LCoR acts as a novel androgen receptor corepressor, inhibits prostate cancer growth and is functionally inactivated by the Src kinase

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Introduction

Prostate cancer (PCa) in men is a serious health problem worldwide and the second leading cause of cancer-related male deaths (1). The growth of normal prostate and PCa is regulated by the androgen receptor (AR) (2, 3), a ligand-activated transcription factor and a member of nuclear hormone receptor (NHR) superfamily. Its modular structure comprises of an N-terminally located activation function 1 (AF1) and an activation function 2 (AF2); AR, androgen receptor; CoR, corepressor; CPA, cyproterone acetate; CtBP, C-terminal binding protein; DBD, DNA binding domain; DHT, dihydrotestosterone; ER, estrogen receptor; HDAC, histone deacetylase; HLH, helix-loop-helix motif; LBD, ligand binding domain; LCoR, ligand-dependent CoR; NCoR, nuclear receptor CoR; NHR, nuclear hormone receptor; NTD, amino-terminal domain; OHF, hydroxy-flutamide; PCa, prostate cancer; SMRT, silencing mediator of retinoid and thyroid receptors; VP16, viral protein 16 activation domain.

Background: Prostate cancer (PCa) growth is promoted by the androgen receptor (AR). Castration resistant PCa is associated with activated signaling pathways.

Results: LCoR represses human PCa growth in vivo. Src kinase inactivates the corepressive function of LCoR in vivo.

Conclusion: LCoR, novel corepressor for AR, inhibits PCa cell growth in vivo. LCoR is inactivated by Src kinase in castration-resistant PCa in castration-resistant PCa.

The activated androgen receptor (AR) promotes prostate cancer (PCa) growth. AR-antagonists repress the AR by recruitment of corepressors. Not much is known about the inactivation of AR by corepressors in the presence of agonists (androgens). Here we show that the corepressor LCoR acts as an androgen-dependent corepressor that represses human PCa growth in vivo. In line with this, progressive decrease of LCoR expression was observed in the PCa TRAMP mouse model with increasing age. LCoR interacts with AR and is recruited to chromatin in an androgen-induced manner. Unexpectedly, the LXXLL motif of LCoR is dispensable for interaction with the AR, rather the data indicate that LCoR interacts with the AR-DBD on DNA. Interestingly, the interaction of LCoR with AR is inhibited by signaling pathways that are associated with androgen-independent PCa. Here we also show that the Src kinase inactivates the corepressive function of LCoR. Interfering with endogenous Src function by a dominant negative Src mutant, the growth inhibitory activity of LCoR is enhanced in vivo in xenograft mouse model system. Thus, our studies indicate the role of LCoR as an AR corepressor and a tumor suppressor. Further, the decreased expression or inactivation of LCoR is as an important step towards PCa carcinogenesis in vivo.

Abbreviations
AF1 and AF2, activation function 1 and 2; AR, androgen receptor; CoR, corepressor; CPA, cyproterone acetate; CtBP, C-terminal binding protein; DBD, DNA binding domain; DHT, dihydro-testosterone; ER, estrogen receptor; HDAC, histone deacetylase; HLH, helix-loop-helix motif; LBD, ligand binding domain; LCoR, ligand-dependent CoR; NCoR, nuclear receptor CoR; NHR, nuclear hormone receptor; NTD, amino-terminal domain; OHF, hydroxy-flutamide; PCa, prostate cancer; SMRT, silencing mediator of retinoid and thyroid receptors; VP16, viral protein 16 activation domain.
transcription activation function (AF1), a central DNA binding domain (DBD) and a C-terminal ligand binding domain (LBD). Binding of natural agonist dihydrotestosterone, (DHT) to the LBD induces conformational changes in AR, leading to its shuttling into the nucleus and subsequent binding to the regulatory elements in the target genes thereby modulating their expression. AR is vital for proper prostate functioning and its mutations have been linked to benign prostatic hyperplasia (BPH) and PCa. Further, increased AR-mediated transactivation has been linked to progression of PCa. Therefore, the AR is a key factor and drug target for PCa.

The transcriptional activity of AR is regulated by interacting proteins termed 'coactivators' which positively modulate receptor function and by 'corepressors', which inhibit AR function (4, 5) such as Alien, (6) SMRT (7) and NCoR (8). Binding of agonist to AR allows a preferential functional interaction of coactivators through their NR-box containing the LXXLL motif, which in turn promotes gene activation (9). Conversely, we have previously shown that corepressors are mostly recruited to antagonist-bound AR and lead to suppression of AR target gene expression (6, 10, 11). LCoR was first identified as a ligand-dependent interaction partner for the LBD of estrogen receptor (ER) requiring the LXXLL of LCoR motif for binding to ER (12).

