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Progression to Androgen Insensitivity in a Novel *In Vitro* Mouse Model for Prostate Cancer

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We have shown previously that the *ras* and *myc* oncogenes can induce poorly differentiated mouse prostate carcinomas *in vivo* with high frequency (greater than 90%) using inbred C57BL/6 mice in the mouse prostate reconstitution model system. To study the androgen sensitivity of these carcinomas, we have developed an *in vitro* model system which includes a cell line from normal urogenital sinus epithelium (CUGE) and cell lines from three *ras + myc* transformed mouse prostate carcinomas (RM-9, RM-1, and RM-2). CUGE cells, as well as all prostate carcinoma cell lines, were positive for cytokeratin 18 mRNA and immunoreactive to cytokeratin-specific antiserum. Two out of three of the early passage carcinoma cell lines were clonal with respect to Zipras/myc 9 retrovirus integration as determined by Southern blot analysis. Whereas significant mitogenic effects of testosterone (10 nM) were not seen in CUGE cells grown in serum-free medium, under similar conditions approx. 2-fold increases in cell number were seen in all low passage prostate carcinoma cell lines. Also, in the presence of growth inhibitory levels of suramin (50 μg/ml), testosterone was capable of significant growth stimulation in the carcinoma cell lines. With further propagation from low passage [20–25 population doublings (PD)] to high passage (75–100 PD), all carcinoma cell lines demonstrated increased and similar growth rate in the presence and absence of testosterone. These cell lines maintained stable androgen receptor numbers and binding kinetics during the transition from testosterone-responsive growth to reduced responsivity over multiple passages in culture (> 150 PD). Overall, our studies indicate that the capacity to bind testosterone is stably maintained through the transition of the androgen-sensitive to insensitive phenotype and raise the possibility that androgen sensitivity can persist throughout progression but is masked by the acquisition of autocrine pathways.

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INTRODUCTION

Treatment of locally advanced prostate cancer, the most commonly diagnosed cancer in U.S. men [1], is restricted almost exclusively to surgical or chemical castration. In the majority of patients castration produces a period where progression of the disease is apparently suppressed, but this response is temporary, with a mean duration of approx. 18 months [2, 3]. In part, the inability to treat these tumors more successfully is due to a lack of information concerning the evolution of these tumors from androgen sensitivity to insensitivity. *In vivo* studies using a transplantable rat prostate cancer model have demonstrated that castration can induce the selection of androgen independent prostate cancer cells [4]. Recent studies using a mouse model demonstrated that castration therapy can induce the expression of a panel of apoptosis-related genes in the absence of increased levels of apoptosis activity or reduced rate of proliferation [5]. These studies indicated that these castration-induced biological activities, which are normally linked, can be dissociated in prostate cancer [5]. Additional studies that address androgen sensitivity and subsequent loss of

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Abbreviations: BSA, bovine serum albumin; PBS, phosphate buffered saline; MPR, mouse prostate reconstitution; FCS, fetal calf serum; UGS, urogenital sinus; HEPES, *N*-[2-hydroxyethyl]piperazine-*N*1[2-ethanesulphonic acid]; SFM, serum free media; R1881, methyltrienolone.

androgen sensitivity of prostate cancer under highly controlled *in vitro* conditions have been hampered by the paucity of androgen responsive tumor cell lines and/or model systems. Until now, it has not been possible to study, in a comparative fashion, testosterone sensitivity and the transition between androgen sensitivity to insensitivity in non-transformed epithelial cells and prostate cancer cells derived from a common tissue type as occurs in human disease.

To address this issue we have established an *in vitro* model system including a non-tumorigenic cell line (CUGE cells) from normal urogenital sinus epithelium (UGE) and three prostate cancer cell lines (RM-9, RM-1 and RM-2) derived from tumors initiated using the mouse prostate reconstitution (MPR) model system. Under the influence of androgens, UGE tissues used to derive CUGE cells normally undergo growth and morphogenesis that culminates in fully differentiated ductal and acinar prostatic epithelia [6, 7]. Prostatic morphogenesis is not inhibited by the removal and dissociation of these cells from day 17 fetuses and subsequent genetic manipulation using recombinant retroviral vectors. This capacity led to the development of the mouse prostate reconstitution model system which allows for the initiation of prostate carcinogenesis *in vivo* via transduction of *ras* and *myc* oncogenes using the recombinant retrovirus Zipras/myc 9 [8–10]. This model system produces mono- or oligo-clonal prostate carcinomas with respect to retrovirus integration in greater than 90% of experiments using C57BL/6 mice. Uninfected or non-oncogenic recombinant retrovirus-infected MPRs have been previously shown to differentiate into normal prostate tissue as evaluated by morphological criteria as well as expression of epithelial cell-specific and prostate-specific antigens [8–10]. Epithelial cell-specific antigens expressed in Zipras/myc 9-induced carcinomas include cytokeratin 18 suggesting that these malignancies arise from luminal epithelial cells. This finding is in general agreement with cytokeratin profiles of human prostate cancer [11, 12]. In addition, primary carcinomas produced in this manner are androgen sensitive *in vivo* (unpublished observation) and closely mimic the human disease in many respects. For example, these tumors demonstrate elevated levels of transforming growth factor-β1 (TGF-β1); reproducibly demonstrate a tetraploid phenotype; and in the early stages of progression exhibit morphological progression patterns similar to human prostate cancer [10, 13–16]. Recent studies further validate the use of *ras* and *myc* oncogenes for the experimental induction of prostate cancer. *Ras* mutations have been identified in up to 25% of primary human prostate cancer specimens [17–20] and elevated *myc* mRNA and protein levels have been demonstrated in the majority of cases of prostate cancer when compared to benign prostatic hyperplasia [21–23]. Although the MPR model allows for studies regarding the molecular and cellular determinants of carcinogenesis *in vitro*, biochemical/pharmacological studies that require highly controlled conditions are more difficult. The establishment of the *in vitro* model described here allows for comparison between the non-tumorigenic and tumorigenic phenotypes under conditions where direct effects of various growth factors and hormones can be studied. We previously demonstrated that the carcinoma cell lines used for these studies are highly tumorigenic and exhibit a stabilized wild-type p53 phenotype, characterized by elevated levels of wild-type p53 protein, suggesting that downstream activities of normal p53 are compromised [24]. In contrast CUGE cells are non-tumorigenic and have a normal p53 protein profile. However, CUGE cells express cytokeratin 18 mRNA indicating a common marker among these cells, normal prostatic luminal epithelial cells and prostate cancer cells induced *in vivo* by *ras* + *myc* in the MPR system [8]. Based on these preliminary studies we have pursued additional comparative studies concerning androgen regulation.

