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Development and Characterization of DP-153, a Nontumorigenic Prostatic Cell Line That Undergoes Malignant Transformation by Expression of Dominant-negative Transforming Growth Factor β Receptor Type II[1]

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ABSTRACT

We have developed a nontumorigenic epithelial cell line, DP-153, from the dorsal prostate of a Lobund/Wistar rat treated with *N*-methyl-*N*-nitrosourea and testosterone propionate. DP-153 cells express cytokeratins 5 and 14, but not cytokeratin 18, consistent with a basal epithelial cell phenotype. Similar to the nontumorigenic NRP-152 prostatic cell line, DP-153 cells do not form tumors in athymic mice and retain many of the properties of normal prostatic cells. They express prostatic acid phosphatase and androgen receptors and require several mitogens (epidermal growth factor, insulin, dexamethasone, and cholera toxin) for sustained growth in culture under serum-containing conditions. DP-153 cells are also growth-stimulated by keratinocyte growth factor and basic fibroblast growth factor and growth-inhibited by *all-trans*-retinoic acid, 1,25-dihydroxyvitamin D_3, and transforming growth factor (TGF)-β1. We demonstrate that expression of dominant-negative TGF-β receptor type II by retroviral transduction of DP-153 cells leads to complete loss of TGF-β1-induced growth inhibition. When transplanted s.c. in athymic mice, DP-153 cells expressing dominant-negative TGF-β receptor type II form tumors as early as 4 weeks, in contrast to the vector control and parental cell line, which do not form tumors even 8 months after transplantation, supporting the observation that TGF-β functions as a tumor suppressor in these cells. Our data further support that DP-153 is a suitable cell line for analysis of normal prostatic growth and carcinogenesis.

INTRODUCTION

Establishment of suitable nontumorigenic or "preneoplastic" cell lines is vital to a rational understanding of the molecular mechanisms for carcinogenesis. Development of such preneoplastic prostatic cell lines remains an important undertaking. Although several nontumorigenic prostatic cell lines have been developed, in almost all cases these cells have been immortalized through genetic manipulations (1–9) and thus may not be representative of cells in premalignant lesions. Our laboratory previously developed a spontaneously immortalized preneoplastic cell line, NRP-152, from the dorsolateral prostate of carcinogen-treated Lobund/Wistar rat (1). NRP-152 is a basal prostatic epithelial cell line phenotypically similar to normal prostatic epithelial cells and exquisitely sensitive to numerous growth factors and steroid hormones. Importantly, NRP-152 cells are AR[3] positive and grow in response to physiological concentrations of androgens (1). Most unique about this line is its stem cell phenotype, as evidenced by a unique ability to form normal prostatic ductal structures when coimplanted with urogenital sinus mesenchyme under the renal capsule of athymic mice (10) and by transdifferentiation toward a luminal phenotype in culture (11).

Similar to normal prostatic epithelium, NRP-152 cells undergo growth arrest and apoptosis by TGF-β. In addition, we have shown that TGF-β is involved in the mechanism of the transdifferentiation of NRP-152 cells from a basal to a luminal phenotype, as observed in culture (11). Impressively, disruption of growth inhibition and apoptosis by TGF-β in NRP-152 cells, which was accomplished by expression of DNR, alone causes their malignant transformation (12). This is consistent with the demonstration that restoration of response to TGF-β in the LNCaP human prostate adenocarcinoma cell line, by transfection of TβRII, suppresses tumor growth in athymic mice (13). Together, these studies support that TβRII functions as a tumor suppressor of the prostate.

TGF-βs, for which there are three mammalian isoforms, are multifunctional autocrine/paracrine growth regulators that belong to a large TGF-β superfamily, which includes bone morphogenic proteins, activins/inhibins, and others related ligands (14, 15). TGF-βs signal through a cooperative interaction with three cell surface receptors, TβRI, TβRII, and TβRIII (15, 16). Although TβRI and TβRII are critical for growth inhibition, TGF-β2 does not bind to TβRII and is believed to require TβRIII for presentation to TβRI (17). TGF-β first associates with TβRII, which recruits TβRI to form a ligand/receptor heteromeric complex. TβRII, which contains a constitutively active kinase domain, activates TβRI by transphosphorylation of the GS box in the cytoplasmic domain (18). The activated TβRI is believed to transmit TGF-β signals by binding and activating Smads 2 and 3 through transphosphorylation of their COOH-terminal SS*X*S serines (19), a process that requires accessory proteins such as Smad anchor for receptor activation protein (20), Hrg (21), Dab-2 (22), and ligand-dependent receptor endocytosis (23). Receptor-activated Smads homodimerize and heterodimerize (24) and then translocate to the nuclear compartment with or without Smad4 (25), where they either directly or indirectly activate transcription through association with Smad response elements (26, 27). Through unknown mechanisms, TGF-β receptors also activate members of the mitogen-activated protein kinase family, which together with activated Smads, are critical for the mediation of growth arrest and apoptosis induced by TGF-β (28).

Loss of response to TGF-β occurs in virtually all cancers examined, including prostate cancer, and is common between adenoma and carcinoma transitions (29). Loss of such response during carcinogenesis has been shown to occur from mutations in TGF-β receptors, loss of TGF-β receptor expression, mutations in Smads, or activation of oncogenes that interfere with TGF-β signal transduction (30, 31). Aquisition of resistance to TGF-β during prostatic carcinogenesis correlates well with loss of TGF-β receptor expression (32–34).

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[3] The abbreviations used are: AR, androgen receptor; TGF, transforming growth factor; TβR, TGF-β1 receptor; DNR, dominant-negative TβRII; AP1, activator protein-1; SRE, serum response element; CRE, cyclic AMP response element; RT-PCR, reverse transcription-PCR; FBS, fetal bovine serum; CS, calf serum; DC-FBS, dextran-coated, charcoal-stripped FBS; EGF, epidermal growth factor; CT, cholera toxin; Dex, dexamethasone; RA, all-*trans*-retinoic acid; KGF, keratinocyte growth factor; HGF, hepatocyte growth factor; bFGF, basic fibroblast growth factor; βNGF, nerve growth factor; DHT, dihydrotestosterone; PAP, prostatic acid phosphatase; HA, hemagglutinin; OH-D_3, 1,25-dihydroxyvitamin D_3; NF-κB, nuclear factor κB; CMV, cytomegalovirus; TBST, TBS +0.1% Tween 20.

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Androgens, which promote the growth and viability of prostatic epithelium, negatively regulate TGF-β ligand and receptor expression and Smad activation through unknown mechanisms (35–39). Our laboratory recently showed that androgens can block TGF-β signaling through the association of AR with Smad3, leading to repression of Smad3-mediated transcriptional activation (39).

In this report we describe the development and characterization of a new rat dorsal prostatic cell line, DP-153. This line was developed with essentially identical methods used for establishing the NRP-152 line, except that in the current study, the horns of the dorsal prostate were used rather than the dorsal-lateral lobes (1). DP-153 cells are exquisitely sensitive to many hormones and growth factors and have a stringent growth factor requirement like NRP-152 cells. Also, similar to the NRP-152 line, DP-153 cells have a basal cell phenotype and do not form tumors in athymic mice. Significantly, blocking TGF-β signaling alone, by overexpression of DNR, causes malignant transformation of DP-153 cells, further supporting a critical role of TGF-β in tumor suppression of the prostate. We conclude that DP-153 cells are useful for studying the mechanism of tumor suppression by TGF-β in prostatic epithelial cells.

