Abstract

The androgen receptor (AR) is a critical effector of prostate cancer development and progression. Androgen-dependent prostate cancer is reliant on the function of AR for growth and progression. Most castration-resistant prostate cancer (CRPC) remains dependent on AR signaling for survival and growth. Ribosomal RNA (rRNA) is essential for both androgen-dependent and castration-resistant growth of prostate cancer cells. During androgen-dependent growth of prostate cells, androgen-AR signaling leads to the accumulation of rRNA. However, the mechanism by which AR regulates rRNA transcription is unknown. Here, investigation revealed that angiogenin (ANG), a member of the secreted ribonuclease superfamily, is upregulated in prostate cancer and mediates androgen-stimulated rRNA transcription in prostate cancer cells. Upon androgen stimulation, ANG undergoes nuclear translocation in androgen-dependent prostate cancer cells, where it binds to the rDNA promoter and stimulates rRNA transcription. ANG antagonists inhibit androgen-induced rRNA transcription and cell proliferation in androgen-dependent prostate cancer cells. Interestingly, ANG also mediates androgen-independent rRNA transcription through a mechanism that involves its constitutive nuclear translocation in androgen-insensitive prostate cancer cells, resulting in a constant rRNA overproduction and thereby stimulating cell proliferation. Critically, ANG overexpression in androgen-dependent prostate cancer cells enables castration-resistant growth of otherwise androgen-dependent cells. Thus, ANG-stimulated rRNA transcription is not only an essential component for androgen-dependent growth of prostate cancer but also contributes to the transition of prostate cancer from androgen-dependent to castration-resistant growth status.

Implications: The ability of angiogenin to regulate rRNA transcription and prostate cancer growth makes it a viable target for therapy.
Effector might mediate the effect of androgen-AR on rRNA transcription, as there has been no evidence that AR binds to rDNA. It is also conceivable that overexpression of this effector may result in overproduction of rRNA and promote castration-resistant growth of prostate cancer.

Angiogenin (ANG) was originally identified as an angiogenic molecule (11) and has been shown to play a role in tumor angiogenesis. Recent evidence indicates that ANG also directly stimulates cancer cell proliferation by enhancing rRNA transcription (12–16). ANG expression is upregulated in many types of cancers (17), particularly in prostate cancer (16, 18–21). Immunohistochemistry (IHC) studies have shown that ANG protein is dramatically enhanced in prostate cancer tissues as compared with normal prostate glands (21). ELISA analysis showed that ANG level in the blood is progressively upregulated in patients with prostate cancer when they transit from androgen-dependent to castration-resistant, hormone-refractory phenotype (19). Immunohistochemical analysis of ANG level in a large cohort of radical prostatectomy samples indicated that ANG expression is progressively upregulated in prostatic epithelial cells as they evolve from a benign to an invasive phenotype in the same patients (18). These results clearly showed that ANG expression is correlated to prostate malignancy.

The biologic activity of ANG is related to its ability to stimulate rRNA transcription. ANG is normally located in the extracellular matrix and plays a role in mediating cell adhesion (22). When cells have a high metabolic demand and need more ribosomes, ANG is taken up by the cells through receptor-mediated endocytosis (23) and is translocated to the nucleus. Upon reaching the nucleus, ANG accumulates in the nucleolus and stimulates rRNA transcription (24). Nuclear translocation of ANG is thus a metabolic requirement for sustained cell growth and is tightly controlled. However, the normally well-controlled nuclear translocation process of ANG is dysregulated in cancer cells to fulfill the higher metabolic requirement of these cells (15). In case of prostate cancer, ANG-mediated rRNA transcription has been shown to be essential for both establishment and maintenance of AKT-induced prostate intraepithelial neoplasia (PIN; refs. 13, 14), as well as for the growth of androgen-insensitive prostate cancer (14, 16, 20, 21, 25). We therefore examined the role of ANG in androgen-stimulated rRNA transcription and in the transition of prostate cancer to castration-resistant growth status. Our results show that ANG is responsible for rRNA transcription in the prostate cancer cells when they are stimulated to proliferate by androgen, indicating an essential role of ANG in prostate cancer growth. Moreover, overexpression of ANG promotes the transition of prostate cancer cells from androgen-dependent to castration-resistant growth status.