Previous studies suggest that in normal tissue androgen does not act directly on prostatic epithelium but rather through a paracrine pathway via the mesenchyme [25 and reviewed in 26]. However, there are some indications that in transformed prostatic epithelium, testosterone can produce a direct effect. For example, the LNCaP cell line which was isolated from a metastatic lesion of a human prostate tumor has been previously reported to be androgen sensitive *in vitro* as determined by thymidine incorporation and direct measurements of cell growth [27]. Similar results demonstrating a direct response to testosterone were reported for the Dunning R3327, a rat prostate cancer cell line, in monolayer culture [28]. However, it is difficult to evaluate the significance of these results in regard to the malignant phenotype in the absence of comparative data obtained from non-tumorigenic control cells.

In this paper we characterize the phenotype of non-tumorigenic CUGE cells as well as RM-9, RM-1 and RM-2 prostate cancer cells with respect to androgen sensitivity. Although CUGE cells did not demonstrate a significant response to testosterone by changes in cell number, all three carcinoma cell lines were stimulated by androgen *in vitro* as indicated by direct measurement of increased cell number. Carcinoma cells were also androgen sensitive in the presence of suramin. After multiple cell divisions the response to androgens is apparently diminished in these cells, mimicking human tumor behavior *in vivo*. Radioligand binding studies indicate the stable presence of androgen receptor in all isolated cell lines at both early and late passage number. These studies raise the possibility that a direct growth response to testosterone is related to the transformed phenotype in prostate cancer and document the use of this *in vitro* model system to study the progression to androgen insensitivity.

MATERIALS AND METHODS

Cell culture

Tumor tissues were derived from Zipras/myc 9 total UGS MPRs as previously described [24] and finely minced and plated in DMEM with 10% fetal calf serum, 10 mM HEPES and penicillin (100 U/ml) and streptomycin (100 μg/ml) in TC media. Cells were maintained with routine media changes and cultures were passaged by trypsinization (0.025% trypsin) approx. every 7 days. Parallel cultures of normal uninfected urogenital epithelial cells were established after manual separation of epithelium and mesenchyme from day 17 fetal urogenital sinuses derived from C57BL/6 mice. Epithelial ductules were plated in collagen I coated flasks in a chemically defined media which consisted of Ham's F12 supplemented with 5 μg/ml insulin, 40 ng/ml epidermal growth factor, 0.5 μg/ml hydrocortisone, extract, 10 mM HEPES, 100 U/ml penicillin and 100 μg/ml streptomycin (EC media [29]). Cell cultures were maintained with routine media changes and passaged by trypsinization as above every 10–14 days. All chemicals for cell culture were obtained from Sigma Chemical Co. (St Louis, MO) with the exception of bovine pituitary extract which was obtained from Collaborative Research Biochemical Products, Inc. (Bedford, MA).

Southern blot analysis

Southern blot analysis was performed as previously described [8]. Briefly, high molecular weight DNA isolated from cell lines was digested with BglII and electrophoresed through 0.8% agarose gels. Following denaturation, DNA was transferred to Zeta-probe membranes (Bio-Rad Labs, Richmond, CA) and UV crosslinked. Membranes were then probed with a 700 bp *Bam*HI fragment from Zipras/myc 9 to determine clonal status of tumor cell lines with respect to virus integration [8, 10].

Northern blot analysis

RNA was isolated from frozen tissues by the guanidinium isothiocyanate method [9] and electrophoresed through 1% agarose–formaldehyde gels. RNA was then transferred to Hybond N (Amersham) by capillary transfer after which filters were sequentially hybridized with random primed ^{32}P-labeled cDNA fragments. Probes included a 1.5 kb *Hind*III fragment containing the cDNA for cytokeratin 18 (Endo B) [30] and a 1.5 kb Pst fragment of the human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA [31].

Immunocytochemical characterization of cultures

Immunocytochemical staining was performed as previously described [32–34]. Isolates of normal epithelial and tumor derived cells were fixed in prechilled (−20°C) absolute methanol/acetone (1:1) and then characterized using specific antibodies to cytokeratins, desmin and vonWillebrands factor (all obtained from DAKO Corporation, Carpinteria, CA) in order to identify vascular smooth muscle, mesenchymal or endothelial cell contamination of cultures.

Growth kinetics studies

Cells were plated at a density of 4 × 10^4 cells/cm^2 in their own media (either EC media or TC media). 24 h after plating cells were cultured within basal media (F12 for CUGE and DMEM for tumor derived cells) with 0.1% BSA in the presence and absence of varying amounts of testosterone, suramin or androgen receptor antagonists (cyproterone acetate, hydroxyflutamide and ICI 176334 or Casodex [35]; kindly provided by Dr Marco Marcelli, Baylor College of Medicine). Controls received the appropriate volume of vehicle only. At this time one plate of each cell type was trypsinized and cell number determined using a Coulter counter model ZF. Further cell number determinations were performed on the remaining cultures every 48 h [36].

In vivo tumorigenicity assay

Assays were performed essentially as described [5, 13]. Cultured cells were assessed for viability using trypan blue and 4 × 10^6 viable cells were injected s.c. in the flanks of adult hosts. Tumors were measured using vernier calipers and the presence of tumors was confirmed by hematoxylin and eosin staining of formalin fixed tissues.

