An AR-Skp2 pathway for proliferation of androgen-dependent prostate-cancer cells

Hongbo Wang¹, Daqian Sun¹, Peng Ji¹, James Mohler² and Liang Zhu¹,*

¹Department of Developmental and Molecular Biology, The Albert Einstein Comprehensive Cancer Center and Liver Center, Albert Einstein College of Medicine, Bronx, NY 10461, USA
²Department of Urologic Oncology, Roswell Park Cancer Institute, Buffalo, NY 14263, USA

Summary

Androgen–androgen-receptor (androgen-AR) signaling in normal prostate epithelium promotes terminal luminal epithelial cell differentiation. In androgen-dependent prostate-cancer cells, androgen-AR signaling gains the ability to promote both differentiation and proliferation. How this signaling promotes proliferation of androgen-dependent prostate-cancer cells and its relationship with the differentiation-promoting functions of the AR are important issues regarding the biology of androgen-dependent prostate-cancer cells. Herein, we report the identification of an AR-Skp2 pathway in prostate-cancer cells that depend on the AR for proliferation; in this pathway, AR is a robust upstream regulator of Skp2 through blocking the D-box-dependent degradation of this protein, and Skp2, in turn, serves as an essential downstream effector of AR in promoting proliferation independently of the differentiation-promoting function of AR. These results provide new knowledge on how AR functions in androgen-dependent prostate-cancer cells and identify strategies to specifically target the proliferation-promoting function of AR without compromising cancer-cell differentiation.

Introduction

Proliferation and differentiation are two fundamental cellular processes in development and cancer. These two processes are generally opposing each other in that inhibition of proliferation is necessary for differentiation and less differentiation is accompanied by more proliferation (Zhu and Skoulitchi, 2001). During development, organ mass is achieved by proliferation of less-differentiated cells, and organ function is subsequently achieved by cessation of proliferation and by terminal differentiation. Generally, cessation of proliferation is achieved by a number of cell-cycle regulators, such as Rb and p27Kip1, whereas differentiation is accomplished by master differentiation transcription factors. In addition to activation of expression of functional genes that confer differentiated status, master differentiation transcription factors might also function to inhibit cell proliferation, which would strengthen further the coupling of proliferation inhibition and differentiation. A hallmark of tumorigenesis is the reversal of these two coupled processes in differentiated cells (less differentiation and more proliferation) (Hanahan and Weinberg, 2000).

In spite of conforming generally to the above principles, androgen–androgen-receptor (androgen-AR) signaling in prostate-gland development and prostatic tumorigenesis has some special properties. In addition to promoting terminal differentiation of prostate luminal epithelium, androgen-AR signaling is required for growth and survival of prostate epithelium during prostate-gland development and homeostasis. How does AR promote both differentiation and proliferation of the normal prostate epithelium? Early research with AR-deficient (testicular feminization mutation with frame shift in AR; Tfm) mice and tissue reconstitution has established that androgen signaling in prostate epithelial cells directly promotes terminal cellular differentiation, not cell proliferation (Cunha et al., 2004; Donjacour and Cunha, 1993; Marker et al., 2003). AR has also been shown to inhibit proliferation and to stimulate differentiation of prostate epithelial cells in many instances (Ling et al., 2001; Whitacre et al., 2002). In this respect, AR functions as a typical master differentiation transcription factor in prostate epithelial cells (it promotes a differentiation program with coupled inhibition of proliferation). Proliferation (as well as maintenance of cell survival) of prostate epithelial cells is promoted indirectly by androgen through androgen signaling in prostate stromal cells, which secrete paracrine peptide growth factors to stimulate proliferation of epithelial cells in response to androgen (Isaacs and Isaacs, 2004).

Similar to all other forms of tumor, a hallmark of prostate cancer is deregulated proliferation. An important feature of prostate cancer is that its growth at early stages of the disease is androgen dependent. However, in contradistinction to benign prostate epithelial cells, proliferation of androgen-dependent prostate-cancer cells does not depend on regulation by stromal signals and androgen signaling can directly stimulate proliferation of androgen-dependent prostate-cancer cells (Isaacs and Isaacs, 2004). Gao et al. used Tfm mice as xenograft hosts to show that the proliferation of normal prostate epithelial cells was dependent on androgen signaling in the host cells, but proliferation of various androgen-responsive prostate-cancer cells was not (Gao et al., 2001). Importantly, differentiation of prostate-cancer cells, at least at early stages, is still promoted by AR, as evidenced by the dependence on androgen for the expression of prostate-specific antigen (PSA). Thus, AR has gained a dual-role of promoting both proliferation and differentiation in androgen-dependent prostate-cancer cells (we call them the proliferation arm and differentiation arm of AR functions).
The prostate-cancer cell line LNCaP is a cell model of androgen-dependent prostate cancer (Horoszewicz et al., 1983). LNCaP cells express functional AR [although it contains a T877A mutation that broadens its ligand specificity (Veldscholte et al., 1992)], and depend on androgen-AR signaling for proliferation and for expression of differentiation markers of prostate epithelium. However, unlike most clinical androgen-dependent prostate cancer, LNCaP cells generally do not depend on androgen for survival, indicating that LNCaP cells do not model all aspects of androgen-dependent prostate cancer and can be more accurately called an androgen-sensitive or androgen-responsive model (because LNCaP cells have been historically called androgen dependent, we will use this term in this manuscript). Another more recently developed prostate-cancer cell line, LAPC4, has similar properties as LNCaP (Klein et al., 1997). Currently, androgen-dependent survival of prostate-cancer cells is best modeled with the prostate-cancer-tissue xenograft model CWR22 (Pretlow et al., 1993). In response to castration of host mice, CWR22 tumors undergo apoptosis, leading to shrinkage of tumor volumes.

