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Pancreatic tumor pathogenesis reflects the causative genetic lesion

(islands of Langerhans/*myc*/oncogene/transgenic mice)

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ABSTRACT **Transgenic mice in which c-*myc* expression is targeted to pancreatic acinar cells develop mixed acinar/ductal pancreatic adenocarcinomas between 2 and 7 months of age. This contrasts with the effect on pancreas of the simian virus 40 tumor antigen or activated ras, which in adult mice causes lesions composed exclusively of acinar-like cells. Furthermore, during an early stage of *myc*-induced pathology, transformed acinar-derived cells appear within islets, suggesting that islet hormones may influence the progression of these exocrine pancreatic tumors. These findings demonstrate that the initial oncogenic alteration can influence the pattern of subsequent tumor pathogenesis and, given that human exocrine pancreatic tumors are predominantly ductal adenocarcinomas, support the suggestion that transformed acinar cells may contribute to the genesis of this serious disease in man.**

For most cells, the differentiated state is phenotypically stable, and even tumors usually display a variable but distinct resemblance to surrounding normal tissue. Nevertheless, under certain conditions, transformed cells appear to lose their differentiated character or undergo metaplastic conversion into a different type of cell (1, 2). Given the considerable diversity among oncogenes and tumor suppressor genes, the targets of the genetic alterations that accompany tumor development (3–5), it is reasonable to suppose that this variability in tumor phenotype may reflect the identity of the underlying genetic lesions. Alternatively, given that critical oncogenic lesions are superimposed upon the genetic program of a (presumably) differentiated cell, differentiated characteristics intrinsic to that cell may direct progression of a tumor regardless of the tumor's cause.

Tumors of the exocrine pancreas illustrate the importance of understanding the relative influence of these potential determinants of tumor phenotype. In the rat or mouse pancreas, both preneoplastic lesions and tumors are generally composed of cells that resemble acinar cells (6, 7). In the hamster, preneoplastic lesions are visible among both pancreatic acinar and duct cells, but tumors are almost exclusively ductal adenocarcinomas (6). These observations suggest that the cell of tumor origin may vary within as well as among species and also that the resultant tumors may not always reflect the identity of that cell. Ninety percent of human pancreatic tumors are classified as ductal adenocarcinomas (8). However, because diagnosis usually identifies only advanced tumors, precursor lesions in man are generally inaccessible. Thus, for the human species, the issues of whether pancreatic tumors necessarily resemble their cell of origin and to what extent the inciting genetic lesions can influence tumor morphology become critical to an understanding of the onset and progression of this lethal disease.

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Transgenic mouse technology (for review, see ref. 9) allows an assessment of the relative contribution of oncogenes versus intrinsic tissue characteristics to tumor pathogenesis. We have examined (10, 11) the effect on pancreas of the simian virus 40 tumor antigen (TAg) and a mutant human Harvey *ras* by using the rat elastase enhancer/promoter to direct the expression of each oncogene specifically to acinar cells in transgenic mice. TAg induced multiple preneoplastic lesions and exocrine pancreatic tumors composed of acinar-like cells in young adult mice (10). *ras* induced tremendous acinar hyperplasia in fetal pancreas and subsequent death of affected neonates (11). However, a small number of founder mice appeared normal and survived, apparently because they expressed a lower level of transgene *ras* in fetal pancreas. Adult offspring of these mice developed focal preneoplastic acinar lesions and, infrequently, acinar cell tumors (E.P.S., unpublished observations). Therefore, in the adult mouse, acinar cell-specific expression of either TAg or *ras* induced tumors that resembled the initially altered cell, consistent with a primary role in tumor pathogenesis for intrinsic tissue characteristics that can act independently of the tumor's initiating genetic cause and that in this example preserve the identity of the transformed cell.

We now report the results of experiments designed to determine the influence on pancreas of dysregulated c-*myc* expression. Rather than reproducing the pathogenesis induced by TAg or ras, myc induces a unique pattern of pancreatic pathology, indicating that the nature of the initiating oncogenic alteration can have a critical influence upon important aspects of tumor pathogenesis.

