

Conversion from a Paracrine to an Autocrine Mechanism of Androgen-stimulated Growth during Malignant Transformation of Prostatic Epithelial Cells¹

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ABSTRACT

Normal adult prostate epithelium of both human and rat origin was transplanted with Matrigel into intact or androgen-ablated (*i.e.*, castrated) nude mice. Within these transplants, an influx of mouse mesenchymal cells was one of the earliest events to occur resulting in the development of a collar of smooth muscle cells and fibroblasts surrounding the transplanted epithelium. A subset of these surrounding stromal cells express androgen receptor (AR). The surrounded transplanted epithelium initially expresses high molecular weight cytokeratins characteristic of prostatic basal cells and AR. In both intact and androgen-ablated hosts, this epithelium subsequently develops a patent lumen producing a rudimentary glandular acini. Only in the nonablated hosts, however, do these rudimentary acini undergo a further proliferative growth phase, as determined by Ki67 immunocytochemical stainings and the development of a low molecular weight cytokeratin positive layer of luminal (*i.e.*, secretory) epithelial cells. Because AR is expressed in both the donor epithelium and host (*i.e.*, mouse) stromal cells, this androgen-stimulated growth response could involve either autocrine pathways initiated within donor normal adult epithelial cells themselves or paracrine pathways initiated within the AR-expressing subset of mouse stromal cells. To resolve this issue, mice carrying the testicular feminized mutation in the X-linked AR gene were cross-bred to AR-wt nude mice to produce AR-null nude male mice. None of the cells in these AR-null nude male mice express functional AR protein. Therefore, these animals can be used to prevent any possibility of host stromal cell paracrine involvement in initiating an androgen-stimulated growth response when normal adult or malignant prostatic epithelial cells are transplanted into these null hosts. In these AR-null nude male mice, the androgen-stimulated growth of normal adult prostatic epithelial cells did not occur (*i.e.*, androgen-induced growth response of normal prostatic epithelial cells requires stromal cell paracrine involvement). In contrast, using four different prostatic cancer models (*i.e.*, human PC-82, human LNCaP, human LAPC-4, and rat R3327G), the androgen-stimulated growth of prostatic cancer cells occurred identically in both AR-null and AR-wt nude male mice (*i.e.*, a direct autocrine mechanism is responsible for androgen-stimulated growth of malignant prostatic epithelial cells). In summary, a fundamental change in the mechanism for androgen-stimulated growth occurs during the transformation from normal to malignant prostatic epithelial cells.

INTRODUCTION

Because prostatic epithelial cells express the AR,³ it was assumed initially that androgen-regulated growth of the gland involved an AR-dependent transcriptional effect directly within these cells. Developmental studies by Cunha *et al.* (1) demonstrated that during the embryogenesis of the prostate, however, androgens do not initiate this regulation directly within the prostatic epithelial cells themselves.

Instead, the critical androgen ligand/AR interactions occur in embryonic prostatic stromal cells inducing these stromal cells to synthesize and release soluble paracrine factors in which its functions are to regulate the growth and development of prostatic epithelial cells (1). Additional studies have suggested that such stromal cell-dependent paracrine pathways may be involved in the regulation of the balance between proliferation and death of prostatic epithelial cells in the adult as well as during development (2–9). Because in prostatic cancer this balance is abnormally disrupted, there is a critical need to resolve whether the role of stromal cells is the same in androgen-regulated normal *versus* malignant prostatic tissue. Resolving this issue is critical because depending on the answer, choices of theoretical as well as practical targets and/or methods for treatment of prostatic cancer are profoundly different.

To resolve this issue, a series of transplantation experiments were performed using both normal adult and malignant prostatic epithelial cells of humans and rat origin. These transplantation studies were based upon the earlier demonstration by Hayward *et al.* (10) that human BPH tissue could be enzymatically dissociated and epithelial organoids isolated from supporting stromal elements using unit gravity sedimentations. When these BPH epithelial organoids were cultivated *in vitro* within collagen gels for 3 days and then these gels xenographed into nude mice, the xenographed glands underwent a further growth phase not observed in the castrated host (10). These results demonstrated that isolated prostatic epithelium could be transplanted into nude mice to study the mechanism for its androgen-stimulated growth. On the basis of such a transplantation approach, the present studies were performed using intact and androgen-ablated (*i.e.*, castrated) AR-wt and AR-null male nude mice as recipients for these epithelial transplants. This allowed the role of host stromal cells in the androgen-stimulated growth of the various prostatic epithelial transplants to be tested using histological analysis to evaluate prostatic morphogenesis and immunocytochemical assays to evaluate proliferative and secretory ability of these transplanted cells.

MATERIALS AND METHODS

Epithelial Organoid Transplantation. Grossly normal areas of peripheral prostate tissue were obtained from radical prostatectomy specimens from patients undergoing surgery for localized prostate cancer. The nonmalignant nature of the harvested tissue was confirmed retrospectively via histological analysis of H&E-stained representative sections. These human tissues were used both to harvest epithelial organoids ($n = 5$) and to establish separate stromal and epithelial cell cultures ($n = 6$) as described later. In addition, ventral prostatic tissues were obtained from male Copenhagen rats (Harlan Sprague Dawley, Indianapolis, IN) that had been castrated 1 month previously. For both the human and rat prostates, the tissues were minced and then dissociated with 0.28% collagenase Type I (Sigma Chemical Co., St. Louis, MO) in RPMI 1640 (Life Technologies, Inc., Bethesda, MD) + 10% FCS (Hyclone, Logan, UT) for 2 h at 37°. The mixture was then filtered using tissue sieves (E-C Apparatus Corp.) to remove large fragments of connective tissue. The filtrate was then allowed to settle for 10 min without centrifugation. The supernatant containing individual (*i.e.*, mostly stromal) cells was discarded, and the sediment containing epithelial organoids was resuspended and resedimented. The second sediment was then resuspended in RPMI 1640 + 10% FCS, and the mixture was placed into tissue culture flasks to allow selective