It is well-established that AR acts as a ligand-dependent transcription factor to regulate the expression of its target genes. Many prostate epithelial cell differentiation genes, exemplified by PSA, have been identified as AR target genes by their containing the androgen-responsive elements (AREs) in their promoters and their activation by androgen-AR signaling, explaining the differentiation-arm function of androgen-AR signaling. By contrast, AR target genes that can account for the proliferation-arm function of androgen-AR signaling have not been clearly identified, despite numerous attempts with expression arrays (DePrimo et al., 2002; Geck et al., 1997; Nelson et al., 2002; Segawa et al., 2002; Velasco et al., 2004), SAGE (Waghray et al., 2001; Xu et al., 2001), and ChIP-on-chip screens (Massie et al., 2007; Wang et al., 2007) with cultured LNCaP cells stimulated with androgen.

By contrast, with the candidate-gene approach, the cyclin-dependent kinase inhibitor p21Cip1 was identified as an AR target with AREs in its proximal promoter (Lu et al., 1999). Activating the expression of a cell-proliferation inhibitor might indeed reflect the nature of AR as a master differentiation factor (promoting differentiation together with inhibition of proliferation).

The F-box protein Skp2 is a substrate-recruiting component of the SCFSkp2-Roc1 E3 ubiquitin ligase complex (Cardozo and Pagano, 2004). The best-established ubiquitylation target is the cyclin-dependent kinase inhibitor p27Kip1, although the list of Skp2 targets has expanded in the last few years. Skp2 also forms stable complexes with cyclin A (Zhang et al., 1995) to directly protect cyclin-A-dependent kinases from inhibition by the p27/p21 family of cyclin kinase inhibitors (CKIs) (Ji et al., 2006). Skp2 fulfills the definition of an oncoprotein by its frequent overexpression in cancer cells and oncogenic activities in various laboratory assays (Nakayama and Nakayama, 2005).

Evidence for a role of Skp2 in prostate cancer is particularly strong (Ben-Izhak et al., 2003; Drobnjak et al., 2003; Yang et al., 2002). One study found Skp2 to be overexpressed in 557 of 622 (89.2%) primary prostate-cancer samples, and Skp2 labeling frequency was positively correlated with preoperative serum-PSA levels and Gleason Score (Yang et al., 2002). More importantly, Skp2 overexpression has been found in pre-malignant high-grade prostatic intra-epithelial neoplasia (HGPIN) lesions (64 of 74 samples, 86.4%), which suggests that Skp2 has a role in the early stages of the disease. In the mouse, overexpression of Skp2 in prostate epithelial cells induces low-grade adenocarcinomas (Shim et al., 2003). The key question is now: can targeting Skp2 treat prostate cancer?

In this study, we report the identification of an AR-Skp2 pathway in prostate-cancer cells that depend on the AR for proliferation; in this pathway, AR is a robust upstream regulator of Skp2 through blocking its D-box-dependent degradation and Skp2, in turn, serves as an essential downstream effector of AR in promoting androgen-dependent proliferation independently of the differentiation-promoting function of AR.

**Results**

LNCaP cells are the most sensitive to Skp2 silencing among a panel of cancer cell lines

Skp2 overexpression is frequently found in various types of cancer. To determine the functional significance of Skp2 overexpression, we used a lentivirus-mediated multi-miRNA hairpin method (Sun et al., 2006) to knockdown Skp2 in a panel of cancer cell lines cultured in media containing 10% fetal bovine serum (FBS), including the osteosarcoma cell lines U2OS and Saos-2, cervical carcinoma cell lines C33A and HeLa, prostate-cancer cell lines LNCaP, 22Rv1 and DU-145, breast-cancer cell lines MDA231, MDA435 and MDA468, and colon-cancer cell line LS174. These cell lines all express significantly higher levels of Skp2 than human diploid fibroblast WI38 (data not shown) and their Skp2 protein levels were all significantly knocked down with Skp2-targeting miRNA (Fig. 1A). The effects of Skp2 knockdown on cell proliferation, as measured by BrdU pulse labeling, are shown in Fig. 1B. In general, Skp2 knockdown induced proliferation inhibition to various degrees from 50% to almost no effect. LNCaP stood out as the only cell line in which the BrdU incorporation rate was inhibited much more significantly, to more than 90%. Although this limited screen does not exclude the possibility that certain other cancer cell lines might be as sensitive to Skp2 silencing as LNCaP cells, these results suggest that Skp2 may play an especially important role in androgen-dependent proliferation, a property that is, among this group of cancer cells, unique to LNCaP cells.

**Skp2 is an important downstream effector of the proliferation arm of AR independently of its differentiation arm**

The above finding and analysis prompted us to compare the effects of Skp2 knockdown with that of AR knockdown in LNCaP cells. As expected, knockdown of AR led to inhibition of cell proliferation (Fig. 2A). Skp2 knockdown led to proliferation inhibition to a similar degree. In both cases, cell death was not observed. Unexpectedly, knockdown of AR led to a reduction in Skp2 protein level nearly as efficiently as Skp2 knockdown itself (Fig. 2B). By comparison, Skp2 knockdown did not affect AR level. AR knockdown also increased p27 protein levels, whereas Skp2 knockdown appeared to lead to more p27 accumulation. Protein levels of cyclin E, Cul1, Cdh1 and Cdk2 were not affected.

