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Short Communication

Long-Term Culture and Immortalization of Epithelial Cells from Normal Adult Human Pancreatic Ducts Transfected by the E6E7 Gene of Human Papilloma Virus 16

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Pancreatic cancer is one of the most lethal cancers in humans. The majority of these cancers arise from the pancreatic duct epithelium. Research into the pathogenesis of pancreatic carcinoma has largely relied on animal models. **In vitro** ***models of pancreatic carcinogenesis using propagable cultured epithelial cells derived from the pancreatic ducts of rats and hamsters have been described. A human model, however, has been nonexistent due to the unavailability of propagable cultured duct epithelial cells derived from normal human pancreas. We report here a reproducible method for the long-term culture of pancreatic duct epithelial cells derived from normal and benign adult human pancreata by infection with a retrovirus containing the E6 and E7 genes of the human papilloma virus 16. One of these cell lines has become immortal and has propagated continuously for more than 20 passages. They remain anchorage dependent in their growth and nontumorigenic in nude mice. These cell lines and the methodology described here to establish them may provide new avenues for*** **in vitro** ***studies of the roles played by duct epithelium in human pancreatic diseases and cancers. (Am J Pathol 1996, 148:1763–1770)***

Pancreatic cancer is one of leading causes of cancer death in the United States and many developed countries.[1,2] Despite significant efforts devoted to the development of animal models of pancreatic carcinogenesis during the last 20 years,[3–5] the etiology and pathogenesis of the human pancreatic cancers remain largely unknown. The majority (90%) of human pancreatic cancers belong to adenocarcinoma of ductal origin.[6] Although tobacco-specific nitrosamines have been implicated as one of the etiological agents of human pancreatic cancer,[7–9] the metabolism and effect of these carcinogens in human pancreatic duct epithelial cells is largely unknown. Based on morphological and molecular studies, it is clear that human pancreatic duct carcinogenesis is a multistage process.[10–15] The preneoplastic nature of hyperplatic and dysplastic human pancreatic duct epithelium has been supported by the common occurrence in these cells of genotypic and phenotypic changes characteristically found in pancreatic duct adenocarcinoma. These include the mutations of the Ki-*ras* and *p53* genes[11–14] and the overexpression of hepatocyte growth factor and the met/hepatocyte growth factor receptor.[15] However, the mechanistic

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Table 1. *Origin of Human Pancreatic Duct Epithelial (HPDE/E6E7) Cell Lines*

Cell line	Patients' characteristics			Number of passages achieved*
	Age (years)	Sex	Primary diagnosis	
HPDE-1/E6E7	75	M	Adenocarcinoma of ampulla of Vater	8
HPDE-4/E6E7	51	F	Acute and chronic pancreatitis	20
HPDE-5/E6E7	54	F	Multiloculated cystadenoma of pancreas	12

M, male; F, female.
*The cells were forzen for storage at these passage numbers.

roles that these biochemical and molecular changes play during the promotion and progession stages of human pancreatic carcinogenesis remain poorly understood. These important questions are difficult to answer in the absence of suitable *in vitro* models. In this paper, we describe a method for achieving the long-term culture and immortalization of epithelial cells that originated from normal human pancreatic ducts. Although these cells have been genotypically altered by infection with a retrovirus vector expressing the E6E7 genes of human papilloma virus (HPV)-16, such non-neoplastic human pancreatic duct epithelial cell lines will remain useful to investigate directly and *in vitro* the multistage pathogenesis of human pancreatic cancers.

Materials and Methods

Duct Explant and Cell Culture

Normal and/or benign pancreatic tissues were obtained from three human pancreatic specimens that had been resected by Whipple's procedure for diseases of the pancreas and duodenum (Table 1). Representative histological sections from the tissue fragments used for primary cultures revealed only pancreatitis or normal pancreatic tissue. Tissue fragments recovered at the end of the explants were also examined histologically and revealed the absence of malignancy.

