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Murine Pancreatic Ductal Adenocarcinoma Produced by *in Vitro* Transduction of Polyoma Middle T Oncogene into the Islets of Langerhans

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Pancreatic islets isolated from juvenile but not aging adult mice, when infected with a retrovirus carrying polyomavirus middle T oncogene, produced cell lines, mPAC, with characteristics both of pancreatic ductal epithelium and neuroendocrine cells of the islets. Following three cycles of single cell cloning, mPAC cells consisted of two subtypes, a null cell, and a double-positive cell that co-expressed cytokeratin, a marker of ductal epithelium, and A2B5, a neuroendocrine ganglioside expressed in developing islet cells. Two islet cell genes, encoding somatostatin and pancreatic polypeptide, were transcribed at low levels in most mPAC clones, whereas the insulin and glucagon genes were not. Upon inoculation of mice, mPAC cells rapidly formed well-differentiated ductal adenocarcinomas that expressed cytokeratin but not the islet cell markers. The mPAC phenotype may result from a specific dedifferentiation of juvenile islet cells or ductal epithelium induced by middle T protein. Alternatively, mPAC cells may arise by transformation of a multipotential progenitor present within or in juxtaposition to juvenile islets. This cell type could therefore represent one of the targets in human cancers of the pancreatic duct. Moreover, signal transduction systems modulated by middle T, including src-related kinases, phosphatidylinositol kinase, and protein phosphatase 2A, may be involved in pancreatic carcinogenesis. (Am J Pathol 1994, 145:671–684)

Pancreatic cancer remains one of the most difficult cancers to diagnose early and treat curatively. Several genetic abnormalities have been detected in pancreatic tumors. There is a high incidence of point mutations in the K-*ras* oncogene, in 60 to 95% of tumors, depending on the survey.[1,2] However, it seems that the K-*ras* activation is an early event in pancreatic tumorigenesis, inasmuch as the mutation does not correlate with malignancy or poor prognosis and has been also observed in pancreatic adenomas.[3] Alterations of *p53* and *Rb* genes have been reported in 40 and 20% of pancreatic cancers, respectively,[4] and expression of c-*erb*B-2 was found to be elevated by immunohistochemical analysis in 19% of pancreatic cancer.[5] Abnormalities on *src* or *src*-family oncogenes have not been reported.

The origin of pancreatic cancer, of which about 90% are ductal adenocarcinomas, has been the focus of considerable controversy. Studies on rodent chemical carcinogenesis in the mid-1970s first suggested that the target in pancreatic carcinogenesis is an acinar cell, which subsequently undergoes transdifferentiation or metaplasia to assume a duct cell phenotype (reviewed in ref. 6). The development of mixed acinar/ductal pancreatic carcinomas in transgenic mice expressing the c-*myc* oncogene driven by the acinar-specific rat elastase 1-enhancer/promoter may support an acinar cell origin for pancreatic duct adenocarcinomas.[7] On the other hand, pancreatic cancers induced in Syrian golden hamsters by *N*-nitroso-bis(2-oxopropyl)amine arise through sequential stages of ductal hyperplasia, atypical hyperplasia, intraductal carcinoma, and invasive ductal carcinoma,[8,9] suggesting an origin in the ductal epithelium. The presence of foci with ductlike profiles has been reported in noncancerous portions of pancreas

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from patients with pancreatic ductal carcinoma. These foci express both acinar-specific and duct-specific antigens,[10] and thus may represent preneoplastic lesions.

Ductal epithelial cells have been proposed to contain potential stem cells for both endocrine and exocrine pancreas.[11,12] The duct seems to contain stem cells with a capability for proliferation and differentiation throughout life, as evidenced by the avid regenerative capacity of the pancreas in adult and elderly mice[13] (and our unpublished observations). Regeneration of the endocrine pancreas has also been observed following massive inflammation of the pancreas induced by expression of interferon-γ; new islet cells could be seen budding out of the ductal epithelium.[14] Moreover, the transdifferentiation of pancreatic duct cells to hepatocyte has been documented, indicating considerable developmental plasticity.[15] Thus, multipotential stem cells in the ductal epithelium should not be overlooked as a potential target cell for pancreatic carcinogenesis.

Progress in understanding the genesis of pancreatic cancer and distinguishing amongst these etiologies has in part been limited by the paucity of animal or *in vitro* models of pancreatic ductal carcinogenesis. Here we report use of a retrovirus vector carrying polyoma middle T (mT) oncogene for reproducible derivation from juvenile endocrine pancreas of cell lines that upon transplantation produce typical pancreatic ductal adenocarcinomas.

Materials and Methods

Isolation of Islets of Langerhans

C3H mice with various ages were sacrificed by cervical dislocation, and their common bile ducts were perfused immediately by retrograde infusion of 300 U/ml of collagenase (Worthington Biochemical, Freehold, NJ, class 4) and 10 μg/ml of DNAse I (Worthington Biochemical) in Hanks' balanced salt solution as described.[16] After incubation at 37 C for 45 minutes, the pancreases were washed three times by pipetting in RPMI 1640 medium supplemented with 10% fetal calf serum, followed by centrifugation at 200 × *g* for 30 seconds. The islets were manually picked up from the digested tissue fragments under a dark-field stereomicroscope (Wild M3Z, Heerbrugg, Switzerland). The islets were cultured in a f3.5 cm bacteriological Petri dish in RPMI 1640 medium with 10% fetal calf serum at least for two days to remove contaminating exocrine cells.[17]

For newborn mice, 20 to 30 pancreases were removed and minced with scissors. Three hundred μl (or microliters) of 750 U/ml collagenase and 15 μg/ml of DNAse I in Hanks' balanced salt solution was added and the pancreases quickly sheared through a 23G needle, followed by incubation at 37 C for 3 minutes with intermittent vortexing. The digested pancreases were cultured in the same medium as above in a bacteriological Petri dish for 1 week. The medium was changed every day; each time the tissue fragments were sedimented by simple 1 × gravity for 1 minute, and the floating small cells, which were mostly acinar cells, were removed before replenishing the medium. After 1 week in suspension culture, those that survived consisted of small balls (less than ~1 mm diameter) of tissue fragments from which one or more islet-like domes protruded. Dithizone staining[18] and immunohistochemistry with insulin and glucagon antibodies proved that these are in fact nascent endocrine islets. We refer to this spheroid as a mother islet (Yoshida and Hanahan, in preparation), in which one or more nascent islets are attached to and budding from the small duct fragment in the body of the structure.

Infection of the Islets with a Polyoma mT Retroviral Vector

The grossly acinar cell-free islets were transferred to a gelatinized tissue culture dish, and 0.1 mol/L of IBMX (3-isobutyl-1-methylxanthine), a cyclic AMP agonist, was added to enhance decapsularization and attachment of the islets to the dish surfaces. The medium was Dulbecco's minimal essential medium (glucose 4.5 g/l) with 2% fetal calf serum and 12% horse serum. Seven to 10 days later, the islet cells were exposed to fresh conditioned medium of GP+EmT cells supplemented with 4 μg/ml of polybrene for 12 hours. The cells, a gift from Dr. Erwin Wagner (I.M.P., Vienna, Austria), produce the retrovirus vector N-TKmT, where the neomycin resistance gene is expressed from the Moloney murine leukemia virus 5′ long terminal repeat, and the polyoma mT gene from the thymidine kinase gene promoter.[19] The titer of the conditioned medium was at least 1×10^5 G418-resistant colonies/ml when assayed on NIH3T3 cells. The infection was repeated three times, and G418 selection at 0.4 mg/ml was started 1 week after the last infection. As a control, we used pXM5 (N2), which is same as N-TKmT, except the former does not contain the thymidine kinase promoter/polyoma mT hybrid gene. A retroviral vector for a temperature-sensitive mutant of simian virus (SV40) large T oncogene, pZIPtsU19, was a generous gift from Dr. Ron McKay (MIT, Cambridge, MA).[20] tsNY68 is a retrovi-

rus carrying a temperature-sensitive *src* oncogene[21] and was kindly provided by Dr. Michael Bishop (UCSF).

