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Transdifferentiation of NRP-152 rat prostatic basal epithelial cells toward a luminal phenotype: regulation by glucocorticoid, insulin-like growth factor-I and transforming growth factor-beta

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SUMMARY

The role of basal epithelial cells in prostatic function, development and carcinogenesis is unknown. The ability of basal prostatic epithelial cells to acquire a luminal phenotype was explored in vitro using the NRP-152 rat dorsal-lateral prostate epithelial cell line as a model system. NRP-152, which was spontaneously immortalized and clonally derived, is an androgen-responsive and nontumorigenic cell line that has a basal cell phenotype under normal growth conditions. However, when placed in mitogen-deficient media, these cells undergo a dramatic morphological change to a luminal phenotype. Under these growth-restrictive conditions, immunocytochemical analysis shows that NRP-152 cells acquire the luminal markers Z0-1 (a tight-junction associated protein), occludin (integral tight-junction protein), and cytokeratin 18, and lose the basal markers cytokeratins 5 and 14. Total protein and mRNA levels of cytokeratins 8, 18, c-CAM 105 (the calcium-independent cell adhesion molecule) and Z0-1, as detected by western and/or northern blot analyses, respectively, are induced, while cytokeratin 5 and 15 are lost, and occludin is unchanged. Concomitant with this differentiation, expression of transforming growth factor-beta 2 (TGF-β2), TGF-β3, and TGF-β receptor type II (TβRII) is induced, while those of TGF-β1 and TβRI remain essentially unchanged. Mitogens, such as insulin-like growth factor-I and dexamethasone inhibit luminal differentiation, while exogenous TGF-β induces such differentiation. These data together with TGF-β neutralization experiments using pan-specific antibody implicate an important role for autocrine TGF-β in the induction of the luminal differentiation.

Key words: Lobund-Wistar rat, Dorsal-lateral prostate, Cytokeratin, Tight junction, Cell growth

INTRODUCTION

Delineation of the basic cellular biology of the various cell types in the prostate is critical to understanding the mechanisms of normal development and carcinogenesis in this organ. Prostatic epithelium, which consists mainly of two basic cell types (luminal and basal), requires androgens not only for development but also for maintaining the structure and function of the mature organ (Hayward et al., 1996a,b; Isaacs, 1987). When deprived of androgen, the adult prostate undergoes rapid atrophy concomitant with extensive apoptotic death, predominantly in the luminal epithelial cells (English et al., 1985, 1987; Kyprianou and Isaacs, 1988; Rouleau et al., 1990; Hayward et al., 1996b). Despite this massive loss of cells, the regressed prostate long retains the ability to regenerate completely following restimulation with androgens (Isaacs, 1987). This indicates that the regressed prostate must have a reservoir of epithelial stem cells that are activated to proliferate and differentiate upon androgen stimulation. The identity or localization of such cells remains controversial, despite over 18 years of research towards this effort.

Based mostly on morphometric analyses of basal and luminal epithelial cells in the prostate and their respective DNA labeling indices (with ^{3}H-thymidine) before and following castration and androgen re-stimulation, a number of studies suggested that basal cells may serve as the regenerating cell type in the prostate (English et al., 1985, 1987; Evans and Chandler, 1987). Most critical to supporting this hypothesis have been the observations that basal cells turn over rapidly, since their numbers do not change following regression and regeneration of the prostate despite their relatively high proliferative indices compared to the luminal cells (English et al., 1987; Bonkhoff et al., 1994a,b; Bonkhoff and Remberger, 1996). Thus, because relatively little apoptosis occurs in basal cells (Kyprianou and Isaacs, 1988; Rouleau et al., 1990; Hayward et al., 1996b), one can speculate that basal cell numbers may be maintained through their continual conversion to luminal variants.

In addition to differences in morphology, luminal epithelial cells can be distinguished from basal epithelial cells by the expression of cytokeratins 8 and 18 and C-CAM-105 in the former and cytokeratin 5, 15, and 14 in the latter cell type (Lane and Alexander, 1990; Sherwood et al., 1991; Hsieh et al., 1992, 1994). The availability of cDNA probes and antibodies specific for these markers (Lane and Alexander, 1990; Roop et al., 1984, 1985; Hsieh et al., 1992) have more recently allowed assessment of subtle and discreet phenotypic changes during prostate development (Hayward et al., 1996) and have been critical to observations that expression of luminal cell specific cytokeratin and cell adhesion markers can be identified in basal cells during prostate development, in the regressed prostate following androgen ablation, and during regeneration of the regressed prostate (Hsieh et al., 1992, 1994; Verhagen et al., 1988, 1992). Such findings have been key to supporting the hypothesis that basal and luminal cells may share a common lineage or are interchangeable (Bonkhoff et al., 1994a,b, 1996; Verhagen, 1992; Hsieh et al., 1992, 1994). However, to date there has been no direct demonstration that a prostatic basal epithelial cell can differentiate into a luminal variant.

The present study is the first report that demonstrates the ability of a prostatic epithelial cell line, NRP-152, to differentiate from a basal to a luminal phenotype. NRP-152, which was derived from rat dorsal-lateral prostate, is a non-tumorigenic and androgen-responsive basal epithelial line (Danielpour et al., 1994) with the unique ability to form fully functional prostatic organoids in vivo, when recombined with rat or mouse urogenital sinus mesenchyme (Hayward et al., 1988). When placed under growth restrictive conditions (Dulbecco's modified Eagle's medium/Ham's F12 (DMEM/F12) + 1% calf serum (CS)) NRP-152 undergoes a morphological change resembling that of luminal cells. Changes in various basal and luminal epithelial cell specific markers, as measured immunocytochemically and by western and northern blot analyses, support the notion that NRP-152 cells transdifferentiate toward a luminal phenotype under these growth-restrictive conditions. Moreover, discreet growth regulatory factors such as glucocorticoids, insulin-like growth factor I (IGF-I) and TGF-β can each selectively modulate the basal-luminal differentiative phenotype of these cells. Thus, NRP-152 is a suitable cell line to study the regulation and mechanism of differentiation of prostatic epithelial cells.

MATERIALS AND METHODS

Materials

Sources were as follows: TGF-β 1, 2 and 3, R&D Systems, Inc. (Minneapolis, MN); DMEM/F12, FBS, CS, and trypsin-EDTA, Gibco/BRL (Grand Island, NY); bovine insulin and mouse epidermal growth factor (EGF), Biofluids, Inc. (Rockville, MD); LR^3-IGF-I, GroPep (Adelaide, SA, Australia); cholera toxin, dexamethasone (Dex), dihydrotestosterone, retinoid acid, Sigma Chemicals (St Louis, MO). Monoclonal antibody against TGF-βs (pan specific), Genzyme, Inc. (Cambridge, MA); FITC-conjugated Texas Red-conjugated, and horseradish peroxidase-conjugated secondary antibodies, Jackson Laboratories (West Grove, PA); hepatocyte growth factor (HGF) and keratinocyte growth factor (KGF), Collaborative Research (Bedford, MD); rat anti-human ZO-1 MoAb, Chemicon International Inc. (Temecula, CA). Affinity purified rabbit antibodies made against specific sequences of mouse cytokeratins 5 and 8 (Roop et al., 1984, 1985), and cDNA to cytokeratin 5 were gifts from Dr Stuart Yuspa. Mouse monoclonal antibody specific for rat cytokeratin 18 (Woodcock-Mitchell et al., 1986) was a gift from Dr Janet Woodcock-Mitchell. Dr Jer-Tsong Hsieh kindly provided cDNA to rat cytokeratins 8 and 15 and antibody to rat C-CAM 105 (Hsieh et al., 1994). Human ZO-1 cDNA (Willott et al., 1993) and guinea pig anti-occludin antisera were gifts from Dr James Anderson.