Binding of [^{3}H]R1881 to androgen receptors and competition of [^{3}H]R1881 with other steroids

Ligand binding was performed as previously described [37–39]. Confluent tumor cell monolayers were incubated for 24 h prior to binding in DMEM/BSA. Cell layers were then incubated for 90 min in the presence of decreasing concentrations of [^{3}H]methyltrienolone (R1881), in the presence and absence of unlabelled ligand (100 nM, and then rinsed repeatedly in PBS). Labeled ligand and receptors were solubilized in absolute ethanol. Scatchard analysis [40] was performed to determine receptor affinity (K_d) and number of binding sites per cell (B_{max}). For competition of androgen receptor sites with other steroids, cell layers were treated essentially as described above, except that [^{3}H]R1881 was used at a standard concentration (10 pM) and other androgenic and nonandrogenic steroids were used at concentrations ranging from 5 pM to 100 nM. Cell layers were incubated for 90 min in the presence of these steroids and washed, solubilized and counted as described above.

RESULTS

When separated mechanically and placed in culture, fetal UGS epithelial ductules attached to collagen type I coated dishes which then led to outgrowth of epi-

thelial cells. Staining of early passage CUGE cells with cytokeratin specific antiserum (Dako A575) [Fig. 1(A)] demonstrated that these cells were a pure population of epithelial cells uncontaminated by either endothelial cells as demonstrated by the absence of staining with monoclonal antibodies specific to vonWillebrands factor or mesenchymal cells/vascular smooth muscle cells as demonstrated by the absence of specific immunocytochemical staining using specific antibodies to desmin (data not shown).

Cell lines derived from Zipras/myc 9-induced carcinomas (RM-9, RM-1, and RM-2) were heterogenous in regard to morphology in culture, yet all were positive for the presence of cytokeratin by immunohistochemical analysis [Fig. 1(B–D, respectively)]. Northern blotting analysis of mRNA derived from these cells using a cDNA probe for mouse cytokeratin 18 (Endo B) [30] was consistent with the results of immunocytochemical staining and further suggests a luminal epithelium derivation of these cell lines (Fig. 2) [41, 42].

CUGE cells as well as RM-9, RM-1 and RM-2 cell lines were also evaluated for their ability to form

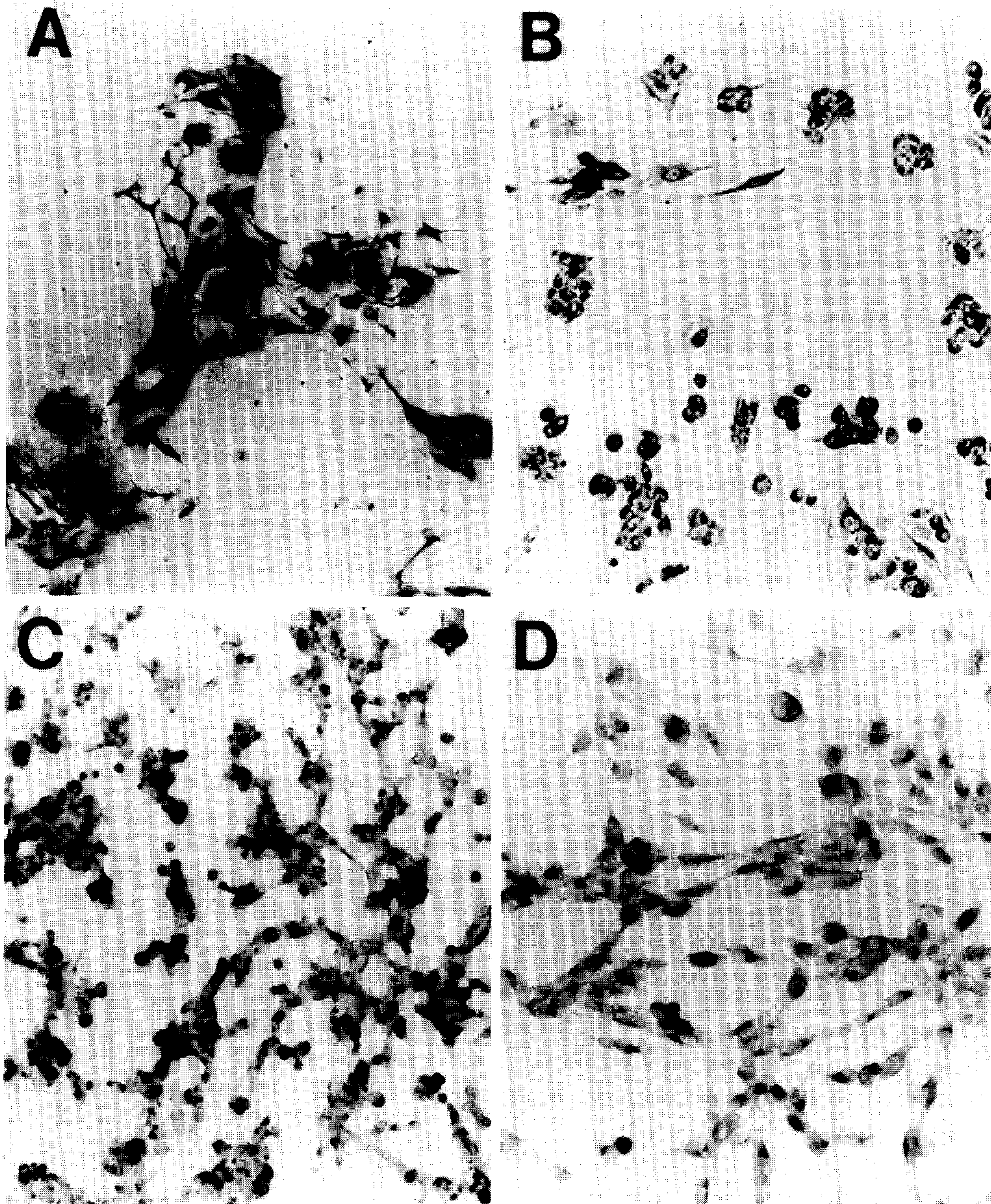

Fig. 1. Immunocytochemical staining of intermediate filaments in normal and transformed prostate cell cultures. Epithelial cells derived from normal tissue (CUGE, panel A) and tumor cells derived from Zipras/myc 9-infected MPRs (RM-9, RM-1, RM-2; panels B, C, and D, respectively) were cultured on collagen I coated multiwell plates, fixed as described and stained with a polyclonal antibody to cytokeratin (see methods).

