuzzi B., Fiechtl M., Malinowska K., Steiner H., Bartsch G. and Culig Z. (2006): androgen receptor pathway is by-passed in prostate cancer cells generated after prolonged treatment with bicalutamide. Prostate Mar 1;66(4):413-420.

Holmes D. S. and Quigley M. (1981): A rapid boiling method for the preparation of bacterial plasmids. Anal Biochem. 114: 193-197.

Hong S. H. and Privalsky M. L. (2000): The SMRT corepressor is regulated by a MEK-1 kinase pathway: inhibition of corepressor function is associated with SMRT phosphorylation and nuclear export. Mol Cell Biol. 20: 6612-6625.

Horlein A. J., Naar A. M., Heinzel T., Torchia J., Gloss B., Kurokawa R., Ryan A., Kamei Y., Soderstrom M., Glass C. K. and Rosenfeld M. G. (1995): Ligand-independent repression by the thyroid hormone receptor mediated by a nuclear receptor corepressor. Nature 377: 397-403.

Hu X. and Lazar M. A. (1999): The CoRNR motif controls the recruitment of corepressors by nuclear hormone receptors. Nature 402: 93-96.

Hu X. and Lazar M. A. (2000): Transcriptional repression by nuclear hormone receptors. Trends Endocrinol Metab. 11: 6-10.

Huang N., vom Baur E., Garnier J. M., Lerouge T., Vonesch J. L., Lutz Y., Chambon P. and Losson R. (1998): Two distinct nuclear receptor interaction domains in NSD1, a novel

SET protein that exhibits characteristics of both corepressors and coactivators. EMBO J. Jun 15;17(12):3398-3412.

Inoue H., Nojima H. and Okayama H. (1990): High efficiency transformation of Escherichia coli with plasmids. Gene Nov 30;96(1):23-28.

Irvine R. A., Yu M. C., Ross R. K. and Coetzee G. A. (1995): The CAG and GGC microsatellites of the androgen receptor gene are in linkage disequilibrium in men with prostate cancer. Cancer Res. May 1;55(9):1937-1940.

lsaacs J. T. (1994): Role of androgens in prostatic cancer. Vitam.Horm. 49:433-502.

Isaacs JT. (1984): Antagonistic effect of androgen on prostatic cell death. Prostate. 5(5):545-557.

Jain A., Lam A., Vivanco I., Carey M. F. and Reiter R. E. (2002): Identification of an androgen-dependent enhancer within the prostate stem cell antigen gene. Mol Endocrinol. Oct;16(10):2323-2337.

Jenster G., van der Korput H. A., Trapman J. and Brinkmann A. O. (1995): Identification of two transcription activation units in the N-terminal domain of the human androgen receptor. J Biol Chem. 1270:7341-7346.

Jenster G., van der Korput H. A., van Vroonhoven C., van der Kwast T. H., Trapman J. and Brinkmann A. O. (1991): Domains of the human androgen receptor involved in steroid binding, transcriptional actvation, and subcellular localization. Mol Endocrinol. 5:1396-1404.

Jenster G. (2000): Ligand-independent activation of the androgen receptor in prostate cancer by growth factors and cytokines. J.Pathol. 191[3]: 227-228.

Jia L. and Coetzee G. A. (2005): Androgen receptor-dependent PSA expression in androgen-independent prostate cancer cells does not involve androgen receptor occupancy of the PSA locus. Cancer Res. Sep 1;65(17):8003-8008.

Johnson P. H. and Grossman L. I. (1977): Electrophoresis of DNA in agarose gels: Optimizing separations of conformational isomers of double- and single-stranded DNAs. Biochemistry 16: 4217-4225.

Khanim F. L., Gommersall L. M., Wood V. H., Smith K. L., Montalvo L., O'Neill L. P., Xu Y., Peehl D. M., Stewart P. M., Turner B. M. and Campbell M. J. (2004): Altered SMRT levels disrupt vitamin D3 receptor signalling in prostate cancer cells. Oncogene Sep 2;23(40):6712-6725.

