0888-8809/03/$15.00/0
Printed in U.S.A.

Molecular Endocrinology 17(9):1726–1737

doi: 10.1210/me.2003-0031

Androgen Receptor Represses the Neuroendocrine Transdifferentiation Process in Prostate Cancer Cells

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Androgen-ablation therapy is an effective method for treating prostate cancer. However, prostate tumors that survive long-term androgen-ablation therapy are classified as androgen-independent as they proliferate in the absence of androgens, and they tend to be enriched for neuroendocrine (NE) cells. Androgen withdrawal causes androgen-dependent prostate cancer cells to adopt a pronounced NE phenotype, suggesting that androgen receptor (AR) represses an intrinsic NE transdifferentiation process in prostate cancer cells. In this report we show that short interfering RNA-induced AR silencing induced a NE phenotype that manifested itself in the growth of dendritic-like processes in both the androgen-dependent LNCaP and androgen-independent LNCaP-AI human prostate cancer cells. Western blot analysis revealed that neuronal-specific enolase, a marker of the neuronal lineage, was increased by AR knockdown in LNCaP cells. The expression levels of the neuronal-specific cytoskeletal proteins β-tubulin III, nestin, and glial acidic fibrillary protein were also characterized in AR knockdown cells. Most interestingly, AR silencing induced β-tubulin III expression in LNCaP cells, while AR knockdown increased glial acidic fibrillary protein levels in both LNCaP and LNCaP-AI cells. Lastly, AR silencing reduced the proliferative capacity of LNCaP and LNCaP-AI cells. Our data demonstrate that AR actively represses an intrinsic NE transdifferentiation process in androgen-responsive prostate cancer cells and suggest a potential link between AR inactivation and the increased frequency of NE cells in androgen-independent tumors. (*Molecular Endocrinology* 17: 1726–1737, 2003)

ANDROGENS ARE CRITICAL for the development and maintenance of normal and cancerous prostate tissue (1). Androgens bind the androgen receptor (AR), a member of the steroid-receptor superfamily of ligand-dependent transcription factors, and induce the transcription of prostate-specific genes associated with cellular growth and maintenance of the differentiated prostate-epithelial phenotype (2). Early-stage prostate cancer that is confined to the prostate gland is normally cured by surgical methods (*i.e.* radical prostatectomy) (3). In contrast, advanced-stage prostate carcinoma that has metastasized beyond the prostatic capsule, is typically treated by androgen-ablation therapies (4). Androgen ablation therapy exploits the androgen dependency of prostate cancer cells by either reducing endogenous androgen levels that circulate within the body or by directly blocking AR activity with chemical inhibitors (5). These therapies cause massive apoptotic cell death in the bulk of the prostate tumor cells (6); however, on occasion, cancer cells escape these treatments and reappear as androgen-independent prostate tumors that proliferate in the absence of androgens *in vivo* (7). This class of prostate tumor is often incurable and ultimately leads to the demise of the patient (8).

AR reactivation under androgen-withdrawal conditions is believed to be a critical step in the development of advanced, hormone-refractory prostate carcinomas *in vivo* (9). Several mechanisms of AR reactivation have been proposed. They include amplification of the *AR* that results in AR overexpression and an increased capacity to bind lower levels of androgen that circulate within the body, structural alterations in the AR that induce promiscuous interactions with other steroids, and hormone-independent AR activation induced by growth-factors (*i.e.* IGF-I, keratinocyte growth factor, epidermal growth factor) and neuropeptides (*i.e.* neurotensin, bombesin) (5). Long-term androgen-ablation therapy also tends to select for prostate tumor populations that are enriched for neuroendocrine (NE) cells (10, 11). NE cells are one of several cell types that occupy the prostate gland and have been hypothesized to have a paracrine role in the growth and differentiation of the normal prostate gland, similar to NE cells that reside within the pancreas, respiratory, and gastrointestinal systems (12). Prostate-localized NE cells are nonproliferative and express a number of neuronal proteins including neuronal-specific enolase (NSE) and chromogranins (12). NE cells also secrete a large class of neuropeptides that includes bombesin, neurotensin, serotonin, calci-

Abbreviations: AR, Androgen receptor; FAS, fatty acid synthase; GFAP, glial acidic fibrillary protein; NE, neuroendocrine; NSE, neuronal-specific enolase; PSA, prostate-specific antigen; RNAi, RNA interference; siRNA, short interfering RNA; TBST, Tris-buffered saline containing 0.1% Tween 20.

tonin, TSH, and PTHrP that possess a wide range of cellular activities associated with tumor proliferation, transformation, and metastasis (12). Therefore, it has been hypothesized that NE cells promote the development and growth of androgen-refractory prostate tumors through the secretion of neuropeptides that induce tumor cell growth within surrounding prostate tissue (12).

The exact role or contribution that NE cells have on the development of androgen-independent prostate carcinomas is highly debatable and is an active area of scientific inquiry (5). Investigators have traditionally used *in vitro* human prostate cancer cell lines to study the molecular processes that drive androgen-independent prostate cancer cell growth (12). For example, the androgen-dependent human LNCaP prostate cancer cell line requires androgen for growth *in vitro* and *in vivo* (13, 14). However, androgen-independent LNCaP sublines have been derived that are capable of growing indefinitely *in vitro* in the absence of androgen (15, 16). Interestingly, the immediate withdrawal of androgen causes androgen-dependent prostate cancer cells to acquire dendritic-like processes and express neuronal-specific proteins *in vitro* and *in vivo* that closely resemble the cellular properties of NE cells located within the prostate gland (12, 14). This suggests that activation of the NE transdifferentiation process represents an early response to AR inactivation induced by androgen withdrawal in prostate tumors. However, over time, AR reactivation may result in androgen-independent prostate tumor cell growth and in conjunction with repression of the NE transdifferentiation process in corresponding tumor cells. Therefore, elucidation of the molecular effectors that control the NE transdifferentiation process in prostate cancer cells may lead to the discovery of new therapeutic target(s) in the treatment of androgen-independent, NE-enriched prostate cancers (17).

The accumulation of NE cells in xenografted LNCaP prostate cancer tumors after androgen withdrawal argues that AR actively represses a NE transdifferentiation process in prostate cancer cells both *in vitro* and *in vivo* (14). We report that short interfering RNAs (siRNAs) directed against AR induced AR silencing and the activation of the NE transdifferentiation process in the androgen-dependent LNCaP and androgen-independent LNCaP-AI prostate cancer cell lines. AR silencing induced the growth of dendritic-like projections from both LNCaP and LNCaP-AI cells. Furthermore, AR knockdown induced higher levels of the neuronal marker proteins NSE, β-tubulin III, and glial acidic fibrillary protein (GFAP) in LNCaP cells, while nestin levels were decreased in these cells. In contrast, AR silencing had no measurable effect on NSE and β-tubulin III levels in LNCaP-AI cells, even though GFAP levels were increased and nestin levels decreased in these cells. Lastly, AR silencing inhibited the growth of LNCaP and LNCaP-AI cells *in vitro*. Our results confirm and expand previous observations that have shown prostate cancer cells have the potential to transdifferentiate in response to androgen withdrawal, suggesting a direct link to AR (14). AR silencing has now confirmed a direct link between AR and inhibition of the NE transdifferentiation process in prostate cancer cells. Overall, we have shown that AR actively represses the NE transdifferentiation process in androgen-responsive prostate cancer cells. AR inactivation may represent a plausible mechanism leading to the accumulation of NE cells in primary and advanced-stage prostate carcinoma *in vivo*.