Grossly visible pancreatic ducts, including main ducts, were isolated by dissection and freed as much as possible from the adjacent acinar tissue. After several washes with ice-cold Mg^{2+}- and Ca^{2+}-free Hanks' balanced salt solution (HBSS; GIBCO-BRL, Grand Island, NY), they were cut into approximately 1-mm-long fragments and subsequently put on to 60-mm tissue culture dishes (Falcon, Becton Dickinson, Mountain View, CA). Some of these dishes had been coated with a thin layer of rat tail collagen gel. The explants were cultured in 2 ml of keratinocyte-serum-free (KSF) medium supplemented with 50 mg/ml bovine pituitary extract and 5 ng/ml epidermal growth factor (GIBCO BRL) at 37°C in a humidified atmosphere containing 5% CO_2. The medium was replaced every 2 to 3 days.

To propagate the monolayer-cultured cells, they were dissociated with the HBSS containing 0.025% trypsin, 0.265 mmol/L EDTA, and 0.5% polyvinylpyrrolidone. After neutralization of the trypsin with KSF containing 0.1% soybean trypsin inhibitor (GIBCO BRL) and 0.1% bovine serum albumin (Böehringer-Mannheim Canada, Dorval, Québec, Canada), the cells were subcultured at 1:4 to 1:8 split ratios.

The PK1, PK8, and PK9 human pancreatic duct adenocarcinoma cell lines were previously established in the laboratory of Dr. Masao Kobari at Tohoku University School of medicine.[16] Soft agar colony assays were performed as previously described.[17]

Infection by Retrovirus

An amphotrophic retrovirus, LXSN16E6E7, containing the E6 and E7 genes of HPV-16 and the neomycin-resistant gene was used to infect the duct epithelial cells in primary cultures. The construction and generation of the retrovirus has been described in detail previously.[18] Infection was carried out soon after a monolayer sheet of cells had formed around the duct fragments, which usually occured approximately 3 to 6 days after the start of explants. Infection was carried out in 2 ml of KSF medium in the presence of 4 μg/ml polybrene (Sigma Chemical Co., St. Louis, MO), and 16 to 18 hours later, the medium was replaced with 5 ml of the fresh KSF medium. The infected cells were subsequently selected by growing them in the presence of 400 μg/ml G-418 (GIBCO BRL). Fresh medium was replaced every 3 days, and passage was made when the cells had reached 80 to 90% confluence. A continuous selection in G-418 was carried out for at least three subcultures. The cells were subsequently expanded and grown in 100-mm culture dishes in 10 ml of medium.

Detection of the HPV E6 and E7 Genes

Genomic DNA was isolated from cultured cells as described previously.[19] The presence of the HPV-16 E6 and E7 genes in the cell lines were studied using polymerase chain reaction (PCR) and Southern blotting.

The PCR was carried out using oligonucleotide primer sets (Research Genetics, Huntsville, AL) for the HPV-16 E6 gene (position 201 to 523; sense, 5′-GCAAGCAACAGTTACTGCG ACGT-3′; antisense, 5′-GCAACAAGACATACATCGACCGG-3′), the HPV-16 E7 gene (position 676 to 818; sense, 5′-GATGGTCCAG CTGGACAAGC-3′; antisense, 5′-GTGCCCA TTAACAGGTCTTC-3′), and the genomic DNA isolated from cell lines after more than five passages as templates. Thirty-five cycles of PCR were carried out in the Thermal Cycler (Perkin-Elmer Corp., Norwalk, CT) with each cycle consisting of denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, and polymerization at 72°C for 2 minutes. The PCR products were electrophoresed in a 1.5% agarose gel and then blotted to a Hybond-N membrane (Amersham, Oakville, Ontario, Canada). To confirm that the PCR products were the E6 and E7 gene fragments, the membrane was hybridized with a ^{32}P-end-labeled internal oligonucleotide probe for the HPV-16 E6 gene (5′-GCTTTTCGGGATTTAT GCATAGT -3′). As the E7 internal oligoprobe is no longer available, the membrane was subsequently also hybridized with a ^{32}P-labeled 604-bp *Kpn*I-*Nde*I fragment of the HPV-16 E6-E7 gene of plasmid p1318.[20]