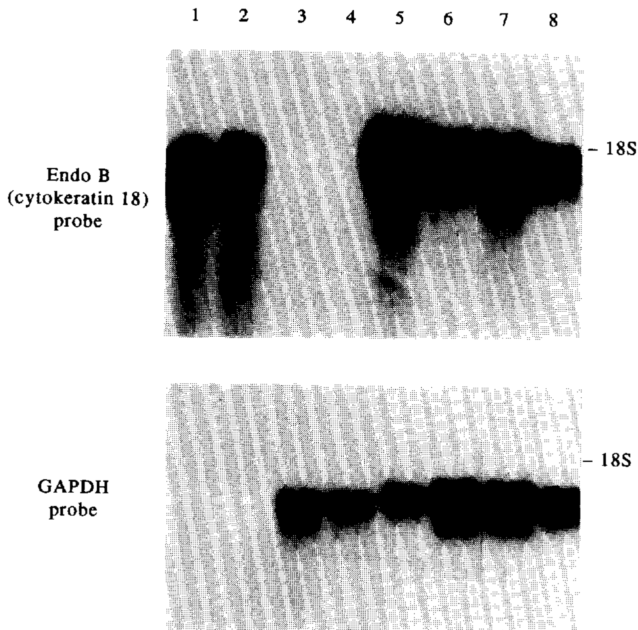

Fig. 2. Detection of cytokeratin mRNA in CUGE, RM-9, RM-1 and RM-2 cells by Northern blotting. Northern blots were probed with a 1.5 kb fragment from pUC9B7 containing the complete mouse cytokeratin 18 (Endo B) cDNA. RNA samples are as follows: normal adult C57BL/6 ventral prostate (lane 1); normal adult C57BL/6 anterior prostate (lane 2); NIH 3T3 cells, uninfected (lane 3) and infected with Zipras/myc 9 (lane 4); CUGE cells (lane 5); and RM-9, RM-1 and RM-2 cells (lanes 6, 7 and 8, respectively).

tumors *in vivo*. In two separate experiments 4×10^6 viable CUGE cells were injected s.c. into the flank of either adult male or female hosts. In the case of male hosts a period of 2 months produced no tumors at the site of injection ($n = 6$). In the case of female hosts a similar 2 month growth period did not result in any tumors ($n = 4$). For RM-9, RM-1 and RM-2 cell lines in all cases (10 out of 10 inoculations into male hosts for each cell line) rapidly growing carcinomas were produced within 7 days of injection (data not shown).

Southern analysis was used to investigate the clonal status of the carcinoma cell lines with respect to Zipras/myc 9 integration sites (Fig. 3). Digestion of cellular DNA with BglII generates unique virus-cell DNA junction fragments that can be detected by hybridization with a v-Ha-*ras* specific probe [8, 10]. Using this approach unique Zipras/myc 9 integration sites were detected in RM-9, RM-1 and RM-2 cells (Fig. 3, lanes 3–5, respectively). Under these conditions RM-9 and RM-1 were confirmed to be monoclonal. RM-2 demonstrated two unique integration sites and could therefore represent two distinct clones, however, multiple Southern blotting analyses at various passages as well as analysis of DNA derived from multiple independent s.c. tumors *in vivo* consistently demonstrated this pattern suggesting that RM-2 is a clonal cell line. Control kidney and CUGE cell DNAs (lanes 1 and 2, respectively) were negative for Zipras/myc 9 proviruses.

Growth kinetic studies were performed by plating either CUGE cells or tumor cell lines at a density of 4×10^4 cells/cm^2 in their respective media. After 24 h, cultures were then changed to DMEM/BSA in the presence or absence of concentrations of testosterone ranging from 10 pM to 100 nM. Cell number determinations were performed every other day with routine media changes. Under our conditions, neither testosterone nor dihydrotestosterone (data not shown) significantly increased cell number of CUGE above those maintained in the absence of these steroids [Fig. 4(A)]. However, RM-2 cells were responsive to testosterone [Fig. 4(B)] as were RM-9 and RM-1 (data not shown) at passage numbers below 8. Testosterone stimulated

