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HK-2: An immortalized proximal tubule epithelial cell line from normal adult human kidney

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HK-2: An immortalized proximal tubule epithelial cell line from normal adult human kidney. Studies assessing mechanisms of proximal tubular cell (PTC) physiology and pathophysiology increasingly utilize cell culture systems to avoid the complexity of whole organ/whole animal experiments. However, no well-differentiated PTC line derived from *adult human kidney* currently exists. Therefore, the goal of this research was to establish such a line by transduction with human papilloma virus (HPV 16) E6/E7 genes. A primary PTC culture from normal adult human renal cortex was exposed to a recombinant retrovirus containing the HPV 16 E6/E7 genes, resulting in a cell line designated HK-2 (human kidney-2) which has grown continuously in serum free media for more than one year. HK-2 cell growth is epidermal growth factor dependent and the cells retain a phenotype indicative of well-differentiated PTCs (positive for alkaline phosphatase, gamma glutamyltranspeptidase, leucine aminopeptidase, acid phosphatase, cytokeratin, $\alpha_3\beta_1$ integrin, fibronectin; negative for factor VIII-related antigen, 6.19 antigen and CALLA endopeptidase). Furthermore, HK-2 cells retain functional characteristics of proximal tubular epithelium (Na^+ dependent/phlorizin sensitive sugar transport; adenylate cyclase responsiveness to parathyroid, but not to antidiuretic, hormone). The E6/E7 genes are present in the HK-2 genome, as determined by PCR. To assess its potential usefulness as a tool for studying injury and repair, HK-2 cells were exposed to a toxic concentration of H_2O_2 ± iron chelation (deferoxamine) or hydroxyl radical scavenger (Na benzoate) therapy. Only the former blocked H_2O_2 cytotoxicity, reproducing results previously obtained with freshly isolated rat proximal tubular segments. In conclusion, an immortalized adult human PTC line has been established by transduction with HPV 16 E6/E7 genes. It appears to be well-differentiated on the basis of its histochemical, immune cytochemical, and functional characteristics, and it can reproduce experimental results obtained with freshly isolated PTCs. Thus, this new PTC line could have substantial research application.

Given the diversity of cell types within the kidney, and because of the complex interplay between its nephronal and vascular elements, cell culture has increasingly been used to study primary events in proximal tubular cell (PTC) physiology, injury, and repair. Primary PTC cultures can be helpful in this regard; however, their usefulness is restricted by their inability to survive serial passage, variability amongst sequential PTC preparations, the labor intensive nature of their isolation, and the need to continuously prove that PTC have, indeed, been obtained. To circumvent these problems, a number of transformed or tumor-derived PTC lines have been established [1]. However, altered gene expression and hence, derangements in cell function and structure typically result, potentially limiting their utility [2–5].

Recently, the E6/E7 genes of a human papilloma virus (HPV 16) have been reported to immortalize epithelial cells of diverse origin without significantly changing their phenotype or function [6–12]. At the molecular level, the E6 and E7 gene products bind to DNA regulatory proteins, the result of which is to facilitate cell proliferation [13–18]. However, these changes do not equate with malignant transformation since the host cells retain features of differentiation, and thus, their growth is anchorage dependent and they remain under the control of many normal regulatory processes. Therefore, this type of cell preparation may represent a useful compromise between primary cultures and highly transformed cell lines: many regulatory processes remain intact, and yet the need for repeated PTC isolation and characterization is eliminated.

The purpose of the present investigation was to ascertain whether HPV 16 E6/E7 viral constructs can be used to produce an immortalized adult human proximal tubular cell line which expresses many of the phenotypic and functional properties of their cellular origin. Once established, some initial studies were undertaken to demonstrate the potential utility of this preparation for the study of PTC injury and repair.

Methods

Organ procurement

Following guidelines established by the Northwest Kidney Foundation (Seattle, Washington, USA), organs were procured for transplantation from a noninfectious adult male. One kidney, deemed unsuitable for transplantation due to an upper pole cortical scar, was released for research purposes.

Cell culture techniques

Cortical proximal tubular segments were isolated using a technique previously described for the rat kidney [19]. The segments, once obtained, were transferred to a T-25 tissue culture flask and maintained at 37°C with 5% CO_2 in keratinocyte serum-free medium (K-SFM, Gibco Life Technologies,

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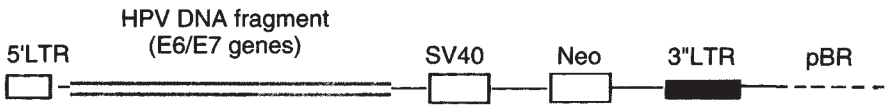

Fig. 1. *The recombinant retrovirus vector, pLXSN 16 E6/E7, used to transduce human proximal tubule cells.* Abbreviations are: LTR, Moloney murine promotor-enhancer; SV 40, simian virus 40 promotor; Neo, gene conferring neomycin resistance; pBR, pBR322 plasmid sequence which contains the origin of replication and the gene conferring neomycin resistance.

Grand Island, New York, USA), supplemented with epidermal growth factor (EGF; 5 ng/ml) and bovine pituitary extract (40 μg/ml). After achieving confluency (approximately 2 weeks), the cells were exposed to a replication-defective recombinant retrovirus (see below) for 48 hours. Then, they were rinsed twice with Hank's balanced salt solution (HBSS) and detached from the flask with trypsin (0.05%)/EDTA (0.6 mM). Trypsin activity was stopped by calf serum addition. The suspended cells were centrifuged, washed in HBSS, re-suspended in hormone supplemented K-SFM, and re-plated in T-25 flasks. Growing cell clones, visualized by phase contrast microscopy, were isolated with a cloning ring. One clone, designated HK-2, (human kidney-2), has been growing continuously for more than one year (approximately 30 passages), aliquots of which have been stored in liquid N_2 with calf serum containing 10% DMSO. Studies to characterize the cell line were conducted between 6 and 12 months after the line was established. All histochemistry was repeated at nine and 12 months.

Recombinant retrovirus

Recombinant virus was generated in the laboratory of Denise Galloway (Fred Hutchinson Cancer Research Center; FHCRC), as previously described [9, 20]. In brief, the ectotropic packaging cell line Psi-2 was transfected with plasmid DNA, containing the HPV 16 E6 and E7 genes. The pLXSN 16 E6/E7 construct is shown in Figure 1. Virus produced by the Psi-2 cells was used to infect the amphotropic packaging cell line PA 317. Virus produced by the PA 317 cells was used to transduce primary cell cultures, as described above. Subsequent outgrowths of immortalized cells were removed by treatment with trypsin and expanded in supplemented K-SFM. Although pLXSN 16 E6/E7 also confers resistance to neomycin, selection in G418 was not used to isolate transduced clones.

Enzyme cytochemistry

Alkaline phosphatase. Since alkaline phosphatase is a normal constituent of the proximal tubular brush border membrane (BBM), its expression by HK-2 cells was ascertained by a previously described technique [21] to help assess their nephronal origin. In brief, HK-2 cells, grown on chamber slides, were washed with phosphate buffered saline (PBS), and fixed in a mixture of 5% formaldehyde/95% methanol for 30 seconds at room temperature. The cells were stained for alkaline phosphatase with a solution of naphthol AS phosphate and Fast Blue BB (Sigma Chemical Co., St. Louis, Missouri, USA) dissolved in 0.2 M Tris, at 37°C for 30 minutes. Control cells were treated in an identical fashion but were not exposed to the naphthol AS phosphate.