Materials and Methods

Cell culture, ELISA, immunofluorescence, Western blotting, and cell counting

RWPE-1 cells were purchased from American Type Culture Collection (ATCC) and were cultured in keratinocyte serum-free medium supplemented with 5 ng/mL EGF (Promega) and 0.05 mg/mL bovine pituitary extract (Sigma). LNCaP cells (ATCC) were cultured in RPMI-1640 supplemented with 10% FBS, PC-3, DU 145 (ATCC), and PC-3M (from Dr. Isaiah J. Fidler, MD Anderson Hospital Cancer Center, Houston, TX) were cultured in Dulbecco’s modified Eagle medium (DMEM) + 10% FBS. ANG secretion levels were determined by ELISA as described previously (26). To test the effect of dihydroxytestosterone (DHT) on nuclear translocation of endogenous ANG, cells were washed in phenol red–free medium containing charcoal/dextran–treated (steroid-stripped) FBS (Sigma), incubated in this steroid-free medium for 1 day, and then stimulated by 1 nmol/L DHT (Sigma) for 2 days. Immunofluorescence and Western blot analyses of nuclear ANG were conducted as described previously (26). ANG monoclonal antibody (mAb) 26–2F was used for immunofluorescence and polyclonal antibody (pAb) R112 was used for Western blot analysis. To examine the effect of exogenous ANG on cell proliferation, cells were prepared as described earlier in steroid-free medium and incubated with 0.1 μg/mL ANG or at the concentrations indicated. Medium was changed and fresh ANG was added every 2 days. Cells numbers were determined by MTS Cell Proliferation Assay (CellTiter 96 AQueous; Promega) or with a Coulter counter.

ANG underexpression and overexpression LNCaP cells

Transient ANG knockdown LNCaP cells were prepared using a plasmid encoding an ANG-specific short hairpin RNA (shRNA; 5′-GTTTCAGAAACGTTGTGA-3′) as previously described (15). Stable transfectants were selected with 0.5 μg/mL puromycin. ANG overexpression cells were prepared by transfecting LNCaP cells with a pcDNA-ANG containing the entire ANG-coding sequence with the signal peptide (15). Stable transfectants were selected with 2 mg/mL G418.

Northern blotting

RNAs were extracted with TRIzol (Invitrogen), separated on an agarose/formaldehyde gel, stained with ethidium bromide, and photographed to show the 18S rRNA level. The gel was then destained and transferred onto a nylon membrane. The probes used for the 47S rRNA (5′-GGAGCCGTTGTCGACGAC-3′) hybridizes with the first 25 nucleotides of the rRNA precursor. The probe used for actin (5′-GGAGCCGTTGTCGACGAGCAGCGCGGCG-3′) hybridizes with nucleotides 56 to 83 of the actin mRNA.

Chromatin immunoprecipitation

Parent and siRNA knockdown LNCaP cells were cultured in steroid-free medium and stimulated with 1 nmol/L DHT for 1 hour. ANG overexpression transfectants were cultured in steroid-free medium and was not stimulated with DHT. Cells were washed with PBS and cross-linked with 1% formaldehyde at 37°C for 10 minutes. After the cross-linking reaction was stopped by 125 mmol/L glycine, cells were collected, washed, and resuspended in the SDS lysis buffer (50 mmol/L Tris, pH 8.1, 10 mmol/L EDTA, and...
1% SDS) containing 1× protease inhibitor cocktail (Roche). The lysates were sonicated five times on a Branson Sonifier 450 with a microtip in 15-second bursts followed by 2 minutes of cooling on ice. Cell debris was cleared and the supernatant was diluted 5-fold in chromatin immunoprecipitation (ChIP) dilution buffer (16.7 mmol/L Tris, pH 8.1, 167 mmol/L NaCl, 1.2 mmol/L EDTA, 1.1% Triton X-100, and 0.01% SDS), followed by incubating with 80 μl of protein A-Sepharose slurry for 1 hour at 4°C with agitation. After a brief centrifugation, 10% of the total supernatant was put aside and one tenth of that material was used as input control. Of the 90% remaining supernatant, half was incubated with R112 polyclonal ANG antibody and the other half with a nonimmune rabbit immunoglobulin G (IgG) overnight at 4°C with rotation. Protein A-Sepharose (GE Healthcare Life Sciences) slurry (60 μL) was added to the sample and the reaction mixtures were incubated for another hour. The Sepharose beads were washed according to the manufacturer’s protocol (Upstate) and DNA fragments were purified with QIAquick Spin Kit (Qiagen). For PCR reactions, 10% of the immunoprecipitated materials were used as the DNA template in 35 cycles of amplification with the following primer sets. ABE1: forward, 5’-CCCTCGCTCGTTTCTTTC-3’; reverse, 5’-ACTGTGGCACGCCCTCGGT-3’. ABE2: forward, 5’-TATTGCTACTGGGCTAGGG-3’; reverse, 5’-AACACAGGAGGGAGA-3’. ABE3: forward, 5’-TCTTAC-TCTGTTCCTCTGGC-3’; reverse, 5’-AGAAACACCACGAAAGAG-3’. UCE: forward, 5’-GCTTGTCCTTGGGTGTCG-3’; reverse, 5’-GGGACAGCGTGTCAGCA-3’. CORE: forward, 5’-CGGGGGAGGTATATC-3’; reverse, 5’-AGACACCGCCGACGAGGAG-3’.