As expected, PSA protein level was nearly abolished in AR-knockdown cells (Fig. 2B). Importantly, Skp2 knockdown did not significantly affect PSA levels. Another well-known differentiation-promoting function of AR in LNCaP cells is to block their transdifferentiation, or de-differentiation and re-differentiation, into a neuroendocrine-cell-like phenotype. Consistent with a previous report (Wright et al., 2003), knockdown of AR in LNCaP cells induced the appearance of long dendritic processes representing a neuron-like morphology, accompanied by significant proliferation inhibition (Fig. 2C). Knockdown of Skp2 resulted in a similar degree of proliferation inhibition but the proliferation-inhibited cells did
not show such neuron-like morphology. These results revealed an AR-Skp2 pathway in LNCaP cells; in this pathway, AR is upstream of Skp2 and acts to maintain Skp2 levels, and Skp2 is an essential effector of AR in the proliferation arm of AR, which is separate from its differentiation arm.

Requirement for Skp2 in prostate-cancer cells corresponds with their requirement for AR

To determine whether the AR-Skp2 pathway is functional in androgen-dependent prostate-cancer cells other than LNCaP cells, we used LAPC4 (the other available androgen-dependent prostate-cancer cell line) (Klein et al., 1997) and the androgen-dependent CWR22 xenograft (as an in vivo system) (Pretlow et al., 1993).

As shown in Fig. 3A, knockdown of AR also significantly reduced protein levels of Skp2 in LAPC4 cells. Inhibition of proliferation of LAPC4 cells by knockdown of AR and Skp2 was not as efficient as the inhibition observed in LNCaP cells (Fig. 2A), but, importantly, proliferation inhibition by Skp2 knockdown was again as efficient as AR knockdown. These results suggest that LAPC4 cells also contain a functional AR-Skp2 pathway.

To learn about the AR-Skp2 relationship in vivo, we used the androgen-dependent CWR22 prostate-cancer xenograft model. Castration led to a significant reduction in AR protein levels in xenograft tumors (Fig. 3B). Skp2 protein levels showed parallel decreases, indicating that Skp2 is also under androgen-AR regulation in CWR22 cells (Fig. 3B). With CWR22 xenografts, we further found that progression of cells to androgen independence after castration was accompanied by re-expression, to high levels, of both AR and Skp2, suggesting that the AR-Skp2 pathway is reactivated in recurrent tumors. It also suggests that overexpression of Skp2 contributes to tumor recurrence.

It has been suggested that, when androgen-dependent prostate-cancer cells progress to androgen independency, some of them might still be dependent on androgen-AR function and the term ‘hormone refractory’ or ‘androgen-ablation-therapy refractory’ might be more accurate (Haag et al., 2005; Li et al., 2007; Zegarra-Moro et al., 2002). We therefore wished to determine whether the AR-Skp2 pathway is still functional in AR-dependent but androgen-independent prostate-cancer cells. The androgen-independent prostate-cancer cell line C4-2 was derived from LNCaP xenografts after progression in a castrated host (Wu et al., 1994). We found that, although C4-2 cells are androgen independent for proliferation as expected (data not shown), AR knockdown still significantly reduced Skp2 protein levels and both AR knockdown and Skp2 knockdown still significantly inhibited cell proliferation (Fig. 3C). In comparison, the androgen-independent 22Rv1 line, derived from CWR22 xenografts after progression in a castrated host (Sramkoski et al., 1999), was not inhibited by AR knockdown (Fig. 3D), indicating that it is AR independent for proliferation. In this line, knockdown of AR did not significantly reduce Skp2 levels and knockdown of Skp2 also did not inhibit cell proliferation (Fig. 3D).

These results reveal that the requirement for Skp2 correlates with the requirement for AR function in prostate-cancer cells, providing further support for the link between AR and Skp2 in prostate-cancer cells.

Skp2 expression in LNCaP cells is regulated by androgen

We next determined whether the AR-Skp2 pathway identified with gene silencing could be reproduced by manipulation of androgen levels in culture media. The androgen antagonist bicalutamide (also known as Casodex) specifically inhibits AR and is currently a preferred clinical anti-androgen because of its high potency and specificity. Prolonged treatment with Casodex, however, can induce mutations in AR, upon which Casodex may act as an agonist (Hara et al., 2003). Another commonly used laboratory method of androgen withdrawal is to treat FBS with charcoal dextran (the CDT media). Because CDT also depletes other steroids and factors, its effects can be broader than depletion of androgen alone. When androgen (or a commonly used synthetic and more-stable form of...
androgen, R1881 (methyltrienolone)] is added to CDT media, cellular response to androgen treatment can be determined, albeit in the absence of many other components, in FBS media. We used these four conditions to study how cellular Skp2 levels respond to androgen in LNCaP cells. As shown in Fig. 4A, proliferation of LNCaP cells, as measured by relative cell number at the end of a 12-day incubation, was regulated by these four conditions as reported previously (Bernard et al., 2003; Chen et al., 2004). Skp2 protein levels in LNCaP cells were reduced when Casodex was present in FBS media and were increased when the synthetic androgen R1881 was included in CDT media; expression of PSA followed the same pattern (Fig. 4B). The degrees of cell proliferation were about twofold higher in FBS media than in CDT+R1881 media, indicating that CDT depleted other proliferation-promoting factors in addition to androgen from FBS. In this respect, it is notable that CDT+R1881 supported higher Skp2 protein levels than FBS, suggesting that Skp2 protein levels were not completely correlated with cell proliferation.