Cell culture

The rat prostatic cell lines used in this study (between passages 15 and 35 of the NRP-152 and NRP-154 lines) were derived from the dorsal-lateral prostate of carcinogen-treated Lobund-Wistar rats without transfection or infection of an immortalizing gene, as described (Danielpour et al., 1994). These cells were grown in GM2 (DMEM/F12, 5% FBS, 0.1 μM Dex, 5 μg/ml insulin, 20 ng/ml EGF, 10 ng/ml cholera toxin), and passaged as described (Danielpour et al., 1994). Unless indicated all differentiation assays were at a seeding density of 2×10^5 cells per ml of medium (DMEM/F12, 15 mM Hepes, 1% CS) in 100 mm Falcon tissue culture dishes or in 8-well Permanox chamber slides (Nunc, Naperville, IL).

Immunostaining

Cytoskeletal markers, ZO-1, and occludin, were assayed immunocytochemically using subconfluent to confluent monolayers grown in chamber slides. Cells on slides were washed twice with PBS and then fixed for 10 minutes at –20°C in 100% methanol. Slides were blocked with BB (PBS +1% BSA + 5% serum matched to that of the secondary antibody) for 30 minutes at RT, treated with primary antibodies or control IgG in BB1 (BB + 0.1% Tween-20) for 1 hour followed by a 1 hour incubation in BB1 with 10 μg/ml of FITC-labeled or Texas Red-labeled secondary antibody against the primary antibody, using extensive washes between each step. Slides were mounted with an anti-quenching aqueous mounting medium (Vector Laboratories, Berlingame, CA) and stored at 4°C for up to two weeks.

Western blot

Cells were lysed with lysis buffer (PBS, 1% SDS, 1 mM EDTA, 1 mM PMSF, 2 μg/ml each of aprotinin, leupeptin, and pepstatin A), passed several times through a 25 gauge needle to sheer DNA. Protein concentration was measured by the BCA protein assay (Pierce Chemical Co., Rockland, IL) to normalize sample loading. Samples were treated with SDS-PAGE loading buffer for 5 minutes at 100°C, electrophoresed at 4°C through a 4 to 20% gradient Novex Tris-glycine polyacrylamide gel (Novex, San Diego, CA), and then electroblotted to a nitrocellulose membrane with transfer buffer containing 10% methanol (Towbin et al., 1979). Membranes were allowed to dry at RT overnight. Dried blots were hydrated with deionized water for 10 minutes, blocked for 30 minutes with 5% (w/w) non-fat milk in TBS (10 mM Tris-HCl, 150 mM NaCl, pH 8.0), followed by 30 minutes in TBSTBG (TBS, 0.1% Tween-20 + 2% BSA V, 0.2% gelatin). Primary antibody was allowed to bind in TBSTBG for 1 hour, and blots were washed three times (10 minutes per wash) with TBSTM (TBS, 0.1% Tween-20, 5% non-fat milk). Horseradish peroxidase-labeled secondary antibody (0.16 μg/ml) was allowed to bind in the latter buffer for 1 hour. Following two washes with TBST and three with TBS (10 minutes/wash), bands were visualized by enhanced chemoluminescence (Pierce Chemical Co, Rockland, IL).

Northern blot

Total RNA was purified using RNeasy™ columns (Qiagen, Chatsworth, CA). 10 μg of total RNA was electrophoresed though 1% agarose-0.66 M formaldehyde gels containing 0.72 μg/ml ethidium bromide. Equal loading and even transfer were 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 minutes, neutralized with 50 mM Tris-HCl, pH 7.4, 10 mM NaCl

for 20 minutes, and then blotted onto Nytran (pore size, 0.45 μM; Scleicher & Scheull, Neene, NH) for 16-24 hours with 10× SSPE. After membranes were cross-linked by UV radiation, they were hybridized, and washed at 65°C as described by Church and Gilbert (1984). The cDNA probes were labeled with [^{32}P]dCTP using a random priming kit (Boehringer Mannheim, Indianapolis, IN). Hybridization was done with 2-3×10^6 cpms/ml (>10^9 dpms/μg DNA). The cDNA probes were the full-length coding regions of rat TGF-β1, mouse TGF-β2 and 3, human TβRI, TβRII, rat cytokeratin 8 and 15 and ZO-1, residues 1-421 of rat cytokeratin 18, residues 107-950 of rat c-CAM-105, and 3′ UTR of mouse cytokeratins 5 and 14.

Detection of internucleosomal DNA ladder

Unless indicated, 1.5 to 2×10^6 cells were plated in 10 cm Falcon tissue culture dishes with 10 ml DMEM/F12 containing 15 mM Hepes, and 1% CS. All factors were added 24 hours after plating, and cells were detached by trypsinization following 24 hours of treatment (3 ml trypsin-EDTA, 7 minutes at 37°C). Internucleosomal DNA ladders (Wyllie, 1980; Rosl, 1992) were detected with a modification of TACS^{TM} apoptotic DNA ladder kit (Trevigen, Gaithersburg, MD). Cell pellets resuspended in 50 μl PBS were lysed by the addition of 50 μl of lysis buffer and purified as described by the kit. The nicked ends of 1 μg of DNA were ^{32}P-labeled with 2.5 units of Klenow fragment of DNA Pol I in the presence of 0.5 μCi of [α-^{32}P]dCTP (3 Ci/μmol, NEN-Dupont, Boston, MA) for 30 minutes at RT. The reaction volume was 10 μl and contained 5 mM $MgCl_2$ and 10 mM Tris-HCl (pH 7.5). One third of this labeled DNA was electrophoresed through 1.8% agarose-1× TAE at 70 V for 2 hours. Gels were then dried directly and exposed to X-Omat AR (Kodak, Rochester, NY) for about 1 hour.

TGF-β assays

NRP-152 cells were plated in 100 mm Falcon tissue culture dishes at 2 ×10^6 cells/well/10 ml of DMEM/F12, 1% CS. Plates were harvested daily, medium stored at 70°C, and cell numbers determined for each plate. TGF-βs 1, 2 and 3 secreted in the conditioned medium were determined by SELISAs (Danielpour et al., 1989; Danielpour, 1993; Danielpour and Roberts, 1995) for these isoforms.