Development of hormone therapy resistant or castration resistant PCa is associated with increased AR mRNA and protein levels that lead to reduced corepressor recruitment to AR target genes in the presence of AR-specific antagonists (2, 3). Moreover, various signaling pathways including Src kinase pathway are activated in androgen-independent/castration resistant PCa leading to activated AR (13). Evidence suggest that also in this situation binding of corepressors to the AR is reduced in the presence of AR antagonists as one underlying molecular mechanism (10, 11).

This study focused on the inhibition of AR in the presence of AR-agonists and identifies LCoR as a novel androgen-dependent corepressor for the human AR that inhibits the growth of PCa. Further, LCoR itself is in turn functionally inactivated by the Src kinase pathway.

**Materials and Methods**

**Reagents** AR antibody, salmon sperm and DNA/protein A agarose 50 % slurry and were obtained from Upstate Biotechnology. LCoR antibody was obtained from Genway Biotechnology. The myc- and non-specific IgG antibodies were from Santa Cruz Biotechnology. Methyltrienolone or R1881 (Perkin Elmer), DHT, CPA, proteinase K, RNase A and Guanidium HCl (Sigma), Casodex and OHF from Schering AG. RNA purification kit was from Qiagen. PP2, U0126 and LY294002 were procured from Calbiochem. Complete mini protease inhibitor and protease inhibitor cocktail from Roche. Matrigel was purchased from BD Biosciences. 3 weeks old athymic male nude mice were obtained from NxGen Biosciences and mice studies were permitted by the animal ethics committee of the University of Wisconsin.

**Plasmid constructs** Mammalian expression vectors for various ARs and luciferase reporters have been described previously (11). pSG5-LCoR and pSG5-LCoR-mut were a kind gift from Dr. John White. pABΔgal, pAB-gal-NCoR, pABgal-Alien, pETE-Hyg and pCMX-VP16, Src-wt and Src mutant were described previously (6, 11, 13). pCS2-MT-LCoR, pAB-VP-DBD and VP-LCoR fusions were generated by standard cloning techniques.

**Cell culture and transient transfection** CV1 and C4-2 cells were grown and transfected as described previously (11, 13). Unless otherwise stated 1 µg of each luciferase reporter, 0.2 µg of AR expression plasmids and 1 µg of pSG5/pSG5-LCoR and 0.4 µg of pCMX-LacZ were used in transfections and 16 h post transfection treated with hormones R1881 at concentrations 10^{-10} M, DHT 10^{-7} M, Casodex 10^{-7} M, CPA 10^{-7} M and OH-F 10^{-7} M and all signaling inhibitors at 1 µM. 72 h post transfection cells were harvested and measured for luciferase and beta-galactosidase activities as described previously (11). Independent triplicate experiments were performed each time and were repeated three times. Error bars represent the deviation of the mean value. Student’s t-test was used to calculate the p-
values. A P-value below 0.05 was considered statistically significant.

**Semi-quantitative polymerase chain reaction (PCR)** PCR reactions were carried out using forward and reverse primer for LCoR (forward 5’-tgcacaatcagaacccgtgtct-3'; reverse 5’-tgccagctgacaatggctttc-3’), and GAPDH (forward 5’-aatcccatcaccatcttcaggag-3; reverse 5’-gcattgctgatgatcttgaggctg-3). PCR reaction standardization kit was obtained from Epicentre Biotechnologies (Madison, WI).

**Chromatin immunoprecipitation (ChIP) assay** ChIP experiments involving PSA enhancer region (ARE III) were performed essentially as described previously (6). ChIP experiments were repeated three times with similar results.

**Real-time RT-PCR** Isolation of mRNA and the real-time PCR was performed as described earlier (6). A total of 200,000 C4-2 cells per well were seeded out in charcoal-stripped serum containing T-media in six-well tissue culture dishes. After 24 h cells were treated with R1881 (10^{-10} M) for 48 h, total cellular RNA was isolated, 1 mg RNA was reverse-transcribed to cDNA and was subjected to amplification by light cycler using specific primers and control primers against actin.

**GST-Pulldown** GST and GST-AR-DBD were expressed in E. coli strain HB101 over night at 16°C after induction with 0.1 mM Isopropyl-β-D-thiogalactopyranosid (Sigma). After bacterial extraction GST proteins were affinity purified via glutathione beads, which were either incubated with 0.5 mg LNCaP whole cell extract as positive control for full-length LCoR binding to AR-DBD or with 10 µg of His-tagged purified LCoR 101-218 or 219-433. His-tagged proteins were expressed in E. coli strain BL21 under the same conditions. Affinity purification was performed with Ni-NTA-beads (Invitrogen). The binding to GST-AR-DBD was analysed via LCoR-western blotting (LCoR antibody was obtained from Cell Signaling). Ponceau staining of proteins served as loading control.

**Generation of stable clones** A total of 200,000 C4-2 cells were transfected with Src or mutant Src along with pETE-Hyg plasmid in 5:1 molar ratio (total amount being 10 µg) using calcium phosphate method. The medium was replaced with fresh T-media 18 h post transfection. Stable clones were selected as described previously (6).