\[^{3}H\]-Uridine, \(^{3}H\)-thymidine, and \(^{3}H\)-leucine incorporation

Control (pCI-Neo) and ANG overexpression (pCI-ANG) LNCaP transfectants were seeded in a 24-well plate in a density of 1 × 10^5 cells/cm² and cultured in phenol red–free RPMI-1640 supplemented with 10% charcoal/dextran–treated FBS for 4 days. Cells were washed three times and incubated with fresh medium containing 1 μCi/mL \(^{3}H\)-uridine (PerkinElmer; NET367250UC), \(^{3}H\)-thymidine (PerkinElmer; NET027250UC), or \(^{3}H\)-leucine (PerkinElmer; NET46250UC) for 6 hours in the absence or presence of 5 μmol/L RAD001 (Novartis). At the end of incubation, the cells were washed three times with PBS, precipitated with 10% trichloroacetic acid (TCA), washed three times with acetone, and solubilized with 0.2 mol/L NaOH plus 0.3% SDS. After neutralization with one fifth volume of 1 mol/L HCl, the radioactivity was determined by liquid scintillation counting. Cell numbers were determined with a Coulter counter from parallel dishes cultured under the same conditions.

Castration

Severe combined immunodeficient mice (SCID) mice (The Jackson Laboratory), 5-week-old, were anesthetized and the surgical area was disinfected using Betadine and rinsed with 70% ethanol. An incision was made in the scrotum. Then, an incision was made in the tunica of the first testicle. The testis, vas deferens, and attached testicular fat pad were pulled out of the incision. The blood vessels supplying the testis were cauterized. The testis, vas deferens and fatty tissue were severed just below the site of the cauterization. The tunica on the contralateral side was similarly penetrated, and the procedure repeated. The scrotum incision was closed using wound clips. The mice were given 0.05 mg/kg buprenorphine s.c. as an analgesic once they began to move around slightly. The animals were allowed to recover from surgery for a week before being used in tumor inoculation experiments.

**Xenograft growth of LNCaP cells in SCID mice**

LNCaP cells were transfected with a control vector pCI-Neo or the ANG expression vector pCI-ANG. Stable transfectants were selected in the presence of 2 mg/mL G418 in complete medium and ANG secretion levels of the pooled transfectants were determined by ELISA. For tumor inoculation, a 0.1 mL of 50% Matrigel (BD Biosciences) containing 3 × 10^6 cells were injected subcutaneously into the lateral flank region of the mouse. Tumor size was measured weekly with a caliper and the volume was calculated as volume = length × width^2. The animals were sacrificed after 42 days and the tumors were removed and weighed.

**IHC**

Xenograft tumor tissue were fixed in formalin and embedded in paraffin. Sections of 4 μm were cut, hydrated, incubated for 30 minutes with 3% H₂O₂ in methanol at room temperature, washed with H₂O and PBS, and microwaved in 10 mmol/L citrate buffer, pH 6.0, for 10 minutes. Sections were then block in 5% nonfat dry milk in PBS for 30 minutes and incubated with human ANG mAb 26-2F at 1 μg/mL in 1% bovine serum albumin in PBS at 4°C for 16 hours. The DakoCytonisation EnVision System was used to visualize the signals.

**Results**

**Androgen stimulates nuclear translocation of ANG in LNCaP cells**

LNCaP cells express a gain-of-function AR and are responsive to androgen stimulation (27). If ANG mediates rRNA transcription of LNCaP cells in response to androgen stimulation, its nuclear translocation should be enhanced when cells are treated with androgen. This is indeed the case.

