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Transgelin Functions as a Suppressor via Inhibition of ARA54-Enhanced Androgen Receptor Transactivation and Prostate Cancer Cell Growth

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The androgen receptor (AR) requires coregulators for its optimal function. However, whether AR coregulators further need interacting protein(s) for their proper function remains unclear. Here we describe transgelin as the first ARA54-associated negative modulator for AR. Transgelin suppressed ARA54-enhanced AR function in ARA54-positive, but not in ARA54-negative, cells. Transgelin suppressed AR transactivation via interruption of ARA54 homodimerization and AR-ARA54 heterodimerization, resulting in the cytoplasmic retention of AR and ARA54. Stable transfection of transgelin in LNCaP cells suppressed AR-mediated cell growth and prostate-specific antigen expression, whereas this suppressive effect was abolished by the addition of ARA54-small interfering RNA. Results from tissue surveys showing decreased expression of transgelin in prostate cancer specimens further strengthened the suppressor role of transgelin. Our findings reveal the novel mechanisms of how transgelin functions as a suppressor to inhibit prostate cancer cell growth. They also demonstrate that AR coregulators, like ARA54, might have dual *in vivo* roles functioning as both a direct coactivator and as an indirect mediator in AR function. The finding that a protein can modulate AR function without direct interaction with AR might provide a new therapeutic approach, with fewer side effects, to battle prostate cancer by targeting AR indirectly. (*Molecular Endocrinology* 21: 343–358, 2007)

PROSTATE CANCER IS the most frequently diagnosed malignancy in aging males, and each year about 31,500 men in the United States lose their lives because of this malignancy (1). Androgen and the androgen receptor (AR) play pivotal roles in the progression of prostate cancer. Surgical or chemical castration in combination with antiandrogens, known as complete androgen blockade, has been widely used in the treatment of this disease (2, 3). However, androgen ablation therapy eventually fails, and prostate cancer progresses to a hormone-refractory state in spite of consistent AR expression in the majority of patients in this androgen-independent stage (4–8). Dissecting the precise molecular mechanisms of how AR signaling is regulated and how it contributes to the prostate cancer progression, therefore, might be extremely helpful in battling this disease.

AR is a ligand-dependent transcription factor that belongs to the superfamily of nuclear receptors (NRs) (9, 10). The proper function of AR requires coregulators for its optimal signaling (11, 12). Several AR coregulators, including the cAMP response element-binding protein (CREB)-binding protein (CBP), steroid receptor coactivator 1 (SRC-1), ARA54, ARA55, ARA67/PAT1, ARA70, hRad9, and phosphatase and tensin homolog deleted on chromosome 10 (PTEN) have been identified, and their potential pathophysiological roles in the prostate cancer progression have been studied (13–27). ARA54 enhances AR function in a ligand-dependent manner, and coexpression of ARA54 with other AR coactivators like SRC-1 or ARA70 additively enhances AR function (17). Our previous study showed that a dominant-negative mutant of ARA54 suppresses the AR-mediated LNCaP cell

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Abbreviations: AR, Androgen receptor; CBP, CREB binding protein; CDFBS, charcoal-dextran-stripped FBS; CT, threshold cycle; DTT, dithiothreitol; DHT, 5α-dihydrotestosterone; E2, 17β-estradiol; FBS, fetal bovine serum; GST, glutathione-*S*-transferase; HF, hydroxyflutamide; MMTV-LUC, mouse mammary tumor virus luciferase; mtART877A, mutant AR derived from prostate cancers, codon 877 mutation threonine to alanine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NR, nuclear receptor; nt, nucleotide; PMSF, phenylmethylsulfonylfluoride; PSA, prostate-specific antigen; SD, synthetic dropout; siRNA, small interfering RNA; SRC-1, steroid receptor coactivator-1.

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growth and the expression of AR target gene, prostate-specific antigen (PSA) (28).

The detailed mechanisms of the AR transcriptional machinery remain to be further elucidated. It is still unclear whether these AR coregulators need interacting proteins to modulate their coregulator activity. Transgelin, also named SM22α, was first isolated from chicken gizzard (29) as a transformation- and shape change-sensitive actin-binding protein, the expression of which was lost in virally transformed cells (30, 31). Recently, Shields *et al.* (32) characterized transgelin as a gene, the expression of which was abolished by Ras, and loss of transgelin expression might represent an early event for the tumor progression in breast and colon cancers. Here we describe the identification of transgelin as a potential prostate cancer suppressor via inhibition of ARA54-enhanced AR transactivation and prostate cancer cell growth.

RESULTS

Identification of Transgelin as an ARA54 Interacting Protein

Full-length ARA54 was used as bait to screen its associated proteins from the human prostate cDNA library using the CLONTECH matchmaker yeast two-hybrid system. Full-length cDNA of three positive clones was then amplified and cloned into the pSG5 expression vector for further characterization. Sequence analysis found that one of the candidate clones matched the transgelin sequence, and its interaction with ARA54 was further confirmed in the yeast growth assay and colony lift assay for β-galactosidase on -3 synthetic dropout (SD) nutrition selection plates (data not shown), as well as mammalian two-hybrid assay. Figure 1A shows ARA54 interacted with transgelin in 3T3-L1 and COS-1 mammalian cells in the presence or absence of 5α-dihydrotestosterone (DHT) (lane 5). In contrast, transgelin showed little interaction with AR (lane 6). The interaction between ARA54 and transgelin was also confirmed using glutathione-*S*-transferase (GST) pull-down assay. As shown in Fig. 1B, in the presence or absence of DHT, the *in vitro* translated [^{35}S]methionine-labeled transgelin was pulled down by GST-ARA54 fusion protein, but not by GST alone. To further confirm their *in vivo* interaction, we demonstrated that endogenous transgelin in LNCaP cells could be coimmunoprecipitated with endogenous ARA54 using an anti-ARA54 antibody (Fig. 1C). Together, results from mammalian two-hybrid assay, GST pull-down assay, and coimmunoprecipitation assay all demonstrated that ARA54 could interact with transgelin both *in vitro* and *in vivo* in an androgen-independent manner. In contrast, transgelin showed little interaction with AR in the mammalian two-hybrid assay system.

Transgelin Suppressed the ARA54-Enhanced AR Transactivation in Mammalian Cells

We then tested the effect of transgelin on ARA54-enhanced AR transactivation. In 3T3-L1, which is an ARA54-negative cell line (see Fig. 3A), addition of ARA54 can further enhance AR transactivation in the presence of 10 nM DHT (Fig. 2A, lanes 5 and 9 *vs.* 2). The ratio of ARA54 to AR we used here was 3:1 to 4:1, which is the usual optimal ratio of coactivators to AR. This ARA54-enhanced optimal AR transactivation was suppressed after addition of transgelin (Fig. 2A, lanes 6, 7, and 8 *vs.* 5, lanes 10, 11, and 12 *vs.* 9). Similar suppression effects were also observed when we replaced 3T3-L1 cells with another ARA54 negative COS-1 cell line (33) (Fig. 2A). We also tested the suppressive effect of transgelin on the ARA54-enhanced mtART877A (mutant AR derived from prostate cancers, codon 877 mutated from threonine to alanine) transactivation in the presence of hydroxyflutamide (HF) or 17β-estradiol (E2). As shown in Fig. 2B, lanes 13 and 14 *vs.* 12, addition of transgelin could effectively suppress the ARA54-enhanced mtART877A transactivation induced by E2 or HF in COS-1 cells. We further tested the effect of transgelin on the AR transactivation enhanced by other AR coregulators such as the ARA70, ARA55, SRC-1, supervillin, gelsolin, and CBP. As shown in Fig. 2C, transgelin showed little effect on the AR transactivation enhanced by these coregulators except for ARA54. These data suggested that transgelin might function as a relatively specific modulator for ARA54.

ARA54 Is Essential for Mediating Transgelin's Suppressive Effect

Because transgelin showed little interaction with AR (Fig. 1A), we hypothesized that transgelin might need to interact with ARA54 to exert the suppressive effect on AR transactivation. As shown in Fig. 2A, lanes 3 and 4, addition of transgelin in the absence of ARA54 showed little suppression of AR transactivation. We further confirmed this result by comparing transgelin's effect on AR transactivation in both ARA54-positive LNCaP cells and ARA54-negative 3T3-L1 cells. Figure 3A showed the detection of endogenous ARA54 using Western blot in LNCaP but not in 3T3-L1 cells. We found that transgelin could significantly repress the AR transactivation in the ARA54-positive LNCaP cells (Fig. 3B) but had only a marginal effect in the ARA54-negative 3T3-L1 cells (Fig. 3C). Retrovirus-delivered ARA54-small interfering RNA (siRNA) constructs against ARA54 were generated to silence the endogenous ARA54 expression in LNCaP cells, as shown by Western blotting assay (Fig. 3D). The suppressive effect of transgelin was abolished after the silencing of endogenous ARA54 (Fig. 3E, *upper panel vs. lower panel*). In addition, the suppression ability of transgelin in ARA54-mediated AR transactivation activity might not be correlated to the actin-binding feature of trans-

