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Androgen responsive adult human prostatic epithelial cell lines immortalized by human papillomavirus 18

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Prostate cancer and benign tumors of the prostate are the two most common neoplastic diseases in men in the United States, however, research on their causes and treatment has been slow because of the difficulty in obtaining fresh samples of human tissue and a lack of well characterized cell lines which exhibit growth and differentiation characteristics of normal prostatic epithelium. Non-neoplastic adult human prostatic epithelial cells from a white male donor were immortalized with human papillomavirus 18 which resulted in the establishment of the RWPE-1 cell line. Cells from the RWPE-1 cell line were further transformed by v-Ki-*ras* to establish the RWPE-2 cell line. The objectives of this study were to: (1) establish the prostatic epithelial origin and androgen responsiveness of RWPE-1 and RWPE-2 cell lines; (2) examine their response to growth factors; and (3) establish the malignant characteristics of the RWPE-2 cell line. Immunoperoxidase staining showed that both RWPE-1 and RWPE-2 cells express cytokeratins 8 and 18, which are characteristic of luminal prostatic epithelial cells, but they also coexpress basal cell cytokeratins. These cell lines show growth stimulation and prostate specific antigen (PSA) and androgen receptor (AR) expression in response to the synthetic androgen mibolerone, which establishes their prostatic epithelial origin. Both cell lines also show a dose-dependent growth stimulation by EGF and bFGF and growth inhibition when exposed to TGF-β, however, the transformed RWPE-2 cells are less responsive. RWPE-1 cells neither grow in agar nor form tumors when injected into nude mice with or without Matrigel. However, RWPE-2 cells form colonies in agar and tumors in nude mice. In the *in vitro* invasion assay, RWPE-1 cells are not invasive whereas RWPE-2 cells are invasive. Nuclear expression of p53 and Rb proteins was heterogeneous but detectable by immunostaining in both cell lines. The RWPE-1 cells, which show many normal cell characteristics, and the malignant RWPE-2 cells, provide useful cell culture models for studies on prostate growth regulation and carcinogenesis.

***Abbreviations:** PSA, prostate specific antigen; AR, androgen receptor; BPH, benign prostatic hyperplasia; HPV 18, human papillomavirus 18; K-SFM, keratinocyte-serum free medium; DMEM, Dulbecco's modified Eagle's medium; PBS Dulbecco's phosphate buffered saline Ca^{2+}/Mg^{2+}-free; FBS, fetal bovine serum; EGF, epidermal growth factors; TGF-β, transforming growth factor β; bFGF, recombinant basic fibroblast growth factor; 5α-DHT, 5-α-dihydrotestosterone; BSA, bovine serum albumin; DAB, 3,3′-diaminobenzidine; BPE, bovine pituitary extract; Ki-MuSV, kirsten murine sarcoma virus; INT, 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride.

Introduction

Prostate cancer and benign prostatic hyperplasia (BPH*) are the two most common neoplastic diseases in men in the US. About 50% of men in their sixth decade (age 51–60) and 90% of men by age 85 will develop BPH (1). It is estimated that 43% of all new cancers in men are prostate cancer, making it the most common cancer and the second leading cause of death in men in the US (2). Research on the causes and treatment of both BPH and prostate cancer has been slow because of the difficulty in obtaining fresh samples of human tissue and a lack of well characterized cell lines which exhibit characteristics of normal prostatic epithelium (3).

Since cellular senescence is a characteristic of normal cells, it is necessary to establish immortalized cell lines which provide useful cell models for *in vitro* and *in vivo* experimentation. We recently reported an adenovirus 12/SV40 immortalized and fully characterized human prostatic epithelial cell line PWR-1E (4). Although several immortalized cell lines of prostatic origin have been recently reported, very few have been fully characterized (3). The ultimate objective of any *in vitro* cell system is to mimic the *in vivo* cell architecture, to retain the ability to perform differentiated function and to respond to growth regulating factors.

We report on characterization of two human prostate epithelial cell lines, a human papillomavirus 18 (HPV 18) immortalized, non-tumorigenic cell line RWPE-1 which was further transformed by v-Ki-*ras* (5) to a tumorigenic cell line RWPE-2. HPV 18, a clinically relevant HPV strain, was used for immortalization for two reasons. The first reason is that HPV immortalized cells are more likely to retain growth and differentiation characteristics of their normal cells of origin and they are non-tumorigenic. This has been demonstrated for human foreskin keratinocytes, and tracheal and cervical epithelium (6,7). The second reason is that HPVs are the most common sexually transmitted disease. Human papillomavirus DNA has been detected in 93% of cervical cancers (8). In bladder cancer, in addition to chemicals and smoking as etiological factors, HPVs are considered to be the most common infectious agent which may play a role in its etiology (9). HPV appears to be a necessary factor but may not be sufficient alone for the development of the majority of cervical, vulvar, penile, perianal and bladder cancers (10,11).

Whether HPVs play a role in prostate cancer is still controversial. Because of the common occurrence of HPVs in urogenital neoplasms in men (12), it is at least logical to consider their potential role in prostate tumors. There is some evidence to suggest that the sexually transmitted HPVs may be involved in the etiology of prostate cancer. In Japan, 41% of prostate carcinomas were found to have HPV 16, 18 or 33 DNAs and the co-occurrence of *ras* mutations and HPV infection increased with disease progression (13,14). A potential role for HPVs in

prostatic carcinoma remains to be established. Studies in the US report only a low prevalence of HPVs in prostate cancer samples (15,16). Concern has been expressed about the large variability and sensitivity in HPV DNA detection. It has been suggested that different methods of sample fixation, DNA preparation and amplification conditions may explain some of the discrepancies reported in the literature (12,17). Because of the low HPV DNA copy number observed in bladder cancer, it was suggested that HPV should ideally be tested in fresh or frozen tissue (12).

The HPV 18 immortalized RWPE-1 cells were further infected with the v-Ki-*ras* oncogene to induce malignant transformation (5). The role of *ras* oncogene in prostate cancer is also controversial. The presence of increased *ras* expression and mutations in a high percentage (41–59%) of prostate cancers in Japanese men have been demonstrated by PCR, DNA hybridization and immunostaining (13,18). Studies in the US have shown a low frequency of *ras* mutations but when they did occur, they were associated with progression (19). Because of a possible association of the *ras* oncogene with prostate cancer, we used v-Ki-*ras* to induce transformation of immortalized, non-tumorigenic RWPE-1 cells to tumorigenic RWPE-2 cells.