Gamma glutamyltranspeptidase. The presence of gamma glutamyltranspeptidase (GGT), another PTC BBM-associated enzyme, was assessed [22]. Briefly, cells grown on chamber slides and washed as noted above were shock-frozen in isopentane over dry ice for five minutes and incubated for 45 minutes in a solution of gamma glutamyl-4-methoxy-2-naphthylamide (GMNA), glycylglycine, Fast Blue BBN (Sigma Chemical), 0.2 M Tris, and 0.85% NaCl. They were rinsed in 0.85% NaCl, incubated in 0.1 M $CuSO_4$ for two minutes, rinsed in distilled water, dried, and mounted. Cells incubated in the above solution, but lacking GMNA, were used as controls.

Acid phosphatase. Acid phosphatase, another PTC BBM-associated enzyme, was sought in HK-2 cells by the technique of Burstone [23]. In brief, HK-2 cells were washed and fixed in 60% acetone in citrate buffer (pH 5.4) for 30 seconds at room temperature, rinsed with water, and air dried. They were incubated for one hour in the dark at 37°C in a solution of naphthol AS-BI phosphate and Fast Garnet GBC (Sigma Chemicals). Cells not exposed to naphthol AS-BI served as controls.

Leucine aminopeptidase. The method of Nachlas et al [24] was used to stain HK-2 cells for a fourth BBM enzyme, leucine aminopeptidase (LAP). Confluent cell monolayers were washed twice in PBS, snap frozen in isopentane over dry ice, and incubated at 37°C for 20 minutes in a solution composed of L-leucyl-4-methoxy-2-naphthylamide HCl (0.4 mg/ml), acetate buffer (0.05 M, pH 6.5), NaCl (0.85%), KCN (0.02 M), and Fast Blue B powder (0.5 mg/ml; Sigma Chemical). This was followed by post-coupling in 0.1 M $CuSO_4$. Cells treated in an identical fashion, but not exposed to L-leucyl-4-methoxy-2-naphthylamide HCl, served as controls.

Immune cytochemistry

To further characterize the HK-2 cell line, its reactivity to a panel of antibodies directed against: cytokeratin, vimentin, fibronectin, basement membrane-associated $\alpha_3\beta_1$ integrin, human type 1 factor VIII-related antigen, CALLA endopeptidase, and 6.19 antigen were assessed. Mouse antibodies against vimentin, $\alpha_3\beta_1$ integrin and fibronectin were provided by Dr. William Carter (FHCRC). Anti-CALLA endopeptidase was a gift from Dr. Paul Martin (FHCRC). Anti-6.19 was provided by C.N. Frantz (University of Rochester School of Medicine, Rochester, New York, USA). Anti-cytokeratin and anti-factor VIII-related antigen were purchased from Sigma Chemical and Calbiochem (La Jolla, California, USA), respectively. Briefly, HK-2 cells, grown to confluency in eight chamber slides, were washed twice in HBSS, fixed for 10 seconds in ice cold acetone, rinsed in PBS, and permeabilized in 1% Triton at room temperature for five minutes. The cells were rinsed again and incubated for 45 minutes at room temperature with one of the above primary antibodies. The presence of bound antibody was detected by fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG. Nuclei were counterstained with propidium

iodide (1 μg/ml). Controls included staining with isotyped matched nonbinding irrelevant primary antibodies.

Functional assays of HK-2 cells

Adenylate cyclase activity. Proximal tubular cells characteristically respond to parathormone (PTH), but not to antidiuretic hormone (ADH), with an increase in adenylate cyclase activity, and hence, in cAMP production [for example, 25–27]. Thus, the responses of HK-2 cells to PTH and ADH were assessed to further characterize their origin and functional characteristics. Four × 10^5 HK-2 cells were placed into each well of a 24 well plate. After one week, when the cells had reached confluency, the medium was removed and the cells were washed twice with HBSS. The incubation medium was replaced with K-SFM supplemented with 1 mM 3-isobutyl-1-methylxanthine (IBMX; an inhibitor of cAMP degradation). Incubations were carried out for 20 minutes at 37°C in the presence of human PTH (2×10^{-7} M), ADH, (1×10^{-6} M), or forskolin (25 μM; which maximally stimulates cAMP production). After the incubation was complete, the entire 24 well plate and the media were frozen over dry ice and ethanol, and then thawed at room temperature. Freeze/thawing was repeated four times, by which time the cells had lifted off the plastic. The cellular debris and media were dried in a vacuum oven, the pellet was resuspended (in a cAMP assay buffer), and the samples were centrifuged for 15 minutes in a microfuge. Then, cAMP levels were determined with a commercially available [^{125}I] cAMP assay kit (Amersham Corporation, Arlington Heights, Illinois, USA) according to the manufacturer's instructions (N = 6 per treatment).

Membrane transport studies. Proximal tubular luminal glucose transport is both Na transport dependent and inhibited by phlorizin [28, 29]. To further assess the origin of the HK-2 cell line and its functional integrity, alpha-methyl glucopyranoside (AMG) cell uptake, a marker of glucose transport [30], was assessed in the presence and absence of NaCl and with phlorizin exposure [31–33]. In brief, 1 × 10^5 cells were plated into each well of a six well plate. Six days later, when the cells were near-confluence, the growth medium was removed and the cells were washed twice in a solution containing 137 mEq NaCl, 5.4 mEq KCl, 2.8 mEq $CaCl_2$, 1.2 mEq $MgSO_4$, and 10 mM Hepes-Tris buffer, pH 7.4. The monolayers were then incubated in 1 ml of the above medium supplemented with alpha-[^{14}C] AMG (0.4 Ci/ml; Amersham Corp.). For the determination of Na^+-independent solute transport, 137 mEq N-methyl-D-glucamine were substituted for the NaCl in the incubation medium. The effect of phlorizin on AMG uptake was determined by the addition of 1.0 mM phlorizin to the Na^+ uptake medium. After 5 or 15 minutes of incubation, AMG transport was terminated by removing the incubation medium and washing the cells four times in an ice cold solution consisting of 137 mEq NaCl and 20 mM Hepes-Tris (pH 7.4). The culture plate was inverted and air-dried and the cells were solubilized in 1 ml of 0.5% Triton X-100. Two hundred microliter aliquots were placed in scintillation fluid (Liquiscent; National Diagnostics, Inc., Advanced Applications Institute, Inc., Sommerville, New Jersey, USA) to determine the amount of accumulated radioactivity (N = 3 for each experiment at each time point).

Assessment of the HK-2 cell line as a tool for studying PTC injury/repair

It has been demonstrated using freshly isolated rat proximal tubular segments that H_2O_2 cytotoxicity is blocked by iron chelation (deferoxamine) but not by hydroxyl radical scavenger therapy [34]. The following experiment attempted to reproduce these findings with the HK-2 cell line, helping to validate its potential utility for studying PTC injury/repair. HK-2 cells were plated in a 96-well flat bottom microtiter plate at a density of 1000 cells/well in a volume of 200 μl of supplemented K-SFM and incubated with 5% CO_2. After 24 hours, the following groups were established: (1) control incubation (no addition); (2) H_2O_2 addition (17 mM); (3) 150 μM deferoxamine addition (for iron chelation); (4) 100 μM Na benzoate addition (for hydroxyl radical scavenging); (5) H_2O_2 + deferoxamine; and (6) H_2O_2 + Na benzoate (N = 7 wells/group). The cells were incubated for four days and then quantified using the MTT assay, as previously described [35]. This assay is based on the principle that MTT (3-[4,5-dimethylthiazol-2-y1]-2,5 diphenyltetrazolium bromide; Sigma Chemicals) is actively transported by respiring mitochondria which leads to intramitochondrial formazin crystal precipitation which can be quantified by ELISA technology. The amount of precipitation reflects the mitochondrial mass, and hence, provides an index of cell number [36, 37]. After completing the four day incubation, the cell media were removed from the wells and replaced with 60 μl of 0.83 mg/ml MTT in culture medium. The plate was incubated at 37°C for 30 minutes, the MTT solution was removed and the precipitated formazin crystals were dissolved in 100 μl DMSO for 60 seconds on a plate shaker. The absorbance was measured on an ELISA reader (Bio Tek Instruments, Burlington, Vermont) at 570 nm using a 630 nm reference wavelength. Serial dilutions of HK-2 cells (10,000, 5,000, 2,500, 1,250, 625, 312 cells per well) indicated a direct relationship between cell number and absorbance (0.073, 0.039, 0.020, 0.010, 0.001, 0.001, respectively) for wells containing greater than 1,000 cells per well. Therefore, an increase in absorbance over time indicates an increase in cell number, whereas a decrease in absorbance indicates a decrease in cell number.

