A Human Pituitary Adenoma Cell Line Proliferates and Maintains Some Differentiated Functions Following Expression of SV40 Large T-Antigen

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Abstract

Human pituitary cells proliferate very slowly in vitro. Only a few cell lines have been established, and these have been used mainly for short-term studies. To obtain immortalized cell lines of human pituitary adenomas for in vitro studies, we infected adenoma cells with a replication-defective recombinant human adenovirus, which contains an SV40 early large T-antigen. One of the cell lines (HP75), which has been studied in culture during 60 passages, has been extensively characterized. It expressed the large T-antigen protein and its mRNA, as well as the genes for FSH-β, LH-β and α-subunit (α-SU) of gonadotropin hormone.

The HP75 cell line also expressed the genes of various members of the chromogranin (Cg)/secretogranin (Sg) family, including CgA as well as the prohormone convertases PC1/3 and PC2. CgA was processed to pancreastatin in vitro, which was secreted into the culture medium. Treatment with phorbol 12-myristate 13-acetate (PMA), TGF-β1, and forskolin increased CgA expression in the cells and stimulated pancreastatin secretion into the medium while inhibiting cell growth. The HP75 cell line also expressed TGF-β mRNA isoforms (β1, β2, β3) and the mRNAs for the receptors for TGF-β (RI, RII, and RIII). The cells responded to TGF-β1 in vitro by increasing CgA protein expression and pancreastatin secretion. TGF-β-RII protein and mRNA expression were both increased by PMA.

Ultrastructural studies showed that the HP75 cells had very few dense-core secretory granules and a poorly developed Golgi complex. After treatment with TGF-β1 and PMA, there was an increase in the development of rough endoplasmic reticulum and the Golgi complex.

This is the first report of the development of an immortalized human pituitary cell line that retains some differentiated functions. HP75 can be used to study TGF-β and CgA functions in pituitary cells. Replication-defective recombinant human adenovirus with an SV40 large T-antigen insert can be used to generate other immortalized human pituitary cell lines for in vitro studies.

Key Words: Pituitary; cell culture; TGF-β; PCR.

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Introduction

Since cultured pituitary adenoma cells have a limited capacity to proliferate, the availability of human pituitary adenoma cell lines that proliferate in vitro should provide excellent models for investigators interested in pituitary biology. Previous attempts to establish functional cell lines from normal or neoplastic human

pituitaries have met with only limited success [1–9]. Such cell lines often discontinue producing hormones or other peptides, and dedifferentiate after several passages in vitro.

Recent studies have shown that the expression of SV40 large T-antigen by hepatocytes and other cells can induce cell proliferation while maintaining regulated expression of differentiated genes, such as albumin and α-fetoprotein [10]. When a recombinant human adenovirus vector was used to express SV40 large T-antigen in primary cultures of terminally differentiated, nondividing rat myocardial cells with a replication-defective adenovirus, there was maintenance of the proliferative capacity of myocardial cells [11,12]. Similar observations were reported for skeletal muscle [13], thyroid epithelial [14], and other cells [15–18]. Some of these studies utilized temperature-sensitive mutants of SV40 for transfection [15–18] with proliferation occurring at 34°C and growth reduction or arrest at 39°C [15–19]. Targeted oncogenesis utilizing the promoter regions from sets of genes expressed at specific stages of differentiation with SV40 T-antigen has also been used to generate various pituitary cell lines in transgenic mice [20,21].

In the present study, we utilized a replication-defective recombinant human adenovirus with an SV40 early T-antigen insert (AD-SVR4) to infect human pituitary adenoma cells. This resulted in immortalized, rapidly proliferating pituitary adenoma cells expressing the large T-antigen. These cells retained some differentiated functions, including expression of chromogranin A (CgA) and processing of CgA to pancreastatin. The cell lines responded to 12-myristate 13-acetate (PMA), forskolin, and to TGF-β1 by increasing CgA production. Over a 2-yr period, one of these cell lines, HP75, retains differentiated functions after more than 60 passages in culture.

Materials and Methods

Cell Culture

A total of 19 pituitary adenomas, 5 growth hormone adenomas, 1 prolactin adenoma, and 13 nonfunctioning adenomas, were used for primary culture as previously described [22]. After dissociation, an aliquot of the cells was placed on glass slides by cytocentrifugation and used for immunostaining to characterize the type of adenomas. The cells were incubated on 35-mm dishes coated with extracellular matrix (ECM) (Accurate, Hicksville, NY) in complete medium with Dulbecco's Modified Essential Medium (DMEM) with 15% horse serum (HS) and 2.5% fetal bovine serum (FBS) (Life Technologies, Grand Island, NY).

Other cell lines used included: human embryonic kidney 293 cells; COS-1 cells (SV40 transformed African green monkey kidney), Mv1Lu mink epithelial cells, and the breast carcinoma cell lines MDA-MB-231 and MDA-MB-468 (all from American Type Culture Collection, Rockville, MD).

Adenovirus—AD-SVR4 Infection

The recombinant adenovirus-SV40 R4 (AD-SVR4) used in this study was kindly donated by R. D. Gerard, University of Texas Southwestern Medical Center, Dallas, TX. The AD-SVR4 contains an SV40 early large T-antigen transcriptional unit (ORI) cloned into the *Xba*I site of the helper-independent human adenovirus vector ΔE1/X [11]. This virus is a replication-deficient human adenovirus in which the adenoviral E1a and E1b genes have been deleted [11], and the expression of T-antigen is under control of the SV40 early promoter. The kidney 293 cell line was used as a positive control for cell infection with AD-SVR4. Incubation for

Table 1. Immunohistochemical Characterization of HP75 Cells

Antibodies[a]	Dilution	Immunostaining results
LH (P)	1/1000	+
FSH (P)	1/1000	+
TSH (P)	1/1000	–
α-Su (M)	1/250	–
ACTH (P)	1/2000	–
PRL (P)	1/2000	–
GH (P)	1/1000	–
CgA (M)	1/1000	+
CgB (P)	1/500	+
PST (P)	1/500	+
PC1/3 (P)	1/1000	+
PC2 (P)	1/500	+
TGF-β^{PAN} (P)	1/200	+
TGF-β-RII (P)	1/250	+
SV40 large T-antigen (M)	1/1000	+
Ki-67 (M)	1/100	+
p53 (M)	1/500	+

[a]α-SU, α-subunit of glycoprotein hormone; Cg, chromogranin; FSH, follicle-stimulating hormone; GH, growth hormone; LH, luteinizing hormone; M, MAb; P, polyclonal antibodies; PC, proconvertase; PRL, prolactin; PST, pancreastatin; TGF-β, transforming growth factor β.

2 to 4 d after infection led to complete lysis of the 293 cells and the release of abundant AD-SVR4 into the medium.

Primary monolayers of pituitary adenoma cells seeded at 1×10^6 cells/2 mL on ECM dishes were incubated in 200 plaque-forming units (PFU)/cell AD-SVR4 in 2% FBS-DMEM. After washing with fresh medium, the cells were incubated in 2% FBS DMEM with 1% antibiotic (100 U/mL penicillin, 100 μg/mL streptomycin and 0.25 μg/mL fungizone), and fresh medium was changed two to three times weekly until the cells began to proliferate and to form colonies (4–5 wk). Cell lines from two adenomas were obtained, one was from a 47-yr-old man with a nonfunctioning plurihormonal adenoma (HP75), and the other from a 39-yr-old man with a gonadotroph adenoma (HP68). The cell clones were then transferred to plastic dishes in completed medium, subcloned, and used for various experiments.