The development of human cell models permits studies on the etiology, progression and prevention of human prostatic neoplasia and on the identification of relevant cellular and molecular alterations in the neoplastic process. In addition to our previously reported adenovirus12/SV40 immortalized PWR-1E cell line (3,4), RWPE-1 and RWPE-2 cell lines, reported here, are the only well characterized, androgen responsive, human prostatic epithelial cell lines available at the present time.

Materials and methods

Materials

Keratinocyte-serum free medium (K-SFM) no. 17005–042, antibiotic/antimycotic mixture no. 15240–013, Gibco-BRL, Grand Island, NY; Dulbecco's modified Eagle's medium (DMEM) no. CC293, RPMI-1640 no. CC261, Celox Laboratories, Hopkins, MN; Dulbecco's phosphate buffered saline Ca^{2+}/Mg^{2+}-free (PBS) no. 28374, Pierce, Rockford, IL; fetal bovine serum (FBS) no. 1020-75, Intergen, Purchase, NY; mibolerone no. W-300, BIOMOL Biomolecular Research Laboratories, Plymouth Meeting, PA; epidermal growth factors (EGF) no. 40001, transforming growth factor β (TGF-β) no. 40039, Collaborative Research, Bedford, MA; recombinant basic fibroblast growth factor (bFGF) no. 234-FS, R & D Systems, Minneapolis, MN; monoclonal antibody to prostate specific antigen no. M-0750, DAKO, Carpinteria, CA; polyclonal antibody to androgen receptor no. PA1–110, Affinity BioReagents, Neshanic Station, NJ; monoclonal antibodies to: cytokeratin 8 no. C-5301, cytokeratin 18 no. C-8541, desmin no. D-1033, Factor VIII no. F-3520, 5-α-dihydrotestosterone (5α-DHT) no. D-5027 and bovine serum albumin (BSA) no. A-2153, Sigma, St Louis, MO; monoclonal antibody to basal cell cytokeratins (Keratin-903) no. C34903, Enzo Diagnostics, Farmingdale, NY; monoclonal antibody to p53 no. OP09, Oncogene Science, Cambridge, MA; monoclonal antibody to pRb no. 14001A, Pharmingen, San Diego, CA; Vectastain Elite ABC Peroxidase Kit no. PK-6102 and 3,3′-diamino-benzidine (DAB) Substrate Kit no. SK-4100, Vector Laboratories, Burlingame, CA; HEMA-3 Stain Set no. 67–56–1, Curtin-Matheson, Wood Dale, IL; 24 well plates (Falcon) no. 08–7721; 12 mm circle coverslips no. 12–545–80, Fisher Scientific, Itasca, IL; Nuclepore membrane, 8 μ pore size no. 1550446, 96 well plates 3596, Costar, Cambridge, MA; Matrigel from Dr H.K. Kleinman, NIH.

Isolation and immortalization

Epithelial cells were derived from a histologically normal prostate of a white male undergoing cystoprostatectomy. The tissue was minced and digested in RPMI-1640 medium containing 5% fetal bovine serum and 400 U/ml collagenase and acini were isolated according to our previously described method (20,21). The isolated acini were plated on fibronectin and type IV collagen coated plates, 10 μg/ml each, in a serum-free medium containing bovine pituitary extract (BPE) and 10 ng/ml EGF (4). Secondary cultures were used for immortalization. The plasmid pSHV-18m, which contains a single copy of the HPV 18 genome inserted into the EcoR1 site of pSV_2neo, was used as described by Woodworth *et al*. (6). Polybrene-induced DNA transfection was carried out as described earlier (5). Cells were transfected with 10 μg of DNA using 10 μg/ml polybrene and incubated at 37°C overnight. The cells were then shocked with 30% dimethyl sulfoxide for 4 min. Five days later the cells were subcultured at 1:2 ratio weekly. Medium was changed 2 times/week. Several clones were isolated. One selected clone, RWPE-1, was further characterized. The RWPE-2 cell line was derived from RWPE-1 after infection with Kirsten murine sarcoma virus (Ki-MuSV), which contains the activated Ki-*ras* oncogene as previously described (5). The RWPE-2 cell line, derived from one selected clone, was characterized.

Cell culture

RWPE-1 and RWPE-2 cells were maintained in the complete K-SFM medium which contains 50 μg/ml of BPE and 5 ng/ml EGF, plus antibiotic/antimycotic mixture (Penicillin, 100 U/ml, Streptomycin 100 μg/ml and Fungizone, 25 μg/ml). Cells were passaged upon confluence and seeded at 2×10^6 cells/ T-75 flask. All experiments were conducted on RWPE-1 cells between passages 30 and 46. For RWPE-2 cells, growth factor experiments used passages 15–25 while other experiments used passages 35–45. DU-145 human prostate carcinoma cell line was maintained in RPMI-1640 medium containing 5% DCS.

Growth in vitro

Cells were plated in 96-well plates at a density of 10 000 cells/well in triplicate. Medium was changed every 48 h. Plates were fixed and stained at 48 h intervals. Test plates were rinsed in PBS, fixed in absolute ethanol and stained with the protein-binding dye methylene blue. The dye was released with 1% SDS and absorbance measured at 620 nm with a Titertek microplate reader using a previously described method where absorbance is used as an indicator of growth (4).

Androgen induced growth

The microplate assay described above was used to establish effects of mibolerone on growth. Mibolerone was dissolved in ethanol, protected from light and stored at –20°C. The final concentration of ethanol in the medium was 0.1%, which did not cause growth inhibition. Ten thousand cells/well were plated in triplicate in 96-well plates in 200 μl of complete K-SFM. Serum-free K-SFM without BPE or EGF but supplemented with 0.1% BSA was used for test media. After 48 h, triplicate wells were switched to test media containing mibolerone concentrations ranging from 0.01–10 nM, and medium was changed every 48 h. After 5 days of treatment, test plates were prepared for absorbance reading.