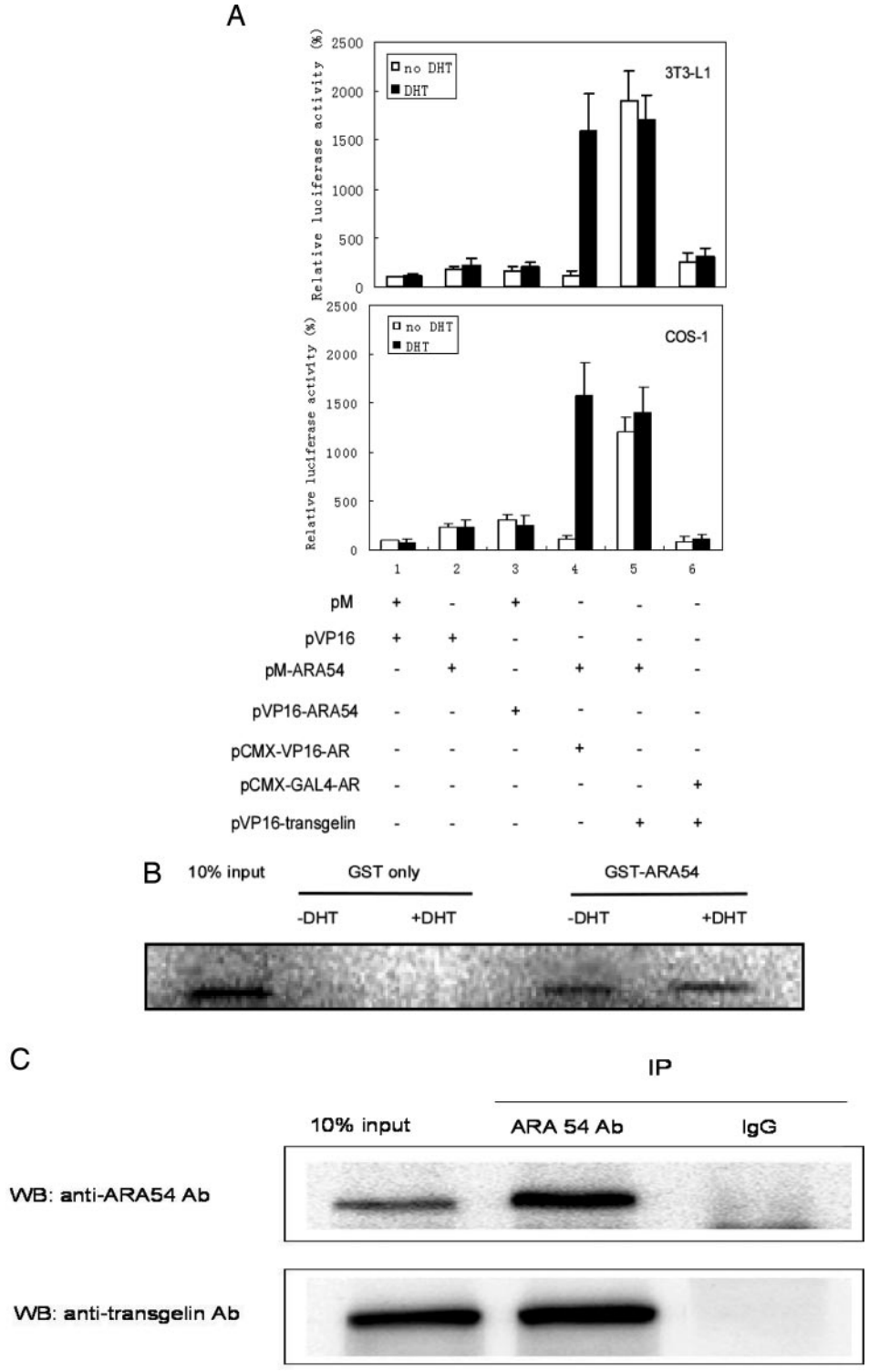

Fig. 1. ARA54 Interacts with Transgelin *in Vitro* and *in Vivo*

A, Mammalian two-hybrid assay: 3T3-L1 and COS-1 cells cultured in 24-well plate were cotransfected with 0.5 μg pVP16, pM, pM-ARA54, pVP16-ARA54, pCMX-GAL4-AR, pCMX-VP16-AR, and pVP16-transgelin as indicated, together with 0.4 μg pG5-LUC reporter plasmid and 0.5 ng pRL-TK internal control plasmid. The luciferase activity of the interaction between pM and pVP16 without ethanol treatment (lane 1) was set as 100%. All values represent the mean ± SD of three independent experiments. B, ARA54 interacts with transgelin *in vitro*. GST pull-down assay was performed with purified GST control protein and GST-ARA54 fusion protein with 5 μl of [^{35}S]methionine-labeled full-length transgelin in the presence and absence of 10 nM DHT. C, Transgelin coimmunoprecipitates with ARA54. LNCaP cells were cultured in RPMI-1640 with 10% non-heat-inactivated FBS. Anti-ARA54 antibody or IgG was added to 500 μg of LNCaP cell lysate for 4 h at 4 C to immunoprecipitate the endogenous ARA54 and transgelin complex. IP, Immunoprecipitation; WB, Western blot.

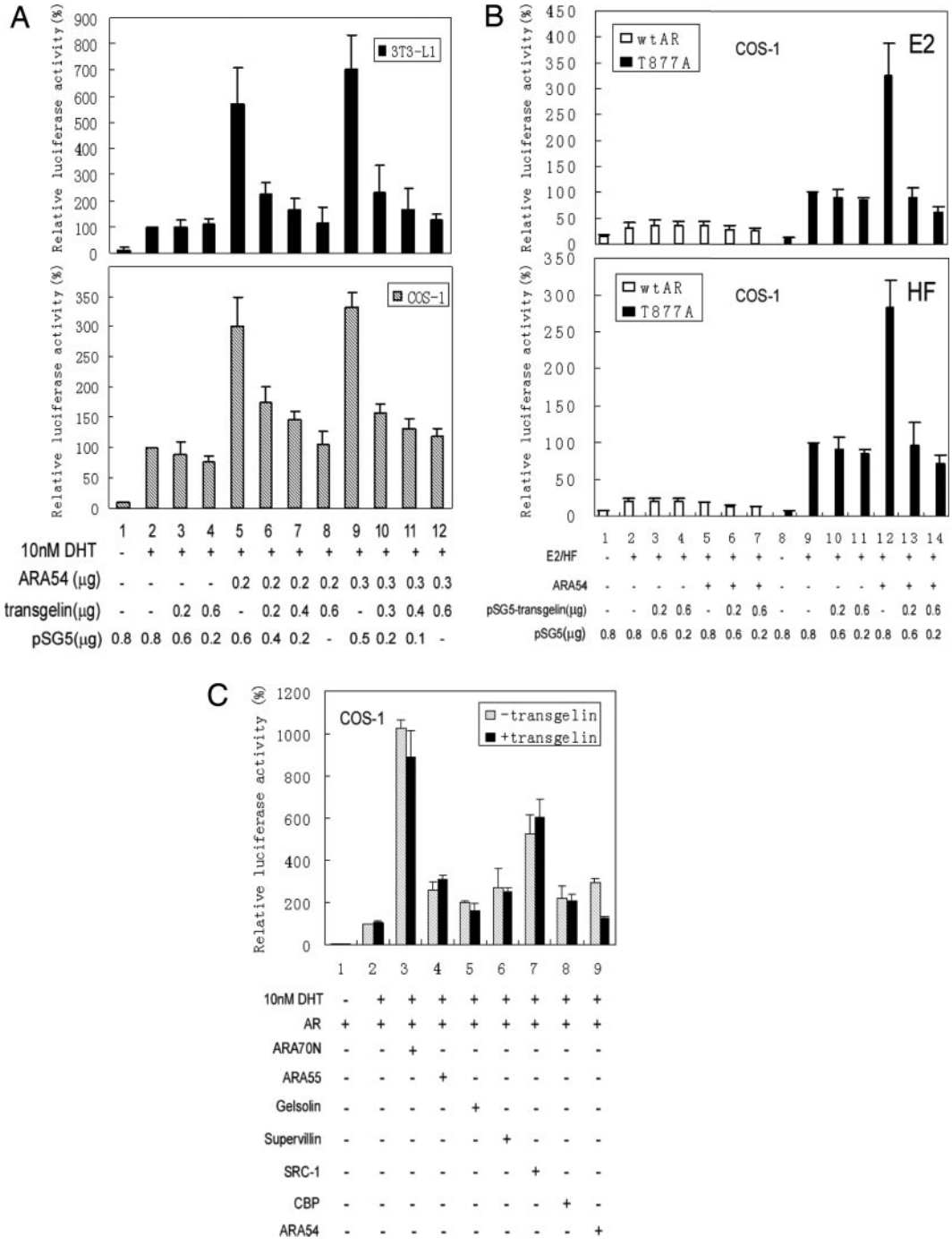

Fig. 2. Transgelin Selectively Suppressed ARA54-Enhanced AR Transactivation

A, 3T3-L1 and COS-1 cells cultured in 24-well plates were cotransfected with 0.075 μg pSG5-AR and 0.2 or 0.3 μg pSG5-ARA54, together with different doses of pSG5-transgelin as indicated. MMTV-LUC (0.225 μg) was used as reporter plasmid and 5 ng of pRL-TK as the internal control. The luciferase activity in the presence of 10 nM DHT without ARA54 or transgelin (lane 2) was set as 100%. Values represent the mean ± SD of at least three determinations. B, COS-1 cells were cotransfected with 0.225 μg MMTV-LUC, 0.075 μg pCMV-mtART877A or pSG5-AR (wild-type AR), 0.2 μg pSG5-ARA54, and different doses of pSG5-transgelin as indicated. After transfection, cells were cultured for an additional 24 h in the presence or absence of 10 nM E2 or 10 μM HF. The luciferase activity is presented relative to that of pCMV-mtART877A with E2 or HF (lane 2), respectively. C, COS-1 cells were transfected with 0.225 μg MMTV-LUC, 0.075 μg pSG5-AR, 0.3 μg of each AR coactivator (ARA70N, SRC-1, gelsolin, supervillin, ARA55, CBP, or ARA54), and 0.4 μg pSG5-transgelin. The luciferase activity is presented relative to that of vector alone with 10 nM DHT in the absence of transgelin (lane 2). Values represent the mean ± SD of at least three determinations.