RESULTS

Morphological evidence for the differentiation of NRP-152 cells

The NRP-152 androgen-responsive and non-tumorigenic basal epithelial cell line, which was derived from the dorsal-lateral prostate of a Lobund-Wistar rat, requires a rich supply of growth factors for optimal growth (Danielpour et al., 1994; Danielpour, 1996). These cells are normally cultured in GM2 (DMEM/F12 + 5% FBS, 20 ng/ml EGF, 5 μg/ml insulin, 0.1 μM dexamethasone, and 10 ng/ml cholera toxin) in our laboratory. Under mitogen-deficient conditions (basal medium (DMEM/F12) supplemented with 1% CS) that cause growth arrest, NRP-152 cells undergo a dramatic change in morphology resembling that of the NRP-154 luminal prostatic epithelial cell line (Fig. 1). Under these conditions NRP-152 cells become flatter, hexagonal, form distinct cell-cell junctions (Fig. 1), and make greatly increased numbers of adherent junctions, desmosomes, hemidesmosome, and microfilaments (data not shown).

Immunocytochemical evidence for luminal differentiation of NRP-152 cells

NRP-152 cells that were maintained in GM2 or under the above differentiation permissive conditions for 4 days were stained for tight-junction associated (ZO-1) and integral (occludin) proteins, to test the acquisition of a luminal phenotype. NRP-152 cells maintained in GM2 exhibit a basal phenotype with respect to the absence of staining for ZO-1 or occludin (Fig. 2A,C). When switch to basal medium containing 1% CS, NRP-152 cells acquire ZO-1 and occludin (Fig. 2B,D) specifically and continuously along cell-cell boundaries, suggesting the formation of a complete tight-junction ring characteristic of luminal cells.

Consistent with a basal phenotype, 100% of the NRP-152 cells cultured in GM2 are positive for cytokeratin 5 and 100% of them are negative for cytokeratin 18 staining (Fig. 3). When shifted to basal medium containing 1% CS about 35% (7 days of treatment) of NRP-152 cells stained for cytokeratin 18, while 46% and 33% of them completely or partially lost, respectively, cytokeratin 5 staining. Similar losses of cytokeratin 14 occurred under these conditions (data not shown). Double immunofluorescence using cytokeratins 5 and 18 antibodies showed that 87% of the cells that acquired cytokeratin 18 lost cytokeratin 5 staining upon differentiation, although about 4% of the cells co-expressed both markers to varying degrees (Fig. 3). Also, 48% of the cells that completely lost cytokeratin 5 did not appear to express cytokeratin 18. The staining pattern of these cytokeratins appeared clearly filamentous at high magnification, typical of intermediate filaments. Thus, the NRP-152 cell line that was clonally derived and is homogeneous (with respect to karotype (triploid), expression of cytokeratin 5 and 14, and absence of cytokeratin 18; Danielpour et al., 1994) under mitogen-rich growth conditions become heterogeneous with respect to basal and luminal cytokeratins when cultured under mitogen-deficient conditions.

Western and northern blot analysis of luminal and basal markers

The ability of NRP-152 cells to change expression of cytokeratins, and tight-junction proteins was examined quantitatively at the protein level by western blot analysis from cells cultured for 1-10 days in 1% CS. Cytokeratin 18 was induced 3-4 days upon switching to 1% CS with >100-fold increased expression following 8 days (Fig. 4A). Expression of the luminal markers cytokeratin 8, 18 and c-CAM 105 were significantly elevated after several days of culture. However, c-CAM 105 levels dropped by day 10, in contrast to that of cytokeratin 8 and 18 that remained elevated. The expression of ZO-1 was induced about 2-fold, whereas that of occludin was not elevated, suggesting that differentiation triggers the assembly of tight junctions rather than the synthesis of their components. Also as expected, cytokeratin 5 expression was significantly down-regulated upon such differentiation, although not as dramatically as predicted by the immunocytochemical results. This discrepancy may be explained by an enhanced expression of cytokeratin 5 in a significant proportion of cells that retained basal phenotype during this differentiation. At day 10 of differentiation the levels of the luminal cytokeratins in these cells were similar to those of the luminal rat prostatic cell line, NRP-154 (Fig. 4B). Northern blot analysis was used to determine whether the above changes were controlled at the level of mRNA. This analysis revealed that, for the most part, the above protein

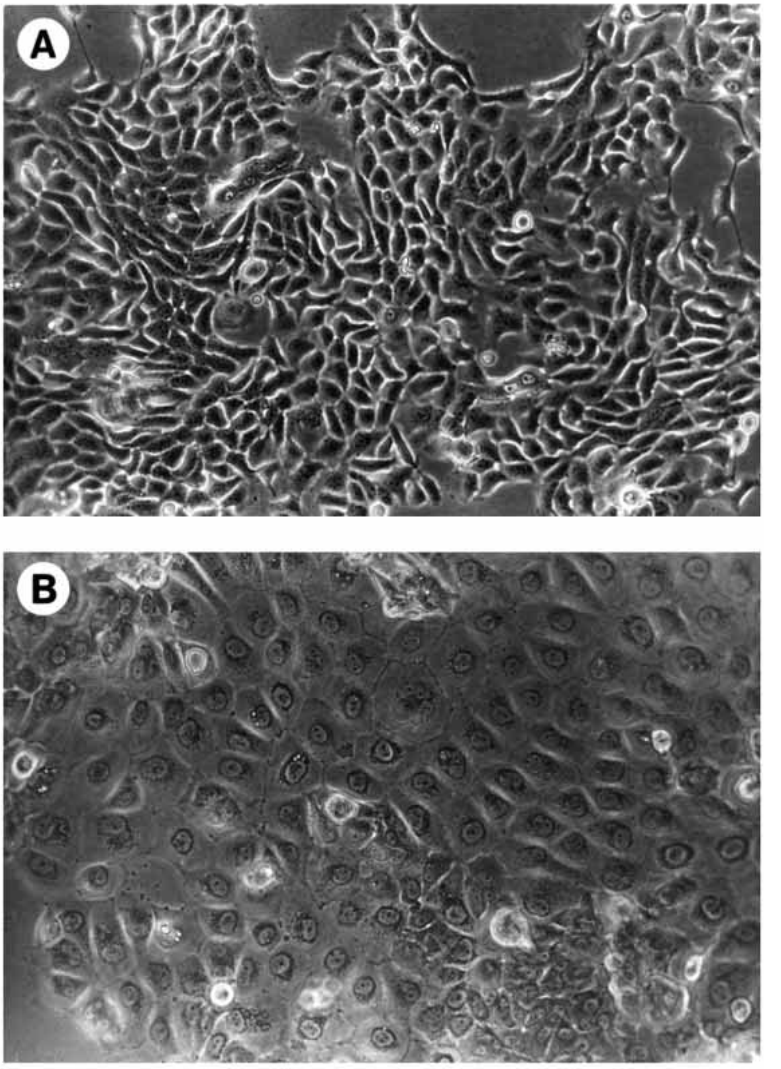


Fig. 1. Morphology of NRP-152 cells cultured in either growth factor-rich or -deficient medium. NRP-152 cells were grown in either GM2 (A) or DMEM/F12 +1% CS (B) for three days in Costar dishes. Cells were photographed directly without fixation at ×100 magnification.