**In vivo tumor xenograft model** 1x10^6 C4-2 stable clones were suspended in 100 µl of complete cell culture media and 100 µl of matrigel. Cells were subcutaneously implanted in the left and right flanks of 5 athymic male nude mice in every experimental group. At different time points, tumors were measured by using digital Vernier caliper.

**Immunohistochemistry** Paraffin-embedded prostate tissues arrays (4 mm) were obtained from US Biomax. Immunostaining was performed using LCoR antibody (dilution 1:50) essentially as described previously (13).

**Results**

**LCoR functionally represses AR transactivation function** A varying degree of LCoR protein expression was observed in a panel of prostate epithelial cells both normal and tumorigenic (Figure 1A). RWPE1, non-tumorigenic normal prostate epithelial cells exhibited higher LCoR protein levels compared to human prostate carcinoma cells such as LNCaP, C4-2, 22RV1, PC3, except DU145 indicating a decreased LCoR expression in most of PCa carcinoma cells compared to normal prostate epithelial cells. To examine the transcriptional effect of LCoR on AR transactivation function, reporter assays employing androgen responsive promoters were performed in CV1 cells lacking endogenous AR expression. Ectopic expression of LCoR (5 to 40 ng) repressed agonist R1881-activated wild type (wt) AR transactivation in a dose-dependent manner (Figure 1B). Also, LCoR repressed wt-AR activated by the natural agonist dihydrotestosterone (DHT), the partial agonist cyproterone acetate (CPA) (Figure 1C). Since wt-AR was not activated
either by Casodex or hydroxy-flutamide (OHF), a repressive effect was not observed. Further, the endogenous mutant AR (AR-T877A, bearing a mutation in the LBD) often found in clinically relapsed disease (14), shown to positively stimulate the growth of PCa (15), was also repressed by LCoR in agonists, R1881- and DHT-dependent fashion (Figure 1D). This AR mutant was also activated by the partial agonist CPA whereas LCoR expression led to its down-regulation. Notably, unlike wt-AR, the T877A mutant was activated by AR antagonist OHF and cotransfection of LCoR also repressed AR transactivation suggesting that LCoR could overcome the T877A-mediated activation through OHF. No significant activation of AR was however seen by Casodex. Thus, this suggests that LCoR down-regulates the transactivation of both activated wt and AR mutant T877A.

We also tested, a double point mutant of AR (termed AR-SUMO), lacking the SUMoylation sites of AR and devoid of interaction sites for the corepressors SMRT and Alien (6, 11, 16). LCoR repressed transactivation of the androgen-induced AR-SUMO mutant (Supplementary figure 1), indicating that LCoR acts on AR in a distinct manner than the corepressor SMRT or Alien.

Similar to repression of AR-mediated transactivation function on MMTV promoter, ectopically expressed LCoR efficiently repressed AR transactivation on GRE, probasin and PSA promoter reporters containing androgen response elements (Figure 1E, 1F) in an agonist-dependent manner indicating the versatility of LCoR in its repression function in a wide androgen responsive promoter context.

Since AR regulates the growth of PCa and interference with AR function leads to growth inhibition of PCa, we tested whether interference with its function by LCoR has an influence on cellular growth. For that purpose, stable clones of the human castration resistant PCa C4-2 cells overexpressing LCoR were generated and colony formation assays were performed. Using the empty expression vector numerous colonies were formed with more cells per colonies. However, cells stably transfected with LCoR exhibited marked decrease in both their colony forming potential and cell number per colony (Figures 1G and H), indicating that LCoR functionally interferes with the growth of PCa cells.

The C-terminus of LCoR is sufficient to repress AR function via targeting its DNA binding domain (DBD)

First, we analyzed whether LCoR requires the previously identified LXXLL motif necessary for interaction with ER also to repress AR in the presence of AR agonist. Interestingly however, the LCoR mutant having NR-box mutated (LCoR mut), also repressed the wt-AR-mediated transactivation in an agonist-dependent manner (Figure 2A). This indicates that NR-box of LCoR is dispensable for AR repression and suggests a different mode of interaction.

To map down the region(s) of AR, which is targeted by LCoR and to define whether a functional interaction between AR and LCoR takes place, various deletion mutants of the AR NTD were analyzed. The N-terminal of AR harbors the major transactivation function. Deleting AF-1 (ΔNTD) therefore renders the intact C-terminus AR transcriptionally incompetent and no repressive effect of LCoR on this AR mutant was observed. Ectopically expressed LCoR repressed various N-terminal AR truncations (Δ39-171, Δ39-328, Δ510-536, and Δ447-536) in an androgen-dependent manner (Figure 2B) indicating that NTD is also dispensable for LCoR-mediated repression of AR.