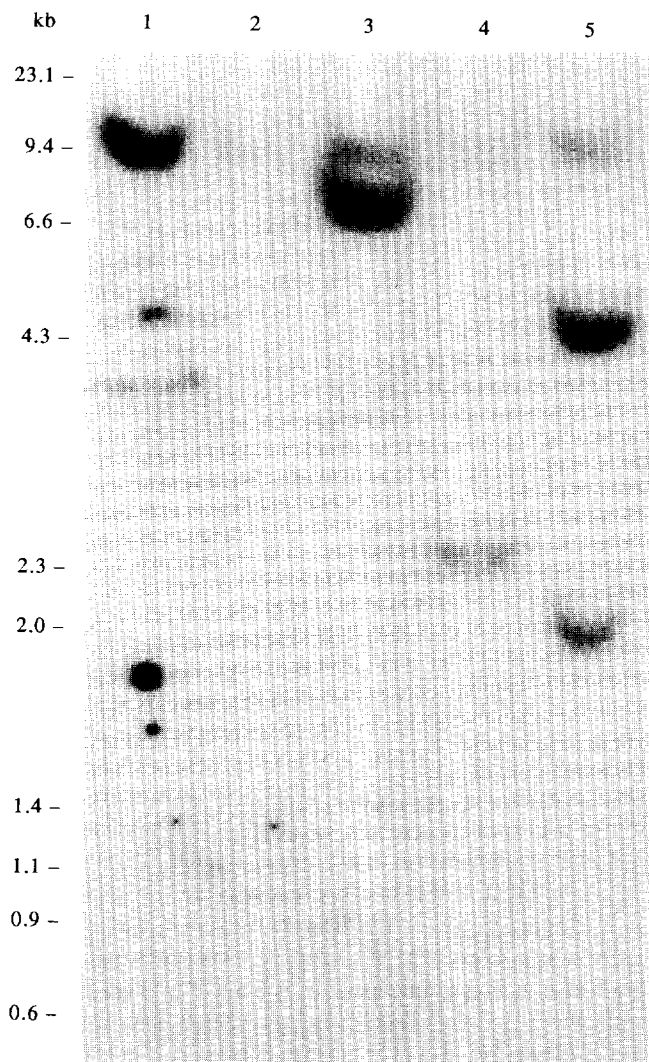

Fig. 3. Clonal status of RM-9, RM-1 and RM-2 cells with respect to Zipras/myc 9 provirus integration sites. Southern transfer of BglII-digested DNA from normal adult male kidney (lane 1), CUGE cells (lane 2), RM-9 (lane 3), RM-1 (lane 4) and RM-2 (lane 5) hybridized with a v-Ha-*ras* probe. The endogenous c-Ha-*ras* fragment of 9–10 kb was seen in all lanes and used to estimate copy number for virus-cell DNA junction fragments.

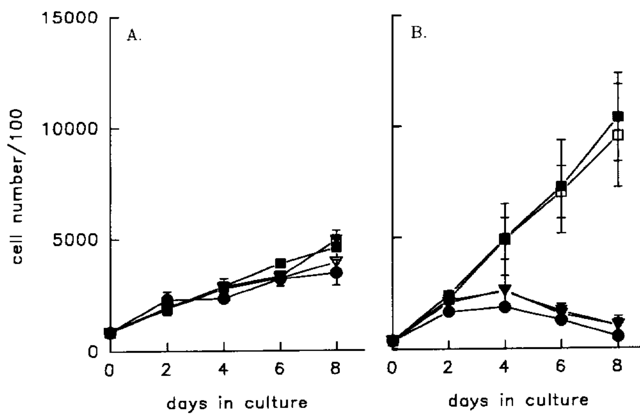

Fig. 4. Dose dependent growth kinetics of CUGE and RM-2 in the presence and absence of testosterone. Non-transformed (CUGE, panel A) transformed and (RM-2, panel B) cells were grown in the presence (10 mM, ▽; 1 nM, ▼; 10 nM, □; 100 nM, ■) or absence (●) of testosterone ($n = 3$). Experiments were performed thrice in triplicate.

maximal growth of these three tumor cell lines at a concentration of 10 nM [Fig. 5(B,D,F)]. At later passages (> 10), all three tumor cell lines either lost or demonstrated reduced testosterone sensitivity for growth stimulation [Fig. 5(C,E,G)]. All three tumor cells examined were capable of proliferating in the absence of exogenous growth factors at early passage and this growth rate increased after multiple passages. The apparent loss of testosterone sensitivity suggested the progressive acquisition of autocrine growth stimulatory properties in this system. Suramin, an anti-malarial drug which binds polypeptide growth factors and inhibits receptor binding and signal transduction [43, 44], reduced constitutive tumor cell proliferation at a concentration of 50 μg/ml (Fig. 6). These data implicate the activities of suramin-sensitive autocrine factors in these prostate cancer cell lines. In order to determine if testosterone-stimulated growth was mediated by these suramin-sensitive factors, suramin was added to testosterone-stimulated cells (Fig. 6). These cells demonstrated a significant androgen growth response in the presence of suramin suggesting that testosterone-induced mitogenesis may occur either directly, or through heparin-insensitive growth factor pathways.

Androgen receptor binding using [^{3}H]R1881 demonstrated the presence of androgen receptors in all cell lines and isolates. Receptor numbers (B_{max}) for CUGE cells were 5×10^3 receptors/cell which was approximately half the receptor number found in testosterone responsive tumor cell lines ($1.0 \times 10^4 \pm 2.8 \times 10^3$ receptors/cell) (Fig. 7). Passage number and/or androgen responsiveness did not have a significant effect on either number of receptors per cell or affinity of these receptors (Table 1). In both CUGE and tumor cells the receptor affinity (K_d) was $9.1 \times 10^{-11} \pm 1.7 \times 10^{-11}$ M. Northern analysis of mRNA isolated from tumor cell lines demonstrated both 10.5 and 6.5 kb androgen receptor mRNA transcripts (data not shown).

Androgen receptor antagonists cyproterone acetate, ICI 176334, and hydroxyflutamide significantly reduced testosterone-stimulated proliferation after 6 days (Fig. 8). However, complete abrogation of androgen-stimulated growth using these antagonists was not observed. Competition studies show that androgen receptors in both tumor cell and CUGE isolates have similar affinities for testosterone, DHT and R1881.