Immunocytochemistry

HP75 and HP68 cells grown in complete medium were harvested, cytocentrifuged onto glass slides, fixed with 4% paraformaldehyde for 20 min, and air-dried. Antibodies used to characterize the cell lines are summarized in Table 1 and included ones directed toward pituitary hormones (LH, FSH, TSH, PRL, and GH) obtained from S. Raiti of the National Pituitary Agency, Bethesda, MD, as well as ACTH (Dako, Santa Barbara, CA), and α-subunit (α-SU) (Biogenex, San Ramon, CA). Others included chromogranin A (Boehringer Manniheim, Indianapolis, IN), pancreastatin (Peninsula Labs, Belmont, CA), the prohormone convertases PC1/3 and PC2 (a generous gift from D. Steiner, University of Chicago), TGF-β^{PAN} (R&D Systems, Minneapolis, MN), TGF-β-RII, SV40 large T-antigen and p53 (Santa Cruz Biotechnology, Santa Cruz, CA), and Ki-67 (AMAC, Westbrook, ME). All immunostains were performed using the avidin-biotin peroxidase (ABC) methods, as previously described [22,23]. Immunocytochemical (ICC) analyses for Ki67, SV40 large T-antigen, and p53 were performed with microwave pretreatment for 5 min. Frozen sections of normal pituitary as well as pituitary adenoma culture cells served as positive controls for pituitary hormones, Cg/secretogranin (Sg), PC1/3, and PC2; mink cells were used for TGF-β and TGF-β-RII, and Cos-1 cells for SV40 large T-antigen. Cells were counterstained with hematoxylin for 2 s. Substitution of normal serum for primary antibodies resulted in no staining of the cells.

Immunoblot Analysis

Cells grown in plastic culture flasks (75 cm^2) were harvested for protein

Table 2. Primers and Internal Probes for PCR

Gene	Sense primer	Antisense primer	Size	Internal probes
hTGF-β-RI	CGG AGG CAG GAG AAG CAG CGT (524–544)	TAG GAT GTT GTC GTG TCT GAG (842–862)	339	ACT GTC CAG GAG GTC CCC CAA CAT GCT GTCC (601–631)
hTGF-β-RIII	CTT TGT CCA GGT GAG AAC ATC (3489–3509)	GTC ATC AGC ATT GGT TTT GGC (3811–3831)	343	AGA CCC CAC AGG TTG TGG CAG CAA GGT CAG (3701–3730)
CgA	AAC CAG AGC AGC CAG GCC GAG (410–430)	TGG CTG GAG GGT GGG TGT TGG (624–644)	235	GAA TCC TCT CTT TTC TCC ATA ACA TCC TTG (460–489)
CgB	CCA GTG GAT AAC AGG AAC CAC (176–196)	GGC TCC TGC CTC TCC CCT GCT (410–430)	254	AGC CCT GGA CTT CGA CAA GGC ATT TGA GAG (233–262)
SG-II	GGT CGT GCT GGG ACT GAG GCC (1320–1340)	TTG CTC ACC GGG GCC AGC TTG (1742–1762)	443	GTT TTC ATA TGC CAT CTG TCT GTT TTC AAC (1521–1550)
SG-V	CAT TTG GGT CCT TTT GGC AAC (278–298)	CGC TTT CGT CTC TCT CCT CCC (544–564)	287	ACA GGA CAG GGA TTT GGA GGG TCT GGG TAC (467–496)
PC-1	CTG GAA GCT GAT TTT GCA CGG (1967–1987)	ATC GCA GGG TAA GGA AGA AGC (2506–2526)	560	CCC TGG ATC CAC CAT CTT CTC CAC CCC TCT (2061–2090)
PC-2	AGA AGG GGG TGC TGA AGG AGT (1826–1846)	GGT GCC TGC CTG AAA CGT GGA (2062–2082)	257	GCA CCA CCT GGT CGA TGT ACG GGG CAC TCT (1871–1900)
hPRL	CCC GAA GAC AAG GAG CAA GCC (284–304)	TGT GAA TCC CTG CGT AGG CA (608–627)	344	CCC TCT AGA AGC GTT TTG GTT TGC TCC TC (446–474)
SV40 large T-antigen	TCC ATT CTT CTA TGT CAG GAG (3656–3676)	ACT AAA CAA GTG TCC TGG AAG (4005–4025)	370	ACA TCC CAA GCA ATA ACA ACA CAT CAT CAC (3944–3973)

extraction. One-dimensional sodium dodecyl sulfate-poly-acrylamide gel electrophoresis (SDS-PAGE) was performed with 7.5% gel using the discontinuous buffer system of Laemmli (Bio-Rad Laboratories, Richmond, CA), as previously reported [23], using 50 μg of total protein from HP75 cells for electrophoresis and then transferred to a polyvinylidene difluoride membrane, and subjected to immunoblot analysis with CgA MAb (1/2,500), TGF-β-RII polyclonal antibody (1/1000), or SV40 T-antigen MAb (1/5,000). The positive control proteins included: mink cells for TGF-β-RII, Cos-1 cells for SV40 T-antigen, and a pheochromocytoma for CgA protein. The reaction was detected by enhanced chemiluminescence (ECL) (Amersham Life Science, Arlington Heights, IL). To check for equal loading of proteins to the gels, the membranes were reblotted with a β-actin MAb (1/2,500; Sigma) after washing.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Total RNA from cultured HP75 cells was extracted with the TRIzol reagent kit (Gibco BRL) and was used for analysis of mRNA expression by RT-PCR. The sequences of primers are shown in Table 2.

First-strand cDNA was prepared from total RNA with oligo dT primers by using a first-strand synthesis kit (Stratagene, LaJolla, CA), following the manufacture's instruction, as previously described [23]. PCR amplification was performed in 100-μL final reaction volumes containing 5 μL of RT reaction as template DNA, 1X PCR buffer, 1.5 mmol/L $MgCl_2$, 0.2 mmol/L each deoxynucleotide, 100 ng of each sense and antisense primer, and 2.5 U of *Taq* DNA polymerase (Promega). Programmable temperature cycling (Perkin-Elmer/Cetus 480, Norwalk, CT) was performed with the following cycle profiles: 95°C for 4 min, followed by 30 cycles (except that 40 cycles was used for FSH-β of 94°C

for 1 min, 60°C for 1 min, and 72°C for 2 min. After the last cycle, the elongation step was extended by 10 min at 72°C. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. A 20- or 30-µL aliquot of PCR product was analyzed by 2% agarose gel electrophoresis and stained with ethidium bromide.

PCR products were transferred to a nylon membrane filter, and Southern hybridization was performed with ^{33}P-labeled internal probes at 42°C for 18 h. After washing with 6X SSC/0.1% SDS at 23°C for 20 min and at 42°C for 10 min, autoradiography was performed at –70°C with Kodak X-Omat-AR film. Primers and internal probes for TGF-β1, TGF-β2, TGF-β3, TGF-β-RII, and GAPDH were recently reported [23] as were primers for GnRH and GnRH receptor as well as for FSH-β and α-SU [24–26]. Other primers and internal probes are listed in Table 2.