Southern and RNA Blot Hybridizations

DNA and poly(A)$^+$ RNA extractions, and blot hybridization procedures were described elsewhere.[22] Hybridization and washing were done under stringent condition. A full-length complementary (c)DNA was used as a probe for polyoma mT gene. Mouse insulin cDNA was provided by Dr. Gerhard Christofori (UCSF). Mouse glucagon, somatostatin, pancreatic polypeptide, and amylase cDNA probes were cloned by polymerase chain reaction (PCR) using the primer sequences listed in.[23]

Immunohistochemistry

The tumors were fixed in 3.7% paraformaldehyde, and then routine paraffin or JB-4 plastic embedding and sectioning was done. Cells were grown in plastic-surface Lab-Tek Chamber Slides (Nunc) and fixed in 3.7% paraformaldehyde for 1 hour. Standard indirect immunocytochemistry using horseradish peroxidase was performed. The following antibodies were used: guinea pig anti-insulin antiserum (Linco Research, St. Louis, MO); rabbit anti-glucagon antiserum (ICN); rabbit anti-carboxypeptidase antiserum (a gift from Dr. William Rutter, UCSF); rabbit anti-trypsin antiserum (a gift from Dr. William Rutter, UCSF); rabbit anti-cytokeratin antiserum (DAKO, Carpinteria, CA); mouse monoclonal hybridoma supernatants A2B5 (Böhringer Mannheim, Mannheim, FRG), RT97 and 2H3 (Developmental Studies Hybridoma Bank, Iowa City, IA). Normal guinea pig and rabbit sera and monoclonal antibody against SV40 large T antigen (made in our laboratory) were used as negative controls; the primary antibodies were diluted at least to the point where the negative controls gave no signal.

Bromodeoxyuridine-Cytokeratin Double Immunostaining

mPAC cells plated into a Chamber Slide were pulsed for 1 hour with 10 μmol/L of 5-bromo-2′-deoxyuridine (BrdUrd). After various times of 0 to 36 hours, the cells were fixed in 3.7% paraformaldehyde for 1 hour, followed by DNA denaturation in 1.5 N HCl for 30 minutes and neutralization in 0.1 N borax (sodium tetraborate) solution for 5 minutes. The cells were first stained with fluorescein isothiocyanate-conjugated anti-BrdUrd monoclonal antibody (Becton-Dickinson, San Jose, CA) at 1:10 dilution. After washing, the cells were incubated sequentially with the rabbit anti-cytokeratin antiserum (1:200) and 7-amino-4-methyl coumarin-3-acetic acid (AMCA)-conjugated goat anti-rabbit immunoglobulins (Jackson ImmunoResearch Laboratories, West Grove, PA).

In Vitro *Kinase Assay for mT Activity*

The assay was performed as described,[24] with minor modifications. Cells were cultured to 80 to 100% confluency, washed twice in ice-cold phosphate-buffered saline, and lysed in RIPA buffer (50 mmol/L Tris [pH 7.5], 150 mmol/L NaCl, 1% NP-40, 0.1% sodium dodecyl sulfate, and 0.5% sodium desoxycholate) supplemented with 100 μmol/L sodium orthovanadate, 20 μg/ml leupeptin, 1% aprotinin, and 0.5 μmol/L phenylmethylsulfonyl fluoride. Protein concentration was determined using the BioRad Protein Assay Reagent. Equal amounts (1.5 mg) of cell protein in 1 ml of RIPA buffer were incubated with 50 μl of normal rabbit serum for 1 hour at 4 C, and precleared by sequential incubation with 50 μl of Pansorbin cells (Calbiochem) for 45 minutes at 4 C, centrifuged for 10 minutes at 12,000 rpm, followed by a similar cycle using 80 μl of a 1:1 suspension of protein G Sepharose (Pharmacia) in RIPA buffer. The cleared lysates were divided into two tubes; one was incubated with the PAb815 antibody for 7 hours at 4 C, and the other treated similarly without added antibody. Then both were precipitated with 50 μl of a 1:1 suspension of protein G Sepharose in RIPA buffer. The immune complexes were resuspended and incubated in kinase buffer (20 mmol/L HEPES (pH 7.0), 10 mmol/L $MgCl_2$, 1 mmol/L dithiothreitol) for 30 minutes at 30 C in the presence of 0.7 MBq of [γ^{32}P]ATP. Then the complexes were washed twice with RIPA buffer, disrupted by the addition of a sample buffer containing sodium dodecyl sulfate and dithiothreitol, boiled, and fractionated by electrophoresis through an 8% sodium dodecyl sulfate-polyacrylamide gel, which was subjected to autoradiography for variable periods. The monoclonal antibody used, PAb815, is specific for polyoma mT oncoprotein.[25]

Results

Infection of Pancreatic Islets with a Polyoma mT Retrovirus Elicits Epithelial Cell Lines

Figure 1 shows the basic scheme used for the generation of mPAC cells. Briefly, islets of Langerhans