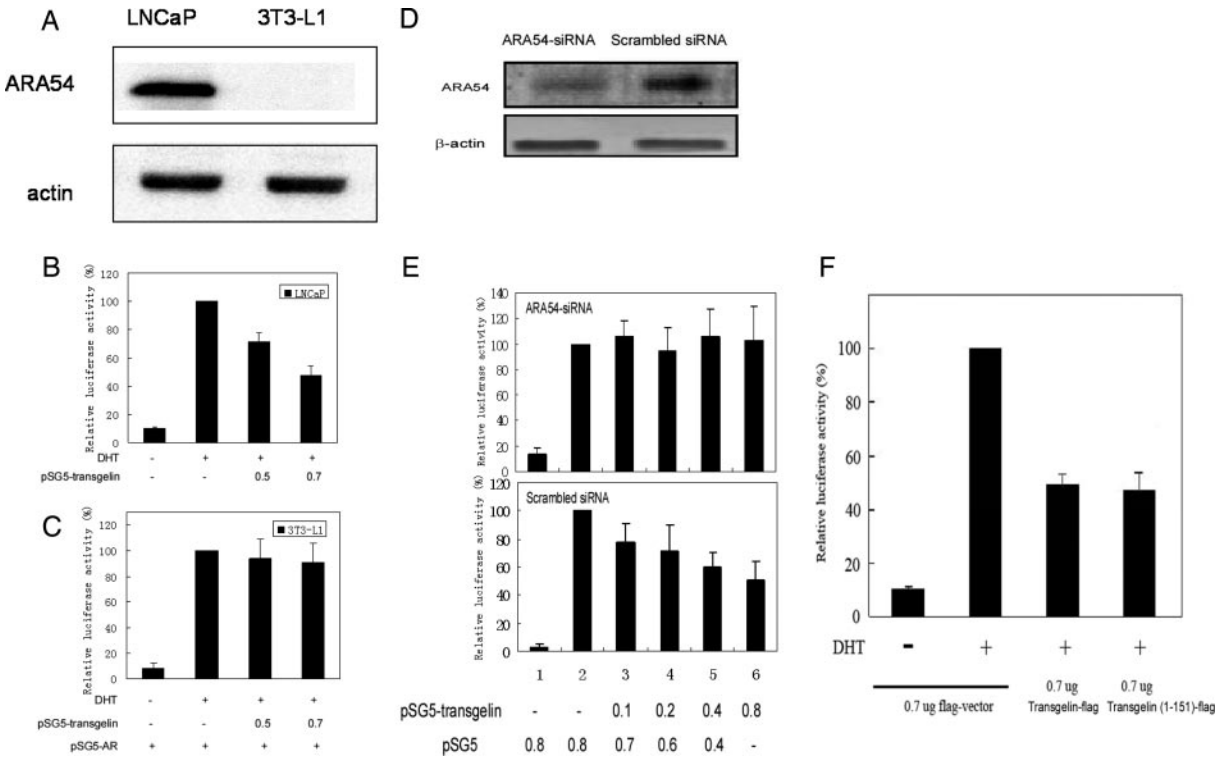

Fig. 3. ARA54 Is Essential for Transgelin's Suppressive Effect on AR Transactivation

A, Western blotting data show the endogenous ARA54 level in LNCaP and 3T3-L1 cells. B, LNCaP cells seeded in 24-well plates were cotransfected with 0.225 μg of MMTV-LUC reporter plasmid, 5 ng pRL-TK internal control plasmid, as well as different doses of pSG5-transgelin as indicated. C, 3T3-L1 cells seeded in 24-well plates were cotransfected with 0.225 μg of MMTV-LUC reporter plasmid, 5 ng pRL-TK internal control plasmid, and 0.075 μg pSG5-AR, as well as different doses of pSG5-transgelin as indicated. The luciferase activity with DHT treatment and vector alone (lane 2) was set as 100%. All values represent the mean ± SD of three independent experiments. D, Western blotting data show the endogenous ARA54 level in ARA54-siRNA and scrambled siRNA-stably transfected LNCaP cells. E, ARA54-siRNA and scrambled siRNA-stably transfected LNCaP cells seeded in 24-well plates were cotransfected with 0.225 μg MMTV-LUC reporter plasmid, and 5 ng pRL-TK internal control plasmid, as well as different doses of pSG5-transgelin as indicated. The luciferase activity with DHT treatment and vector alone (lane 2) was set as 100%. All values represent the mean ± SD of three independent experiments. F, LNCaP cells seeded in 24-well plates were cotransfected with 0.225 μg of MMTV-LUC reporter plasmid, 5 ng pRL-TK internal control plasmid, and 0.7 μg wild-type transgelin, truncated form transgelin (1–151), or vector control as indicated. The luciferase activity with DHT treatment was set as 100%. All values represent the mean ± SD of three independent experiments.

gelin because truncated transgelin with defective actin binding could still suppress the AR transactivation (Fig. 3F). Together, results from Fig. 3, A–E, clearly demonstrated that transgelin might need the interaction with ARA54 to suppress AR transactivation. Silencing of endogenous ARA54 via its siRNA abolished transgelin's suppressive effect on AR function, which further confirmed the essential role of ARA54 as a mediator for transgelin's suppressive function.

Transgelin Blocked the Interaction between AR and ARA54 as Well as the ARA54 Homodimerization

To dissect the possible molecular mechanisms of how transgelin suppresses ARA54-enhanced AR transactivation, we applied mammalian two-hybrid assay to test whether transgelin has any effect on the interaction between AR and ARA54 as well as ARA54 homodimerization. In Fig. 4A, cotransfection of pcDNA3-Flag-transgelin significantly suppressed the homodimerization interaction between pM-ARA54 and pVP16-ARA54 (lanes 3, 4, and 5 *vs.* 2). Androgen-dependent interaction between ARA54 and AR was also suppressed by the addition of transgelin in a dose-dependent manner (lanes 8, 9, and 10 *vs.* 7). As control, the addition of transgelin showed little influence on the interaction between pVP16-T and pM-53 (lanes 11–14), indicating the effect of transgelin is not the result of squelching effect on transcription. These results suggested transgelin might be able to suppress ARA54-enhanced AR transactivation via interruption of interaction between ARA54 and AR as well as ARA54 homodimerization.

Transgelin Influences the Subcellular Distribution of ARA54 and AR

AR can translocate from the cytoplasm into the nucleus upon androgen binding, resulting in the activa-

