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Immortalized bovine pancreatic duct cells become tumorigenic after transfection with mutant k-ras

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Abstract Mutation of the K-ras gene is thought to be an early and important event in pancreatic carcinogenesis. In order to study the role of this molecular alteration in the transition from the normal to the neoplastic pancreatic cell, bovine pancreatic duct cells were first immortalized by SV40 large T antigen (Ag) complementary (c)DNA transfection and then transfected with a mutated K-ras gene. As did primary duct cells, the immortalized duct cells (more than 100 passages) expressed cytokeratins, carbonic anhydrase type-II, cystic fibrosis transmembrane conductance regulator (CFTR), and multidrug resistance (mdr). They grew as a single layer after transplantation under plastic domes and formed three-dimensional structures resembling ducts when grown on Matrigel. Cell growth was stimulated by insulin, epidermal growth factor (EGF), transforming growth factor (TGF)-α, but cells did not respond to gastrin and CCK-8. They did not form colonies in soft agar nor did they form tumors in nude mice. Immortalized cells transfected with mutated K-ras acquired the ability to form tumors after orthotopic injection into the nude mouse pancreas. It is concluded that SV 40 immortalized bovine pancreatic duct cells retain the features of normal duct cells and gain tumorigenicity by transfection with mutated K-ras. This suggests an important role for K-ras in this pancreatic carcinoma model.

Keywords Pancreatic duct cell · SV40 · Immortalization · K-ras transfection · Tumorigenicity · Carcinogenesis

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Introduction

Most pancreatic carcinomas are of ductal phenotype and presumably derive from ductal cells [40]. Although a genetic profile has been established for pancreatic ductal carcinomas, it is not known which of the gene alterations turn the normal duct cell of the pancreas into a neoplastic cell. Since K-ras mutations are very common in these adenocarcinomas and are also detected in ductal changes that are thought to be precursor lesions [43, 44], they are considered to be an early event in pancreatic carcinogenesis. To understand the initial steps of carcinogenesis in the pancreas, the availability of a model for the transition of a normal duct cell to a neoplastic cell would be of great importance.

We recently succeeded in isolating and cultivating bovine and human pancreatic duct epithelial cells (PDEC) [19]. Here we report our results in establishing a stable pancreatic duct cell line by transfection with the large T antigen (Ag) of SV40 [36] and creating a tumorigenic clone by a second transfection with a mutated K-ras gene.

Materials and methods

Isolation and culture of primary pancreatic duct cells

Bovine pancreas was obtained from the slaughterhouse. Preparation and cultivation of primary pancreatic duct cells were performed as described previously [46]. In brief, the main pancreatic duct was opened and dissected. The excised duct was freed from

surrounding tissue and minced into 1 mm pieces. After repeated washes with ice-cold solution 1 [Hanks' balanced salt solution (Life Technologies) containing 0.01% soybean trypsin inhibitor, type-II-S (Sigma) and 0.02% bovine serum albumin, fraction V (Boehringer Mannheim)], the pieces were digested in solution 2 [solution 1 with 1000 U/ml collagenase, type-XI (Sigma) and 0.02% α-chymotrypsin, type-II (Sigma)] at 37°C for 1–2 h. After several washes with solution 1 and finally with culture medium, the resulting cell clumps were seeded into 60-mm plastic dishes (Nunc). These primary cells were grown in RPMI-medium (Life Technologies) supplemented with 2 mM L-glutamine (Life Technologies), 1 mM sodium pyruvate (Biochrom), 100 IU/ml penicillin, 100 µg/ml streptomycin (Life Technologies), 1 mg/ml dexamethasone (MSD), 100 µg/ml gentamycin/netilmycin (Essex Pharma), and 15% FCS (Life Technologies) in a humidified 5% CO_2 incubator at 37°C. After 5 days, fibroblasts were removed by scraping with a toothpick under microscopic guidance. The procedure was repeated every 2 days thereafter [46]. Cells were characterized using phase contrast and electron microscopy as described previously [46]. The pancreatic adenocarcinoma cell line Panc-1 (ATCC, Rockville, Md.) and human foreskin fibroblasts served as control cell lines [26].

Transfection with SV40 large T

Using the calcium–phosphate (CaP) method [50] with the plasmid pSV3neo [41], cells were transfected 6–8 days after seeding. The plasmid contains the DNA of SV40 large T Ag, under control of the SV40 early promoter and also carries the neomycin-resistance gene, which enables selection with G 418. Unlinearized CaP-precipitated DNA (10 µg) was added dropwise to each culture dish. After a transfection time of 5 h, the medium was removed and, to improve the transfection rate, the cells were shocked by the addition of 10% glycerol for 3 min. After that, the cells were washed twice with phophate-buffered saline (PBS) and re-fed with fresh medium.

From day two, G 418 (Life Technologies) was added for the purpose of selecting transfectants to a final concentration of 300 µg/ml. No toothpick removal of fibroblasts was performed thereafter. Transfection resulted in the establishment of five G418-resistant cell clones. Three of them had an epithelial-like morphology in phase contrast microscopy (V-A, X-C, and X-D), one appeared to be fibroblastic (UE-B), and one was of indefinite morphology (X-E). Transfection was confirmed by means of Northern blot, Western blot, and immunocytochemistry. The V-A clone was further examined.