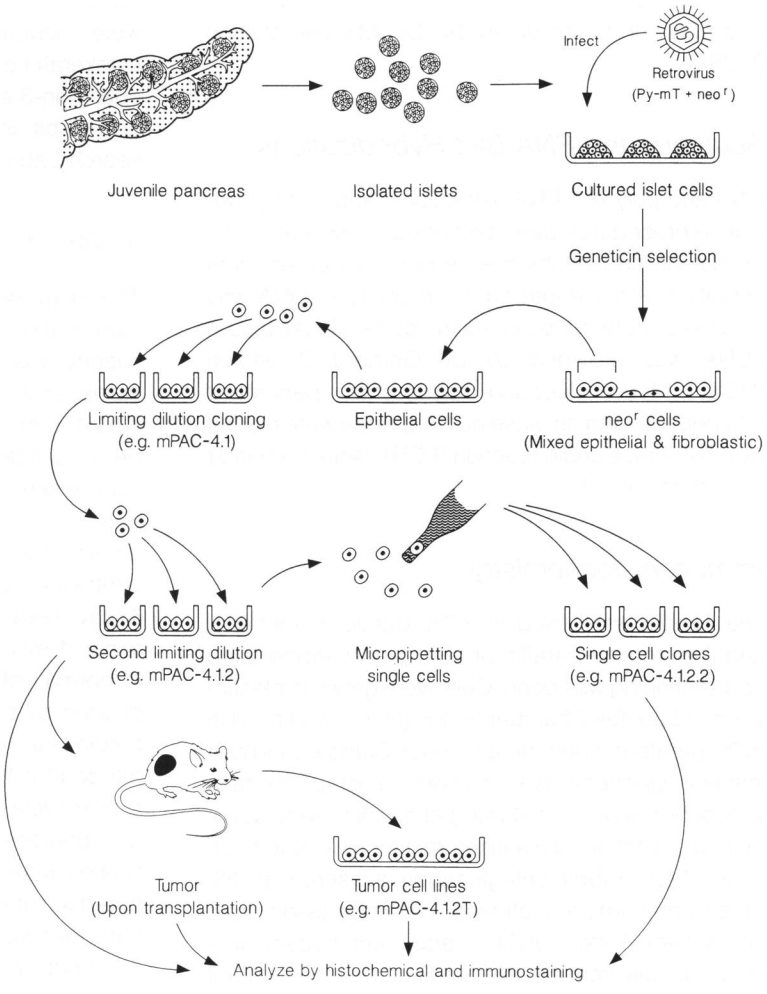

Figure 1. *Generation and cloning of mPAC lines. Pancreatic islets were isolated from 2-week-old mice without detectable acinar cell contamination when examined under a dark-field stereomicroscope. The islet cells were cultured as adherent cells and infected three times with a polyoma mT-transducing retrovirus. G418 selection gave rise to a mixture of nontumorigenic fibroblasts and tumorigenic epithelial mPAC. Two rounds of limiting dilution and a micropipetting were performed to remove fibroblast and to assure monoclonality of mPAC cells, which show stable heterogeneity regarding expression of ductal and neuroendocrine markers (see text).*

were prepared from normal C3H murine pancreases by perfusing the common bile duct with collagenase and DNAse I. The islets were clearly identifiable under a dark-field stereomicroscope and handpicked individually. Subsequent short-term suspension culture in bacterial plastic Petri dishes effectively removed contaminating acinar cells. The islets were then placed in gelatinized tissue culture dishes, where they decapsulated and the islet cells became attached to the surface. The islet cells were infected in three cycles by a retrovirus carrying polyoma mT gene.[19] Two types of cells were recognized in the infected islet cultures, about 2 to 3 weeks after the beginning of G418 selection (Figure 2a). One was fibroblastic and the other appeared epithelial and resembled βTC or αTC cells, which are insulinoma and glucagonoma cell lines, respectively, derived from SV40 T antigen transgenic mice.[26,27] After trypsinization of the whole G418^r population, several nonfibroblastic islands were picked up and designated mPAC-1 to mPAC-4. A second independent cycle of infections produced mPAC-5, etc. Subsequently, two rounds of limiting dilution were performed to remove contaminating fibroblasts and to confirm the monoclonality of each line. Cell lines obtained by the first limiting dilution were termed mPAC-1.1, mPAC-2.3, and so on. Those from the second limiting dilution are mPAC-1.1.1, mPAC-2.3.4, etc. Some mPAC cells were further cloned by direct micropipetting of single cells under a phase microscope, generating definitive monoclonal cells, such as mPAC-4.1.1.1 or mPAC-4.1.2.2. All the mPAC clones showed essentially similar morphology under a phase-contrast microscope (Figure 2b). Floating spheroid cell masses were observed in mPAC cultures even before they reached confluency (Figure 2, a and b, arrows).

Both the fibroblastoid cells and the epithelioid mPAC cells were apparently immortalized and grew rapidly in culture, with comparable doubling time of 12 to 13 hours. When 1×10^6 cells were injected subcutaneously into syngeneic C3H mice, all of the mPAC clones formed ~0.5- to 1.0-cm diameter ul-

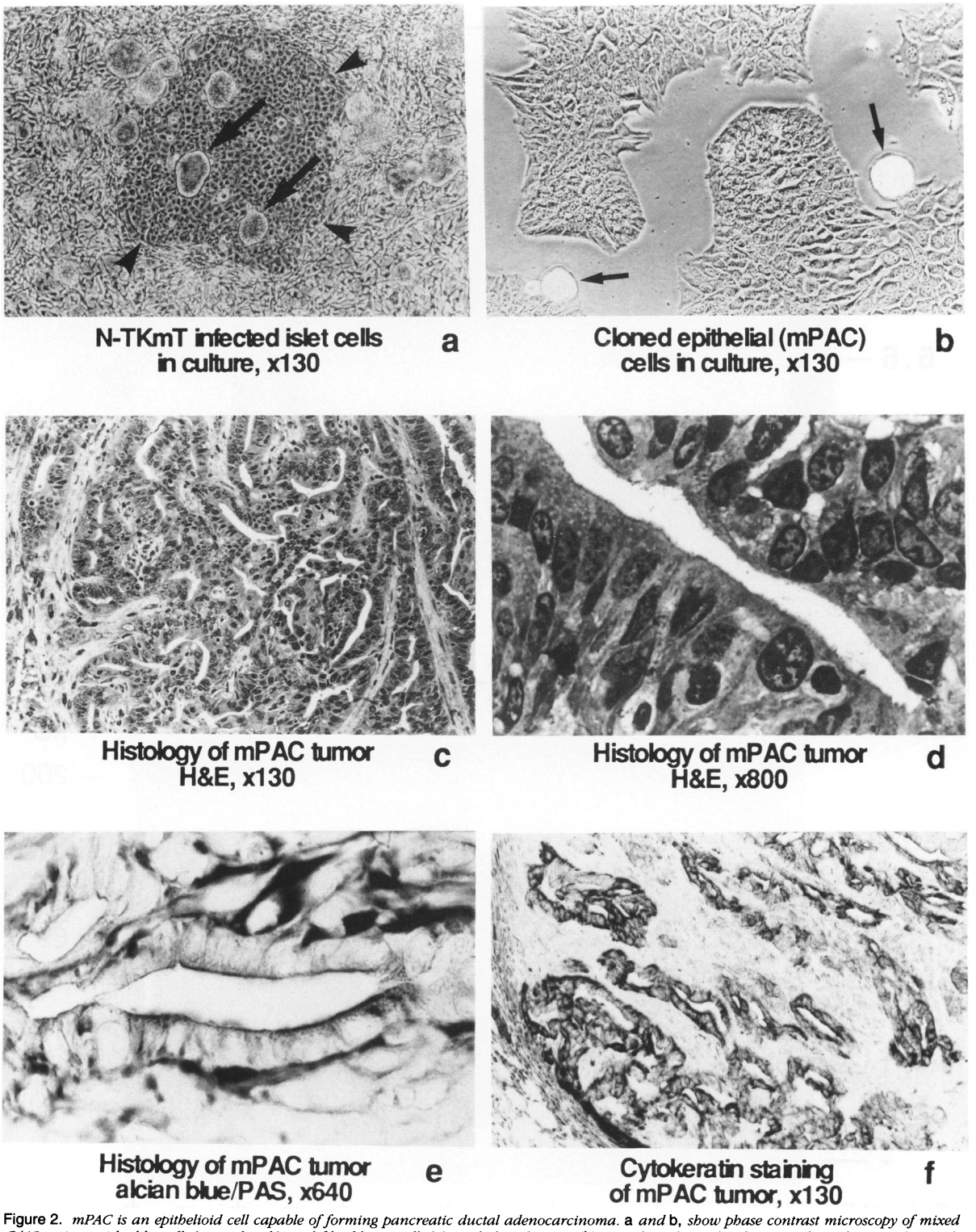

Figure 2. *mPAC is an epithelioid cell capable of forming pancreatic ductal adenocarcinoma.* a *and* b, *show phase contrast microscopy of mixed G418-resistant islet-like cells (arrowheads) and fibroblastic cells* (a) *and cloned mPAC* (b) *in culture (×110). Floating spheroid masses (arrows) were observed both in primary cultures* (a) *and in mPAC clones* (b), *even in the subconfluent culture.* c *exemplifies the histology of a tumor formed by subcutaneous injection of mPAC cells into a syngeneic mouse (H&E staining, ×110). Under a high-power view (×680,* d), *fuzzy structures along the luminal surface can be visualized, suggesting the presence of microvilli. In* e, *Alcian blue/periodic acid-Schiff staining shows mucin-like substance on the apical surface of the duct epithelium (×540). In* f, *an mPAC tumor was stained for cytokeratin; the tumor was surrounded by a fibrous capsule and contained a considerable noncancerous stroma (×110).*