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³ The abbreviations used are: AR, androgen receptor; BPH, benign prostatic hyperplastic; VP, ventral prostate; PSA, prostate-specific antigen; Tfm, testicular feminized; wt, wild type.

attachment of fibroblasts overnight. The next morning, the unattached organoids were collected and mixed with liquid Matrigel (Collaborative Biomedical Products, Bedford, MA) at 4° at a ratio of 600 organoids/ml Matrigel. Aliquots (200 μ l) of this Matrigel mixture were injected s.c. in the flank of mice using an 18-gauge sterile needle.

Epithelial Single Cell Transplantation. For human single cell transplantation experiments, surgical tissue was used to establish separate stromal and epithelial cell cultures as we have described previously (11). In brief, this involves mincing the tissue with sterile scalpel blades and digesting of the minced tissue with collagenase Type I for 2 h at 37°C, followed by differential sedimentation and selective adherence in defined medium. For the epithelial cells, the medium used was PREGM, containing the standard prostatic epithelial cell media additives (PREGM Bullet Kit), including bovine pituitary extract, insulin, transferrin, epidermal growth factor, hydrocortisone, retinoic acid, epinephrine, and tri-iodothyronine, obtained from Clonetics/Bio Whittaker (Walkersville, MD). Stromal cells were grown in RPMI 1640 with 10% FCS and 10 mg/liter insulin, 5.5 mg/liter transferrin, and 5 μ g/liter selenium (Sigma Chemical Co.).

Single cells were also isolated from VPs of male rats castrated 1 month previously and from PC-82 human prostate cancers grown in intact nude male mice. For each of these tissue types, tissue (*i.e.*, 50–100 mg for VPs and 500–1000 mg for PC-82) was initially minced with sterile scalpel blades. The mince was then incubated in 50 ml of 1 mM DTT (Sigma Chemical Co.) in PBS for 30 min with gentle rotation of 30 cycles/min at 37°, and then the mixture was allowed to settle without centrifugation for 10 min. The supernatant fraction was discarded. Dissociation solution (10 ml) containing 1% DNase Type I (Sigma Chemical Co.) and 0.28% collagenase Type I (Sigma Chemical Co.) in RPMI 1640 with 10% FBS was added to the mince and rotated at 37° for 30 min. The mince was allowed to settle for 5 min, the supernatant fraction was collected, and another 10 ml of dissociation solution were added to the settled mince for another 30 min of rotation at 37° as before. A second supernatant was collected, as described, and combined with the first. The pooled supernatant was then passed through a series of tissue sieves with pore sizes ranging from 230 to 46 μ m. The sieved cell suspension was then washed twice with RPMI 1640 containing 10% FBS, passed through a 25-gauge needle twice, and inoculated into tissue culture flasks in RPMI 1640 containing 10% FBS to allow selective attachment of the fibroblast cells overnight. The next morning, the unattached cells (floaters) were collected and mixed with Matrigel (*i.e.*, 10⁷ cells/ml of Matrigel). Aliquots (200 μ l) were then injected s.c. in the flank of nude mice.

Single cells were also injected from: (a) LNCaP human prostatic cancer, (b) LAPC-4 human prostatic cancer, and (c) R3327G rat prostatic cancer cells. The characteristics of these prostatic cancer sublines have been published previously (11, 12). Each of these sublines were maintained as continuous cell lines in culture in RPMI 1640 + 10% FCS. Semiconfluent cultures were trypsinized, and single cells were recovered and mixed with Matrigel at a ratio of 10⁷ cells/ml of Matrigel. Aliquots (200 μ l) were injected s.c. into the flanks of nude mice.

Animal Studies. AR-wt homozygous nude male mice (*i.e.*, *nu/nu* at the nude locus and AR/Y at the AR locus) were obtained from Harlan Sprague Dawley. AR-null homozygous nude male mice (*i.e.*, *nu/nu* at the nude locus and *tfm/Y* at the AR locus) were bred in-house and genotyped using a PCR-based method as described previously (13). All of these AR-null male nude mice had their peritoneum opened under anesthesia to confirm their genotype by identifying the undescended testes, which are characteristic of the male mice with *tfm* mutation in their AR genes (14). These undescended testes were then removed from all of the AR-null male nude mice, and a 1-cm long polydimethylsiloxane (Silastic) sealed capsule packed with testosterone was implanted s.c. in the flank as described (13). The peritoneum and skin were then sutured, and the mice were allowed to recover. Where indicated, AR-wt male nude mice (AR/Y, *nu/nu*) were androgen ablated via castration using a scrotal route under anesthesia as described previously (13). Where indicated, AR-null male mice were androgen ablated via removal of their testosterone implants. Tumor volume doubling times were determined as described previously (12).

Assays. H&E histological analysis was performed as described previously (13). Immunocytochemical analysis for the AR, PSA, low and high molecular cytokeratins, and α -smooth muscle actin were as described previously (13). The antibodies used were obtained from DAKO Corp. (Carpinteria, CA)

except for those for AR, which were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Cells in the proliferative cell cycle were determined immunocytochemically as described previously (13) using the M1B5 monoclonal antibody (Immunotech, Cedex, France), which detects the Ki67 antigen present in nuclei of all phases of the cell cycle except G₀ (15, 16). The mouse *versus* rat or human origin of specific cells was determined on histological sections using the Hoescht 33258 stain (Calbiochem-Novabiochem Corp., La Jolla, CA) technique of Cunha and Vanderslice (17). Serum testosterone was determined as described previously (18). Western blotting was performed using antibodies for α -smooth muscle actin or AR using antibodies obtained from DAKO Corp. and Santa Cruz Biotechnology, respectively.