Effects of growth factors

Effects of EGF, bFGF and TGF-β on growth were examined. EGF was dissolved in sterile distilled water, bFGF in D-PBS containing 0.1% BSA and TGF-β in 4 mM HCl in D-PBS containing 1 mg/ml BSA. All growth factor stocks were stored at –20°C. Growth factor dilutions were made in the final medium at 1:1000. The microplate assay was used as described above and 5000 cells were plated/well for EGF and bFGF experiments in complete K-SFM medium. After 48 h, cells were switched to test media consisting of K-SFM with BPE only and supplemented with EGF or bFGF at concentrations ranging from 0.625–10 ng/ml. For TGF-β experiments, 10 000 cells/well were plated in complete K-SFM. TGF-β was added, 48 h after plating, at concentrations ranging from 0.625–10 ng/ml in complete K-SFM. The medium was changed every 48 h. Test plates were prepared for absorbance readings 5 days after treatment as described above.

Isozyme and chromosome analysis

To establish that RWPE-1 and RWPE-2 cells were of human male origin, chromosome and isozyme phenotype analyses were performed by Dr B.Hukku at the Children's Hospital of Michigan, Detroit, MI.

Growth in nude mice

Cells were tested for tumorigenicity by injecting 1×10^7 early passage cells subcutaneously into nude mice and maintained for 5 months. In addition, 500 000 passage 18 RWPE-1 or passage 46 RWPE-2 cells, mixed with or without Matrigel (22), were injected subcutaneously into nude mice.

Immunostaining for cytokeratins, desmin, Factor VIII, p53 and pRb

Protein expression was detected by a modified avidin-biotin immunoperoxidase Vector protocol, using monoclonal antibodies. Control coverslips lacked primary antibody. In 24-well plates, 20 000 cells/well were plated on coverslips. At 75% confluency, cells were fixed in 50/50 methanol/acetone at room temperature and processed the same day or stored at –80°C. Antibody dilutions were made in normal horse serum. The following sequential steps were conducted at room temperature and cells were rinsed twice with PBS between steps after application of the primary Ab: cells were blocked with normal horse serum for 1 h; incubated with the appropriate specific antibody for 1 h except pRb which was incubated for 24 h, followed by biotinylated secondary Ab (1:200) for 30 min; treated with 3% H_2O_2 for 3 min to quench endogenous

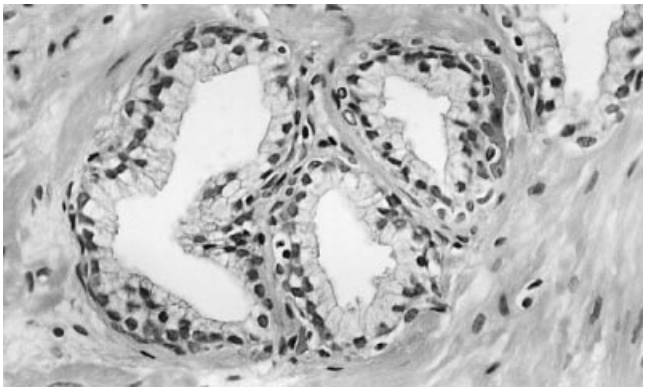

Fig. 1. A histological section of the prostate from which RWPE-1 cells were derived. H & E, ×154.

peroxidase activity; incubated with the avidin-biotin-peroxidase complex for 30 min, developed with DAB–nickel chloride for 5 min and dehydrated and mounted on acid/alcohol washed slides. Primary antibody dilutions were: for CK 8, 1:200; CK 18 1:800; desmin 1:50; Factor VIII 1:1000; p53 1:20; and pRB 1:100.

Immunostaining for PSA and AR expression

Cells grown on coverslips for immunostaining were pretreated in K-SFM medium containing 5 nM mibolerone, a non-metabolizable androgen, for 6 days, beginning 48 h after plating. Controls consisted of ethanol treated cultures. Cells on coverslips were processed as described above. The primary Ab dilutions, incubation temperature and times were: PSA (monoclonal) 1:20, 4°C, 24 h; and AR (polyclonal) 1:100, room temperature, 2 h.

Invasion assay

The ability of RWPE-1 and RWPE-2 cells to invade through Matrigel, a reconstituted basement membrane, was examined using a Boyden chamber *in vitro* invasion assay (23) modified in our laboratory. DU-145 cells, which show high invasive ability in this assay, were used as controls. The modified assay was conducted as follows: on the day prior to running the invasion assay, the following cells were plated in 100 mm plates in 10 ml of the appropriate medium. RWPE-1 and RWPE-2 cells were plated in complete K-SFM at 3×10^6 /plate and DU-145 cells in 5% DCS at 2×10^6 cells/plate. Matrigel was diluted 1:20 with cold (4°C), sterile distilled water on ice for a final concentration of 500 µg/ml. Each Nuclepore filter was coated with 25 µg Matrigel in 50 µl and left to dry overnight at room temperature under sterile conditions. Cells were rinsed with D-PBS 24 h after plating and incubated with 3 ml of 1 mM EDTA for 8–10 min, dislodged by tapping, suspended in 3 ml K-SFM or serum-free RPMI containing 0.1% BSA and recovered by centrifugation. Pellets were resuspended to obtain 2 million cells per ml in K-SFM or RPMI medium containing 0.1% BSA. The bottom chamber contained 220 µl of NIH/3T3 cell conditioned medium which served as the chemoattractant (24). For preparing conditioned medium, subconfluent NIH/3T3 cultures in 100 mm plates were fed with 7 ml of serum-free DMEM containing 50 µg/ml ascorbic acid. Conditioned medium was collected 24 h later, centrifuged to remove cell debri and stored at –20°C. A cell suspension containing 400 000 cells in 200 µl medium was added to the top chamber and allowed to remain undisturbed for 5 min before overlaying with 650 µl of K-SFM for RWPE-1 and RWPE-2 cells or serum-free RPMI medium for DU-145 cells, containing 0.1% BSA. Controls consisted of K-SFM medium containing 0.1% BSA. Cells were allowed to invade for 24 h at 37°C at which time the filters were prepared for absorbance reading. The migrated cells were fixed, stained with HEMA-3 and allowed to hydrate in distilled water. Nuclear stain was extracted by placing filters individually in wells of a 24-well plate containing 300 µl of 0.1 N HCl for 15 min and 200 µl from each well were then placed in a 96-well plate and the absorbance measured at 620 nm using a Titertek microplate reader. In addition to evaluating the number of migrated cells on the filter, cells that had migrated to the bottom chamber were also counted. Medium in the bottom chambers was triturated five times and 100 µl were taken from each of the three chambers, pooled into cuvettes containing 10 ml Isoton, triturated ten times and counted. The DU-145 cells that invaded and were attached to the underside of the filter and those counted in the bottom well were each taken as 100%. The two percentages were added to obtain total invasion. Percent absorbance and cell counts for RWPE-1 and RWPE-2 cells were calculated using DU-145 as 100%. Three replicate invasion chambers were prepared per treatment and the mean values for three such experiments were plotted.