The LBD deletion (ΔLBD) of AR was activated ligand-independently and was also repressed by LCoR. This suggests that in contrast to many other NHRs, where intact LBD is required for LCoR-mediated repression, the LBD of AR is dispensable for LCoR mediated repression. Employing the LCoR mut, devoid of the LXXLL motif, revealed similar pattern to repress the AR mutants, AR deletions repressing the T877A, and AR ΔLBD mutant well (Figure 2C). Taken together, these experiments rule out the involvement either of NR-box of LCoR or of the LBD of AR in repression by LCoR. The involvement of DBD-LBD cannot be entirely ruled out since deletion of AF-1 bearing N-terminal results in transcriptionally incompetent AR and
therefore repressive effects of LCoR could not be tested in these experiments.

To analyze the involvement of the DBD-LBD of AR in LCoR-mediated repression, NTD was exchanged with the potent transactivation domain of VP16 to generate VP-DBD-LBD. This AR mutant was activated ligand-dependently demonstrating specificity and cotransfection either of LCoR-wt or LCoR-mut repressed the activation of this AR mutant (Figure 3A and 3B) ruling out the possibility of the involvement of NTD of AR in LCoR-mediated repression. All these experiments deleting or replacing one or both activation functions suggest that in order to repress AR function, LCoR does not require the NTD or LBD of AR.

Therefore, the involvement of the DBD of AR in repression by LCoR was tested. The AR chimera (VP-DBD), deleting both the NTD and the LBD was generated, possessing only the intact DBD of AR. This fusion protein was strongly activated ligand-independently compared to empty vector VP16 control and VP16-Gal-DBD (VP16-Gal) (Figure 3C). Interestingly, cotransfection, of either LCoR or LCoR-mut led to the repression of this AR mutant, strongly implicating that LCoR targets specifically the DBD of AR, and not of Gal, to mediate repression. Albeit a strong repressive action of LCoR was not observed with this AR-fusion as compared to full length AR, which may be due to the exchange of the NTD with VP16. On the other hand, to map down the region of LCoR that interacts with AR, various chimeras of LCoR deletions fused to the VP16 transactivator, were generated (Figure 4A) and employed in the mammalian-one-hybrid assays. This assay is based on the interaction of VP16-LCoR fusion to the full length AR. The activation of the reporter by the empty vector VP16 control was therefore set as 1, further activation of the reporter is possible if VP16 is brought into close proximity of the promoter. The full length LCoR (LCoR f.l.), owing the intact CtBP and HDAC interaction domain (12), fused to VP16 transfectected into CV1 cells (Figure 4B) indicating a possible interaction of LCoR with AR that was further strengthened using LCoR deletions that lack the N-terminal domain. Whereas the LCoR 1-90 fusion did not reveal an activation of the reporter presumably due to the presence of two repressive CtBP interaction domains that may counterbalance the transactivation function of VP16. Notably however, both LCoR 101-218 and the non-overlapping LCoR 219-433 fragments exhibited ligand-dependent interaction with AR suggesting that LCoR potentially uses two independent interaction domains to bind to AR. This was confirmed by using bacterially expressed and affinity purified LCoR fragments and the AR-DBD (Fig. 4D). The data suggest that each of the two LCoR fragments interact independently and directly with the AR-DBD in vitro. Interestingly, the C-terminus of LCoR 101-360 and LCoR 101-433 (VP-cLCoR), both of which harbor the HLH interaction motif, were found to functionally interact with wt-hAR in a ligand-dependent manner. VP-cLCoR was able to interact with both CPA- and R1881-bound wt-hAR and exhibited 4- and 7-fold higher activity over empty vector VP16 respectively (Figure 4C).

Taken together, these results indicate that in order to repress AR function, LCoR uses two independent domains to interact with the AR-DBD and that this interaction was independent of the NR-box of LCoR.

**Src activity opposes repressive action of LCoR on AR**

To test the functional relevance of LCoR expression in human PCa cells, the ability of LCoR to repress endogenous T877A mutant AR in PCa C4-2 cells was tested. LCoR was ectopically overexpressed leading to only a slightly (2-fold) repression of AR-mediated transactivation (Figure 5A) in a ligand-dependent manner. The degree of repression was lower as compared to robust repression observed in CV1 cells (Figures 1A and 1B) suggesting that even with forced overexpression, LCoR mediated repression is weaker compared to that observed in CV1 cells. Our hypothesis was that this marginal decrease could be due to the activation of signal transduction pathways in PCa cells that weaken the repressive effect of LCoR on AR. To test this hypothesis we employed a battery of signalling cascade inhibitors to block specific signal transduction pathways, which are overexpressed in PCa such as Src kinase (13). Interestingly, blocking Src function by a specific inhibitor PP2, 4-amino-5- (4-chlorophenyl)-7-(t-buty1) pyrazolo[3,4-d] pyrimidine, enhanced LCoR-mediated AR
repression in presence of agonist (Figure 5A, right panel). This suggests that the Src inhibitor reduced agonist-induced AR transactvation in the presence of LCoR. It is noteworthy that agonist-induced empty vector transfected cells (in the absence of LCoR expression) treated with PP2 yielded also a decreased ligand-induced induction. This effect is presumably due to the activation of endogenous LCoR (Figure 5A). Thus, the potency to inhibit the AR is enhanced by treatment with the Src kinase inhibitor PPS.