MATERIALS AND METHODS

Materials. Sources of materials were as follows: Lobund/Wistar rat, National Cancer Institute Animal Production Program (Frederick, MD); recombinant human TGF-β1, R&D Systems, Inc. (Minneapolis, MN); anti-cytokeratin 5 and 14 antibodies, Dr. Stuart Yuspa and Adam Glick; anti-cytokeratin 18 antibodies, Janet Woodcock-Mitchell; AP1-luciferase, NF-κB-luciferase, SRE-luciferase, CRE-luciferase, and p53-luciferase cis-acting PathDetect constructs, Stratagene (La Jolla, CA); DMEM:Ham's F-12 (1:1; v/v), CS (Invitrogen, Carlsbad, CA), insulin, bovine bFGF, and mouse EGF, BioSource International (Camarillo, CA); CT, Dex, RA, and βNGF, Sigma (St. Louis, MO); KGF, Dr. Jeffrey Rubin; HGF, Becton Dickinson (Franklin Lakes, NJ); OH-D_3, BioMol (Plymouth Meeting, PA); and characterized FBS and DC-FBS, HyClone Inc. (Logan, UT).

Development of DP-153 Cell Line. Lobund/Wistar rats were treated with *N*-methyl-*N*-nitrosourea (30 mg/kg body weight, injected into the intralingual vein). One week later, they received a s.c. implant of testosterone proprionate (50 mg) encased in a silastic capsule, and these implants were replaced at 2.5-month intervals. Seven months after initial carcinogen treatment, rats were killed by suffocation in carbon dioxide. The horns of the dorsal prostate were excised, washed extensively with sterile PBS, and placed in 60-mm dishes containing 2 ml of HBSS supplemented with 10% FBS, where tissue was minced to <1-mm fragments with fine scissors. Minced tissue was then distributed evenly among ten 80-mm^2 flasks, each with 15 ml of GM1 [DMEM:Ham's F-12 (1:1; v/v) supplemented with 10% FBS, 20 ng/ml EGF, 10 ng/ml CT, 5 μg/ml insulin, and 0.1 μM Dex]. Cells appeared to attach to plastic within a week, at which time the medium was replaced with fresh GM1 and then replaced similarly every 2 days thereafter. At 3–5-day intervals, fibroblasts were removed by treatment with trypsin-EDTA (2–10 min, 37°C), and the adherent epithelial monolayer was washed several times with PBS. This process was continued until a subconfluent monolayer of essentially 100% epithelial cells was obtained. These cells were then passaged after a 15-min treatment with trypsin-EDTA at 37°C and cultured in GM1 for the first four passages.

Extra care was taken at all stages of this procedure to ensure the absence of any cross-contamination of cells from other tissues or cell lines. This included (*a*) keeping separate fresh stock bottles and tubes of culture medium and trypsin for each culture and (*b*) working with only one culture at a time in the tissue culture laminar flow hood, separated by at least 20 min of laminar flow ventilation with UV light.

Culturing the Established Cell Line. The established DP-153 line was maintained in GM2 (GM1 with 5% FBS) in 80-cm^2 Nunc tissue culture flasks and passaged every 3–4 days at subconfluence, seeding at 2–5 × 10^5 cells/flask. For passaging, a flask of cells was treated with 5 ml of trypsin-EDTA (Invitrogen) for 10 min at 37°C. Cells were dislodged by gentle tapping, trypsin was neutralized with an equal volume of GM1, and cells were recovered after centrifugation (1000 × *g*, 4 min).

Athymic Mouse Studies. Cells trypsinized off near-confluent monolayers were washed once with DMEM:Ham's F-12 containing 10% FBS and twice with DMEM:Ham's F-12 by centrifugation, resuspended in DMEM:Ham's F-12 to a final cell density of 5 × 10^7 cells/ml, and kept at 4°C until transplantation in mice. Six-week-old intact male athymic (NC:NU) mice (5 animals/group) were inoculated s.c. with 200 μl/site on opposite flanks of each animal. Tumor growth (in volume) was monitored once or twice a week using precision calipers. Animal experiments were repeated three times.

Cell Proliferation Assays. DP-153 cells (passage 19–25) were plated in 12-well dishes at a density of 10^4 cells/1.0 ml/well with appropriate culture medium and incubated overnight for attachment. Cells were then incubated with a combination of mitogens and hormones as indicated. For cell enumeration, wells were washed with PBS and treated with 0.5 ml of trypsin/EDTA solution for 10 min. The detached cells were combined with 0.5 ml of 5% FBS + DMEM:Ham's F-12 and triturated with a 1.0-ml tip. Cell suspensions (0.5 ml) were transferred to cuvettes containing 9.5 ml of isotonic solution, and cell number was determined using a Coulter counter (Coulter Electronics, Inc., Hialeah, FL).

RT-PCR. Total RNA was prepared using RNeasy column (Qiagen, Valencia, CA) according to the manufacturer's instructions. Three μg of total RNA were used for generation of cDNA using Superscript reverse transcriptase (Invitrogen). The primers applied to detect AR expression were 5′-ATGAAGCTTCTGGGTGTCACTATG-3′ (forward) and 5′-TCACT-GGGTGTGGAAATAGATGGGCTT-3′ (reverse). PCR amplification was performed for 30 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 2 min. β-Actin served as an internal control, using primers 5′-TCGTCGTCGA-CAACGGCTCCGGCAT-3′ (forward) and 5′-CCTGCCAGGTCCAGA CG-CAGGATGG-3′ (reverse). The PCR products were electrophoresed through 0.7% agarose-TAE (10 mM Tris-acetate and 0.5 mM EDTA buffer) gels and visualized by ethidium bromide staining.

Transient Transfection and Luciferase Reporter Assays. Cells (8 × 10^4) were plated overnight in 12-well dishes and cotransfected with 1 μg of the indicated luciferase reporter construct and 12.5 ng of CMV-*Renilla* plasmid (CMV-PRL) using a calcium phosphate coprecipitate method for 3 h in 1 ml of GM2. Cells were washed once with PBS, glycerol shocked (15% glycerol in 1× HEPES-buffered saline) for 90 s, washed twice with PBS, and allowed to recover overnight in 1 ml of GM3 [DMEM:Ham's F-12 (1:1; v/v) supplemented with 1% CS, 15 mM HEPES, and 0.1 μM Dex (40)]. TGF-β was then added, and luciferase was measured 24 h later using Promega's Dual Luciferase Assay Kit and ML3000 Microtiter Plate Luminometer.

Retroviral Infection of DNR. DNR, consisting of nucleotides 1–687 encoding the entire cytoplasmic domain of TβRII together with seven amino acids of the intracellular domain followed by a stop codon, was expressed as a polycistronic message of neomycin resistance gene (*Neo*) in the pMFG vector (12). Infectious viral particles were generated by transfection of amphotropic packing cell line BOC 23 (12, 41) with pMFG-*neo* (control) or pMFG-DNR-*neo*. Transfection was done by calcium phosphate precipitation and cotreatment with chloroquine (100 μM) for 6 h, and viral supernatants (in DMEM:Ham's F-12 + 10% FBS) were collected every 12 h between 24 and 72 h and passed through a 0.4-μm-pore filter. DP-153 cells were plated in GM2 at 2.5 × 10^5 cells/100-mm dish and infected the next day with 3 ml of virus supernatant diluted 2-fold with GM2 and with a final concentration of 4 μg/ml protamine sulfate. After 24 h of infection, cells were washed with PBS, and was replaced with 10 ml of GM2 containing 200 μg/ml G418. After 8 days of selection, cells were maintained in GM2 + 100 μg/ml G418.