Agar assay for anchorage independence

Cell suspensions of RWPE-1 and RWPE-2 cells were filtered through sterile 40 µ cell strainers to ensure a single cell suspension. Cells were resuspended in K-SFM medium supplemented with 10% FBS and added to 0.5% agar to yield a final concentration of 25 000 or 50 000 cell/ml in 0.33% agar. One ml of this cell suspension was layered over a 0.5% agar base and maintained for 15 days for colony formation. Colonies in agar were stained with sterile, 1.0 mg/ml 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride (INT) in distilled water as follows: 1 ml of the INT was added to each dish and the dishes were incubated at 37°C for 12 h. Stained colonies were fixed in 0.5 ml of 10% buffered formalin and counted using a colony counter.

Results

Histology

Figure 1 shows histology of the prostate tissue specimen from which RWPE-1 cells were derived. The tissue architecture and epithelial cells in the acini were determined to be normal.

Isozyme and chromosome analysis

RWPE-1 cells in passage 32 have the following isozyme profile: LDH, human; G6PD, B; PGM1, 2; PGM3, 1; ESD, 2; Me-2, 0; AK-1, 1; GLO-1 1–2. The phenotypic frequency of this phenotype was calculated to be 0.00012, which means that <0.1% of cell lines might be expected to have an identical isozyme phenotypic profile. The cell line is aneuploid human male (XY). One karyotype with 51 chromosomes is shown in Figure 2A. Cells other than RWPE-1 in passage 32 were not detected. RWPE-2 cells in passage 14 have an identical isozyme profile. This cell line is aneuploid human male, with most chromosome counts in the hyperdiploid range. One karyotype with 51 chromosomes is shown in Figure 2B. Cells other than RWPE-2 passage 14 were not detected. Several marker chromosomes common to both cell lines are present.

Growth characteristics in vitro

We have successfully isolated and established HPV 18 immortalized and *ras*-transformed cell lines. Growth curves for RWPE-1 and RWPE-2 cells show (Figure 3A) that the *ras*-transformed, tumorigenic RWPE-2 cells grow at a faster rate than the immortalized non-tumorigenic RWPE-1 cells with a doubling time of ~48 and 58 h, respectively.

Effects of androgen on growth

RWPE-1 and RWPE-2 cells were grown in the presence of mibolerone concentrations varying from 0.01–10 nM. Both cell lines show (Figure 3B) a similar dose-dependent increase in growth in response to mibolerone, with an increase to 149% and 144% of control for RWPE-1 and RWPE-2 cells, respectively, after five days of treatment (Figure 3B).

Expression of prostatic epithelial cell markers of differentiated function

RWPE-1 and RWPE-2 cells have an epithelial cell morphology (Figures 4a and 5a). Prostatic epithelial cell-associated PSA and AR expression are considered to be the functional markers for prostatic epithelium. We were successful in inducing PSA and an increase in AR expression in both cell lines by treatment with 5 nM mibolerone for 6–8 days. Immunostaining shows PSA expression (Figures 4b and 5b) as well as strong nuclear staining for AR (Figures 4c and 5c). Figures 4d and 5d are controls lacking the primary antibody. In other control cultures treated only with ethanol, no detectable staining for PSA and only weak staining for AR was observed (data not shown).