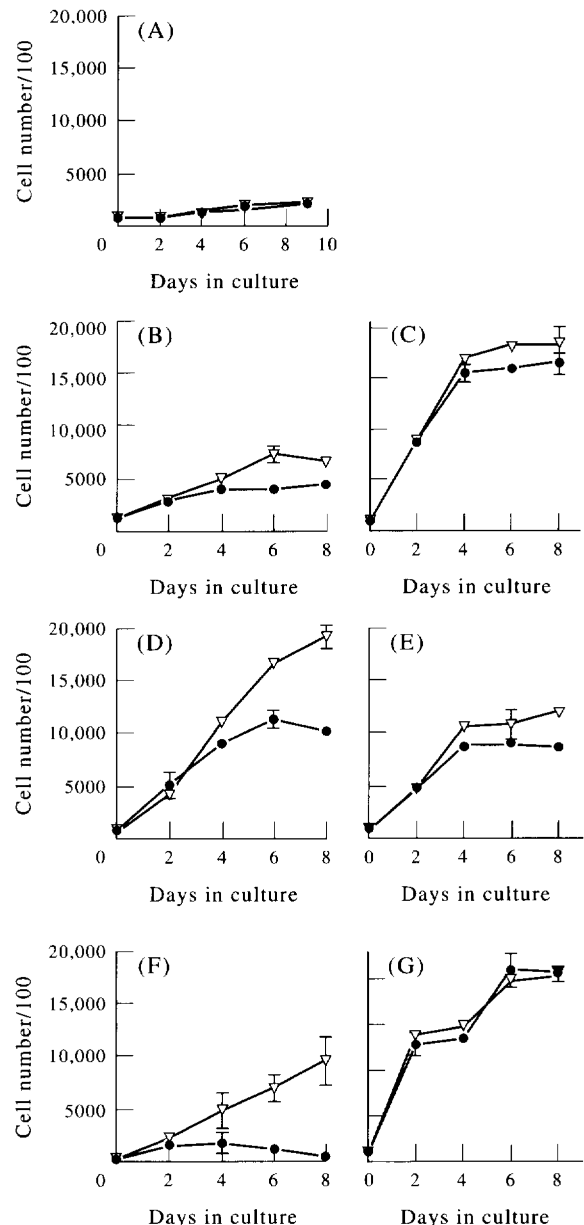

Fig. 5. Growth kinetics of CUGE, RM-9, RM-1 and RM-2 in the presence and absence of testosterone at early and late passage. Non-transformed (CUGE, panel A) cells and transformed (RM-9, panels B and C; RM-1, panels D and E; and RM-2, panels F and G) and were maintained in the presence (▽) or absence (●) of 10 nM testosterone ($n = 3$) with routine media changes. Cell numbers were determined as described in methods and all experiments were performed thrice in quadruplicate.

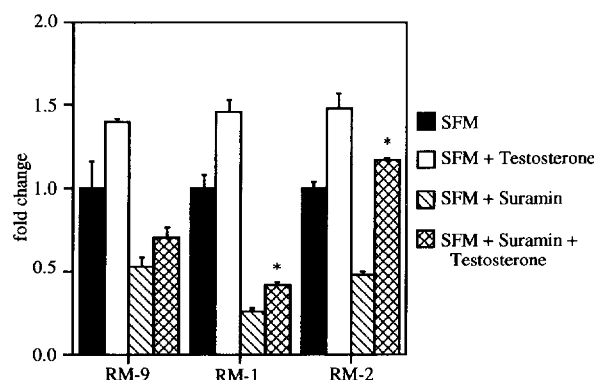

Fig. 6. Testosterone responsiveness in the presence of 50 μg/ml suramin. Cell number for RM-9, RM-1 and RM-2 were determined after 6 days in the presence and absence of 50 μg/ml suramin as described in methods. Determinations for each treatment were normalized to cell number in SFM. Experiments were performed thrice and values represent mean ± SD of triplicate wells. (■, SFM; □, SFM + 10 nM testosterone; ▧, SFM + suramin (50 μg/ml); ▩, SFM + 10 nM testosterone + suramin (50 μg/ml). All values were significant ($P \leqslant 0.05$) by Student's t-test except* which denotes higher significances ($P \leqslant 0.001$).

Estrogen bound with 100-fold less affinity than these steroids and hydrocortisone and progesterone had essentially no affinity for this receptor (Fig. 9). Competition experiments agree with saturation binding experiments with K_d for R1881 between 50 and 100 pM and were similar for both CUGE cells and carcinoma cell lines.

DISCUSSION

To study the androgenic response of non-tumorigenic prostate epithelial cells and prostate cancer cells we have developed an *in vitro* model system using non-tumorigenic urogenital sinus epithelial cells (CUGE cells) and cell lines derived from *ras* + *myc*-induced carcinomas (RM-9, RM-1 and RM-2). We have demonstrated that CUGE cells do not exhibit a significant growth response to testosterone *in vitro*. This lack of testosterone response may be the result of selection *in vitro*, yet the absence of direct growth response by normal prostate epithelial cells to androgen has been previously described [25, 45] and there is extensive evidence that androgen response in normal urogenital sinus epithelial cells is mediated by the mesenchyme *in vivo* [26, 46]. The lack of significant testosterone responsiveness in CUGE cells is in contrast to RM-9, RM-1 and RM-2 carcinoma cell lines which at early passage number show significant increases in cell number in culture in the presence of 10 nM testosterone. Proliferation of the low passage prostate cancer cell lines as defined by increased growth rate in culture, demonstrates a direct response to testosterone by these cells in the absence of a mesenchymal component. RM-9 and RM-1 are monoclonal with respect to Zipras/myc 9 integrations and, although RM-2 demonstrates two independent Zipras/myc 9 integration sites, this integration pattern has been maintained over many generations indicating that our carcinoma cells lines are clonal and free from mesenchymal cell contamination, and therefore, not influenced by stromal cell secreted growth factors with respect to androgen stimulation. Therefore, as in human LNCaP cells and rat Dunning 3327-H cells, it appears that these prostate cancer cells are directly responding to the growth promoting effects of androgens and do not require mesenchymal–epithelial interactions for promotion of proliferation. Although all three early passage carcinoma cell lines were sensitive to testosterone, they also uniformly demonstrated a continuous increase in cell number in the absence of any exogenous growth factors and therefore were not dependent on testosterone for this basal growth activity. This increase in cell number suggests that these cells may produce autocrine growth factors which might confer testosterone independence. These putative autocrine growth factors may increase in activity as increasing passage number ultimately led to significantly higher basal growth activity for all three