MATERIAL AND METHODS

Fusion Gene Construction and Production of Transgenic Mice. To construct the *Ela-1–myc* transgene, the 2.7-kilobase (kb) *Xba* I–*Xho* I fragment of murine c-*myc*, which includes the entire protein coding region within exons 2 and 3 (12), was cloned between (*i*) the 3-kb rat elastase 1 (*Ela-1*) gene *Stu* I–*Kpn* I fragment that includes the enhancer and promoter (13) and (*ii*) the 0.3-kb human growth hormone gene *Sma* I–*Sph* I fragment that includes the 3′ untranslated and poly(A) addition sequences (14). The *Kpn* I site is at about position +25 relative to the elastase transcription start site. The construct was built up in pUC vectors such that unique sites would flank the transgene. The 6-kb *Eco*RI–*Hin*dIII fragment was isolated and microinjected into fertilized (C57BL/6 × SJL)F_2 mouse eggs to produce transgenic mice, as described (15). Founder mice were identified using a nick-translated human growth hormone probe. The three lines described in this paper were assigned the following genetic designations: 1194-2, Tg(*Ela-1*,Myc)Bri158; 1195-3, Tg(*Ela-1*,*Myc*)Bri159; 1195-4, Tg(*Ela-1*,*Myc*)Bri160.

Abbreviation: TAg, simian virus 40 tumor antigen.

mRNA Determination. Total nucleic acid was isolated from frozen tissue samples, as described (16). The abundance of transgenic myc or endogenous elastase transcripts was assayed by solution hybridization (16) using ^{32}P-labeled oligonucleotide probes.

Histology and Immunohistochemistry. Tissues were fixed in Bouin's fixative for 1–4 hr, transferred to 70% ethanol, then processed, embedded in paraffin, cut at 5 μm, and stained with hematoxylin and eosin or alcian blue/periodic acid Schiff. Some fixed tissues were embedded in glycol methacrylate and cut at 2 μm prior to staining. Immunohistochemistry was performed on 5-μm deparaffinized tissue sections using a peroxidase-antiperoxidase kit that detects rabbit antibodies (Dako, Santa Barbara, CA). Incubations with primary antisera were performed overnight at room temperature. The following primary antisera were employed: rabbit anti-mouse amylase at a dilution of 1:100 (kindly provided by Miriam Meisler, University of Michigan, Ann Arbor); rabbit anti-mouse laminin at a dilution of 1:100 (Sigma); and prediluted rabbit anti-bovine muzzle keratins, guinea pig anti-porcine insulin, rabbit anti-porcine glucagon, and rabbit anti-human somatostatin (Dako). As a negative control, tissues were incubated overnight with nonimmune rabbit serum (Dako). Staining was detected using diaminobenzidine or aminoethylcarbazole as chromogenic substrates.

RESULTS

Generation of *Ela-1–myc* Transgenic Mice and Analysis of Transgene Expression. Transgenic founder mice were produced bearing the elastase 1 (*Ela-1*)–*myc* fusion gene shown in Fig. 1. This construct differs from an earlier reported elastase 1–*myc* construct (11) in that the 3′ noncoding region including the poly(A) addition site of the *myc* gene, which is associated with instability of the resulting mRNA (17), has been replaced by a similar region from the human growth hormone gene that encodes a more stable mRNA. In addition, the construct was injected into mouse eggs without adjoining plasmid DNA, which can interfere with transgene expression (18). A portion of the pancreas was surgically removed from each founder mouse at 4 weeks of age and assayed for the presence of transgene mRNA. The three mice displaying highest expression, 1194-2, 1195-3, and 1195-4, were mated to generate lines of offspring for additional study.

Pancreatic nucleic acid was isolated from mice in lines 1195-3 and 1195-4 at several ages, and transgene mRNA was measured (Fig. 2). Transgene expression in line 1194-2 was not examined in detail but was similar to line 1195-3 at 1 month of age (data not shown). Pancreatic *myc* expression severely inhibited the accumulation of endogenous elastase mRNA (Fig. 2), suggesting that myc interferes with postnatal acinar cell differentiation. No transgene expression could be detected in any other tissue assayed, including liver, kidney, spleen, lung, and stomach (data not shown).