Kim D., Gregory C. W., French F. S., Smith G. J. and Mohler J. L. (2002): Androgen receptor expression and cellular proliferation during transition from androgen-dependent to

recurrent growth after castration in the CWR22 Prostate Cancer Xenograft. Am J Pathol. 160: 219-226.

Knudsen K.E., Cavenee W. K. and Arden K. C. (1999): D-type cyclins complex with the androgen receptor and inhibit its transcriptional activity. Cancer Res. 59: 2297-2301.

Koelle M. R., Talbot W. S., Segraves W. A., Bender M. T., Cherbas P., and Hogness D.S. (1991): The Drosophila EcR gene encodes an ecdysone receptor, a new member of the steroid receptor superfamily. Cell Oct 4;67(1):59-77.

Kousteni S., Bellido T., Plotkin L. I., O'Brien C. A., Bodenner D. L., Han L., Han K., DiGregorio G. B., Katzenellenbogen J. A., Katzenellenbogen B. S., Roberson P. K., Weinstein R. S., Jilka R. L. and Manolagas S.C. (2001): Nongenotropic, sex-nonspecific signaling through the estrogen or androgen receptors: dissociation from transcriptional activity. Cell 104:719–730.

Kuil C. W. and Brinkmann A. O. (1996): Androgens, antiandrogens and androgen receptor abnormalities. Eur Urol 29 Suppl 2:78-82.

La Spada A. R., Wilson E. M., Lubahn D. B., Harding A. E. and Fischbeck K. H. (1991): Androgen receptor gene mutations in X-linked spinal and bulbar muscular atrophy. Nature 352:77-79

Le Douarin B., Zechel C., Garnier J. M., Lutz Y., Tora L., Pierrat P., Heery D., Gronemeyer H., Chambon P., and Losson R. (1995): The N-terminal part of TIF1, a putative mediator of the ligand-dependent activation function (AF-2) of nuclear receptors, is fused to B-raf in the oncogenic protein T18. EMBO J. May 1;14(9):2020-2033.

Lee C. H., Chinpaisal C., and Wei L. N. (1998): Cloning and characterization of mouse RIP140, a corepressor for nuclear orphan receptor TR2. Mol Cell Biol. Nov;18(11):6745-6755.

Liao X., Tang S., Thrasher J. B., Griebling T. L., and Li B. (2005): small-interfering RNA-induced androgen receptor silencing leads to apoptotic cell death in prostate cancer. mol Cancer Ther. Apr;4(4): 505-515.

Li J., Wang J., Wang J., Nawaz Z., Liu J. M., Qin J. and Wong J. (2000): Both corepressor proteins SMRT and N-CoR exist in large protein complexes containing HDAC3. EMBO J. Aug 15;19(16):4342- 4350.

Liao G., Chen L. Y., Zhang A., Godavarthy A., Xia F., Ghosh J. C., Li H. and Chen, J. D. (2003): Regulation of androgen receptor activity by the nuclear receptor corepressor SMRT. J Biol Chem. 278: 5052-5061.

Liu Z., Auboeuf D., Wong J., Chen J. D., Tsai S. Y., Tsai M. J., and O'Malley B. W. (2004): Coactivator/corepressor ratios modulate PR-mediated transcription by the selective receptor modulator RU486. Proc Natl Acad Sci USA. Jun 11;99(12):7940-7944.

Lu S., Liu M., Epner D. E., Tsai S. Y., and Tsai M. J. (1999): Androgen regulation of the cyclin dependent kinase inhibitor p21 gene through an androgen response element in the proximal promoter. Mol Endocrinol. 13:376-384.

Lu S., Tsai S. Y. and Tsai M. J. (1997): Regulation of androgen-dependent prostatic cancer cell growth: androgen regulation of CDK2, CDK4, and CM p16 genes. Cancer Res. 57: 4511-4516.