Western Blot Analysis. Cells were lysed at 4°C with ice-cold radioimmunoprecipitation assay buffer (PBS, 1% NP40, 0.1% SDS, and 0.5% sodium deoxycholate) containing 1 mM sodium orthovanadate, Complete Mini-EDTA-free Protease Inhibitor Mixture (Roche, Indianapolis, IN), and 1 mM phenylmethylsulfonyl fluoride, and lysates were clarified at 14,500 rpm for 20 min at 4°C. Protein concentration was measured using BCA test (Pierce, Rockford, IL), and 50 μg of protein were separated by electrophoresis through 4–12% NuPAGE BisTris gel (Invitrogen) with 1% 4-morpholinepropanesulfonic acid buffer (Invitrogen) and transferred to nitrocellulose membrane (Invitrogen). For detection of TGF-β receptors after deglycosylation, 20 μg of protein lysates were deglycosylated by incubation with peptide-*N*-glycosidase F according to the manufacturer's instructions (New England Biolabs, Beverly,

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MA). Deglycosylated samples were separated by electrophoresis through 4–12% NuPAGE BisTris gels (Invitrogen) in 4-morpholineethanesulfonic acid running buffer (Invitrogen) and transferred to nitrocellulose membrane (Invitrogen). After 1 h of blocking in TBS [10 mM Tris-HCl (pH 8.0) and 150 mM NaCl] + 5% nonfat dried milk, membranes were incubated with the indicated primary antibodies and appropriate horseradish peroxidase-conjugated secondary antibody (1:5,000; Jackson Laboratory, West Grove, PA) in TBST + 5% nonfat dried milk. Each incubation was followed by three 10-min washes with TBST. Signals were then detected by enhanced chemiluminescence (Pierce). Antibodies were anti-cytokeratin 5 (1:40,000), anti-cytokeratin 18 (1:40,000), anti-β-actin (1:5,000; Sigma), anti-HA (Y-11; 1:1000; Santa Cruz Biotechnology), and proliferating cell nuclear antigen (0.4 μg/ml; Oncogene Science, Inc.).

Northern Blot Analysis. Total RNA was purified using RNeasy columns (Qiagen). Ten μg of total RNA were electrophoresed through 1% agarose-0.66 M formaldehyde gels containing 0.72 μg of ethidium bromide. Equal loading was assessed by visualization of the 18S and 28S rRNAs. To facilitate equal and complete transfer of RNA, gels were treated with 60 mM NaOH for 20 min, neutralized with 50 mM Tris-HCl (pH 7.4) and 10 mM NaCl for 20 min, and blotted onto Nytran (0.45-μm pore) for 16–20 h with 10× saline-sodium phosphate-EDTA. Membranes were then cross-linked with UV light and hybridized to ^{32}P-labeled cDNA probes under Church hybridization conditions. The cDNA probes used for Northern blot hybridization were rat AR (1), rat PAP (1), dorsal protein-1 (42), SVS II (insert from pRWB vector), and probasin (insert from pM-40.1 vector). The pRWB and pM-40.1 constructs were gifts from Dr. Robert Matusik.

Karyotype and Isozyme Analysis. Karyotype and isozyme analyses were performed by Drs. Ward D. Peterson and Joseph Kaplan at the Cell Culture Laboratory, Children's Hospital of Michigan (Detroit, MI).

***Mycoplasma* and Viral Testing.** The absence of *Mycoplasma* contamination in DP-153 cells was confirmed by an indirect Hoechst method and by a direct agar method (American Type Culture Collection). DP-153 cells were shown to be free of the following viruses by the RAP test: Tolans H-1; GDVII (mouse encephalomyelitis virus); KRV (kilham rat virus); PRV (pseudorabies virus); Reo; Sendai; RCV/SDA (rat coronavirus/sialodacryoadenitis virus); and Hantaan (Microbiological Associates).

Immunohistochemistry. Slides containing paraffin-embedded tumor sections were baked for 1 h at 60°C and deparaffinized in xylene for 10 min twice and in 100% ethanol for 2 min twice. Sections were rehydrated in 95% and 70% ethanol and rinsed twice in distilled water for 2 min each. Antigen retrieval was then performed by placing slides in distilled water at 95°C for 30 s and incubating them in a steamer for 20 min with 10 mM sodium citrate buffer (pH 6.0) at 95°C. After 20 min of cooling to room temperature, slides were rinsed three times with distilled water and soaked in TBS for 3 min. Slides were then blocked with 10% donkey serum in TBS for 1 h at room temperature and incubated overnight at 4°C with 3% BSA + TBST containing 2 μg/ml of either rabbit preimmune IgG or rabbit anti-HA solution (Y-11; sc-805; Santa Cruz Biotechnology). After 5-min sequential washes twice with TBST and once with TBS, slides were incubated for 1 h at room temperature with 5% donkey serum + TBST containing 2.5 μg/ml Rhodamine Red-conjugated donkey antirabbit IgG (Jackson Laboratory, West Grove, PA) and then washed twice with TBST and twice with TBS (5 min each). After mounting with 4′,6-diamidino-2-phenylindole Vectashield medium (Vector Laboratories, Burlingame, CA), slides were examined for signal and triple beam fluorescence using a Nikon Eclipse E800 microscope (Nikon Inc., Melville, NY).

RESULTS

Derivation of DP-153 Cell Line. Lobund/Wistar male rats are well known to develop a high incidence of spontaneous neoplasias of the prostate, seminal vesicle, and possibly the ampulary gland (43–46). These animals were treated with the carcinogen methyl-*N*-nitrosourea and the promoting agent testosterone propionate, which significantly reduce the lag time and increase the incidence of such neoplasias. To obtain preneoplastic cells, the dorsal horns of preneoplastic prostates (7 months after carcinogen treatment) were minced to <1-mm fragments and placed in tissue culture under highly mitogen-enriched conditions (GM1 medium), as done to develop NRP-152 (1). We succeeded in establishing a cell line from one of the four rats used in this study. Explants of the ventral prostate, ampulary glands, and seminar vesicles from each of these rats were also cultured this way; however, none of these epithelial explants grew beyond a couple of passages. By passage 4, DP-153 cells were completely free from contaminating fibroblasts and displayed a uniform cobblestone-like morphology (Fig. 1), similar to primary cultures of normal prostatic epithelial cells (47, 48).

Karyotype and Isozyme Analysis. Karyotype and isozyme analyses at passage 8 indicated that the DP-153 line is of a male rat and aneuploid, with most chromosomes in the diploid range. All chromosomes could be assigned as normal. The most consistent feature of this cell line is trisomy or tetrasomy of chromosome 12. Trisomy of chromosome 7 was also observed in one of seven karyotypes. Monosomy was observed in chromosomes 14, 15, and 18 in three, one, and one of seven metaphases, respectively. Ninety-eight of 100 metaphases were observed to have 41–44 chromosomes (2N = 42), with two metaphases with 80+ chromosomes. A typical karyotype is shown in Fig. 2. Isozyme analysis showed that the electrophoretic mobilities of glucose-6-dehydrogenase, purine nucleoside phosphorylase, and malate dehydrogenase were comparable with that of a normal rat cell control preparation. No extra bands were observed on electrophoretic film that may suggest the presence of cells of different species in cell culture.

Expression of Cytokeratins, PAP, and the AR. DP-153 cells were stained with pan-cytokeratin antibody to confirm their epithelial phenotype. Our observation that 100% of these cells stained with pan-cytokeratins confirmed that the DP-153 cell line consists of a pure population of epithelial cells (data not shown). Further screening of these cells for expression of basal *versus* luminal cytokeratins was done by Western blot analysis, using NRP-152 and NRP-154 cells as positive controls for basal and luminal cells, respectively. Our data demonstrate that DP-153 cells are similar to NRP-152 cells because they express the basal cell cytokeratin, cytokeratin 5, but not the luminal cytokeratin, cytokeratin 18 (Fig. 3*A*). Further immunostaining analysis indicated that 100% of the DP-153 cells also stained positive for another basal cell cytokeratin, cytokeratin 14 (data not shown).

The prostatic markers PAP and AR were used to further characterize the DP-153 cell line. The expression of AR in DP-153 cells was shown by RT-PCR (Fig. 3*B*) and Northern blot (Fig. 3*C*) analyses. Both assays compared expression of these markers in DP-153 cells

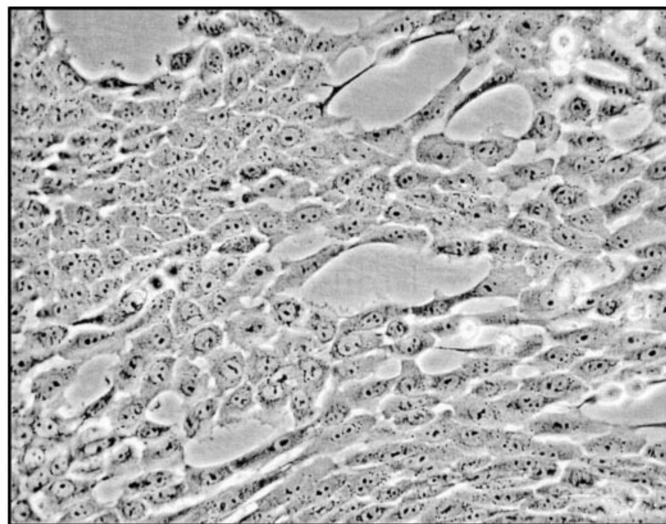

Fig. 1. Morphology of DP-153 cells grown on monolayer plastic in GM2.