Immunocytochemistry

Indirect immunocytochemistry was used in native and transfected PDECs as previously described [46]. The primary antibodies were directed against SV40 large T (Calbiochem, 1:100), broad-spectrum cytokeratin (CK; Sigma; 1:200), CK 7 (Sigma, 1:100), CK 8 and 18 (Enzo Diagnostics, 1:100), CK 19 (Sigma, 1:200), vimentin (Boehringer Mannheim, 1:100), carbonic anhydrase type-II (Pan Systems, 1:200), and fibroblasts (Dianova, 1:200) [46]. Secondary and tertiary antibodies used for immunocytochemical staining were horse radish peroxidase (HRP)-conjugated rabbit anti-mouse, rabbit anti-goat, and swine anti-rabbit immunoglobulin (Ig)Gs (all: Dako, 1:100) with AEC as a color substrate.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis and Western blot analysis

Protein extracts were prepared by scraping the cells into 100 µl of ice-cold buffer A [10 mM Hepes (pH 7.9); 1.5 mM $MgCl_2$; 10 mM KCl; and 0.5 mM dithiothreitol (DTT)] and forcing the suspension through a 28-G needle to disrupt cell membranes. Nuclei were pelleted in a microcentrifuge for 10 s at 4°C and 15,000×*g*. The supernatant was used to analyze cytoplasmatic proteins, and the nuclei were used in experiments not described in this work. The samples were boiled in Laemmli denaturing buffer [23] and analyzed by means of Western blotting. Primary antibodies used were: carbonic anhydrase II (Chemicon), K-ras (Santa Cruz, sc-30), extracellular signal regulated kinase (ERK) 1/2 (sc-94), ERK 1/2-P (sc-7383), proliferating cell nuclear antigen (PCNA; sc-56), p21/WAF1 (sc-6246), p27 (sc-1641), Bcl-2 (sc-509), Bcl-xL (sc-1690), Caspase 3 (sc-1226), and ELK-P (New England Biolabs). The primary antibodies were used at a working dilution of 1:1000. Secondary and tertiary antibodies were rabbit anti-mouse Ig (Dako, 1:5,000) and swine anti-rabbit Ig-alkaline phosphatase (AP)-labeled (Dako, 1:5000).

For detection, CDP-star was used as substrate, and resulting signals were hard-copied to X-ray film (Kodak). For the CFTR (cystic fibrosis transmembrane conductance regulator) and mdr (multidrug resistance) P-glycoprotein blots, cells were harvested and solubilized by means of sonication for 30 s in 'lysis' buffer containing 50 mM mannitol, 0.1% Triton-X 100, 10 µg/ml leupeptin, 1 mM benzamidine, 0.2 mM Pefabloc SC, and 10 mM Tris (pH 7.0). Detergent-insoluble material was removed by centrifugation at 12,000×g for 5 min, and equal amounts of membrane proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 7.5% acrylamide minigels and transferred onto PVDF membranes. Blots were blocked with 3% nonfat dry milk in Tris-buffered saline containing 0.05% Tween 20 for 6 h and incubated with primary antibody against mdr P-glycoprotein [5 µg/ml C219 mAb [21] from Alexis Deutschland (Grünberg, Germany)] or CFTR (1 µg/ml mouse monoclonal antibody M3A7) [22] overnight. Following incubation with horseradish peroxidase-conjugated secondary antibody (Amersham; 1:6000 dilution) for 60 min, blots were developed in enhanced chemiluminescence reagents (Amersham), and signals were visualized on X-ray films.

Reverse slot blot

The expression of several proteins [growth factors, extracellular matrix (ECM) proteins, oncogenes, and tumor suppressor genes] known to play a role in pancreatic carcinoma was investigated using a reverse slot blot, as previously reported [16]. In brief, cDNA probes corresponding to 500 ng insert were immobilized by baking (120°C, 30 min) onto a nylon membrane (Qiagen) using a slot blot apparatus (Schleicher and Schuell), following the instructions of the manufacturer. Total RNA (7.5 µg) of clone V-A and of the tumor cell line Panc 1 were reversely transcribed, and cDNA was DIG-dUTP labeled with 400 U superscript II (Life Technologies). Blots were hybridized with the labeled cDNA in Dig Easy Hyb (Boehringer, Mannheim) overnight at 42°C and hybrids were detected with the DIG nucleic acids detection kit (Boehringer Mannheim) according to the manufacturer's instructions and hard-copied to X-ray film (Kodak). The cDNAs were as follows: collagen type-I and collagen type-III [37]; fibronectin (FN) [38]; vitronectin (VN) [39]; carbonic anhydrase type-II [8]; fibroblast growth factor (FGF)2 [1]; int2 [3]; epidermal growth factor (EGF; ATCC); platelet-derived growth factor (PDGF)-A, PDGF-B (Amersham); transforming growth factor (TGF)-β1 [52]; *K-ras* (ATCC); *p53* [15]; *fos* (Amersham); *raf* (ATCC); and S6 used as a ribosomal control gene [17].

Growth response to peptides

Bovine pancreatic duct cells (3–4×10^4 per well) were seeded onto 6×35 mm multiwell plates (Corning, N.Y.) containing 3 ml of RPMI 1640 medium supplemented with 10% FCS and 2 mM L-glutamine (all Life Technologies). After 24 h, cells were washed twice with 1 ml of PBS, and serum-free medium containing growth peptides at the desired concentrations was added: bombesin (BBS; Peninsula Laboratories), EGF (Serva), non-sulfated cholecystokinin (CCK-8; Peninsula Laboratories) and TGF-α (Gibco) at 1×10^{-11}, 1×10^{-10},

and 1×10^{-9} M; human gastrin-1 (Peninsula Laboratories) and secretin (Ferring, Berlin, Germany) at 1×10^{-9}, 1×10^{-8}, and 1×10^{-7} M, and insulin (Sigma) at 1×10^{-8}, 1×10^{-7}, and 1×10^{-6} M. The synthetic somatostatin analogue (SMS 201–9950) was used at concentrations of 10, 100, and 1000 ng/ml in the presence of 1% FCS; bombesin stimulation was performed both in serum-free conditions and in 1% FCS supplemented medium [7]. For each peptide, a minimum of nine separate experiments was performed. Cell numbers were counted after incubation for 5 days in a Coulter counter (Coulter Electronics, Inc.). For statistical analysis, the Mann–Whitney U test was employed. Two-sided *P* values less than 0.05 were considered statistically significant.