Northern Hybridization

Forty micrograms denatured total RNA were electrophoresed through a 1% agarose/formaldehyde gel and were blotted onto a nylon filter. A TGF-β-RII riboprobe (obtained from R. Weinberg, MIT, Cambridge, MA) was labeled with ^{32}P by T_7 (antisense) RNA polymerase provided in the riboprobe labeling kit following the manufacturer's instruction (Promega). Labeled probe 1×10^6 cpm/mL was used for Northern hybridization for 18 h at 50°C, and was then washed for 30 min at 50°C. The filter was air-dried and exposed for 2 d with Kodak X-OMAT film at –70°C with intensifying screens. Equal loading onto the gel was checked by rehybridization with a ^{32}P-labeled β-actin oligonucleotide probe [27].

Cytogenetics Analysis

Cells were prepared for cytogenetic analysis using standard methods of colcemid (0.1 µg/mL) pretreatment, hypotonic swelling (FCS:distilled H_2O, 1:2) and methanol:acetic acid (3:1) fixation followed by Leishman staining.

P53 Gene Analysis

Genomic DNA was extracted from HP75 cells. Thereafter, exons 5, 6, 7, and 8 of the p53 gene were screened for the presence of mutations using published primers [28]. After PCR amplification, single bands of predicted size for each p53 exon were obtained by agarose-gel electrophoresis. The PCR products were then purified from contaminating primers and nucleotides by ultrafiltration (Centricon-100, Amicon, Beverly, MA). Automated sequencing of upper and lower strands of PCR products was done in the Molecular Core Facility at Mayo Clinic. Breast carcinoma cell lines 468 and 231 as well as a human pituitary ACTH carcinoma metastatic to the liver served as positive controls.

Growth Curves

Plastic dishes (35 mm) were seeded with 0.25×10^5 HP75 cells each in complete medium and were allowed to attach for 4 h. The cells were then refed with either complete medium (with serum) or serum-free medium, and the cells were harvested and counted at intervals of up to 14 d.

Tumorigenicity

Six athymic (nude) mice were given sc injections of 1×10^7 cells suspended in phosphate-buffered saline and were monitored for the gross appearance of tumor for 4 mo. MDA-MB-231 cell lines served as a positive control.

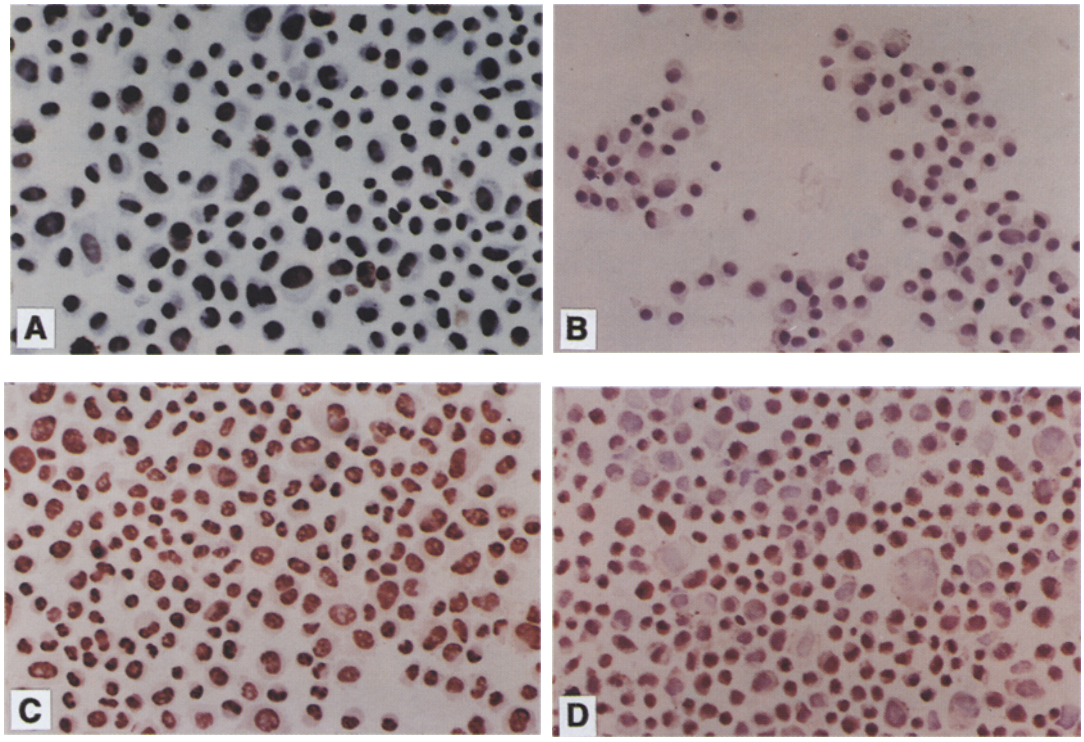

Fig. 1. Immunocytochemical analysis of HP75 cells from passage 8: **(A)** SV40 large T-antigen with brown nuclear staining. **(B)** The adenoma cells in the control dishes without AD-SVR4 were negative for T-antigen; the blue nuclear staining is from the hematoxylin counterstain. **(C)** Ki67 for HP75 cells with brown nuclear staining. **(D)** FSH-β staining was present in the cytoplasm of some of the smaller cells. Cells were counterstained with hematoxylin for 2 s. Magnification was ×250 for each figure.

Analysis of FSH and CgA Regulation

HP75 cells were plated onto 35-mm ECM dishes at 0.25×10^6 cells each in complete medium. Cells were treated with gonadotropin-releasing hormone (GnRH, 10^{-7} *M*), phorbol 12-myristate 13-acetate (PMA, 10^{-7} *M*), forskolin (10^{-6} *M*) (Sigma Chemical Co., St. Louis, MO), and porcine TGF-β1 (10^{-9} *M*) (R&D Systems, Minneapolis, MN) for 6 d and were then harvested for ICC analysis. The optimal concentration of secretagogs was determined by titration of each drug: PMA (10^{-11}, 10^{-9}, and 10^{-7} *M*), TGF-β1 (10^{-13}, 10^{-11}, and 10^{-9} *M*), and forskolin (10^{-8}, 10^{-6}, and 10^{-4} *M*). The culture medium was collected and immunoassays for FSH and pancreastatin were performed as previously described [22].

Thymidine Incorporation

Incorporation of ^{3}H-thymidine was performed as previously reported [29,30]. Briefly, HP75 cells were grown in complete DMEM on plastic 35-mm dishes for 6 d. The medium was changed, and 10 μCi ^{3}H-thymidine (SA 15 Ci/mmol; Dupont, Boston, MA) were added for 4 h. The cells were harvested, washed three times in PBS, and then lysed with 0.5 *N* NaOH + 1% SDS. A 100-μL aliquot was used for liquid scintillation counting (Beckman Instruments, Inc., Palo Alto, CA).

Electron Microscopy

Cells were fixed in 2% formaldehyde in phosphate-buffered glutaraldehyde in 0.1 *M* cacodylate buffer, pH 7.2, and processed for electron microscopy.