For hybridization with the E6 oligoprobe, the membrane was prehybridized in 5X SSPE (1X SSPE contained 0.3 mol/L NaCl, 20 mmol/L phosphate buffer, pH 7.4, and 2 mmol/L EDTA), 5X Denhardts', 0.5% sodium dodecyl sulfate (SDS), and 100 mmol/L sodium pyrophosphate for 20 minutes at 58°C. Hybridization was carried out overnight in the same buffer and at 55°C. The membrane was then washed twice with 6X standard saline citrate (1X SSC contained 150 mmol/L sodium chloride, 15 mmol/L sodium citrate, pH 7.0) at room temparature for 20 minutes each and then with 3 mol/L tetramethyl ammonium chloride at room temperature for 10 minutes and at 61°C for 20 minutes. After a final rinse in 6X SSC for 5 minutes at room temperature, the membrane was autoradiographed with Kodak XAR-5 film. Hybridization with the E6-E7 cDNA probe was carried out as described below.

A Southern analysis for stable integration of the E6-E7 gene into the genomic DNA was also carried out. Ten micrograms of DNA was digested with *Eco*RI and then separated in 1% agarose gel in TAE buffer and transferred onto the Hybond-N membrane. After cross-linkage by exposure to ultraviolet light, it was hybridized to the *Kpn*I-*Nde*I fragment of the E6-E7 cDNA, which had been labeled with [^{32}P]dCTP using the random primer labeling kit (Pharmacia Biotech, Piscataway, NJ). Hybridization was carried out overnight in the QuickHyb hybridization solution (Strategene, La Jolla, CA) at 68°C and then washed twice at room temperature in 2X SSC solution containing 0.1% SDS and twice at 60°C in 0.1X SSC solution containing 0.1% SDS. The membranes were then exposed to Kodak XAR film.

Immunohistochemistry

The cells were cultured on tissue culture chamber slides (Miles Laboratories, Naperville, IL). When the cultures were still subconfluent, the cells were fixed with 70% ethanol for 10 minutes at 4°C and then air dried and stored at −20°C. Immunohistochemistry was performed with the avidin-biotin immunoperoxidase technique, using the Histostain broad spectrum kit (Zymed, San Franscisco, CA). Primary antibodies included CAM-5.2 against human cytokeratins 8 and 18 (Becton Dickinson, San Jose, CA), the anti-human cytokeratin 19 antibody (Dako Corp., Carpinteria, CA), and DO7 anti-p53 antibody (Dako). The antibodies were used at the dilutions suggested by the manufacturers.

*Point Mutational Analysis for the Ki-*ras *Gene*

Exons 1 and 2 of the human Ki-*ras* gene were amplified from 250 ng of genomic DNA using the PCR and the human Ki-*ras* amplimers (Clonotech, Palo Alto, CA). The PCR products were dot-blotted onto replicate Hybond-N membranes (Amersham) and then hybridized to the appropriate panels of mutant-specific oligonucleotide sequences for codons 12, 13, and 61 of the human Ki-*ras* gene, as contained in the *ras*-ONCO-LYZER kit (Clonotech). PCR, hybridization, and washing were carried out according to the company's technical instructions.

Tumorigenicity Assay in Nude Mice

Trypsin-dissociated cells were suspended at a concentration of 4×10^6 cells/0.5 ml in the KSF medium containing a 10% volume of basement membrane extract Matrigel (Collaborative Biomedical). Cells (2×10^6) were injected into the dorsal interscapular fat