Nuclear ANG was detectable only in 9% ± 2% of untreated LNCaP cells (Fig. 1A, left). The percentage of nuclear ANG-positive cells increased to 76% ± 8% (Fig. 1A, middle, indicated by arrows) after treatment with DHT. Exogenous ANG (0.1 μg/mL) was added to the cells as a positive control (Fig. 1A, right), which resulted in a 93% ± 5% positive staining for nuclear ANG. The mAb 26-2F used in this study has a defined epitope recognition site and is specific only to human ANG (28). Western blot analysis confirmed that nuclear translocation of endogenous ANG in LNCaP cells is stimulated by DHT (Fig. 1B). Moreover, we found that
DHT promotes ANG transcription in LNCaP cells. Quantitative real-time PCR (qRT-PCR) analysis indicated that ANG mRNA level was increased by 1.5-fold after DHT treatment (Fig. 1C, top). Accordingly, ANG protein level in the medium (secreted ANG) increased from 0.54/C6 0.10 to 1.08/C6 0.19 pg/10^3 cells per day (Fig. 1C, bottom) after DHT treatment. Thus, androgen enhances the expression as well as nuclear translocation of ANG in LNCaP cells. However, antiandrogen has no effect on ANG expression in prostate cancer cells. As shown in Fig. 1C, treatment of LNCaP cells with flutamide did not change the mRNA and the protein levels of ANG. These results resemble the earlier findings that ANG expression in estrogen-dependent breast cancer is stimulated by estrogen but is not affected by antiestrogen (29). However, antiestrogen has been shown to inhibit nuclear translocation of ANG in endothelia cells (29) and regulates ANG-induced angiogenesis in vitro and in vivo (30). We have not observed any effect of androgen or antiandrogen on nuclear translocation of ANG in endothelial cells and on the angiogenic activity of ANG. Thus, although both androgen and estrogen seem to upregulate ANG in hormone-dependent prostate and breast cancer cells, respectively, the outcome of hormone ablation therapies in prostate and breast cancers might be different, partially due to the differential response of ANG to antiandrogen and to antiestrogen.

**Figure 1.** Effect of DHT on ANG. A, DHT stimulates nuclear translocation of ANG in LNCaP cells. Cells were cultured in phenol red-free RPMI-1640 medium + 10% charcoal/dextran-stripped FBS, and incubated in the absence (left) or presence of 1 nmol/L DHT (middle) or 0.1 µg/mL ANG (right) for 30 minutes. Cells were fixed and ANG localization was determined by immunofluorescence staining with ANG mAb 26-2F and Alexa Fluor 555-labeled goat F(ab')2 anti-mouse IgG. ANG-positive cells were counted in a total of 200 cells in five randomly selected areas. B, Western blot analysis of nuclear ANG. Nuclear proteins were extracted and analyzed by Western blotting (150 µg per lane) with ANG pAb R112. Histone H1 was used as a loading control. C, effect of androgen and antiandrogen on ANG expression. Cells were treated with DHT (1 nmol/L) or flutamide (1 µmol/L) for 2 days. ANG mRNA and secreted ANG protein were determined by qRT-PCR and ELISA, respectively. D, ANG binds to the promoter region of rDNA. Left, ChIP analyses of ANG binding to ABE1, ABE2, ABE3, UCE, and CORE regions of the rDNA promoter. PCR primers were designed using MacVector software. The input control in each panel contains 1% of the total DNA. Left, ImageJ analysis of the ChIP bands. *, P < 0.01; **, P < 0.001. Bottom, a schematic view of ANG occupancy in the rDNA promoter.
ANG mediates rRNA transcription in androgen-stimulated LNCaP cells

We next examined the role of endogenous ANG in rRNA synthesis in LNCaP cells. Northern blotting analysis was used to detect the steady-state level of 47S rRNA. Figure 2A shows that DHT and exogenous ANG enhanced rRNA transcription by 2.23- and 1.53-fold, respectively. Significantly, ANG mAb 26-2F inhibited basal and DHT-induced rRNA transcription by 33% and 67%, respectively. These data showed that ANG plays a role in both basal level and DHT-stimulated rRNA transcription in LNCaP cells. Because antibody does not enter the cells, the inhibitory effect of ANG mAb also indicates that it is necessary for ANG to be secreted first to mediate rRNA transcription, suggesting that ANG undergoes nuclear translocation via an autocrine or paracrine route.