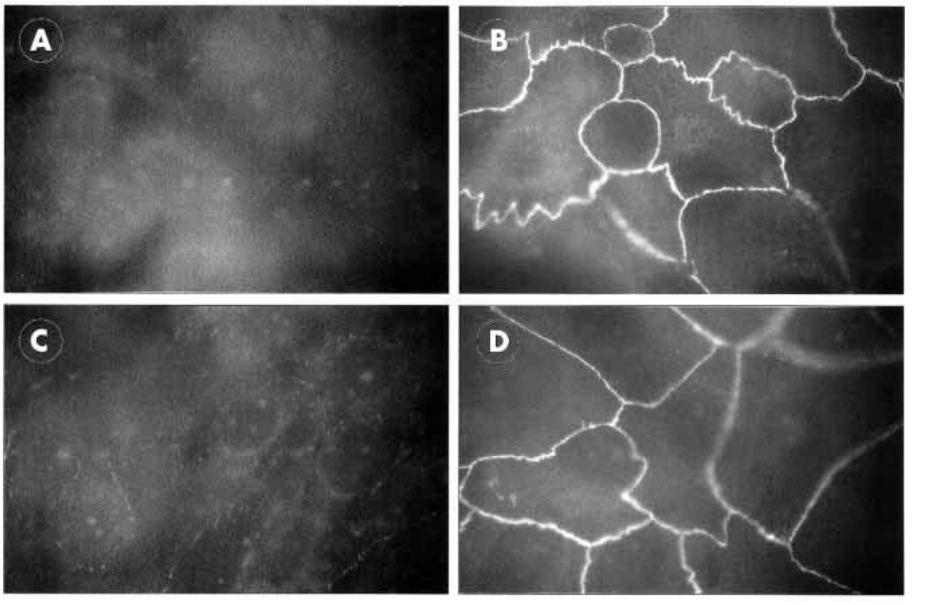


Fig. 2. Appearance of tight-junctions upon differentiation of NPR-152 cells. NRP-152 cells cultured in either GM2 (A,C) or DMEM/F12 + 1% CS (B,D) for 4 days were examined by immunofluorescence (×100 objective) for Z0-1 (A,B) and occludin (C,D).

changes followed changes in their mRNAs (Fig. 4C). Thus, the expression of these proteins is regulated by either a transcriptional or message stabilization mechanism. NRP-152 cells did not produce any c-CAM-105 protein when cultured in GM2 even though they expressed the message for this adhesion molecule. This suggests that expression of c-CAM-105 is also regulated at the translational level.

Dedifferentiation of NRP-152 cells

To test whether the differentiation of NRP-152 cells toward the luminal phenotype was reversible, NRP-152 cells were allowed to differentiate for 12 days in 1% CS, at which time they were switched to GM2. Under these conditions, expression of mRNAs for cytokeratins 8 and 18 were partially to completely lost and that for cytokeratins 5 and 15 were induced (data not shown). Since loss of cytokeratin 18 occurred as early as day 1 and preceded any significant increases in cell proliferation, which did not occur until day 5, early losses in cytokeratin 18 are more likely due to either dedifferentiation or/and selective death of luminal cells rather than the selective expansion of basal cells. In two out of three experiments this dedifferentiation was incomplete as such cultures retained some cytokeratin 18 expression even after two weeks in GM2. This suggests that some differentiated cells did not dedifferentiate and also acquired proliferative potential. Moreover, the remaining dedifferentiated cells retained the ability to redifferentiate upon mitogen removal (data not shown).

Effect of dexamethasone and IGF-I on differentiation of NRP-152 cells

To define the key factors essential for maintaining the basal phenotype of NRP-152 cells, the ability of Dex, IGF-I, EGF, KGF, HGF, dihydrotestosterone (DHT) and all-trans-retinoic acid (RA) to maintain the basal phenotype was examined in basal medium containing 1% CS. As shown, Dex and IGF-I (in the form of LR^3-IGF-I) were most effective in maintaining basal phenotype (although essentially all the above factors seemed to block cytokeratin 18 expression to some extent) (Fig. 5). This analogue of IGF-I was used because it binds poorly to IGF-I binding proteins, and has been shown to be about 500-fold more active that IGF-I on NRP-152 cells (Hsing et al., 1996). Dex and IGF-I, which synergize to stimulate NRP-152 growth, also synergistically maintained the basal epithelial phenotype of these cells. These results support the suggestion that differentiation may be triggered by growth arrest signals.

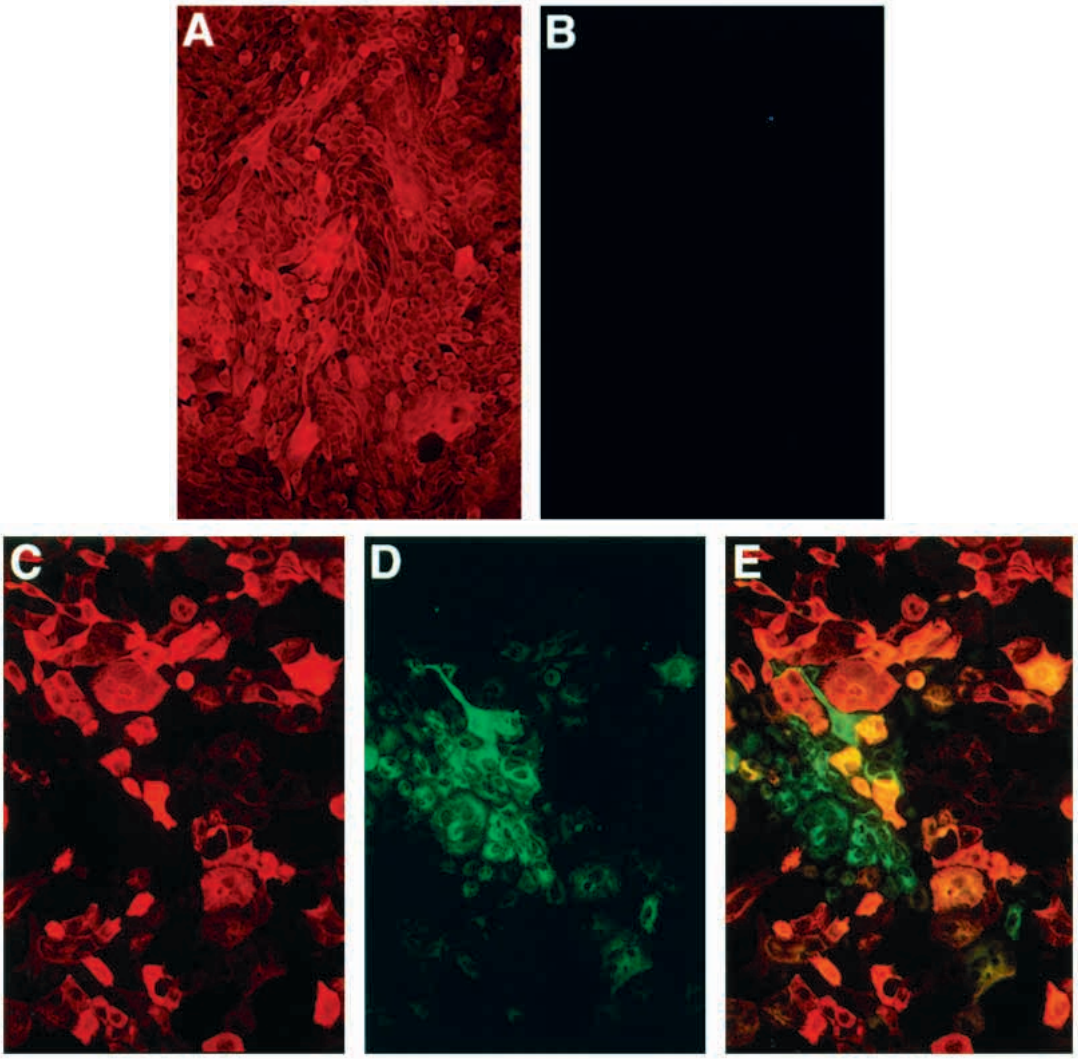
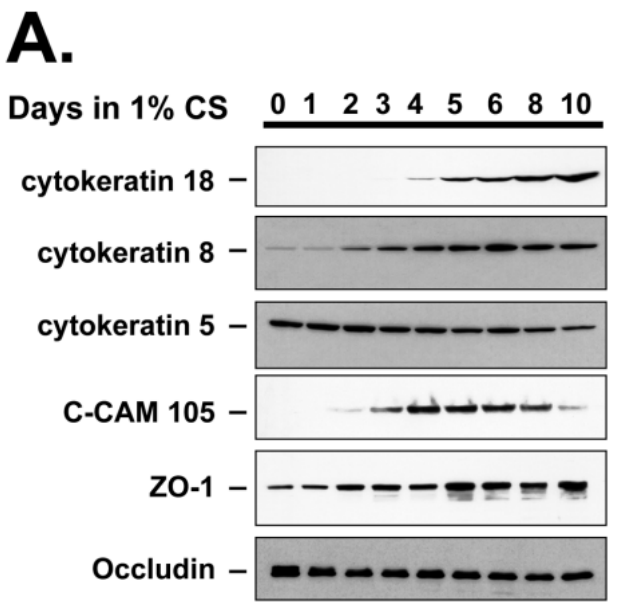