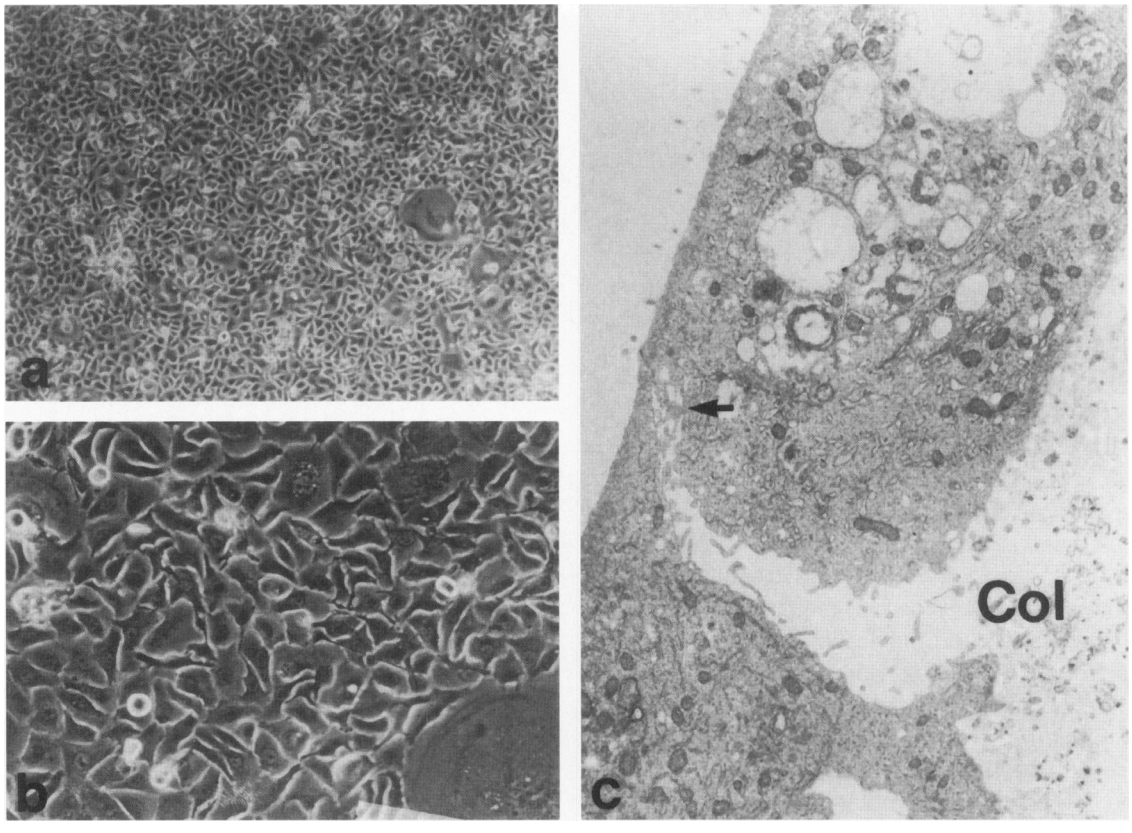

Figure 1. *Representative microscopic appearance of propagable culture human pancreatic duct epithelial cells.* a: *A confluent culture of HPDE4/E6E7 at passage 12.* b: *A higher magnification showing the polygonal epithelial cells growing in a cobblestone pattern.* c: *The ultrastructural appearance of these cells when placed on collagen gel (Col). Note the presence of occasional surface microvilli and lateral interdigitating microvillous processes* (arrow) *mimicking the appearance of duct epithelium* in vivo. *Magnification, ×40* (a); *×100* (b); *and ×8970* (c).

pad of each of the 6-week-old male nude mice (strain *nu/nu*, Charles River Laboratories, Wilmington, MA). Two nude mice were injected with each cell line, and tumor formation was monitored for up to 6 months.

Results

We discovered that, when pancreatic duct fragments were explanted either on tissue culture plastic surface or on collagen gel, 3 to 5 days later, epithelial cells will migrate out of the edges of the tissue and grow to form a sheet of monolayer cobblestone-appearing cells. These cell colonies will continue to expand centrifugally for several more days, and the colony may reach 1 to 2 cm in diameter.