Expression of *myc* in Acinar Cells Induces Acinar and Ductal Neoplasia. *Ela-1–myc* transgenic mice became moribund between 2 and 7 months of age (Fig. 3). Upon necropsy, the majority of mice in each line displayed several pale, generally firm pancreatic masses up to 2 cm in diameter. Remaining portions of the pancreas appeared atrophied, and in some mice only a shrunken pancreatic remnant was present. Most

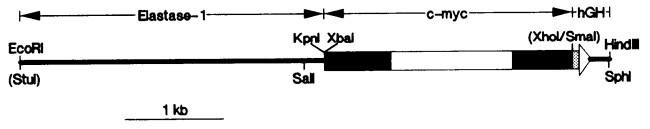

FIG. 1. Restriction map of elastase–*myc*. hGH, human growth hormone.

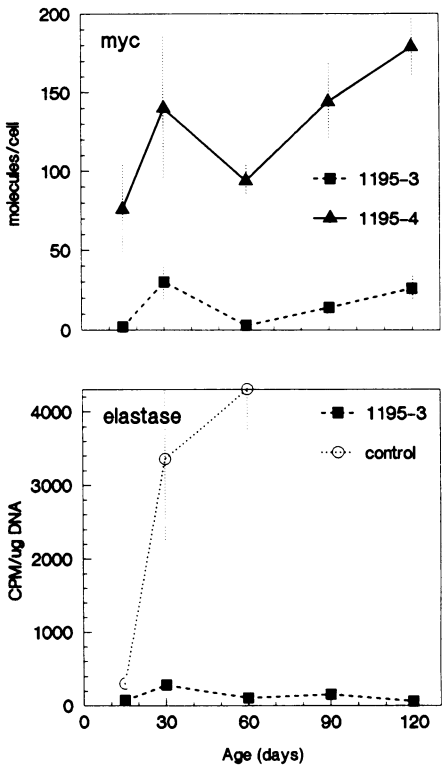

FIG. 2. Pancreatic mRNA. Transgene myc mRNA (molecules per cell, ref. 16) and endogenous elastase mRNA (cpm/μg of DNA, proportional to molecules per cell) were measured in pancreases of transgenic and control mice at several ages. Data are presented as mean ± SEM. Control mouse pancreases were all negative for transgene mRNA.

mice at this stage had yellowish–tan intestinal contents and feces, a few were jaundiced, and 1 in 10 mice displayed tumor spread to peritoneal surfaces or liver.

Approximately one-half of the tumors representing end-stage disease that were examined microscopically displayed features typical of acinar cell carcinomas (Fig. 4*a*). Unexpectedly, the remaining one-half of the tumors were composed of a mixture of acinar-like and duct-like cells embedded in a dense stroma (Fig. 4*b*). Focal areas in some of these tumors exhibited squamous metaplasia or adenosquamous carcinoma. The tumors were frequently associated with a marked inflammatory cell infiltrate.

Two approaches were used to further examine the molecular characteristics of tumor cells. When stained with alcian blue/periodic acid Schiff, many of the ductal cells present in

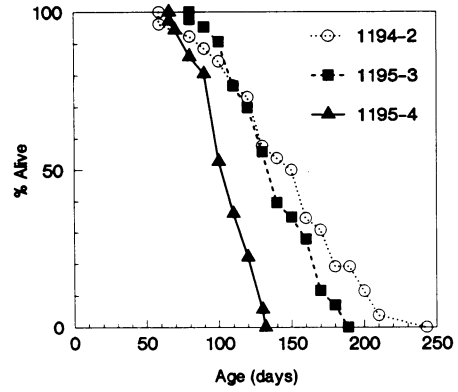

FIG. 3. *Ela-1–myc* transgenic mouse survival curves. Each curve illustrates the range of ages at which transgenic mice died or became moribund (n = 26 to 43 for each line).