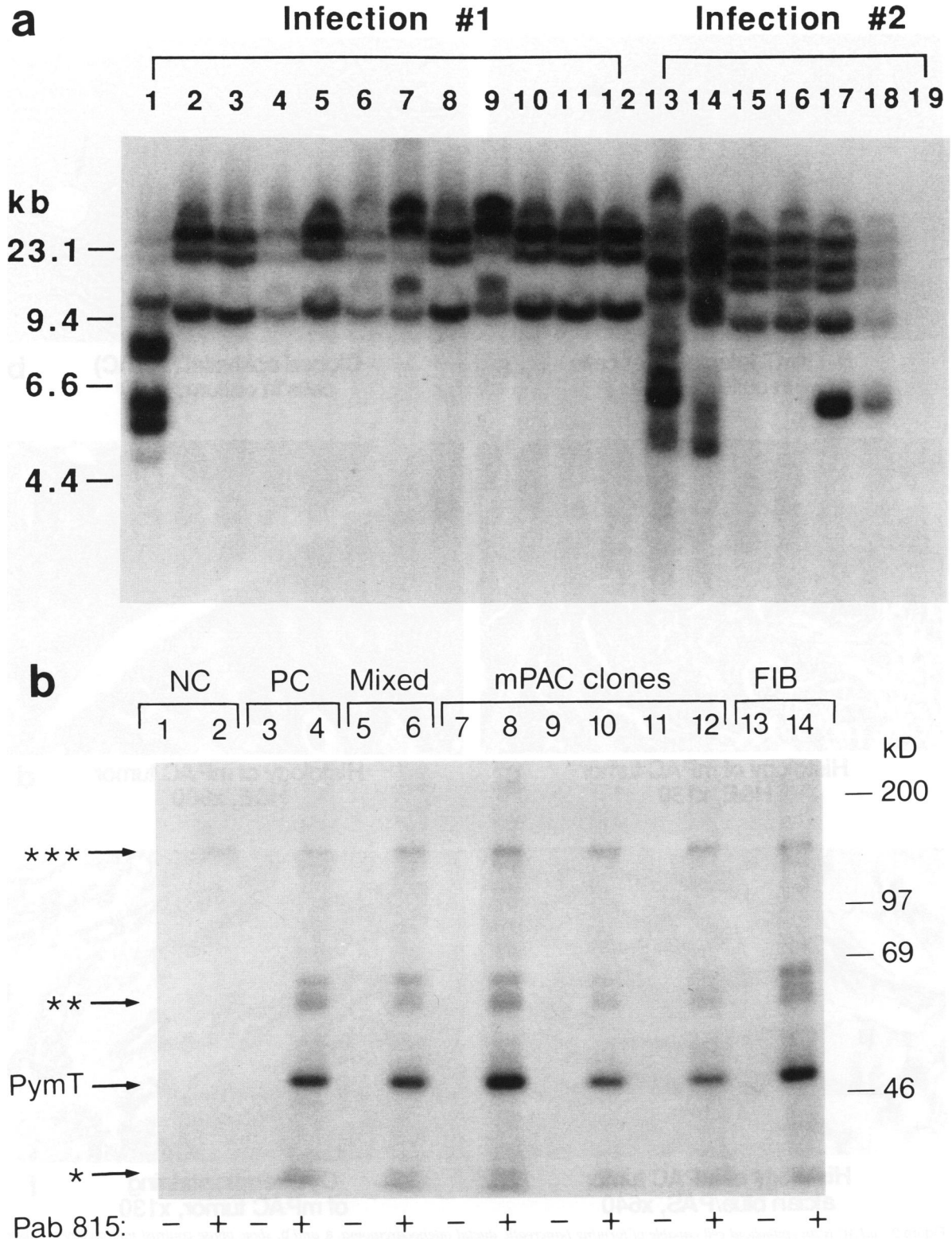

Figure 3. *mPAC cells contain the N-TKmT provirus and express functional mT protein.* a: *Southern blot analysis for N-TKmT provirus in mPAC. DNAs were digested with* BamHI, *which does not cut the vector, and hybridized with the polyoma mT cDNA probe.* Lanes 1 *to* 12 *are cells obtained by the first infection:* lane 1 *is the original uncloned and mixed G418-resistant population;* lanes 2 *to* 12 *are various clones derived from cells in* lane 1. Lane 2: *mPAC-1;* lane 3: *mPAC-2;* lane 4: *mPAC-2 tumor tissue;* lane 5: *mPAC-3;* lane 6: *mPAC-3 tumor tissue;* lane 7: *mPAC-4;* lane 8: *mPAC-4.1;* lane 9: *mPAC-4.1.2.1;* lane 10: *mPAC-4.1.2.2;* lane 11: *mPAC-4.3;* lane 12: *mPAC-4.4.* Lanes 13 *to* 18 *represent cells derived from another independent infection:* lane 13: *mixed fibroblastic and epithelial cells;* lane 14: *mPAC-5.1;* lane 15: *mPAC-5.2;* lane 16: *mPAC-5.3;* lane 17: *mPAC-5.4;* lane 18: *mPAC-5.4 tumor tissue.* Lane 19 *is DNA from NIH3T3 cells. Migration of a molecular weight marker, phage* λ *DNA digested*

cerating tumors within 2 weeks. Two of four fibroblastoid cell lines tested formed anaplastic tumors with a fibrosarcoma morphology at a similar growth rate to the ductal adenocarcinomas produced by the epithelioid mPAC cells, whereas the other two failed to do so by 3 months, when the observation was terminated. There were no gross metastases for either cell type.

The reproducibility of the above method of mPAC generation was confirmed by repeating the experiment. As a negative control, we employed pXM5 (N2), which is the backbone vector used to construct the N-TKmT retrovirus.[28] This neo-only virus was used to infect islets from 2-week-old mouse pancreas. Fibroblastoid cells were rendered G418-resistant, but no mPAC-like cells appeared.

Two-week-old mouse islets were also infected with a retrovirus, pZIPtsU19, carrying a temperature-sensitive mutant of SV40 large T oncogene.[20] The culture was maintained at permissive temperature of 32.5 C. All of the infected dishes showed fibroblast overgrowth after 1 month, and no mPAC-like epithelioid cells could be recovered. Similarly, infection with tsNY68, a retrovirus transducing a temperature-sensitive mutant of the *src* oncogene,[21] also failed to generate mPAC-like epithelial cells (data not shown).

Ductlike fragments that were found among the collagenase-digested pancreatic fragments were also handpicked under a stereomicroscope and infected with the N-TKmT virus. However, the duct fragments did not attach to the tissue-culture surface with or without gelatinization, and no G418-resistant cells were obtained.