HPV 16 E6/E7 detection

PCR amplification was used to prove that the HPV 16 E6/E7 viral construct had, indeed, been incorporated into HK-2 genomic DNA. To this end, a confluent layer of HK-2 cells was treated with trypsin, washed twice in HBSS, and the cell pellet (containing 1 × 10^6 cells) was transferred to a 500 μl tube. Genomic DNA was extracted with 40 μl of 50 mM NaOH and incubated at 95°C for 10 minutes. The two primers used in the PCR amplification were oligomers derived from the HPV 16 genome [18]. The first primer was a 25 mer, extending from base pair 253 to 277, 5'-ATGCATAG TATATAGAGA TGGAAT. The second primer was the complimentary strand to base pairs 880 to 859, 5'-CTGCAGGATC AGCCATGGTAGA. The amplification was done in a solution composed of 5 μl buffer (500 mM KCl, 1% Triton X-100, 100 mM Tris, pH 9), 5 μl of 2 mM dNTP, 5 μl each of the two primers, 0.1 μl Taq-Polymerase (5000 U/ml), 25 μl of distilled water and 5 μl of the sample DNA from 10^5 cells. A clone of B cells, transformed with EBV virus instead of E6/E7, was used as a negative control for the PCR

reaction. One hundred picograms of pLXSN 16 E6/E7 vector was used as a positive control. Genomic DNA from the untreated donor kidney was also tested. The amplification was done in a PCR machine (Perkin Elmer, Norwalk, Connecticut, USA), and involved 30 cycles (denaturation for 60 seconds at 94°C, annealing for 60 seconds at 55°C, and extension for 120 seconds at 72°C). PCR products were run for two hours on a 2% agarose gel, and stained with ethidium bromide.

Analysis of clonality

Southern analysis. DNA extracted from 10^7 HK-2 cells as described above was digested with ECO RI. Digested DNA (10 μg) was run on a 0.5% agarose gel, transferred and cross linked to a nylon filter. The blotted DNA was prehybridized in 50% foramide at 42°C for eight hours, and then hybridized to a ^{32}P-labeled DNA fragment complementary to HPV 16 E6/E7 (nucleotides 56 to 875), washed two times with 2 × SSPE/0.5% SDS at room temperature for 20 minutes, followed by a stringent wash of 0.1 × SSPE/0.1% SDS at 65°C for one hour. The film was exposed at −80°C for three days.

Fluorescence in situ *hybridization (FISH).* HK-2 cells were recovered from tissue culture flasks after treatment with trypsin, fixed in methanol:acetic acid (1:3) and dropped onto clear glass slides. The slides were denatured through an ethanol series and air dried. Hybridization mix (10 μl) containing 20 μg of biotin labeled pLYSN probe, and 5 μg salmon sperm DNA were denatured at 70°C for five minutes, applied to slides, coverslipped, sealed with rubber cement, and incubated overnight at 37°C in a moist chamber. The slides were washed in 50% foramide then 2× SCC at 42°C (5 min ×3). Detection of the labeled probe was done using Fluorescein-Avidin (Vector), 5 μg/ml, followed by amplification with biotinylated goat anti-avidin, 5 μg/ml (Vector; Burlingame, California, USA). Nuclei were counterstained with 0.25 μg/ml 4,6-diamidino-2-phenylindole-dihydrochloride(DAPI) in 1,4-Dazabicyclo (2,2,2) octane antifade (Eastman Kodak, Rochester, New York, USA).

Analysis of these cells was done using a Nikon fluorescence microscope in the laboratory of Dr. Donald VanDevanter at the Swedish Medical Center Tumor Institute. Pseudocolor fluorescence *in situ* hybridization images were generated by digitally capturing individually excited chromophores in black and white using a Nikon Optiphot-2 epifluorescence microscope coupled to a charge coupled device camera and a SAMBA 4000 (Imaging products International, Chantilly, Virginia, USA) imaging system. Exact registration of individually excited chromophores was achieved by using individual chromophore excitation filters (DAPI, FITC, or Texas Red; Chroma Corporation) carried on an externally driven filter wheel coupled with a triple band pass dichroic and barrier filter set. Individual grey-levels were adjusted for background (by substraction of a blank field) and then by linear rescaling when appropriate using the SAMBA 4000 system.

Pseudocolor images were generated by first subtracting black and white localization signals (either FITC or Texas Red) from black and white counterstain images and then transferring all raw black and white images from the SAMBA 4000 system to the program Photostyler 1.1a (Aldus Corporation, Seattle, Washington, USA), where they were combined and saved as color RGB (red, green, and blue channel) files using the TIFF format. These color TIFF files were then transferred to the Macintosh-based program Photoshop 2.5 (Adobe Systems, Mountain View, California, USA), from which they were printed as hard copies using a Phaser IISD color printer (Tektronix, Beaverton, Oregon, USA).

Results

Culture characteristics of HK-2 cells

Addition of human proximal tubular segments to supplemented K-SFM produced a cell monolayer in approximately one week which, by phase contrast microscopy, demonstrated cuboidal morphology, contact inhibition and dome formation characteristic of cultured tubular epithelial cells. After exposure to the replication defective retrovirus, multiple clones were established (Fig. 2A) and one, designated HK-2, was selected for repetitive expansion (as well as storage at liquid N_2 temperature). These cells have grown continuously as a monolayer for more than one year in supplemented K-SFM. Growth of HK-2 cells is dependent on the presence of EGF, with the optimal concentration between 10 and 20 ng/ml. However, HK-2 cells will not grow in methylcellulose, soft agar, or suspension, regardless of EGF concentration. They will grow, albeit at a much reduced rate, in glucose-free K-SFM, indicating that their viability is not extracellularly glucose dependent.

Electron microscopy reveals HK-2 cells form junctional complexes and microvilli (Fig. 2B) resembling those observed in primary cultures of PTC [38–41]. Electron microscopy suggested that HK-2 cells form glycogen (Fig. 3), the presence of which was confirmed by the observation that HK-2 cells contain intracellular, periodic acid Schiff positive, deposits which can be removed by pretreating the cells with α amylase [42].

Enzyme markers of proximal tubular cells

As shown in Figure 2C, confluent HK-2 monolayers stained for alkaline phosphatase. However, the intensity of the staining was variable, with areas of intense blue staining being separated by areas of less intense, or no, staining. The proportion of alkaline phosphatase positive cells varied with both the age and confluency of the culture, as reported previously for primary rabbit PTC cultures [38]. In contrast, all HK-2 cells consistently demonstrated uniform staining for GGT (Fig. 2D), LAP (Fig. 2F), and acid phosphatase (not depicted).