In order to explain the restoration of strong repressive effect of LCoR on AR in the presence of PP2, we analyzed the influence of blocking Src kinase function on the interaction of LCoR with endogenous AR employing VP16-cLCoR chimera. Treatment of C4-2 PCa cells with PP2 in the presence of the AR-specific agonist suggested that the interaction of endogenous LCoR with endogenous AR is enhanced (Figure 5B). In line with this, chromatin immunoprecipitation (ChIP) assays revealed that endogenous LCoR is co-recruited to the enhancer of PSA gene in presence of agonist R1881 in C4-2 cells and the recruitment was enhanced by PP2 (Figure 5C; compare lane 1 with 3 & 2 with 4). Further, to test for the expression of the endogenous AR target gene PSA, real-time RT-PCR (qRT-PCR) experiments were performed with and without PP2 treatment. The data suggest that the androgen-induced PSA mRNA levels are decreased by PP2 treatment (Figure 5E). Taken together, the SRC kinase pathway inhibits LCoR-mediated repression of AR presumably by inhibiting the LCoR-AR interaction.

However, we have further addressed also the possibility that the LCoR-mediated silencing may be inhibited by the activated Src kinase pathway. For that purpose Gal4-LCoR chimera were generated. The Gal4-LCoR repressed a cognate reporter much strongly in CV1 cells (approx. 20 folds) as compared to repression observed in C4-2 cells (approx. 4 fold), indicating that the LCoR-mediated silencing is much weaker in the PCa C4-2 cells (Supplementary figure 2). Similarly, the corepressor NCoR chimera exhibited a potent silencing function in CV1 cells compared to C4-2 cells. Conversely, the AR corepressor Alien repressed the induction of the reporter to the same degree in both of these cell lines. This suggests that the repression function of different corepressors is regulated in a cell type dependent context.

Therefore, our hypothesis was that LCoR could be functionally attenuated by activating signaling pathways, which may play a role in PCa progression (16, 17, 18). A battery of specific signalling inhibitors was therefore employed to test whether signalling kinases influence the autonomous repression function of LCoR. Inhibitors of MAPK (U0126), PI3K (LY294002) and Src kinase (PP2) signalling, all of which have been reported to be overexpressed in PCa, were therefore included. Interestingly, blocking Src activity restored strong repression function of LCoR in C4-2 cells (Figure 5D). Also, blocking PI3K activity led to a partial enhancement of LCoR repression function suggesting that LCoR could potentially be targeted by signalling kinases or a circuit of signalling cascades. Notably, blocking MAPK function did not significantly modulate the repressor function of LCoR.

The data suggest that the LCoR-mediated gene silencing is inhibited by the Src kinase pathway. Thus taking together, inhibiting Src kinase pathway using PP2 enhances strongly both the interaction of LCoR with AR and LCoR-mediated gene silencing.

**Src regulates LCoR-mediated growth repression in PCa in vivo & in vitro**

The influence of Src kinase pathway on LCoR was further investigated by cellular growth analyses. For that purpose, stable clones of C4-2 cells overexpressing LCoR and/or Src, wt or mutant were generated. Cells stably transfected with LCoR expression vector showed marked decrease (3.5-fold) in their colony forming potential as compared to control cells transfected with empty vector (Figure 6A). Moreover, interference with endogenous Src function using a Src mutant (Src-mut) further potentiated the repressive effect of LCoR on cellular growth. In contrast, coexpression of Src along with LCoR resulted in increase in colony number as compared to LCoR alone. These results further indicate that LCoR represses PCa cell growth and its function is largely governed by Src kinase activity in PCa cells.

Moreover, the tumorigenic potential of C4-2 cells overexpressing LCoR and/or Src an in
vivo xenograft experiments was analyzed. Both C4-2 wt and C4-2/Src cells developed large tumors in athymic male nude mice (Figures 6B and C). Notably, mice bearing C4-2 cells stably overexpressing either LCoR alone or in combination with Src-mut did not develop any tumor. An interesting finding of the experiment was that cells co-expressing LCoR did not develop any tumor through the entire course of the six week-study and in combination with Src only moderate sized tumors were developed. This indicates on one hand the importance of functionally active corepressors to reduce tumor growth and on the other hand the negative influence of Src activity on corepressor LCoR-mediated repression of prostate tumorigenesis. In agreement with cell culture data, expression of Src-mut alone also developed moderate sized tumors.

To summarize, the results indicate that LCoR acts as a corepressor for ligand-activated AR in vivo, represses the transactivation of AR and subsequently the growth of PCa and that the overexpression of Src negatively regulates its corepression function and therefore also has a positive influence on tumor development.