Karyotype analysis

Chromosome preparation was performed according to standard procedures. Colcemid (final concentration 0.1 mg/ml; Life Technologies) was added to the culture for 2 h. The medium was replaced by a hypotonic solution (0.4% KCl) and incubated at 37°C for 20 min. Thereafter, 0.6 ml fixative (3:1 methanol:acetic acid, 4°C) was added to 10 ml hypotonic solution and gently mixed by inverting the tube. The supernatant was removed, the was pellet resuspended, and 3:1 fixative was added dropwise. Three washes in fixative were performed. The cell suspension was dropped on cold wet slides. The preparations were air-dried, aged at 90°C for 1 h and GTG-banded [12].

K-ras transfection of immortalized V-A cells

The day before transfection, V-A cells were seeded with 5 ml Dulbecco's modified Eagle's medium (DMEM)/10% FCS into 60-mm plates (Nunc). CaP transfection [19] was performed using the plasmid pCMV/K-ras. This plasmid contains the complementary (c)DNA of the human K-ras gene mutated in codon 12 (GGT to GTT) under control of the cytomegalovirus (CMV) promoter and also carries the hygromycin-B phosphotransferase gene, thus enabling selection with hygromycin B. In brief, 5 µg plasmid DNA was diluted in 250 µl 0.25 M $CaCl_2$, mixed with 250 µl 2×BBS and incubated for 20 min at room temperature. Culture medium (4.5 ml) was added, and the cells were re-fed with this CaP-DNA solution. After overnight incubation (35°C, 3% CO_2), cells were washed twice with PBS, re-fed with fresh culture medium, and incubated for another 2 days at 37°C, 5% CO_2. For selection of transfectants, cells were split 1:5 into culture medium containing hygromycin B (125 µg/ml). Selection was carried out for 14 days, and resistant clones were expanded. Successful introduction of the mutated K-ras gene was checked using RE-mismatch polymerase chain reaction (PCR; see below) analysis, and the expression of the transfected K-ras cDNA was analyzed using Western blot analysis.

K-ras PCR

High-molecular DNA was prepared directly from fresh cells according to standard PCR protocols. The *K-ras* PCR was performed as described previously [18]. In brief, the codon 12 flanking region was spanned by two primers covering a 99-bp fragment. A modification on the 3′ end of the upstream (5′) primer lies on the last base of codon 11, creating an artificial *Msp*I restriction endonuclease site in case the wild-type *K-ras* gene is present [18]. A 20-µl aliquot of the 50-µl PCR reaction product was used in a *Msp*I enzyme (Boehringer Mannheim) digest for 2 h. If the wild type is present, the reaction yields a 78-bp and a 21-bp fragment. If the *K-ras* gene is mutated in codon 12, a double band will result, representing the undigested original 99 bp and 78 bp in addition to the 21-bp fragment. The result is visualized on a standard 3% agarose gel (NuSieve; FMC).

Growth in soft agar and tumorigenicity in athymic mice

Cells were plated onto a sublayer of medium containing 0.5% agar and covered with 0.3% agar-containing medium (Bactoagar; Life Technologies) [42]. The formation of colonies was observed for 5 weeks. Tumorigenicity was assayed using subcutaneous or intraperitoneal injection of $1–2\times10^6$ cells in a small volume (100 µl) of plain RPMI medium into athymic nude mice. In a second set of experiments, the cells were diluted 1:1 in Matrigel (EHS-matrix, Serva) and then injected. The growth was observed over a period of 10 weeks. Panc-1 tumor cells (ATCC) were used as controls [26, 27]. Alternatively, 1×10^6 immortalized cells in 100 µl plain medium were injected into pre-established plastic domes put on skin defects (1.5 cm^2) on the back of nude mice, as described [10]. Cells were allowed to grow for 12 weeks. The domes were then removed, and the tissue was examined under a microscope [hematoxylin and eosin (HE) stain]. For orthotopic implantation, $1–5\times10^6$ SV40/ras^{mut} (V-A^{ras}) transfected cells were injected directly into the pancreas in about 100 µl culture medium [34]. The mice were checked once weekly until a solid tumor was palpable. Then, the mice were killed, and pancreas, liver, and spleen were removed, paraffin embedded, microdissected, and stained with HE. The resulting specimens were screened for tumor cells.

Results

Morphology, growth, and proliferation of transfected bovine PDECs

Resistant colonies appeared 5–9 weeks after transfection. A successful passage into new culture dishes was accomplished 10 weeks after transfection, and then five G418-resistant cell clones were isolated. Three of them had an epithelial-like morphology in phase contrast microscopy (V-A, X-C and X-D). The V-A clone was further examined (Fig. 1A). In contrast to untransfected normal cells, V-A cells could be trypsinized and re-plated. They were frozen, re-thawed, and re-cultured, and they are currently

Table 1 Comparison of life span, growth, and tumorigenic characteristics of untransfected cells (nl bPDEC), SV40-transfected cells (V-A), K-ras-transfected V-A cells (V-A^{ras}), pancreatic tumor cells (Panc-1), and bovine fibroblasts. *nl bPDEC* normal bovine pancreatic duct epithelial cells; *ND* not done; + positive characteristic; – negative characteristic; +/– a low number of three-dimensional (3D) structures appeared after 65 h of cultivation

	nl bPDEC	V-A	V-A^{ras}	Panc-1	Fibroblasts
Large T antigen expression	–	+	+	ND	ND
Life span (passage)	<2	>100[a]	>40[a]	>100	>30
Soft agar colonies	ND	–	+	+	–
Nude mice tumors	–	–	+	+	ND
3D structures on Matrigel	ND	+	ND	+/–	–