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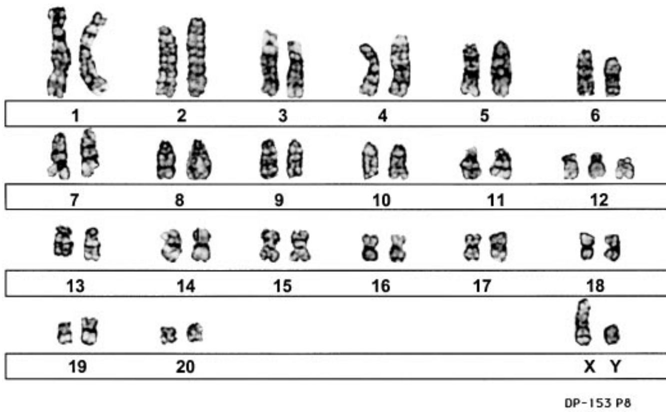

Fig. 2. Karyotype of a typical mitotic figure of DP-153 cells taken at passage 8.

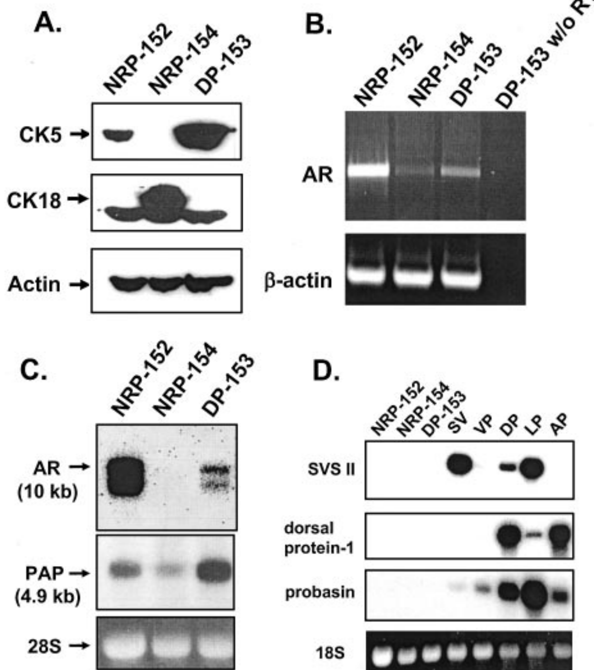

Fig. 3. Expression of luminal and basal epithelial markers, PAP and AR in NRP-152, NRP-154 and DP-153 cells. *A,* expression of cytokeratins 5 and 18 (*CK5* and *CK18*) in DP-153 cells was compared by Western blot analysis with the levels of these markers in NRP-152 and NRP-154 cells. Fifty μg of protein from whole cell lysate were subjected to Western blot analysis, using β-actin as a loading control. *B,* AR mRNA expression was determined by RT-PCR using primers that yield a 1-kb fragment. Total RNA (0.3 μg) extracted from DP-153 cells was subjected to RT-PCR, and the resulting products were resolved through 0.7% agarose gel. The level of AR in DP-153 cells was compared with the levels in NRP-152 and NRP-154 cells, using β-actin as loading control. *C,* expression of AR and PAP mRNAs in these cell lines was also measured by Northern blot analysis, using 10 μg of total RNA. *D,* expression of seminal vesicle secretory protein (*SVS II*), probasin, and dorsal protein I in prostate cell lines cultured for 4 days in GM2 with 10 nM DHT and in adult rat seminal vesicles (*SV*), ventral prostate (*VP*), dorsal prostate (*DP*), lateral prostate (*LP*), and anterior prostate (*AP*) was detected by Northern blot hybridization.

with the NRP-152 and NRP-154 prostatic cell lines. DP-153 cells express significantly higher levels of AR than NRP-154 cells but lower levels of AR than NRP-152 cells. Northern blot analysis also revealed that DP-153 cells express high levels of PAP mRNA relative to that of NRP-154, NRP-152 (Fig. 3*C*), or whole prostate (1).

We also screened DP-153 cells for the expression of mRNA for specialized secretory protein markers of the mature adult prostate, probasin, the seminal vesicle secretory protein II, and dorsal protein-1 (42, 49). Similar to NRP-152 and NRP-154 prostatic cell lines, the DP-153 line did not express any of these markers in monolayer plastic dishes (Fig. 3*D*). These results are consistent with lack of the expression of these markers in primary cultures of rat prostatic epithelium and without the proper mesenchyme (10, 50–52). However, expression of dorsal protein-1 occurred in tissue recombinants of NRP-152 cells with mouse urogenital sinus mesenchyme grown under the renal capsule of athymic mice (10). Thus, it is likely that expression of dorsal protein-1 by DP-153 cells, similar to NRP-152 cells, will require interaction with normal prostatic mesenchyme, appropriate extracellular matrix, and proper hormonal milieu.

Conditions for Optimal Growth of DP-153 Cells. DP-153 cells were unable to grow in DMEM:Ham's F-12 supplemented with 5% FBS alone and required additional growth factors and hormones for both their growth and maintenance. Thus, conditions optimal for the growth of these cells were monitored using components of GM2, which was used to develop both the DP-153 and NRP-152 cell lines. We first studied the contribution of each of the components in GM2 for growth of DP-153 cells after 4 days (Fig. 4). When added alone, none of them significantly stimulated cell growth, except for Dex. Data in Fig. 4 were further analyzed to assess the effects of each mitogen under the indicated conditions (data not shown). EGF worked synergistically with Dex, CT, or Dex + CT on promoting cell growth, whereas the inclusion of insulin blunted these synergistic effects of either Dex or CT. Insulin was mitogenic with Dex or CT alone, but not in the presence of EGF. Dex stimulated growth with all combinations of the above-mentioned mitogens, but the best stimulation by Dex occurred with EGF + insulin, and small stimulation occurred with CT or CT + insulin. CT was growth stimulatory in the presence of EGF or insulin, but not with Dex. Likewise, Dex also blocked growth by CT in the presence of insulin but not with insulin + EGF. These results point to a rather complex pattern of growth factor interaction and synergism in DP-153 cells.

Regulation of DP-153 Cell Growth by Growth Factors and Steroid Hormones. We assayed for mitogenic effects of other growth factors believed to also function in the prostate. KGF was highly mitogenic for DP-153 cells in DMEM:Ham's F-12 supplemented with 5% FBS, whereas other growth factors tested here did not promote growth under these conditions (data not shown). The above-mentioned growth factors were also tested in medium with 5% FBS, insulin, Dex, and CT. Under this condition, KGF markedly enhanced DP-153 cell growth, and EGF and bFGF stimulated a 2-fold increase

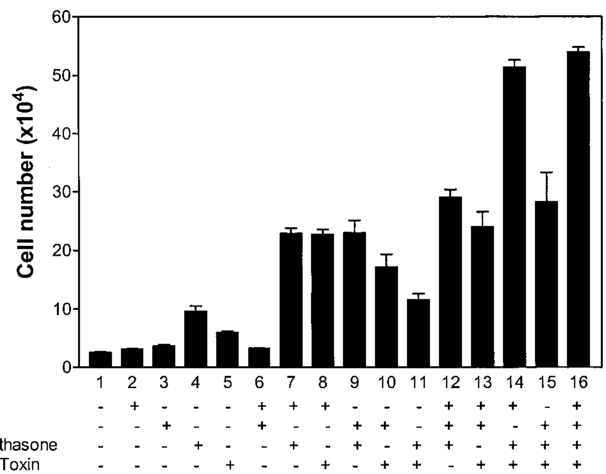

Fig. 4. Growth of DP-153 in response to regulatory molecules. *A,* growth of DP-153 cells in DMEM:Ham's F-12 containing 5% FBS with all combinations of 20 ng/ml EGF, 5 μg/ml insulin, 0.1 μM Dex, and 10 ng/ml. Cell number was measured after 4 days using a Coulter counter as detailed in "Material and Methods." Each *bar* represents the mean ± SE of triplicate determinations.