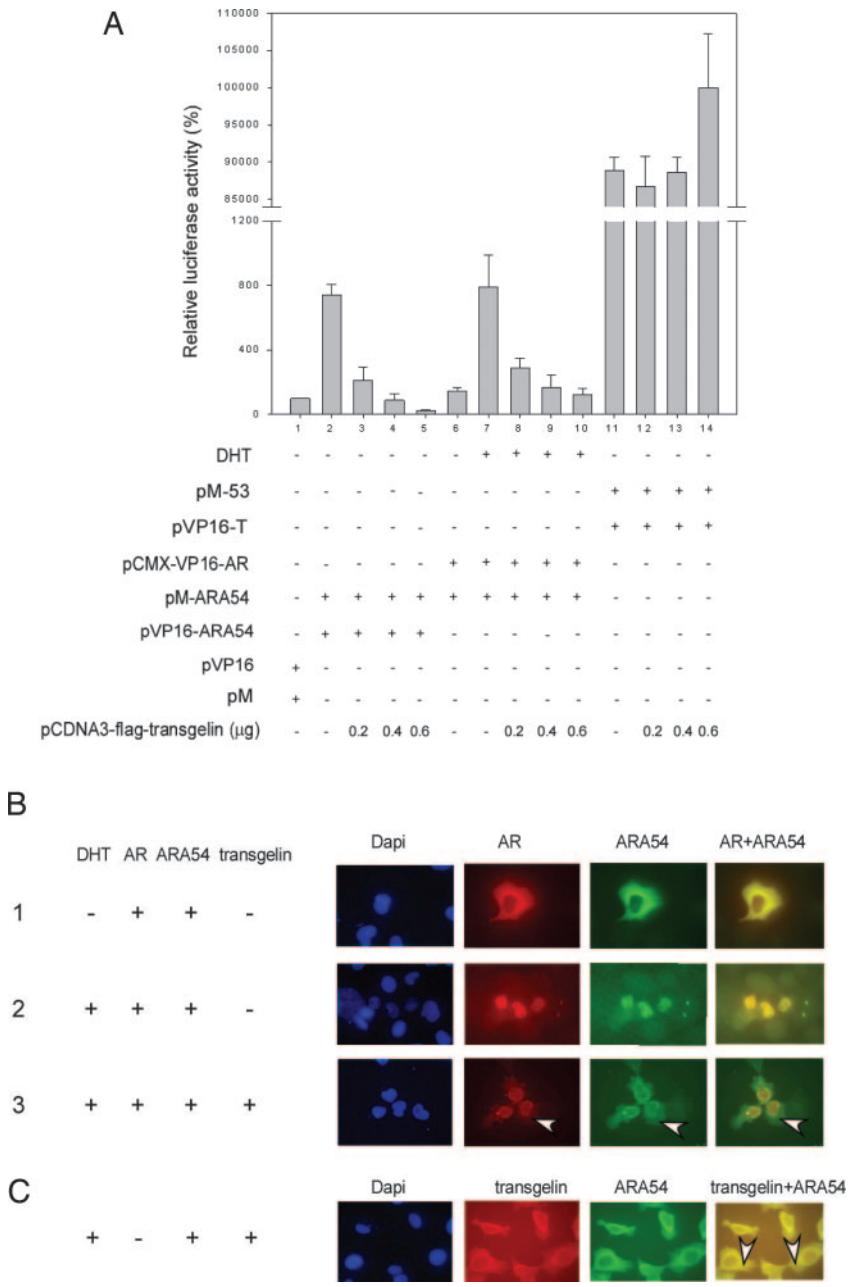

Fig. 4. Molecular Mechanisms of Transgelin's Suppressive Effect on AR Function

A, Transgelin blocked the interaction between ARA54 and AR as well as the homodimerization of ARA54, but not the interaction between pM-53 and pVP16-T. COS-1 cells were transiently cotransfected with 0.4 μg pG5-LUC reporter plasmid, 0.5 ng pRL-TK internal control plasmid, 0.2 μg pM-ARA54 and pVP16-AR, or 0.2 μg pM-ARA54 and pVP16-ARA54, or 0.2 μg pM-53 and pVP16-T, with different doses of pcDNA3-Flag-transgelin as indicated. The luciferase activity of the sample transfected with pM and pVP16 without DHT treatment (lane 1) was set as 100%. All values represent the mean $\pm$ SD of three independent experiments. B, Transgelin influenced the subcellular distribution of ARA54 and AR. H1299 cells were cotransfected with 0.2 μg pSG5-AR and 0.2 μg pSG5-ARA54 in combination with 1.6 μg pcDNA3-Flag-transgelin or pcDNA3-Flag vector. *Arrowheads* point out that ARA54 and AR were prevented from entering the nuclei. C, Colocalization of transgelin and ARA54 in the cytoplasm. H1299 cells were cotransfected with 1 μg pcDNA3-Flag-transgelin and 1 μg pSG5-ARA54. *Arrowheads* point out the colocalization of ARA54 and transgelin in the cytoplasm. Dapi, 4′6-Diamidino-2-phenylindole.

tion of AR target genes. The AR coregulator ARA67 has been reported to suppress the AR function via interruption of its nuclear translocation (18). Because transgelin blocks the interaction between AR and ARA54, it is possible that interruption of AR-ARA54 interaction by transgelin might result in the altered subcellular distribution of AR and ARA54. To test this hypothesis, we performed immunofluorescence stain-

ing analysis. AR-negative H1299 cells were cotransfected with AR and ARA54 in the presence or absence of transgelin with or without DHT. The subcellular localization of AR was examined as red fluorescence and ARA54 as green fluorescence under the Nikon Eclipse E800 fluorescence microscope (Nikon, Melville, NY). In the absence of DHT, the unliganded AR and ARA54 signals were mainly in the cytoplasm (Fig. 4B, row 1). Upon treatment with DHT, AR and ARA54 translocated into the nucleus (Fig. 4B, row 2). Only a very little AR and ARA54 signals remained in the cytoplasm. Interestingly, the addition of transgelin suppressed the nuclear translocation of ARA54 and AR. As shown in Fig. 4B, row 3, a considerable amount of ARA54 signals was detained in the cytoplasm. The AR signal in the nucleus was relatively weak in the presence of transgelin (Fig. 4B, row 3). Therefore, this suggests transgelin interrupts the interaction between AR and ARA54, resulting in the enhanced cytoplasmic retention and impaired nuclear translocation of ARA54 and AR. In contrast, in the absence of AR, transgelin colocalized with ARA54 in the cytoplasm (Fig. 4C).

siRNA Suppression of Endogenous Transgelin Enhanced AR Transactivation in LNCaP Cells

To minimize the potential artificial effects linked to exogenous transfection of transgelin and to demonstrate that endogenous transgelin can function as an AR suppressor via its interaction with ARA54, we applied siRNA to silence the endogenous transgelin expression. As shown in Fig. 5, A and B, transgelin expression was suppressed by more than 60% as demonstrated in real-time RT-PCR and Western blot assay. A vector containing the scrambled sequence served as negative control. As shown in Fig. 5C, AR transactivation was further enhanced by 2- to 4-fold when the endogenous transgelin was suppressed. These results suggest that the silencing of endogenous transgelin via its siRNA might reduce transgelin's suppressive effect on AR function, which further confirmed the suppressor role of transgelin on the AR transactivation.

The Suppression of Transgelin on LNCaP Cell Growth and PSA Expression Is ARA54 Dependent

We then studied the consequence of transgelin function as a suppressor via inhibition of ARA54-enhanced AR transactivation in prostate cancer cell growth. We transfected pcDNA3-Flag-transgelin or pcDNA3-Flag into AR-positive LNCaP and AR-negative PC-3 cells with neomycin to select the stably transfected cells. The overexpression of transgelin in the selected clones was confirmed by real-time RT-PCR (data not shown) and Western blotting (Fig. 6B). As shown in Fig. 6, A and B, we found the mRNA and protein expression of the AR target gene, PSA, but not AR, was significantly decreased in the transgelin-stably transfected LNCaP subline after 1 nM DHT treatment for 48 h. The quantitation of Western blotting showed a 43% decrease of PSA (Fig. 6B, *lower panel*). In the 7-d 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) growth assay, the growth rate of stably-transfected-transgelin LNCaP subline was significantly suppressed as compared with subline stably transfected with vector alone (Fig. 6C). Similar growth suppression effect was also observed in two other subclones (data not shown here). In contrast, the growth of transgelin-stably transfected PC-3 cells, which are AR negative and ARA54 positive, showed little influence as compared with cells stably transfected with vector alone (Fig. 6D). Because the suppressive effect of transgelin on the AR function is ARA54-dependent (Fig. 3), we were interested to know whether the growth-suppressive effect of transgelin on the LNCaP is also ARA54 dependent. We used retrovirus-delivered siRNA to silence the endogenous ARA54 in transgelin- and vector-stably transfected LNCaP cells. Stable ARA54-siRNA-transfected cells were selected by puromycin. In the 7-d growth assay, we found that the growth suppression effect of transgelin was reduced upon the silencing of endogenous ARA54 (Fig. 6E). These data indicate that transgelin functions to suppress the ARA54-enhanced AR-mediated prostate cancer cell growth.

Silencing of Transgelin via siRNA Enhanced Growth of LNCaP Cells and Expression of PSA

We then used siRNA to silence the endogenous transgelin in LNCaP cells to confirm its suppressive effect. We stably transfected transgelin siRNA into the LNCaP cells and selected the stable clones with puromycin. After the stable clones were selected, their expression of transgelin was confirmed by Western blotting (Fig. 7A, *upper panel*). After 1 nM DHT treatment for 48 h, the PSA expression in the transgelin siRNA-transfected LNCaP cells was significantly higher compared with the scrambled siRNA-transfected cells (Fig. 7A, *upper panel*). Quantitation of Western blotting showed PSA expression increased to 157% (Fig. 7A, *lower panel*). We then seeded the transgelin-siRNA and scrambled-siRNA LNCaP sublines in 24-well plates and treated them with 1 nM DHT for 7 consecutive days. As expected, stable transfection of transgelin siRNA enhanced LNCaP cell growth (Fig. 7B). Together, both addition and silencing of transgelin demonstrate that transgelin might suppress prostate cancer cell growth via inhibition of ARA54-enhanced AR activity and AR-mediated LNCaP cell growth.

The Differential Expression of Transgelin in Human Normal *vs.* Malignant Prostate Tissues

To strengthen the above findings that transgelin functions as a suppressor of AR-mediated prostate cancer cell growth, we analyzed the expression of transgelin in human prostate samples under normal or malignant