Fig. 3. Concomitant acquisition of cytokeratin 18 with loss of cytokeratin 5. NRP-152 cells cultured in either GM2 (A,B) or in DMEM/F12 + 1% CS (C,D,E) for 7 days were examined expression of both cytokeratin 5 by immunofluorescence with Texas Red (A,C,E), and cytokeratin 18 by immunofluorescence with FITC (B,D,E). Cells coexpressing both markers are shown in yellow in E.



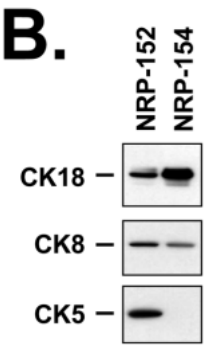
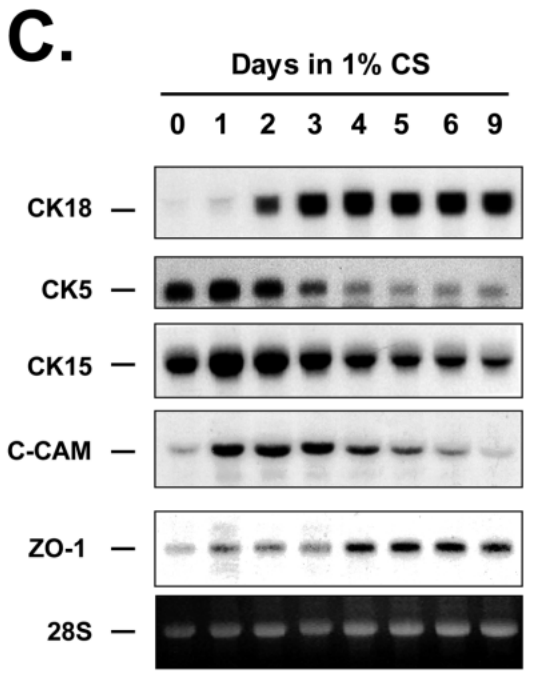


Fig. 4. Temporal changes in the expression of luminal and basal markers in differentiating NRP-152 cells as measured by western blot and northern blot analyses. NRP-152 cells were allowed to differentiate in the presence of DMEM/F12 containing 1% CS from 1 to 10 days, and changes in both the protein and mRNA levels for various luminal and basal markers were determined by western blot (A) and northern blot (C) analysis. The expression of some of these markers in NRP-152 cells at 10 days of differentiation were compared by western blot analysis to the levels of these markers in the NRP-154 luminal cell line cultured in GM2 (B).



TGF-βs 2, 3 and TGF-β type II receptors are induced during differentiation of NRP-152 cells. The expression of TGF-βs and their receptors was measured as a function of NRP-152 cell differentiation. When switched to 1% CS, these cells expressed elevated levels of TGF-β2, TGF-β3, TGF-β receptor type II (TβRII) mRNA, while that of TGF-β1 and TβRI were unchanged (Fig. 6). Changes in the protein levels of TGF-βs 1, 2, and 3 (as assayed by SELISA), reflected that of their message levels, whereas that of TGF-β3 was dramatically suppressed relative to the induction of its mRNA (Fig. 7). The effects of individual mitogens on the expression of TGF-βs were also explored. Both Dex and IGF-I, which help maintain NRP-152 cells in a basal epithelial state, also down-regulated TGF-β expression along with TβRII (data not shown). This suggests a link between the expression of TGF-β and the induction of luminal differentiation.

TGF-βs regulate the differentiated phenotype of prostatic epithelial cells

The correspondence of changes in TGF-β expression with cellular differentiation suggested that TGF-β might play a role associated with the luminal differentiation of NRP-152 cells. The ability of TGF-β to induce differentiation of NRP-152 cells was examined by the acquisition of luminal cytokeratins and loss of basal cytokeratins. NRP-152 cells were allowed to first differentiate in 1% CS for 5 days, a time in which there was no further increase in cytokeratin 18 mRNA. At this time a two day treatment of these cells with 10 ng/ml of TGF-β1 dramatically induced the expression of cytokeratin 18 and inhibited expression of cytokeratins 5 and 15, either in the presence or absence of Dex or LR^3-IGF-I (Fig. 8). However, the simultaneous addition of Dex and LR^3-IGF-I, which stimulate growth and block differentiation (Fig. 7), blocked the ability of TGF-β to induce differentiation. This effect of TGF-β on differentiation can be seen to varying degrees, regardless of the time of its addition. In a previous report we showed that TGF-β also induces apoptosis of these cells under similar conditions (Hsing et al., 1996). However, the conditions that are optimal for this differentiation are different from those optimal for the induction of apoptosis by TGF-β. For example, Dex, which enhances the ability of TGF-β to induce apoptosis, does not enhance the ability of TGF-β to promote differentiation (Fig. 9). It is likely that TGF-β signal transduction pathways that lead to differentiation and apoptosis are distinct, with the latter pathway being selectively enhanced by Dex. Whether or not Dex, which by itself inhibits differentiation, actually blunts the ability of TGF-β to transduce a signal for the induction of differentiation remains to be determined. The possibility that the differential sensitivity of these two processes to Dex may result from selective death of the luminally-differentiated cells is weakened by the fact that Dex, which inhibits differentiation, would rather be expected to also inhibit TGF-β-induced apoptosis.