[a] Ongoing monitoring

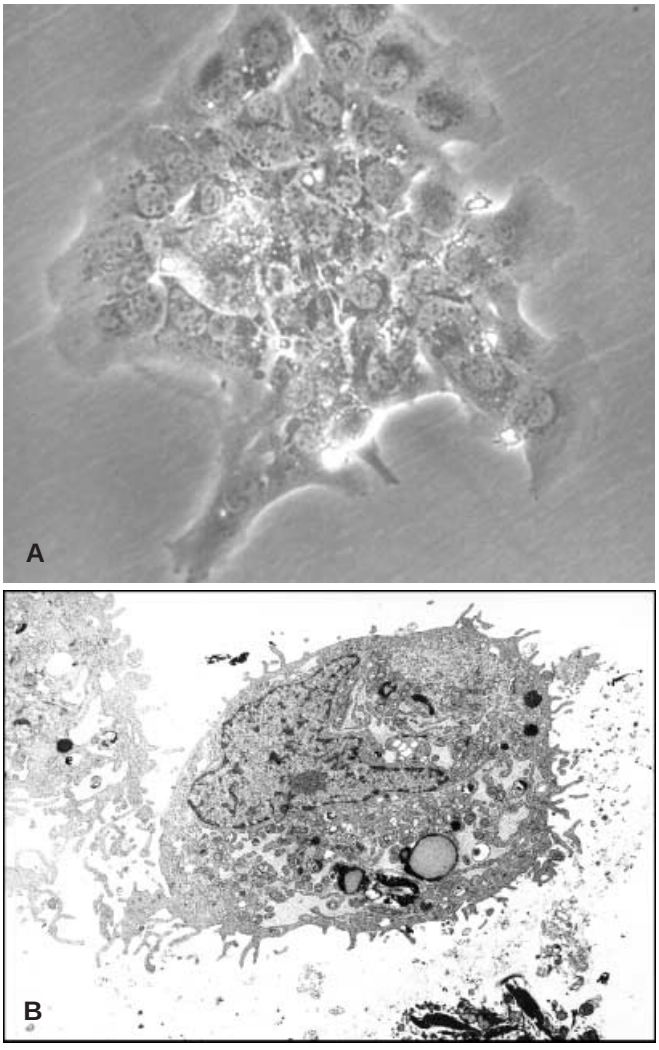

Fig. 1 **A** Epithelial morphology of the SV40 large T antigen-transfected bovine pancreatic duct cell clone V-A (320×). **B** Electron microscopy of bovine SV40 immortalized pancreatic duct cell clone V-A showing microvilli and secretory granules (7500×)

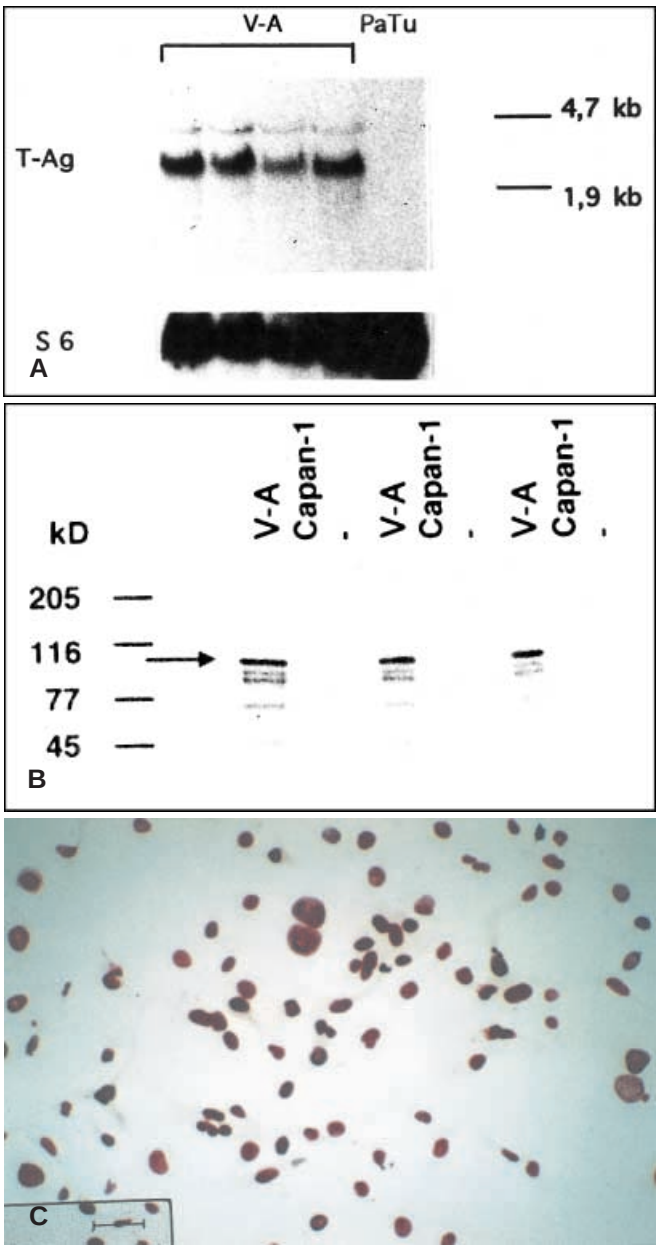

Fig. 2 Expression of SV40 large T antigen in bovine SV40 immortalized pancreatic duct cells (clone V-A). **A** Northern blot demonstrating the 2.2-kb large T transcript (*top panel*), with the ribosomal S6 transcript serving as loading control (*lower panel*). **B** SV40 large T protein (*arrow*) detected by means of Western blot. **C** Strong nuclear immunostaining for SV40 large T (250×)

growing in their 100th passage (Table 1). The doubling time was 40 h and was not influenced by G 418. The immortalized bovine duct cells expressed the typical ultrastructural features of normal pancreatic duct epithelial cells, e.g., polar organization and polymorphous secretory vesicles (Fig. 1B).

Expression of large T Ag

A 2.2-kb transcript corresponding to SV40 large T RNA was detected in the transfected cells (Fig. 2A). Western blot analysis revealed an 89-kDa protein (Fig. 2B) that was found using indirect immunocytochemistry to be located in the nuclei of all cells (Fig. 2C). Control cells (untransfected duct cells, carcinoma cells, and fibroblasts) did not express large-T antigen at either the RNA or the protein level.