Immune cytochemistry

A proximal tubular origin of the HK-2 cell line, strongly suggested by the above histochemistry results, was also supported by the immune cytochemistry studies. The cells stained positively for cytokeratin, an intermediate filament family protein which is characteristic of epithelial cells [41, 43–45] (Fig. 2E). Vimentin staining (typical for cells of mesenchymal origin, but also observed in expanding cultured epithelial cells) [46], was seen (not shown). Anti- $\alpha_3\beta_1$ integrin stained the cells in a pericellular pattern (Fig. 2G). Since this integrin (which mediates cell adhesion to basement membranes, thereby allowing them to undergo morphogenesis and differentiation) is widely expressed in epithelial tissue/cultured epithelial cells [47], its production by HK-2 cells further supports its epithelial origin.

In contrast, HK-2 cells did not stain for factor VIII-related antigen, an endothelial cell marker (Fig. 2H). Similarly, the

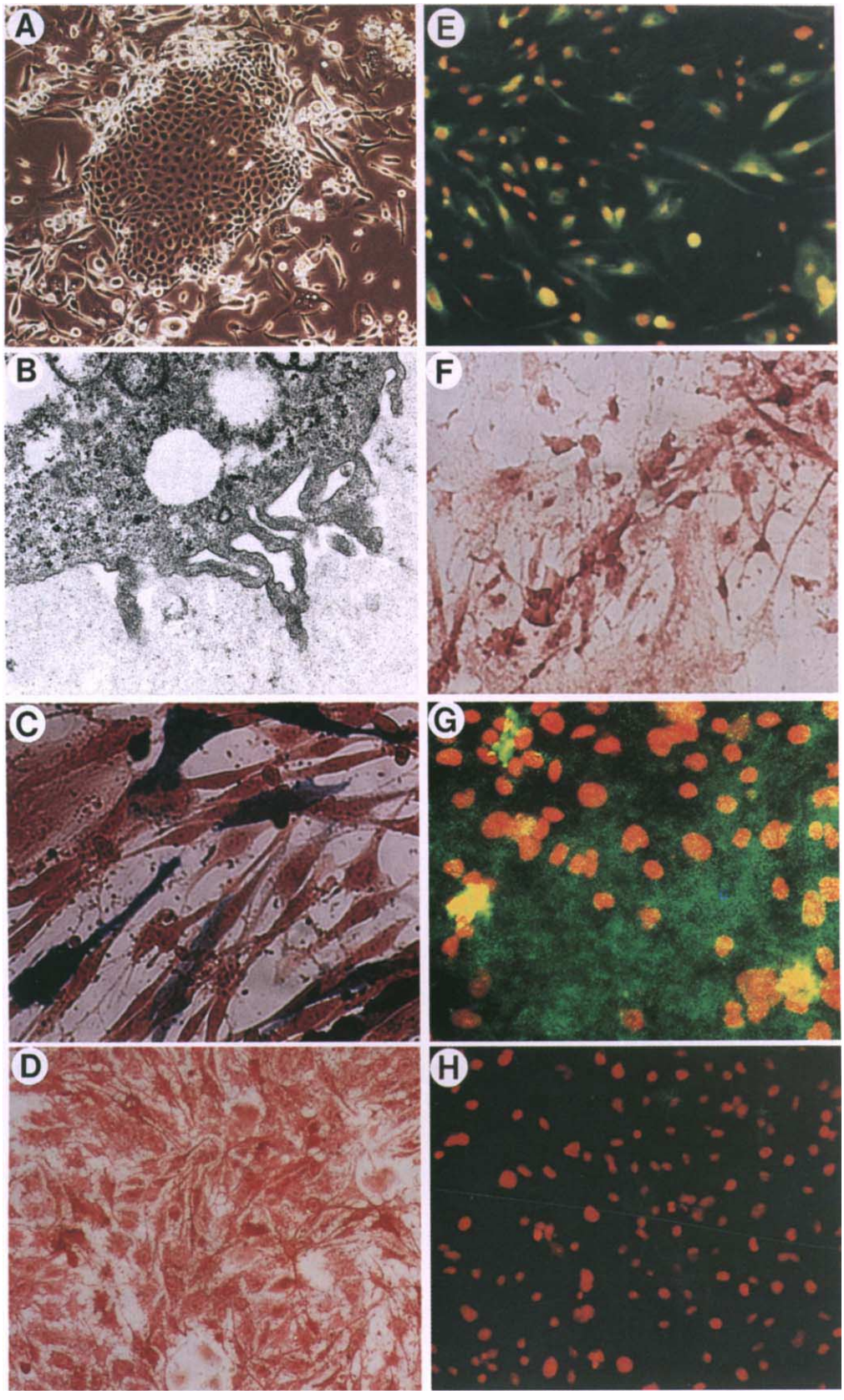

Fig. 2. *Morphology, cytochemistry, and immune cytochemistry studies.* (**A**) Example of a growing clone of transduced proximal tubular cells. (**B**) Electron microscopy (8000×) of HK-2 cell demonstrating microvilli reminiscent of brush border; (**C, D**) histochemical demonstration of alkaline phosphatase and gamma glutamyltranspeptidase, respectively, in HK-2 cells. (**E**) Immune cytochemical demonstration of cytokeratin; (**F**) histochemical demonstration of leucine aminopeptidase. (**G**) Immune cytochemical demonstration of $\alpha_3\beta_1$ integrin in HK-2 cells; and (**H**) negative staining for factor VIII-related antigen (nuclei stained positive with propidium iodide). Publication of this figure in color was made possible by a grant from Amgen Corporation, Thousand Oaks, California, USA.