Integration and Expression of the Polyoma mT Gene

The cloning process of mPAC cells was monitored by Southern blot analysis for the mT gene. As shown in Figure 3a, the mPAC clones derived from the first round of limiting dilution (eg, mPAC-4.1, -4.3, and -4.4) already showed the same pattern of provirus integration as the triple-cloned cells (eg, mPAC-4.1.2.2), implying early clonal outgrowth. The difference in the band pattern between mPAC-4.1.2.1 and -4.1.2.2 is probably due to partial digestion of the former genomic DNA, because the same pattern was observed in the parental mPAC-4, but not in its immediate subclones, mPAC-4.4, -4.3, or mPAC-4.1, from which mPAC-4.1.2.1 or -4.1.2.2 were derived. Because the multiple cloning procedure means higher passage number and may be prone to change of cellular phenotype *in vitro*, the clones from the first limiting dilution, such as mPAC-4.3 and -4.4, were used preferentially in most of the studies.

All the mPAC cell lines were found to express abundant and similar levels of retroviral RNA, including the mT messenger RNA (data not shown). RNAs isolated from tumors (see below) had reproducibly lower levels of retroviral transcripts than the parental cells in culture, likely due to contaminating host-derived stromal cells in the tumor tissue. The pattern of transcripts was similar to that reported in previous analyses of the retrovirus.[19]

To assess the presence of functional mT protein, *in vitro* kinase assays were performed, because this oncoprotein can elicit self-phosphorylation through its association with *src*-like tyrosine kinases, as well as autophosphorylation of those kinases. Cell lysates of three cloned mPAC lines, one fibroblastic line, and one mixed uncloned population were immunoprecipitated with a monoclonal antibody specific for polyoma mT antigen (PAb815[25]). The immunoprecipitates were incubated with [γ^{32}P]ATP such that any kinases bound to the mT protein and co-precipitated with it would radiolabel themselves and the mT protein. As shown in Figure 3b, all the cell lines expressed functional mT protein, and at similar levels. Two other labeled bands may well represent the *src*-like tyrosine kinase(s) bound, as well as another known substrate of the protein, protein phosphatase 2A[29,30]; to date confirmation of the identity of these bands has not been pursued.

A Window of mPAC Generation

To assess the effect of developmental age of the mice on successful generation of mPAC cells, adult islets, newborn islets (both mature islets and mother islets, see Materials and Methods) and fetal pancreatic epithelium from mice of different ages were subjected to the same protocol as described above. The results are summarized in Table 1. The data show that mPAC lines are most efficiently generated from 2-week-old

with Hind*III, is indicated.* **b:** In vitro *kinase assay for functional mT protein. Duplicate cell lysates were immunoprecipitated with an mT-specific monoclonal antibody (+PAb815, even numbered lanes) or similarly treated without the specific antibody (−PAb815, odd lanes). The complexes were incubated with [γ^{32}P]ATP, disrupted, and fractionated by 8% polyacrylamide gel electrophoresis. The cells analyzed were: a negative control (NC), NIH3T3; a positive control (PC), NSLXMT, the same NIH3T3 transformed by mT; a primary mPAC culture following infection of islets and G418 selection (mixed,* lanes 5 *to* 6*); three mPAC clones, mPAC-4.3* (lanes 7 *to* 8*), mPAC-4.4* (lanes 9 *to* 10*), and mPAC-5.1* (lanes 11 *to* 12*); and a fibroblastic clone from the mT infection of islets (FIB,* lanes 13 *to* 14*). Bands marked by * may represent proteins associated with mT protein (see text).*

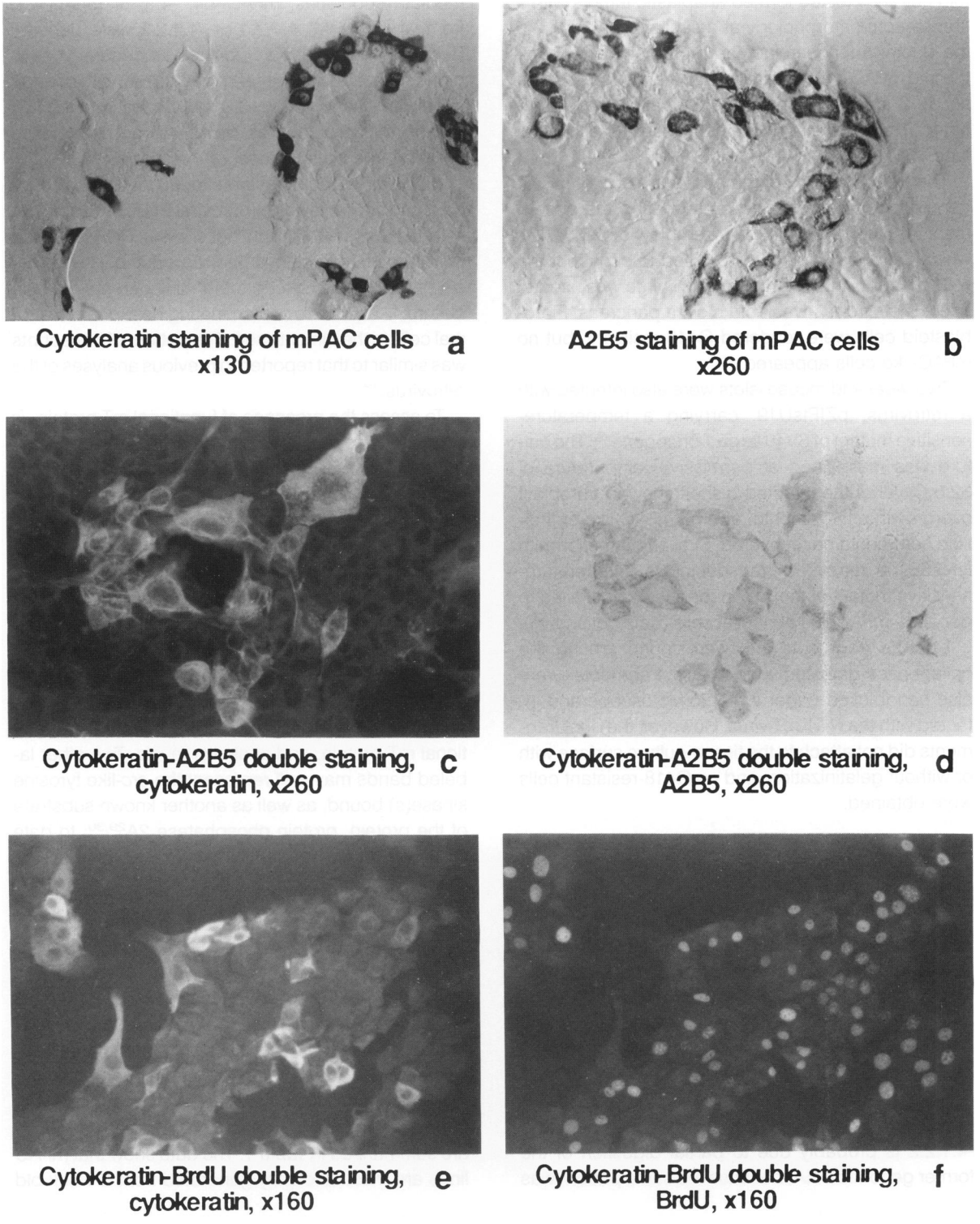

Figure 4. *A dual null/double-positive phenotype for mPAC and tumor re-derived mPAC-T lines. Analysis of representative mPAC lines. The mixed null and double-positive phenotype of the cloned mPAC lines is shown in* **a** *to* **d**: **a** *and* **b**: *separate immunostaining of mPAC-4.4 for cytokeratin* (**a**, ×*110*) *and A2B5* (**b**, ×*230*); **c** *and* **d**: *double immunostaining of mPAC-4.3 for cytokeratin* (**c**, *fluorescein isothiocyanate fluorescence*) *and A2B5* (**d**, *diaminobenzidine precipitate*), (×*230*). **e** *and* **f** *show that both null and double-positive cells proliferate, as measured by incorporation of BrdUrd: double staining of mPAC-4.4 for cytokeratin* (**e**, *7-amino-4-methylcumarin 3-acetic acid fluorescence*) *and BrdUrd* (**f**, *fluorescein isothiocyanate fluorescence*), (×*140*).