Figure 2. ANG mediates DHT-stimulated rRNA transcription and cell proliferation. A, ANG stimulates, and ANG mAb inhibits, rRNA transcription in LNCaP cells. Top, Northern blotting analysis of 47S rRNA. Cells were cultured in phenol red–free medium and charcoal/dextran–stripped FBS and incubated with DHT (1 nmol/L), ANG (0.1 µg/mL), ANG mAb 26-2F (80 µg/mL), or a mixture of DHT and 26-2F for 2 hours. The level of 47S rRNA was determined by Northern blotting with a probe specific to the initiation site sequence of the rRNA precursor. Ethidium bromide staining of 18S rRNA and Northern blotting of actin mRNA were used as the loading controls. The bar graph at the bottom shows the relative intensity of 47S rRNA to actin mRNA determined by ImageJ. B, ANG stimulates LNCaP cell proliferation in the absence of androgens. Cells were culture in phenol red–free RPMI-1640 for another 2 days. The culture media were collected and PSA level was determined by ELISA. F, ANG mAb inhibits PC-3 cell proliferation. PC-3 cells were cultured in DMEM plus 10% charcoal/dextran–stripped FBS. The cells were treated with 0.1 µg/mL of ANG or 60 µg/mL of 26-2F for another 2 days. The culture media were collected and PSA level was determined by ELISA. F, ANG mAb inhibits PC-3 cell proliferation. PC-3 cells were cultured in DMEM plus 10% FBS in the presence of 30 µg/mL 26-2F or a control nonimmune IgG for the time indicated. **; P < 0.01; ***; P < 0.001.
ANG stimulates LNCaP cell proliferation in the absence of androgen

The findings that ANG stimulates rRNA transcription in the absence of androgen and mediates androgen-stimulated rRNA transcription in LNCaP cells prompted us to look at the effect of ANG on cell proliferation. LNCaP cells, which could not proliferate in steroid-free medium, were stimulated to proliferate by exogenous ANG (Fig. 2B) in a dose-dependent manner (Fig. 2C). DHT was used as a positive control for cell proliferation in these experiments. No additive or synergistic effect was observed when ANG and DHT were added simultaneously, indicating a functional overlap between ANG and DHT. ANG-stimulated rRNA transcription might present a mechanism by which prostate cancer cells bypass androgen-AR signaling pathway.

Endogenous ANG is required for androgen-stimulated LNCaP cell proliferation

The essential role of ANG in rRNA transcription in response to androgen-AR signaling suggested that ANG may be required for androgen to induce cell proliferation. Figure 2D shows that ANG mAb 26-2F inhibited DHT-induced proliferation of LNCaP cells in a dose-dependent manner, indicating that ANG is required for LNCaP cells to proliferate in response to androgen-AR signaling. Both exogenous ANG and ANG mAb did not alter prostate-specific antigen (PSA) expression in LNCaP cells (Fig. 2E), suggesting that AR-stimulated gene transcription is not regulated by ANG. ANG mAb also inhibited proliferation of androgen-independent PC-3 cells (Fig. 2F). These results show that ANG is essential for cell growth of both androgen-dependent and castration-resistant prostate cancer.

ANG siRNA inhibits DHT-stimulated rRNA transcription and proliferation of LNCaP cells

To confirm the role of endogenous ANG in rRNA transcription, we used a plasmid-mediated siRNA (15) to knockdown ANG expression in LNCaP cells and examined the resultant changes in rRNA transcription. ELISA analysis indicated that the amount of secreted ANG in control and ANG-specific siRNA-transfected cells was 0.55 ± 0.1 and 0.12 ± 0.05 pg/10^3 cells per day (Fig. 3A), representing a 79% knockdown efficiency. ANG knockdown is accompanied by a decrease in DHT-stimulated nuclear translocation of ANG (Fig. 3B). Only 12% ± 5% of the ANG knockdown cells were positive for nuclear ANG upon DHT stimulation (Fig. 3B, middle), which is 6.3-fold lower than that in control LNCaP cells (76% ± 8%; Fig 1A). The percentage of cells with a positive nuclear ANG in the presence of exogenous ANG was 90% ± 7% (Fig. 3B, right), which is not significantly different from that of the parent cells (93% ± 5%; Fig. 1A, right), indicating that the ability of the cells to uptake exogenous ANG was not altered after endogenous ANG was knocked down. A decrease in nuclear ANG resulted in a lower occupancy of ANG at rDNA promoters (Fig. 3C), and is accompanied with a reduced capacity of the cells to respond to DHT-stimulated rRNA transcription (Fig. 3D). ANG occupancy in the UCE was not detectable in ANG knockdown cells and that at AB1 and AB2 was 0.35% and 1.38% (Fig. 1C), which is 7.7- and 13.3-fold lower than that in control cells (Fig. 1D). Knockdown of ANG completely abolished the activity of DHT in stimulating rRNA transcription (Fig. 3D). DHT was able to stimulate transcription of 47S rRNA in cells treated with a control siRNA but failed to do so in cells transfected with an ANG-specific siRNA (Fig. 3D). The reasons that the basal transcription of rRNA in unstimulated cells did not decrease in ANG knockdown cells (Fig. 3D, lanes 1 and 3) could be due to the remaining 21% of the ANG after siRNA treatment. This amount of ANG might suffice in maintaining the basal transcription but might be inadequate to meet the metabolic demand imposed by DHT treatment. The efficiency of the mAb in inhibiting the biologic activity of ANG was thus better than that of siRNA. The concentration of the antibody used in these experiments was 60 μg/mL, which was at least 100-fold higher than the maximum possible concentration of endogenous ANG secreted from the cells. It is conceivable that all the secreted ANG could be potentially neutralized by the antibody. However, 21% of the endogenous ANG remained in the siRNA knockdown cells. The discrepancy seen between ANG mAb and siRNA is most likely caused by the difference in the remaining ANG activities in the two systems. These results confirmed that ANG is necessary for enhanced rRNA transcription in LNCaP cells when they are stimulated to grow by androgen. Consistently, DHT is no longer able to stimulate proliferation in ANG knockdown LNCaP cells (Fig. 3E), further showing that ANG is necessary for the growth-stimulatory function of androgens.