RESULTS

Transfection of an siRNA Targeted to the AR Results in AR Silencing and Induces a NE Phenotype in LNCaP Cells

AR signaling is paramount to the development, growth, and maintenance of early and advanced-stage prostate carcinoma (18). Despite our detailed knowledge of AR function as a nuclear steroid hormone transcription factor, studies detailing the biochemical and morphological events associated with loss of AR function in prostate cancer cells have been minimal (19, 20). The development and maturation of RNA interference (RNAi) technologies, specifically siRNAs, have given researchers a powerful tool for silencing mammalian genes in cultured cells in an attempt to elucidate their function (21, 22). To gain a better understanding of the cellular processes affected by inhibiting AR expression in prostate cancer cells, we decided to silence AR in the human LNCaP prostate cancer cell line by transfecting a siRNA duplex that targeted nucleotides 293–313 of the AR mRNA (AR1 siRNA). Transfection of AR1 siRNA efficiently silenced AR expression in LNCaP cells 72 h post transfection (Fig. 1A, compare lanes 4 and 5). Androgen starvation also caused a slight reduction in AR in comparison to 72-h androgen (10 nM R1881, an androgen analog)-treated cells (Fig. 1A, compare lanes 2 and 3), which is in agreement with previous studies that have shown that the androgen hormone promotes AR stabilization in prostate cancer cells (23). Transfection of control siRNA decreased target protein levels [*e.g*. AR, prostate-specific antigen (PSA), fatty acid synthase (FAS)] when compared with untransfected LNCaP cells (compare lane 4 to lanes 1–3); however, due to the obvious differences in the experimental manipulations (*e.g*. transfection conditions) between androgen starvation (lanes 1–3) and AR silencing (lanes 4 and 5), a direct comparison of protein levels is only meaningful within lanes 1–3 and lanes 4–5 in all subsequent panels.

AR inactivation induced by androgen withdrawal induces the NE transdifferentiation process in androgen-dependent prostate cancer cells as measured by an increase in neuronal-specific protein expression and the growth of dendrite-like processes (12). Therefore, we decided to silence AR in an androgen-depen-

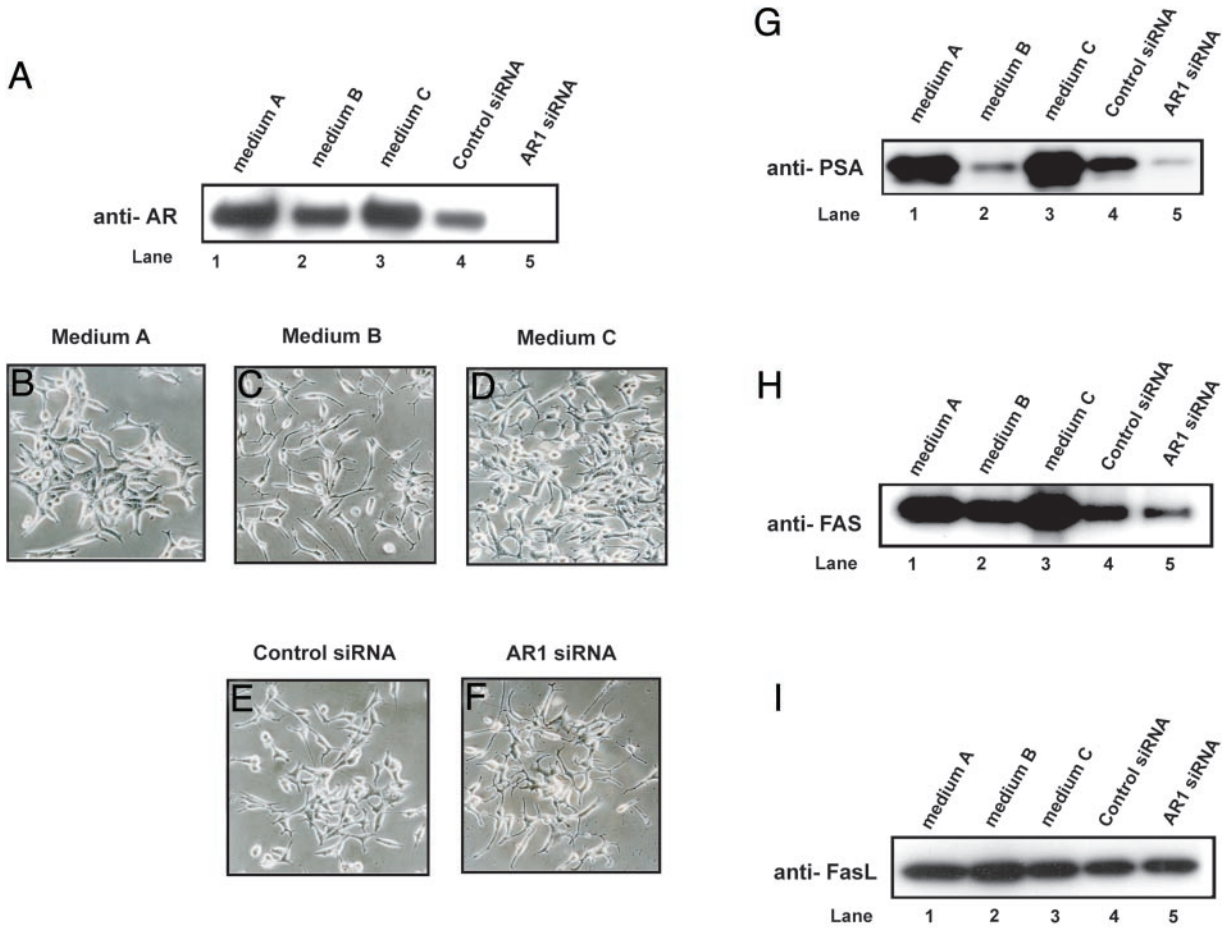

Fig. 1. siRNAs Targeted to the AR Silence AR in LNCaP Prostate Cancer Cells

A, AR expression in LNCaP cells. On d 0, LNCaP cells were seeded at 5000 cells/cm^2 into six-well tissue culture dishes and incubated in medium A (phenol red-deficient RPMI 1640 medium supplemented with 10% FBS lacking antibiotics). On d 1, cells were fed and incubated in either medium A (lane 1), medium B (phenol red-deficient RPMI 1640 medium supplemented with 10% charcoal-stripped FBS minus antibiotics) (lane 2), medium C (phenol red-deficient RPMI 1640 medium containing 10% charcoal-stripped FBS supplemented with 10 nM R1881 minus antibiotics) (lane 3), transfected with control siRNA (lane 4), or transfected with AR1 siRNA (lane5) as detailed in *Materials and Methods*. On d 4, the cells were washed with PBS and solubilized in buffer A. Total protein lysate (4 μg) from each treatment were subjected to SDS-PAGE (4–20% gradient gels) and immunoblot analysis with 1 μg/ml anti-AR IgG AR441 as detailed in *Materials and Methods*. B–F, Photographs of LNCaP cells on d 4 of experimental treatments as detailed in panel A. B, LNCaP cells grown in medium A; C, LNCaP cells grown in medium B; D, LNCaP cells grown in medium C; E, LNCaP cells transfected with control siRNA; F, LNCaP cells transfected with AR1 siRNA. Cells were viewed at $\times$10 and imaged as detailed in *Materials and Methods*. G–I, Immunoblot analyses of PSA, FAS, and fas-ligand (FasL). Total protein lysate (4 μg) derived from the cell extracts described in panel A was subjected to SDS-PAGE (12%) and immunoblot analysis using 1 μg/ml anti-PSA IgG RDI-PSA85abm/RDI-PSA85abm-1 (panel G), 1 μg/ml anti-FAS IgG clone 23 (panel H), 1 μg/ml anti-FasL IgG clone 33 (panel I). The blots were processed as described in *Materials and Methods*. Data are representative of at least three independent experiments.

dent prostate cancer cell line to directly test whether the NE transdifferentiation process was directly AR dependent. This prompted us to compare the biochemical and morphological changes associated with AR inactivation induced by androgen starvation and AR silencing in LNCaP cells. As demonstrated previously (14, 24), a 72-h withdrawal of androgen induced the growth of dendritic-like processes in LNCaP cells (Fig. 1, compare B and C). A 72-h transfection of LNCaP cells with AR1 siRNA also promoted the growth of dendritic-like projections when compared with control transfected cells (Fig. 1, compare E and F). The neuronal appearance of androgen-starved and AR-silenced cells was strikingly similar, and their cellular morphology was quite distinct from LNCaP grown in standard medium or androgen-depleted medium containing androgen (Fig. 1, compare B and D). These results demonstrated that AR silencing promoted the growth of dendritic-like processes similarly to AR inactivation induced by androgen starvation, suggesting that AR actively represses the NE transdifferentiation process in LNCaP prostate cancer cells (14).