Statistics. All of the values are presented as means \pm SE. Statistical analysis was performed by a one-way ANOVA with the Newman-Keuls test for multiple comparison.

RESULTS

Androgen Responsiveness of Normal Human Prostatic Epithelial Cells When Transplanted as Organoids. The Hayward *et al.* studies were repeated using human prostatic organoids prepared from normal ($n = 5$) adult prostatic tissue, instead of BPH tissue. An additional difference between the present studies and those of Hayward *et al.* was that once isolated, these normal prostatic epithelial organoids were immediately mixed at 4° with liquid Matrigel, which is the extracellular matrix obtained from the EHS mouse sarcoma (19). This mixture was allowed to gel at 37°, and then 0.2-ml aliquots of the gelled mixture containing 120 organoids/aliquot were xenografted into either intact or castrated male nude mice. The physiological androgen level in the intact nude male mice was determined to be 2.8 ± 0.4 ng of testosterone/ml serum *versus* the androgen-ablated level of <0.1 ng of testosterone/ml serum in castrated nude male mice.

During the first 2 weeks *in vivo*, the Matrigel pellets were invaded by host mesenchymal cells and endothelial cells to produce a significant angiogenic response. The organoids during this time period were composed of medullary balls of epithelial cells with no glandular lumen in either intact or castrated hosts (Fig. 1A). Between the second and third week, several events occurred in both intact and castrated hosts. These included the development of a collar of smooth muscle cells around the rudimentary organoid balls and subsequent development of a glandular lumen producing a discernable glandular acini (Fig. 1B). The cells which surround the acini were determined to be mouse smooth muscle cells on the basis of: (a) immunocytochemical staining which demonstrated their positive expression of α -smooth muscle actin and (b) the Hoechst 33258 staining which demonstrated that the nuclei of the stromal cells had the punctate feature characteristic of mouse cells, whereas the nuclei of the epithelial cells had the uniformly diffuse staining characteristic of human cells (17).

Between the third and fifth weeks after inoculation, differences began to develop between transplants in intact *versus* castrated hosts. In the castrated hosts with serum testosterone <0.1 ng/ml, the glands were composed of epithelial cells which expressed high molecular weight cytokeratins (*i.e.*, cytokeratins 1, 5, 10, and 14) characteristic of prostatic basal cells (20), surrounded by a well-developed stromal component composed of both smooth muscle and fibroblasts of mouse origin. These rudimentary glands did not express low molecular cytokeratins (*i.e.*, cytokeratins 8 and 18) characteristic of prostatic luminal cells (20). These glands remained rudimentary with a low rate of epithelial cell proliferation (*i.e.*, percentage of epithelial cells in the proliferative cell cycle as determined by Ki67 immunocytochemical staining was $<0.1\%$). In contrast, in intact male hosts with a physiologically normal level of serum testosterone (*i.e.*, 2.8 ng/ml), the glands increased in a cross-sectional area between the third and fifth weeks with the glands now being composed of a distinct basal and luminal layer of epithelial cells surrounded by well-developed mouse