Lubahn D. B., Joseph D. R., Sullivan P. M., Willard H. F., French F. S. and Wilson E. M. (1988): Cloning of human androgen receptor complementary DNA and localization to the X chromosome. Science 240:327-330.

Ma H., Hong H., Huang S. M., Irvine R. A., Webb P., Kushner P. J., Coetzee G. A. and Stallcup M. R. (1999): Multiple signal input and output domains of the 160-kilodalton nuclear receptor coactivator proteins. Mol Cell Biol. Sep;19(9):6164-6173.

MacLean H. E., Wame G. L. and Zajac J. D. (1997): Localization of functional domains in the androgen receptor. J Steroid Biochem Mol Biol. 62: 233-242.

Mangelsdorf D. J., Thummel C., Beato M., Herrlich P., Schutz G., Umesono K., Blumberg B., Kastner P., Mark M. and Chambon P. (1995): The nuclear receptor superfamily: the second decade. Cell 83, 835-839.

Metzger E., Muller J. M., Ferrari S., Buettner R. and Schule R. (2003): A novel inducible transactivation domain in the androgen receptor: implications for PRK in prostate cancer. EMBO J. 22: 270-280.

Metzger E., Wissmann M., Yin N., Muller J. M., Schneider R., Peters A. H., Gunther T., Buettner R., and Schule R. (2005): LSD1 demethylates repressive histone marks to promote androgen-receptor-dependent transcription. Nature 437: 436-439.

Moore R. (1944): Benign hypertrophy and carcinoma of the prostate. Surgery. 16: 152-167.

Moehren U., Dressel U., Reeb C. A., Vaisanen S., Dunlop T. W., Carlberg C. and Baniahmad A. (2004): The highly conserved region of the co-repressor Sin3A functionally interacts with the co-repressor Alien. Nucleic Acids Res. 2004 Jun 1;32(10):2995-3004.

Moras D. and Gronemeyer H. (1998): The nuclear receptor ligand-binding domain: structure and function. Curr Opin Cell Biol. 10, 384-391.

Murillo H., Schmidt L. J., and Tindall D. J. (2001): Tyrphostin AG825 triggers p38 mitogen-activated protein kinase-dependent apoptosis in androgen-independent prostate cancer cells C4 and C4-2. Cancer Res. Oct 15;61(20):7408-7412.

Nettles K. W., Sun J., Radek J. T., Sheng S., Rodriguez A. L., Katzenellenbogen J. A., Katzenellenbogen B. S. and Greene G. L. (2004): Allosteric control of ligand selectivity between estrogen receptors alpha and beta: implications for other nuclear receptors. Mol Cell Feb 13;13(3):317-327.

Newmark J. R., Hardy D. O., Tonb D. C., Carter B. S., Epstein J. I., Isaacs W. B., Brown T. R. and Barrack E. R. (1992): Androgen receptor gene mutations in human prostate cancer. Proc Natl Acad Sci USA. Jul 15;89(14):6319-6323.

Nickerson T., Chang F., Lorimer D., Smeekens S. P., Sawyers C. L. and Pollak M. (2001): In vivo progression of LAPC-9 and LNCaP prostate cancer models to androgen independence is associated with increased expression of insulin-like growth factor I (IGF-I) and IGF-I receptor (IGF-IR). Cancer Res. Aug 15;61(16):6276-80.

O'Malley B.W. (1990): The steroid receptor superfamily: more excitement predicted for the future. Mol Endocrinol. 4:363-369.

Ordertlich P., Downes M., and Evans R. M. (2001): Corepressor and nuclear hormone receptor function. Curr Top microbial Immunol. 254:101-116.

Perissi V. and Rosenfeld M. G. (2005): Controlling nuclear receptors: The circular logic of cofactor cycles. Nat. Rev. Mol Cell Biol July 6:542-554.

Perry J. E., Grossmann M. E. and Tindall D. J. (1996): Androgen regulation of gene expression. Prostate Suppl. 6: 79-81.