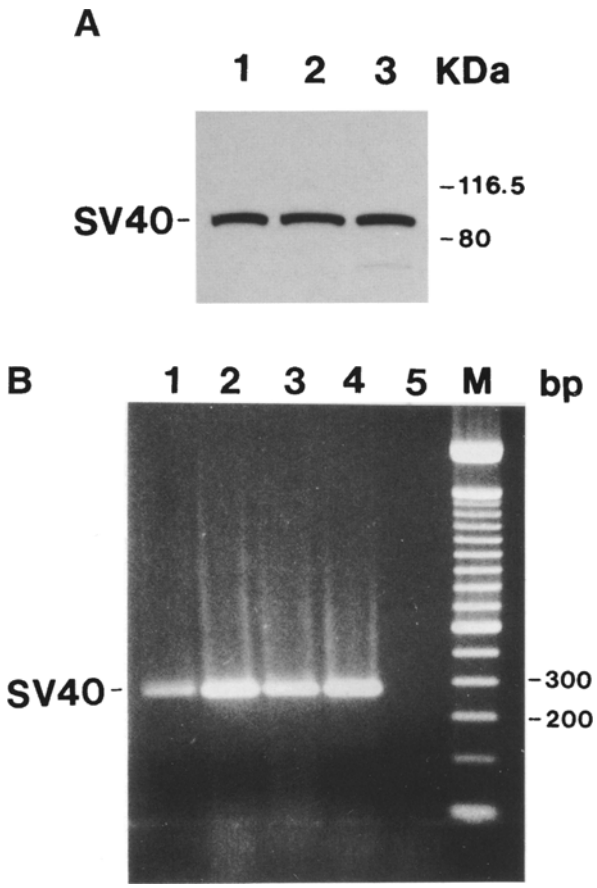

Fig. 2. (A) Immunoblot detection of SV40 large T-antigen (SV40) identified a 94-kDa band in HP75 cells. Lanes 1 and 2: HP75 cells at passage 10 and 14, respectively; lane 3: COS-1 cells used as a positive control, 50 μg of protein were used for electrophoresis. **(B)** RT-PCR detected a predicted 370-bp band of SV40 large T-antigen mRNA (SV40) in HP75 cells. Lanes 1, 2, 3: HP75 cells passages 8, 12, and 40, respectively; lane 4: COS-1 cells; lane 5: negative control without reverse transcriptase for HP75 cells.

Statistics

Each experiment was repeated between two and five times. Results were expressed as the mean ± SEM; *T*-test was used for statistical comparisons.

Results

Isolation of Cell Lines

Six weeks after infection of pituitary adenoma cells with AD-SVR4, 2 of 19 adenomas demonstrated proliferation of cells. One adenoma, HP75, was subcloned and passaged for more than 60 passages over a 2-yr period, whereas the second cell line, HP68, was studied for a few months only because the proliferation rate decreased after 10 passages. The other 17 adenomas failed to proliferate after infection even after 16 wk in culture.

Both the HP75 and HP68 cell lines consisted of epithelioid and spindle-shaped cells, which grew equally well on plastic or ECM dishes. After cytocentrifugation onto glass slides, the HP75 cells consisted of small cells 20–30 μ in diameter and larger multinucleated cells of up to 50 μ in diameter. The HP68 cells consisted mostly of small 20–30 μ cells.

Expression of SV40 Large T

Immunostaining of both HP75 and HP68 cells showed positive nuclear staining for large T-antigen in more than 90% of the cells, but the control cells were negative (Fig.1A, B). Western blotting identified a 94-kDa band in the HP75 cell line, which was also detected in the positive control COS-1 cell line (Fig. 2A). RT-PCR detected a 370-bp amplified band of the predicted size in both the HP75 and COS-1 cell lines (Fig. 2B)

Cytogenetic Analysis

The HP75 cells analyzed after 10 passages consisted of diploid and hypertetraploid cells and showed a few clonal abnormalities, including additions affecting chromosome 19 (p13.1, and q13.3). The HP68 cell line also had clonal abnormalities including additions on 12 p11.2 and 15 p11.2 as well as dicentric (9;19) (p13, q13.1).

p53 Protein and Mutation Analysis

Immunostaining showed that most of the HP75 and HP68 cells overexpressed

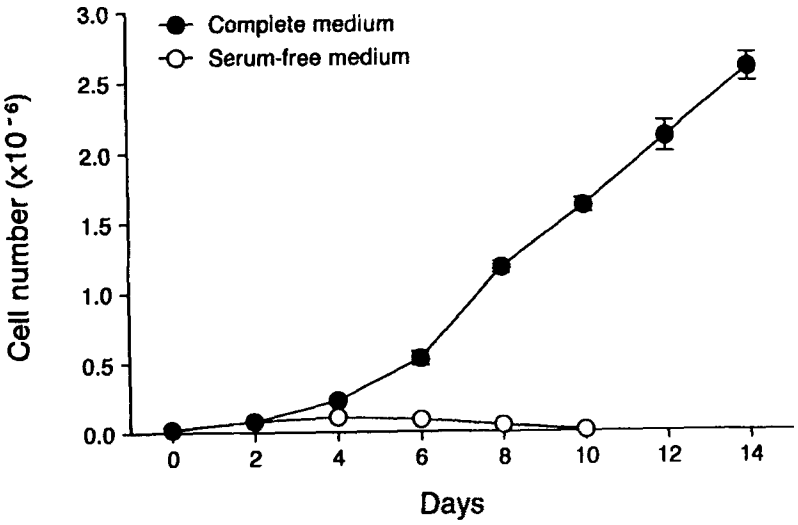

Fig. 3. Growth curves of HP75 cells showing serum dependence. Cells were cultured in either complete medium or serum-free DMEM. Data are shown as mean cell count ± SEM for triplicate dishes at each time-point.

p53 protein (data not shown). However, analysis for mutations in exons 5–8 was negative. The positive controls of two breast carcinoma cell lines 468 and 231 had point mutations in exon 8 codon 273 (CGT → CAT) and exon 8 codon 280 (AGA → AAA), respectively. In addition, a human pituitary ACTH-producing carcinoma cell line with liver metastases had a point mutation in exon 7 codon 248 (CGG → CAG) (data not shown).

Proliferation in Culture

HP75 cells proliferated rapidly in complete medium with a doubling time of approx 2 d. However, when grown in serum-free defined medium, only minimal cell proliferation was observed (Fig. 3). Proliferation rate as indicated by immunostaining for Ki67 with antibody MIB1 showed staining in more than 90% of the nuclei of HP75 (Fig. 1C) compared to <1% of nuclei in the same pituitary adenoma before infection.