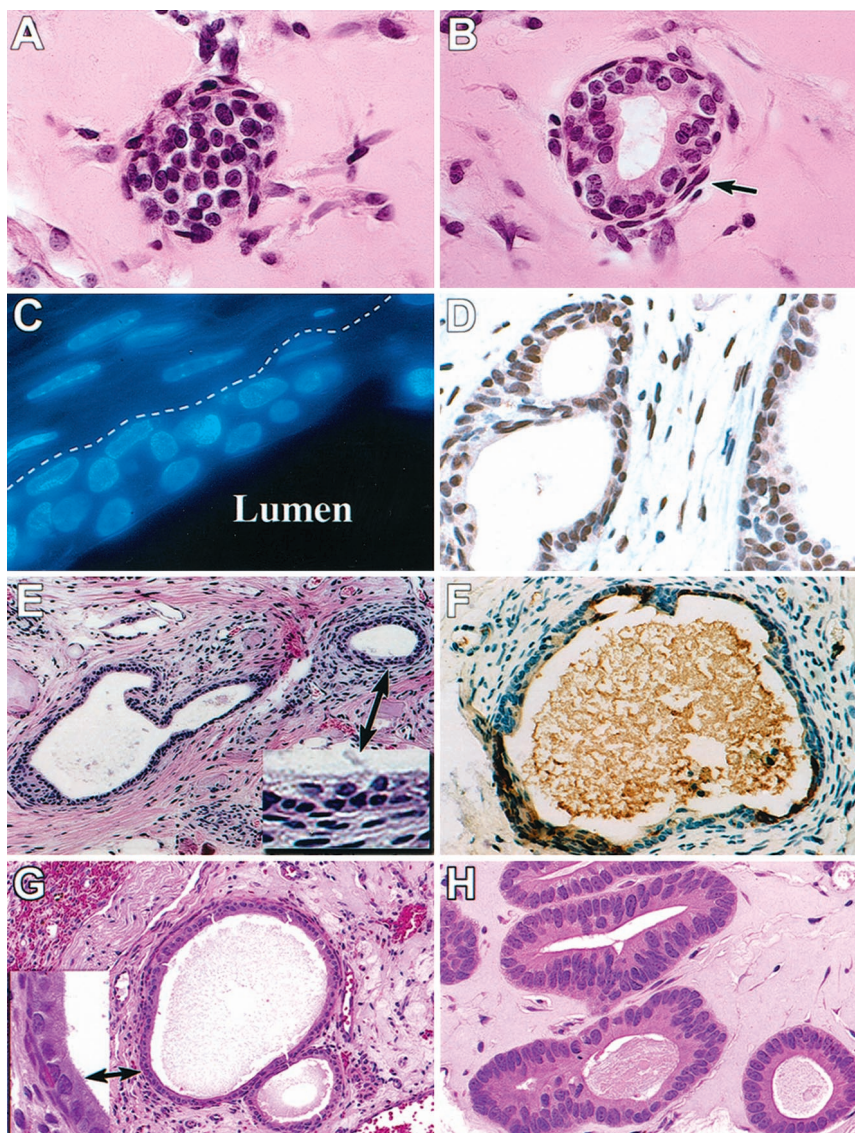


Fig. 1. A, H&E histology of normal human adult prostatic epithelial organoid within Matrigel 10 days after transplantation in an intact nude male mouse ($\times 400$). B, H&E histology of normal human adult prostatic organoid within Matrigel 20 days after transplantation in an intact nude male mouse. Arrow, smooth muscle cell ($\times 400$). C, fluorescence image of Hoechst 33258 staining of normal human adult prostatic epithelial organoid 4 weeks after transplantation in intact male nude mice. ---, basement membrane separating the mouse stromal cells (i.e., above line) and human epithelial cells (i.e., below line). Black area, the glandular lumen ($\times 600$). D, immunocytochemical staining for AR expression in normal human adult prostatic epithelial organoid 4 weeks after transplantation into an intact male nude mouse. Note positive expression (i.e., brown stain) in both stromal and glandular cells ($\times 200$). E, H&E histology of normal adult prostatic epithelial organoid 5 weeks after transplantation into an intact male nude mouse. Arrow, the magnified insert of a specific area in a well-developed glandular acini demonstrated the dual (i.e., basal and luminal) layer of the epithelium (i.e., characteristic of the human prostate) surrounded by a well-developed stromal compartment ($\times 100$). F, immunocytochemical staining for PSA expression in a well-developed glandular acini produced 5 weeks after transplantation of normal human adult prostatic epithelial organoid. Note the positive brown stain filling the lumen and glandular cells ($\times 100$). G, H&E histology of well-developed glandular structure produced 15 days after transplantation of normal adult rat ventral prostatic epithelium as single cells. Arrow, the magnified insert which illustrates that the well-developed glandular acini are composed of a continuous luminal layer and a sporadic number of basal epithelial cells, characteristic of the rat prostate ($\times 100$). H, H&E histology of human PC-82 prostatic cancer cell within Matrigel 1 month after inoculation in intact AR-wt nude male mice ($\times 200$).

stromal cells (Fig. 1E). This was documented by: (a) immunocytochemical staining with antibodies which recognized either high molecular weight cytokeratins characteristic of basal cells or antibodies which recognized low molecular weight cytokeratins characteristic of prostatic luminal cells (20) and (b) Hoechst 33258 staining (Fig. 1C) which demonstrated that the surrounding stromal cells had the punctate staining characteristic mouse cells, whereas the epithelial layer composed of luminal and basal cells had the diffuse staining characteristic of human cells. The fact that the increase in the cross-sectional area of the glands between 3 and 5 weeks after transplantation was attributable predominantly to an increase in epithelial cell proliferation was documented by $1.0 \pm 0.2\%$ of epithelial cells being Ki67 positive immunocytochemically (i.e., at least a 10-fold higher percentage of Ki67 than in castrated hosts). The Ki67-positive cells were nearly exclusive to the basal and not the luminal layer of epithelial cells. In addition, there was also a measurable level of Ki67 staining (i.e., $0.3 \pm 0.1\%$) in the stromal cells surrounding the prostate acini in the intact male *versus* no detectable (i.e., $<0.1\%$) Ki67 staining in stromal cell surrounding acini in castrated hosts. Of special significance, by 3 weeks after transplantation, a significant fraction of the mouse stromal cells surrounding the growing acini expressed AR, as did the majority of the human epithelial cells (Fig. 1D).

The growth phase in the intact male essentially stopped by 5 weeks after inoculation (Fig. 1E; i.e., at this time point, $<0.1\%$ of epithelial cells are Ki67 positive). At this time point, the epithelial cells in the intact male nude mice expressed abundant amounts of PSA, which was present within the luminal contents of the acini (Fig. 1F). In contrast, in castrated nude mice even at 5 weeks, prostatic acini remain rudimentary and did not produce PSA. Two conclusions can be drawn from these results. The first is that when prostatic organoids are transplanted in Matrigel, prostatic glandular morphogenesis was stimulated. This involved migration of mouse mesenchymal cells into the Matrigel transplants where they formed a histologically normal stromal interface for the transplanted epithelial cells, as well as provided an angiogenic supply for the acini. This morphogenic process occurred even without a physiologically normal level of androgenic stimulation. The second conclusion is that whereas stromal-epithelial morphogenesis did not require physiological androgen, without such stimulation, the resultant glandular acini remained rudimentary, being composed of only basal-like epithelial cells with few luminal cells being present. Only when there was a physiologically normal level of androgen did these rudimentary glands undergo further cell proliferation and develop a luminal layer of secretory epithelial