To further document the role of AR activity in LNCaP cells, we wanted to know whether the neuronal phenotype of AR-silenced LNCaP cells correlated with changes in the levels of known androgen-regulated proteins. Consistent with previous studies (25), the androgen-regulated serine protease, PSA, was reduced in androgen-starved LNCaP cells in comparison to normal or androgen-treated cells (Fig. 1G, compare lanes 1–3). Similarly, PSA levels also decreased in AR knockdown cells in comparison to control transfected cells (Fig. 1G, compare lanes 4 and 5). Androgens induce the expression of a number of enzymes involved in cellular growth and metabolism (26). For example, FAS is an indirect target of AR transcription (27). We found that androgen-starved cells had lower FAS levels in comparison to androgen-treated cells (Fig. 1H, compare lanes 2 and 3). Similarly, FAS levels were also decreased in AR knockdown cells in comparison to control transfected cells (Fig. 1H, compare lanes 4 and 5).

To show that AR knockdown did not cause general fluctuations in gene expression, we decided to follow the expression levels of membrane-bound FasL after androgen removal and AR knockdown. FasL, a ligand of the TNF superfamily of receptors, is constitutively expressed and secreted by prostate cancer cells (28). To date, FasL has not been implicated as an androgen-regulated protein in prostate cancer cells. We found that FasL levels remained relatively constant during androgen starvation, androgen treatment, or AR silencing (Fig. 1I, lanes 1–5). In total, our results demonstrate that AR silencing, similar to androgen withdrawal, led to reductions in PSA and FAS expression in LNCaP cells, and that FasL was not a target of androgen regulation in LNCaP cells.

AR Regulates Cell Shape in LNCaP Cells and Represses the Expression of Neuronal-Specific Marker Proteins

We sought to better characterize the shape changes of LNCaP cells in response to androgen treatment and also document all androgen-mediated shape changes as they related to AR expression in these cells. Therefore, we decided to observe 72-h androgen-starved LNCaP cells after a 24-h incubation with androgen. Androgen-starved cells contained dendritic-like processes (Fig. 2A) that readily disappeared after a 24-h incubation in androgen (Fig. 2B), demonstrating the plasticity of the NE phenotype in LNCaP cells. AR knockdown cells also grew dendritic-like processes (Fig. 2C); however, AR knockdown cells maintained their dendritic-like processes after the 24-h incubation with androgen (Fig. 2, compare C and D), while cells transfected with the control siRNA acquired a very round and circular morphology in response to androgen treatment (Fig. 2, compare E and F). These results demonstrate that AR expression is critical for androgen-mediated shape changes in LNCaP cells.

AR knockdown LNCaP cells have a distinct neuronal appearance, which prompted us to document the expression pattern of a target protein that is restricted to the neuronal lineage. We decided to measure neuronal-specific enolase (NSE), the expression of which is generally restricted to neuronal cells and its levels increase in prostate cancer cells after they undergo the NE transdifferentiation process (14). Consistent with these studies, we found that NSE levels increased in androgen-starved LNCaP cells (Fig. 2G, compare lanes 1–3). NSE levels also increased in AR knockdown cells (Fig. 2G, compare lanes 4 and 5), demonstrating that AR negatively regulates NSE expression in LNCaP cells.

To further characterize the pronounced neuronal phenotype of androgen-starved and AR-silenced LNCaP cells, we decided to determine the expression levels of several neuronal-specific cytoskeletal proteins. These proteins included nestin, which defines immature neurons, neuron-specific β-tubulin class III, which defines mature neurons, and GFAP, which demarcates mature astrocytes (29–32). We found that β-tubulin levels were relatively constant in response to androgen treatment or AR knockdown (Fig. 2H, compare lanes 1–3; compare lanes 4 and 5). However, the TUJI antibody that specifically recognizes the neuronal-specific isoform III of β-tubulin revealed an increase in β-tubulin III in androgen-starved and AR knockdown LNCaP cells (Fig. 2I, compare lanes 1 and 2; compare lanes 4 and 5). Surprisingly, β-tubulin III levels were also increased in androgen-treated cells, demonstrating that exogenous androgen was not able to repress β-tubulin III expression in cells that were exposed to androgen-depleted growth medium (Fig. 2, compare lanes 2 and 3). Androgen treatment also had little effect on nestin levels (Fig. 2J, compare lanes 2 and 3), while nestin levels were decreased in AR knockdown cells (Fig. 2J, compare lanes 4 and 5). Androgen treatment had little effect on GFAP levels (Fig. 2K, compare lanes 2 and 3), while GFAP levels increased in AR knockdown cells (Fig. 2K, compare lanes 4 and 5). In total, these data demonstrate that androgens and AR elicit protein abundance changes in specific neuronal cytoskeletal proteins in LNCaP cells.

AR Represses the NE Transdifferentiation Process in the Androgen-Independent LNCaP-AI Prostate Carcinoma Cell Line

Androgen-dependent prostate cancer cell lines require androgens for growth *in vitro* and *in vivo* (9). An unresolved issue is whether androgen-independent prostate cancer cell lines also require AR for growth (33). Androgen-independent prostate cancer cells are capable of proliferating in androgen-deficient microenvironments, suggesting these cells no longer require AR for growth. This led us to test whether AR silencing would have any effect on the morphology or protein expression of a previously classified androgen-inde-

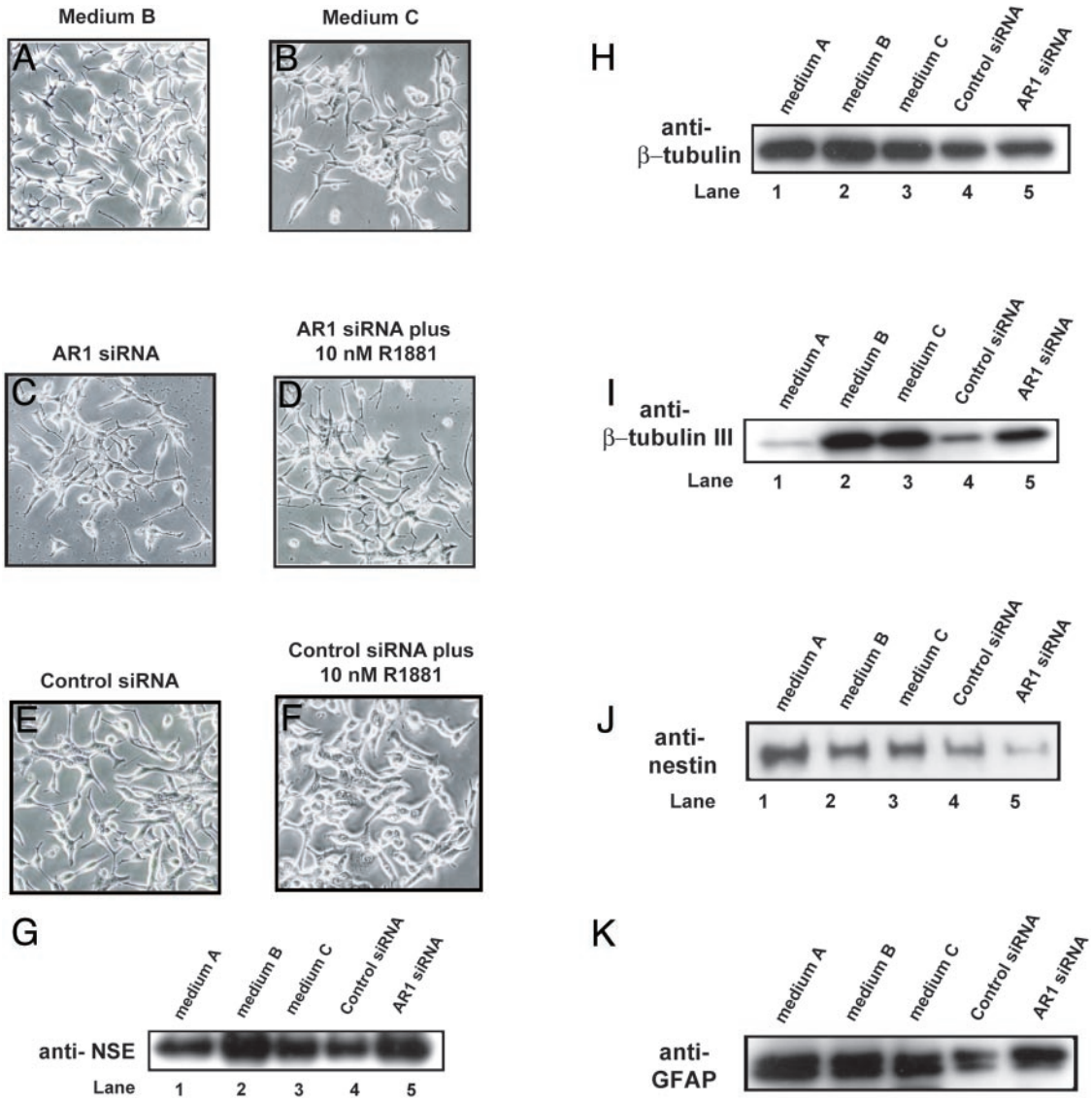

Fig. 2. AR Represses the NE Phenotype and Decreases Neuronal-Specific Protein Marker Expression in LNCaP Cells