Table 1. *Effect of Mouse Age on Generation of mPAC Cells by mT Oncogene*

Target*	Result
e11 pancreatic epithelium	No G418-resistant cells
Mother islets from 0.5–1.5 day-old pancreas	Fibroblastoid overgrowth
Nascent islets† from 0.5–1.5 day-old pancreas	No G418-resistant cells
Islets from	
2 week-old pancreas	mPAC + fibroblastoid cells
8.4 week-old pancreas	Fibroblastoid cells
9.1 week-old pancreas	mPAC + fibroblastoid cells
9.5 month-old pancreas	No G418-resistant cells
11 month-old pancreas	No G418-resistant cells

* Each experiment was performed in duplicate, and each time about 10 to 40 islets or fetal pancreatic epithelia were infected. Although not all of these infections were carried out on the same day, there was no substantial difference in the titer of the retrovirus produced by the GP+EmT producer cells as assessed by induction of G418-resistant foci on NIH3T3 cells.

† Nascent islets from 0.5 to 1.5 day-old pancreas were obtained by excision and isolation of "buds" from corresponding mother islets (*see Materials and Methods*).

juvenile islets by the current protocol of infection. Islets from newborn and aged mice could not be transformed, and young adults (about 2 months old) sporadically produced mPACs.

mPAC Form Ductal Adenocarcinomas in Vivo

Upon inoculation of histocompatible mice, mPAC clones produced solid tumors within 2 weeks. The tumors showed the typical morphology of well-differentiated ductal adenocarcinomas (Figure 2, c and d). On higher magnification, the microvilli were recognized on the luminal side of the cells, and alcian blue/periodic acid-Schiff staining showed the presence of mucin-like substance in the apical portion of cells lining ducts, albeit in low amounts (Figure 2e). The obvious duct structures tend to be lost and replaced with a more poorly differentiated pattern in tumors older than 1 to 2 months. Immunohistochemistry did not detect expression of insulin, glucagon, somatostatin, pancreatic polypeptide, carboxypeptidase, or trypsin (data not shown). Cytokeratin, which constitutes a family of intermediate filament proteins present in epithelia, was expressed in mPAC cells. The DAKO anti-cytokeratin antibody we used in this study stained ductal epithelium but not other cell types in the pancreas,[7] and this duct and ductule specificity was confirmed on a pancreas from a 2-week-old normal mouse using our immunohistochemistry protocol (data not shown). The cytokeratin staining pattern in the tumors was not homogeneous but rather patchy; both cytokeratin-positive and -negative cells were observed in the same tumor (Figure 2f).

Immunocytochemistry of mPAC Cells

Similar to tumor tissues, immunocytochemistry revealed clear expression of cytokeratin in the mPAC cells in culture (Figure 4a). However, because many animal sera contain natural antibodies against cytokeratin, the intense expression of cytokeratin caused false positivity for other polyclonal antisera. This was not a problem for the immunostaining of tumor tissues, where a higher dilution of the primary antibodies was possible. Hybridoma supernatant (which does not contain cytokeratin autoantibodies) containing a monoclonal antibody recognizing A2B5, a neuroendocrine ganglioside, also stained mPAC cells (Figure 4b). Other monoclonal antibodies recognizing neurofilaments (RT97 and 2H3), or the HNK-1 carbohydrate antigen, did not react (data not shown).

Cytokeratin and the ganglioside A2B5 were expressed in some, but not all, of the cells in a given clonal mPAC culture. These two markers were simultaneously visualized by the use of an fluorescein isothiocyanate-conjugated secondary antibody for cytokeratin and a peroxidase-conjugated secondary antibody for A2B5. A majority of the cytokeratin-positive cells were also A2B5-positive, and vice versa (Figure 4, c and d). However, a few cells (c. <5%) expressed either cytokeratin or A2B5, but not both. Even after rigorous serial single-cell cloning involving micropipetting (see Figure 1 above), the culture always contained both null cells and expressers. The proportion of positive and negative cells seemed to vary from clone to clone; typically, expressers are about 10 to 30% of the population.

Cytokeratin Expression and Proliferation Status

The presence of both null cells and cytokeratin/A2B5 double-positive cells as a stable heterogeneity of the monoclonal mPAC lines raised a question regarding the lineage relationship between these two types of cells. To address this question, mPAC cultures were labeled with BrdUrd for 1 hour and then double-stained at various time points after the BrdUrd pulse with antibodies recognizing BrdUrd and cytokeratin. Four types of cells were observed: cytokeratin-positive cells in S phase, cytokeratin-positive cells not in S phase, and null cells either in or out of S phase

(Figure 4, e and f). The results suggested that both the expressers and null cells are in the proliferative compartment.

Moreover, several pairs of adjacent cells were found after a chase of 24 or 36 hours and judged to be daughter cells derived from a single cell division. Their intensity of BrdUrd incorporation was identical to each other, but clearly differed from the other surrounding cells. Three types of expression patterns were observed for such pairs of daughter cells: 1) both daughters negative for cytokeratin, 2) both positive for cytokeratin, and 3) only one of the two daughters positive for cytokeratin. The presence of the third combination supports the monoclonality of the mPAC cells and suggests that null cells can produce expressers and vice versa.

Tumor-Derived Cell Lines Maintain the Original Phenotype

Cell lines were derived from five tumors representing four different mPAC clones and designated mPAC-T series. These cells grew in epithelioid clusters, much as the original mPAC cells. The mPAC-T cultures were analyzed by immunocytochemistry, which revealed a staining pattern indistinguishable from that of the parental cell lines (Figure 5). The morphology and growth kinetics are also indistinguishable from the original lines, suggesting stability of the transformed phenotype of mPAC upon transplantation and recovery. Therefore, mPAC cells in culture can be regarded as valid representatives of the ductal adenocarcinoma cell type seen *in vivo*.