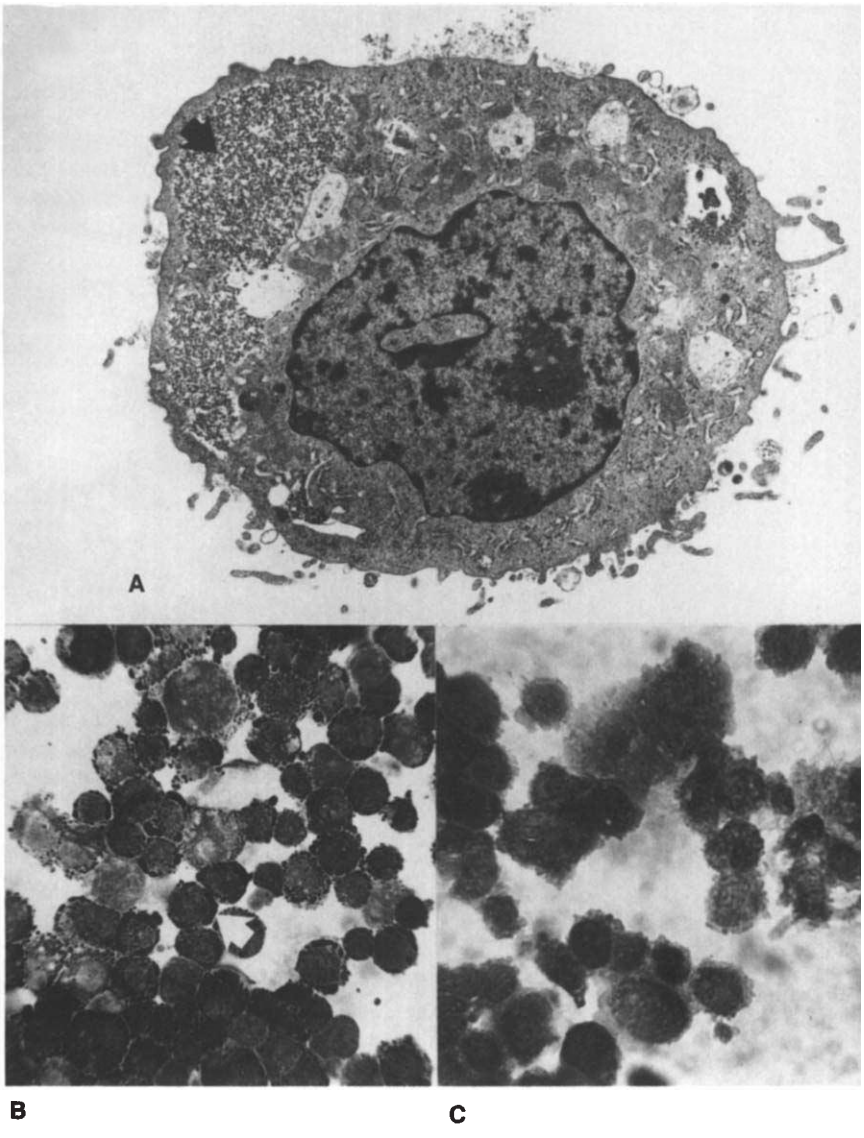

Fig. 3. *Top: HK-2 cell in suspension (after trypsin digestion) demonstrating large granular deposits (arrow) characteristics of glycogen.* These cells demonstrated intracellular black (PAS positive) deposits (bottom left), again consistent with glycogen (arrow). Pre-treatment of the cells with α amylase prior to PAS application prevented their staining, confirming the presence of glycogen.

cells did not stain with antibodies for CALLA endopeptidase or 6.19 (a marker for fibroblasts, adipocytes, and endothelial cells) [48]. Thus, these findings are consistent with an HK-2 cell tubular origin.

Functional characteristics of HK-2 cells

Adenylate cyclase activity. As depicted in Figure 4, HK-2 cells developed an approximate 50% increase in cAMP concentrations in response to PTH addition. Conversely, no cAMP increment resulted from ADH exposure. This differential response supports the cell's proximal tubular origin and indicates an intact receptor-activated enzyme system [25–27]. Forskolin increased cAMP by over 20-fold, serving as a positive control.

AMG transport. AMG transport was extracellular Na^+ dependent since removing Na^+ from the buffer (N-methyl-D-glucamine substitution) depressed its uptake by 57% (assessed at both 5 and 15 min; Fig. 5). Phlorizin, an inhibitor of Na^+ dependent glucose transport, decreased AMG uptake by 70% and 80% at 5 and 15 minutes, respectively. Since Na^+ dependent sugar transport is unique to the proximal tubular apical membrane, the above findings indicate that: (a) the HK-2 cells are of proximal tubular origin; and (b) that they retain this BBM-associated transport system.

H_2O_2 mediated cytotoxicity: Effect of iron chelation/hydroxyl radical scavenger therapy

As shown in Figure 6, the MTT assay could be used to quantify HK-2 cell proliferation and their response to H_2O_2 addition in the presence and absence of potential cytoprotective agents. H_2O_2 markedly inhibited HK-2 proliferation (undoubtedly due to H_2O_2-induced cell killing). DFO almost completely blocked this cytotoxicity despite the fact that, in the absence of H_2O_2, it exerted a modest anti-proliferative effect ($P < 0.001$; probably due to decreased iron availability, necessary for normal proliferation). Na benzoate did not mitigate H_2O_2 cytotoxicity, suggesting hydroxyl radical-independent cell killing. That these findings are exactly analogous to those observed

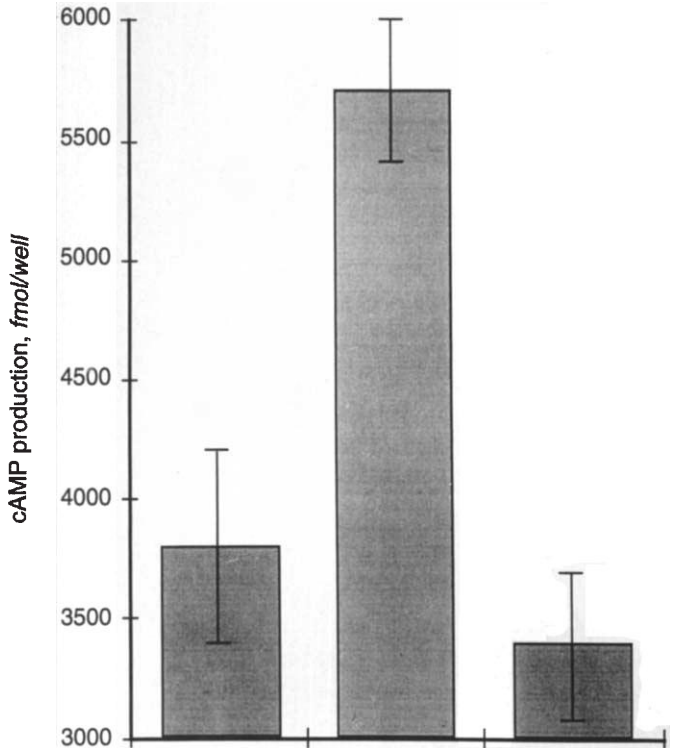

Fig. 4. *Biochemical determination of adenylate cyclase activity, as assessed by cAMP concentrations.* PTH, but not ADH, induced a significant increase in cAMP over control values after a 20 minute incubation ($P < 0.03$; paired Student's *t*-test; Bonferroni correction). Forskolin raised cAMP 20-fold over control values (results not depicted). Values = absolute amounts of cAMP per incubation well (N = 6/treatment).

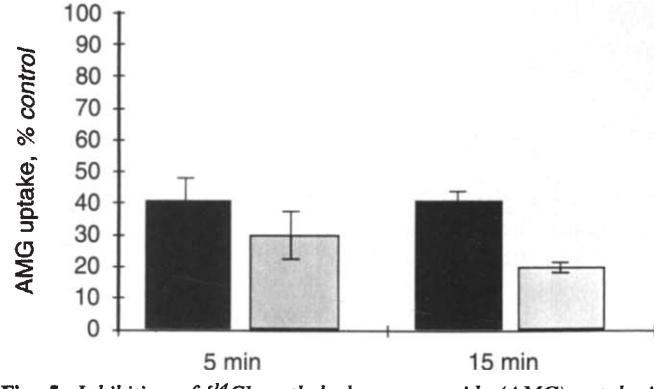

Fig. 5. *Inhibition of [^{14}C] methyl glucopyranoside (AMG) uptake induced by removing Na^+ (No Na^+) or adding phlorizin to HK-2 cells.* Values given = % inhibition, compared to co-incubated controls (that is, in the presence of Na^+/no phlorizin; $N = 6$ each). Both Na^+ removal and phlorizin addition significantly inhibited AMG uptake at both 5 and 15 minutes (overall, $P < 0.03$ for each; paired Student's *t*-test, Bonferroni correction).

with freshly isolated rat proximal tubular segments [34] suggests that: (a) HK-2 cells retain at least some of the injury responses observed with freshly isolated PTC; and (b) the HK-2