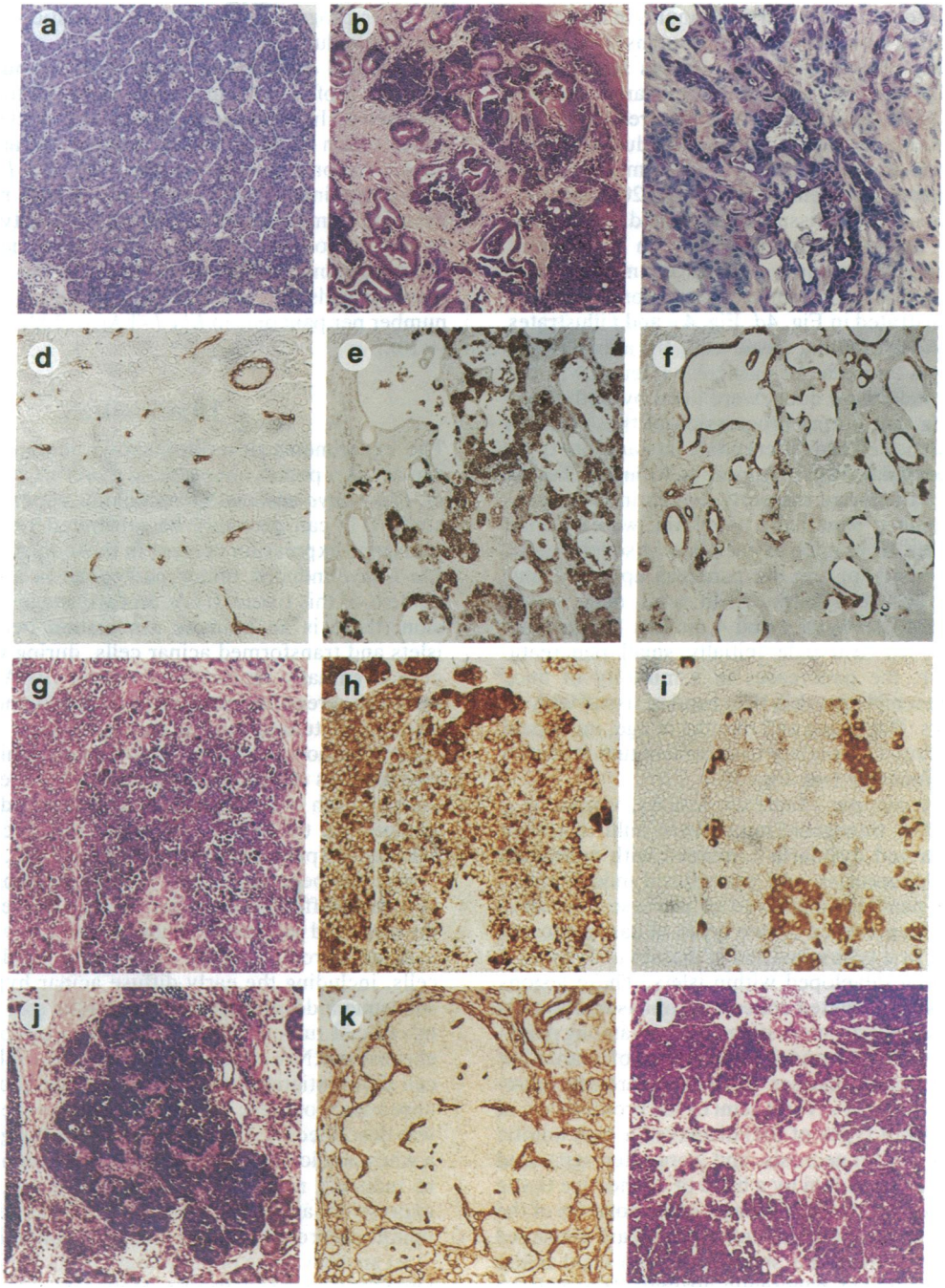

FIG. 4. Histopathology and immunohistochemistry of *myc*-induced pancreatic lesions. (*a*) Acinar cell carcinoma present in a 116-day-old line 1195-3 transgenic male. Tumor cells are not arranged in typical acini, but otherwise resemble differentiated acinar cells. (Hematoxylin and eosin; ×30.) (*b*) Mixed acinar/ductal pancreatic adenocarcinoma present in a 130-day-old line 1195-3 transgenic female. Note the marked fibroplasia. A focus of squamous metaplasia is present at the upper right. (Hematoxylin and eosin; ×24.) (*c*) Mixed acinar/ductal pancreatic adenocarcinoma from a 116-day-old line 1195-3 male stained with alcian blue/periodic acid Schiff. The deep purple stain in the apical portion of cells lining ducts indicates the presence of mucins. (×60.) (*d*) Normal mouse pancreas treated with anti-keratin antiserum. Only ductal and centroacinar cells stain positively with this antiserum. (×60.) (*e*) Mixed pancreatic adenocarcinoma from a 132-day-old line 1194-2 transgenic female treated with anti-amylase antiserum. Note that cells lining many of the ducts as well as cells within solid masses stain positively with this antiserum. (×24.) (*f*) Parallel section to that shown in *e* treated with anti-keratin antiserum. Note that only cells lining ducts stain positively with this antiserum. (×24.) (*g*) Basophilic cell masses in the pancreas of a 61-day-old line 1195-4 transgenic female. Several relatively normal-appearing acini are present at the extreme upper left in the figure. A mass of cells surrounding isolated pale-pink islet cells is present to the right and center. The many small clear spaces that contain debris represent sites of focal cell death. To the left is a solid mass of acinar-like cells that lack the usual pancreatic architecture. (Hematoxylin and eosin; ×60.) (*h*) Parallel section to those shown in *g* and *i* treated with anti-amylase antiserum. Note the intense cytoplasmic staining in the relatively normal acinar cells above and the reduced staining in basophilic cells below. (×60.) (*i*) Parallel section to those shown in *g* and *h* treated with anti-insulin antiserum. Remaining β cells are present only in small clumps or as single cells. (×60.) (*j*) An islet largely replaced by basophilic