We have previously used the HPV-16 E6-E7 genes to immortalize cultured normal human bronchial epithelial cells.[21] Our previous experience with primary cultures of normal colonic crypt epithelial cells, however, suggested that explanted epithelial cells from the human gastrointestinal tract might not survive long (1 to 2 days) in primary cultures (unpublished results). Hence, to obtain continuously proliferating duct cells, we decided to infect the human pancreatic duct epithelial cells early during primary cultures. This was done usually as soon as monolayer colonies of epithelial cells had formed, 5 to 7 days after the start of duct explants. Infection was only for 24 hours, and the cells were subsequently cultured in the supplemented KSF medium, which was replaced every 3 days. Using this strategy, we obtained propagable epithelial cell lines HPDE-1/E6E7, -4/E6E7, and -5/E6E7 (Figure 1, A and B). One of these cell lines, HPDE-4/E6E7, has undergone crisis at passage 12 and developed an immortal cell line that has been propagated up to 20 passages (Table 1).

These LXSN16E6E7-infected cell lines remained as monolayer culture. The cells demonstrated some pleomorphism in size and shape, although they clearly maintained the polygonal appearance typical of cultured epithelial cells. When these cells were plated on to collagen gels, they demonstrated polarization with surface microvilli, apical junctional complexes, and lateral interdigitations (Figure 1C), fea-

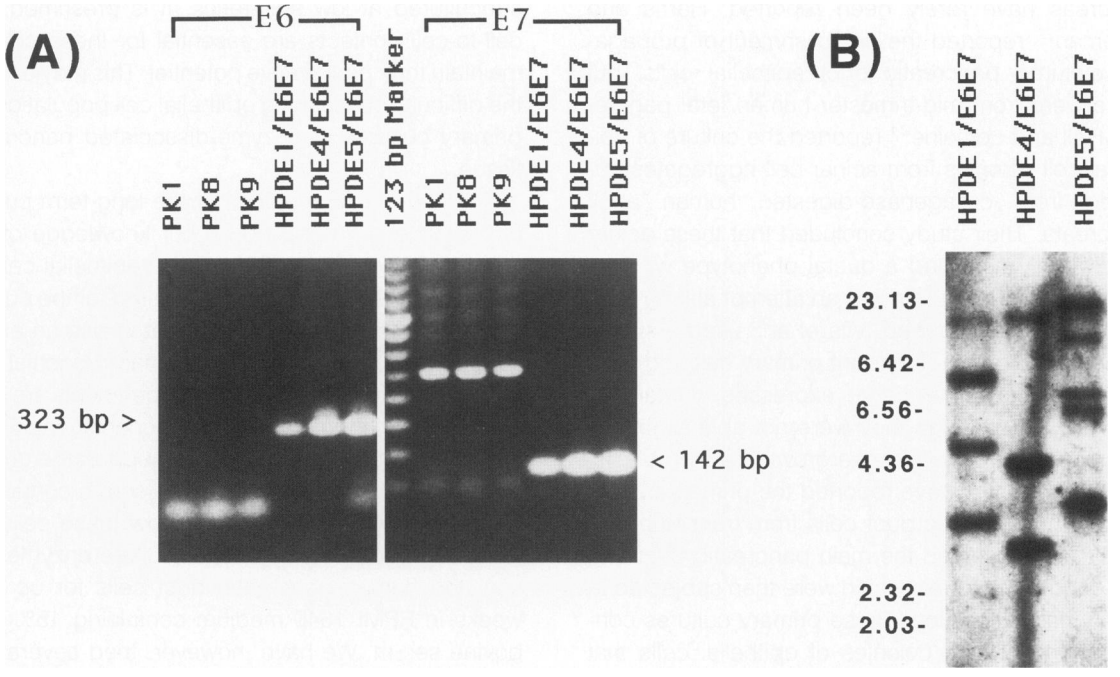

Figure 2. *The detection of the HPV-16 E6 and E7 genes in the genomic DNA.* A: *Polymerase chain reaction analyses yielded the expected 323-bp DNA fragment with the E6 primer set and the 142-bp DNA fragment with the E7 primer set in the transfected HPDE/E6E7 cell lines but not in the nontransfected PK adenocarcinoma cell lines.* B: *A Southern blot of* EcoRI*-digested genomic DNA of these cell lines, which has been hybridized with the HPV-16 E6-E7 cDNA, demonstrated multiple bands in each cell line, consistent with the presence of multiple clones of HPDE/E6E7 cells containing randomly integrated E6E7 genes.*

tures typically seen in duct epithelium *in vivo*. Zymogen and endocrine granules were not seen. These cells in monolayer or on collagen were positive for cytokeratins 8/18 and 19 by immunohistochemistry. They did not form colonies in soft agar, and all of them were nontumorigenic in nude mice for up to 6 months.