RNA-PCR and RNA Blot Analysis of Endocrine and Exocrine Markers

The presence of cytokeratin autoantibodies in most preparations of rabbit and guinea pig antisera, as well as in mouse hybridoma ascites fluid, and the high level expression of cytokeratin in the mPAC cells precluded an immunostaining analysis to assess expression of other pancreatic markers in these cells. Therefore, expression of genes encoding major endocrine and exocrine markers was evaluated by RNA-PCR and RNA blotting analyses. Among the four pancreatic hormones, the amounts of insulin and glucagon transcripts were similar to those in negative control cells, such as fibroblasts, as assessed by RNA-PCR. However, both somatostatin and pancreatic polypeptide (PP) seemed to be expressed in the mPAC clones, as revealed by RNA-PCR and by RNA blot analysis. The expression level was low and variable among different clones of mPAC especially for the PP gene (Figure 6). Regarding PP, we have sequenced six independent clones of the PCR product and confirmed that all of them contain a sequence representing the icosapeptide that is specific to the PP gene product,[31] and not present in the closely related genes, NPY[32] and PYY[33] (data not shown). Two exocrine-specific genes, carboxypeptidase A1 and amylase, were negative by RNA-PCR and RNA blotting analysis, respectively (data not shown).

Discussion

We report that epithelial cell lines capable of producing typical pancreatic ductal adenocarcinomas upon

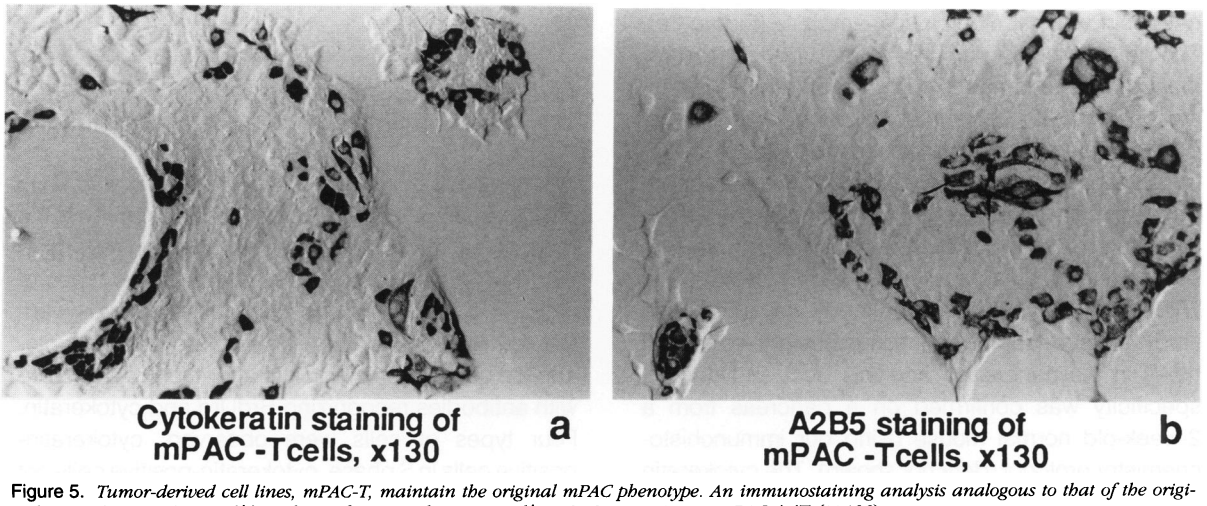

Figure 5. *Tumor-derived cell lines, mPAC-T, maintain the original mPAC phenotype. An immunostaining analysis analogous to that of the original mPAC (Figure 4,* **a** *and* **b**) *is shown for* **a**, *cytokeratin, and* **b**, *A2B5 expression in mPAC-4.4T, (×130).*

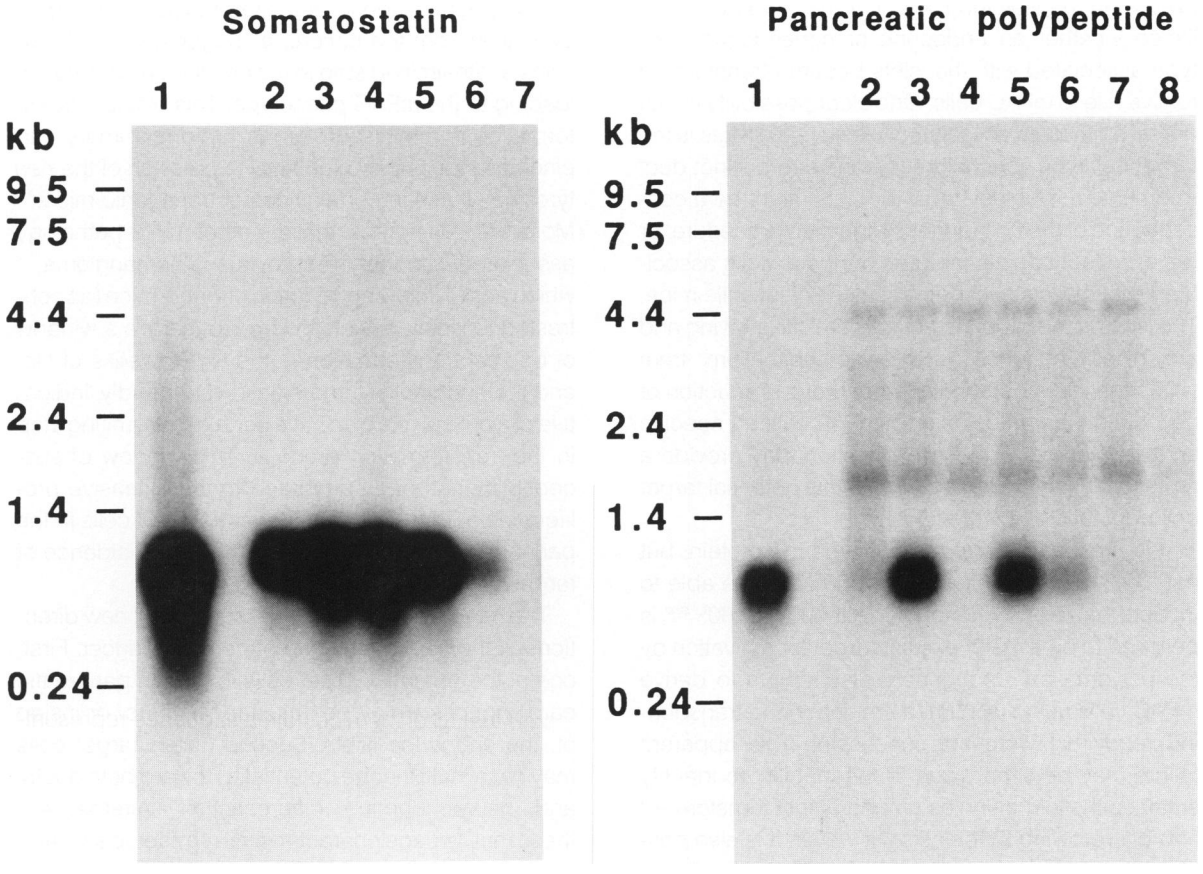

Figure 6. *RNA blotting analysis demonstrates that mPAC cells express somatostatin (left) and PP (right). In the somatostatin panel:* lane 1, *3 μg of total RNA from adult pancreas;* lanes 2 *to* 7, *2 μg of poly(A)$^+$ RNAs from mPAC-5.3, -5.1, -4.4, -4.3, -4.1, and NIH3T3 cells, respectively. The PP panel analyzes the following:* lane 1, *1 μg of total RNA from adult pancreas;* lanes 2 *to* 8, *2 μg of poly(A)$^+$ RNAs from mPAC-5.3, -5.1, -4.1.2.1, -4.4, -4.3, -4.1, and NIH3T3 cells, respectively. The blots were probed with radiolabeled cDNAs for the respective hormone genes. Molecular weight size markers are indicated on the side. The faint bands at 4.4 and 1.8 kb on* lanes 2 *to* 8 *may represent ribosomal RNAs. PP-specific transcript was detected very weakly in clones in* lanes 2 *and* 7.