We also determined whether androgen could stimulate Skp2 expression in LNCaP cells that were starved of androgen. For this purpose, LNCaP cells were first incubated in CDT media for 2 days, which inhibited proliferation of LNCaP cells, as indicated by a decrease in BrdU-labeling rate from about 30% in FBS media to about 2% in CDT media. R1881 was then added to these androgen-starved cells for 1, 2 or 3 days. A parallel set of cultures was processed without the addition of androgen. As shown in Fig. 4C, at 24 hours after the addition of androgen, when BrdU labeling increased to about 5%, Skp2 protein expression was clearly increased, with a further increase observed at 48 hours (at which point BrdU labeling had increased to 8%). Skp2 protein levels were...
maintained, but did not show further increase, at the 72-hour time point (at which point BrdU labeling was 10%). p27 protein level showed a corresponding decrease. Expression of cyclin-A protein exhibited similar increases as Skp2, whereas expression of a number of other cell-cycle regulators, such as cyclin E, Cdh1, Cdk4 and Cdk2, did not change noticeably. PSA expression was gradually increased in the same cells but with clearly different kinetics than Skp2. Taken together, these results indicate that androgen can regulate Skp2 protein levels in LNCaP cells, consistent with the results obtained with AR silencing.

Androgen-AR signaling regulates Skp2 expression at mRNA- and protein-stability levels
To determine whether androgen regulates Skp2 protein levels at transcription or post-transcription levels, Skp2 protein and mRNA levels were measured during treatment of LNCaP cells with CDT and CDT+R1881. At 24 and 48 hours after LNCaP cells were transferred from FBS media to the indicated conditions, Skp2 protein was more abundant in CDT+R1881-treated cells than in CDT-treated cells (Fig. 5A), whereas Skp2 mRNA levels were not different in these two conditions as measured by semi-quantitative reverse-transcriptase (RT)-PCR (Fig. 5B). After 72 hours of treatment, the difference between Skp2 protein abundance in the two conditions became greater and Skp2 mRNA levels also became different. Thus, androgen can regulate Skp2 expression at both the mRNA level (with a rather slow kinetics) and post-transcriptional level (because Skp2 protein levels reduced with a faster kinetics than the reduction in Skp2 mRNA levels).

The proteasome inhibitor MG-132 mostly abolished the regulation of Skp2 protein levels by androgen (Fig. 5C), indicating that regulation of Skp2 protein degradation by proteasomes is a significant component in the overall regulation. Protein degradation was measured after inhibiting protein synthesis with cycloheximide (CHX) after cells were transferred from FBS media to CDT or CDT+R1881 media. Androgen slowed Skp2 degradation (Fig. 5D), directly suggesting that androgen signaling can stabilize Skp2 protein. Similar results were also obtained when androgen-starved LNCaP cells were stimulated with R1881 (supplementary material Fig. S1).

Androgen-AR signaling can stabilize Skp2 protein that is overexpressed from a CMV promoter
The findings that (1) Skp2 mRNA levels responded to androgen withdrawal with slower kinetics than Skp2 protein levels, (2) the Skp2 gene promoter does not contain AREs, and (3) a previous study demonstrated that high concentrations of androgen actually decreased Skp2 mRNA levels (Lu et al., 2002) suggest that the effects of androgen on Skp2 mRNA expression are not direct. We therefore focused on the effects of androgen on Skp2 protein stability. To study the effects of androgen-AR signaling on the regulation of Skp2 protein levels independent of Skp2 mRNA expression from the Skp2 promoter, we expressed a FLAG-tagged Skp2 in LNCaP cells from the constitutive CMV promoter using lentivirus transduction. In these LN-FLAG-Skp2 cells, Skp2 was expressed at levels more than tenfold higher than endogenous Skp2 protein. Ducing cell proliferation and induction of CMV-expressed FLAG-Skp2 protein accumulation by high concentrations of androgen. High concentrations of androgen inhibit cell proliferation and induce accumulation of CMV-expressed FLAG-Skp2. (A) BrdU labeling of LN-FLAG-Skp2 cells cultured in various concentrations of R1881 as indicated (20p, 20 pM; 2n, 2 nM). (B) Western blots of LN-FLAG-Skp2 cells cultured at the indicated conditions for 3 days. (C) CHX experiment as in Fig. 5D. (D) LN-FLAG-Skp2 cells were transduced with lentiviruses expressing control or AR miRNA and treated with various concentrations of R1881 to determine their effects on FLAG-Skp2 protein levels as in B. These results are representative of three independent experiments. (E) LAPC4 and DU145 prostate-cancer cell lines were similarly treated with increasing concentrations of R1881 for 3 days and subjected to western blot analysis. These experiments were performed twice with identical results.
levels in LNCaP cells cultured in FBS media, whereas LNCaP cells transduced with empty lentiviruses did not show an increase in Skp2 level (Fig. 6A). Proliferation of LN-FLAG-Skp2 cells was not inhibited in FBS+Casodex media, and the difference between cell proliferation in CDT media and CDT+R1881 media was reduced (compare Fig. 6B with Fig. 4A). Thus, artificial overexpression of Skp2 could induce androgen-independent proliferation. Together with the finding that Skp2 protein levels are significantly elevated in recurrent CWR22 tumor in castrated hosts (Fig. 3B), these results suggest that increases in Skp2 expression play a role in progression to androgen independency, but the proliferation effects of artificially overexpressed Skp2 must be interpreted with caution.