Peterziel H., Mink S., Schonert A., Becker M., Klocker H., Cato A. C. (1999): Rapid signalling by androgen receptor in prostate cancer cells. Oncogene. Nov 4;18(46):6322-6329.

Poukka H., Karvonen U., Jänne O. A. and Palvimo J. J. (2000): covalent modification of the androgen receptorby small ubiquitin-like modifier I (SUMO-1). Proc Natl Acad Sci USA 97:14145-14150.

Poukka, H., Aarnisalo, P., Karvonen, U., Palvimo, J. J. and Janne, O. A. (1999): Ubc9 interacts with the androgen receptor and activates receptor-dependent transcription. J Biol Chem. 274: 19441-19446.

Protopopov A. I., Li J., Winberg G., Gizatullin R. Z., Kashuba V. I., Klein G. and Zabarovsky E. R. (2002): Human cell lines engineered for tetracycline-regulated expression of tumor suppressor candidate genes from a frequently affected chromosomal region, 3p21. J Gene Med. Jul-Aug;4(4):397-406.

Putz T., Culig Z., Eder I. E., Nessler-Menardi C., Bartsch G., Grunicke H., Uberall F. and Klocker H. (1999): Epidermal growth factor (EGF) receptor blockade inhibits the action of EGF, insulin-like growth factor I, and a protein kinase A activator on the mitogen-activated protein kinase pathway in prostate cancer cell lines. Cancer Res. Jan 1;59(1):227-233.

Radloff R., Bauer W. and Vinograd J. (1967): A dye-buoyant-density method for the detection and isolation of closed circular duplex DNA: the closed circular DNA in HeLa cells. Proc Natl Acad Sci USA 57: 1514-1521.

Reiter R. E., Gu Z., Watabe T., Thomas G., Szigeti K., Davis E., Wahl M., Nisitani S., Yamashiro J., Le Beau M. M., Loda M. and Witte O. N. (1998): Prostate stem cell antigen: a cell surface marker overexpressed in prostate cancer. Proc Natl Acad Sci USA. Feb 17;95(4):1735-1740.

Reutens A.T., Fu M., Wang C., Albanese C., McPhaul M. J., Sun Z., Balk S. P., Janne O. A., Palvimo J. J. and Pestell R. G. (2001): Cyclin D1 binds the androgen receptor and regulates hormone-dependent signaling in a p300/CBP-associated factor (P/CAF)-dependent manner. Mol Endocrinol. May;15(5):797-811.

Roche P. J., Hoare S. A. and Parker M. G. (1992): A consensus DNA-binding site for the androgen receptor. Mol Endocrinol. 6: 2229-2235.

Rovin J. D., Frierson H. F. Jr, Ledinh W., Parsons J. T. and Adams R. B. (2002):Expression of focal adhesion kinase in normal and pathologic human prostate tissues. Prostate. Oct 1;53(2):124-132.

Ruizeveld de Winter J. A., Janssen P. J., Sleddens H. M., Verleun Mooijman M. C., Trapman J., Brinkmann A. O., Santerse A. B., Schroder F. H. and van der Kwast, T. H. (1994): Androgen receptor status in localized and locally progressive hormone-refractory human prostate cancer. Am J Pathol. 144[4]: 735-746.

Russell P. J., Bennett S., Stricker P. and Djakiew D. (1998) Growth factor involvement in progression of prostate cancer. Clin Chem 44:705-723 Dysregulated expression of growth factors and their receptors in the development of prostate cancer. Prostate 2000 42:150-160.

Russell, D. W. and Wilson, J. D. (1994): Steroid 5 alpha-reductase: two genes/two enzymes. Ann Rev Biochem. 63:25-61.

Sadar M. D. (1999): Androgen-independent induction of prostate-specific antigen gene expression via cross-talk between the androgen receptor and protein kinase A signal transduction pathways. J Biol Chem. 274: 7777-7783.

Saffran D. C., Raitano A. B., Hubert R. S., Witte O. N., Reiter R. E. and Jakobovits A. (2001): Anti-PSCA mAbs inhibit tumor growth and metastasis formation and prolong the survival of mice bearing human prostate cancer xenografts. Proc Natl Acad Sci USA. Feb 27;98(5):2658-63.