Differential expression of LCoR in prostate tumorigenesis

A further link between LCoR and PCa progression arose analyzing the expression of LCoR in TRAMP mice that exhibit age-dependent progression stages of PCa, similar to human disease (19). The data suggest that the expression of LCoR decreased as the tumor progressed from well-differentiated carcinoma at 16 weeks to a poorly differentiated carcinoma at 32 weeks (Figure 7A). The levels of LCoR expression was also determined in situ in TRAMP mouse PCa tissue where LCoR expression was highest in PCa tissue in 12-week old mice, which represent well differentiated carcinoma. The LCoR levels were further reduced in moderately differentiated carcinoma derived from 20 weeks old mice and the lowest expression of LCoR was observed in the poorly differentiated carcinoma of mice with 34 weeks of age (Figure 7B). These data strongly suggest a link between LCoR expression and PCa progression.

Discussion

In this study we have characterized LCoR as a novel androgen-dependent AR corepressor, which regulates the growth of PCa cells in vitro and in vivo. Our data demonstrate that the LCoR-mediated repression of AR transactivation is associated with the inhibition of PCa growth in vivo and further linking Src kinase activity to LCoR function.

The corepressors SMRT and Alien act preferentially in the presence of AR antagonists and inhibit AR-mediated transactivation (6, 11). Interestingly, SMRT was shown to interact with AR in the presence of AR agonist but did not exhibit repression. Whereas, in the presence of the partial antagonist CPA, SMRT binds to AR and inhibits AR-mediated transactivation. Alien, on the other hand, seems to interact with AR only in the presence of AR antagonists and not in the presence of agonist (6). This suggests that these corepressors repress AR only in the presence of antagonists. Here, we focused on whether AR transactivation and PCa growth can be repressed in the presence of agonists.

A major activator of AR seems to be activating signaling pathways. Several underlying possibilities can be envisaged how these signaling pathways activate the AR. One of them would be the inactivation of corepressor function. Therefore the work focuses on one of several molecular mechanisms how corepressor function is modulated by signal transduction pathways. Repression of AR function by LCoR highlights its physiological relevance, which may play a protective role against the onset and progression of the disease by repressing AR-mediated gene activation and thereby the growth of PCa.

We demonstrate that the NR-box of LCoR, although required to interact with various other NHR members, is not required to interact with AR and our data suggest that at least the AR-DBD is targeted by LCoR. In vitro protein-protein interaction assays with purified LCoR fragments and the AR-DBD confirm that two independent sites in LCoR exist that interact with the AR-DBD. All the N terminal AR truncations are significantly repressed by LCoR (Fig. 2B), which does not confirm but suggest that LCoR may not
target the AR NTD, which is the case for the interaction of the ligand-sensitive CoR such as SMRT, NCoR or Alien with the AR (6, 11). Some coactivators like Ubc9 have been shown to target the DBD of the AR in order to activate its function (16). Therefore, it is possible that corepressors can also target the same domain to modulate AR transactivation and so can compete with the coactivators such as Ubc9 to interact with AR. This kind of mechanism could help to counterbalance the activated AR in order to fine tune its activation.

Rendering LCoR functionally attenuated could be beneficial for PCa growth in order to attain desirable constitutive activation of AR. Post-translational regulation by Src kinase activity indicates that, in addition to directly activating AR function (13, 20), Src kinase can repress interaction of corepressors such as LCoR and thereby indirectly activating AR function in PCa cells. This report also provides a mechanistic explanation for Src kinase family-mediated involvement in the AR pathway, where Src inhibits corepressors such as LCoR from repressing AR function. In fact, many other signal transduction pathways are overexpressed in androgen-independently growing PCa cells, which may potentially decrease interaction of corepressors with AR in order to activate AR function. In C4-2 cells, introduction of dominant-negative Ras restores sensitivity to Casodex (21). Similarly, treatment of C4-2 cells with Her2 tyrosine kinase inhibitor, AG825 leads to apoptosis in C4-2 cells indicating that overexpression of Her2 may confer androgen-independence to C4-2 cells (22). Similar to that we have found that Src kinase is overexpressed in PCa cells where it promotes the transactivation function of AR (13).

The rationale for using C4-2 cells in the present study is that although they represent androgen insensitive or hormone refractory tumor cells, their growth is dependent on functional androgen receptor mediated signaling (23). Moreover, in these cells as well, LCoR marginally interacts with AR and this is enhanced in presence of a Src kinase inhibitor. Therefore these cells have been used as a model system to tease apart the role of LCoR on AR.

One way to promote AR transactivation by Src kinase could involve attenuation of the corepressor LCoR to repress AR function. In this study we found a pivotal mode of Src kinase action against LCoR by (I) interfering with the binding of LCoR to AR in vivo and (II) by decreasing the autonomous repression potential of LCoR in C4-2 PCa cells. This indicates a novel mechanism that PCa can utilise to weaken specific corepressors at multiple levels to constitutively activate AR function. Both of these LCoR inactivating mechanisms may work in synergy to functionally attenuate LCoR from acting as a potent corepressor for AR in PCa cells.