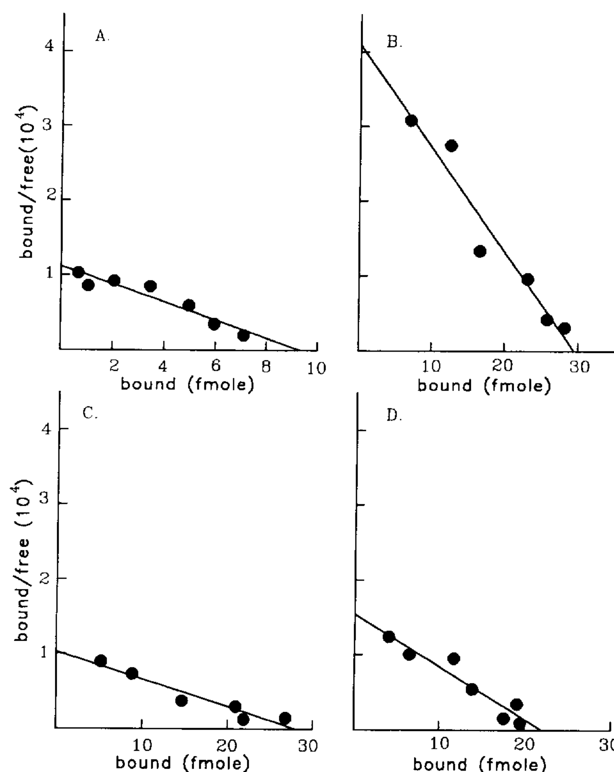

Fig. 7. Scatchard analysis of androgen receptors in untransformed and transformed cell lines. Saturation binding of [^{3}H]methyltrienolone and subsequent Scatchard analysis was performed on untransformed (CUGE, panel A) and transformed (RM-9, RM-1, RM-2, panels B, C and D, respectively) cell lines as described in methods. Experiments were performed three times in triplicate and data shown is representative.

Table 1. Androgen receptor status of transformed and untransformed cell lines

Cell strain/line	Determination	Passage #	K_d	B_{max}
CUGE	1	7	110 pM	6249
	2	25	77 pM	5180
RM-9	1	5	130 pM	7740
	2	6	87 pM	9400
	3	13	70 pM	16732
RM-1	1	7	77 pM	11700
	2	7	99 pM	7730
	3	15	74 pM	9663
RM-2	1	7	100 pM	10120
	2	15	94 pM	8370

Saturation ligand binding using [^{3}H]methyltrienolone was performed on transformed (RM-9, RM-1 and RM-2) and untransformed (CUGE) cell lines. Affinity (K_d) and number of receptors/cell (B_{max}) was determined by Scatchard analysis (see Methods).

carcinoma cell lines and apparent testosterone insensitivity in late passage cultures. To further determine whether testosterone was acting directly or indirectly via a second polypeptide growth factor, we used suramin, an anti-malarial drug which is also capable of blocking growth factor interactions with cells [42, 43, 47, 48]. Testosterone responsiveness of the tumor derived cell lines was not reduced in the presence of 50 μg/ml suramin, a concentration sufficient to inhibit cell proliferation, suggesting that testosterone was not acting through a suramin-sensitive polypeptide growth factor, such as a member of the fibroblast growth factor family. Thus, it appears that in these early passage carcinoma cells testosterone and some autocrine growth factor(s) can function independently to promote cell proliferation.

Recent evidence suggests that alterations in the expression of specific fibroblast growth factor isoforms as well as the expression of specific fibroblast growth factor receptor splice variants may underlie the progression of prostate cancer to stromal cell independence [49]. These studies were carried out using isolated epithelial and mesenchymal cell types derived from Dunning hormone responsive prostate cancer [50]. Further studies in the Dunning model system indicate that androgen-induced growth of normal prostate epithelium and prostate cancer is mediated specifically by FGF-7 which is androgen regulated and derived exclusively from stromal cells [51]. Similar differences in the expression of FGF isoforms and/or FGF receptor subtypes may also underlie differences in the growth and malignant potential of CUGE cells and *ras* + *myc*-induced carcinoma cell lines. Furthermore, it is conceivable that additional alterations in the FGF system may play a role in the progression of RM-9, RM-1, and RM-2 from the clearly androgen sensitive but mesenchyme-independent state to the more rapidly growing and apparently androgen insensitive status.

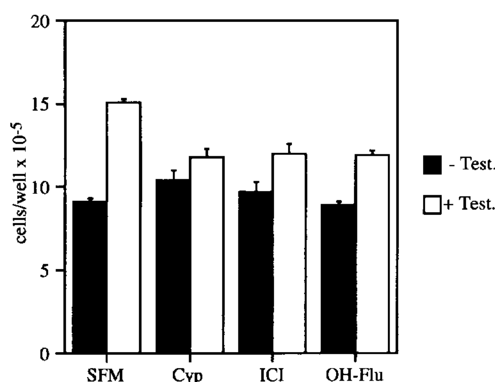

Fig. 8. Effect of androgen receptor antagonists on testosterone-stimulated growth of RM-2 cells. RM-2 cells were grown in SFM for 6 days in 12 well plates in the presence or absence of 10^{-8} M testosterone with and without a 100-fold excess of androgen receptor antagonists cyproterone acetate (Cyp), ICI 176334 (ICI), or 4-hydroxyflutamide (OH-Flu). Cell numbers were determined with a Coulter Counter and are expressed as total cells per well. Testosterone-stimulated growth was significantly reduced ($P < 0.05$) by all three androgen receptor antagonists compounds (■, −testosterone; □, +testosterone).

Although the mechanism(s) and nature of the autocrine growth pathways remain to be established, it is clear that at high passage number basal growth rates for all three carcinoma cell lines are exceedingly rapid. It is only under these conditions that the cell lines apparently lose sensitivity to testosterone. Interestingly, analysis of androgen receptor numbers or affinities did not reveal changes during progression to androgen insensitivity. Both normal and tumor cells demonstrated the presence of androgen receptors as determined by specific radioligand binding assays using [^{3}H]R1881 and Northern blotting using cDNA probes for androgen receptor. Neither androgen receptor number nor affinity for ligand changed after time in culture. The presence or absence of androgen response in tumor cell lines had no effect on receptor affinity or number of receptors/cell. Competition studies with both tumor and CUGE cell lines show that androgen receptors found in these cell lines will bind the syn-