A and B, Light microscope photographs of LNCaP cells grown in medium B. On d 0, LNCaP cells were seeded at a cell density of 5000 cells/cm^2 into six-well tissue culture dishes and incubated in medium A. On d 1, the cells were washed with PBS and incubated in medium B. A, on d 4, the cells were photographed and R1881 was added to the culture at a final concentration of 10 nM R1881. B, On d 5, the cells were photographed as detailed in *Materials and Methods*. C and D, Light microscope photographs of LNCaP cells transfected with the AR1 siRNA. On d 0, LNCaP cells were seeded at 5000 cells/cm^2 into six-well tissue culture dishes and transfected with AR1 siRNA as detailed in *Materials and Methods*. C, On d 4, the cells were photographed and R1881 was added to the culture at a final concentration of 10 nM. D, On d 5, the cells were photographed as detailed in *Materials and Methods*. E and F, Light microscope photographs of LNCaP cells transfected with the control siRNA. On d 0, LNCaP cells were seeded at a cell density of 5000 cells/cm^2 into six-well tissue culture dishes and incubated in medium A. On d 1, the cells were transfected with the control siRNA as detailed in *Materials and Methods*. E, On d 4, the cells were photographed and R1881 was added to a final concentration of 10 nM. F, On d 5, the cells were photographed as detailed in *Materials and Methods*. G–K, Immunoblot analyses of NSE, β-tubulin, β-tubulin III, nestin, and GFAP expression in LNCaP cells. Total protein lysate (4 μg) derived from the cell extracts described in panel A were subjected to SDS-PAGE and immunoblot analysis using 1:10 dilution of anti-NSE rabbit polyclonal serum (panel G), 4 μg/ml anti-β-tubulin IgG clone TUB 2.1 (panel H), 1 μg/ml anti-β-tubulin III IgG clone TUJI (panel I), 0.5 μg/ml anti-nestin IgG clone 25 (panel J), 1 μg/ml anti-GFAP IgG clone 52 (panel K). The blots were processed as described in *Materials and Methods*. Data are representative of at least three independent experiments.

pendent prostate cancer cell line. We decided to silence AR in the LNCaP-AI prostate cancer cell line, an androgen-independent LNCaP subline that proliferates in androgen-deficient growth medium, expresses AR at levels comparable to LNCaP cells, and undergoes increased proliferation in response to androgen treatment *in vitro* (16). LNCaP-AI cells typically grow in tight cell clusters in androgen-deficient growth medium (Fig. 3A). However, a 72-h incubation with androgen produced a noticeable shape change in these cells in comparison to untreated cells (Fig. 3, compare A and B), as androgen-treated cells adopted a distinct spindle-like shape and tended to spread out on the dish, thus demonstrating LNCaP-AI cells responsiveness to androgen (16). To test whether LNCaP-AI cells required AR, we decided to silence AR and look for any detectable morphological and biochemical changes that were a result of this treatment. Microscopic examination of AR knockdown cells revealed the growth of dendritic-like projections in LNCaP-AI cells that mirrored the cellular processes that were seen in AR knockdown LNCaP cells (compare Figs. 1G and Fig. 3D). Similar to LNCaP cells, Western blot analyses of total cell lysates revealed that AR levels

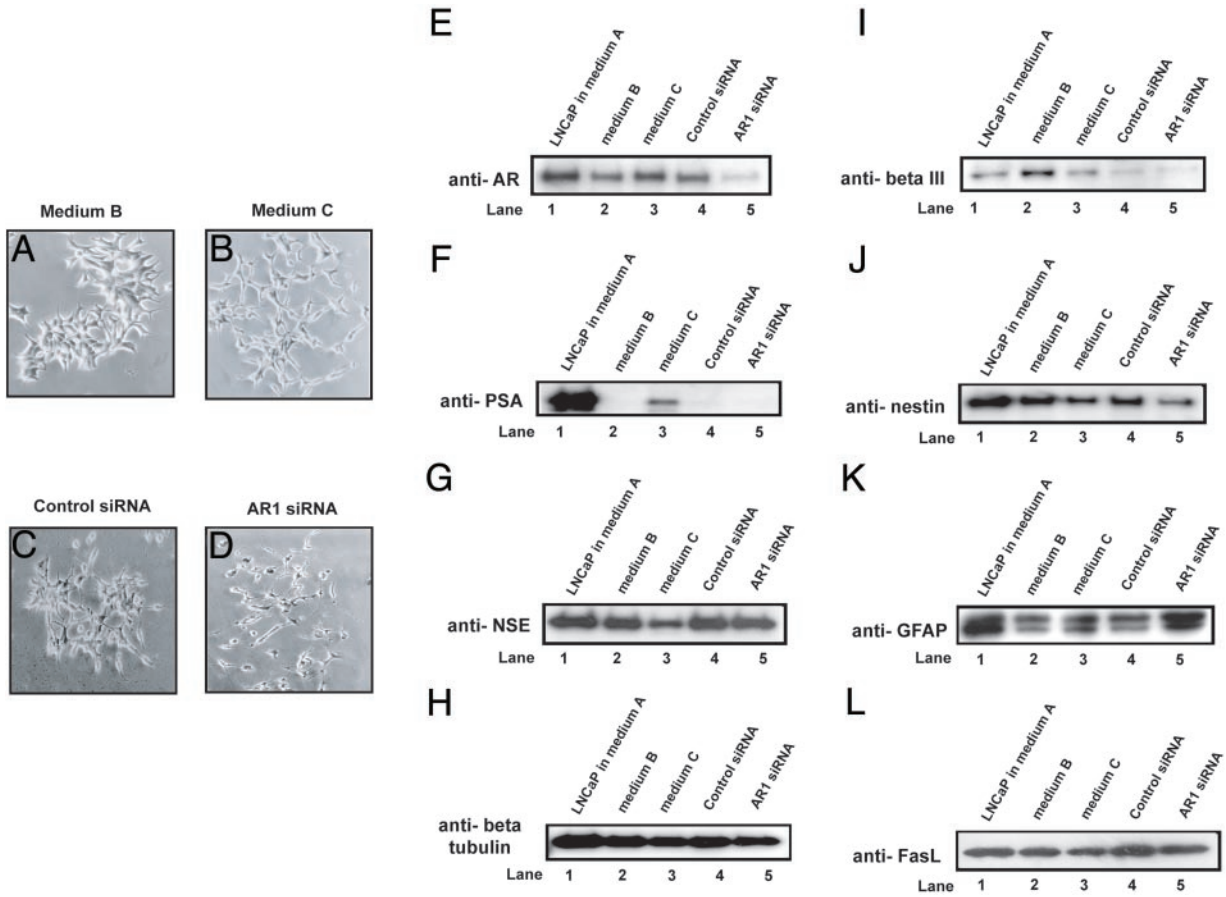

Fig. 3. AR Represses the Neuronal Phenotype and Modulates Neuronal-Specific Marker Protein Expression in the Androgen-Independent LNCaP-AI Prostate Cancer Cell Line