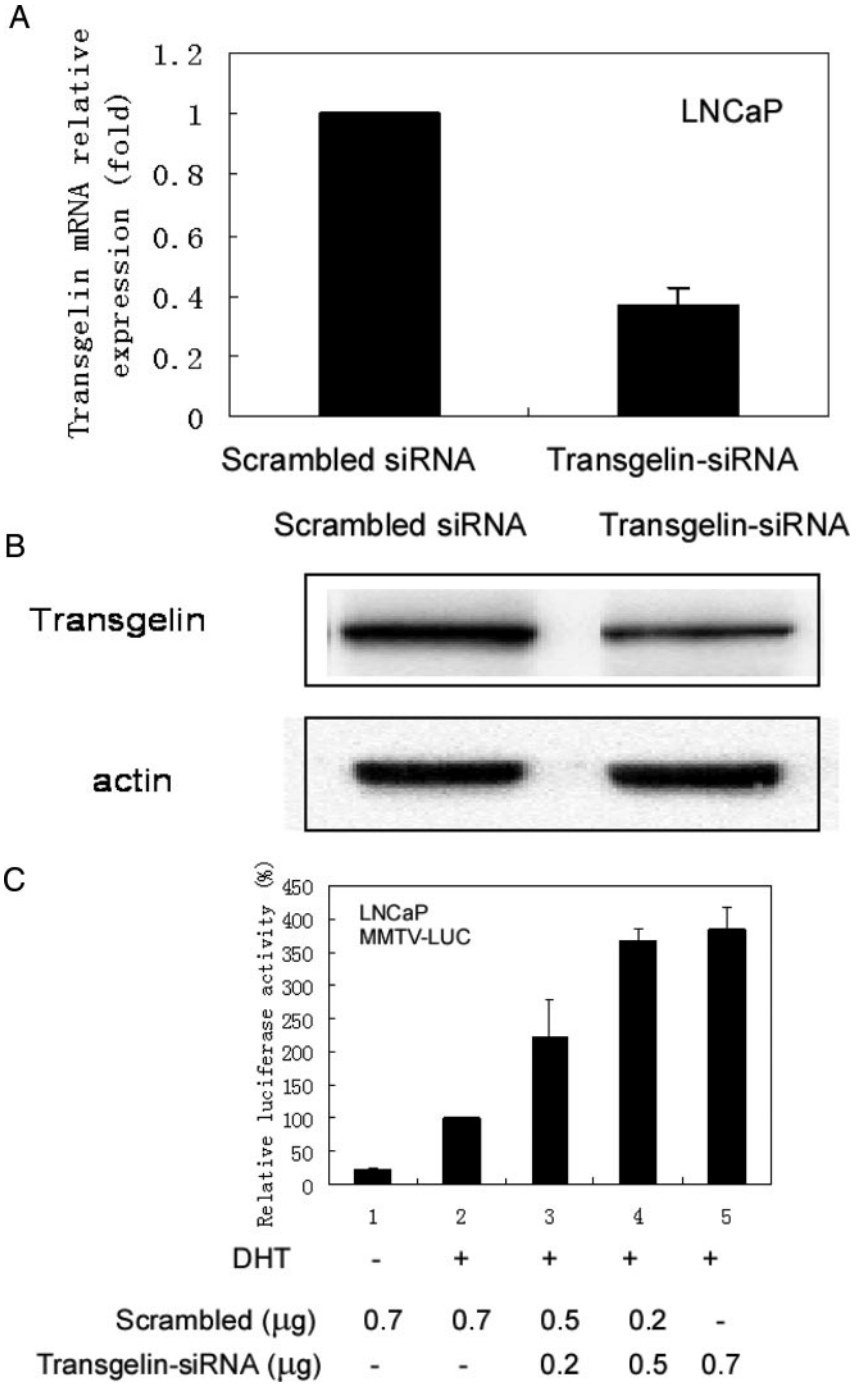

Fig. 5. Suppression of Endogenous Transgelin by siRNA Resulted in Enhanced AR Transactivation in LNCaP Cells

A, Expression of transgelin by real-time RT-PCR in the siRNA-transiently transfected LNCaP cells. Scrambled siRNA or transgelin-siRNA (10 μg) was transfected into LNCaP cells cultured in 10-mm dishes. Total RNA was extracted, and real-time RT-PCR was used to assay the relative expression level of transgelin mRNA. B, Expression of transgelin by Western blot in the siRNA-transiently transfected LNCaP cells. Scrambled siRNA or transgelin-siRNA (10 μg) was transfected into LNCaP cells cultured in 10-mm dishes. C, LNCaP cells were cultured in a 24-well plate and transfected with 0.225 μg MMTV-LUC and 5 ng pRL-TK internal control plasmid, together with scrambled control siRNA or siRNA against transgelin as indicated. Values represent the mean ± SD of at least three determinations.

conditions. First, three prostate tumor samples and adjacent normal tissues were obtained from patients with high-grade prostate adenocarcinoma (Gleason score >7). Using real-time RT-PCR to detect the mRNA level in human prostate samples, we found the transgelin mRNA expression level was much lower in

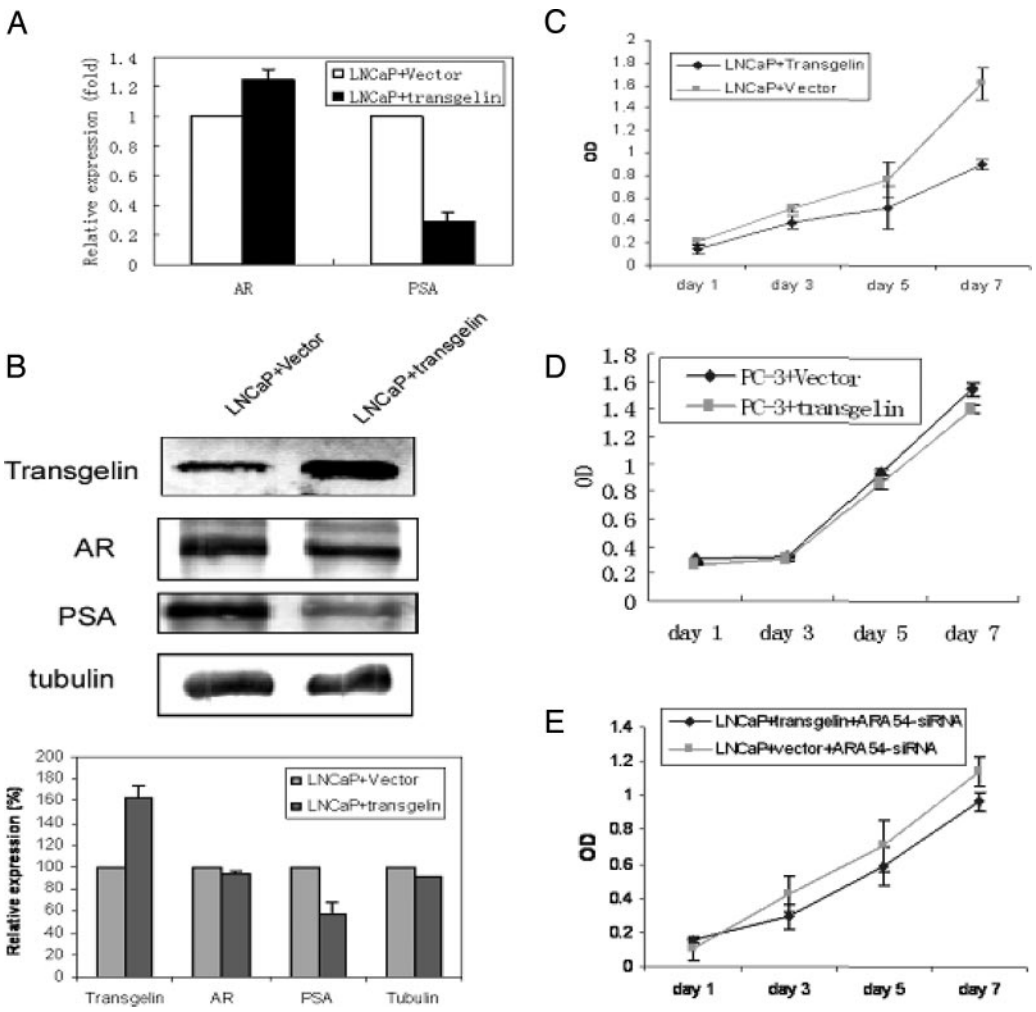

Fig. 6. The ARA54-Dependent Suppression of Transgelin on LNCaP Cell Growth and PSA Expression

A, LNCaP cells stably transfected with pcDNA3-Flag-transgelin and pcDNA3-Flag were treated with 1 nM DHT for 48 h. The mRNA levels of AR and PSA were assayed using real-time RT-PCR. Values represent the fold differences in gene expression relative to LNCaP cells stably transfected with pcDNA3-Flag. B, LNCaP cells stably transfected with pcDNA3-Flag-transgelin and pcDNA3-Flag were treated with 1 nM DHT for 48 h. The protein levels of AR, transgelin, and PSA were assayed using Western blot. The stable LNCaP (C) and PC-3 (D) clones with pcDNA3-Flag-transgelin or pcDNA3-Flag were cultured with RPMI 1640 medium supplemented with 10% CDFBS and 1 nM DHT. Cell growth was determined using MTT assay. E, Transgelin- and vector-stably transfected LNCaP cell lines were further stably transfected with retrovirus-delivered ARA54-siRNA and selected with puromycin. Cells were cultured in RPMI 1640 medium supplemented with 10% CDFBS and 1 nM DHT. Cell growth was determined using MTT assay.

malignant prostate tissue as compared with the adjacent normal prostate (Fig. 8A). Also, a total of 80 cases of archival prostatic adenocarcinoma were evaluated for the expression levels of ARA54 and transgelin using standard tissue microarray and immunohistochemistry techniques. As shown in Fig. 8, B and C, the intensity of transgelin immunostaining and the percentage of transgelin positive-staining (Table 1) were significantly higher in the normal tissue compared with prostate cancer. In contrast, the intensity of ARA54 immunostaining (Fig. 8, D and E) and the percentage of ARA54 positive staining (Table 2) were significantly lower in the normal prostate tissue compared with prostate cancer. These data, at both mRNA and protein levels, further support our conclusion that transgelin might function as a suppressor via inhibition of ARA54-enhanced AR function and loss of transgelin, and its suppressor function in prostate cancer might contribute to the progression of prostate cancer.

DISCUSSION

Sustained blocking of the AR signaling pathway is critical for controlling prostate cancer. Although multiple strategies have been used to reduce serum levels of androgens or interfere with their function via AR,

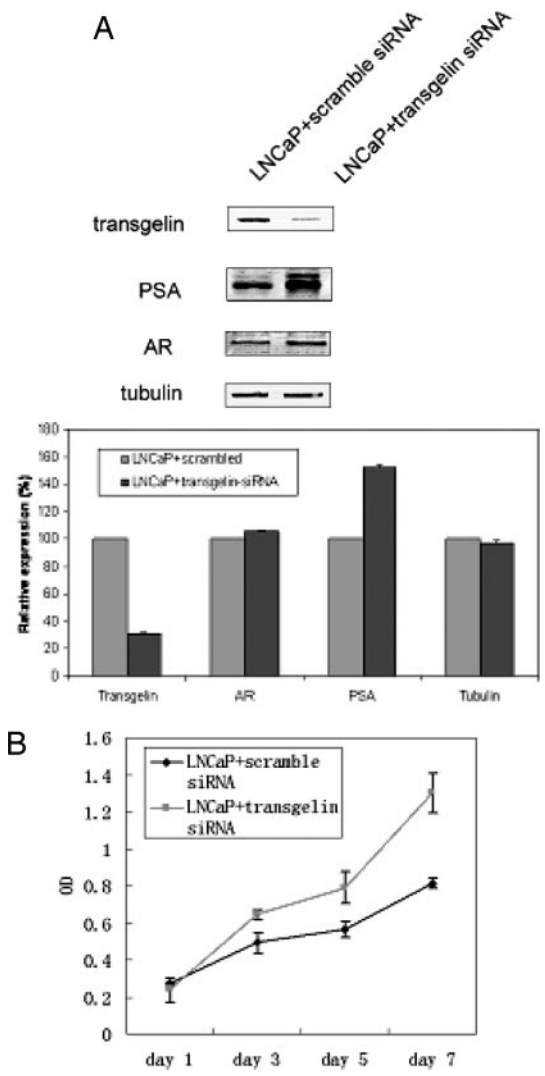

Fig. 7. Silencing of Endogenous Transgelin by siRNA Enhanced the LNCaP Cell Growth and PSA Expression