Expression of markers specific to PDEC

Native and immortalized PDECs were positive for broad spectrum CK (Fig. 3A) and carbonic anhydrase type-II (Fig. 3B, C, and Table 2). Western blot analysis revealed that V-A cells expressed CFTR at lower levels than primary PDEC and mdr P-glycoprotein at similar levels (Fig. 3D). At the RNA level (reverse slot blot), clone V-A exhibited a similar expression pattern to that of the tumor cell line Panc-1 (Fig. 4). Both cell lines expressed

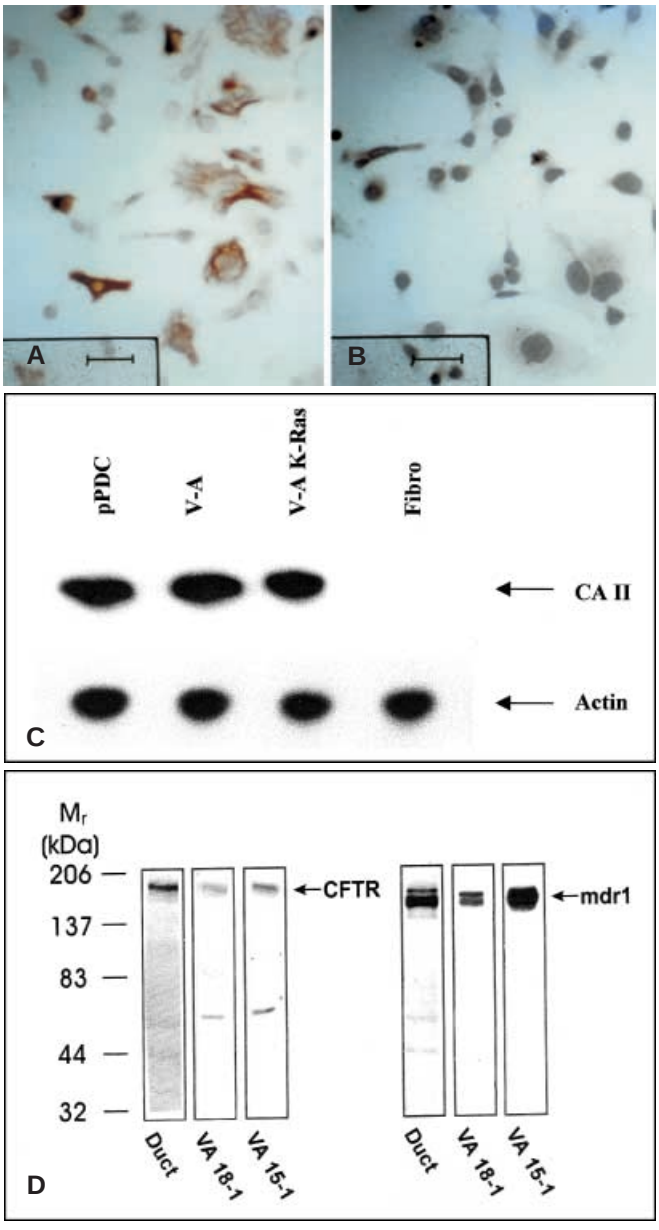

Fig. 3 Expression of pancreatic duct markers in SV40 transformed bovine pancreatic duct cells (clone V-A) and in cells additionally transfected with mutated K-ras (V-A^{ras}). **A** Positive immunostaining for broad-spectrum cytokeratin and carbonic anhydrase II (**B**). **C** Western blot analysis for carbonic anhydrase II. *pPCD* primary pancreatic duct cells; *Fibro* fibroblasts serving as negative control. Actin was used as a loading control protein. **D** Western blot analysis of bovine main pancreatic duct cells grown as primary culture (duct) and of immortalized bovine main pancreatic duct cell lines at passages 15 and 18 (V-A 15-1 and V-A 18-1) for cystic fibrosis transmembrane conductance regulator (CFTR) and multidrug resistance (mdr)1

carbonic anhydrase type-II, a marker enzyme for pancreatic duct cells. Of the growth factors, TGF-β1, bFGF, and PDGF were expressed at low levels. Furthermore, a messenger (m)RNA signal for the ECM proteins, VN and FN, was detectable, and raf, fos, and p53 were expressed in both cell lines.

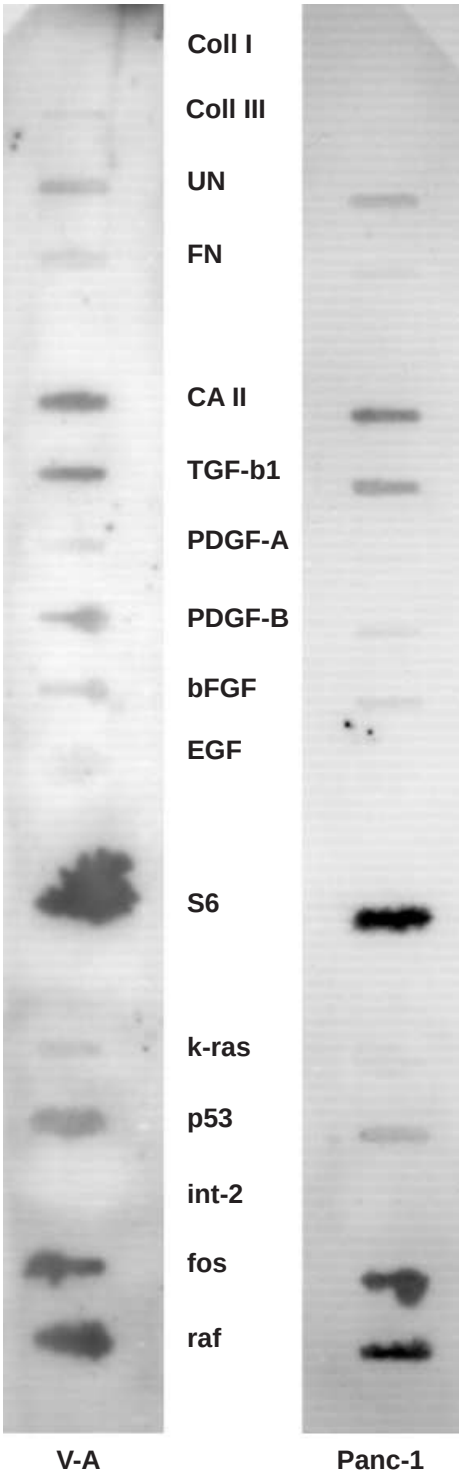

Fig. 4 RNA expression of SV40 immortalized bovine pancreatic duct cells. Reverse slot blot with labeled cDNA from V-A cells (*left*) and Panc-1 human pancreatic carcinoma cells (*right*) for selected growth factors, matrix proteins, and oncogenes. *Coll I* collagen type-I; *Coll III* collagen type-III; *VN* vitronectin; *FN* fibronectin; *CA II* carbonic anhydrase type-II; *S6* ribosomal protein (positive control)