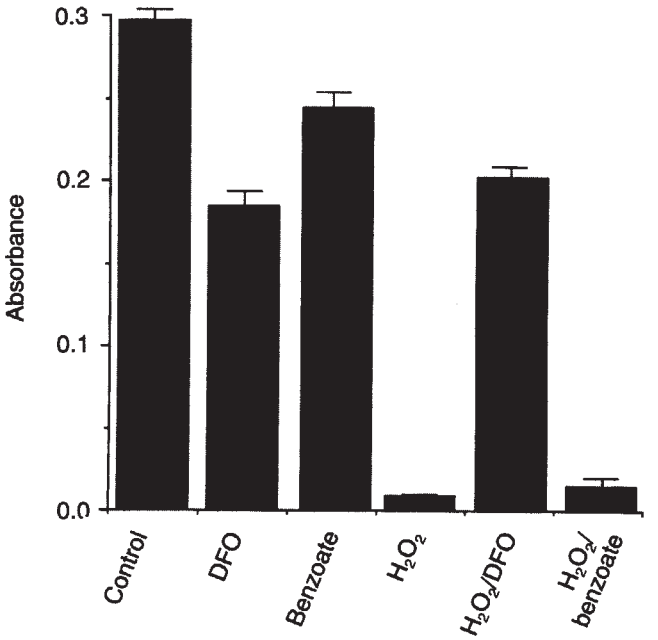

Fig. 6. *HK-2 cell proliferation, as assessed by the MTT assay.* H_2O_2 prevented cell proliferation, a result which was mitigated by deferoxamine (DFO; $P < 0.001$ vs. H_2O_2 alone) but not by benzoate therapy. DFO's protective effect occurred despite the fact that it, by itself, exerted an anti-proliferative effect ($P < 0.001$ vs. controls). Benzoate also decreased HK-2 proliferation ($P < 0.001$ vs. controls). Statistics by unpaired Student's *t*-test with Bonferroni correction.

cell line has potential utility for studying mechanisms of PTC injury and repair.

Detection of HPV 16 E6/E7 DNA; Assessments of clonality

The PCR products amplified from the plasmid containing the E6/E7 viral construct yielded a discreet band, slightly larger than 603bp. This is in agreement with the expected size of the PCR product amplified from intact HPV, which should be 627bp. Likewise, the PCR products from the HK-2 genomic DNA resulted in an identical band, indicating that the HK-2 cells contain the E6/E7 gene construct. This band was not evident in the control lanes. The E6/E7 gene construct was also not detected by PCR in untreated tissue obtained from the donor kidney (data not shown). The results of the PCR amplification are shown in Figure 7.

Figure 8 shows nonamplified HK-2 genomic DNA, cut with ECO RI, blotted and probed for the E6/E7 insert. The presence of two bands suggests that either the HK-2 cell line is derived from two cells each with a single viral insert, or from a single cell with two viral inserts. FISH detection of E6/E7 in individual cells indicated that HK-2 cells predominantly contained two inserts (Fig. 9), lending support to the second hypothesis, that is, that the HK-2 cell line is clonally derived.

Discussion

To gain mechanistic insights into PTC physiology, injury, and repair, investigators have increasingly utilized isolated cell systems, thereby avoiding the complexities inherent to whole animal or whole organ experiments. Freshly isolated rat or rabbit proximal tubular segments have been widely used in this

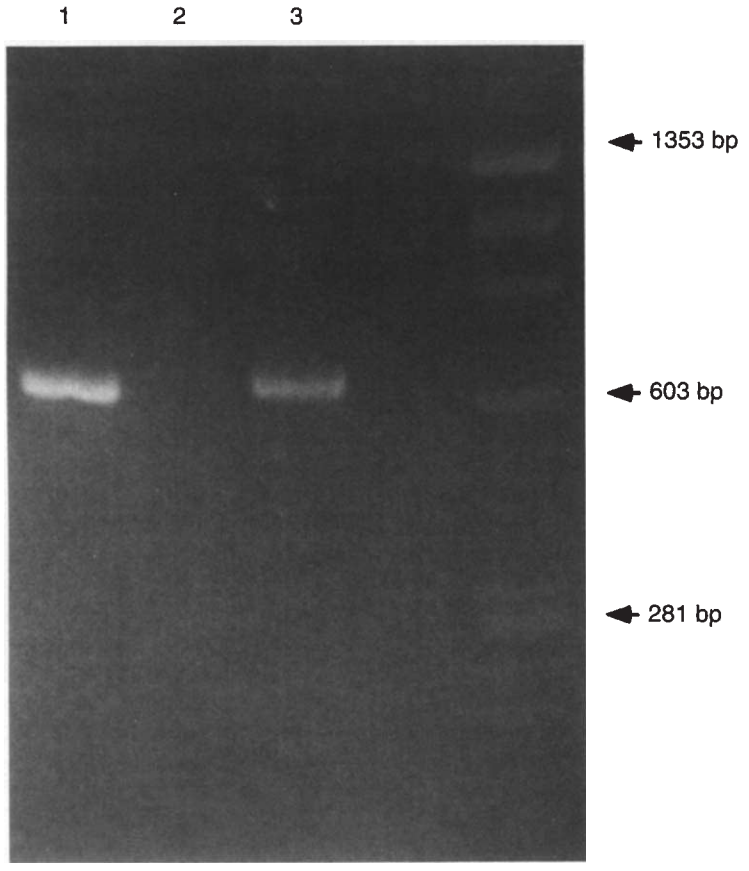

Fig. 7. *Ethidium bromide stained agarose gel of PCR amplification products from E6/E7 region primers.* Lane 1 is a positive control from a plasmid with the E6/E7 insert, showing the expected 672 bp product also found in HK-2 cells (lane 3). Lane 2 contains a negative control showing no amplification from B cell DNA. Molecular weight markers (X174/HaeIII) are on the right.

regard. However, during their isolation, substantial cell injury results, limiting experimentation to only a few hours because of progressive cell death. Furthermore, the resulting data must be interpreted with caution since this background damage may either alter, or make possible, the results obtained. Because of these problems, primary PTC cultures are an attractive alternative: since these cells are metabolically stable, neither background injury nor rigid time constraints are imposed. However, the need for repeated isolation and confirmation of preparation uniformity are major limitations. Furthermore, if human proximal tubular cells are desired, lack of ready tissue availability represents a formidable problem.

To circumvent these difficulties, transformed human embryonic tubular cell lines have been established. For example, in 1977, Graham et al [4] reported the transformation of human embryo kidney (HEK) cells by transfection with sheared fragments of adenovirus type 5. The resulting cells appeared to be of epithelial origin since, by direct immunofluorescence, they expressed keratin-associated antigens. However, these cells are also tumorigenic in mice and exhibit foci formation and growth in soft agar, all characteristics of highly transformed cells. Other HEK cell lines are ST-1i and ST-4i, generated by transfection with plasmids encoding for SV40 large T antigen and SV40 small T antigen, respectively [1]. These lines express a number of proximal tubular enzymes (such as LAP, GGT, maltase), suggesting that they have retained differentiated properties. However, since they are derived from embryonic tissues, they may not be ideal for studying mechanisms of adult PTC physiology and disease. Lastly, Nanus et al [3] reported an HEK cell line transformed with an *src*-containing retrovirus. However, these cells exhibit deletion of chromosomal region 3p14-21 (a defect commonly seen in human renal tumors) and they are highly transformed, as evidenced by altered morphology, loss of contact inhibition, and tumorigenicity. Thus, each of these changes could limit their utility for broad-based experimentation.

Given the above considerations, the goal of the present study was to establish an immortalized adult human kidney proximal tubular cell line, while retaining relatively normal phenotypic expression. To achieve this goal, a primary human proximal tubular cell culture was transformed with the HPV 16 E6/E7 genes, resulting in a cell line which has survived for more than one year in culture and which can withstand liquid nitrogen freeze/thawing. In addition, its growth is anchorage dependent and requires EGF, indicating that the line is not truly autonomous, but rather, retains some normal regulatory controls. Of note, similar results have been described for other E6/E7 transduced epithelial cells [10]. HPV 16 E6/E7 transformation is believed to occur because the E6/E7 proteins interact with two important negative regulators of cell proliferation: E6 binds to and induces degradation of the P53 protein and E7 inactivates the retinoblastoma tumor-suppressor gene product, p105-RB [8, 15, 17, 18]. By so doing, E6 and E7 proteins functionally eliminate two negative regulators of cell proliferation, allowing an immortalized cell line to develop.