A, Cell lysates from transgelin-siRNA and scrambled siRNA-stably transfected LNCaP cells were Western blotted for endogenous transgelin, AR, PSA, and tubulin. B, The stable cell lines transfected with transgelin siRNA and scrambled siRNA were cultured with RPMI 1640 medium supplemented with 10% CDFBS and 1 nM DHT. Cell growth was determined using MTT assay.

combined androgen blockade using medical or surgical castration with antiandrogens is currently a standard treatment for advanced prostate cancer (34). One serious problem for current androgen ablation therapy is the severe side effects caused by the systemic suppression of AR signaling in all types of cells expressing functional AR, whereas regulated AR signaling is essential for the proper function of many important organs, like male fertility, cardiovascular system, and bone. Severe side effects, such as the loss of libido, erectile dysfunction, male infertility, osteoporosis, and increased incidence of cardiovascular diseases, usually result from the undifferentiated and sustained blocking of AR signaling in these organs. How to suppress AR function more specifically in the malignant cells with minimal influence in normal cells and organs poses a severe challenge to successful treatment. With the knowledge of differential distribution and expression of AR coregulators and their modulators in different cell types, it is possible to modulate AR signaling in a cell type-specific manner. Accumulating evidence showed that the progression of prostate cancer and its acquisition of androgen independence were accompanied by changing expression profiles of AR coregulators. In our study, we showed the increased expression of ARA54 in prostate cancer tissue, whereas the normal tissue expresses much lower levels of ARA54. If the therapy targets specific AR coregulators, such as ARA54, and their modulators instead of targeting AR directly, only prostate cancer cells expressing specific AR coregulators will be suppressed whereas the AR signaling in normal cells with low or no expression of this coregulator would be unaffected. The identification and characterization of AR coregulators and their interacting proteins might pave the way to finding potential therapeutic strategies of suppressing AR function by interrupting the interaction between AR and its coregulators. Transgelin is the first of such modulators that showed suppressive effects on AR function via selective suppression of ARA54-enhanced AR transactivation and AR-mediated prostate cancer cell growth. Molecular mechanistic dissection showed that this suppressive effect is via the interruption of AR-ARA54 heterodimerization and ARA54 homodimerization. AR transactivation in cancer cells expressing ARA54 was inhibited by transgelin, whereas AR signaling in fibroblast 3T3-L1 cells without ARA54 expression was not affected. These findings suggested that new approaches, targeting AR coregulator modulator without directly affecting AR, might have better therapeutic effects with fewer side effects in the treatment of prostate cancer.

Tissue array and quantitative RT-PCR showing reduced expression of transgelin in the high-grade prostate tumor further suggested that transgelin might potentially function as a tumor suppressor. Finding low transgelin levels in prostate cancer specimens has several implications for the diagnosis and treatment of this disease. The most obvious benefit is for the pathologists diagnosing prostate cancer because low transgelin expression might be used as a molecular marker for diagnosis. Second, increasing the transgelin expression to suppress prostate cancer cell growth might be used as a gene therapy strategy for the treatment of prostate cancer. Previously, Shields *et al.* (32) reported that transgelin expression is lost in human-derived breast and colon tumors and hypothesized that loss of transgelin expression could be an early event in the development of these tumors. Our findings of decreased expression of transgelin in human prostate cancer samples suggest its potential suppressor roles in prostate cancer development.

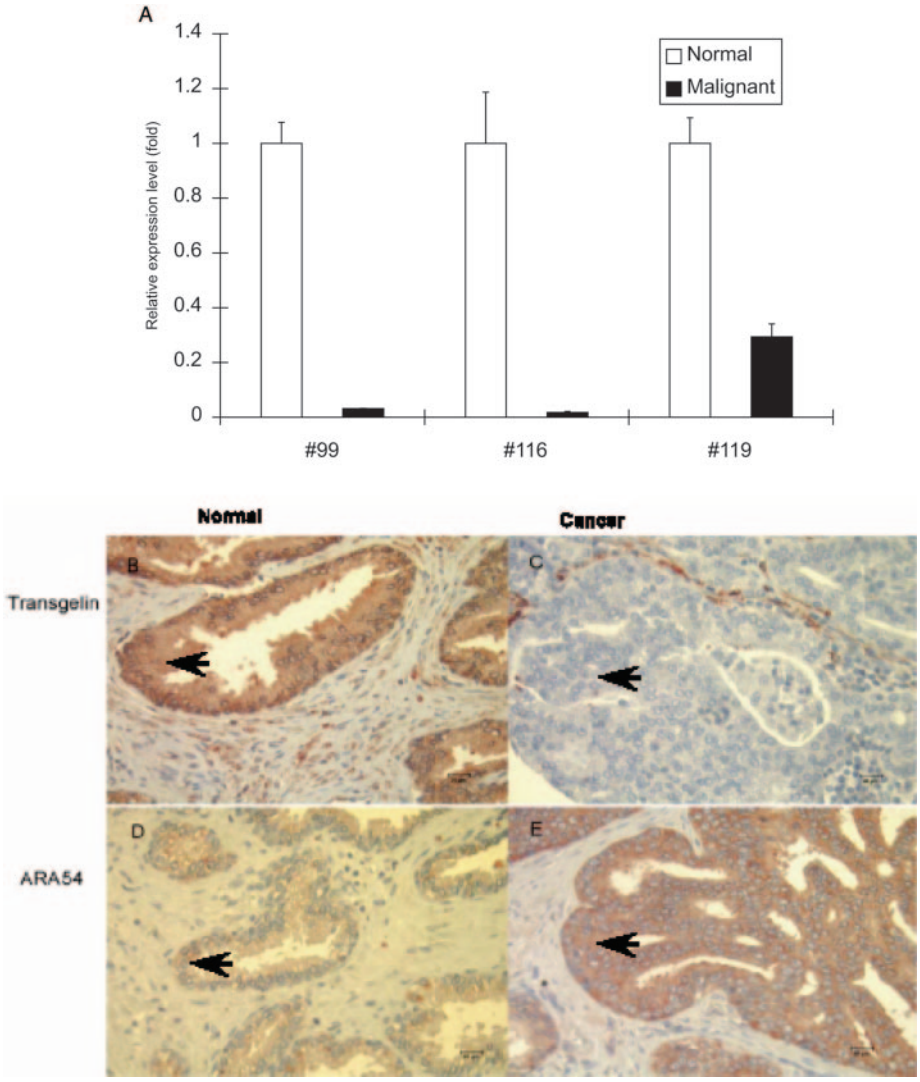

Fig. 8. Differential Expressions of Transgelin and ARA54 in Benign *vs.* Malignant Prostate Tissues

A, Total RNA was extracted using Trizol from three cases of patient-derived high-grade prostate carcinoma and matched adjacent histologically normal tissues from the same patients. Real-time RT-PCR was performed to determine the relative expression of transgelin. Values represent the fold difference in transgelin expression level relative to adjacent normal tissue set as 1.00. B and C, Immunohistochemistry staining of transgelin in benign and malignant prostate tissue. *Arrows* indicate the expression of transgelin in the prostate epithelium. D and E, Immunohistochemistry staining of ARA54 in benign and malignant prostate tissue. *Arrows* indicate the expression of ARA54 in the prostate epithelium.

Table 1. Expression Level of Transgelin in Normal and Malignant Prostate Tissue

	Positive (n)	Negative (n)	Total (n)	Positive (%)	*P* Value (*vs.* Normal)
Normal	33	7	40	82.5	
Cancer	56	52	108	51.85	<0.001

Total androgen blockade therapy used in the treatment of prostate cancer, although initially effective, eventually fails to sustain its efficacy when continued beyond 1–2 yr. Cancer cells become resistant to ablation therapy, and AR loses its specificity and becomes activated by antiandrogens. Earlier studies suggested AR mutations and aberrant expression of AR coregulators as reasons for the antiandrogen withdrawal syndrome (21, 35–38). Our previous study showed that ARA54, in addition to enhancing the DHT-induced wild-type AR transactivation, also enhanced mtART877A transactivation induced by E2 and HF (17), suggesting its role in the development of this syndrome. Interestingly, in this study, the ARA54-en-

Table 2. Expression Level of ARA54 in Normal and Malignant Prostate Tissue

	Positive (n)	Negative (n)	Total (n)	Positive (%)	*P* Value (*vs.* Normal)
Normal	22	18	40	55.0	
Cancer	93	16	109	85.32	<0.001

hanced mtART877A transactivation induced by E2 and HF was abrogated by transgelin, suggesting that the transgelin effect might be helpful in the suppression of mutant AR transactivation induced by antiandrogens.