When Skp2 protein levels in LN-FLAG-Skp2 cells were examined in the same four media conditions, high levels of FLAG-Skp2 were still subject to effective regulation by androgen (Fig. 6C). CHX experiments confirmed that androgen treatment still slowed protein degradation of FLAG-Skp2 (Fig. 6D). The ability of androgen to induce FLAG-Skp2 protein accumulation when it was expressed from a constitutive promoter at levels much higher than endogenous Skp2 demonstrates that both the degradation of Skp2 in the absence of androgen and the ability of androgen to stabilize Skp2 protein are robust.

Stabilization of Skp2 by AR is not a consequence of cell-cycle progression

Because Skp2 mRNA expression and protein stabilization are more active in proliferating cells (Bashir et al., 2004; Wei et al., 2004; Wirbelauer et al., 2000; Yung et al., 2007; Zhang and Wang, 2005) and androgen-AR promotes proliferation of LNCaP cells, it is important to determine whether androgen-AR has direct effects on Skp2 mRNA expression and protein stabilization or whether these effects are secondary consequences of androgen-mediated cell proliferation via other mechanisms.

An important property of LNCaP cells is that, whereas low concentrations of androgen promote proliferation, high concentrations of androgen can potently inhibit proliferation, which is thought to reflect a physiological response to androgen in promoting terminal differentiation (Lu et al., 2002; Tsihlias et al., 2000). Interestingly, it has already been reported that inhibition of proliferation of LNCaP cells by high concentrations of androgen lead to a significant reduction in Skp2 mRNA levels (Lu et al., 2002). Thus, androgen can regulate Skp2 mRNA levels in opposite directions corresponding to the effects of androgen on cell proliferation. When a low concentration of androgen promotes proliferation, it maintains Skp2 mRNA levels (Fig. 5B); when a high concentration of androgen inhibits proliferation, it represses Skp2 mRNA levels (Lu et al., 2002). Coupled with the fact that the Skp2 promoter does not contain typical AREs, these results suggest that the regulation of Skp2 mRNA levels by androgen is indirect to its effects on cell proliferation.

We took this opportunity to determine how high concentrations of androgen affected protein levels of FLAG-Skp2 expressed from a CMV promoter in LNCaP cells. High concentrations of R1881 (2 nM) caused potent inhibition of proliferation of LN-FLAG-Skp2 cells (Fig. 7A), similarly to LNCaP and LN-empty cells (data not shown). This result demonstrated that, although overexpression of Skp2 was able to induce androgen-independent proliferation (Fig. 6A), it was not sufficient to override the proliferation inhibition imposed by high concentrations of androgen. This latter observation was also reported by Lu et al. (Lu et al., 2002). Importantly, in cells treated with 2 nM R1881, FLAG-Skp2 accumulated to a greater extent than in cells stimulated to proliferate with 20 pM R1881 (Fig. 7B), and CHX experiment confirmed that FLAG-Skp2 degradation was slowed by treatment with 2 nM R1881 (Fig. 7C). These results demonstrate that androgen-AR can mediate Skp2 stabilization independently of cell proliferation. This effect of high-concentration androgen was dependent on AR function, because knockdown of AR abolished the accumulation of FLAG-Skp2 that was induced by a high concentration of androgen (Fig. 7D), and similar responses to high-concentration androgen were also observed in AR-expressing LAPC4 cells, but not in AR-negative DU-145 cells (Fig. 7E).

AR stabilizes Skp2 protein through a D-box-dependent mechanism

An important aspect in the study of protein degradation is to identify and isolate a degron domain or sequence that can transfer the degradation of the protein under study to an unrelated stable protein, which is exemplified by the study of cyclin-B degradation (Glotzer...
et al., 1991). To provide this type of evidence for the ability of androgen-AR to stabilize Skp2, we sought to determine whether we could transfer the ability of androgen-AR to stabilize Skp2 to GFP. Skp2 was first shown to be regulated by protein degradation in fibroblasts in the G0-G1 phases by an auto-ubiquitylation mechanism in the SCF Skp2-Roc1 complex in a Cul1-dependent manner (Wirbelauer et al., 2000). More recently, a D-box-dependent Skp2-ubiquitylation mechanism was identified that promotes Skp2 degradation in the G0-G1 phases in a Cdh1-dependent manner. Furthermore, the pocket protein p107 has been reported to promote Skp2 degradation independent of either Cul1 or Cdh1 (Rodier et al., 2005). These findings suggest that regulation of Skp2 degradation is complex and involves multiple mechanisms. Because Skp2 contains a D-box sequence and the D-box sequence in cyclin B has been shown to be transferable, we focused on determining whether the D-box in the Skp2 N-terminus could transfer androgen-AR-mediated regulation to GFP. The 97-residue Skp2 N-terminus (sequences before the F box) was fused to GFP to generate the fusion protein NGFP (Fig. 8A). To determine the role of the D box in this sequence, 20NGFP was generated, in which the D box at the beginning of N-terminus is deleted. For comparison, a GFP fusion protein (BGFP) was created with the N-terminus of cyclin B, which contains a well-established portable degron (Glotzer et al., 1991) (Fig. 8B). After transduction of LNCaP cells with lentiviruses expressing NGFP or these three GFP-fusion proteins, the Skp2 N-terminus conferred degradation to GFP in CDT media, because NGFP protein level increased significantly when the proteasome inhibitor MG-132 was added to CDT media (Fig. 8C). The cyclin-B N-terminus showed a similar effect on GFP (the BGFP protein). Addition of R1881 to CDT media increased NGFP level to a similar extent as FBS, which demonstrated that the N-terminus degron of Skp2 is androgen-dependent and -responsive (Fig. 8D). Levels of GFP and 20NGFP did not show such degradation and stabilization, indicating that the effects of androgen on NGFP were through a D-box-dependent mechanism. The effects of androgen were much less significant on BGFP, suggesting that androgen has a specific effect on the D-box-dependent degron of Skp2.