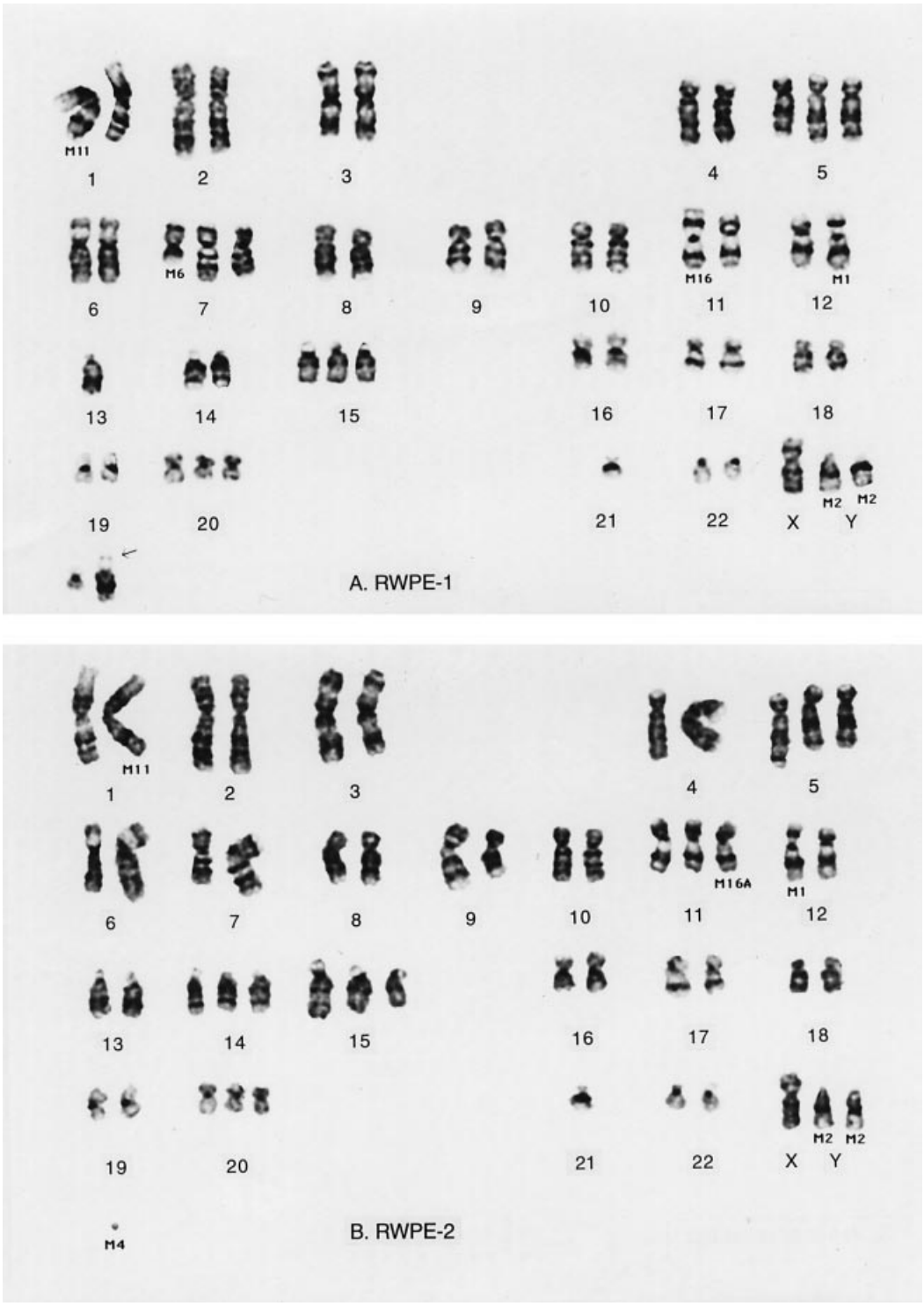

Fig. 2. (**A**) Karyotype of RWPE-1 cells at passage 32 and of (**B**) RWPE-2 cells at passage 14**.** The karyotype is human, male (XY). Karyotypes with 51 chromosomes and several marker chromosomes (M) are shown.

Markers of epithelial origin

RWPE-1 and RWPE-2 cells express CKs 8 and 18 (Figures 4e,f and 5e,f), which confirms their epithelial origin and excludes the possibility of a fibroblastic origin. The presence of CKs 8 and 18 also demonstrates that these cells arose from a secretory cell type of the prostate. It is interesting to note that both cell lines also showed a weak positive staining for basal cell cytokeratin (data not shown). A monoclonal antibody which recognizes basal cell cytokeratins 5 and 14, was used. RWPE-1 and RWPE-2 cells do not express desmin (Figures 4g and 5g) or Factor VIII (Figures 4h and 5h), thus excluding the possibility of their origin from muscle or endothelial cells, respectively. As a positive control for desmin, sections of prostate tissue were stained with antibody to desmin (results not shown) where only muscle cells showed positive staining.

Suppressor gene expression

Both cell lines show positive but heterogeneous nuclear staining for p53 and Rb proteins (Figures 4i,j and 5i,j), however, the

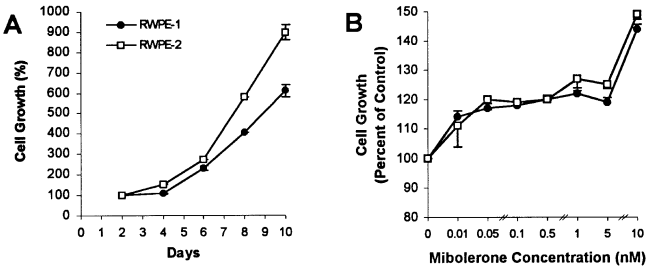

Fig. 3. (**A**) Growth curves for RWPE-1 and RWPE-2 cells. Cells were plated at a density of 10 000 cells per well in K-SFM in 96-well plates. (**B**) Effects of mibolerone on cell growth**.** 5000 cells were plated per well in 96-well plates and cells were treated for 5 days. Absorbance values measured at 620 nm, were plotted.

pRb nuclear staining in the v-Ki-*ras* transformed RWPE-2 cells is punctate and sporadic.

Effects of growth factors

Cells were treated with the growth factors for 5 days. EGF caused a dose-dependent increase in the growth of RWPE-1 cells to 150% of control at a concentration of 10 ng/ml (Figure 6A). The RWPE-2 cells showed an initial increase in growth to 117% of control at 0.625 ng/ml EGF and a further increase did not occur up to 5 ng/ml, reaching a maximum of 133% of control at 10 ng/ml EGF (Figure 6A). Results shown for EGF are the average of three experiments for each cell line. RWPE-1 cells showed a dose-dependent increase in growth at bFGF concentrations >2.5 ng/ml, reaching a maximum of 152% of control at 10 ng/ml (Figure 6B). On the other hand, RWPE-2 cells did not show growth stimulation at concentrations <10 ng/ml, with a maximal increase to 115% of control at this concentration (Figure 6B). Results shown represent the average of two experiments for each cell line. Both cell lines showed inhibition of growth when exposed to TGF-β (Figure 6C). A marked inhibition of cell growth in RWPE-1 cells was observed at 0.625 ng/ml with maximal growth inhibition to 63% of control at 10 ng/ml. Similar inhibition of cell growth in RWPE-2 cells was also observed at 0.625 ng/ml, with a maximal growth inhibition to 68% of control at 10 ng/ml. However, the overall decrease in RWPE-1 cells was slightly greater than that of RWPE-2 cells. Results shown for TGF-β are the average of three experiments for each cell line.

Anchorage independent growth

The ability of RWPE-1 and RWPE-2 cells to form colonies in soft agar was examined. RWPE-1 cells did not form colonies whereas RWPE-2 cell formed small colonies in agar with an average colony forming efficiency of 0.09% which varied with the plated cell density.

In vitro invasion

The invasive ability RWPE-1 and RWPE-2 cells was compared with that of the prostate carcinoma cell line DU-145 where invasion by DU-145 cells was set at 100%. Results show (Figure 7) that in comparison with the invasive DU-145 cell line, RWPE-1 cells were not invasive (1%) even after a 24 h assay period whereas RWPE-2 cells showed 49% invasion.

Growth in nude mice

After five months, none of the five nude mice injected with 10 million RWPE-1 cells developed tumors whereas, 4/5 mice injected with RWPE-2 cells developed tumors. In addition, none of the four sites injected with 500 000 RWPE-1 cells in Matrigel developed tumors. However, tumors developed at 4/4 sites injected with 500 000 RWPE-2 cells with or without Matrigel.