Because TGF-β is induced during differentiation and can itself stimulate differentiation of NRP-152 cells, we speculated that autocrine/paracrine production of TGF-β induced by mitogen-depletion might induce differentiation of these cells. To test this, NRP-152 cells were allowed to differentiate in the presence of either control mouse IgG, or an antibody (1D11.16 MoAb) that neutralizes all three mammalian isoforms of TGF-β. The induction of cytokeratin 18 was significantly inhibited in the presence of anti-TGF-β IgG_1 with respect to control IgG_1 or without IgG_1 addition (Fig. 10), suggesting that autocrine TGF-β plays a role in the induction of luminal differentiation triggered by mitogen withdrawal.

DISCUSSION

This is the first in vitro demonstration that a prostatic epithelial cell line, NRP-152, can differentiate from a basal toward a luminal phenotype. This observation lends direct support to the hypothesis that basal epithelial cells or a certain class of such cells can serve as precursors to luminal cells in the adult prostate (Bazer, 1980; Timms et al., 1976; Bonkoff and Remberger, 1996). Our findings suggest that basal-luminal differentiation of prostatic epithelial cells is a dynamic process that is tightly coupled to mitogenic stimulation. Luminal differentiation of NRP-152 cells, which is either promoted by mitogen depletion and partially reversed by mitogen repletion, is either enhanced or/and mediated by autocrine TGF-β. Moreover, such differentiation may be under transcriptional or message stability control, as suggested by the expression of various specific markers.

Isaacs (1987) proposed a stem cell model, that postulates the presence of an expanding hierarchy of three epithelial cell populations in the prostate: *stem cells* (unlimited proliferative capacity, do not require androgen for survival) generate *amplifying cells* (limited growth capacity, do not require androgen for survival) that in turn become *transit cells* (limited proliferative capacity, require androgen for survival). NRP-152 fits the definition of a stem cell in being pleuripotent and having the potential for virtually unlimited proliferation. Moreover, as expected of stem cells that generate proliferating non-stem cells by virtue of unequal cell division, the pleuripotency of NRP-152 cells is lost or significantly diminished at late passage (passage >50; data not shown). Whether the NRP-152 cell line originated from a stem cell, or acquired stem cell-like behavior concomitant with immortalization cannot be assessed at this time. Nevertheless, these cells are nontumorigenic, have retained the functional properties of normal prostatic epithelial cells, and are capable of developing fully differentiated and functional prostatic

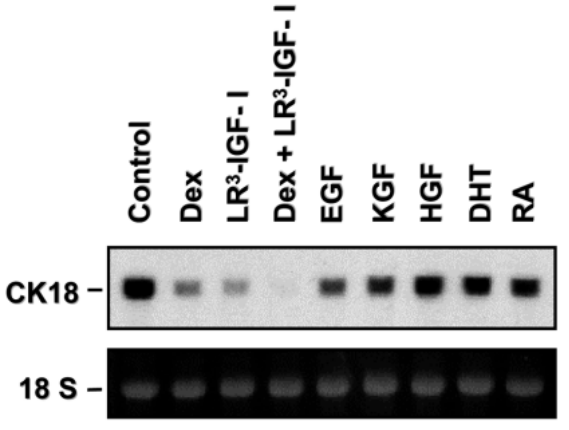


Fig. 5. Regulation of differentiation by specific hormones and mitogens. NRP-152 cells were allowed to differentiation for 4 days in DMEM/F12 + 1% CS supplemented with 0.1 μM Dex, 2 nM LR^3-IGF-I, 20 ng/ml each of EGF, keratinocyte growth factor (KGF), hepatocyte growth factor (HGF), 0.1 μM *all-trans*-retinoic acid (RA), and 10 nM DHT, and the expression of cytokeratin 18 mRNA was determined by northern blot analysis.

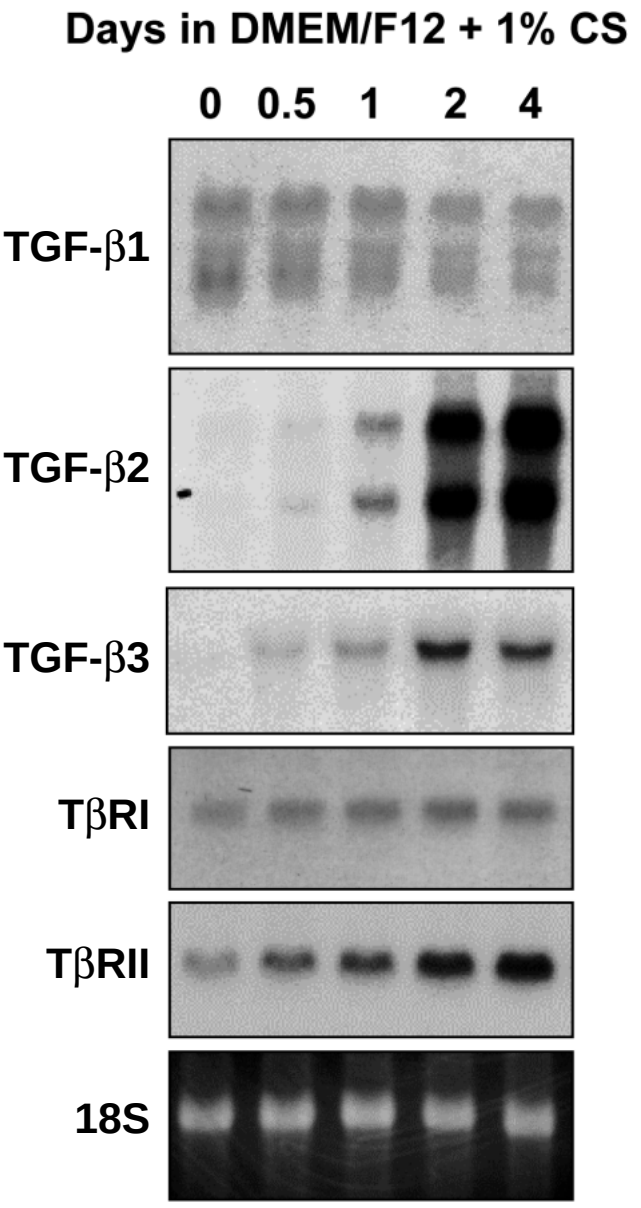


Fig. 6. Expression of TGF-βs, TGF-βII receptor by NRP-152 cells during luminal differentiation. Effect of differentiation of NRP-152 cells in DMEM/F12 + 1% CS on the expression of mRNAs for TGF-β, TGF-βII receptor were determined as a function of time, as assessed by northern blot analysis.