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in cell number. Neither HGF nor βNGF was mitogenic for these cells in this experiment (Fig. 5*A*).

Attempts to demonstrate growth effects of steroid hormones were performed at day 5 in DMEM:Ham's F-12 with 5% DC-FBS alone or with EGF + insulin. Ten nM dihydrotestosterone, progesterone, testosterone, and β-estradiol did not alter growth of DP-153 cells, whereas 100 nM Dex resulted in a 3-fold increase in cell number over control (data not shown). To explore whether growth response of DP-153 cells to androgens was masked by the above-mentioned factors or required additional factors, we examined androgenic effects in the presence or absence of various components of GM2. DP-153 did not grow in response to 10 nM DHT, even when treated daily under a variety of phenol red-free conditions (Fig. 5*B*), in contrast to stimulation of NRP-152 cell growth examined in parallel (Fig. 5*C*).

Growth Inhibition by RA, OH-D_3, and TGF-β1. Retinoids and OH-D_3 inhibit cell proliferation and have been shown to prevent the development of prostate cancer in rodent models (43). Here we show that 5 days of treatment with 100 nM RA or OH-D_3 significantly inhibited growth of DP-153 cells stimulated by EGF + insulin under conditions depleted of steroid hormones, DMEM:Ham's F-12 + 5% DC-FBS (Fig. 6*A*). We also observed a substantial amount of cell death by these treatments (data not shown). Because our cell number determinations do not distinguish between live and dead cells, we estimate the number of remaining live cells is substantially lower than

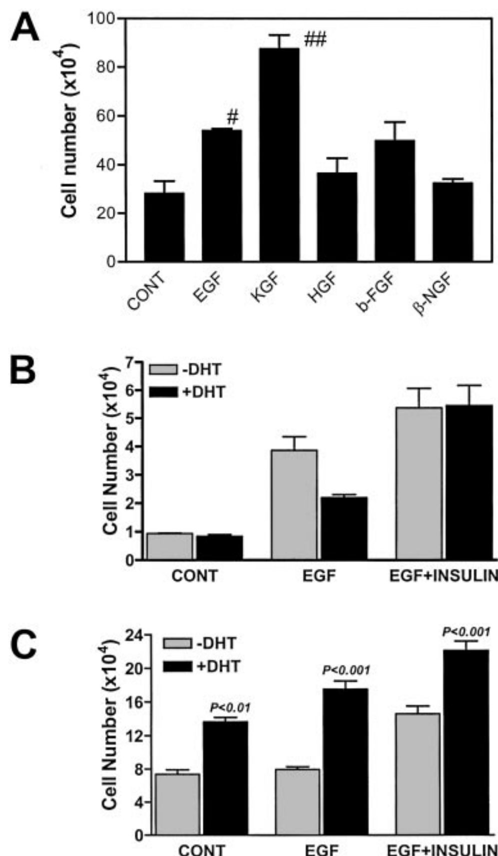

Fig. 5. Growth of DP-153 cells in response to growth factors and hormones. The effect of growth factors including 20 ng/ml EGF, 10 ng/ml KGF, 10 ng/ml HGF, 10 ng/ml bFGF, and 10 ng/ml βNGF on growth of DP-153 cells was determined after 4 days in DMEM:Ham's F-12 containing 5% FBS, 5 μg/ml insulin, 0.1 μM Dex, and 10 ng/ml CT (*A*). The effect of DHT on DP-153 (*B*) and NRP-152 (*C*) cell growth was examined after 7 days in the absence or presence of 10 nM DHT in phenol red-free DMEM:Ham's F-12 supplemented with 5%DC-FBS or with 5% DC-FBS ± 20 ng/ml EGF ± 5 μg/ml insulin. Each *bar* represents the mean ± SE of triplicate determinations. $P < 0.05$ (#) and $P < 0.001$ (##) were considered statistically significant compared with control. Each *point* represents the mean ± SE of triplicate determinations.

the total number scored for in the RA or OH-D_3 groups. The latter response is consistent with high expression of vitamin D receptor mRNA in DP-153 cells relative to rat prostate or NRP-152 cells (data not shown). Further work is under way to better characterize these responses.

We have shown that TGF-β1 is a potent inhibitor of the proliferation of nontumorigenic prostatic epithelial cells (1). We first tested the effect of TGF-β on growth of DP-153 cells in DMEM:Ham's F-12 supplemented with 5% FBS supplemented with three to four of the following mitogens: EGF; insulin; Dex; and CT (Fig. 6*B*). After 4 days of treatment, 10 ng/ml TGF-β1 inhibited growth of these cells optimally in the presence of EGF + insulin + CT. When tested in 5% DC-FBS instead of 5% FBS, the effect of TGF-β1 on cell growth was more pronounced (data not shown). We next assessed the effect of 10 ng/ml TGF-β1 on growth inhibition of DP-153 cells as a function of time over the course of 6 days and as a function of TGF-β1 dose at 6 days of treatment under the above-mentioned optimal conditions, except that DC-FBS was used instead of FBS (Fig. 6, *C* and *D*). As shown, response to TGF-β was substantially greater at day 6 relative to day 4, and inhibition of growth was several-fold greater from 0.1–1 ng/ml than between 10 and 30 ng/ml TGF-β1. For comparison with conditions shown to be optimal for growth inhibition by TGF-β in NRP-152 cells (53), effects of TGF-β on growth of DP-153 cells were also examined as described above in DMEM:Ham's F-12 + 1% CS +0.1 μM Dex (Fig. 6, *E* and *F*). Although cell growth was substantially less under these conditions, the overall effect of TGF-β on inhibition of growth was of similar magnitude and occurred at a lower concentration than that seen in Fig. 6*D*. Under both sets of conditions (Fig. 6, *C*–*F*), the effects of TGF-β were also biphasic, with several-fold less inhibition at 10–30 ng/ml than at 0.1–1 ng/ml TGF-β1.

Regulation of Reporter Activity by TGF-β. DP-153 cells can be transfected with cDNA expression constructs using either LipofectAMINE plus or calcium phosphate precipitation methods. To study the possible signal transduction pathways activated by TGF-β1 in DP-153 cells, we transfected them with various PathDetect cis-acting basic promoter element-luciferase reporter constructs, using a standard calcium phosphate transfection protocol. Cells were treated with 10 ng/ml TGF-β1 the next day, and luciferase was measured 24 h later. Our results show that TGF-β1 activates several reporter constructs including AP1-luciferase, NF-κB-luciferase, 3TP-Luciferase, and SRE-luciferase, but not p53-luciferase or CRE-luciferase, in DP-153 cells (Figs. 7 and 8*C*). These data suggest that TGF-β activates several TGF-β signaling pathways that lead to the activation/induction of mitogen-activated protein kinase kinase kinase 1/c-Jun-NH_2-terminal kinase, NF-κB, and c-fos in these cells.