A and B, Light microscope photographs of LNCaP-AI cells after R1881 treatment. On d 0, LNCaP-AI cells were seeded at 5000 cells/cm^2 into six-well tissue culture dishes and incubated in medium B (A and B). On d 1, R1881 was added to (B) at a final concentration of 10 nM. On d 4, untreated LNCaP-AI cells (panel A) and 10 nM treated LNCaP-AI cells (panel B) were viewed and imaged at ×10 as detailed in *Materials and Methods*. C and D, Light microscope photographs of LNCaP-AI transfected with control and AR1 siRNAs. On d 0, LNCaP-AI cells were seeded at 5000 cells/cm^2 into six-well tissue culture dishes and incubated in medium B. On d 1 the cells were transfected with the control (panel E) and AR1 (panel F) siRNAs as detailed in *Materials and Methods*. On d 4, the control (panel E) and AR1 (panel F) siRNA-transfected cells were viewed and imaged as detailed in *Materials and Methods*. E–L, Immunoblot analysis of AR, PSA, NSE, β-tubulin, β-III-tubulin, nestin, GFAP, and FasL in LNCaP-AI cells. Total protein lysates (4 μg) derived from LNCaP grown in medium A were run as a positive control in lane 1. LNCaP-AI cells exposed to medium B (lane 2), medium C (lane 3), transfected with control siRNA (lane 4), or transfected with AR1 siRNA (lane 5) as detailed in *Materials and Methods*. Total protein lysate (4 μg) was subjected to SDS-PAGE and immunoblot analysis using 1 μg/ml anti-AR IgG AR441 (panel E), 1 μg/ml anti-PSA IgG RDI-PSA85abm/RDI-PSA85abm-1 (panel F), 1:10 dilution of anti-NSE rabbit polyclonal serum (panel G), 4 μg/ml anti-β-tubulin IgG clone TUB 2.1 (panel H), 1 μg/ml anti-β-tubulin III IgG clone TUJI (panel I), 0.5 μg/ml anti-nestin IgG clone 25 (panel J), 1 μg/ml anti-GFAP IgG clone 52 (panel K), 1 μg/ml anti-FasL IgG clone 33 (panel L). The blots were processed as described in *Materials and Methods.* Data are representative of at least three independent experiments.

increased after androgen treatment (Fig. 3E, compare lanes 2 and 3). We found that AR levels were significantly decreased in AR knockdown cells in comparison to control-transfected cells (Fig. 3E, compare lanes 4 and 5). The appearance of the NE phenotype in the LNCaP-AI cells prompted us to further characterize the same group of androgen-regulated proteins that were documented in LNCaP cells as described above. We found that PSA levels were undetectable in LNCaP-AI cells when they were grown in androgen-deficient medium (Fig. 3F, lane 2). However, PSA expression was detectable after a 72-h incubation with androgen (Fig. 3F, compare lanes 2 and 3). Similar to LNCaP cells, androgen reduced NSE levels in LNCaP-AI cells (Fig. 3G, compare lanes 2 and 3). However, NSE levels remained relatively unchanged in AR knockdown cells (Fig. 3G, compare lanes 4 and 5). Similar to LNCaP cells, β-tubulin levels remained unchanged in androgen-treated or AR knockdown cells (Fig. 3H, compare lanes 2 and 3 and 4 and 5). However, in contrast to LNCaP cells, β-tubulin III levels were reduced in androgen-treated cells (Fig. 3I, lanes 2 and 3), while AR silencing had little effect on β-tubulin III levels (Fig. 3I, compare lanes 4 and 5). We also found that nestin levels were decreased in androgen-treated and AR knockdown cells (Fig. 3J, compare lanes 2 and 3 and lanes 4 and 5). Interestingly, in accordance with LNCaP cells, GFAP levels increased in AR knockdown cells (Fig. 3K, compare lanes 4 and 5), while GFAP levels were slightly increased in androgen-treated cells (Fig. 3K, compare lanes 2 and 3). Lastly, in agreement with LNCaP cells, FasL levels remained unchanged by androgen treatment or AR silencing (Fig. 3L, compare lanes 2 and 3 and 4 and 5). In summary, AR expression is required to block the morphological aspects of the NE transdiffentiation process in LNCaP-AI cells, and AR can modulate neuronal cytoskeletal protein expression in LNCaP-AI cells similarly to LNCaP cells.

AR Silencing Inhibits the Growth of LNCaP and LNCaP-AI Prostate Cancer Cells

AR is critical for the growth of prostate cancer cells (9). In contrast, NE cells are devoid of AR and are typically nonproliferative (12). This prompted us to determine whether activation of the NE transdifferentiation process induced by AR silencing correlated with a decrease in cell growth in LNCaP and LNCaP-AI cells. Both control and AR1 siRNA-transfected LNCaP and LNCaP-AI cells were exposed to siRNAs for 120 h and switched to fresh medium that lacked siRNAs and then incubated for another 120 h. Under these experimental conditions, AR knockdown decreased the cell growth of both LNCaP and LNCaP-AI cells when compared with control transfected cells (Fig. 4, compare A and B and C and D). LNCaP cell growth was reduced to 38.2% (SD $\pm$ 5.2) of control siRNA-transfected cells (Fig. 4E), while LNCaP-AI cell growth was reduced to 65.1% (SD $\pm$ 3.3) of control siRNA-transfected cells (Fig. 4E). These results demonstrate that LNCaP and LNCaP-AI cell growth is inhibited by AR silencing and demonstrate that AR expression is required for normal *in vitro* cell growth in the absence or presence of androgens. Although the androgen-independent LNCaP-AI prostate cancer cell line proliferates in the absence of androgens, it also requires AR expression for growth in androgen-deficient growth medium, which is consistent with the previous report that LNCaP-AI cell growth is androgen independent, but can be enhanced by androgen treatment (16).

DISCUSSION

Defining the cellular composition of prostate tumors will be critical in determining how to treat and cure this disease (34). Androgen-ablation therapies are an effective method for treating prostate cancers (4). However, they tend to select for androgen-refractory tumors that become enriched for NE cells (11). NE cells have a normal role in the physiology of the prostate and they secrete neuropeptides that can impart both survival and proliferative signals upon prostate cancer cells *in vitro* (35). It has been hypothesized that NE cells contribute to the development of androgen-independent prostate cancers through the secretion of neuropeptides that function in a paracrine fashion to impart androgen-independent growth properties upon prostate tumors *in vivo* (12). The proclivity of androgen-dependent prostate cancer cells to undergo the NE transdifferentiation process in an androgen-specific manner suggests that AR is central to regulating this process (14). This prompted us to directly test whether AR controlled the NE transdifferentiation process in both androgen-dependent and androgen-independent prostate cancer cells. We found that AR silencing induced the growth of dendritic-like appendages from both androgen-dependent LNCaP and androgen-independent LNCaP-AI prostate cancer cell lines. AR silencing increased NSE and β-tubulin III expression in LNCaP cells and impaired the normal *in vitro* cell growth of LNCaP and LNCaP-AI cells. Our analysis has clearly demonstrated that AR is required to repress the NE transdifferentiation process in prostate cancer cells *in vitro* and suggests that AR may actively repress an analogous NE transdifferentiation process in prostate cancer cells *in vivo*. This intrinsic repression of the NE transdifferentiation process by AR may provide an explanation for the increased number of NE cells in androgen-refractory tumors that have undergone long-term exposure through androgen-ablation therapies (11).

Inactivation of AR through microinjection of a neutralizing antibody or targeting the AR mRNA using a hammerhead ribozyme inhibited LNCaP cell growth and the growth of both the LNCaP-C4 and LNCaP-Rf androgen-refractory prostate cancer cell line derivatives (36). This study was the first to demonstrate that