Exogenous ANG has no effect on androgen-independent prostate cancer cells

The finding that ANG stimulates LNCaP cell proliferation in the absence of androgen, led us to hypothesize that upregulation of ANG contributes to the development of androgen-independent prostate cancer. To test this hypothesis, we first examined the effect of exogenous ANG on cell proliferation of androgen-independent prostate cancer cells including PC-3, PC-3M, and DU145. We found that exogenous ANG had no effect on proliferation of these cells (data not shown). Because endogenous ANG has already been shown to play a role in PC-3 cell proliferation (Fig. 2F; ref. 16), a plausible explanation would be that these androgen-independent cells already express adequate amount of ANG and that their nuclei have already been saturated by endogenous ANG. We therefore measured ANG expression by ELISA in these cells as well as in the normal prostate epithelial cells (RWPE-1 and PrEC). Indeed, androgen-independent PC-3, PC-3M, and DU145 cells secreted more ANG than did androgen-dependent LNCaP cells (Fig. 4A). Moreover, all four prostate cancer cell lines secreted a significantly higher amount of ANG than did the normal prostate epithelial cells RWPE-1 and PrEC.
ANG is constitutively translocated to the nucleus of androgen-independent prostate cancer cells

We next examined nuclear translocation of ANG in those cells in the presence or absence of androgen. ANG was found to be constitutively translocated to the nucleolus of the three types of androgen-independent cells both in the absence and presence of DHT (Fig. 4B). Western blot analysis (Fig. 4C) showed that in PC-3, PC-3M, and DU145 cells, ANG protein was detected in the nuclear proteins extracted from cells cultured in the absence (top) or presence (bottom) of androgen. LNCaP cells were included in this experiment as a control for androgen-stimulated nuclear translocation of ANG, and the results confirmed the finding in Fig. 1A that androgen stimulates nuclear translocation of ANG in LNCaP cells. Taken together, these results support the hypothesis that upregulation of ANG and constitutive nuclear translocation is associated with castration-resistant growth status of prostate cancer cells. Consistently, we found that ANG expression in LNCaP cells was upregulated in prolonged culture under hormone- and steroid-free culture (Fig. 4D). The level of secreted ANG in LNCaP cells cultured in phenol red–free, charcoal/dextran–treated FBS was 0.75 ± 0.15 pg per10^3 cells per day on day 21, which is 47% higher than that on day 7 (0.51 ± 0.05 pg/10^3 cells/day; Fig. 4D).

Overexpression of ANG stimulates castration-resistant growth of LNCaP cells

To determine whether upregulation of ANG is correlated with the development of castration resistance of prostate cancer, we examined the effect of ANG overexpression on LNCaP cell proliferation in the absence of androgens. ANG expression vector pCI-ANG that carries the human ANG cDNA under cytomegalovirus (CMV) promoter and the
control vector pCI-Neo were transfected into LNCaP cells and stable transfectants expressing 6.6-times higher ANG were selected (Fig. 5A, left) and were shown to have increased nuclear accumulation of endogenous ANG (Fig. 5A, right, indicated with arrows). The percentage of positive cells for nuclear ANG increased from 4% in pCI-Neo vector control transfectants to 98% in pCI-ANG transfectants in the absence of DHT. ANG overexpression stimulated LNCaP proliferation in culture in the absence of androgen (Fig. 5B).