Sartor O., Zheng Q. and Eastham J. A. (1999): Androgen receptor gene CAG repeat length varies in a race-specific fashion in men without prostate cancer. Urology 53[2]: 378-380.

Seeger M., Kraft R., Ferrell K., Bech-Otschir D., Dumdey R., Schade R., Gordon C., Naumann M. and Dubiel W. (1998): A novel protein complex involved in signal transduction possessing similarities to the 26S proteasome subunits. FASEB J. 12:469–478.

Shah Y. M. and Rowan B. G. (2005): The Src kinase pathway promotes tamoxifen agonist action in Ishikawa endometrial cells through phosphorylation-dependent stabilization of estrogen receptor (alpha) promoter interaction and elevated steroid receptor coactivator 1 activity. Mol Endocrinol. Mar;19(3):732-48.

Shang Y. and Brown M. (2002): Molecular determinants for the tissue specificity of SERMs. Science Mar 29;295(5564):2465-8.

Shang Y., Myers M. and Brown M. (2002): Formation of the androgen receptor transcription complex. Mol Cell 9: 601-610.

Slack J. K., Adams R. B., Rovin J. D., Bissonette E. A., Stoker C. E. and Parsons J. T. (2001): Alterations in the focal adhesion kinase/Src signal transduction pathway correlate with increased migratory capacity of prostate carcinoma cells. Oncogene Mar 8;20(10):1152-1163.

Southern E. (1979): Gel electrophoresis of restriction fragments. Methods Enzymol. 68: 152-176.

Steinsapir J., Socci R. and Reinach P. (1991): Effects of androgen on intracellular calcium of LNCaP cells. Biochem Biophys Res Commun. Aug 30;179(1):90-96.

Sun C., Shi Y., Xu L. L., Nageswararao C., Davis L. D., Segawa T., Dobi A., McLeod D. G. and Srivastava S. (2006): Androgen receptor mutation (T877A) promotes prostate cancer cell growth and cell survival. Oncogene. Jun 29;25(28):3905-3913.

Suzuki H., Sato N., Watabe Y., Masai M., Seino S. and Shimazaki J. (1993): Androgen receptor gene mutations in human prostate cancer. J Steroid Biochem Mol Biol. 46: 759-765.

Tabor S. and Richardson C. C. (1987): DNA sequence analysis with a modified bacteriophage T7 DNA polymerase. Proc Natl Acad Sci USA 84: 4767-4771.

Tora L., White J., Brou C., Tasset D., Webster N., Scheer E. and Chambon P. (1989): The human estrogen receptor has two independent nonacidic transcriptional activation functions. Cell Nov 3;59(3):477-487.

Treuter E., Albrektsen T., Johansson L., Leers J. and Gustafsson J. A. (1998): A regulatory role for RIP140 in nuclear receptor activation. Mol Endocrinol. Jun;12(6):864-881.

Ueda T., Mawji N. R., Bruchovsky N. and Sadar M. D. (2002): Ligand-independent activation of the androgen receptor by interleukin-6 and the role of steroid receptor coactivator-1 in prostate cancer cells. J Biol Chem. 277: 38087 – 38094.

Unni E., Sun S., Nan B., McPhaul M. J., Cheskis B., Mancini M. A. and Marcelli M. (2004): Changes in androgen receptor nongenotropic signaling correlate with transition of LNCaP cells to androgen independence. Cancer Res. Oct 1;64(19):7156-7168.

Veldscholte J., Berrevoets C. A., Ris-Stalpers C., Kuiper G. G., Jenster G., Trapman J., Brinkmann A. O. and Mulder E. (1992): The androgen receptor in LNCaP cells contains a mutation in the ligand binding domain which affects steroid binding characteristics and response to antiandrogens. J Steroid Biochem Mol Biol. 41: 665-669.