transplantation can be readily derived from isolated murine islets of Langerhans following infection with a retrovirus carrying polyomavirus mT oncogene. Remarkably, the cells co-express A2B5, a neuroendocrine marker exclusive to islet cells in normal mouse pancreas,[34] and cytokeratin, a marker expressed in the pancreatic duct epithelium. Even triply single cell cloned mPAC lines show a stable heterogeneity of expression of these markers: both double-positive cells and null (or double-negative) cells are evident. Both null and double-positive cells proliferate. In addition, expression of two islet cell type-specific genes, somatostatin and PP, was detectable by RNA blot analysis, albeit at relatively low and variable levels among different clones. In contrast, insulin, glucagon, and several exocrine enzyme genes were not expressed. The implication of these results is that mPAC cells may represent a previously unrecognized stage or cell type in the pancreatic cell lineage(s). Alternatively, the cellular phenotype induced by mT may result from a specific dedifferentiation of mature islet or ductal cells into cells with the observed multi-lineage phenotype.

The "amphicrine" or multi-lineage potential of duct adenocarcinoma has been suggested by case reports of human pancreatic cancers with both exocrine and neuroendocrine characteristics.[35–37] Among established adenocarcinoma cell lines, the azaserine-induced rat pancreatic acinar carcinoma cell line AR42J has been the only example of combined exocrine and neuroendocrine properties; this cell line contains zymogen granules as well as synaptophysin-positive small neuroendocrine vesicles and typical neurotransmitters, including γ-aminobutyric acid.[38]

It is possible that the target cell of polyoma mT transformation represents the one of the *in vivo* target cells of human pancreatic ductal carcinogenesis, which seems to arise with several etiologies through distinct pathways. Because small ducts can be

present in the isolated islets, it remains to be established whether an endocrine or nonendocrine cell type associated with the islets became transformed to give rise to mPAC cells. One clear possibility is that ductal epithelium associated with juvenile islets is the target cell type. Our failure to transform distinct duct fragments not associated with islets might be a consequence of the collagenase digestion procedure, or rather reflect special features of ductal cells associated with the islets, especially those of juvenile mice. The islets in these young animals are still growing and are initially attached to the duct epithelium, from which the islets bud out in the *de novo* production of new islets. Characterization of mPAC cells for specific surface markers or gene expression may provide a route to identify and characterize this potential target cell population.

It is provocative that the polyoma mT protein, but not SV40 large T antigen nor pp60$^{c\text{-}src}$ were able to induce the mPAC phenotype. Although pp60$^{c\text{-}src}$ is believed to be a major cellular target for activation by the polyoma mT oncogene, our attempts to derive mPAC cells using tsNY68, a *src* oncogene transducing retrovirus, were not successful. This apparent failure may be simply due to the inability to identify small epithelioid colonies among avidly transformed and overgrowing fibroblasts. However, it is also possible that activation of c-*src* is not sufficient to obtain mPAC cells, implicating other cellular signal transduction pathways mobilized by the polyoma mT oncogene.[39] In this context, it is noteworthy that a recent report described the successful transformation of *src*-deficient fibroblasts and induction of hemangiomas in *src*-deficient mice by polyoma mT oncoprotein.[40] It has been reported that the polyoma mT protein is complexed predominantly to serine/threonine phosphatase 2A,[29,30] as well as to an 85-kd protein that is the regulatory subunit of phosphatidylinositol-3 kinase[41–43] and the family of *src*-like tyrosine kinases.[44–47] The mT oncoprotein may well serve to organize these proteins into an active signaling complex. In addition to *src*, *yes* and *fyn* become phosphorylated in association with mT.[44,46,47] It will be of interest to determine which of these kinases are expressed in mPAC cells and activated by mT, as well as to assess expression and activity of serine/threonine phosphatase 2A and phosphatidylinositol-3 kinase.

The reported alterations of the p53, *RB*,[4] or K-*ras*-1[11] genes in human pancreatic ductal carcinoma cells motivate examination of these genes in mPAC cells. However, the high frequency with which mPAC lines arise, and their stability upon 1) single cell cloning and continuing passage, 2) generation of adenocarcinomas upon inoculation into mice, and 3) rederivation from the tumors, all suggest that mT satisfies a rate-limiting step in the cellular transformation leading to the mPAC phenotype. This efficient transformation is reminiscent of the rapid mammary carcinomas induced by high level expression of the *neu* tyrosine kinase in certain lines of transgenic mice.[45] Moreover, it is notable that the other tumor pathology associated with this mT-retrovirus is hemangioma,[19] which arises following infection of whole mice (as contrasted to isolated islets). Here too there is a window of optimal transformation in the first 2 weeks of life, and the endothelial tumors arise very rapidly, indicative of a critical role of mT at a certain rate-limiting step in the transformation process. This window of susceptibility has been attributed to the extensive proliferation and differentiation of endothelial cells in the perinatal period, suggesting a maximal incidence of target cells for transformation by mT.[19]

The mPAC phenotype suggests several new directions in the study of human pancreatic cancer. First, one of the potential target cells in human pancreatic carcinogenesis may be in juxtaposition to, or inside of, the endocrine islets. Second, these target cells may have multilineage potentiality, toward both ductal and neuroendocrine differentiation. Alternatively, these multilineage characteristics may serve as markers for the cell type whose dedifferentiation results in pancreatic ductal carcinogenesis. Third, 2-week-old juvenile islets were most sensitive to transformation by the polyoma mT retrovirus to generate mPAC. This result suggests that one of the possible mechanisms of human pancreatic carcinogenesis entails a carcinogenic insult that hits the transitory target cells associated with islet neogenesis from the duct in juveniles, initiating a pathway toward eventual development of ductal cancer in adults. Such a model would require that some aspect of the pancreatic environment normally suppresses the initial adenocarcinoma proliferation until adulthood (perhaps until other necessary events relieve that suppression). Such putative suppression is not evident in the isolated islet cultures from which mPAC cells rapidly arise, implicating a paracrine or endocrine suppressor mechanism *in vivo*. In this context, it is notable that pancreatic tumors (or other pathology) have not been observed in transgenic mice expressing mT under control of its natural promoter,[48] the immunoglobulin heavy chain enhancer,[49] or the insulin-promoter (V Bautch and DH, unpublished observations). Moreover, infection of newborn or adult mice with the same polyoma mT retrovirus used in this study also fails to elicit pancreatic pathology.[19] Altogether these negative results, albeit circumstantial, are consistent with

the suggestion that expression of the mT oncogene is not sufficient to initiate *in vivo* tumorigenesis in the pancreas, in lieu of additional requisite events. In conclusion, this study implicates a transitory cell type associated with juvenile islets that may represent both a progenitor to differentiated pancreatic cell types and a target for pancreatic cancer. The rapid and reproducible elaboration by the mT oncogene of mPAC cells evidencing a ductal adenocarcinoma phenotype suggests that signal transduction systems activated by this oncogene might play an important role in human pancreatic carcinogenesis. The candidates include the *src*-related tyrosine kinases, phosphatidylinositol-3 kinase, and serine/threonine phosphatase 2A, none of which have been thoroughly analyzed in human pancreatic cancers to date.

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