cells in a 72-day-old line 1194-2 transgenic female. (Hematoxylin and eosin; ×32.) (*k*) Parallel section to that shown in *j* treated with anti-laminin antiserum. Note that the laminin-positive islet border appears intact. (×32.) (*l*) Focus of ductal cells present within an acinar cell carcinoma in a 143-day-old line 1195-3 transgenic male. Note the association of the ductal structures with a localized increase in connective tissue. (Hematoxylin and eosin; ×24.)

tumors displayed intense blue to purple granules in the apical cytoplasm, indicating the presence of mucins (Fig. 4*c*). Mucins are a family of complex glycoproteins secreted by cells lining epithelial surfaces, including some larger pancreatic ducts (19). Cells containing mucins are frequently observed within human and hamster pancreatic ductal adenocarcinomas, but acinar cells and their transformed counterparts do not express these molecules (19, 20). Second, unstained sections of tumor tissue were treated with rabbit antisera directed against either amylase, an acinar cell marker, or certain cytokeratins, a duct cell marker. The specificity of the anti-cytokeratin antiserum for pancreatic duct cells is demonstrated in Fig. 4*d*. Fig. 4 *e* and *f* illustrates representative staining of tumor tissue using the anti-amylase and anti-cytokeratin antisera. Of particular interest was the finding that some individual ducts were lined by both types of cells, suggesting that during progression of these tumors one type of pancreatic cell can differentiate into another type.

Islets and Extracellular Matrix Appear to Influence Tumor Progression. To characterize the early events in the pathogenesis of *myc*-induced pancreatic neoplasia, we examined pancreases collected from mice in each line at several ages. In general, by 1 month of age the pancreas appeared uniformly thickened and firm, although in some mice it was smaller than normal. Between 1 and 4 months of age most mice developed 1–10 firm pale initially small pancreatic nodules. However, the pancreases in a minority of mice remained small, resembling a thin firm band of tissue with no focal enlargements. Beginning at 2 months of age, individual mice became moribund secondary to excessive tumor burden or pancreatic dysfunction (see Fig. 3).