Discussion

Immortalization is a necessary step in the multistep process of carcinogenesis. The cellular senescence exhibited by normal cells can be overcome by transfection with viral immortalizing genes of DNA tumor viruses, which include gene encoding the SV40 large T antigen, E1a and E1b proteins of adenoviruses and the E6 and E7 proteins of HPV 16 and 18 (4,10,25). Gene products of two tumor suppressor genes, pRb and p53, bind to these proteins. The SV40T antigen, E1a and E7 proteins bind pRb while SV40T antigen, E1b and E6 proteins bind p53 (10). It has been generally believed that in the case of human papillomaviruses, both E6 and E7 genes are required for immortalization of human cells. However, recent studies show that either E6 or E7 alone is sufficient for immortalization of certain types of human cells, although the ability of E7 to immortalize is considerably less than that of E6 and E7 together (9,25). Immortalization of human uroepithelial and mammary epithelial cells has been achieved by either E6 or E7 (9,26). The E6 immortalized cells show normal levels of pRb but p53 is almost undetectable because rapid degradation of p53 is promoted by E6 with a decrease in its half life (9,25). However, in E7 immortalized cells, E7 seems to modulate pRb levels rather than inactivate its activity but E7 stabilizes p53 levels (25). The E6 or E7 immortalized cell types also show different characteristics in that the E7 immortalized cells have a relatively stable karyotype and resemble normal cells in growth and morphology as compared to E6 immortalized cells (9). Tracheal epithelial cells immortalized by E7 also show the ability to differentiate similar to native epithelium (7). Our RWPE-1 cells express E7 gene product (5) and show nuclear staining for both p53 and pRb. The staining is heterogeneous in that one cell population stains for p53 and the other for pRb. Chromosome analysis showed that the RWPE-1 cell line consists of two populations, one with chromosome counts in the 40–48 range and the other in the 50 plus range. Further cloning of these populations needs to be done to isolate and characterize the two cell populations.

RWPE-1 cells have retained a normal epithelial morphology. Their ability to respond to androgens, by the expression of PSA and upregulation of AR, is consistent with the observation that E7 immortalized cells retain characteristics of normal cells. RWPE-1 cells show a response, similar to normal cells, to EGF, bFGF and TGF-β while the v-Ki-*ras* transformed RWPE-2 cells are less responsive. It is for these reasons that RWPE-1 cell line is considered to be invaluable for studies on growth regulation at the cell and molecular level. It was generally believed that immortalization with HPVs results in the loss of p53 protein due to the binding of E6 to p53 leading to its degradation. However, immortalization of epithelial cells with E7 does not necessarily lead to loss of p53 (9,27). In transitional cell carcinomas, infection with high risk HPV 16 and 18 and overexpression of p53 have been used as prognostic indicators where high p53 expression was associated with an invasive phenotype (28). Further, decreased expression of pRb in bladder carcinomas was associated with an aggressive phenotype (29). The RWPE-1 cells show heterogeneous nuclear staining for p53 and pRb by immunostaining while RWPE-2 cells show p53 nuclear staining similar to that seen in RWPE-