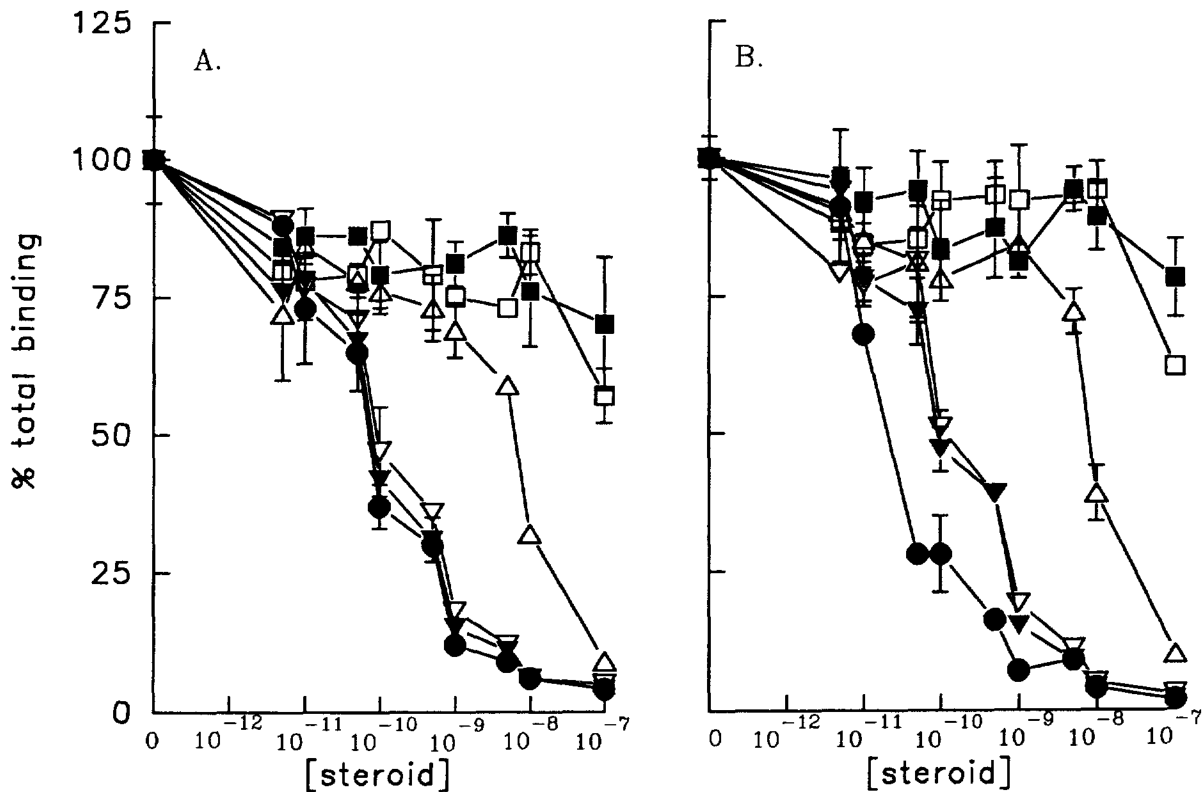

Fig. 9. Androgen receptor competition with other steroid ligands. Androgen receptor competition was performed with radiolabelled methyltrienolone at 100 pM ($K_d \simeq 90$ pM) and varying concentrations of cold steroid (●, R1881; ▽, testosterone; ▼, DHT; □, progesterone; ■, hydrocortisone; △, estrogen) on normal (CUGE, panel A) and transformed cells (RM-2 panel B). Experiments were performed thrice in triplicate.

thetic androgen R1881, dihydrotestosterone and testosterone with equal affinity. Progesterone and estrogen were also found to bind these receptors but with much lower affinities (100- and 1000-fold less, respectively) as has been previously described by Tilley *et al.* [52].

Overall the receptor binding and competition studies indicate that the presence or absence of androgen responsiveness in these cell lines is not due to changes in the receptor number or affinity. A related phenomenon has been previously reported in a study in which it was demonstrated that functional AR as defined by transcriptional activity of a reporter construct was a feature of androgen insensitive as well as androgen sensitivity S115 breast cancer cells [53]. These results are complementary to ours and together suggest that apparent androgen insensitivity is downstream of AR function. Recently, we demonstrated that in the absence of a measurable impact on tumor growth, castration therapy resulted in the induction of multiple growth and apoptosis related gene activities in a transplantable and androgen-independent *ras* + *myc*-induced mouse prostate carcinoma *in vivo* [5]. These results together with those reporting a persistent androgen sensitivity of human prostate cancer cells that are clearly androgen independent in terms of growth [54, 55], strongly suggest that androgen-independent prostate cancer retains well-defined biological endpoints of androgen action. The perpetuation of a functional androgen receptor system throughout progression to apparent androgen sensitivity raises the possibility that androgens could continue to impact on the behavior of cancer cells throughout advanced stages of progression. Although the data presented here are consistent with the progressive involvement of autocrine mechanisms as increased proliferative capacity was seen during continued growth of these cells in culture, these additional growth stimulatory pathways do not preclude the continued action of androgens in these cells. In fact, because of the profoundly increased growth of these cell lines with sequential passage, the cells may be proliferating at such a maximal rate that the effects of androgens are masked.

In summary, we have derived a novel model system to study prostate cancer *in vitro*. This system includes *ras* + *myc*-transformed clonal prostate cancer cell lines and a non-tumorigenic control urogenital sinus epithelial cell line. Using this system we show that at early passage all three cancer cell lines demonstrate apparent direct growth promoting effects of testosterone *in vitro* and that these effects are either lost or diminished during continued passage of these cells. The testosterone response is in direct contrast to the lack of prolif-

erative response to this hormone seen in CUGE cells. As the prostate cancer cells are passaged, increased growth rate is apparent and androgen sensitivity is apparently lost. Overall, this progression pattern is consistent with the acquisition of autocrine pathways. However, during progression androgen receptor binding with respect to both K_d and B_{max} remains constant and relatively high in all three cell lines. In addition, receptor levels in carcinoma cells appear to be approximately twice that of CUGE cells as would be consistent with the observed response differential. These data and the results of previous studies raise the possibility that androgen sensitivity could remain patent throughout progression and simply be masked by a maximal proliferative response induced by the acquisition of autocrine pathways. Furthermore, our data are consistent with other reports demonstrating the absence of a direct mitogenic response to testosterone by normal prostatic epithelium or its fetal precursor cells. Finally, our data together with previous studies suggest that a direct mitogenic response to androgen may be characteristic of the transformed phenotype and could play a role in the progression of prostate cancer. Additional studies will be necessary to confirm the relevance of our results to prostate cancer in man.

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