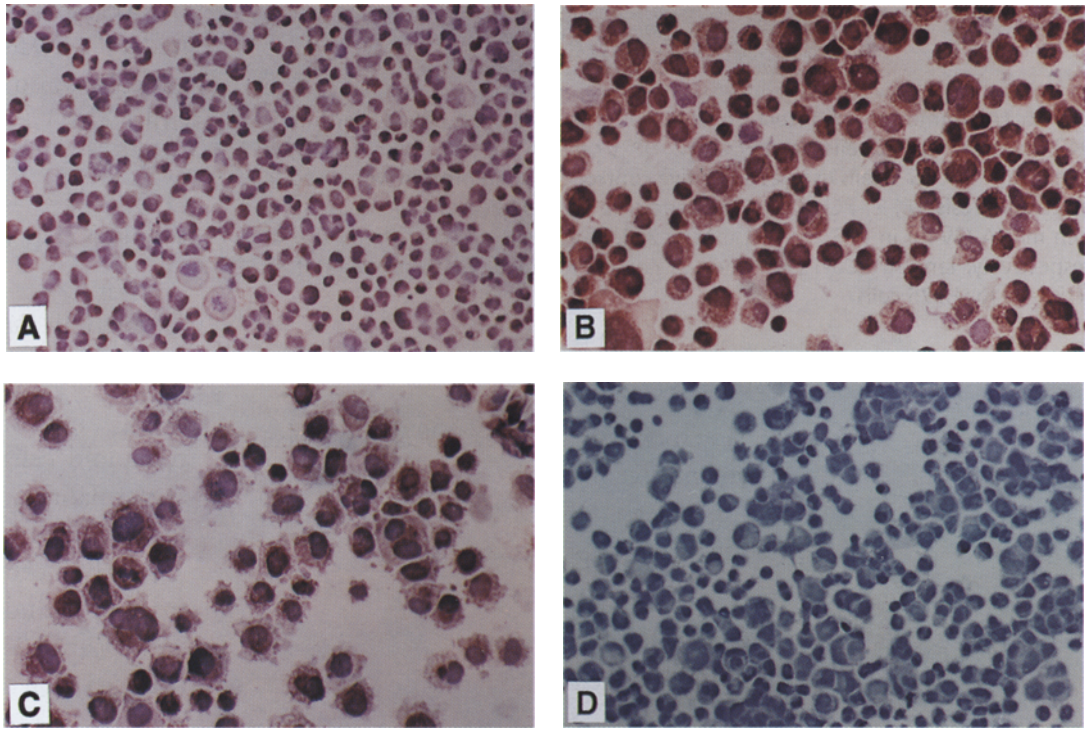

Fig. 4. Immunocytochemical analysis of HP75 cells showed expression of **(A)** CgA in control cells; very few cells are positive. **(B)** CgA in PMA-treated cells; there is an increase in the number of positive cells compared to the control. **(C)** CgA in forskolin-treated cells; moderate cytoplasmic staining is present. **(D)** Negative control for forskolin-treated cells; magnification was 200× for A, and 250× for B, C, and D. Cells were counterstained with hematoxylin for 2 s.

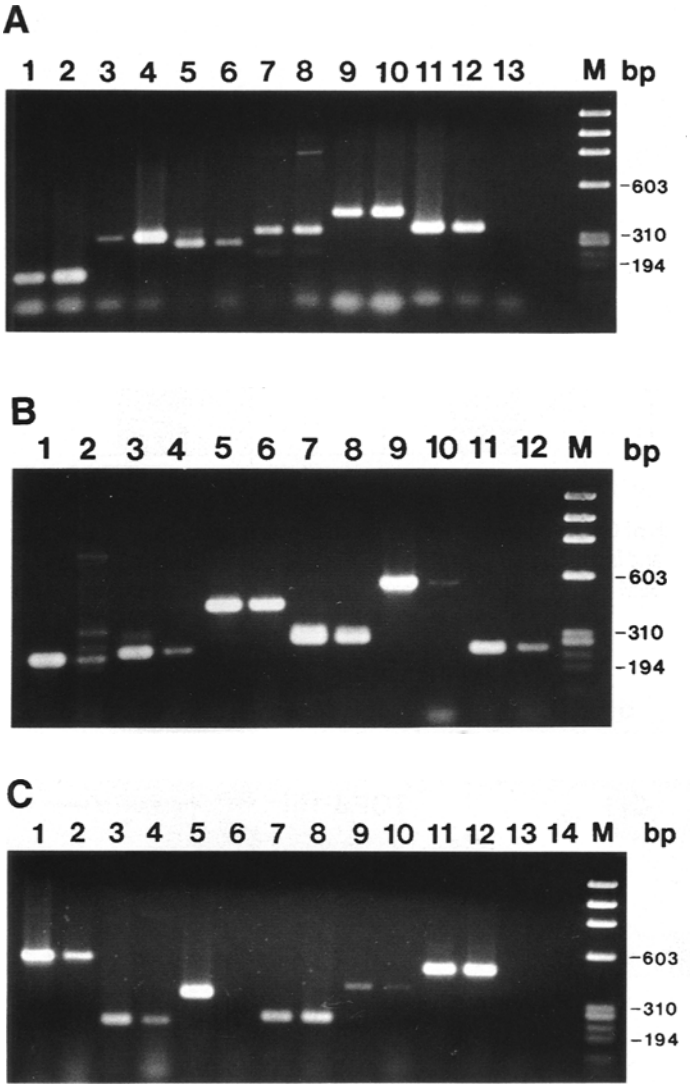

Fig. 5. RT-PCR analysis was performed with HP75 cells (lanes 2, 4, 6, 8, 10, 12). A gonadotroph adenoma (lanes 1, 3, 5, 7, 9, 11) and non-neoplastic human pituitary (lanes 1, 3, 5 in Fig. 5A only) were used as positive controls. Ethidium bromide-stained gels showing: **(A)** FSH-β (lanes 1, 2), α-SU (lanes 3, 4), PRL (lanes 5, 6), GnRH (lanes 7, 8), GnRH-R (lanes 9, 10), GAPDH (lanes 11, 12). A negative control of FSH-β (lane 13) and CgA (lane 14) for HP75 cells are shown. **(B)** CgA (lanes 1, 2), CgB (lanes 3, 4), SGII (lanes 5, 6), SGV (lanes 7, 8), PC1/3 (lanes 9, 10), PC2 (lanes 11, 12). **(C)** TGF-β1 (lanes 1, 2), TGF-β2 (lanes 3, 4), TGF-β3 (lanes 5, 6), TGF-β-RI (lanes 7, 8), TGF-β-RII (lanes 9, 10), TGF-β-RIII (lanes 11, 12), and negative control of TGF-β-RII for HP75 cells (lane 13). The sizes of the amplified products are shown in Table 2.

No tumors were seen in six nude mice injected with 1×10^7 cells after 4 mo of observation. The control 231 breast carcinoma cells produced tumors in <5 wk.

Hormone and Chromogranin Production

Both HP75 and HP68 cell lines were immunoreactive for FSH-β, LH-β, CgA, PC1/3, and PC2 (Figs. 1D, 4A–C) (Table 1).

RT-PCR analysis showed expression of FSH, α-SU mRNA, but not PRL in HP75 cells (Fig. 5). Both GnRH of GnRH receptors were expressed by the HP75 cells. Analysis of Cg/Sg family members showed CgA, CgB, SgII, and SgV mRNA expression (Fig. 5). The mRNAs for the prohormone convertases PC1/PC3 and PC2 were also expressed in HP75. RT-PCR also detected the mRNAs of TGF-β1, β2, β3, and TGF-β receptors I, II, and III (Fig. 5). Southern hybridization with probes internal to the PCR primers confirmed the identities of the PCR amplified products (data not shown). Although multiple bands were observed for CgA after ethidium bromide staining, Southern hybridization showed only a 235-bp band.