Androgen can induce accumulation of NGFP independently of serum peptide growth factors
In cultured fibroblasts, Skp2 protein is destabilized by serum starvation and stabilized by re-addition of serum (Bashir et al., 2004; Wei et al., 2004; Wirbelauer et al., 2000). The ability of serum to stabilize Skp2 and NGFP in fibroblasts is fully retained in CDT media (data not shown), indicating that peptide growth factors and their signaling through tyrosine kinase receptors (TKRs) mediate stabilization of the Skp2 protein in fibroblasts (because charcoal-dextran treatment of FBS does not deplete peptide growth factors).

AR can crosstalk with signaling pathways initiated from various TKRs and their ligand peptide growth factors. Keratinocyte growth factor (KGF), insulin-like growth factor 1 (IGF1) and epidermal growth factor (EGF), as well as an activated TKR, Neu, have been shown to activate AR transactivation in androgen-depleted media, or ‘super-activate’ AR in the presence of low concentrations of androgen (Craft et al., 1999; Culig et al., 1994; Gregory et al., 2001; Yeh et al., 1999). The TKR pathways also play important roles in the transactivation activity of AR, because a dominant-negative version of MEKK1 inhibited androgen-mediated activation of the PSA promoter (Abreu-Martin et al., 1999). Conversely, AR has been shown to bind Src and is important for functions of the EGF-EGFR signaling pathway to promote S-phase entry of LNCaP cells (Migliaccio et al., 2005). It was therefore possible that peptide growth factors that are present in CDT media play an important role in, or are responsible for, the stabilization of NGFP in response to androgen.

To investigate the role of peptide growth factors in androgen-mediated stabilization of Skp2, the effects of androgen on NGFP accumulation were determined in LNCaP cells cultured in serum-free media. To more quantitatively analyze the effects of androgen on NGFP accumulation, flow cytometry was used to measure GFP levels. As shown in Fig. 9A, addition of R1881 to CDT media caused a right-shift of GFP fluorescence intensity of NGFP.
In this scenario, target genes of the Ets family of transcription factors, now under regulation by androgen-AR, might be responsible for androgen-AR-mediated Skp2 protein stabilization in androgen-dependent prostate-cancer cells. However, protein levels of Cdh1 and Cul1, two proteins known to be involved in Skp2 protein degradation, did not show changes in response to AR knockdown or androgen stimulation in LNCaP cells (Fig. 2B and Fig. 4C), suggesting that expression of certain cofactors or inhibitors of Cul1 or Cdh1 that are necessary for Skp2 ubiquitylation are regulated by Ets transcription factors in an androgen-AR-dependent manner in androgen-dependent prostate-cancer cells (but not in various androgen-independent cells).

By contrast, gene fusion to Ets-family transcription factors does not readily explain why androgen-dependent prostate-cancer cells lost the ability to stabilize Skp2 protein when treated with serum peptide growth factors. In this respect, how serum peptide growth factors promote Skp2 protein stability in various androgen-independent cells also remains to be determined.

Interestingly, stimulation of Skp2 protein levels by androgen has been observed in ventral prostate glands after administration of androgen to castrated rats, and the Skp2 protein levels inversely correlated with p27 protein levels (Waltregny et al., 2001), suggesting that the AR-Skp2 pathway is also present in normal prostate. Importantly, however, whereas p27 expression was determined in prostate epithelium with immunohistochemical staining, Skp2 expression was determined by western blots of the prostate glands, in which androgen might signal proliferation of prostate epithelium indirectly via its direct action on stromal cells (see Introduction). Further studies are needed to determine whether androgen treatment induces Skp2 accumulation in epithelium, stroma or both.

Our finding that androgen-AR functions at the level of protein stabilization to increase Skp2 expression, which in turn promotes androgen-dependent proliferation, raises the issue of how Skp2 mRNA is expressed. It seems to be necessary for androgen-AR to induce Skp2 mRNA expression or promote cell-cycle progression to a stage at which Skp2 mRNA is expressed via E2F activation (Yung et al., 2007; Zhang and Wang, 2005) for the Skp2-protein-stabilization function to be effective. It is also possible that there are low basal levels of Skp2 mRNA expression that are sufficient for androgen-AR to initiate Skp2 protein accumulation, which promotes cell-cycle progression and, in turn, leads to higher expression of Skp2 mRNA, forming an amplifying loop of Skp2 protein accumulation.

The finding that androgen-dependent prostate-cancer cells are particularly sensitive to Skp2 knockdown compared with a number of other cancer cell lines points to a biochemical vulnerability of androgen-dependent prostate-cancer cells that could be exploited to treat prostate cancer at androgen-dependent and early androgen-independent stages. It is also important that inhibition of proliferation of LNCaP cells by Skp2 knockdown did not affect their differentiation. This finding suggests that targeting Skp2 could be a better therapeutic strategy for androgen-dependent prostate-cancer than androgen-deprivation therapy, because androgen deprivation inhibits both proliferation and differentiation of androgen-dependent prostate-cancer cells, and de-differentiation is a major component in cancer-cell progression.