Consistently, rDNA promoter occupancy by ANG was significantly enhanced in ANG overexpression cells (Fig. 5C, left). ChIP analyses indicate that the percentage of ANG-bound ABE1, ABE2, and UCE was 8.6% ± 0.8%, 8.2% ± 0.2%, and 12.2% ± 0.14%, respectively (Fig. 5C, right), which is similar to the promoter occupancy in DHT-stimulated parent LNCaP cells (Fig. 1D). 3H-uridine incorporation experiments indicated that ANG overexpression cells have enhanced rRNA synthesis rate as compared with that of vector control (Fig. 5D). DNA and protein synthesis rates were concurrently enhanced in ANG overexpression cells as shown by 3H-thymidine and 3H-leucine incorporation experiment (Fig. 5D, left). RAD001, a water-soluble analog of rapamycin, inhibited ANG-induced protein and DNA synthesis but not RNA synthesis (Fig. 5D, right). These results suggest that ANG-stimulated RNA transcription is mTOR-insensitive but that of DNA synthesis and protein translation require the mTOR activity. This is consistent with our hypothesis that ANG-mediated rRNA transcription and mTOR-mediated ribosomal protein synthesis are both necessary for cell proliferation.

When these cells were inoculated into SCID mice, both the vector and ANG transfectants were able to establish tumors with a 100% tumor take rate in uncastrated mice (n = 6). It is noticeable that the tumor growth rate in animals inoculated with ANG transfectants was much greater than in those inoculated with the vector control transfectants (Fig. 6A). Consistently, the tumor weight derived from ANG overexpression cells (0.62 ± 0.35 g) was 3.6-times bigger than that derived from the vector transfectants.

**Figure 4.** Constant nuclear translocation of ANG in androgen-insensitive prostate cancer cells. A, upregulation of ANG in human prostate cancer cells. Secreted ANG proteins in normal human prostate epithelial cells and prostate cancer cells were determined by ELISA. B, nuclear translocation of ANG in prostate cancer cells. LNCaP, PC-3, PC-3M, and DU145 cells were cultured in their respective media supplemented with 10% FBS for 2 days. FBS was then replaced with charcoal/dextran-stripped serum and the cells were cultured in the absence or presence of DHT (1 nmol/L) for 2 days. Immunofluorescence of ANG was conducted with ANG mAb 26-2F (50 μg/mL) and Alexa Fluor 488-labeled goat F(ab')2 anti-mouse IgG (1:100 dilution) as described in the legend to Fig. 1A. Nucleolar ANG was indicated by arrows. C, Western blot analysis of nuclear ANG. Nuclear proteins were extracted from the cells cultured in the absence (top two) and presence (bottom two) of DHT and analyzed by Western blotting (150 μg per lane) with ANG pAb R112. Histone H1 was used as a loading control. D, ANG expression is upregulated in ANG knockdown LNCaP cells after prolonged culture in steroid-free medium. Control and ANG siRNA transfectants were continuously cultured in steroid-free medium for 1 or 3 weeks without subculture. Medium was changed every 2 days. ANG secreted into the medium between day 6 and 7, and between day 20 and 21 was determined by ELISA. Cell number was determined from parallel dishes by a Coulter counter. **, P < 0.001.
To determine whether ANG overexpression promotes androgen-independent proliferation in vivo, these transfectants were inoculated into castrated SCID mice. None of the animals (n = 6) inoculated with vector transfectants had palpable tumors in castrated mice, whereas 5 of 6 castrated mice developed tumors when inoculated with ANG transfectants, indicating that ANG overexpression permits LNCaP tumor establishment in the absence of androgen. We noted that the tumor growth rate in castrated mice was slower than that in uncastrated mice (Fig. 6A), and that the tumor weight from the castrated animals (0.14 ± 0.95 g) was lower than that from the uncastrated animal (0.62 ± 0.35; Fig. 6B), indicating that androgen still has an effect on in vivo growth of ANG transfectants. IHC shows that ANG expression and localization in the tumors derived from ANG transfectants grown in uncastrated (Fig. 6C, left) and castrated (Fig. 6C, center) mice are indistinguishable. Both nuclear (arrows) and extracellular ANG (stars) were prominent and much stronger than that in the tumor tissues derived from vector control transfectants grown in uncastrated mice (Fig. 6C, right). These results indicate that ANG overexpression results in constant nuclear translocation of ANG in LNCaP cells even in castrated mice. They also indicate that ANG overexpression enables LNCaP cells to proliferate in the absence of androgen both in vitro and in vivo, suggesting that overexpression of ANG contributes to the transition of prostate cancer to castration resistance. These results, together with the finding that ANG is enhanced in the nucleus of androgen-independent cells, suggest that upregulation and enhanced nuclear translocation of ANG will result in an excessive supply of rRNA, which may contribute to the development of castration resistance.
Discussion