Analysis of hormone secretion showed low levels of FSH secreted into the culture medium (0.11 ± 0.02 IU/mL/10^6 cells/d). This level of secretion was much lower than that seen with gonadotroph tumors cultured under the same conditions (6.8 ± 1.3 IU/mL/10^6 cells/d), but was in the same range of that of such nonfunctional adenomas as null cell tumors [22]. The HP75 cells also secreted pancreastatin into the medium ($2.0 + 0.3$ fmol/mL/10^6 cells/d), which was in the same range as null cell adenomas (2.9 ± 0.2 fmol/mL/10^6 cells/d), but lower than gonadotroph tumors (7.4 ± 1.3 fmol/mL/10^6 cells/d) [22].

Regulation of FSH and CgA Production

When the HP75 cells were cultured in the presence of GnRH, PMA, and TGF-β1, there was a significant increase in the number of cells expressing FSH-β and CgA with a fourfold increase in FSH and a 1.5- to 2-fold increase in CgA after PMA and TGF-β1 treatment (Figs. 4 and 6). West-

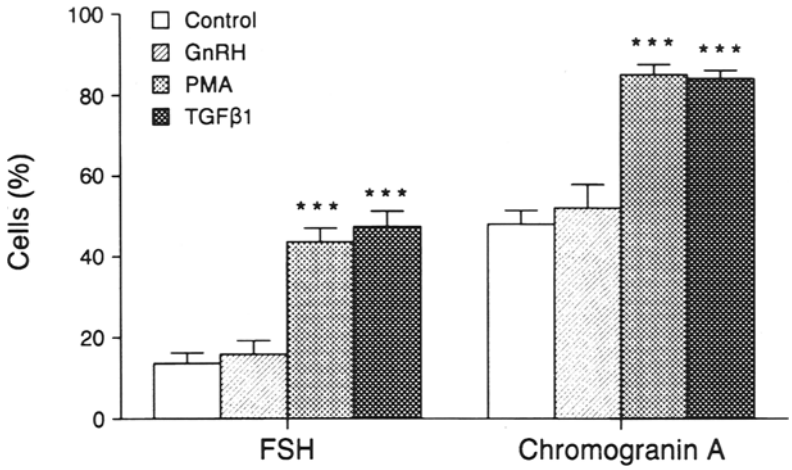

Fig. 6. Immunocytochemical analysis of CgA and FSH-β in HP75 cells after treatment with GnRH, PMA, and TGF-β1. Cultured cells were cytocentrifuged and immunostained with CgA or FSH-β antibody. The percentage of positive cells was expressed as mean cell count ± SEM from 5 independent experiments with duplicate dishes (***$p < 0.001$).

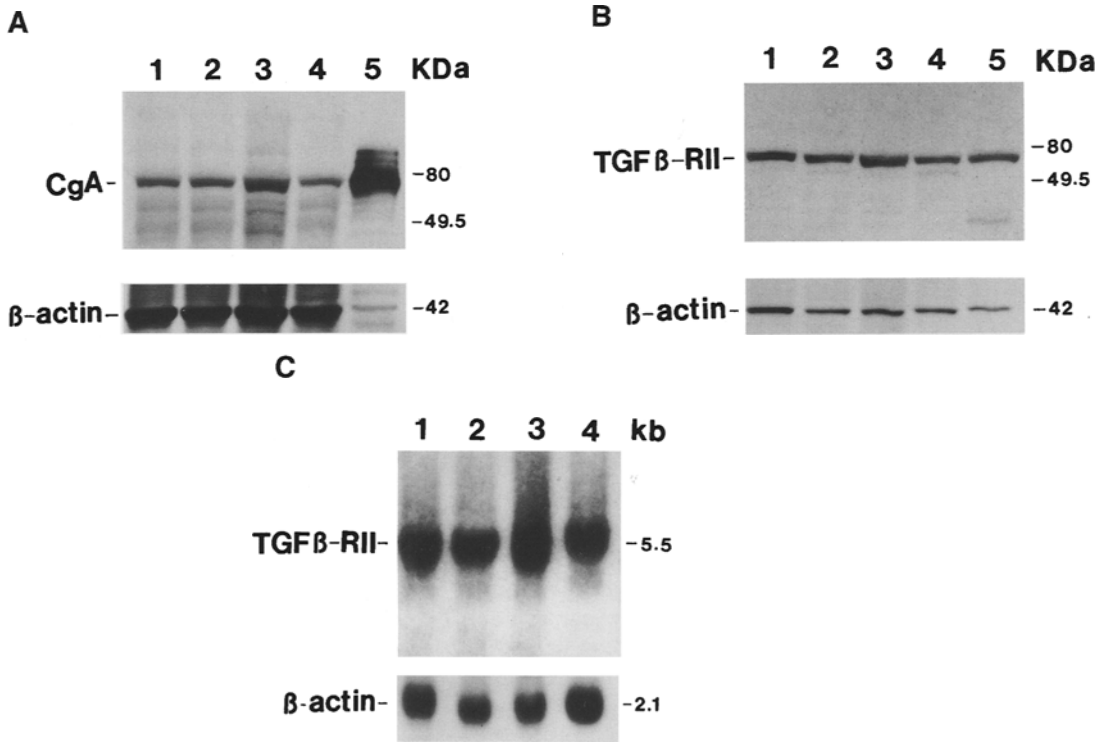

Fig. 7. (A) Immunoblot analysis showed a 75-kDa band of CgA protein in HP75 cells. CgA was upregulated after PMA treatment. Lane 1: control cells; lane 2: GnRH-treated cells; lane 3: PMA-treated cells; lane 4: TGF-β1-treated cells; lane 5: pheochromocytoma tissue used as a positive control; 50 μg of proteins were loaded for lanes 1–4, and 10 μg for lane 5. Lower panel: immunoblot of β-actin to check for equal protein loading of the gel. **(B)** Upper panel: immunoblot analysis of TGF-β-RII protein (70 kDa) in HP75 cells indicated that PMA treatment increased the TGF-β-RII expression. Lane 1: Control cells; lane 2: GnRH-treated cells; lane 3: PMA-treated cells; lane 4: TGF-β1-treated cells; lane 5: mink Mv1Lu cells were used as a positive control; 50 μg of protein were used for each lane. Lower panel: immunoblot of β-actin to check equal protein loading on gel. **(C)** Upper panel: Northern hybridization of TGF-β-RII mRNA with a ^{32}P-labeled riboprobe showing a band of 5.5 kb in HP75 cells. PMA treatment increased TGF-β-RII expression. Lane 1: control cells; lane 2: GnRH-treated cells; lane 3: PMA-treated cells; lane 4: TGF-β1 treated cells; 40 μg of total RNA were loaded in each lane. Lower panel: rehybridization with β-actin oligonucleotide probe.