In contrast to the CoR Alien (6), cells overexpressing LCoR produce fewer colonies in the presence of AR agonist indicating the potential growth inhibitory effect of LCoR on cellular growth.

It is possible that ratio of coactivators to corepressors, which has been shown to modulate NHR function (24) gives competitive advantage to coactivators to bind to AR in PCa cells. This increase in ratio of coactivators to corepression may arise from (I) the overexpression of coactivators or decreased expression of corepressors or (II) functional attenuation of corepressors by signal transduction pathways, which are known to be activated in PCa cells.

These results are suggestive of the existence of multi-modal ways where, in addition to directly activating AR function, as has been reported previously (13, 20), corepressor function can be repressed by activating signalling cascades decreasing both, its autonomous repression potential and interaction with AR, which together allow AR to become more active in PCa.

Taken together, our data characterized a novel transcriptional corepressor for AR, which plays an important role in regulating PCa cell growth in vitro and tumor progression in vivo. It suggests the role of LCoR to fine-tune the androgen receptor transcriptional activity in the presence of androgen agonists. Further, the data indicate that activated or overexpressed Src kinase in PCa cells inhibits LCoR binding and LCoR-mediated gene silencing. Thus, we suggest the existence of signal transduction pathways, which may indirectly regulate corepressor function. Employment of chemotherapeutics based on the inhibition of specific kinases that regulate
corepressor action on AR could therefore prove beneficial in the treatment of therapy resistant PCa.

References


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**Figure legends**

**Figure 1** Functional repression of AR by LCoR.

(A) Western blot analysis showing LCoR proteins in a panel of prostate cell lines. β -actin was used as a loading control. (B) CV1 cells were transfected with pMMTV-Luc, wt-hAR and increasing amount of pSG5-LCoR (5 to 40 ng). Cells were transfected with 1 µg either of (C) hAR wt or (D) T877A mutant. (E) CV1 cells were transfected with each of GRE-Luc, pARR3-Luc (probasin) or (F) PSA-Luc along with wt-hAR and pSG5-LCoR. Graph represents the fold hormone induction with standard deviation between triplicates. LCoR mediated AR repression was significant in 1C - F (t-test: p < 0.005). LCoR-mut represents the NR-box mutant of LCoR (12). AR ligands: agonists: DHT, R1881; antagonists: OHF, Cas, CPA. (G) Stable clones of C4-2 cells overexpressing either empty vector control (left panel) or overexpressing LCoR (right panel) and (H) number of colonies obtained from the corresponding stable clones.

**Figure 2** Mutational analysis of AR-LCoR mediated repression.

(A) CV1 cells were transfected with pMMTV-Luc reporter along with pSG5 or pSG5-LCoR expression vectors and treated with agonist R1881. LCoR mediated AR repression was significant (t-test: p < 0.001).

(B) Various AR deletion plasmids that lead to truncated AR proteins were cotransfected with LCoR and later treated with agonist R1881. LCoR mediated AR repression was significant (t-test: p < 0.01).

(C) CV1 cells were transfected with pMMTV-Luc along with pSG5-LCoR-mut. In addition various AR mutant expression plasmids were also transfected and cells were treated with R1881. The
graph represents the fold hormone induction. LCoR-mutant mediated AR repression was significant (t-test: p < 0.05).

**Figure 3** Interaction analysis in mammalian cells: NTD and DBD are required.

(A and B) CV1 cells were transfected with pMMTV-Luc, VP16 or VP-DBD-LBD (0.1 μg) and 1 μg each of either pSG5 or pSG5-LCoR or pSG5-LCoR-mut and were incubated with R1881. Graph represents fold hormone activity. (C) Cells were transfected with MMTV-Luc, VP16 empty and VP16-Gal-DBD (called VP-Gal) as controls or VP-DBD (1 μg), pSG5 alone, or LCoR, or LCoR-mut and treated with R1881. LCoR mediated repression of the VP-AR deletion mutants was significant (t-test: p < 0.005). The graph represents the fold hormone induction.

**Figure 4** LCoR deletion analysis: Requirement of two interaction domains.

(A) LCoR is a 433 aa protein with a single NR-box for interaction with selected members of nuclear hormone receptor superfamily. The C-terminal part of LCoR harbors a HLH interaction motif. VP16-cLCoR was constructed by cloning last 332 C-terminus aa into pCMX-VP16 empty vector. (B) CV1 cells were transfected with MMTV-Luc, VP16 or various VP16-LCoR fusions (1 μg) and were treated with CPA. Data were plotted setting each empty vector control VP16 in the absence of hormone arbitrarily as one and represents fold reporter activation. (C) CV1 cells were transfected with pMMTV-Luc, pCMX-VP16-cLCoR (2 μg) and hAR (50 ng) treated with R1881 or CPA. The graph depicts the fold hormone induction. LCoR binding to AR was significant for (B) and (C) (t-test: p < 0.05). (D) Bacterially expressed GST or GST-AR-DBD were affinity purified and incubated with either 0.5 mg LNCaP whole cell extract, as positive control for full-length LCoR (f.l.) binding to the AR-DBD, or with 10 μg of His-tagged bacterially expressed and purified LCoR 219-433 or 101-218 fragments that were detected by western blotting. Ponceau staining serves as loading control. Both LCoR minimal domains show significant binding to the AR-DBD.