Although the utility of this newly established human cell line

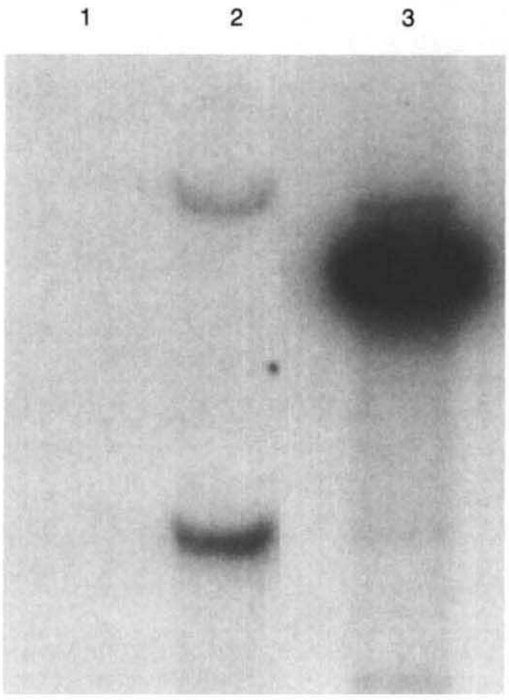

Fig. 8. *Southern analysis of genomic DNA digested with ECO RI, electrophoresed on an agarose gel and transferred to nylon.* The blot was hybridized to a ^{32}P-labeled DNA fragment containing nucleotides 56-875 of the HPV 16 E6/E7 genes. Lane 1 contains DNA from noninfected fibroblasts, lane 2 contains HK-2 DNA, and lane 3 contains plasmid pLXSN 16 E6/E7.

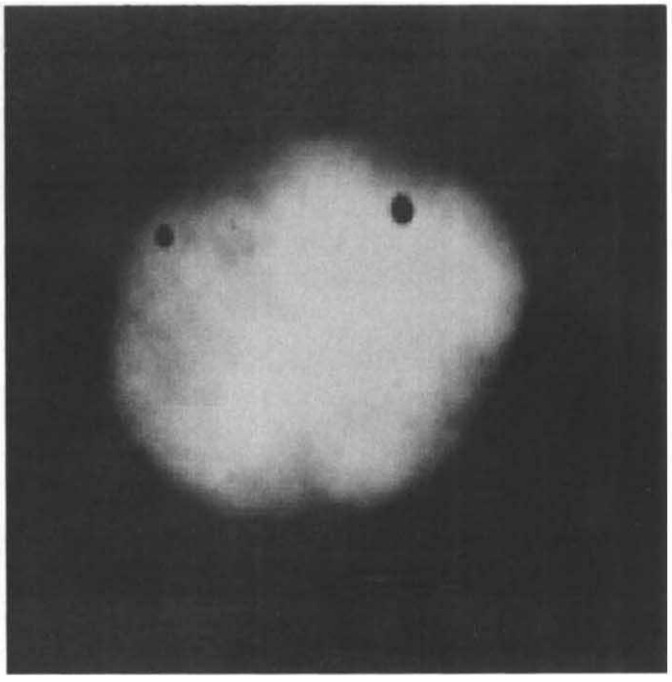

Fig. 9. *Fluorescence* in situ *hybridization image of an HK-2 cell interphase nucleus probed for the E6/E7 insert.* Two distinct signals detected in this nucleus suggest two viral insertions. This image was contributed by Dr. Donald VanDevanter at the Swedish Medical Center Tumor Institute, Seattle, Washington, USA.

remains to be elucidated, its characteristics documented to date suggest great potential for the following reasons: (1) HK-2 cells possess typical BBM-associated enzymes (acid and alkaline phosphatase, LAP, GGT), indicating a proximal tubular origin and suggesting normal phenotypic expression; (2) HK-2 cells are recognized by antibodies directed against cytokeratin, vimentin, fibronectin, and $\alpha_3\beta_1$ integrin, but not against factor VIII, CALLA endopeptidase and 6.19 antigen, further supporting a proximal tubular origin and a relatively normal phenotype; (3) the HK-2 cells retain functional characteristics of *in vivo* proximal tubules and primary PTC cultures; its adenylate cyclase content is stimulated by PTH, but not by ADH, and Na^+ dependent glucose uptake is present (indicating that at least some receptor-activated hormonal responses and characteristic transport mechanisms are intact). (4) Although HK-2 cells almost certainly possess glycolytic activity (as do all previously cultured tubular cells), they can be maintained in glucose-free medium; (5) they are capable of gluconeogenesis, as evidenced by their ability to make and store glycogen; (6) HK-2 cells are anchorage dependent and require EGF, suggesting that these cells are immortalized, rather than highly transformed; (7) the present H_2O_2 cell injury experiments exactly reproduce results previously obtained with freshly isolated proximal tubular segments, indicating their potential utility for the study of cell injury and repair; and (8) that HK-2 is derived from adult human kidney represents a major potential advantage over currently available animal or human embryonic-derived cell lines.

In summary, the present report describes a novel, immortalized adult human proximal tubular cell line, HK-2, induced by transduction with the HPV 16 E6/E7 genes. It appears to be derived from a single cell based on Southern and FISH analysis, and it has grown continuously in culture for over one year retaining functional and morphologic characteristics of normal adult human proximal tubular epithelium. Given these characteristics, HK-2 cells could prove to be a powerful new tool for the study of PTC physiology/pathophysiology and mechanisms of cell injury and repair.

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References

1. Abcouwer S, Robinson PS, Goochee CF, Crow MT: Generation of human embryonic kidney cells with extended *in vitro* life span through viral oncogene transfection. *Bio/Technol* 7:939–946, 1989
2. Chang SE: *In vitro* transformation of human epithelial cells. *Biochim Biophys Acta* 823:161–194, 1986
3. Nanus, DM, Lynch SA, Pulivarthi HR, Anderson SM, Jhanwar SC, Albino AP: Transformation of human kidney proximal