**Discussion**

Androgen-dependent proliferation, differentiation and survival are important properties of androgen-dependent prostate cancer. In this study, we used prostate-cancer cell lines that model the androgen-dependent-proliferation aspect of prostate cancer in culture in response to various manipulations of androgen concentrations in the culture media to study how androgen-AR promotes proliferation. Importantly, androgen-dependent proliferation of androgen-dependent prostate-cancer cells in culture distinguishes them not only from androgen-independent prostate-cancer cells, but also from untransformed prostate epithelial cells and from normal fibroblasts because proliferation of these cells does not depend on androgen (these cells together can be considered as androgen-independent cells for the purpose of this Discussion). Androgen-dependent prostate-cancer cells have lost the ability to proliferate in response to peptide growth factors in serum in the culture media, whereas peptide growth factors are sufficient to stimulate proliferation of the androgen-independent cells; androgen-AR signaling does not stimulate proliferation of androgen-independent cells but gained the ability to stimulate proliferation of androgen-dependent prostate-cancer cells. These two differences between androgen-dependent prostate-cancer cells and androgen-independent cells indicate that androgen-dependent prostate-cancer cells have undergone certain ‘hardwiring changes’ (Isaacs and Isaacs, 2004) during prostate tumorigenesis that disrupted peptide growth-factor signaling to the cellular proliferation machinery and, at the same time, connected androgen-AR signaling to it.

Findings of this study suggest that the ‘hardwiring changes’ in androgen-dependent prostate-cancer cells are biochemically linked to how Skp2 protein stability is maintained. In cultured fibroblasts, Skp2 protein is stabilized by serum peptide growth factors (Bashir et al., 2004; Wei et al., 2004; Wirbelauer et al., 2000). Our results show that, in androgen-dependent prostate-cancer cells, serum peptide growth factors lost the ability to stabilize Skp2, whereas androgen-AR signaling gained this ability.

Our results therefore suggest that elucidating the underlying mechanisms for the loss of ability of serum peptide growth factors to support Skp2 protein levels and for the gain of ability of androgen-AR to support Skp2 protein levels in androgen-dependent prostate-cancer cells promises to yield fundamental insights on prostate tumorigenesis. Regulation of gene expression by androgen-AR is complex, involving both direct effects (on ARE-containing promoters) and indirect effects (on non-ARE-containing promoters). The finding that Ets-family transcription factors fuse to a number of direct AR target genes in about 80% of prostate-cancer specimens and a number of prostate-cancer cell lines, including the LNCaP cell line (which contains the MIPOL1-ETV1 fusion) (Tomlins et al., 2007), might provide the most concrete suggestion as to how androgen-AR signaling gains new androgen-dependent effects in prostate-cancer cells, enabling it to take on the new ability to directly promote proliferation of androgen-dependent prostate-cancer cells. In this scenario, target genes of the Ets family of transcription factors, now under regulation by androgen-AR, might be responsible for androgen-AR-mediated Skp2 protein stabilization in androgen-dependent prostate-cancer cells. However, protein levels of Cdh1 and Cul1, two proteins known to be involved in Skp2 protein degradation, did not show changes in response to AR knockdown or androgen stimulation in LNCaP cells (Fig. 2B and Fig. 4C), suggesting that expression of certain cofactors or inhibitors of Cul1 or Cdh1 that are necessary for Skp2 ubiquitylation are regulated by Ets transcription factors in an androgen-AR-dependent manner in androgen-dependent prostate-cancer cells (but not in various androgen-independent cells).

By contrast, gene fusion to Ets-family transcription factors does not readily explain why androgen-dependent prostate-cancer cells lost the ability to stabilize Skp2 protein when treated with serum peptide growth factors. In this respect, how serum peptide growth factors promote Skp2 protein stability in various androgen-independent cells also remains to be determined.

Interestingly, stimulation of Skp2 protein levels by androgen has been observed in ventral prostate glands after administration of androgen to castrated rats, and the Skp2 protein levels inversely correlated with p27 protein levels (Waltregny et al., 2001), suggesting that the AR-Skp2 pathway is also present in normal prostate. Importantly, however, whereas p27 expression was determined in prostate epithelium with immunohistochemical staining, Skp2 expression was determined by western blots of the prostate glands, in which androgen might signal proliferation of prostate epithelium indirectly via its direct action on stromal cells (see Introduction). Further studies are needed to determine whether androgen treatment induces Skp2 accumulation in epithelium, stroma or both.

Our finding that androgen-AR functions at the level of protein stabilization to increase Skp2 expression, which in turn promotes androgen-dependent proliferation, raises the issue of how Skp2 mRNA is expressed. It seems to be necessary for androgen-AR to induce Skp2 mRNA expression or promote cell-cycle progression to a stage at which Skp2 mRNA is expressed via E2F activation (Yung et al., 2007; Zhang and Wang, 2005) for the Skp2-protein-stabilization function to be effective. It is also possible that there are low basal levels of Skp2 mRNA expression that are sufficient for androgen-AR to initiate Skp2 protein accumulation, which promotes cell-cycle progression and, in turn, leads to higher expression of Skp2 mRNA, forming an amplifying loop of Skp2 protein accumulation.