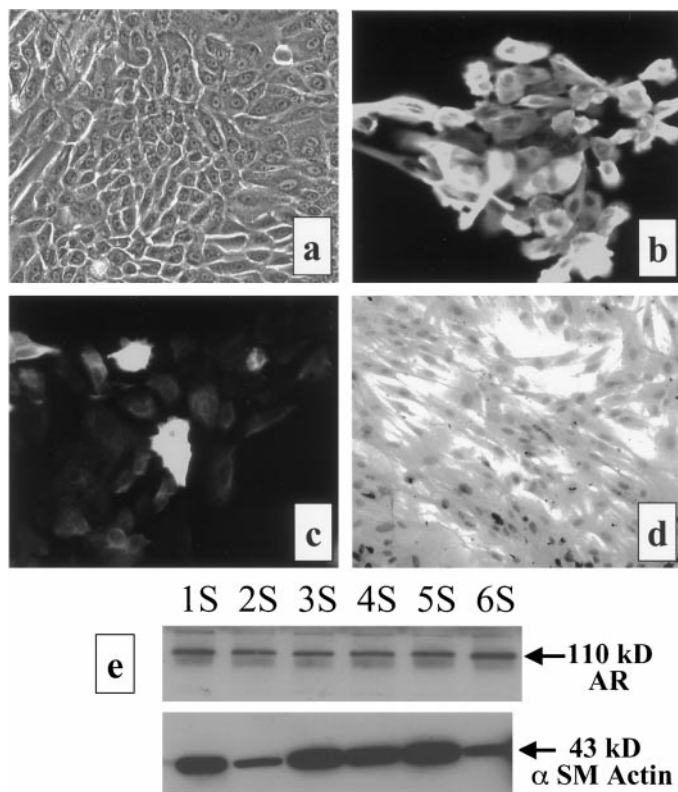


Fig. 2. *a*, phase contrast morphology of human prostatic epithelial cells in early *in vivo* passage ($\times 200$). *b*, immunocytochemical staining for high molecular weight cytokeratins (*i.e.*, cytokeratins 1, 5, 10, and 14). *c*, immunocytochemical staining for low molecular weight cytokeratins (*i.e.*, cytokeratins 8 and 18). *d*, phase contrast morphology of human prostatic stromal cells in early *in vitro* passage ($\times 200$). *e*, Western blot detection of expression of AR and α -smooth muscle actin in a series of human prostatic stromal cells *in vitro* from six different patients.

cells. Thus, these transplanted rudimentary glands retain an androgen stimulation of their growth.

Androgen Responsiveness of Normal Prostatic Epithelial Cells When Transplanted as Single Cells. On the basis of the success of the organoid experiments, human prostatic tissue from multiple patients was dissociated into epithelial organoids and single cell suspensions of stromal cells. These were used separately to establish early passage *in vitro* prostatic epithelial and stromal cell cultures. The stromal cells grown in 10% FCS-containing media (Fig. 2*d*) consistently express both AR and α -smooth muscle actin regardless of the donor source (Fig. 2*e*). In contrast, none of the multiple prostatic epithelial cell cultures grown in serum-free-defined media (Fig. 2*a*) expressed AR or PSA, although immunocytochemical staining of the epithelial cells in the original tissue from surgery was positive for both of these markers. The majority of the cultured epithelial cells did express high molecular weight cytokeratins (*i.e.*, cytokeratins 1, 5, 10, and 14; Fig. 2*b*) characteristic of prostatic basal epithelial cells (20). In addition, a subpopulation of these epithelial cells expressed the low molecular weight cytokeratins (cytokeratins of 8 and 18; Fig. 2*c*), characteristic of prostatic luminal cells (20). A million of these human prostatic epithelial cells from early passage culture were mixed with 0.2 ml of Matrigel and inoculated into intact male nude mice. At 5 weeks after inoculation, the gelled pellets were removed and analyzed histologically. Only nests containing three to five human prostatic epithelial cells each were detectable, and these nests were not surrounded by infiltrating stromal cells. Interestingly, the epithelial cells in these nests did not express AR but did express high molecular weight cytokeratins characteristic of basal cells. Additional experi-

ments were repeated in which 10^6 human prostatic epithelial cells and 2×10^6 human prostatic stromal cells, both from early passage cultures, were coinoculated in 0.2 ml of Matrigel into intact nude male mice. After 5 weeks, histological analyses using Hoechst 33258 staining demonstrated again only scattered nests of three to five AR nonexpressing human epithelial cells each within a diffuse background of Matrigel containing both mouse and human stromal cells.

These negative results suggested two possibilities: (*a*) the presence of a minimal epithelial (*i.e.*, organoid) structure in which the junctional complexes between epithelial cells are retained might be required in order for morphogenesis to occur subsequently when prostatic epithelial cells are inoculated into nude mice, and (*b*) during even limited *in vitro* culture when prostatic epithelial cells are separated from their stromal support, changes occur causing these cells to lose their ability to subsequently undergo normal morphogenesis when inoculated *in vivo*. Consistent with this latter possibility is the observation that within the first *in vitro* passage of prostate epithelial cells, these cells stop expressing both AR and PSA and do not re-express either of these markers when inoculated with Matrigel *in vivo*.

To test for the first possibility, advantage was taken of the enhanced proliferative potential of epithelial cells obtained from regressed prostates from animals castrated 3 weeks earlier. For example, the proliferation rate of the epithelial cells of the VP in an intact male rat is $<0.2\%$ per day (21). If these rats were castrated and allowed to go untreated for 1 month, $\sim 80\%$ of the total cells were eliminated via apoptosis (22). This death response removed the androgen-dependent transit (*i.e.*, luminal) epithelial cells without the elimination of the androgen-sensitive amplifying (*i.e.*, basal) and androgen-independent (*i.e.*, basal) stem cells (23). If androgen was replaced in these long-term castrated rats, the amplifying cells underwent >5 population doublings to restore the original epithelial cell number within 2 weeks of androgen replacement (23).