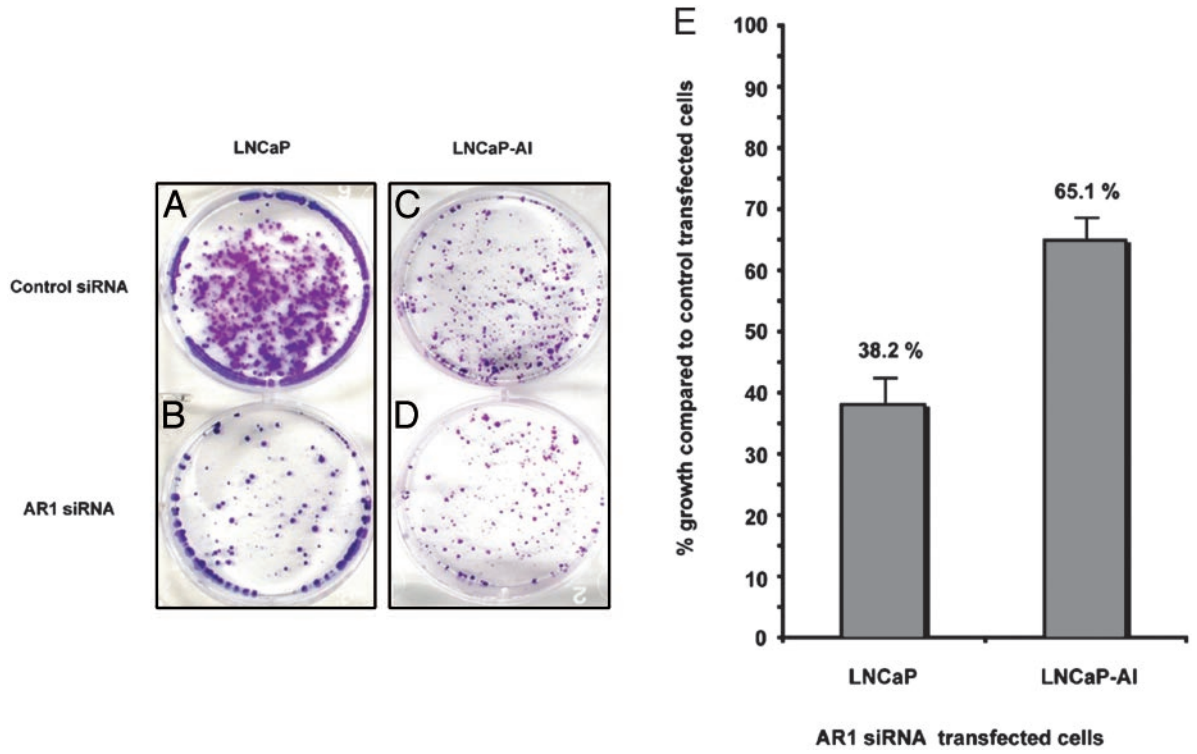

Fig. 4. AR Silencing Severely Inhibits the Growth of LNCaP and LNCaP-AI Cells

A and B, On d 0, LNCaP cells were seeded at 5000 cells/cm^2 into six-well tissue culture dishes and incubated in medium A. On d 1, cells were fed medium A and transfected with the control siRNA (panel A) or AR1 siRNA (panel B). On d 6, the cells were washed with PBS and fed medium A. On d 11, the cells were washed with PBS, fixed in methanol, and stained with the crystal violet dye. The dishes were viewed and imaged with a digital camera as detailed in *Materials and Methods*. C and D, LNCaP-AI cells were transfected with control siRNA (panel C) or AR1 siRNA (panel D) using the exact experimental conditions described in panel A, except that LNCaP-AI cells were grown in medium B during the analysis. On d 11, cells were washed with PBS, fixed in methanol, and stained with the crystal violet dye. The dishes were viewed and imaged with a digital camera as detailed in *Materials and Methods*. Data are representative of at least three independent experiments. E, Quantitative measurement of cell growth in control and AR siRNA-transfected LNCaP and LNCaP-AI cells. Cells were treated as detailed in panels A–D. Cells were trypsinized on d 11, and the total number of viable cells were counted as detailed in *Materials and Methods*. The total number of AR1 siRNA-transfected cells (x-axis) was plotted relative to the total number of control siRNA-transfected cells (y-axis).

both androgen-dependent and androgen-refractory prostate cancer cell lines require AR for normal cell growth. Our AR siRNA studies in LNCaP and LNCaP-AI cells are in complete agreement and expand the results of this earlier study (36). Not only did we find that androgen-dependent and androgen-independent prostate cancer cell lines require AR expression for growth, but we also found that AR expression is necessary for repressing neuronal-specific protein expression in these cells. Interestingly, Zegarra-Moro *et al.* (37, 38) also showed that the androgen-independent DU145 prostate cancer cell line, which lacks detectable AR, was insensitive to AR inactivation; this suggests that androgen-independent prostate cancer cell lines that are devoid of AR expression are insensitive to AR inactivation, while androgen-independent prostate cancer cell lines that retain AR expression most likely require AR for cell growth. Future studies that inactivate AR in androgen-independent prostate cancer cell lines that are either AR positive or negative using RNAi will be required to test this hypothesis more robustly. Multiple mechanisms could explain the hormone-independent AR-dependent growth phenotype of LNCaP-AI cells. For example, these cells may have acquired a hypersensitivity phenotype to residual levels of androgens that remain in the charcoal-stripped serum that was used in our study. This phenotype was first reported in the androgen-dependent CWR22 human xenograft prostate cancer cell line where recurrent CWR22 tumors had increased AR stabilization that was associated with an increased hypersensitivity to lower levels of androgen both *in vitro* and *in vivo* (39). However, other signaling mechanisms, including growth factors, activation of the protein kinase A pathway, and neuropeptides, have been shown to activate AR in a hormone-independent fashion (5). Future studies will be required to define the underlying mechanism of AR activation in LNCaP-AI cells.

One of the goals of this study was to establish a direct link between AR and the NE transdifferentiation process in prostate cancer cells. We found that AR

silencing induced biochemical and morphological changes associated with the NE transdifferentiation process that mirrors those that occur when AR is inactivated by androgen withdrawal. AR silencing increased NSE levels in LNCaP cells, thereby demonstrating AR has a repressive effect on NSE expression. AR silencing had no effect on NSE expression in LNCaP-AI cells even though NSE levels decreased upon the addition of exogenous androgen. This result demonstrates that AR still retained its repressive activity toward NSE expression in LNCaP-AI cells. However, these results suggest there is a strong possibility that AR represses neuronal-associated target genes and related neuronal shape changes at different thresholds. For example, in LNCaP-AI cells, which were derived from a long-term exposure to androgen-depleted growth medium (16), AR may have lost its ability to repress certain subsets of neuronal genes. As a result, removal of AR by RNAi would have no effect on the expression levels of certain neuronal targets such as NSE, even though the expression levels of other neuronal targets may be removal of AR. This may explain why the NE transdifferentiation process is not fully activated in LNCaP-AI cells without the complete removal of AR by RNAi. However, future studies will be required to test these hypotheses more directly.

The dendritic-like protrusions caused by AR silencing in LNCaP and LNCaP-AI cells strongly suggested that the levels of specific neuronal cytoskeletal proteins were altered in these cells. For example, the expression pattern of neuronal cytoskeletal proteins is commonly used to classify the differentiation state of neurons (40–42). The *in vitro* differentiation of immature neurons into mature neurons entails the growth of dendritic processes and a concomitant decrease in nestin immunoreactivity and a subsequent increase in β-tubulin III immunoreactivity (42). However, testosterone has been shown to block this differentiation process by preventing dendrite cell growth and preventing β-tubulin III immunoreactivity (42). We found that activation of the NE transdifferentiation process by AR silencing led to expression level changes in neuronal-specific cytoskeletal proteins β-tubulin III, nestin, and GFAP in both LNCaP and LNCaP-AI cells. Androgen withdrawal and AR silencing led to increases in β-tubulin III expression in LNCaP cells. However, we also found that β-tubulin III levels increased in the presence of androgen even though the cells were absent of dendritic-like processes. This result suggests that the charcoal-stripped calf serum used in this experiment was deficient in one or several factors that cooperate with androgen to repress β-tubulin III expression in LNCaP cells. However, despite this discrepancy, AR silencing clearly demonstrated that AR has a negative effect on β-tubulin III expression in LNCaP cells. Conversely, AR silencing had no effect on β-tubulin III expression, although androgen had a repressive effect on β-tubulin III expression. We also found that AR expression was required to sustain basal nestin expression in both LNCaP and LNCaP-AI cells. However, androgen had no effect on nestin expression in LNCaP cells, while it had a slight repressive effect on nestin expression in LNCaP-AI cells. Lastly, GFAP, which maintains the morphology and motility of astrocytes (43), was negatively regulated by AR in both LNCaP and LNCaP-AI cells, while GFAP levels were unaffected by androgen treatment in either LNCaP or LNCaP-AI cells. Overall, AR silencing led to expression level changes in neuronal cytoskeletal proteins in LNCaP and LNCaP-AI cells. Functional roles for these expression level changes in the context of the NE transdifferentiation process in prostate cancer cells *in vitro* and *in vivo* will require further investigation.

Table 1 summarizes the androgen- and AR-mediated protein expression changes in NSE, β-tubulin III, nestin, and GFAP proteins in LNCaP and LNCaP-AI cells. It is hoped that future analyses will expand this list of androgen- and AR-responsive target proteins. A comprehensive protein expression map that details all androgen- and AR-responsive neuronal cytoskeletal protein expression may facilitate the identification of new markers in the classification of neuroendocrine-enriched prostate cancers.

Our results demonstrate that AR functionally represses the NE transdifferentiation process in prostate cancer cells *in vitro* and may repress an analogous NE transdifferentiation process of prostate cancer cells *in vivo*. Systematic application of RNAi directed against the AR-mediated signal transduction pathway in prostate cancer cells will provide a unique opportunity to dissect the specific roles of AR and its associated proteins in the growth of androgen-dependent and androgen-independent growth in prostate cancer cells.