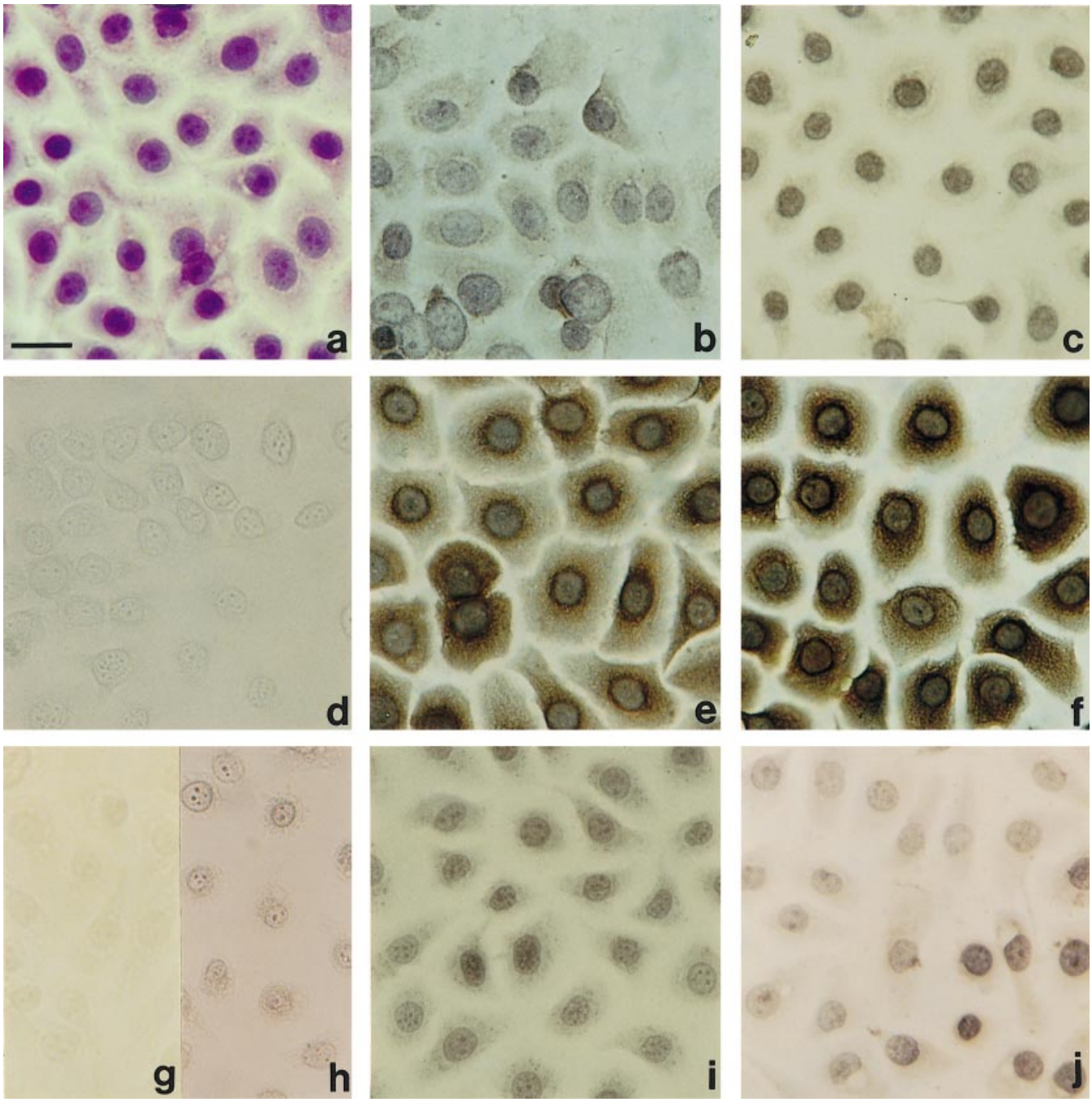

Fig. 4. Characterization of RWPE-1 cells on the basis of cellular proteins. Proteins were detected by immunoperoxidase staining. (**a**) hematoxylin and eosin staining; (**b**) positive staining for PSA; (**c**) positive staining for nuclear androgen receptor. Cells for (b) and (c) were pretreated for 6 days with 5 nM mibolerone; (**d**) a control lacking primary antibody; (**e**) and (**f**) positive staining for cytokeratin 8 and 18, respectively; (**g**) and (**h**) absence of staining for desmin and Factor VIII, respectively; (**i**) heterogeneous staining for p53, (**j**) heterogeneous staining for pRb. Scale bar is 20 μM. ×625.

1 cells but pRb staining is much weaker and sporadic. These observations are consistent with the reported *in vivo* results in bladder cancer (28,29).

We were able to induce malignant transformation by the introduction of v-Ki-*ras* oncogene into RWPE-1 cells to derive the RWPE-2 cell line. Similar malignant transformation with Ha-*ras* has been achieved in human cervical epithelial cells immortalized with HPV-16 (30). The RWPE-2 cells grow in agar but form only small colonies. This anchorage independent growth represents a step beyond immortalization. The RWPE-2 cells are tumorigenic and are moderately invasive in the *in vitro* invasion assay, when compared to DU-145 cells. Cloning of colonies in agar may provide cells with low and high invasive ability. The low invasive ability of RWPE-2 cells may partly relate to the continued *p53* expression in RWPE-2 cells. A negative correlation between *p53* gene dosage and growth, tumorigenicity and malignant progression has been shown in mouse keratinocytes (31). Further studies on RWPE-1 and RWPE-2 cells should examine *p53* and pRb gene dosage.

In skin, the squamous epithelial cells are considered to be the natural host for HPV infection and the lytic replication of viral DNA is restricted to the highly differentiated cells in the upper layers of the epithelium where the viral replicative cycle