Table 2 Comparison of expression of pancreatic duct cell antigens. *V-A* SV40-transfected cells; *V-A^{ras}* K-ras-transfected V-A cells; *Panc-1* pancreatic tumor cells; *nl bPDEC* normal bovine pancreatic duct epithelial cells; + positive characteristic; – negative characteristic

	nl bPDEC	V-A	V-A^{ras}	Panc-1
Cytokeratin	+++	++	++	++
Carbonic anhydrase II	++	+	+	(+)
Vimentin	–	+	+	+
Anti-fibroblast	–	–	–	–

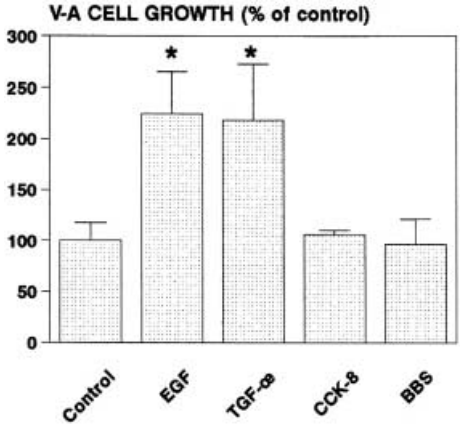

Fig. 5 Proliferation of SV40-transfected bovine pancreatic duct cells (clone V-A) after stimulation with epidermal growth factor (EGF), transforming growth factor (TGF)-α, cholecystokinin (CCK-8), and bombesin (BBS). *Significant difference to controls

Growth responses

As an example of the proper functioning of the SV40 large T-immortalized PDEC, the response to physiological stimuli was investigated. V-A cells grew well both in serum-free medium and in the presence of 1% FCS (data not shown). Insulin at 10^{-7} M induced a 2.2- to 2.4-fold increase in the number of V-A cells ($P<0.0004$; Fig. 5). EGF and TGF-α at 10^{-9} M exerted a similar effect ($P<0.0001$; Fig. 5). Secretin at 10^{-9} M induced a moderate (20%) increase in cell number. Gastrin, CCK-8, bombesin, and somatostatin (SMS 201–995) at 10^{-11}–10^{-7} M concentrations did not exert any trophic effect on the immortalized pancreatic duct cells. The addition of EGF (10^{-9} M) plus insulin (10^{-7} M) displayed an additive effect (data not shown). SMS (1000 ng/ml) reverted the insulin-mediated increase by 50% ($P<0.02$) but did not modify the EGF-induced cell action (data not shown).

Chromosome aberrations

Of the examined cells, 100% had chromosomal aberrations, mostly variations of chromosomal numbers (up to 90 chromosomes) and dicentric chromosomes (data not shown).

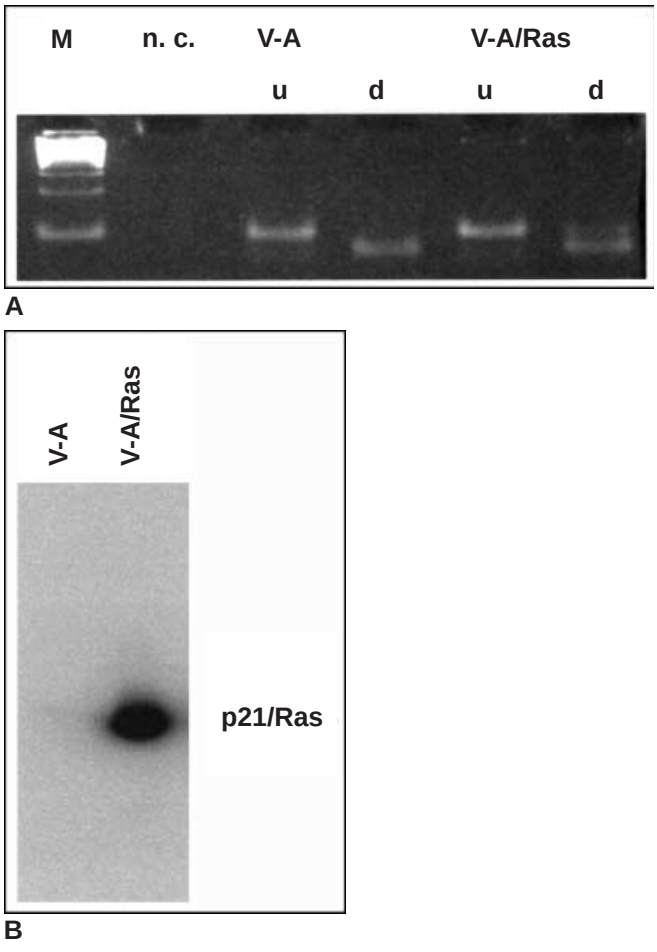

Fig. 6 K-ras transfection of SV40 large T immortalized pancreatic duct cells. **A** Stable uptake of mutated ras complementary (c)DNA in V-A^{ras} cells verified using RE-mismatch polymerase chain reaction (PCR) for ras oncogen. Agarose gel electrophoresis of ras PCR products from bovine pancreatic duct cell lines before and after *Msp*I digestion. The undigested PCR product is 99 bp in size; after *Msp*I digestion, the fragment measures 78 bp. *Lane 1* marker (100 bp DNA ladder); *lane 2* negative control (H_20); *lanes 3 and 4* PCR product from clone V-A, undigested and after *Msp*I digestion and *lane 5 and 6* PCR product from clone V-A^{ras}, undigested and after *Msp*I digestion. Samples with ras mutations demonstrate a double band after digestion, indicative of the mutant and wild-type allele. **B** Western blot of protein preparations from bovine SV40 immortalized pancreatic duct cells clone V-A and clone V-A^{ras}, stained for K-ras