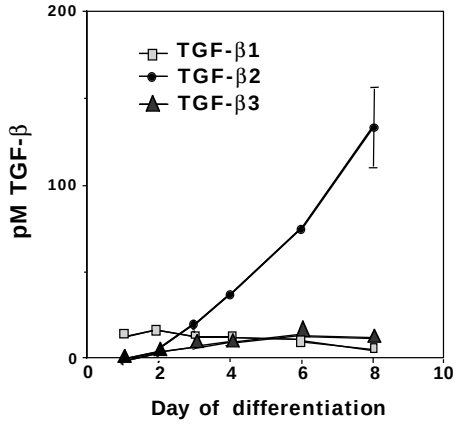


Fig. 7. Secretion of TGF-βs 1, 2 and 3 during the differentiation of NRP-152 cells. NRP-152 cells were plated in DMEM/F12 + 1% CS for 1 to 10 days and the concentration of TGF-βs 1, 2 and 3 were assayed by SELISAs for each isoform, as described in Materials and Methods.

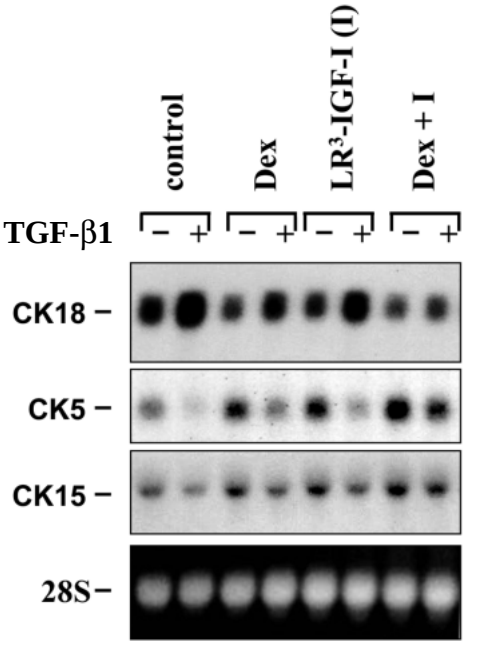


Fig. 8. Regulation of luminal and basal markers by TGF-β. NRP-152 cells were first allowed to differentiate for 5 days in DMEM/F12 + 1% CS, supplemented with either 0.1 μM Dex, 2 nM LR^3-IGF-I, or the combination of Dex and LR^3-IGF-I. 10 ng/ml TGF-β1 was then added to even cultures and mRNA levels of cytokeratins in these cultures were determined 48 hours later.

organoids in vivo, suggesting that differences between them and their putative normal counterpart may be subtle.

In contrast with the prostatic stem cell model proposed by Isaacs (1987), NRP-152 cells (Danielpour et al., 1994; Lucia et al., 1998) may be representative of the small fraction of the basal cells in the prostate that are androgen receptor positive (Prins et al., 1991). This alternative supports the proposal of Bonkhoff et al. (1994a,b) that epithelial stem cells in the prostate may belong to a special class of basal cells that are androgen responsive (not dependent), or have androgen receptors, and further suggests the presence of androgen receptors in such cells is important for cell proliferation and/or differentiation. However, the observation that mitogenic stimulation inhibits luminal differentiation of NRP-152 cells suggests that the mitogenic action of androgens may inhibit rather than promote such differentiation, and castration would thereby induce differentiation. This possibility is supported by the in vivo observations that castration induces the acquisition of a luminal phenotype in basal prostatic cells, as revealed by the induction of cytokeratins 8, 18, and CAM-105 mRNA in basal cytokeratin positive cells following androgen ablation (Hsieh et al., 1992, 1994). Both basal and luminal cell markers are co-expressed in a more substantial number of epithelial cells during regeneration of the regressed prostate (Verhagen et al., 1988, 1992). Thus, androgens may indirectly promote transdifferentiation following castration by stabilizing and

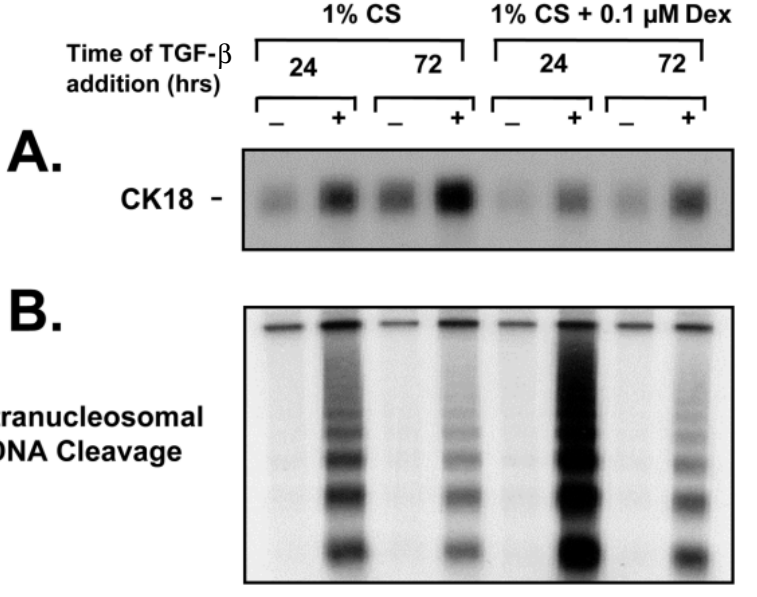


Fig. 9. TGF-β induces differentiation and apoptosis optimally under different conditions. NRP-152 cells were treated with 10 ng/ml TGF-β1 for 48 hours either 24 hours after plating (lanes 1,2,5,6) or 72 hours of plating (lanes 3,4,7,8) in DMEM/F12 + 1% CS in the absence of Dex (lanes 1-4) or with 0.1 μM Dex (lanes 5-8). The expression of cytokeratin 18 mRNA was measured by northern blot (A), and apoptosis by intranucleosomal DNA fragmentation (B) analyses, as described in Materials and Methods.

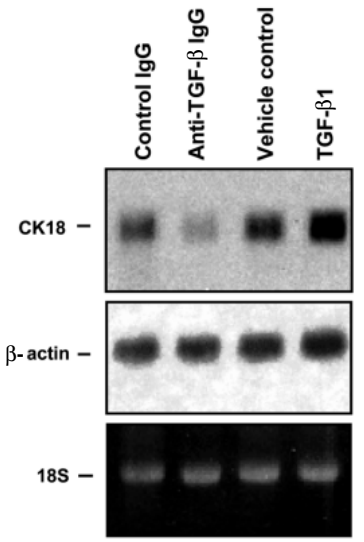


Fig. 10. Role of autocrine TGF-βs in luminal differentiation of NRP-152 cells. NRP-152 cells were allowed to differentiate for 4 days in DMEM/F12 + 1% charcoal-stripped CS in the presence of 60 μg/ml of either non-immune mouse IgG1 or anti-TGF-β IgG1 (1D11.16), and expression of cytokeratin 18 mRNA was measured by northern blot analysis. The blot was stripped and reprobed with rat β-actin, as a negative control.

expanding of the pool of basal cells that co-express luminal markers rather than by directly triggering their differentiation. This should be distinguished from the direct action of androgens on the expression of differentiated functions of luminal cells, such as the induction of secretory proteins (i.e. PSA).