There are two major findings of this article. The first is that ANG mediates DHT-stimulated rRNA transcription in androgen-sensitive LNCaP cells. The second is that ANG overexpression permits LNCaP cell growth in the absence of androgen. These results indicate that ANG is a critical player in the growth of both androgen-dependent and castration-resistant prostate cancer. More importantly, these results also indicate that ANG plays an active role in the transition of prostate cancer from androgen-dependent to castration-resistant growth status.

Transition to castration-resistant growth is a life-threatening development of prostate cancer. The median survival of castration-resistant prostate cancer is 10 to 20 months (33). Recent advancements in chemotherapy have provided only palliative but not survival benefit (34, 35). A major challenge in developing effective therapeutics is the heterogeneous genetic, epigenetic, and cellular pathogenesis of prostate cancer (36, 37). For examples, constitutive AR function (1), AKT activation and PTEN loss (38), aberrant activation of multiple growth factor signaling pathways (36), upregulation of the antiapoptotic protein Bcl-2 (39), loss of cell-cycle regulatory proteins (40), and epigenetic alterations (37) have all been identified as prostate cancer etiology. Therefore, targeting multiple pathways involved in cancer development and progression are thought to provide better chances for success (41). An alternative approach would be to identify a downstream target that is common to multiple upstream pathways. The results we present in this article have identified ANG-mediated rRNA transcription as such a target.

rRNA is essential for ribosome biogenesis that is critical for protein translation (42). Ribosomes have been traditionally considered as merely having house-keeping functions (43). In fact, ribosomes are essential for correctly and efficiently producing all proteins in the cells and their abnormality has been associated to cancers (44). For cancer cells, ribosome biogenesis needs to be enhanced to meet the high metabolic...
and transcriptional activity of AR requires the participation of ANG in the absence of androgen and whether ANG is able to stimulate nuclear translocation of ANG in LNCaP cells (Fig. 1A), but also upregulates ANG expression (Fig. 1C). We also show that nuclear ANG binds to both ABE and UCE at the promoter region of DNA (Fig. 1D) and promotes RNA transcription (Fig. 2A). These results indicate that in androgen-dependent prostate cancer cells, it is ANG that stimulates rRNA transcription so that ribosome biogenesis can take place.

Thus, ANG is a permissive factor for DHT to induce cell proliferation. This contention has been proven by the findings that both ANG mAb (Fig. 2A) and siRNA (Fig. 3D) inhibit DHT-stimulated rRNA transcription in LNCaP cells. Consistently, both mAb and siRNA of ANG inhibit DHT-stimulated LNCaP cell proliferation (Fig. 2D and E).

Another significant finding of this article is that ANG is a contributing factor for the development of castration-resistant prostate cancer. We have found that ANG overexpression enable androgen-independent growth of otherwise androgen-dependent LNCaP cells both in vitro (Fig. 5B) and in vivo (Fig. 6A). These results strongly suggest that elevated level of ANG contributes to the transition of prostate cancer from androgen-dependent growth to castration-resistant growth. It is unclear at present whether or not AR continues to play a role in ANG-stimulated growth of castration-resistant prostate cancer. We are currently investigating whether ANG is able to stimulate nuclear translocation of AR in the absence of androgen and whether the transcriptional activity of AR requires the participation of ANG action. A more in-depth understanding of the crosstalk between the ANG pathway and the AR pathway will likely reveal the mechanism by which ANG overexpression stimulates androgen-independent growth of prostate cancer. Nevertheless, the present evidence has clearly shown that upregulation of ANG is able to promote androgen-independent growth of androgen-sensitive prostate cancer cells. Thus, the progressive upregulation of ANG observed in prostate cancer (18) may not only be a passive response of the cells to an increased metabolic demand, but also an active participant in the development and progression of this disease.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: G.-F. Hu
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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Ibaragi, G. Sun, K. Kishimoto, G.-F. Hu
Writing, review, and/or revision of the manuscript: S. Ibaragi, J. Sheng, G.-F. Hu
Administrative, technical, or material support (i.e., reporting and organizing data, constructing databases): M.G. Hu, N. Yoshioka
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