The earliest microscopic pancreatic lesions, visible by 1 month in line 1195-3 transgenic mice, were mild to severe acinar hyperplasia and dysplasia combined with multifocal single-cell death (apoptosis), and generalized mild fibrosis. By 2 months, the pancreas displayed an increase in interacinar stroma plus the presence of two types of basophilic cell foci (Fig. 4*g*). The first type consisted of masses of dysplastic acinar-like cells that developed within islets. These lesions first appeared as small isolated collections of basophilic cells lining the islet's periphery, suggesting an origin among neighboring acinar cells. The second type consisted of similar but generally less basophilic masses of cells apparently representing the coalescence of several acini or the proliferation of cells within one. Both types of foci displayed a high mitotic index, developed central necrosis, and were often separated from surrounding acini or foci by a band of connective tissue of variable thickness. Also in this stage, other portions of the pancreas (and in some mice the entire pancreas) displayed aggregates of normal-appearing small ducts with scant acinar tissue, evidence of acinar pancreatic atrophy. When tissue sections were treated with anti-amylase antiserum, the acinar nature of cells within the basophilic foci became evident (Fig. 4*h*). Anti-insulin antiserum identified scattered β cells within islets that had been largely replaced by the basophilic acinar-derived cells (Fig. 4*i*). Similar results were obtained using anti-glucagon and anti-somatostatin antisera (data not shown). The islet-associated change is also illustrated in Fig. 4*j*. The islet's original border appears to have remained largely intact, as indicated by the continuous band of immunoreactive laminin surrounding the islet (Fig. 4*k*).

Focal acinar cell adenomas and carcinomas developed in mice between 2 and 6 months of age. These lesions generally resembled those present in adult *Ela-1*–TAg and *Ela-1–ras* transgenic mice, in that most neoplastic cells were distinctly acinar-like. However, unlike tumors in those models, the *myc*-induced tumors frequently displayed small to large foci of variably severe fibrosis, and it was within this microenvironment of stromal proliferation that the earliest duct-like lesions were observed (Fig. 4*l*). At no stage during the course of tumor progression were preneoplastic lesions evident in pancreatic ducts.

As a final experiment, pancreatic cell nuclei from transgenic mice of several ages were analyzed for ploidy by flow cytometry. In contrast to pancreatic nuclei of *Ela-1*–TAg mice, which display first a uniform tetraploidy and then unique tumor nodule aneuploidy (10), *Ela-1–myc* pancreatic nuclei remained diploid (data not shown), ruling out a contribution of marked chromosomal instability to the development of tumors in this model. Since genomic alterations may promote tumor progression (21, 22), this discrepancy between models may explain the ≈10-fold difference in tumor number per pancreas in *Ela-1*–TAg (≈100) versus *Ela-1–myc* (≈10).

DISCUSSION

The experiments described above demonstrate that c-*myc* can act as a potent oncogene in exocrine pancreas. The lack of *Ela-1–myc*-associated pathology reported in an earlier study (11) can probably be attributed to the low level of transgene expression present in those mice. Two features of *Ela-1–myc*-induced tumor pathogenesis are distinct in the context of this tissue. (*i*) At an early stage of tumor progression, there is an intimate association between pancreatic islets and transformed acinar cells, during which acinar-like cells eventually replace islet tissue. (*ii*) At a late stage of tumor progression, neoplastic ductal elements arise within acinar cell tumors and progress to form mixed or ductal adenocarcinomas. Although the relationship between these two features of pathogenesis is unclear, the consistent presence of both only in those tumors induced by *myc* demonstrates that the initiating genetic lesion can influence the subsequent pattern of tumor development.

myc has been implicated in the regulation of cell proliferation and differentiation (for review, see ref. 23). Several of the observed pancreatic lesions in *Ela-1–myc* transgenic mice indicate chronic growth stimulation of developing acinar cells, including the early diffuse acinar hyperplasia and the subsequent development of basophilic foci and of acinar and mixed-cell tumors. The extreme depression of endogenous elastase mRNA content may reflect an inhibition of acinar cell differentiation, consistent with the poorly differentiated appearance of these cells. An *in vivo* parallel to these findings was described by Andres *et al.* (24) in transgenic mice bearing a whey acidic protein promoter–*myc* fusion gene. In addition to inducing mammary adenocarcinomas, initiation of *myc* expression appeared both to promote replication and to inhibit differentiation of mammary epithelium.