Ras transfection

Transfection of SV40-immortalized V-A with the plasmid pCMV/K-ras resulted in the isolation of a hygromycin-resistant clone. Introduction and expression of the mutated K-ras gene was confirmed using RE-mismatch PCR (Fig. 6A) and Western blotting (Fig. 6B). The SV40/ras^{mut} (V-A^{ras})-transfected PDEC also expressed CK and carbonic anhydrase type-II (Table 2). The transfection of the mutated K-ras cDNA resulted in activation of the mitogen-activated protein (MAP) kinases ERK 1 and 2 and of the kinase ELK1 in the V-A^{ras} cells (Fig. 7). The cell cycle inhibitors, p21/WAF1 and p27, were up-

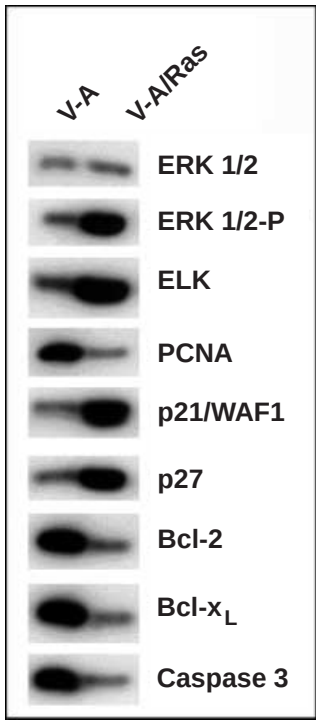

Fig. 7 Western blot analysis of cell cycle genes and kinases of SV-40 immortalized bovine pancreatic duct cells (V-A, *left*) and of immortalized bovine pancreatic duct cells transfected with mutant K-ras (V-A^{ras}, *right*)

regulated in these cells and, as a consequence, PCNA expression was downregulated. The anti-apoptotic proteins, bcl-2 and bcl-xL, and, conversely, the proapoptotic caspase 3 were downregulated in V-A^{ras} cells (Fig. 7). Fluorescence-activated cell sorter (FACS) analysis of propidium iodide-labeled cells revealed no difference in the apoptotic rate of both cell clones (data not shown).

Growth in soft agar and tumorigenicity

V-A cells seeded onto soft agar for up to 5 weeks did not form colonies. Injection of transfected cells into a preformed plastic dome on a skin defect resulted in growth of single-layer epithelial cells (Fig. 8A) and small nests of cells. The V-A cells were not tumorigenic in nude mice when injected s.c., i.p., or orthotopically into the pancreas.

In contrast, V-A^{ras} cells formed colonies in soft agar but at a lower rate than the control Panc-1 cells, which formed easily visible large colonies. Orthotopic injection of V-A^{ras} cells into nude mice revealed a tumorigenic potential of this clone. More than 50% of the nude mice developed slowly growing tumors (Fig. 8B) and, in one case, the liver was infiltrated

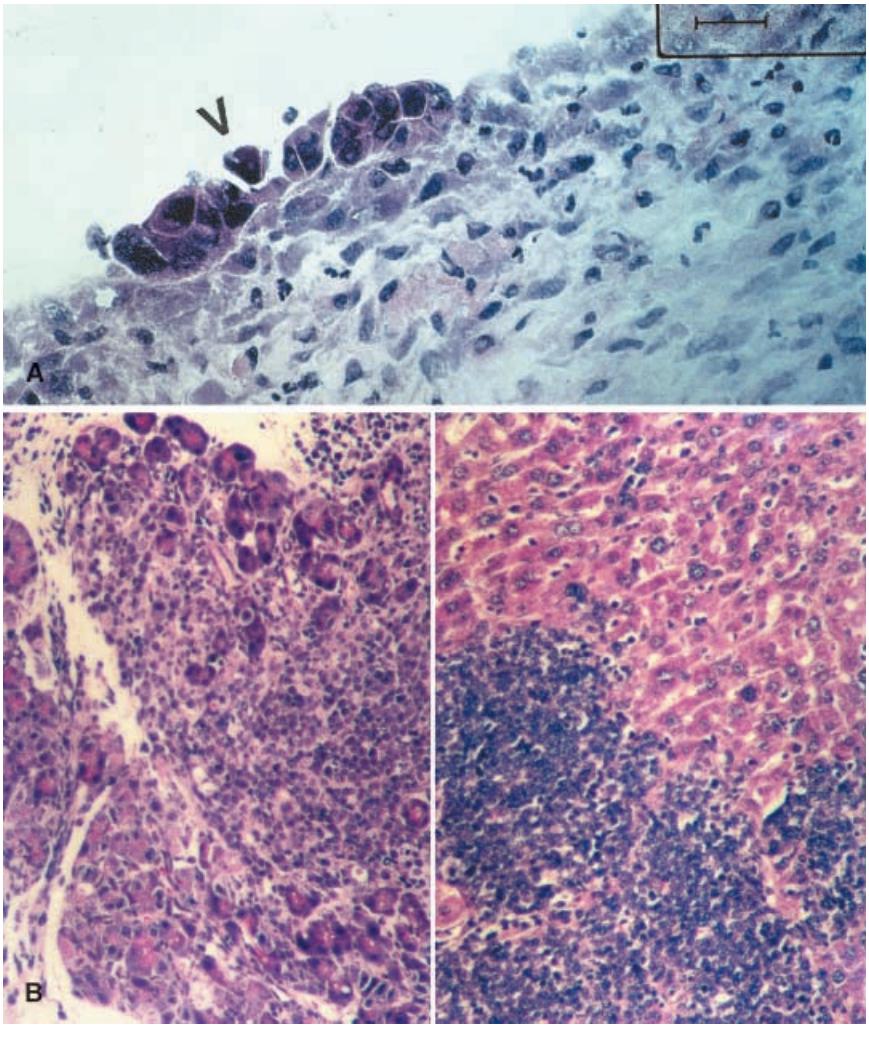

Fig. 8 **A** Formation of a single-layer epithelial lining (*arrowhead*) of SV40-transformed bovine pancreatic duct cells (clone V-A) transplanted under plastic domes in nude mice. Hematoxylin and eosin (HE; 400×). **B** Development of tumors after orthotopic xenotransplantation of SV-40 immortalized and K-ras-transfected bovine pancreatic duct cells (clone V-A^{ras}) in the nude mouse pancreas. *Left* intrapancreatic tumor infiltrating and destroying acinar tissue; *right* metastasis in the liver. HE (250×)

with tumor cells. No tumor development occurred with injection of the parental cells.