Table 3. Effects of Various Treatments on Cell Proliferation

Treatment	^{3}H-Thymidine uptake, CPM/10^4 cells[a]	Cell number, $\times 10^6$ cells[b]
None	5040 ± 283	1.6 ± 0.1
GnRH	5039 ± 347	1.6 ± 0.2
PMA	2584 ± 256[c]	0.9 ± 0.2[d]
Forskolin	3595 ± 469[c]	1.2 ± 0.1[c]
TGF-β1	3062 ± 415[d]	0.9 ± 0.1[d]

[a]^{3}H-thymidine incorporation was done as described in materials and methods in two separate experiments with triplicate dishes.
[b]Mean cell count/dish for three experiments with duplicate dishes.
[c]$p < 0.05$.
[d]$p < 0.01$.
[e]$p < 0.001$.

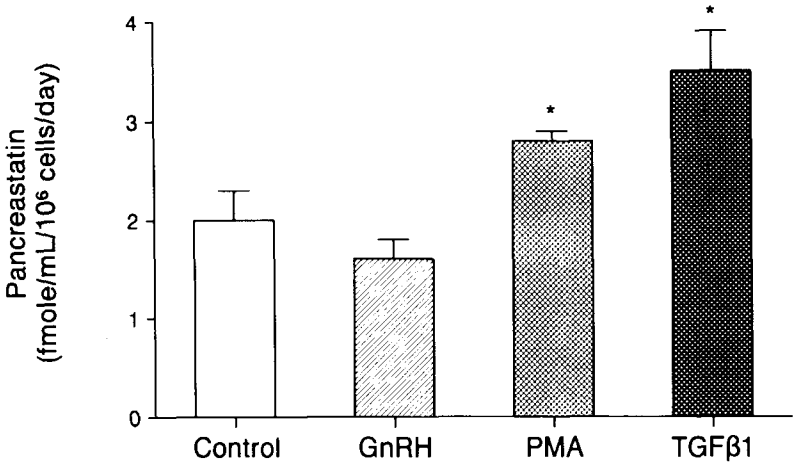

Fig. 8. Pancreastatin secretion detected by immunoassay in the culture medium from HP75 cells. Data were from two separate experiments with triplicate dishes (*$p < 0.05$).

ern blots also demonstrated a 1.8-fold increase in CgA by PMA and a 1.2-fold increase in TGF-β1 by densitometric analysis (Fig. 7A). Forskolin treatment (10^{-6} *M*) increased both FSH expression (control 19.6 ± 1.3% vs treated 34.2 ± 7.3%) and CgA expression (control 52 ± 2.4% vs treated 75 ± 3%, $p < 0.01$) by ICC.

After PMA treatment, TGF-β-RII protein was increased 1.5-fold by Western blotting. Both GnRH and TGF-β1 produced a slight increase in TGF-β-RII proteins (Fig. 7B). Northern hybridization analysis of TGF-β-RII showed an increase in the mRNA after PMA treatment (Fig. 7C).

After 6 d in culture, there was no change in cell number in GnRH-treated cells compared to controls (Table 3). However, PMA, forskolin, and TGF-β1 treatment significantly decreased the total cell numbers. Both direct counting of the cells and [^{3}H]-thymidine incorporation studies showed inhibitory effects on cell proliferation after PMA and TGF-β1 treatment (Table 3). Both PMA and forskolin treatment increased the size of HP75 cells (Fig. 4).

Pancreastatin Secretion

The presence of PC1 and PC2 mRNAs and proteins in the HP75 cell line prompted us to investigate processing of CgA to pancreastatin in the cell line. Treatment of this cell line with GnRH, PMA, and TGF-β1 on ECM dishes for 3 or 6 d led to an increase in the secretion of pancreastatin (Fig. 8). Forsokolin treatment also increased pancreastatin secretion from HP75 cells (control, 1.9 ± 0.5 vs 2.5 ± 0.6 fmol/mL/10^6 cells/d; $p < 0.05$). After PMA treatment of cells grown in 75-cm^2 plastic flasks, there was an increase in both the intracellular content of pancreastatin (4.4 ± 0.5 vs 6.4 ± 0.5 fmol/mL/10^6 cells/d, $p < 0.05$) as well as secretion of this peptide into the medium (0.52 ± 0.08 vs 0.99 ± 0.07 fmol/mL/10^6 cells/d, $p < 0.01$).

Electron Microscopy

Ultrastructural studies showed cells with well-developed rough endoplasmic reticulum, but only rare secretory granules of 100–250 nm were present. Stimulation of the cells with TGF-β and PMA increased the Golgi region and rough endoplasmic reticulum (Fig. 9).

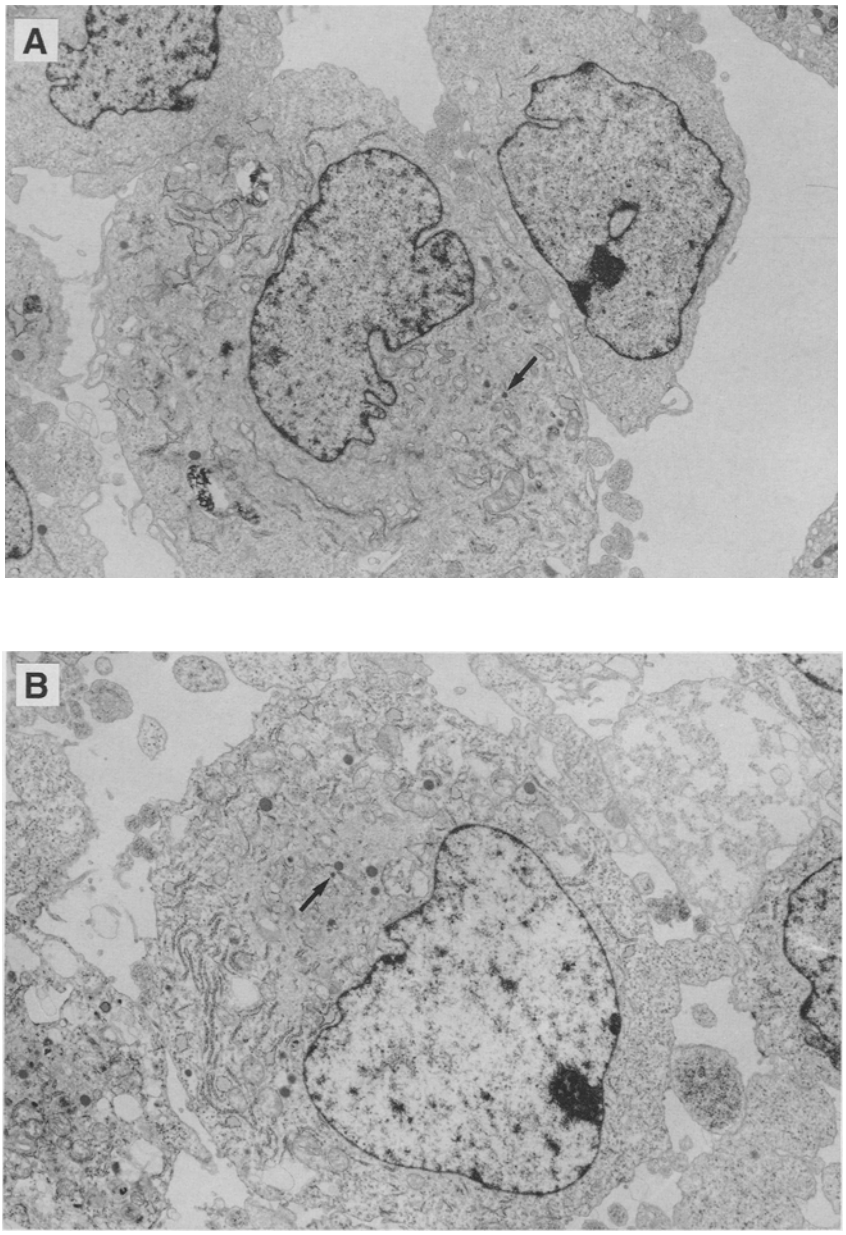

Fig. 9. Ultrastructural examination of HP75 cell line. **(A)** The tumor cells have rare secretory small granules with limiting membranes (arrow) and moderate amounts of rough endoplasmic reticulum (×5,700). **(B)** After treatment with TGF-β and PMA for 7 d, the rough endoplasmic reticulum and Golgi regions are more prominent. Small secretory granules 100–150 nm in diameter with limiting membranes (arrow) are also present (×5,700).