Initial studies demonstrated that if prostate organoids were isolated from the VPs of 1-month castrated rats and these were inoculated with Matrigel into intact or castrated male nude mice, rudimentary glandular formation occurred in both types of hosts. Again, however, glandular growth and development of low molecular weight cytokeratin expressing luminal cells occurred over a 2–3 week period, only in intact hosts, not in castrated nude mice. The resultant acini were composed of functional active (*i.e.*, secretory) epithelial cells surrounded by a ring of smooth muscle cells and fibroblasts. To test if single prostatic epithelial cells with an enhanced growth potential could undergo full organogenesis when transplanted into nude mice, VPs from 1-month castrated rats were treated initially with 1 mM DTT to reduce the disulfide bonds in E-cadherin molecules responsible for homotypic binding between the prostate epithelial cells, and then the tissue was treated with collagenase Type I. This treatment induced the complete dissociation of the epithelial cells into a single cell suspension. These suspensions were placed in tissue culture flasks overnight to allow single stromal cells present to attach to the flasks. The media containing nonadherent single epithelial cells were then collected and centrifuged, and 10^6 of these dissociated epithelial cells were mixed with 0.2 ml of Matrigel and inoculated into intact or castrated male nude mice. Within the first 5 days of inoculation, small nests of epithelial cells were detectable in both types of hosts. The epithelial cells in these nests expressed AR as did sporadic stromal cells surrounding them. By day 8, in both types of hosts, these nests developed into a glandular acini with a patent lumen, and the acini were surrounded by a continuous ring of smooth muscle cells, which by Hoescht 33258 staining were of mouse origin. In the castrated host, this acinar development essentially stopped by day 8 with the production of rudimentary acini, which were essentially identical in size to the rudimentary acini presented in Fig. 1*B*. These rudimentary acini

Table 1 *In vivo* androgen responsiveness of prostatic cancer sublines utilized

Prostatic cancer type	Androgen receptor	<i>In vivo</i> doubling time (days)	
		Intact host	Castrated host
Human PC-82	Wild type	20 ± 2	— ^{a,b}
Human LNCaP	Mutant	15 ± 2	21 ± 2 ^b
Human LAPC-4	Wild type	12 ± 2	17 ± 2 ^b
Rat R3327G	Wild type	10 ± 2	18 ± 3 ^b

^a PC-82 cancers do not grow in castrated hosts.

^b $P < 0.05$ compared with value for intact host group.

had a Ki67 index of $<0.1\%$. In contrast, on day 8 in intact male hosts, the epithelial Ki67 index was $1.8 \pm 0.4\%$ with the acini being two to four times larger in cross-section than those in inoculations in time-matched castrated mice. By day 15 in intact nude mice, the Ki67 index of the acini was now $<0.1\%$. At this time, however, the acinar lumen were filled with secretions, and the acinar compartment was composed of a continuous predominantly columnar luminal epithelium with a sporadic layer of basal epithelium (Fig. 1G), characteristic features of the fully androgen-stimulated rat ventral prostatic in an intact male host.

Resolving the Paracrine Versus Autocrine Nature of the Androgen-responsive Growth of Normal Prostatic Epithelial Cells.

The previous studies demonstrated that both human and rat normal prostatic epithelial cells undergo androgen-responsive growth when transplanted with Matrigel into nude male mice. Because AR is expressed in both the epithelial and stromal cells of such transplants, this raises the issue of whether this response involves an autocrine pathway initiated within epithelial cells themselves or involves paracrine pathways initiated within the AR-expressing stromal cells. To resolve between these two possibilities, a method was required to prevent functional AR expression in the invading mouse stromal cells. This is experimentally possible by taking advantage of a naturally occurring mutation carried in the germ-line of testicular feminized mice. The Tfm mutation involves single-base deletion in the coding region of the X-linked AR resulting in a frame shift mutation in the amino-terminal domain of the receptor (24, 25). The change decreases the stability of the Tfm-encoded AR mRNA (24, 25). In addition, the expressed Tfm-AR protein lacks transcriptional activity (24, 25).

Thus, it is possible to cross-breed homozygous nude/AR-wt (*i.e.*, nude/male- X_{wt}/Y) male mice with homozygous non-nude heterozygous Tfm female mice (*i.e.*, wt/wt- X_{Tfm}/X_{wt}) to produce hybrid female mice which are either *nu*/WT- X_{wt}/X_{wt} or *nu*/WT- X_{Tfm}/X_{wt} . By breeding hybrid females of this latter genotype (*i.e.*, *nu*/WT- X_{Tfm}/X_{wt}) with homozygous nude/AR-wt male mice, it is possible to obtain homozygous nude/AR-null male mice (*i.e.*, *nu/nu*- X_{Tfm}/Y) which completely lack the ability to express functional AR in any of their cells. The genetics at every breeding step are quality controlled using a PCR-based restriction digestion method to identify the AR-wt *versus* AR- X_{Tfm} mutant alleles (13).