Malignant Transformation of DP-153 Cells by DNR. A role of TGF-β in tumor suppression of the prostate has been supported by a limited number of studies (12). Further confirmation using other cell lines and models remains critical to establishing the significance of this hypothesis. The DP-153 cell line was useful for this purpose because it is highly responsive to TGF-β and nontumorigenic in athymic mice. Retroviral particles carrying either pMFG or pMFG-DNR transduced DP-153 cells with high efficiency (approximately 80%). After selection with G418, expression of HA-tagged DNR in DP-153 cells was detected by Western blot analysis using anti-HA antibodies (Fig. 8*A*). Immunocytochemical analysis with HA antibody demonstrated that >90% of the stably transduced cells expressed DNR (data not shown); thus, cloning these cells was not necessary. The biological significance of DNR in blocking TGF-β effects was next tested by reversal of growth inhibition by TGF-β. DP-153 cells expressing DNR were virtually resistant to growth inhibition by 10 ng/ml TGF-β1 (Fig. 8*B*). In fact, the line expressing DNR grew faster than control cells, suggesting that DNR also blocked the effects of

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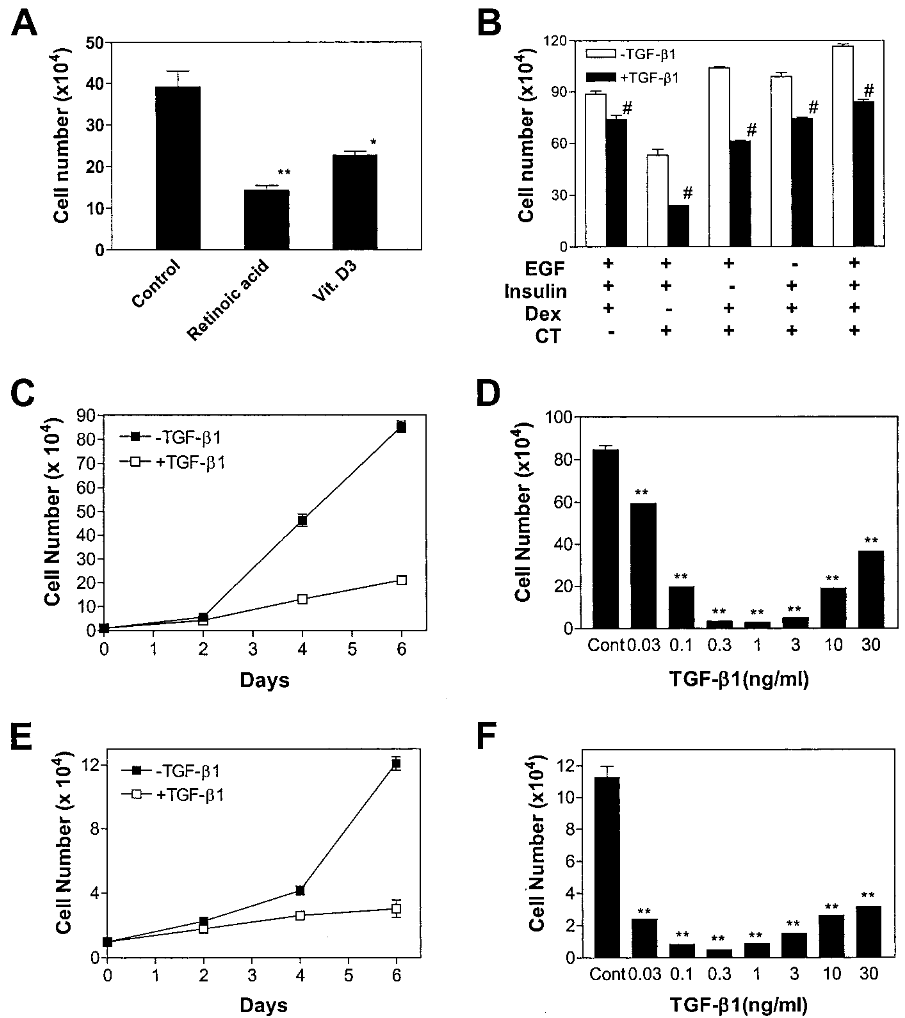

Fig. 6. Growth inhibition by RA, OH-D$_3$, and TGF-β1. *A,* growth inhibition was determined by cell number assay in DP-153 cells cultured for 5 days in the absence or presence of 100 nM RA or 100 nM OH-D$_3$ in DMEM:Ham's F-12 supplemented with 5% DC-FBS, 20 ng/ml EGF, and 5 μg/ml insulin. *B,* DP-153 cells were cultured for 4 days in the absence or presence of 10 ng/ml TGF-β1 with DMEM: Ham's F-12 and 5% FBS and supplemented with the listed combinations of 20 ng/ml EGF, 5 μg/ml insulin, 0.1 μM Dex, and 10 ng/ml CT. Effect of TGF-β1 on growth of DP-153 cells was determined as a function of time (*C* and *E*) using 10 ng/ml TGF-β1, and as a function of TGF-β dose (*D* and *F*) after 6 days under the following conditions. Cells were cultured in DMEM:Ham's F-12 supplemented with either 5% DC-FBS + 20 ng/ml EGF + 5 μg/ml insulin + 10 ng/ml CT (*C* and *D*) or 1% CS and 0.1 μM Dex (*E* and *F*). Each *point* represents the mean ± SE of triplicate determinations, and $P < 0.05$ (#), $P < 0.01$ (*), and $P < 0.001$ (**) were determined for statistically significant comparisons with control.

autocrine TGF-β on these cells. In contrast, control DP-153-pMFG cells were just as sensitive to inhibition of growth by TGF-β as was the noninfected line (Fig. 8*B*). For confirmation that loss of TGF-β in cells expressing DNR was not limited to growth effects, we measured activation of 3TP-Lux by TGF-β in these lines by transient transfection assays (Fig. 8*C*). The DNR line is resistant to activation of 3TP-Lux by TGF-β, in contrast to the control lines.

We next tested whether such resistance to TGF-β could be translated into acquisition of a malignant phenotype, similar to NRP-152 cells (12). Six-week-old athymic male mice were inoculated s.c. with 10^7 DP-153 cells (control or DNR). Cells expressing DNR formed tumors by 4–6 weeks, with an average doubling time of approximately 2 weeks (Fig. 9*A*). In three independent experiments, tumors appeared with 30% incidence as early as 4 weeks and with 60% incidence by 6 weeks in the DNR group. In contrast, there was no tumor growth in the control group (Fig. 9*A*). Histological analysis revealed that DP-153 tumors resulting from DNR were anaplastic, as indicated by large and pleomorphic nuclei, frequent mitotic figures, and prominent nucleoli in the epithelial compartment (Fig. 9*B*). The appearance of extensive squamous pearls underlying a layer of invasive epithelium is indicative of squamous carcinoma.

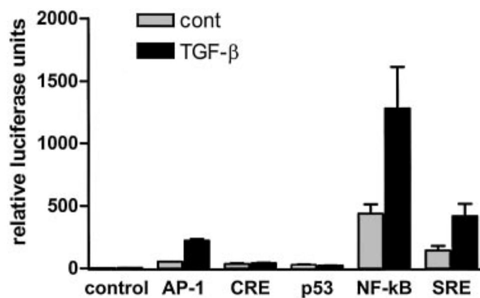

Fig. 7. Activation of cis-acting response element luciferase reporters by TGF-β in DP-153 cells. DP-153 cells plated in 12-well dishes in GM3 were transiently cotransfected with 12.5 ng of CMV-*Renilla* vector and 1 μg of either pLuc-MCS (control) or pLuc-MCS containing various response elements (AP-1, CRE, p53, NF-κB, and SRE). Cells were then treated the following day with either vehicle (control) or 10 ng/ml TGF-β1, and firefly luciferase and *Renilla* luciferase were assayed 24 h later using a dual luciferase assay kit (Promega) and a dual beam luminometer. Relative firefly luciferase values, normalized to *Renilla* luciferase, are the average ± SE of triplicate determinations.