In this study, we demonstrated the dual functional roles played by ARA54 in the AR signaling regulation. In addition to its direct AR coregulator function, ARA54 also mediates the suppressive effect of transgelin on the AR function. These findings further strengthened the *in vivo* coregulator role of ARA54. AR coregulators such as ARA54 might influence AR function via direct coregulator activity as well as by mediating its interacting proteins' modulator function. Recent evidence showed that, in addition to the coregulators that interact directly with NRs, including AR, there are a few protein factors that interact with these coregulators to modulate the NR transactivation (39). For example, the CBP/p300 and coactivator-associated arginine methyltransferase-1 can interact with p160 to modulate the NR transactivation (40, 41); they can also function as coregulators to modulate AR directly (42). This is in contrast to our findings that transgelin can suppress the ARA54-enhanced AR transactivation, but has only little effect on the AR transactivation in the absence of ARA54. Additional identification and characterization of modulators for AR coregulators and their effect on the AR signaling pathway might shed light on the complicated molecular mechanism of AR signaling regulation. The detailed molecular mechanistic dissection may pave the way to better understanding of the functional roles played by AR signaling pathway in androgen-related diseases like prostate cancer.

In conclusion, we demonstrated the dual functional roles played by ARA54 in the AR signaling regulation via direct coregulator activity as well as by mediating its interacting proteins' modulator function. Transgelin functions as an ARA54-associated negative regulator of AR transactivation by interrupting the interaction between AR and ARA54 as well as ARA54 homodimerization that results in the suppression of the cotranslocation of AR and ARA54 into the nucleus. Addition of transgelin suppresses the AR-mediated prostate cancer cell growth and PSA expression in LNCaP cells. The expression of transgelin is down-regulated in human prostate cancer specimens. These results provide insights into the malignant process and potentially generate both diagnostic and prognostic markers as well as future treatment strategies.

MATERIALS AND METHODS

Materials and Plasmids

DHT, progesterone, and E2 were obtained from Sigma (St. Louis, MO) and HF was from Schering-Plough Corp. (Kenilworth, NJ). The pACT2-prostate cDNA library and the Marathon-Ready prostate cDNA library were purchased from CLONTECH Laboratories, Inc. (Palo Alto, CA) and pGEMTeasy vector was from Promega Corp. (Madison, WI). pAS2-ARA54 containing the full-length ARA54 fused to the GAL4 DNA binding domain (amino acids 1–147) was constructed by inserting the *Bam*HI fragment of ARA54 in-frame into the pAS2 plasmid. pGEX-GST-ARA54 was constructed as described previously (43). pM-53 and pVP16-T were purchased from CLONTECH. pCMX-GAL4-AR and pCMX-VP16-AR were constructed for mammalian two-hybrid assay (15, 44). pCMV-mtART877A was constructed in our laboratory (38). pM-ARA54 and pVP16-ARA54 were constructed by inserting the *Bam*HI fragment of ARA54 in frame to the pM and pVP16 plasmids, respectively. pSG5-ARA54, mouse mammary tumor virus luciferase (MMTV-LUC), pG5-LUC, and pRL-TK have been described previously (28). The Transgelin (1-151) plasmid was a gift from Julian Solway (Department of Medicine, University of Chicago, Chicago, IL). Full-length transgelin cDNA was amplified from Marathon-Ready prostate cDNA library using PCR. The PCR product was cloned into the pGEMTeasy vector and subcloned into pSG5 vector via *Bam*HI site using blunt-end ligation. pcDNA3-Flag-transgelin and pVP16-transgelin were generated by PCR of the full-length human transgelin cDNA with primers containing sites *Bam*HI and *Xba*I flanking the 5′- and 3′-ends and cloned into pcDNA3-Flag and pVP16 vectors (CLONTECH). All the constructs were confirmed by sequencing. Anti-ARA54 and anti-PSA antibody were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and anti-Flag antibody was from Sigma. Antitransgelin antibody was purchased from Abcam (Cambridge, MA). pSG5-ARA70N, pSG5-ARA55, pSG5-gelsolin, pSG5-supervillin, pSG5-SRC-1, and pSG5-CBP have been used previously (14, 15, 45–48). The LNCaP, 3T3-L1, PC-3, H1299, and COS-1 cells were obtained from the American Type Culture Collection (Manassas, VA). Cell culture media RPMI-1640 and DMEM were obtained from Life Technologies, Inc. (Gaithersburg, MD).

Yeast Two-Hybrid Screening

A fusion protein (GAL4-ARA54) containing GAL4 DNA-binding domain and full-length ARA54 was used as bait to screen 1.26×10^6 transformants of MATCHMAKER human prostate cDNA library (CLONTECH). Transformants were selected for growth on nutrition selection plates containing SD media lacking histidine, leucine, and tryptophan (−3SD) with 20 mM 3-aminotriazole. Colonies were also filter assayed for β-galactosidase activity. DNA from positive clones was recovered from yeast using PCR. The PCR primers were sense: 5′-GGG TTT TTC AGT ATC TAC GAT TCA TAG A-3′; and antisense: 5′-CTA TTC GAT GAT GAA GAT ACC CCA CCA AAC CC-3′. The annealing temperature was 58 C. The PCR products were sequenced and then digested with *Bgl*II and cloned into pVP16 vector via *Bam*HI site. The full-length cDNA of the candidate gene was generated by PCR using the CLONTECH

marathon-ready cDNA library as template, the PCR primers were sense: 5′-CAC AAG TCT TCA CTC CTT CCT GC-3′; and antisense: 5′-CTC AGT GAC AGA GCC TCA AAG CT-3′. The PCR product was cloned into the pGEMTeasy vector and then subcloned into the pSG5 and pVP16 vector.

Cell Culture, Transient Transfections, and Reporter Gene Assays

COS-1, 3T3-L1, PC-3, and LNCaP cells were grown in appropriate medium at 1–4 $\times$ 10^5 cells in 24-well plates. Transfections were performed using SuperFect (QIAGEN, Chatsworth, CA) according to the manufacturer's procedure. After incubation for 2–3 h, the medium was changed, and cells were treated with ethanol, DHT, or other ligands for another 24 h and then harvested for luciferase assay. In each experiment, the total amount of transfected DNA per well was maintained as a constant by the addition of empty backbone vectors (pSG5, pVP16, or pcDNA3-Flag) or scrambled siRNA. The MMTV-LUC plasmid was used as the reporter gene. pRL-TK was used as an internal control. The Dual-luciferase reporter 1000 assay system (Turner Designs, Sunnyvale, CA) was employed to measure luciferase activity. The activity of the experimental reporter was normalized to the activity of the internal control to minimize the experimental variability caused by differences in cell viability or transfection efficiency.

Mammalian Two-Hybrid Assay

3T3-L1 and COS-1 cells were transiently cotransfected with a GAL4-hybrid expression plasmid, a VP16-hybrid expression plasmid, the reporter plasmid pG5-LUC, and the pRL-TK internal control plasmid. Cells were treated with DHT or ethanol for 24 h and then harvested for luciferase assay. The activities of *Renilla* luciferase were used to normalize any variations in transfection efficiency.

In Vitro GST Pull-Down Assay

GST pull-down assays were carried out as described previously (19). Briefly, the full-length transgelin was *in vitro* translated in the presence of [^{35}S]methionine using T7 polymerase and the coupled transcription/translation kit (Promega). pGEX-GST-ARA54 plasmids expressing the GST-ARA54 fusion protein were transformed into BL21(DE3) bacteria strain, and 0.4 mM isopropyl-β-D-thiogalactopyranoside was added into LB medium containing transformed bacteria when the OD_{600} reached 0.5. Bacteria were further cultured at 30 C for 3 h and lysed by four cycles of freezing-thawing in NENT buffer [20 mM Tris/pH 8.0, 0.5% Nonidet P-40, 100 mM NaCl, 6 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol (DTT), 8% glycerol, and 1 mM phenylmethylsulfonyl fluoride (PMSF)]. The GST-ARA54 fusion proteins were purified with glutathione-beads at 4 C. For *in vitro* interactions, mixtures of glutathione bead-bound GST fusion proteins and 5 μl of [^{35}S]methionine-labeled input proteins in 100 μl of interaction buffer [50 mM HEPES, 100 mM NaCl, 20 mM Tris-Cl (pH 8.0), 0.1% Tween 20, 10% glycerol, 1 mM DTT, 0.5 mM PMSF, 1 mM NaF, and 0.4 mM sodium vanadate] with or without 10 nM DHT at 4 C for 2 h. The beads were then washed with NENT buffer four times, resuspended in SDS-PAGE loading buffer, and resolved on 12% SDS-PAGE followed by autoradiography.

Coimmunoprecipitation of Transgelin and ARA54

Coimmunoprecipitation of transgelin and ARA54 was performed as described previously (49). Briefly, LNCaP cells cultured in RPMI-1640 supplemented with 10% non-heat inactivated fetal bovine serum (FBS) were lysed using lysis buffer (1% Nonidet P-40, 10% glycerol, 135 mM NaCl, 40 mM Tris/pH 7.4, 1 mM PMSF, 1 mM DTT, and 1$\times$ protease inhibitor cocktail) (Roche, Indianapolis, IN). Lysates were clarified by centrifugation, 500 μg protein were incubated with 2 μg anti-ARA54 antibody (G18, Santa Cruz) or normal goat IgG for 4 h at 4 C with agitation, and protein A/G plus agarose (35 μl) was added to each sample and incubated for 1 h. After washing three times with radioimmune precipitation assay buffer, the complex was resolved on a 10% SDS-polyacrylamide gel. The separated proteins were transferred to a polyvinylidene difluoride membrane and then blotted with anti-ARA54 and antitransgelin antibody. The bands were resolved by an alkaline phosphatase detection kit (Bio-Rad Laboratories, Hercules, CA).