tubule cells by a *src*-containing retrovirus. *Oncogene* 6:2105–2111, 1991
4. Graham FL, Smiley J, Russell WC, Nairn R: Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J Gen Virol* 36:59–72, 1977
5. Robinson PS, Goochee CF: Kidney-specific enzyme expression by human kidney cell lines generated through oncogene transfection. *J Cell Physiol* 148:54–59, 1991
6. Howley PM: Role of human papillomaviruses in human cancer. *Cancer Res* 51:5019s–5022s, 1991
7. Vousden K: Human papillomavirus oncoproteins. *Semin Cancer Biol* 1:415–424, 1990
8. Hawley-Nelson P, Vousden KH, Hubbert NL, Lowy DR, Schiller JT: HPV16 E6 and E7 proteins cooperate to immortalize human foreskin keratinocytes. *EMBO J* 8:3905–3910, 1989
9. Halbert CL, Demers GW, Galloway DA: The E7 gene of human papillomavirus type 16 is sufficient for immortalization of human epithelial cells. *J Virol* 65:473–478, 1991
10. Pirisi L, Creek KE, Doniger J, DiPaolo JA: Continuous cell lines with altered growth and differentiation properties originate after transfection of human keratinocytes with human papillomavirus type 16 DNA. *Carcinogenesis* 9:1573–1579, 1988
11. Dürst M, Dzarlieva-Petrusevska RT, Boukamp P, Fusenig NE, Gissmann L: Molecular and cytogenetic analysis of immortalized human primary keratinocytes obtained after transfection with human papillomavirus type 16 DNA. *Oncogene* 1:251–256, 1987
12. Pirisi L, Yasumoto S, Feller M, Doniger J, DiPaolo JA: Transformation of human fibroblasts and keratinocytes with human papillomavirus type 16 DNA. *J Virol* 61:1061–1066, 1987
13. Phelps WC, Yee CL, Münger K, Howley PM: The human papillomavirus type 16 E7 gene encodes transactivation and transformation functions similar to those of adenovirus E1A. *Cell* 53:539–547, 1988
14. Storey A, Pim D, Murray A, Osborn K, Banks L, Crawford L: Comparison of the in vitro transforming activities of human papillomavirus types. *EMBO J* 7:1815–1820, 1988
15. Münger K, Werness BA, Dyson N, Phelps WC, Harlow E, Howley PM: Complex formation of human papillomavirus E7 proteins with the retinoblastoma tumor suppressor gene product. *EMBO J* 8:4099–4105, 1989
16. Levine A, Momand J, Finlay CA: The p53 tumor suppressor gene. *Nature* 351:453–456, 1991
17. Scheffner M, Werness BA, Huibregtse JM, Levine AJ, Howley PM: The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. *Cell* 63:1129–1136, 1990
18. Blanton RA, Coltrera MD, Gown AM, Halbert CL, McDougall JK: Expression of the HPV16 E7 gene generates proliferation in stratified squamous cell cultures which is independent of endogenous p53 levels. *Cell Growth Differ* 3:791–802, 1992
19. Zager RA, Schimpf BA, Gmur DJ: Physiological pH effects on posthypoxic proximal tubular injury. *Circ Res* 72:837–846, 1993
20. Miller AD, Bender MA, Harris EAS, Kaleko M, Gelinas RE: Design of retrovirus vectors for transfer and expression of the human β-globin gene. *J Virol* 62:4337–4345, 1988
21. Rutenburg AM: Histochemical demonstration of alkaline phosphatase using naphthol AS phosphate: Comparison with the substituted naphthol AS phosphates. *Annal D Histochimie* 11:139–149, 1966
22. Rutenburg AM, Kim H, Fischbein JW, Hanker JS, Wasserkrug HL, Seligman AM: Histochemical and ultrastructural demonstration of γ-glutamyl transpeptidase activity. *J Histochem Cytochem* 17:517–526, 1969
23. Burstone MS: Histochemical comparison of napthol AS phosphates for the demonstration of phosphatases. *J Natl Cancer Inst* 20:601–616, 1958
24. Nachlas MM, Monis B, Rosenblatt D, Seligman AM: Improvement in the histochemical localization of leucine aminopeptidase with a new substrate L-leucyl-4-methoxy-2 naphthylamide. *J Biophys Biochem Cytol* 7:261–264, 1960
25. Morel F: Sites of hormone action in the mammalian nephron. *Am J Physiol* 240:F159–F164, 1981
26. Marx SJ, Fedak SA, Aurbach GD: Separation and characterization of a hormone-responsive renal plasma membrane reaction. *J Biol Chem* 24:3913–3918, 1972
27. Chung SD, Alavi N, Livingston D, Hiller S, Taub M: Characterization of primary rabbit kidney cultures that express proximal tubule functions in a hormonally defined medium. *J Cell Biol* 95:118–126, 1982
28. Beck JC, Sacktor B: The sodium electrochemical potential-mediated uphill transport of D-glucose in renal brush border membrane vesicles. *J Biol Chem* 253:5531–5535, 1978
29. Kinne R, Murer H, Kinne-Safran E, Thees M, Sachs G: Sugar transport by renal plasma membrane vesicles. Characterization of the systems in the brush-border microvilli and basal-lateral plasma membranes. *J Membr Biol* 21:375–395, 1975
30. Inman WH, Colowick SP: Growth factor stimulation of sugar uptake. *Meth Enzymol* 146:399–402, 1987
31. Malström K, Stange G, Murer H: Identification of proximal tubular transport functions in the established kidney cell line, OK. *Biochim Biophys Acta* 902:269–277, 1987
32. Misfeldt DS, Sanders JM: Transepithelial glucose transport in cell culture. *Am J Physiol* 240:C92–C95, 1981
33. Wilson PD, Anderson RJ, Breckon RD, Nathrath W, Schrier RW: Retention of differentiated characteristics by cultures of defined rabbit kidney epithelia. *J Cell Physiol* 130:245–254, 1987
34. Zager RA: Combined mannitol and deferoxamine therapy for myohemoglobinuric renal injury and oxidant tubular stress. *J Clin Invest* 90:711–719, 1992
35. Price P, McMillan TJ: Use of the tetrazolium assay in measuring the response of human tumor cells in ionizing radiation. *Cancer Res* 50:1392–1396, 1990
36. Mossman T: Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J Immunol Meth* 65:55–63, 1983
37. Slater TF, Sawyer B, Strauli UD: Studies on succinate-tetrazolium reductase systems. III. Points of coupling of four different tetrazolium salts. *Biochim Biophys Acta* 77:383–393, 1963
38. Toutain H, Vauclin-Jacques N, Fillastre JP, Mollin JP: Biochemical, functional, and morphological characterization of a primary culture of rabbit proximal tubule cells. *Exp Cell Res* 194:9–18, 1991
39. Kempson SA, McAteer JA, Al-Mahrouq HA, Dousa TP, Dougherty GS, Evan AP: Proximal tubule characteristics of cultured human renal cortex epithelium. *J Lab Clin Med* 113:285–296, 1989
40. Wilson PD, Dillingham MA, Breckon R, Anderson RJ: Defined human renal tubular epithelia in culture: Growth, characterization, and hormonal response. *Am J Physiol* 248:F436–F443, 1985
41. Romero MF, Douglas JG, Eckert RL, Hopfer U, Jacobberger JW: Development and characterization of rabbit proximal tubular epithelial cell lines. *Kidney Int* 42:1130–1144, 1992
42. Luna LG (editor): *Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology* (3rd ed), New York, McGraw Hill, 1968, p. 171
43. Franke WW, Weber K, Osborn M, Schmid E, Freudenstein C: Antibody to prekeratin: Decoration of tonofilament-like arrays in various cells of epithelial character. *Exp Cell Res* 116:429–445, 1978
44. Schlegel R, Banks-Schlegel S, Pinkus GS: Immunohistochemical localization of keratin in normal human tissues. *Lab Invest* 42:91–96, 1980
45. Gigi O, Geiger B, Eshhar Z, Moll R, Schmid E, Winter S, Schiller DL, Franke WW: Detection of a cytokeratin determinant common to diverse epithelial cells by a broadly based crossed reacting monoclonal antibody. *EMBO J* 11:1429–1437, 1982
46. Lazarides E: Intermediate filaments: A chemically heterogeneous, developmentally regulated class of proteins. *Annu Rev Biochem* 51:219–250, 1982
47. Carter WG, Ryan MC, Gahr PJ: Epiligrin, a new cell adhesion ligand for integrin $\alpha_3\beta_1$ in epithelial basement membranes. *Cell* 65:599–610, 1991
48. Frantz CN, Duerst RE, Ryan DH, Gelsomino NL, Constine LS, Gregory PK: Anti-neuroblastoma monoclonal antibodies which do not bind to bone marrow cells. *Prog Clin Biol Res* 175:485–499, 1985