The finding that androgen-dependent prostate-cancer cells are particularly sensitive to Skp2 knockdown compared with a number of other cancer cell lines points to a biochemical vulnerability of androgen-dependent prostate-cancer cells that could be exploited to treat prostate cancer at androgen-dependent and early androgen-independent stages. It is also important that inhibition of proliferation of LNCaP cells by Skp2 knockdown did not affect their differentiation. This finding suggests that targeting Skp2 could be a better therapeutic strategy for androgen-dependent prostate-cancer than androgen-deprivation therapy, because androgen deprivation inhibits both proliferation and differentiation of androgen-dependent prostate-cancer cells, and de-differentiation is a major component in cancer-cell progression.
obtained from both lines. LNCaP cells were cultured in RPMI 1640 supplemented with 5% FBS (GIBCO). LAPC4 cells were obtained from Shiv Srivastava (Uniformed Services University of the Health Sciences, MD) with permission from UCLA Technology Transfer Office, and were cultured in Isosove medium supplemented with 10% FBS. MDA231, MDA435, MDA468 and LS174 cell lines were obtained from Sridhar Mani (Albert Einstein College of Medicine, NY). C4-2 cells were purchased from UroCor. HeLa, C33A, DU145, U2OS, Saos2 and 22RV1 cells were purchased from ATCC. These cells were cultured in DMEM medium supplemented with 10% FBS. CDT media consisted of RPMI 1640 with 5% charcoal-stripped, dextran-treated FBS (Omega Scientific). R1881 was purchased from NEN Life Sciences, and Casodex was a gift from AstraZeneca. Treatment with Casodex or R1881 was controlled with their solvent ETOH. CHX was purchased from Sigma and MG-132 was purchased from Calbiochem. Antibodies against AR (441), Cul (H-213), cyclin E (M20), Cdk2 (M2) and GFP (FL) were purchased from Santa Cruz Biotechnology. Antibody against Skp2 (32-3300) was purchased from Zymed Laboratories. Antibody against PSA (A 0562) was purchased from DakoCytomation. Antibody against FLAG (F-3165) was purchased from Sigma Aldrich, and BrdU antibody (NA20) and Cdh1 antibody (Ab-2) were purchased from Calbiochem.

CWR22 xenograft tumors

The CWR22 xenograft tumor model has been maintained by continuous passages in nude athymic mice. Castigation of host mice was performed when xenograft tumors reached a volume of 1 cm³. Samples from the same specimens characterized in our previous studies for their response to castration (Kim et al., 2002) were used in western blot analysis.

DNA constructs

Lenti-CMV-FLAG-Skp2 was described previously (Ji et al., 2006). Lenti-CMV-NGP was constructed by in-frame fusion of a 1-97 of human Skp2 (Skp2 numbering is based on NCBI accession number U33761) to the N-terminus of GFP. Lenti-CMV-20NGFP was constructed similarly, except 20 to 97 were fused to GFP. Lenti-CMV-BGP was constructed by fusing aa 1-134 of human cyclin B to GFP. Construction of Skp2 and AR microRNA constructs were previously described (Sun et al., 2006). The target sequence for Skp2 and AR are 5'-CTTCTAGACCTC-3' and 5'-CAGCAGATATGCATTATGCT-3', respectively. An irrelevant sequence, 5'-GTACAAACAGGAGGTAAA-3', which is derived from the 3' untranslated region of mouse p27 and does not match any human sequence in the databases, was used as a control. Lentivirus vector and its helper constructs were gifts from L. Naldini and A. Follenzi (Follenzi et al., 2002). Various lentivirus stocks were made according to an established protocol (Sun et al., 2006). Lentivirus stocks were concentrated as necessary to achieve an infection efficiency of more than 95% of various cells (for FACS analysis of GFP expression, infection efficiencies of about 75% were used). Concentration of virus stocks was accomplished by centrifugation at 112,400 g for 2 hours at 4°C. The virus pellets were resuspended in media overnight.

Cell-proliferation assay and BrdU-incorporation assay

Cell-proliferation assay was according to protocols described previously (Bernard et al., 2003; Chen et al., 2004). Twenty thousand cells were seeded into each well of 96-well plates in various conditions (Casodex was used at 10 µM and R1881 was used at 20 µM, unless otherwise indicated). Media were refreshed every 3 days. After 12 days, cells were fixed and stained using 2% ethanol and 0.2% crystal violet, and OD595 nm readings above background).

Cell proliferation analysis was accomplished by using an automated cell counter. Plate reader was used to determine absorbance reading at 595 nm (note that 20,000 cells, the starting point, do not generate an absorbance reading at 595 nm).

For BrdU-incorporation assays, cells were pulse-labeled with 10 µM BrdU for 2 hours, whereas a 14-hour pulse-labeling was used for LNCaP cells owing to the slow-proliferation nature of these cells.

Western blot, CHX chase and semi-quantitative RT-PCR

Western blot assays were performed using standard protocols (Ji et al., 2004). For CHX chase assays, LNCaP or LAPC4-LNCaP-Skp2 cells were split from 5% FBS medium into 5% CDT medium with or without R1881 in the presence of 100 µM of CHX. Cells were harvested for western blot at the indicated time points. For semi-quantitative RT-PCR, LNCaP cells were cultured in CDT medium with or without 100 µM R1881 for 24, 48 or 72 hours. Total-RNA samples were prepared by RNeasy kit (Qiagen). Primers used were: Skp2 Fw, 5'-TCCACGCACGATCTGGTCTACG-3', Rv, 5'-GGGCAAATTCAGAGAATCCA-3'. GAPDH Fw, 5'-GGGTTTACGAGGGGTACACC-3', Rv, 5'-GGGTTTACGAGGGGTACACC-3'.

Flow cytometry analysis

Various LNCaP cells were cultured for 3 days in the conditions indicated in Fig. 9. Cells were washed by PBS once, trypsinized and resuspended in PBS for flow-cytometry analysis.
AR-Skp2 pathway in prostate-cancer cells