Using both PCR and Southern blot analyses, we confirmed that the genomic DNA of these HPDE cell lines contained the HPV-16 E6 and E7 genes, which were randomly integrated (Figure 2). All cell lines demonstrated wild-type codons 12, 13, and 61 of the Ki-*ras* gene by allele-specific oligonucleotide analysis (Figure 3). In contrast, all three human pancreatic ductal carcinoma cell lines we tested showed Ki-*ras* mutation in codon 12. Immunohistochemical analyses for p53 protein was negative in all HPDE cell lines.

Discussion

We have described a new method to establish immortalized cultured epithelial cells derived from normal and non-neoplastic ductal fragments of adult human pancreas. The fact that none of these cell lines harbor a mutation in their Ki-*ras* gene also strongly supports their origin from the normal pancreatic duct epithelium. Although Ki-*ras* mutation at codon 12 is extremely common (>80%) in human pancreatic adenocarcinoma,[22] several investigators have also demonstrated a high frequency (60%) of Ki-*ras* mutation occurring in hyperplastic pancreatic duct epithelium.[10–13] The explant method that we have described is simple, highly reproducible, and yields pure duct epithelial cell populations. We believe this is the direct result of several new initiatives that were introduced into our culture methodology.

Previous efforts at establishing propagable cultured duct epithelial cells from normal adult human

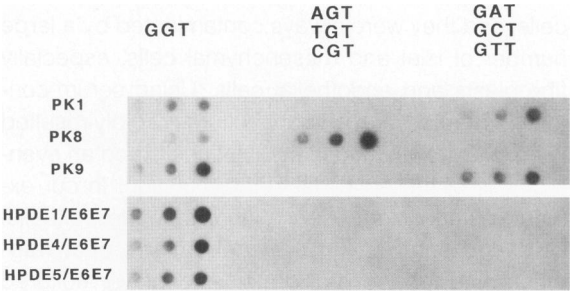

Figure 3. *Analyses for the presence of point mutation at codon 12 of the Ki-*ras *gene. The allele-specific oligonucleotide hybridization technique was used. Except for the wild-type probe, mutant probes were combined into two groups of three oligos to simplify the analyses. All three human pancreatic adenocarcinoma cell lines (PK1, -8, and -9) were positive for the Ki-*ras *mutation, whereas all three HPDE/E6E7 cell lines were negative.*

pancreas have rarely been reported. Harris and Coleman[23] reported the establishment of propagable cultured pancreatic duct epithelial cells, but these were from mid-trimester human fetal pancreata. Hall and Lemoine[24] reported the culture of epithelial cell colonies from acinar cell aggregates obtained from collagenase-digested human adult pancreata. Their study concluded that these acinar cells rapidly assumed a ductal phenotype within 4 days of primary cultures, but an attempt at long-term culture was not reported. Vilá et al[25] used a similar technique to obtain abundant primary cultured pancreatic epithelial cells that expressed ductal cell phenotypes, although they were not able to propagate these cells due to overgrowth by fibroblasts. Trautmann et al[26] have reported the primary culture of human pancreatic duct cells from transplant donors. They dissected the main pancreatic duct and first-degree branches, which were then subjected to collagenase digestion. These primary cultures contained proliferating colonies of epithelial cells that expressed ductal phenotypes, including the production of mucin and the expression of cytokeratin 19 and carbonic anhydrase. Contamination by fibroblasts was a problem, which was partly solved by passaging the epithelial cell colonies using cloning cylinders. They reported the maintenance of these duct epithelial cells for up to 5 weeks in culture, but details on cell passaging were not reported.