Visakorpi T., Hyytinen E., Koivisto P., Tanner M., Keinänen R., Palmberg C., Palotie A., Tammela T., Isola J. and Kallioniemi O. P. (1995): In vivo amplification of the androgen receptor gene and progression of human prostate cancer. Nat Genet. 9:401-406.

Vo N., Fjeld C. and Goodman R. H. (2001): Acetylation of nuclear hormone receptor-interacting protein RIP140 regulates binding of the transcriptional corepressor CtBP. Mol Cell Biol. Sep;21(18):6181-6188.

Wang L., Hsu C. L., Ni J., Wang P. H., Yeh S., Keng P. and Chang C. (2004): Human checkpoint protein hRad9 functions as a negative coregulator to repress androgen receptor transactivation in prostate cancer cells. Mol Cell Biol. Mar;24(5):2202-2213.

Watabe T., Lin M., Ide H., Donjacour A. A., Cunha G. R., Witte O. N. and Reiter R. E. (2002): Growth, regeneration, and tumorigenesis of the prostate activates the PSCA promoter. Proc Natl Acad Sci USA. 2002 Jan 8;99(1):401-406.

Weston A. D., Blumberg B. and Underhill T. M. (2003): Active repression by unliganded retinoid receptors in development: less is sometimes more. J Cell Biol. Apr 28;161(2):223-228.

Whitaker H.C., Hanrahan S., Totty N., Gamble S. C., Waxman J., Cato A. C., Hurst H. C. and Bevan C. L. (2004): Androgen receptor is targeted to distinct subcellular compartments in response to different therapeutic antiandrogens. Clin Cancer Res. Nov 1;10(21):7392-7401.

White J. H., Fernandes I., Mader S. and Yang X. J. (2004): Corepressor recruitment by agonist-bound nuclear receptors. Vitam Horm 68: 123-143.

Wigler M., Pellicer A., Silverstein S. and Axel R. (1978): Biochemical transfer of single copy eukaryotic genes using total cellular DNA as donor. Cell 19:725.

Wright M. E., Tsai M. J. and Aebersold R. (2003): Androgen receptor represses the neuroendocrine transdifferentiation process in prostate cancer cells. Mol Endocrinol. Sep;17(9):1726-1737.

Wu H. C., Hsieh J. T., Gleave M. E., Brown N. M., Pathak S., and Chung L. W. (1994): Derivation of androgen-independent human LNCaP prostatic cancer cell sublines: role of bone stromal cells. Int J Cancer. May 1;57(3):406-412.

Xu L., Glass C. K. and Rosenfeld M. G. (1999): Coactivator and corepressor complexes in nuclear receptor function. Curr Opin Genet Dev. 9(2):140-147.

Young C. Y., Andrews P. F., Montgomery B. T. and Tindall D. J. (1992): Tissue-specific and hormonal regulation of human prostate-specific glandular kallikrein. Biochemistry Jan 28;31(3):818-824.

Zhang J., Thomas T. Z., Kasper S. and Matusik R. J. (2000): A small composite probasin promoter confers high levels of prostate-specific gene expression through regulation by androgens and glucocorticoids in vitro and in vivo. Endocrinology Dec;141(12):4698-4710.

Zhang M., Magit D., and Sager R. (1997): Expression of maspin in prostate cells is regulated by a positive ets element and a negative hormonal responsive element site recognized by androgen receptor. Proc Natl Acad Sci USA. May 27;94(11):5673-5678.

Zhou Y., Gross W., Hong S. H. and Privalsky M. L. (2001): The SMRT corepressor is a target of phosphorylation by protein kinase CK2 (casein kinase II). Mol Cell Biochem. Apr;220(1-2):1-13.

Zhu N., Pewit E. B., Cai X., Cohn E. B., Lang S., Chen R. and Wang Z. (1998): Calreticulin: an intracellular Ca++-binding protein abundantly expressed and regulated by androgens in prostatic epithelial cells. Nucleic acids res. 139 (10): 4347-4344.

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