We next confirmed that tumors from DP-153/DNR cells implanted s.c. were derived from DP-153 cells rather from the host and that DNR expression was retained in tumors, by both Western blot analysis (Fig. 10*A*) and immunohistochemical staining (Fig. 10, *B*–*E*) with anti-HA antibody. Lysates of DP-153 cells expressing either DNR or empty vector control and the above-mentioned DP-153 tumors were first treated with peptide-*N*-glycosidase F to deglycosylate DNR. Expression of the truncated receptor was then analyzed by Western blot using rabbit anti-HA IgG, and the blot was reprobed with anti-proliferating cell nuclear antigen as a loading control. As expected, nearly all of the anti-HA binding shifted toward the single M_r 25,000 deglycosylated DNR in both the DNR-expressing DP-153 cells and tumors, and vector control DP-153 cells did not express this

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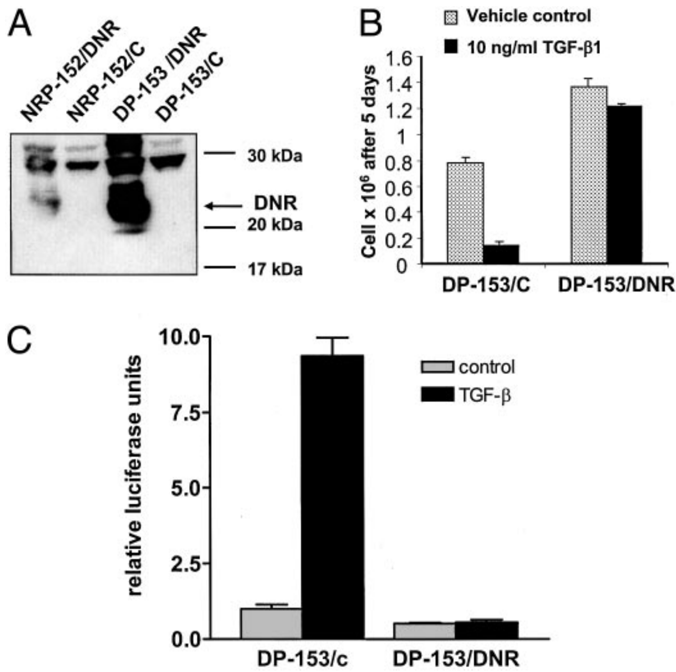

Fig. 8. Blocking of TGF-β responses by retroviral expression of DNR in DP-153 cells. DP-153 cells were infected with retroviral supernatants expressing pMFG or pMFG-DNR and then selected with G418 as detailed in "Materials and Methods." *A,* expression of DNR in infected cells after selection with G418 was determined by Western blot analysis using an HA antibody because the DNR is HA tagged. DP-153 cells expressing DNR are resistant to growth suppression by TGF-β after 5 days of treatment (*B*) and the activation of the 3TP-Lux (TGF-β-responsive promoter reporter construct) after 24 h of treatment with 10 ng/ml TGF-β1 in GM3 (*C*).

band (Fig. 10*A*). Immunohistochemical staining of DNR was also conducted with both DP-153 cells and tumors by formalin fixation followed by antigen retrieval using a steam-based protocol (see "Materials and Methods"). Representative tissue sections containing normal and tumor tissue are shown (see Fig. 10*B* for H&E staining). HA staining, detected by Rhodamine Red immunofluorescence, was very intense in DP-153 tumor epithelium (Fig. 10, *B* and *D*). Adjacent normal tissue (stroma) staining with anti-HA (Fig. 10, *B* and *D*), if any, was very faint and equivalent to control IgG alone (Fig. 10*E*). To further support the specificity of this staining, we subjected control and DP-153/DNR cells to this entire staining protocol, and observed similar intensity of staining with anti-HA in DP-153/DNR, with no specific staining in DP-153/C cells (data not shown). Together, these results confirm that the tumor epithelium retained for expression of DNR and thus originated from DP-153 cells and not from host epithelium.

DISCUSSION

Progress in prostate cancer research has been greatly limited by the availability of suitable cell lines, both tumorigenic and nontumorigenic ones. Because rodents are fundamental to *in vivo* models of prostate cancer, the mechanistic translation of these models to the human disease requires rodent cell lines. Here we report the development of a spontaneously immortalized and nontumorigenic prostatic epithelial cell line we named DP-153, derived from the preneoplastic dorsal prostate of the Lobund/Wistar rat. Importantly, DP-153 cells express markers of normal prostatic epithelial cells and are exquisitely sensitive to a variety of growth factors and hormones. Of particular interest to us are high sensitivities of DP-153 cells to EGF, TGF-β, RA, and OH-D_3. We show that TGF-β functions as a tumor suppressor of DP-153 cells because blocking TGF-β responses by expression of DNR promotes their malignant transformation. Thus, these findings further support that loss of TGF-β receptor function, observed to occur in human prostate cancer (33, 34), may play a role in the etiology of prostate cancer. Consistent with our model, transgenic mice overexpressing DNR targeted to the prostate exhibit hyperproliferation and reduced apoptosis relative to normal controls (54) and are likely sensitized to carcinogenesis of the prostate.

The stringent growth factor requirement of DP-153 cells and their high sensitivity to TGF-β are consistent with their *in vivo* nontumorigenic phenotype. Analysis of interactions between various mitogens (Figs. 4 and 5) clearly illustrates that certain mitogens can be either permissive for or inhibitory to the effects of other mitogens. For example, EGF or insulin alone had no effect on the growth of DP-153 cells, whereas each was mitogenic in the presence of Dex or CT. On the other hand, the ability of Dex or CT to synergize with EGF was greatly blunted in the presence of insulin and *vice versa*, suggesting that EGF and insulin stimulate growth of these cells through overlapping pathways.

Although DP-153 cells have ARs, we have not yet detected a growth-stimulatory response to androgens under several conditions used in this study. Our preliminary data suggest that androgens may instead inhibit their growth under certain conditions (*i.e.*, in the presence of EGF). Further work awaits determination of whether these cells can be growth stimulated by androgens under a different set of conditions, or whether androgens can regulate gene expression in these cells. Candidates for androgen regulation of interest to us include TGF-βs, TGF-β receptors, and Smads, as shown *in vivo* (38, 39). Our laboratory has recently shown that the ligand-occupied AR can inhibit TGF-β transcriptional responses through association with Smad3, preventing association of Smad3 to Smad binding element (39). Thus, it is likely that this can contribute to the greater sensitivity of growth suppression of DP-153 cells to TGF-β in the presence of charcoal-stripped serum compared with normal serum.

Even though the DP-153 cell line shares many characteristics with the NRP-152 cell line (basal cytokeratins, nontumorigenic phenotype, and high responsiveness to growth factor), their karyotype and relative

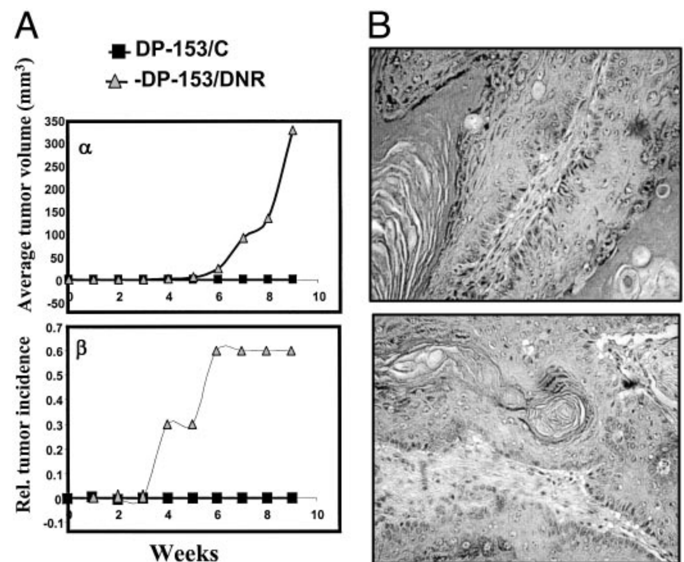

Fig. 9. Malignant transformation of DP-153 cells by expression of DNR. DP-153 cells infected with either empty pMFG or pMFG-DNR were transplanted s.c. in 6-week-old athymic male mice, using 10^7 cells/transplant, 10 transplants/group, and 2 transplants/mouse. *A,* tumor growth (volume and incidence, α and β, respectively) was monitored weekly, measuring tumor volume with precision calipers. *B,* histological analysis (hematoxylin staining) of two representative tumors is shown. Data shown are representative of three independent experiments conducted similarly.