Immunofluorescence Staining

H1299 cells were seeded on two-well Lab-Tek Chamber slides (Nalge) in DMEM containing 10% charcoal-dextran-stripped FBS (CDFBS) 18 h before transfection. DNA was transfected with SuperFect. After transfection, cells were treated with either 10 nM DHT or vehicle for 24 h. Then cells were fixed with fixation solution (3% paraformaldehyde and 10% sucrose in PBS) for 20 min on ice, followed by permeabilization with methanol for 10 min at $-$20 C. Slides were washed and blocked with 2% BSA in PBS for 15 min at room temperature. Then the cells were stained with 1 μg of rabbit polyclonal anti-AR antibody C19 (Santa Cruz Biotechnology) per ml together with 1 μg of goat polyclonal antibody for ARA54 or 1 μg of mouse monoclonal anti-Flag antibody per ml at room temperature for 1 h. After the primary antibody incubation, cells were washed and incubated with Texas Red-conjugated goat antirabbit IgG and fluorescein isothiocyanate-conjugated donkey antigoat IgG or Texas Red-conjugated goat antimouse IgG (ICN Diagnostics, West End, NC). Stained slides were mounted with coverslips and visualized with a fluorescence microscope.

Construction of DNA Vector-Based RNA Interference Plasmids

siRNA target sites were selected by scanning the cDNA sequence of transgelin and ARA54 using the Oligoengine siRNA designing software and BLAST search to eliminate any sequence with significant homology to other genes. The siRNA inserts, containing selected 19-nucleotide (nt) coding sequences, followed by a 9-nt spacer, an inverted repeat of the coding sequence, plus 5 Ts, were generated as double-stranded DNAs with *Hin*dIII and *Bgl*II sites and then cloned into plasmid pSUPERIOR.retro.puro (Oligoengine). The corresponding oligos for generating transgelin siRNA were 5′-GAT CCC CAG ACT GAC ATG TTC CAG ACT TCA AGA GAG TCT GGA ACA TGT CAG TCT TTT TTA-3′ and 5′-AGC TTA AAA AAG ACT GAC ATG TTC CAG ACT CTC TTG AAG TCT GGA ACA TGT CAG TCT GGG-3′ (position 322–341). A nonfunctional scrambled siRNA was constructed as a negative control, which contains nt substitutions at the 19-nt targeting sequence of siRNA. The corresponding oligos for generating this scrambled siRNA control were 5′-GAT CCC CGA GAG TGT CCA CCT ACA TAT TCA AGA GAT ATG TAG GTG GAC ACT CTC TTT TTA-3′ and 5′-AGC TTA AAA AGA GAG TGT CCA CCT ACA TAT CTC TTG AAT ATG TAG GTG GAC ACT CTC GGG-3′. The corresponding oligos for generating ARA54 siRNA sequences were: 5′-GAT CCC CAT GCC TCA ACT GCC CAG AAT TCA AGA GAT TCT GGG CAG TTG AGG CAT TTT TTA-3′ and 5′-AGC TTA AAA AAT GCC TCA ACT GCC CAG AAT CTC TTG AAT TCT GGG CAG TTG AGG CAT GGG-3′ (position 782–801).

Establishment of Cell Lines Stably Transfected with the Plasmid Encoding Transgelin

The pcDNA3-Flag vector contains a selective marker conferring resistance to neomycin for the generation of stable cell lines. We first constructed pcDNA3-Flag-transgelin and then transfected it into LNCaP cells using SuperFect transfection reagent. After transfection, cells were cultured in the presence of 300 μg/ml neomycin (Invitrogen, Carlsbad, CA) to select for stably transfected cells that had incorporated the pcDNA3-Flag based construct. After selection for an additional 2 wk, individual clones were picked. We then confirmed the stable expression of transgelin in the selected clones using real-time RT-PCR.

Establishment of Cell Lines Stably Transfected with the Plasmid Encoding siRNA against Transgelin and ARA54

The pSUPERIOR.retro.puro (Oligoengine) vector-based siRNA against the ARA54 and transgelin were constructed and delivered into LNCaP cells using retrovirus-mediated transfection. Briefly, 10 μg siRNA was transfected into Phi-NX packaging cells (developed by Nolan Laboratory at Stanford University) using SuperFect. After 48 h transfection, cell culture medium was filtered through a 0.45-μm filter. The viral supernatant was used for infection of cells after addition of 4 μg/ml polybrene. Cells were infected for 6 h and allowed to recover for 24 h with fresh medium. Infected cells were selected with 3 μg/ml puromycin for 48–72 h.

Cell Proliferation Assay

LNCaP and PC-3 cells were seeded in 24-well tissue culture plates at a density of 4000 cells/cm^2 in medium containing 10% CDFBS with 1 nM DHT. At the indicated time points, the medium was replenished and cell proliferation was determined by MTT assay. Serum-free medium containing MTT (0.5 mg/ml, Sigma) was added to each well. After 4 h incubation at 37 C, the stop solution was added to solubilize the formazan product and the absorbance was recorded.

Western Blotting Assay

Protein samples collected from the cells were separated on SDS-10% PAGE and transferred to polyvinylidene difluoride membrane. Membranes were blocked with 5% nonfat milk in TBST buffer (150 mM NaCl; 10 mM Tris/pH 8.0; and 0.5% Tween 20) at room temperature for 1 h. Then the membranes were immunoblotted with primary antibodies for 2 h at room temperature or overnight at 4 C, followed by incubation with secondary antibodies for 1 h at room temperature. Blots were developed with the alkaline phosphatase color developing reagents (Bio-Rad). The Western data were further quantified by the Bio-Rad Quantity-One software.

Real-Time RT-PCR

Total RNA was extracted from tissue or cells using Trizol (Invitrogen). We carried out reverse transcription with the SuperScript II kit (Invitrogen) and PCR amplifications with SYBR Green PCR Master Mix on an iCycler IQ multicolor real-time PCR detection system (Bio-Rad). The PCR was performed as follows: initial denaturation at 95 C for 10 min, and 45 cycles of denaturation at 95 C for 30 sec, annealing at 60 C for 30 sec, and extension at 72 C for 30 sec. Primer sequences were as follows. PSA: sense, 5′-CCA GAG GAG TTC TTG ACC CCA AA-3′; antisense, 5′-CCC CAG AAT CAC CCG AGC AG-3′. AR: sense, 5′-CCT GGC TTC CGC AAC TTA CAC-3′; antisense, 5′-GGA CTT GTG CAT GCG GTA CTC-3′. Transgelin: sense, 5′-GGA CCC TGA TGG CTT TGG-3′; antisense, 5′-CCT CTC CGC TCT AAC TGA TG-3′. ARA54: sense, 5′-CTA GTC TTC TAC CTC ATC TTC-3′; antisense, 5′-CGA AAT GAA TAC CTG CAA GCG-3′. β-actin: sense, 5′-TGT GCC CAT CTA CGA GGG GTA TGC-3′; and antisense, 5′-GGT ACA TGG TGG TGC CGC CAG ACA-3′. Δ Threshold cycle (ΔCT) values were calculated by subtracting the CT value from the corresponding β-actin CT (internal control) value from each time point. The absence of nonspecific amplification products was confirmed by agarose-gel electrophoresis.

Tissue Microarray and Immunohistochemistry

Formalin-fixed and paraffin-embedded tumor (n = 109) and adjacent normal control (n = 40) specimens of radical prostatectomy were retrieved from archives of the Department of Pathology, University of Rochester and the Strong Memorial Hospital with Research subject Review Board approval. All tumors and controls were reviewed by one pathologist. Tumor and normal tissue were selected from the representative paraffin block and three to four tissue cores (0.6 mm in diameter) were sampled from this area in the donor block and mounted into recipient paraffin block by the use of a custom-made instrument (Beecher Instruments, Silver Spring, MD), and 5-μm paraffin sections were then made by standard technique. The sections were then deparaffinized according to routine procedures and quenched with 3% hydrogen peroxide for 6 min followed by clearing in running water and a final rinse in Tris buffer at pH 7.6. Sections were heat retrieved using Dako Target Retrieval Solution (citrate buffer solution at pH 6.1) (DAKO Corp., Carpinteria, CA) for 30 min in a Black and Decker (Baltimore, MD) rice steamer at a temperature of 95–99 C. After retrieval, sections were cooled at room temperature for 15 min followed by Tris buffer rinses for 5 min. Slides were mounted on the Dako autostainer and covered with fresh Tris buffer to prevent drying. Sections were stained for 60 min using antibodies for ARA54 (1:50) and transgelin (1:400) followed by 30-min incubation in rabbit antigoat IgG-Biotin (ARA54 and transgelin) (Vector Laboratories, Inc., Burlingame, CA) followed by streptavidin-horseradish peroxidase (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). All slides were developed with AEC+ (DakoCytomation, Carpinteria, CA) for 10 min. Slides were removed from stainer and rinsed in running distilled water and then counterstained in modified Mayer's hematoxylin blue in 0.3% ammonia water followed by a tap water rinse. The slides were mounted using an aqueous media and viewed with a light microscope. Appropriate tissue sections were used as positive controls. For negative controls, Universal negative control (DAKO) was used in place of the primary antibodies.

Evaluation of Staining Results

ARA54 and transgelin expression was evaluated subjectively by one pathologist, who estimated the staining intensity and percentage of positivity. Tissue cores with less than 50% of the original tissue remaining on the slides after immunohistochemistry were not used for the scoring of the stains. In cores that remained intact after staining, the intensity of staining (0 to 3+) and the percentage of positively stained cells in the normal and malignant epithelial cells of the prostatic tissue were recorded. Staining in all cores from the same case was examined and combined to derive the average percentage of positive staining. A core was considered positive only when the staining intensity was 2+ or more in more than 10% of the epithelial cells, whereas cases with less than 10% staining in the epithelial cells or staining intensity below 2+ were recorded as negative, following the scoring method of HercepTest, which is widely used for the evaluation of Her2-Neu overexpression in breast cancer (50, 51). Fisher's Exact Test was per-

formed to compare the statistic significance of the staining results among different groups.

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