The experiments using ventral prostatic epithelial cells isolated from 1-month castrated rats were repeated using these intact *versus* castrated *nu*/AR-null nude male mice as hosts. These studies demonstrated that when 10^6 rat prostatic epithelial cells were inoculated in 0.2 ml of Matrigel into intact or castrated AR-null nude male mice initially, rudimentary prostate acini developed like that presented in Fig. 1B. These rudimentary acini are surrounded by mouse stromal cells, as determined by Hoescht staining (*i.e.*, rat cells have a diffuse nuclear Hoescht staining like human cells *versus* the punctate staining of mouse cells). Unlike the situation in AR-wt nude male mice, the mouse stromal cells surrounding the rudimentary acini in these AR-null nude male mice were negative by immunocytochemical staining for AR expression. In addition, the epithelial cells in these rudimentary acini in intact AR-null nude male mice only sporadically expressed AR. This is in contrast to the nearly universal expression of

detectable AR in the transplanted acini in intact male AR-wt mice. These rudimentary glands did not grow in the intact AR-null nude male mice (*i.e.*, $<0.1\%$ Ki67-positive epithelial cells at 8, 15, or 35 days after transplantation). In summary, these results are consistent with androgen-stimulated growth of normal adult prostatic epithelial cells being indirect via the androgen dependent production of paracrine growth factors secreted by the surrounding AR-positive stromal cells. In addition, these results suggest that androgen-stimulated factors liberated from these AR-positive stromal cells are required to maintain full expression of the AR in normal prostate epithelial cells.

Autocrine Nature of the Androgen-responsive Growth of Malignant Prostatic Epithelial Cells. To determine whether a similar stromal-dependent paracrine mechanism was responsible for the growth-stimulating efforts of androgen on malignant prostatic epithelial cells, four different androgen-responsive xenograph models were used (Table 1). Each of the cancers, except the PC-82, was serially passageable as an *in vitro* permanent cell line and which when injected into nude mice as single cells, produced continuously growing cancers. The PC-82 has not been established as an *in vitro* line but is serially passageable *in vivo* in nude mice as a xenograph. Each of these four models express AR with only the LNCaP having a mutant AR gene (12, 26–28). This mutation allows the AR of LNCaP cells to bind estrogens and progestins, as well as androgens as ligand for its transcriptional activity (28). Interestingly, LNCaP, LAPC-4, and the R3327G cells express AR detectable by Western Blot (data not shown) even in cultures without stromal cells, unlike the situation with the cultured normal human prostate cells described previously. All of these cancers express detectable AR when grown as xenographs in nude mice. When single PC-82 cells were inoculated with Matrigel into intact mice, there was an ~2-month lag period before cancers became palpable. Once palpable, these PC-82 cancers grew as moderately well-differentiated prostatic adenocarcinomas composed of multiple malignant glandular acini (Fig. 1H) with a tumor volume doubling time of ~3 weeks (Table 1). In contrast, inoculation of single PC-82 cells into castrated nude male mice did not result in the development of growing cancers. Thus, the human PC-82 prostatic cancer cells are androgen dependent (29). Because of this dependence, if PC-82 cancers were allowed to develop in intact males and then these animals were castrated, the rate of PC-82 proliferation nearly completely stopped by day 4 after androgen ablation as determined by Ki67 immunocytochemical staining (Table 2). Castration, however, did not simply stop the continuous growth of PC-82 cancers. These PC-82 cancers regressed after castration with 27 ± 4 days required for the loss of 50% of the starting tumor volume. We have demonstrated previously that this regression is attributable to androgen ablation-induced apoptosis of these PC-82 cancer cells (29). The other three prostatic cancer cells lines used were not androgen dependent for their

Table 2 *Androgen responsiveness of the various prostatic cancers in AR-wild-type versus AR-null male nude mice*

Cancer type	AR status of host	Percentage of cancer cells proliferating (% Ki67 positive)	
		Intact	Castrated
Human PC-82	AR wild type	18 ± 3	0.3 ± 0.1 ^{a,b}
	AR null	21 ± 2	0.8 ± 0.1 ^{a,b}
Human LNCaP	AR wild type	46 ± 6	30 ± 3 ^b
	AR null	48 ± 9	32 ± 4 ^b
Human LAPC-4	AR wild type	55 ± 8	44 ± 9 ^b
	AR null	50 ± 2	41 ± 8 ^b
Rat R3327G	AR wild type	45 ± 2	20 ± 3 ^b
	AR null	42 ± 4	23 ± 5 ^b

^a Because PC-82 does not grow in castrated hosts, it was initially inoculated into intact hosts. Eleven weeks later, these animals were castrated, and 4 days later, the tissue was harvested for analysis.

^b $P < 0.05$ compared with the percentage of Ki67 in the corresponding intact group.

survival but were sensitive to androgenic stimulation of their rate of *in vivo* growth (*i.e.*, doubling time when inoculated as single cells with Matrigel; Table 1). This was because of an androgenic stimulation of their rate of cell proliferation (Table 2).

To determine whether the androgen dependence of the PC-82 and the androgen sensitivity of the other three prostatic cancer lines was because of a paracrine effect of stromal cells, the percentage of cancer cell proliferation was compared between untreated and castrated AR-wt *versus* AR-null nude male mice (Table 2). These results demonstrated an essential identical androgen responsiveness in both AR-wt and AR-null nude male mice. Histological analysis also demonstrated that regardless of the type of host, none of these four different prostatic cancers stimulated an influx of mouse stromal cells, and none of the cancers had a surrounding collar of smooth muscle cells as part of the stroma of these cancers. Fig. 1H provides a representative example of the PC-82 cancer cells growing in an intact AR-wt nude male mouse illustrating the lack of a defined smooth muscle component surrounding growing malignant acini. These consistent results document that the androgen responsiveness of the growth of both human and rat prostate cancers is regulated by direct involvement of the AR expressed in the cancer cells themselves and not indirectly attributable to AR-activated stromal cell-dependent paracrine pathways.