**Figure 5** Involvement of Src kinase pathway in LCoR mediated repression of AR.

(A) C4-2 cells transfected with MMTV-Luc, pSG5 or pSG5-LCoR and were treated with R1881 and Src kinase inhibitor PP2. Graph represents the fold hormone induction. (B) C4-2 cells transfected with MMTV-Luc, VP16 or VP16-cLCoR (1 μg), were treated with R1881 and PP2. Graph represents the fold hormone induction. The influence of PP2 on LCoR mediated AR repression (A) and on LCoR binding to AR (B) was significant (t-test: p < 0.001). (C) ChIP analysis was performed with C4-2 cells. Before lysis, samples were treated with PP2 for 48 h and with 10^-8 M R1881 for 1 h. The harvested lysate was diluted, immunoprecipitated with antibodies against AR, or anti-LCoR or non-specific anti IgG antibody. The DNA was eluted from the immuno precipitates, amplified by primers spanning the PSA enhancer. As equal starting material prior to immunoprecipitation the input is shown. (D) C4-2 cells transfected with p(UAS)4TATA-Luc reporter along with 1 μg Gal-LCoR plasmid and were treated with each of U0126, Rapamycin, LY294002 and PP2 and data were plotted with respect to values obtained for each empty gal and represents fold reporter repression over empty vector control. (E) In total 200000 C4-2 cells per well were seeded out in hormone-depleted FBS containing T-media in six-well tissue culture dishes. After 24 h cells were treated with R1881 10^{-10}M) for 48 h. Afterwards total cellular RNA was isolated, reverse transcribed to cDNA and amplified by light cycler using specific primers and control primers for actin. The graph represents the actin-normalized values of the PSA transcript.

**Figure 6** LCoR represses PCa growth in vitro and in vivo and is attenuated by Src kinase signaling.

(A) Number of stable clones obtained from C4-2 cells overexpressing either empty vector control, or LCoR, or dominant negative, with mutation in the kinase cassette, Src-mut., or Src, or LCoR+Src mut. or LCoR+Src. LCoR mediated repression of colony formation and potentiation by Src mutant was significant (t-test: p < 0.01).

(B) A representative photograph of the athymic nude mice bearing the xenograft of the C4-2 cell clones overexpressing LCoR and/or Src. (C) The tumor size of xenografts of the above mentioned stable clones of C4-2 cells in athymic nude mice representing the growth of tumor over six weeks (n=5).
Figure 7 Progressive decrease in LCoR expression *in vivo*.
(A) LCoR transcript expression is progressively decreased in TRAMP mice as function of increase in age. Prostate tissue from 16 wk old mice expression higher LCoR mRNA expression than in 24 wks and is further decreased at 24 and 32 wks. (B) Immuno-histochemical analysis for LCoR expression in prostate tissue of TRAMP mice of different age viz. 12, 20 and 34 weeks. Experiments were conducted from five randomly selected tumor tissues from TRAMP mice in each group with similar results and only representative result shown.
Figure 1

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LCoR

GAPDH

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Fold activation

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AR wt

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AR T877A

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Figure 1

G

**control**

**LCoR**

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Figure 2

A

AR wt

Fold activation

R1881

-  +  -  +

control
LCoR
LCoR-mut
Figure 2

B

Fold activation

LCoR

AR wt  ΔNTD  Δ39-171  Δ39-328  Δ510-536  Δ447-536  ΔLBD

C

Fold activation

LCoR-mut

AR wt  AR-T877A  ΔNTD  ΔLBD
Figure 3
Figure 4

A

NR box
LXXLL motif

1

HLH
433

LCoR
LCoR 1-90
LCoR 101-218
LCoR 219-433
LCoR 101-360
cLCoR 101-433

B

Fold activation

Empty vector LCoR 1-433 LCoR 1-90 LCoR 101-218 LCoR 219-433 LCoR 101-360 LCoR 101-433 c-SMRT

no hAR
hAR
hAR and ligand

VP-16 Fusion

C

Fold activation

control VP-cLCoR

CPA R1881
Figure 7

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B

12 wk  
Well differentiated carcinoma

20 wk  
Moderately differentiated carcinoma

34 wk  
Poorly differentiated carcinoma
LCoR acts as a novel androgen receptor corepressor, inhibits prostate cancer growth and is functionally inactivated by the Src kinase
Mohammad Asim, Bilal Bin Hafeez, Imtiaz Ahmad Siddiqui, Claudia Gerlach, Michaela Patz, Hasan Mukhtar and Aria Banaihmad

J. Biol. Chem. published online August 19, 2011

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