Table 1. Androgen and AR-Mediated Protein Abundance Changes of Selected Neuronal Proteins in LNCaP and LNCaP-AI Prostate Cancer Cells

Protein	Androgen Regulation		AR Silencing	
	LNCaP Cells	LNCaP-AI Cells	LNCaP Cells	LNCaP-AI Cells
NSE	−	−	+	NC
β-Tubulin III	NC	−	+	NC
Nestin	NC	−	−	−
GFAP	NC	+	+	+

Summary of Western blot analyses. +, Protein increases; −, protein decreases; NC, no change.

MATERIALS AND METHODS

Cell Culture and Cell Lines

LNCaP cells were obtained from American Tissue Type Culture Collection (Manassas, VA) and maintained in phenol red-deficient RPMI 1640 (Invitrogen, Carlsbad, CA) medium supplemented with 10% FBS (Hyclone Laboratories, Inc., Logan, UT). LNCaP-AI cells were maintained in phenol red-deficient RPMI 1640 medium supplemented with 10% charcoal/dextran-treated FBS (Hyclone Laboratories Inc.) (16). All cultures were supplemented with penicillin/streptomycin/glutamine and maintained in a 37 C incubator in 5% CO_2.

Reagents

The AR agonist R1881 (methyltrienolone) was purchased from Perkin Elmer Life Sciences (Boston, MA). Double-stranded siRNAs were purchased from Dharmacon Research (Lafayette, CO). Oligofectamine reagent, 4–20%, and 12% SDS-PAGE gels were purchased from Invitrogen. Prestained protein molecular weight markers were purchased from MBI Fermentas (Hanover, MD). Corresponding antibodies used for these analyses included monoclonal AR antibody (AR441) from Santa-Cruz Biotechnology, Inc. (Santa Cruz, CA); monoclonal PSA cocktail (RDI-PSA85abm/RDI-PSA85abm-1) from Research Diagnostic, Inc. (Flanders, NJ); monoclonal FAS (clone 23), monoclonal nestin (clone 25), monoclonal GFAP (clone 52), monoclonal FasL (clone 33) from BD Transduction Laboratories (San Diego, CA); monoclonal β-tubulin (clone TUB 2.1) from Sigma (St. Louis, MO); monoclonal β-tubulin III (clone TUJI) from Covance (Princeton, NJ); and rabbit polyclonal NSE (product no. PC23) from Oncogene (La Jolla, CA). Crystal violet dye was purchased from Sigma. The BCA protein assay kit was purchased from Pierce Biotechnology (Rockford, IL).

siRNA Assay

One 21-nucleotide double-stranded siRNA duplex generated against the amino terminus of the AR at nucleotides 293–312 (5′-aagcccatcgtagaggcccca-3′) will be referred to in the following as the AR1 siRNA. A single control nucleotide double-stranded siRNA duplex, referred to as the control siRNA, was generated to the inverted sequence of AR at nucleotides 293–313 (5′-accccggagatgctacccgaa-3′) and functioned as a nonspecific control siRNA for the RNAi experiments where indicated. On d 0, LNCaP cells were seeded at 5000 cells/cm^2 into Falcon (Becton Dickinson Laboratories, Franklin Lakes, NJ) six-well tissue culture dishes and incubated in medium A (phenol red-deficient RPMI 1640 medium supplemented with 10% FBS lacking antibiotics) for 24 h. On d 1, the cells were washed with PBS, refed medium A, and transfected with control or AR1 siRNAs at a final concentration of 100 nM using the Oligofectamine reagent according to the manufacturer's instructions. Cells were transfected with siRNAs in medium A. The cells were incubated with siRNAs for 72 h. The guidelines for siRNA silencing were followed as detailed in the instructions provided at the Dharmacon Research website (http://www.dharmacon.com/).

Hormone Starvation Treatment

On d 0, LNCaP cells were seeded at 5000 cells/cm^2 into six-well tissue culture dishes and incubated in medium A (phenol red-deficient RPMI 1640 medium supplemented with 10% FBS lacking antibiotics). On d 1, cells were fed and grown in either medium A, or medium B (phenol red-deficient RPMI 1640 medium supplemented with 10% charcoal-stripped FBS minus antibiotics), or medium C (phenol red-deficient RPMI 1640 medium containing 10% charcoal-stripped FBS supplemented with 10 nM R1881 minus antibiotics).

Western Blot Analysis

On d 4, cells were washed once with PBS and solubilized in 0.2 ml of buffer A [50 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA (pH 7.4), 1% sodium dodecyl sulfate] and boiled for 5 min. Total protein lysates were quantified using the Pierce BCA protein assay kit. Total protein lysates were subjected to SDS-PAGE (4–20% gradient precast gels, Invitrogen). Gels were transferred to polyvinylidine difluoride membranes, incubated in Tris-buffered saline containing 0.1% Tween 20 (TBST) and 5% nonfat milk (wt/vol) for 1 h. The blots were subsequently incubated for 1 h using the appropriate primary antibody at the following antibody dilutions: 1 μg/ml anti-AR IgG (Santa Cruz Biotechnology, Inc., AR441), 4 μg/ml anti-β-tubulin IgG (Sigma, clone TUB 2.1), 1 μg/ml anti-β-tubulin III IgG (Covance, clone TUJI), 1:10 dilution of anti-NSE rabbit polyclonal serum (Oncogene, catalog no. PC23), 0.5 μg/ml antinestin IgG (BD Transduction Laboratories, clone 25), 1 μg/ml anti-GFAP IgG (BD Transduction Laboratories, clone 52), 1 μg/ml anti-PSA IgG (Research Diagnostic, Inc., RDI-PSA85abm/RDI-PSA85abm-1); 1 μg/ml anti-FAS IgG (BD Transduction Laboratories catalog no. 610962, clone 23); or 1 μg/ml anti-FasL IgG (BD Transduction Laboratories catalog no. 610410, clone 33). The blots were washed three times for 5 min in TBST and incubated with either a goat antimouse or goat antirabbit horseradish peroxidase-conjugated secondary antibody at 1:10,000 dilutions in TBST and incubated for 1 h at room temperature. The blots were washed three times for 5 min in TBST, and immunoreactive bands were developed and visualized using enhanced chemiluminescence reagents kit (Amersham Pharmacia Biotech, Arlington Heights, IL). The blots were exposed to Hyperfilm enhanced chemiluminescence film (Amersham Pharmacia Biotech) for less than 1 min.

Cell Growth Assay

On d 0, LNCaP or LNCaP-AI cells were seeded at 5000 cells/cm^2 into six-well tissue culture dishes and incubated in medium A and medium B, respectively. On d 1, LNCaP cells were fed medium A, and LNCaP-AI cells were fed medium B and subsequently transfected with the control siRNA or AR1 siRNAs. On d 6 the LNCaP and LNCaP-AI cells were washed once with PBS and incubated in medium A or medium B, respectively. On d 11, the cells were washed once with PBS, fixed in 100% methanol for 10 min, and stained with crystal violet dye. The dishes were viewed and photographed with a Nikon Coolpix digital camera (Nikon Inc., Melville, NY). Cell growth of control and AR1 siRNA-transfected LNCaP and LNCaP-AI cells were quantified on d 10 after using trypan blue dye exclusion (44).

Microscopy

Light microscope photographs were taken of LNCaP and LNCaP-AI cells after the appropriate treatments described in the corresponding figure legends. Cells were viewed and imaged with Fujifilm Super HQ 35-mm film (100-speed film) at $\times$10 and on an Olympus iX70 inverted microscope.

Acknowledgments

We thank our colleague David K. Han for helpful discussions and critical reading of the manuscript.

Received January 28, 2003. Accepted May 21, 2003.

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This work was supported by grants from the NIH to R.A. (1R33CA-93302) and M.J.T. (DK62821) and prostate Specialized Program of Research Excellence (CA58204). A gift from Merck and Co. to support research at the Institute for Systems Biology is gratefully acknowledged. M.E.W. was supported in part by a NIH Postdoctoral Genome Training Fellowship Grant. M.E.W. is a United Negro College Fund/Merck postdoctoral research fellow.