Discussion

The present study shows that human pituitary adenoma cells can be immortalized and induced to proliferate while maintaining some differentiated functions following expression of SV40 large T-antigen. Various rat and mouse pituitary cell lines, such as GH_3, AtT 20, and GHRH-CL1, have been available for in vitro studies [31–33]. Recent developments in targeted oncogenesis have led to the propagation of transgenic mouse pituitary tumor cell lines expressing large T-antigen [21,34], but only a few human pituitary cell lines have been available for experimental studies [1–7]. Thus, human pituitary cell lines, such as HP75, which has retained various differentiated functions after more than 60 passages over a 2-yr period should provide a model to study hormonal regulation of human pituitary growth and function in vitro.

In our experiments, only 2 of 19 pituitary adenomas were immortalized after infection with AD-SVR4. Human cells that are semipermissive for SV40 can produce the virus at an efficiency rate of about 1% compared with permissive monkey cells [35], which leads to death of the infected cell when the virus replicated in the cell. Cells infected with wild-type SV40 rarely retain the ability to differentiate. The origin-defective adenovirus can infect cells without cell lysis and, in most, cases is associated with retention of certain differentiated traits [10,12,13]. In our study, the infected cells showed a marked increase in proliferation with a doubling time of about 2.5 d during the rapid growth phase. The accelerated growth and the expression of T-antigen by the pituitary cells indicated that immortalization was the result of infection and expression of exogenous T-antigen.

The HP75 cell line lost some differentiated functions as shown by the paucity of secretory granules, low levels of secreted FSH-β, and lack of a response to GnRH treatment. However, this cell line should be useful to study other regulatory functions of human pituitary cells.

Previous studies of hepatocyte cell lines have shown that cells with SV40 large T-antigen continued to express genes

associated with differentiation, such as albumin and α_1-antitrypsin [10]. Similarly, both human myogenic cell lines transfected with origin-defective SV40 DNA [13] and neonatal myocardial cells transiently expressing SV40 large T-antigen [12] show differentiated functions, indicating that the presence of SV40 large T-antigen does not interfere with some differentiated functions. Similarly, the immortalized HP75 cell line expressed mRNA for various hormones and for various Cg/Sg proteins and responded to the PKC stimulator phorbol ester PMA and to the PKA stimulator forskolin by increasing CgA expression. These findings indicate that functional PKC and PKA pathways are retained in these adenoma cells. Furthermore, the presence of a PKC and a PKA pathway in the HP75 cells suggests that there may be "crosstalk" between these pathways. This was recently reported for the αT_{3-1} clonal gonadotroph cell line derived from expressing the transgenic mice SV40 large T-antigen [36].

The inhibition of cell growth by PMA, forskolin, and TGF-β1 in this cell line is consistent with the increased expression of CgA, which was considered to be evidence of differentiated functions in these studies. Failure of the HP75 cell line to grow in nude mice is similar to previous reports with immortalized thyroid cell lines [14] and suggests that the cells are not highly malignant.

The overexpression of p53 protein detected by immunostaining was not related to p53 mutations in exons 5–8. Previous studies have shown that interaction of T-antigen and wild-type 53 increased the half-life of the latter and its steady-state level within cells [37], and that the binding of p53 to the T-antigen led to functional inactivation of p53 [38].

CgA is a marker of neuroendocrine cells [39–41] and is expressed by most cells of the adenohypophysis. Our HP75 cell line expressed CgA and secreted pancreastatin into the culture media. Recent studies have suggested that CgA is converted to pancreastatin by the neuroendocrine-specific prohormone convertases (PC) PC1/3 and PC2 which also process other neuropeptides [42–45]. Since our study showed that PC1/3 and PC2 proteins and their mRNAs were expressed by HP75, it is likely that these PCs converted CgA to pancreastatin. The effects of treating HP75 cells with PMA, forskolin, and TGF-β1 were an increase in pancreastatin secretion, a finding consistent with the observed increase in CgA within the cells. Our observations of increased pancreastatin secretion and increased intracellular pancreastatin after PMA treatment differ from those of Udupi et al., who recently reported a decrease in pancreastatin secretion in a neuroendocrine (carcinoid) cell line under the influence of a phorbol ester (TPA). The effect was reportedly accompanied by a decrease in the active from of the PC1/3 enzyme [44]. Instead, our analyses suggested an increase in PC activity in HP75 owing to PMA treatment, which may be related to the specific cell line used. We plan to investigate these changes in future experiments.

The presence of all three major isoforms of mRNAs for TGF-β, (β1, β2, β3), as well as mRNAs for receptors TGF-β-RI, TGF-β-RII, and TGF-β-RIII in the HP75 cell line supports our recent observations about the expression of this growth factor and its receptors in human pituitary tumors [23]. Studies have shown the importance of TGF-β signaling in regulating epithelial cell growth and secretion [45–51]. Thus, the HP75 human pituitary cell line should serve as a good model for studying the regulatory effects of TGF-β on pituitary cell signaling.

Castro et al. recently used recombinant adenovirus vectors (Rads) encoding β-galactosidase driven by various viral promoters to infect normal rat pituitary cells, and the GH_3 and AtT20 cell lines [52]. They found that the transgenes encoded by Rads were expressed in all cell types of the anterior pituitary, in GH_3, and in AtT20. They also observed that the ability of anterior pituitary cells to secrete ACTH or LH decreased after infection with Rads at high multiplicity of infection, but cell viability was not affected [52]. A similar decrease in secretion of FSH-β and pancreastatin was observed in our studies of infected human pituitary adenoma cells in this study.

In summary, we have developed and characterized, HP75, a human pituitary adenoma cell line using a replication-defective recombinant human adenoma containing an SV40 early large T-antigen insert. The HP75 cell line retains some differentiated functions of pituitary adenoma cells, including expression of FSH mRNA, chromogranin A and pancreastatin. The tumor cells responded to PKC and PKA stimulators as well as to TGF-β1 by increasing CgA protein synthesis, as well as TGF-β-RII mRNA and protein. These findings indicate that HP75 can be used as an in vitro model to study the functions of human pituitary cells.

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Note Added in Proofs

Another report immortalizing normal human pituitary cells with a temperature-sensitive mutant of SV-40 large T-antigen has been published [53] while this article was in press.

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