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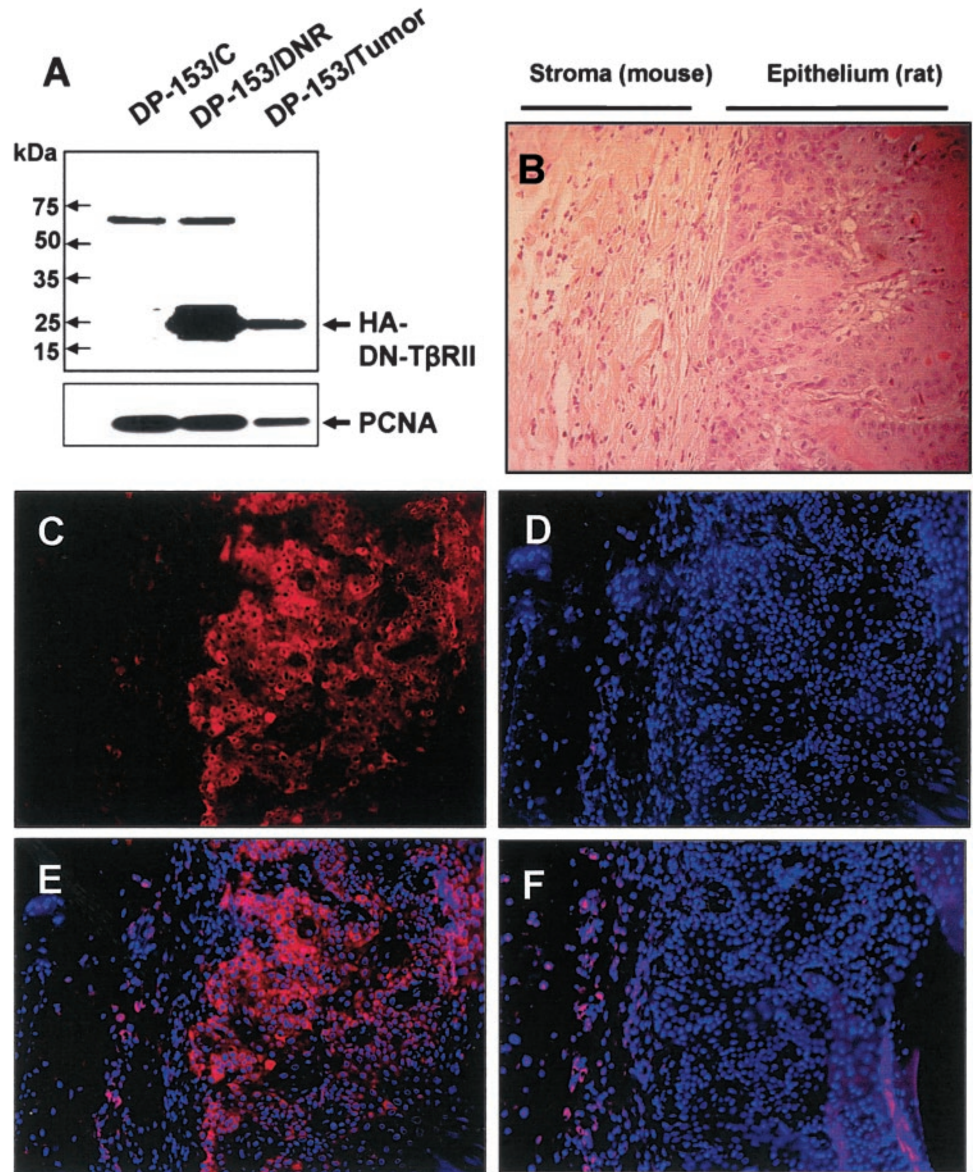

Fig. 10. DP-153 tumors retain expression of DNR. Expression of DNR in the DP-153 cell lines and in the resulting DP-153 tumors was demonstrated by (*A*) Western blot analysis of glycosidase-treated cell and (*B*−*F*) tissue lysates using anti-HA antibody and by immunofluorescence after antigen retrieval. To distinguish mouse from rat tissues in these tumors, a parallel section containing both stroma (mouse) and epithelium (rat and/or mouse) as demonstrated by H&E staining (*B*) was stained for expression of HA-DNR with rabbit anti-HA IgG [or control IgG (*F*)] + Rhodamine Red-conjugated donkey antirabbit IgG, nuclei counterstained with 4′,6-diamidino-2-phenylindole and observed for fluorescence by pseudo-confocal fluorescence microscopy using either single beam (*C* and *D*) or dual beam (*E* and *F*) filters.

levels of AR, cytokeratin 5, and PAP distinguish these cell lines apart. For example, NPR-152 cells have essentially a triploid karyotype (1), whereas DP-153 cells are essentially diploid. Although NRP-152 cells express more AR than DP-153 cells, while the latter cells express much higher levels of cytokeratin 5 and PAP, and DP-153 cells do respond to androgens with increased proliferation under conditions that promote growth response of NRP-152 cells to androgens.

Response to TGF-β is highly dependent on culture conditions in DP-153 cells, as in other cell lines. Such differences may reflect changes in inactivation of Smads by mitogen-activated protein kinase kinase kinase 1 or Ras activated by growth factors (55–57). Our dose-response data suggest that the effect of TGF-β on growth suppression is biphasic in DP-153 cells. It is possible that high concentrations of TGF-β induce a mitogenic response over an inhibitory response occurring chiefly at lower doses. This biphasic response may also result from negative feedback loops, such as down-regulation of TGF-β receptors or induction of Smad7 and NF-κB (53, 54) at the higher TGF-β levels.

Infection of DP-153 cells with pMFG-DNR was very efficient, uniformly expressed, and caused complete loss of growth inhibition and 3TP-luciferase activity by TGF-β. The induction of tumors in cells expressing DNR is consistent with our previous results in NRP-152 cells (12), further strengthening our model that endogenous TGF-β functions as a tumor suppressor of prostatic epithelial cells. Although the mechanism of tumor suppression by TGF-β in this model system cannot be determined at this point, DP-153 cells offer a unique and powerful tool to approach this goal. Data from the cis-acting PathDetect reporter constructs (Fig. 7) suggest that tumor suppression by TGF-β in this model may be independent of signals that lead to the activation of CRE and p53, but may involve the activation of AP1, c-Jun-NH_2-terminal kinase, NF-κB, or c-fos by TGF-β.

An important and remarkable property of DP-153 cells is the relative ease by which they can be transfected or infected with cDNAs, promoting a significant proportion of cells stably expressing high levels of a desired protein. This property, which is unlike other prostatic epithelial cell lines, should greatly facilitate the success of studying the molecular mechanisms of signal transduction and prostatic carcinogenesis.

Histological analysis revealed that DP-153-DNR were squamous carcinoma. Although squamous carcinomas have been reported to account for 0.5–1% of primary tumors of the prostate in humans (58,

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59), this incidence may be underestimated due to poor detection from lack of association with elevated blood levels of prostate-specific antigen or PAP (59, 60). Squamous prostate tumors, which are usually highly malignant and refractory to conventional therapy (58, 60–62), are often observed after hormonal and radiation treatment of classical adenocarcinoma of the prostate (63–65). Thus, although the cellular origin of squamous carcinomas of the prostate is not clear, such neoplasias are likely to originate by differentiation from adenocarcinomas, and may be on the incline due to increased radiation seed therapy. Whether DP-153 cells in culture have squamous cell properties or differentiate toward this phenotype upon stimulation by s.c. morphogens when implanted s.c. remains to be determined. The latter possibility would be likely if DP-153 cells resemble NRP-152 in having stem cell-like behavior (10, 11). Interestingly, NRP-152 tumors induced by expression of DNR were mixed adenocarcinoma and squamous carcinoma (12). Additional studies on implantation under the renal capsule will reveal whether DP-153 cells in tissue recombinants with urogenital sinus mesenchyme are able to form prostatic organelles, similar to NRP-152 cells (10, 11).

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