At an early stage of pathology, islets are infiltrated by cells apparently derived from neighboring acini. Because of the existence of an islet–acinar portal blood system, hormone-rich blood bathes the islet-associated or periinsular acinar cells, which in turn are larger and exhibit greater synthesis of selected acinar cell products than more distant acinar cells (25). Insulin is believed to be the hormone responsible for this effect (25). In *Ela-1–myc* transgenic mice, insulin may increase elastase enhancer/promoter activity and *Ela-1–myc* expression and it may also cooperate with myc to stimulate acinar cell mitosis (26). Periinsular acinar cells could thus have a marked growth advantage, perhaps accounting for the directed growth of these cells into islets. This finding raises the possibility that an association between islets and periinsular tumor cells may be an important mediator of the progression of certain spontaneous pancreatic tumors in other species that arise in the vicinity of islets.

Our findings also address the issues of tumor cell plasticity and the origin of pancreatic cancer. There is a longstanding debate concerning whether transformed pancreatic acinar cells can generate ductal neoplasms. In part this stems from

the observation that, in man, adenocarcinomas with a ductal appearance account for >90% of exocrine pancreatic tumors despite the fact that only ≈10% of all pancreatic cells are duct cells, compared to the 80% that are acinar cells (27). The several points of view regarding the cell of origin in the hamster model, in which carcinogen-induced neoplasms are also ductal, are succinctly presented by Flaks (28), who favors an acinar cell origin, by Pour (29), who favors a duct cell origin, and by Scarpelli *et al.* (30), who argue that both acinar and duct cells can give rise to ductal tumors. In the *Ela-1–myc* model, there is no evidence of early lesions involving pancreatic ducts. Nevertheless, at a later stage in tumor progression, ductal elements appear within acinar cell tumors. The histological and immunohistochemical features of tumors confirm the ductal character of these cells but suggest as well that they are derived from transformed acinar cells, a reversal of the duct to acinar cell transition that occurs during pancreatic organogenesis (31). The alterations we have observed do not follow *in vivo* transformation of the pancreas by TAg or *ras* (10, 11), indicating a specific contribution of *myc* to this process. We note, however, that among mice inoculated at birth with a murine retrovirus encoding v-*myc* a small fraction were shown to develop pancreatic tumors composed exclusively of acinar-like cells (32).

The mechanism underlying this apparent plasticity of acinar cell differentiation in *Ela-1–myc* transgenic mice remains obscure. It is intriguing that, in the whey acidic protein–*myc* transgenic model (24), *myc*-transformed mammary adenocarcinoma cells induced surrounding stromal cells to produce the matrix protein tenascin, which could in turn influence the behavior of neoplastic epithelium. In the *Ela-1–myc* model, stromal remodeling within tumors may be sufficient to redirect acinar differentiation, providing a dramatic example of how tumor-associated matrix might affect the pattern of differentiation of transformed epithelium. Alternatively, the association between *myc*-transformed periinsular acinar cells and islets may affect the cells' eventual behavior. Finally, the tumors initiated by *myc* are focal and thus presumably reflect collaboration of additional genetic or epigenetic lesions that may also influence pathogenesis.

A final consideration concerns the relevance of this model to the study of human pancreatic neoplasia. Pancreatic tumors in man are primarily ductal adenocarcinomas, but it has been difficult to identify the cell type of origin because this disease is usually diagnosed at a late stage of progression. It is clear that ductal adenocarcinomas can arise from transformed duct cells (29). The major issue is whether the appearance of ductal tumors in man might reflect in some cases the neoplastic progression of an initiated acinar cell (33). In view of our findings, this possibility cannot be excluded, and *Ela-1–myc* transgenic mice should provide an outstanding model of this process.

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