Discussion

We immortalized bovine PDECs by transfection with an SV40 large T Ag-coding plasmid. This resulted in three cell clones with epithelial morphology, one of which was further characterized. The immortalized cell clone V-A is a cell line representative of a pancreatic cell lineage expressing pancreatic duct-specific genes, such as carbonic anhydrase II, CFTR, mdr, and CKs, and responding to physiological stimuli.

Our results confirm and extend the recent report of the immortalization of bovine pancreatic duct cells [28]. While these authors demonstrated CFTR expression, no further analysis of the morphology and duct cell markers was performed, as in our study. The immortalization of human pancreatic duct cells has also been reported [9]. The starting material for these transformation experiments employing the papilloma virus E6E7 gene were macroscopically normal ducts, however, obtained from pancreases with pancreatic cancer, preventing a good comparison with animal models employing cells from normal ducts. Furthermore, there was no detailed morphological or functional characterization [9]. In all cases, the immortalized PDEC exhibited different properties from those of primary PDECs, which are unable to grow for more than 6 weeks and cannot be passaged more than once [46].

The expression of CKs, carbonic anhydrase type-II, CFTR, and mdr in the immortalized cell clone V-A proved its pancreatic duct origin and continuous differentiation in this direction [29, 45]. The fact that V-A cells did not respond to gastrin and CCK-8 additionally confirms their ductal origin, since pancreatic duct epithelial cells lack the appropriate receptors [2]. The response to growth factors, including EGF and secretin, was similar to that previously reported for normal pancreatic duct cells [6, 53]. When transplanted under preformed plastic domes, V-A cells retained the ability to form a single epithelial layer and small epithelial nests, as described for papilloma-transformed keratinocytes [10]. SV40 large T Ag clearly affects genetic stability in the transfected cells, inducing a large number of chromosome aberrations. This has been previously observed [32] and might be directly related to the functional inactivation of p53 protein [48]. It has been shown that a truncated large-T protein not capable of binding p53 can induce immortalization [47], thus the immortalizing properties of SV40 large T are not only due to functional inactivation of p53. The absence of a normal p53 protein is obviously insufficient to induce the appearance of a tumorigenic phenotype. In contrast, the accumulation of chromosome aberrations is assumed to be the foundation for immortalization and subsequent carcinogenesis in SV40-infected cells [32].

The additional transfection of mutated *K-ras* into the SV40 large T immortalized cells resulted in a cell clone (V-A^{ras}) that retained the morphological features and differentiation markers of pancreatic duct cells. The constitutively activated *K-ras* resulted in activation of the MAP kinases ERK1 and 2, demonstrating the proper expression and function of the transfected k-ras cDNA. Contrary to the role of the MAP kinases as a mediator of mitogenic signals, in V-A^{ras} cells, the cell cycle inhibitors p21 and p27 were upregulated, with the consequence of a decrease in PCNA expression. Comparable results have been obtained by overexpression of raf [25, 51] or by altering the cellular ratio of ras and rho proteins [30]. Overexpression of SV40 large T antigen or mutated K-ras may exert different effects on the induction of apoptosis, depending on the cellular context and amount of expression [5, 20]. Consequently we analyzed the expression pattern of bcl-2, bcl-xL, and caspase 3 and the rate of apoptosis in both cell lines. Downregulation of the anti-apoptotic proteins bcl-2 and bcl-xL in V-A^{ras} cells seemed to be counterbalanced by a simultaneous decrease in the pro-apoptotic caspase 3, since no differences in the apoptotic rate of the V-A and V-A^{ras} cells was detectable, though a shift to M1 was observable in the V-A^{ras} cells.

V-A^{ras} cells gained tumorigenic potential when injected orthotopically into nude mice, suggesting a key role for this oncogene in pancreatic carcinogenesis. Thus, cooperation of large T and mutated K-ras is apparently necessary for transformation and tumorigenicity, as has been demonstrated for other cell lineages [4, 11, 13, 24, 31, 33, 35, 49]. This is the first report of malignant transformation of terminally differentiated pancreatic duct cells by a mutated *K-ras* gene leading to obvious tumors. Recently, the retroviral transfer of the catalytic subunit of telomerase (hTERT), SV large T, and *h-ras* in human embryonic kidney cells resulted in a tumorigenic cell line that formed tumors in irradiated nude mice [14]. Our experiments revealed the intermediate state of clone V-A. The expression pattern of several genes (TGF-β1, bFGF, PDGF, VN, FN, *raf*, *fos*, and *p53*) was similar to that of the pancreatic carcinoma cell line Panc-1, and thus quite different to normal pancreatic cells [46]. The immortalized clone itself exhibited no signs of tumorigenicity; only after the additional transfection with a mutated K-ras was the clone V-A^{ras} gene able to induce tumor growth.

In summary, we have immortalized a bovine PDEC line by SV40 T Ag transfection and made it tumorigenic by further introducing mutated *K-ras*. This V-A cell line retains morphological and functional characteristics of the ductal phenotype. These cells may now be further employed as an in vitro model for pancreatic duct cell biology, tissue-specific gene expression, and as a target for further transfection steps on the way to an artificial system of fully invasive malignant pancreatic duct cells.

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