REFERENCES

1. Balk SP 2002 Androgen receptor as a target in androgen-independent prostate cancer. Urology 60:132–138; discussion, 138–139
2. Prins GS 2000 Molecular biology of the androgen receptor. Mayo Clin Proc 75(Suppl):S32–S35
3. Walsh PC 1980 Radical prostatectomy for the treatment of localized prostatic carcinoma. Urol Clin North Am 7:583–591
4. Huggins C, Hodges CV 1972 Studies on prostatic cancer. I. The effect of castration, of estrogen and androgen injection on serum phosphatases in metastatic carcinoma of the prostate. CA Cancer J Clin 22:232–240
5. Navarro D, Luzardo O, Fernandez L, Chesa N, Di az-Chico B 2002 Transition to androgen-independence in prostate cancer. J Steroid Biochem Mol Biol 81:191–201
6. Isaacs JT, Lundmo PI, Berges R, Martikainen P, Kyprianou N, English HF 1992 Androgen regulation of programmed death of normal and malignant prostatic cells. J Androl 13:457–464
7. Craft N, Chhor C, Tran C, Belldegrun A, DeKernion J, Witte ON, Said J, Reiter RE, Sawyers CL 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
8. Klotz L 2000 Hormone therapy for patients with prostate carcinoma. Cancer 88:3009–3014
9. Debes J, Tindall D 2002 The role of androgens and the androgen receptor in prostate cancer. Cancer Lett 187: 1–7
10. Abrahamsson PA, Lilja H 1989 Partial characterization of a thyroid-stimulating hormone-like peptide in neuroendocrine cells of the human prostate gland. Prostate 14: 71–81
11. Guate JL, Escaf S, Menendez CL, del Valle M, Vega JA 1997 Neuroendocrine cells in benign prostatic hyperplasia and prostatic carcinoma: effect of hormonal treatment. Urol Int 59:149–153
12. Abrahamsson PA 1999 Neuroendocrine differentiation in prostatic carcinoma. Prostate 39:135–148
13. van Steenbrugge GJ, van Uffelen CJ, Bolt J, Schroder FH 1991 The human prostatic cancer cell line LNCaP and its derived sublines: an *in vitro* model for the study of androgen sensitivity. J Steroid Biochem Mol Biol 40: 207–214
14. Burchardt T, Burchardt M, Chen MW, Cao Y, de la Taille A, Shabsigh A, Hayek O, Dorai T, Buttyan R 1999 Transdifferentiation of prostate cancer cells to a neuroendocrine cell phenotype *in vitro* and *in vivo*. J Urol 162: 1800–1805
15. Kokontis JM, Hay N, Liao S 1998 Progression of LNCaP prostate tumor cells during androgen deprivation: hormone-independent growth, repression of proliferation by androgen, and role for p27Kip1 in androgen-induced cell cycle arrest. Mol Endocrinol 12:941–953
16. Lu S, Tsai SY, Tsai MJ 1999 Molecular mechanisms of androgen-independent growth of human prostate cancer LNCaP-AI cells. Endocrinology 140: 5054–5059
17. Saffran DC, Reiter RE, Jakobovits A, Witte ON 1999 Target antigens for prostate cancer immunotherapy. Cancer Metastasis Rev 18:437–449
18. Gelmann EP 2002 Molecular biology of the androgen receptor. J Clin Oncol 20:3001–3015
19. Eder IE, Culig Z, Ramoner R, Thurnher M, Putz T, Nessler-Menardi C, Tiefenthaler M, Bartsch G, Klocker H 2000 Inhibition of LncaP prostate cancer cells by means of androgen receptor antisense oligonucleotides. Cancer Gene Ther 7:997–1007
20. Caplen NJ, Taylor JP, Statham VS, Tanaka F, Fire A, Morgan RA 2002 Rescue of polyglutamine-mediated cytotoxicity by double-stranded RNA-mediated RNA interference. Hum Mol Genet 11:175–184
21. Montgomery MK, Xu S, Fire A 1998 RNA as a target of double-stranded RNA-mediated genetic interference in *Caenorhabditis elegans*. Proc Natl Acad Sci USA 95: 15502–15507
22. Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T 2001 Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature 411:494–498
23. Kemppainen JA, Lane MV, Sar M, Wilson EM 1992 Androgen receptor phosphorylation, turnover, nuclear transport, and transcriptional activation. Specificity for steroids and antihormones. J Biol Chem 267:968–974
24. Zelivianski S, Verni M, Moore C, Kondrikov D, Taylor R, Lin MF 2001 Multipathways for transdifferentiation of human prostate cancer cells into neuroendocrine-like phenotype. Biochim Biophys Acta 1539:28–43
25. Balk SP, Ko YJ, Bubley GJ 2003 Biology of prostate-specific antigen. J Clin Oncol 21:383–391
26. Lindzey J, Kumar MV, Grossman M, Young C, Tindall DJ 1994 Molecular mechanisms of androgen action. Vitam Horm 49:383–432
27. Heemers H, Maes B, Foufelle F, Heyns W, Verhoeven G, Swinnen JV 2001 Androgens stimulate lipogenic gene expression in prostate cancer cells by activation of the sterol regulatory element-binding protein cleavage activating protein/sterol regulatory element-binding protein pathway. Mol Endocrinol 15:1817–1828
28. Liu QY, Rubin MA, Omene C, Lederman S, Stein CA 1998 Fas ligand is constitutively secreted by prostate cancer cells *in vitro*. Clin Cancer Res 4:1803–1811
29. Eng LF 1985 Glial fibrillary acidic protein (GFAP): the major protein of glial intermediate filaments in differentiated astrocytes. J Neuroimmunol 8:203–214
30. Frederiksen K, McKay RD 1988 Proliferation and differentiation of rat neuroepithelial precursor cells *in vivo*. J Neurosci 8:1144–1151
31. Lendahl U, Zimmerman LB, McKay RD 1990 CNS stem cells express a new class of intermediate filament protein. Cell 60:585–595
32. Zimmerman L, Parr B, Lendahl U, Cunningham M, McKay R, Gavin B, Mann J, Vassileva G, McMahon A 1994 Independent regulatory elements in the nestin gene direct transgene expression to neural stem cells or muscle precursors. Neuron 12:11–24
33. Eder IE, Culig Z, Putz T, Nessler-Menardi C, Bartsch G, Klocker H 2001 Molecular biology of the androgen receptor: from molecular understanding to the clinic. Eur Urol 40:241–251
34. Abate-Shen C, Shen MM 2000 Molecular genetics of prostate cancer. Genes Dev 14:2410–2434
35. Jongsma J, Oomen MH, Noordzij MA, Romijn JC, van Der Kwast TH, Schroder FH, van Steenbrugge GJ 2000 Androgen-independent growth is induced by neuropep-

tides in human prostate cancer cell lines. Prostate 42: 34–44
36. Zegarra-Moro OL, Schmidt LJ, Huang H, Tindall DJ 2002 Disruption of androgen receptor function inhibits proliferation of androgen-refractory prostate cancer cells. Cancer Res 62:1008–1013
37. Webber MM, Bello D, Quader S 1997 Immortalized and tumorigenic adult human prostatic epithelial cell lines: characteristics and applications. II. Tumorigenic cell lines. Prostate 30:58–64
38. Chlenski A, Nakashiro K, Ketels KV, Korovaitseva GI, Oyasu R 2001 Androgen receptor expression in androgen-independent prostate cancer cell lines. Prostate 47:66–75
39. Gregory CW, Johnson Jr RT, Mohler JL, French FS, Wilson EM 2001 Androgen receptor stabilization in recurrent prostate cancer is associated with hypersensitivity to low androgen. Cancer Res 61:2892–2898
40. Lendahl U, McKay RD 1990 The use of cell lines in neurobiology. Trends Neurosci 13:132–137
41. Donahue LM, Coates PW, Reinhart AJ 1996 Characterization of developmental stage and neuronal potential of the rat PNS-derived stem cell line, RT4-AC. Brain Res Dev Brain Res 94:67–80
42. Zhang L, Chang YH, Barker JL, Hu Q, Maric D, Li BS, Rubinow DR 2000 Testosterone and estrogen affect neuronal differentiation but not proliferation in early embryonic cortex of the rat: the possible roles of androgen and estrogen receptors. Neurosci Lett 281:57–60
43. Laping NJ, Teter B, Nichols NR, Rozovsky I, Finch CE 1994 Glial fibrillary acidic protein: regulation by hormones, cytokines, and growth factors. Brain Pathol 4:259–275
44. Freshney RI 2000 Culture of animal cells : a manual of basic technique. New York: Wiley; xxvi, 577