DISCUSSION

Both the original report of Hayward *et al.* (10) and studies reported here document that when transplanted, prostatic epithelial cells have a major chemotactic effect upon host mouse mesenchymal cells. In the present study, Matrigel was used as the matrix for transplantation of the prostatic epithelial cells. Matrigel is solubilized from the extracellular matrix isolated from the EHS mouse sarcoma grown in mice (19). Soluble Matrigel solidifies above 4° and contains a variety of growth factors as well as fibillar material (*i.e.*, collagen type IV, laminin, and entactin) and heparan sulfate proteoglycans (30). In the Hayward studies, the matrix used for transplantation was collagen type I. Thus, the ability of the transplanted prostatic epithelium to stimulate the influx of mouse mesenchymal cells is not matrix dependent. The observations that the infiltrating mesenchymal cells eventually surround the rudimentary glandular acini to produce a stromal collar composed of both smooth muscle cells and fibroblasts and that a subset of these stromal cells express AR are specific responses. This conclusion is based upon two observations: (*a*) when Matrigel alone without the prostatic epithelium is inoculated into nude male mice, there is little influx of mouse cells into the gelled pellet, and the limited number of cells which are present do not express α -smooth muscle actin or AR, and (*b*) when prostatic cancer cells are transplanted with Matrigel, there is an influx of endothelial cells into the gelled pellets as part of an angiogenic response induced by the cancer cells; however these endothelial cells do not express AR. These results lead to the conclusion that normal but not malignant prostatic epithelium can induce both the infiltration of initially AR-negative mouse mesenchymal cells and a shift in the phenotype of these infiltrating cells to smooth muscle cells and fibroblasts, which now express AR. Along these lines, it is significant that when human adult prostatic epithelial cells grown in early *in vitro* passage without their stromal cells were transplanted, no induction of mesenchymal cell infiltration or shift in stromal phenotype occurred. In addition, no development of even rudimentary glandular acini or androgen-stimulated growth occurred. These results suggest that there is an important reciprocal interaction and sensitivity between normal prostatic epithelium and mesenchyme during prostatic morphogenesis and

growth and that this sensitivity is rapidly lost during *in vitro* culture as separated populations.

Indeed, Cunha *et al.* (31) have emphasized this point previously. These previous studies, however, have used embryonic urogenital sinus mesenchyme and suggested that differentiation of urogenital sinus mesenchyme requires an inductive signal from epithelium and androgen. In contrast, in the present studies, the mesenchyme was adult and not embryonic, and the development of smooth muscle cells occurred even in the androgen-ablated hosts. Leaving these differences aside, both the Cunha *et al.* (31) and present studies clearly document that malignant prostatic epithelial cells lose their stromal-inductive ability. This may be attributable to the limitation in both studies of using human and rat prostate cancer cells xenografted into stroma of mouse origin. The fact that there is a total loss of AR expression in the stromal cells surrounding prostatic cancer cells in malignant tissue taken directly from patients (32), however, argues against this interruption. Alternatively, this loss of stromal-inductive ability by prostatic cancer cells appears to be related to the conversion from a stromal-cell-dependent paracrine pathway to a direct autocrine mechanism for the androgen stimulation of their growth *in vivo* as demonstrated in the present studies.

These combined results document that during transformation of androgen-responsive normal prostatic epithelial to malignant cancer cells, a shift in the AR axis from stromal-cell-dependent paracrine pathways to autocrine-dependent pathways occurs. These results do not mean, however, that stromal cells are not required for the growth of such androgen-responsive cancer cells. Indeed, such *in vivo* growth requires the development of a blood supply and an adequate micro-environment, both of which are provided by the stromal cells. Each of the prostate cancer models used in the present studies induced a robust angiogenic response in either AR-wt or AR-null male nude mice. In previous studies, this angiogenic response has been demonstrated to be via the androgen-stimulated production of the potent angiogenic factor vascular endothelial growth factor by prostatic cancer cells (33–35). Thus, there is a paracrine interaction between the androgen-induced secretion of vascular endothelial growth factor by androgen-responsive prostate cancer cells and the migration and proliferation of stromal endothelial cells to produce new blood vessels. This androgen-induced paracrine interaction, however, is initiated by androgen occupancy of functional AR within the nucleus of these cancer cells and not the stromal cells (13, 33, 34).

The demonstration that malignant transformation of prostate epithelial cells involves the acquisition by these cells of an autocrine mechanism for androgen-stimulated proliferation and survival has several significant implications. These include the realization that specific molecular changes in these transformed epithelial cells must occur which allow AR protein to regulate a series of genes that effect proliferation and survival, the expression of which is not under AR control in normal prostatic epithelial cells. *E.g.*, recent studies have demonstrated that normal mouse prostatic epithelium acquire direct responsiveness to the growth-promoting effects of occupied AR if *p53* genes are homogeneously “knocked out” in the cells (36, 37). Presumably, such molecular changes allow AR ectopically to bind to and stimulate the expression of genes encoding a series of peptide growth and survival factors, which once secreted by these malignant cells, bind to cell surface receptors activating an autocrine signaling pathway. These factors could be the same ones produced in a paracrine manner by the AR-stimulated stromal cells in the normal prostate, or they could be different. In addition, the response to these secreted peptide factors would also be fundamentally different in malignant as opposed to normal prostatic epithelial cells. For example, nerve growth factor is secreted by stromal cells, but it is not a survival factor for the normal prostatic epithelium (11). In contrast, nerve growth

factor is directly secreted by malignant prostatic epithelial cells to regulate an acquired survival pathway (11). Identifying additional growth and survival factors whose production by malignant prostatic cells is under AR control and the molecular mechanisms underlying this acquired expression are critically needed. Such information will be essential in prioritizing targets for drug development approaches for this devastating disease.

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