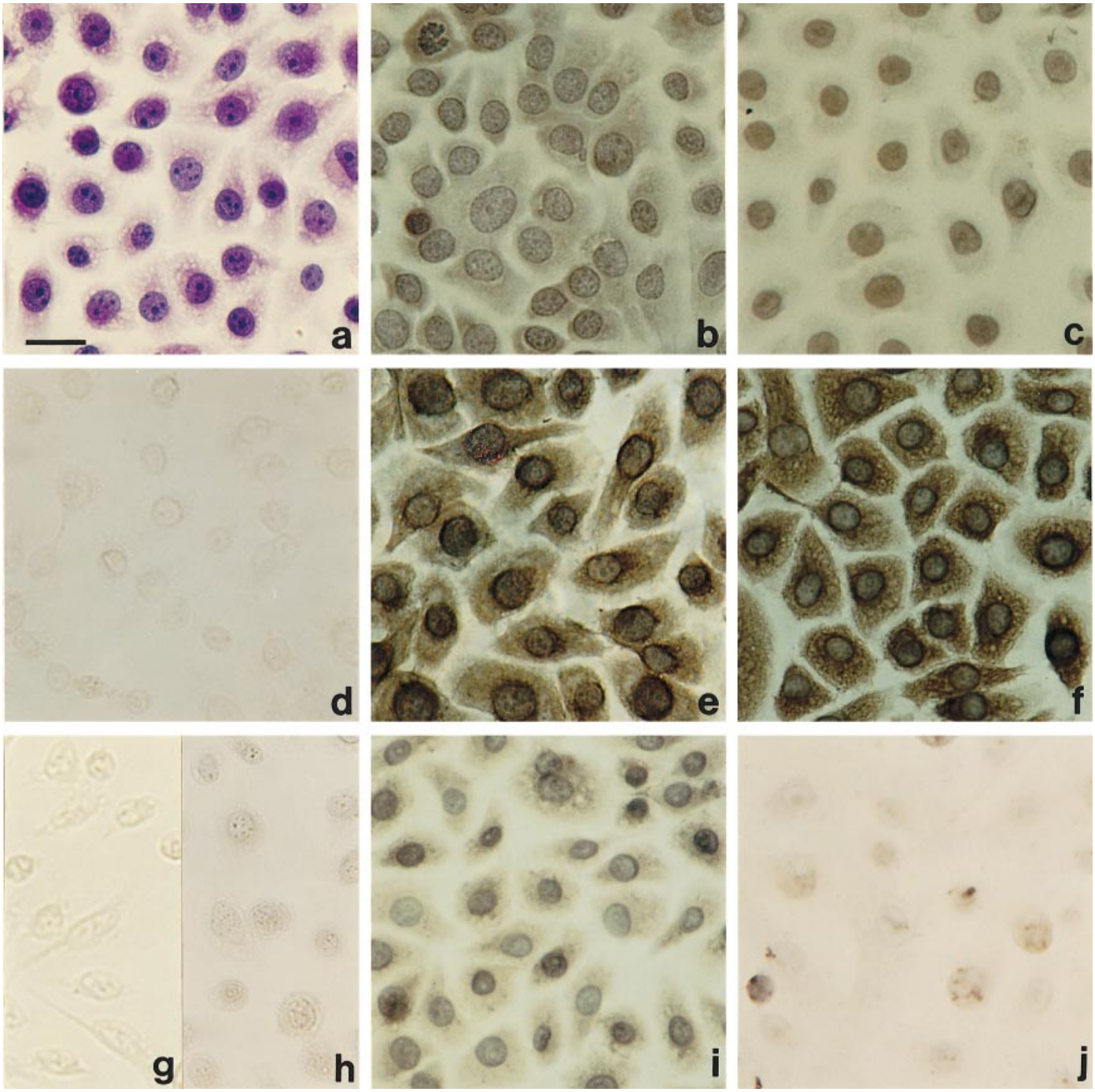

Fig. 5. Characterization of RWPE-2 cells on the basis of cellular proteins. Proteins were detected by immunoperoxidase staining. (**a**) Hematoxylin and eosin staining; (**b**) positive staining for PSA; (**c**) positive staining for nuclear androgen receptor. Cells for (b) and (c) were pretreated for 6 days with 5 nM mibolerone; (**d**) a control lacking primary antibody; (**e**) and (**f**) positive staining for cytokeratin 8 and 18, respectively; (**g**) and (**h**) absence of staining for desmin and Factor VIII, respectively; (**i**) heterogeneous staining for p53; (**j**) heterogeneous, weak staining for pRb. Scale bar is 20 μM. ×625.

is closely associated with the differentiation status of the cell (25). It appears that immortalization with E6 or E7 may be related to the cell differentiation status because distinct human keratinocyte and mammary epithelial cell populations are immortalized by E6 or E7 (25,26). Cells sensitive to lytic infections are generally not immortalized (4). Immortalization is the result of the integration of HPV DNA into the host cell. Cells with proliferative ability rather than the non-proliferative, terminally differentiated cells are more likely to integrate the viral DNA. It is therefore, interesting to note that although RWPE-1 cells express the luminal cell type cytokeratins, they also show weak staining for the basal cell cytokeratins. This coexpression suggests that these cells may be a sub-luminal or intermediate cell type which co-expresses both luminal and basal cell-type cytokeratins (32). This provides an important opportunity to examine the stepwise differentiation process of prostatic epithelial cells from stem cells to terminally differentiated secretory cells and to establish the identity of the prostatic epithelial cell type that gives rise to carcinoma cells. It is generally believed that prostatic carcinoma cells

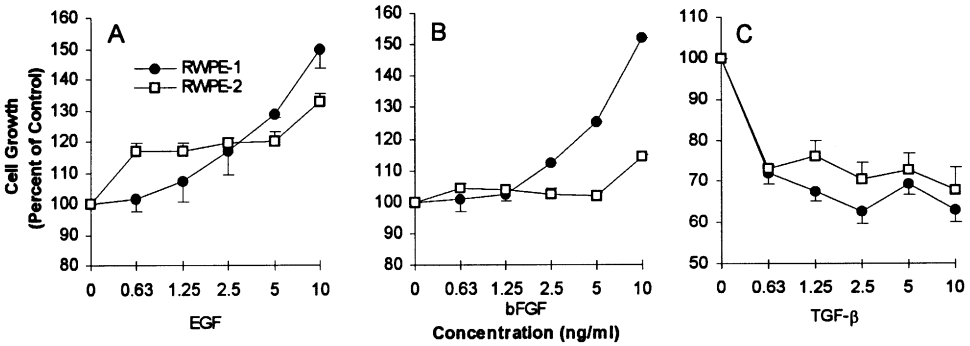

Fig. 6. Effects of growth factors on RWPE-1 and RWPE-2 cell growth. (**A**) EGF (**B**) bFGF (**C**) TGF-β. For EGF and bFGF 5000 cells and for TGF-β 10 000 cells were plated per well in 96-well plates and cells were treated for 5 days. Absorbance values measured at 620 nm, were plotted.

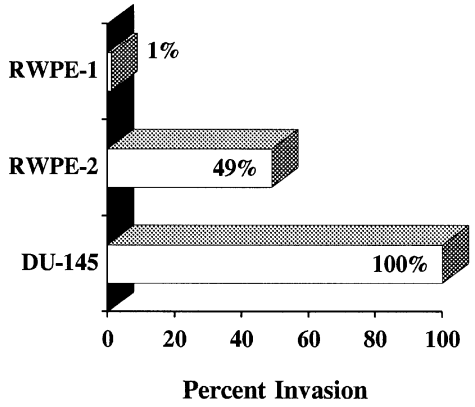

Fig. 7. The invasive ability of RWPE-1 and RWPE-2 cells was compared with that of DU-145 and LNCaP cells by a modified Boyden chamber *in vitro* invasion assay. Cells were plated at 400 000 cells per chamber on a Matrigel coated filter and allowed to invade for 24 h. Invasion by DU-145 cells was set at 100%.

arise from differentiated luminal secretory cells. Since these are terminally differentiated cells, it is more likely that carcinoma cells arise from an intermediate epithelial cell type with proliferative ability and which may coexpress cytokeratins associated with basal and luminal cells rather than from a terminally differentiated cell type lacking proliferative ability and which is destined to undergo cell death. In view of the coexpression of basal and luminal cytokeratins in RWPE-1 cells, they may serve as prototype precursor cells for prostatic carcinoma cells.

In summary, we have established one immortalized and a second, v-Ki-*ras* transformed cell line derived from adult human prostatic epithelium. The parent normal prostatic epithelial cells could not be maintained beyond five passages in culture. The RWPE-1 and RWPE-2 cells express cytokeratins 8 and 18, which are normally expressed by luminal prostatic epithelial cells and they coexpress basal cell cytokeratins. They do not express desmin, a marker for smooth muscle cells or Factor VIII, a marker for endothelial cells. These results establish epithelial origin of RWPE-1 cells. The androgen responsiveness of both cell lines is demonstrated by the expression of PSA and upregulation of the androgen receptor. The non-tumorigenic, non-invasive RWPE-1 cells do not grow in agar and respond to growth factors similar to normal cells. On the other hand, the v-Ki-*ras* transformed, tumorigenic RWPE-2 cells form colonies in agar, show invasion and are less responsive to growth factors than RWPE-1 cells. The use of human prostatic epithelial cells capable of differentiated function is highly desirable for *in vitro* studies on prostate cancer and BPH. They provide the opportunity to dissect and separate the various actions and interactions involved in growth regulation, differentiation and neoplastic transformation. Well characterized, human, epithelial cell lines, such as the RWPE-1 and RWPE-2, provide uniform, standardized and a reproducible resource for study.

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