Recent studies suggest the TGF-β family of autocrine/paracrine growth regulatory peptides play important roles in prostatic growth, development and carcinogenesis (see Barrack, 1997, for review). Expression of TGF-β and TGF-β signaling receptors is induced dramatically in the prostate during regression of this organ (Kyprianou and Isaacs, 1989; Kim et al., 1996), and has been speculated to mediate apoptosis of prostatic epithelium (Martikainen et al., 1990). The data presented here suggest that TGF-β can also promote the differentiation of basal cells to luminal cells following prostate regression. The nature of the intracellular signals that induce TGF-β expression during differentiation of NRP-152 cells in culture and following prostate regression in vivo may be similar, as each is elicited by the abrogation of mitogenic stimulation. We have recently shown that androgens down-regulate the expression of TGF-βs 1, 2, and 3 directly in NRP-152 prostatic epithelial cells in culture (Lucia et al., 1998). This suggests that the induction of TGF-β following castration may be mediated, at least partly, through androgen receptors in the epithelium, rather than mediated entirely through the binding of androgen to androgen receptors in stroma cells. The nature of the secondary signals that regulate TGF-β expression and differentiation in these settings remain to be explored.

The data showing that TGF-β expression is induced during differentiation, that TGF-β induces luminal differentiation, and that neutralizing antibodies against TGF-β partially block such differentiation, suggest a critical role of autocrine/paracrine TGF-β in the regulation of luminal differentiation promoted by mitogen-depletion. We have recently obtained confirmatory evidence that induction of luminal differentiation may be mediated through autocrine TGF-β, as interference with receptor signaling by overexpression of a dominant-negative TGF-β type receptor II caused loss of the ability of NRP-152 cells to differentiate in culture (De Castro et al., 1997). Thus, the regulation of TGF-β expression and activity may be critical determinants of cellular behavior in the prostate. The finding that TGF-β regulates transdifferentiation is not new. Previous reports have shown this cytokine to trigger the transdifferentiation of a variety of cell types, such as mammary epithelial cells to fibroblasts (Miettinin et al., 1994; Oft et al., 1996; Cui et al., 1996), certain endothelial cells to fibroblast-like cells (Potts and Runyan, 1989), fat storage cells of the liver to myofibroblasts (Bachem et al., 1993) and prostatic fibroblasts to smooth muscle cells (Peehl and Sellers, 1997). With respect to the latter, TGF-β secreted by luminally differentiating cells, as evidenced in the present study, may also induce the differentiation of smooth muscle cells through a paracrine mechanism. Alternatively, TGF-β made in prostatic stromal cells may induce transdifferentiation of the epithelium.

The expression of TGF-β1 is invariably induced in prostatic tumors relative to the normal gland (Perry et al., 1997; Truong et al., 1993). Such enhanced expression of this growth inhibitor is most likely the manifestation of normal homeostatic negative-feeback mechanisms to protect against excessive or uncontrolled cell proliferation. Support for this comes from demonstration that numerous oncogenes and proto-oncogenes that are activated during carcinogenesis, such as *fos*, *jun*, *ras*, IGF-I and TGF-α, transcriptionally activate TGF-β1 expression (Kim et al., 1989; Geiser et al., 1991; Cosgaya and Aranda, 1996). This induced expression of TGF-β by tumorigenic prostatic cells may promote the differentiation of adjacent normal basal cells to luminal variants, and thus explain the apparent obliteration of the basal layer that occurs almost invariably during prostatic carcinogenesis (Verhagen et al., 1992). In view of the induction of TGF-β1 during carcinogenesis, the ability of TGF-β to promote basal to luminal transdifferentiation is consistent with the possibility that prostatic carcinoma may be derived from basal cells that transdifferentiate to a luminal phenotype during carcinogenesis (Bonkhoff et al., 1994b, 1996). Overexpression of TGF-β1 may also be selected for later during carcinogenesis, as tumor suppressive actions of TGF-β are lost (i.e. via loss of TGF-β receptor function in tumor cells) and thus overwhelmed by TGF-β's tumor promoting activities (i.e. angiogenesis and immunosuppression, via paracrine and endocrine mechanisms) (Wakefield et al., 1991; Barrack, 1997).

In addition to inducing luminal differentiation of NRP-152 cells, we have shown that TGF-β also induces apoptosis (Hsing et al., 1996), and arrests growth of these cells, albeit each optimally under different conditions. Although Dex potentiates the ability of TGF-β to induce NRP-152 cell death, it opposes the effect of TGF-β in promoting differentiation (Fig. 9). Moreover, IGF-I, which completely blocks apoptosis induced by TGF-β (Hsing et al., 1996), only partially blocks the effect of TGF-β on luminal differentiation (in the absence of Dex), and has little effect on the ability of TGF-β to arrest cell growth (D. Danielpour and A. Hsing, unpublished). These data suggest that the local environment may be critical to the function of TGF-β in the prostate. As such, differences in the local environment, including the context of cytokines and other regulatory molecules, along the prostatic ductal network (from proximal to distal) (Lee et al., 1990; Nemeth and Lee, 1996) may be critical not only for the expression of TGF-β, but also for the ability of TGF-β to inhibit cell growth, promote

differentiation and/or induce apoptosis. Data presented here suggest that IGF-I, which is produced by prostatic stroma (Peehl et al., 1996; Cohen et al., 1994), may be critical to the retention of the basal phenotype and its absence for the ability of TGF-β to induce apoptosis, although paracrine factors such as KGF, HGF and EGF, may also contribute to these effects. As such, the greater proximity of basal cells to stroma (Hayward et al., 1996b) may be a key determinant in the maintenance of the basal phenotype as well as the resistance of basal cells to apoptosis. With respect to tumorigenesis, the increased levels of circulating IGF-I associated with prostate cancer risk (Chan et al., 1998) may promote tumor development by blocking the ability of TGF-β to induce full differentiation and/or apoptosis of initiated epithelium.

Importantly, the ability of NRP-152 cells to differentiate from a basal toward a luminal phenotype is not limited to in vitro, as these cells also form fully differentiated and functional prostatic organoids (with normal secretory activity) when recombined with mouse urogenital sinus mesenchyme in vivo (S. W. Hayward et al., unpublished). These organoids are architecturally similar to the normal gland, with complete segregation of basal and luminal epithelial cells, both of which are of NRP-152 cell origin. Thus, the ability of NRP-152 cells to be stably-infected with specific genes (De Castero et al., 1997) suggests that this line will serve as a unique and powerful model for studying the role of specific genes in stromal-epithelial interactions in vivo, during normal prostatic development and carcinogenesis.

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