The explant method we have reported yields several advantages over the enzyme dissociation method commonly used to establish primary cultures of duct epithelial cells from human, rat, cow, and hamster pancreata.[27–29] First, it is simple to perform. Second, it ensures that the cultured epithelial cells originate exclusively from the ductal epithelium. Before using the method described here, we have also made many attempts to culture human pancreatic ductal cells from collagenase-dissociated pancreatic tissue. Such methods yielded very few epithelial cells, and they were always contaminated by a large number of islet and mesenchymal cells, especially fibroblasts and endothelial cells. Using serum-containing media, such primary cultures rapidly resulted in an overgrowth of mesenchymal cells and an eventual loss of the epithelial cell population. In our explant method, we observed that only the epithelial cells spontaneously migrated from the duct fragments and formed proliferating monolayer cultured cells. The passage of these primary colonies of duct epithelial cells never yielded an outgrowth of fibroblasts. Furthermore, we observed during subsequent passages that these epithelial cells maintained their proliferative activity only when they were subcultured at low split-ratios. It is presumed that cell-to-cell contacts are essential for these cells to maintain their proliferative potential. This may explain the difficulty of enriching epithelial cell populations in primary cultures of enzyme-dissociated pancreatic tissue.

The use of KSF medium for the long-term culture of these cells was based on our knowledge of the culture of normal human bronchial epithelial cells.[21] This medium was originally formulated for the culture of normal human keratinocytes but is also an excellent medium for the growth of human bronchial epithelial cells *in vitro*.[30] Additional experiments are necessary to determine which components/features of KSF are critical for their ability to support the growth of HPDE cells. It is possible that serum-containing medium may also be used to grow these cells, as Trautmann et al[26] were able to culture enzyme-dissociated human pancreatic duct cells for up to 5 weeks in RPMI 1640 medium containing 15% fetal bovine serum. We have, however, tried several serum-containing media, including one formulated to culture human biliary duct epithelial cells,[31] and have found that they yielded a poorer growth for these cells. Interestingly, primary cultured cells and their subcultures grow equally well on tissue culture plates with or without coating by type I collagen or matrigel, suggesting that these extracellular matrices are not essential for the continuous proliferation of these cells *in vitro*.

The HPV-16 and -18 and their E6-E7 genes have been extensively used to immortalize human epithelial cell lines of various origins.[32] The E6 protein of cancer-associated HPV inhibits the transcriptional regulatory function of normal p53 protein by forming a complex with it and promoting its degradation.[33] The E7 protein also complexes with the $p105^{rb}$, the protein product of the retinoblastoma tumor suppressor gene.[34] These interactions putatively form the basis for their immortalizing effects.[32] In contrast to the human fetal pancreatic duct epithelial cell lines, which could be subcultured for only up to 5 passages before senescence,[23] all of our HPDE/E6E7 cell lines have been subcultured for at least 9 passages before being frozen, and one of them has become immortal and been propagated for up to 20 passages. We are currently analyzing the effect of the HPV-16E6E7 gene transfection on the phenotypic expression of these cells, including their ability to maintain the differentiated pancreatic duct cell phenotypes.

The duct epithelium represents only a small proportion of the epithelial cell population in the human pancreas.[35] Furthermore, it is difficult to obtain fresh

normal adult human pancreata for experimental research. These potential barriers have imposed a significant limitation on our ability to study the role of pancreatic duct epithelium in various human pancreatic diseases. We believe the ability to establish primary culture and propagable cell lines from adult human pancreatic duct epithelium may revolutionize the study of diseases putatively related to human pancreatic ducts, including carcinogenesis. These cells could potentially be used to develop *in vitro* models of multistage human pancreatic carcinogenesis to study, for example, the biological effects of various abnormal genes/molecules putatively associated with human pancreatic carcinogenesis. The knowledge derived from these investigations may resolve some of the controversies regarding the cell of origin of human pancreatic ductal adenocarcinomas[36,37] and facilitate the development of strategies for the chemoprevention of human pancreatic cancers.

Addendum

Since the initial submission of this manuscript and using the procedure described, we have successfully established another immortal HPDE-6/E6E7 cell line from the normal pancreas portion of the specimen of a 63-year-old female undergoing partial pancreatectomy for a cystadenocarcinoma in the tail of her pancreas. This cell line has presently been propagated to passage 18.

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