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Acinar-to-ductal metaplasia accompanies c-myc-induced exocrine pancreatic cancer progression in transgenic rodents

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Several important characteristics of exocrine pancreatic tumor pathogenesis remain incompletely defined, including identification of the cell of origin. Most human pancreatic neoplasms are ductal adenocarcinomas. However, acinar cells have been proposed as the source of some ductal neoplasms through a process of acinar-to-ductal metaplasia. The oncogenic transcription factor c-myc is associated with human pancreatic neoplasms. Transgenic mice overexpressing c-myc under control of acinar cell-specific elastase (Ela) gene regulatory elements not only develop acinar cell carcinomas but also mixed neoplasms that display both acinar-like neoplastic cells and duct-like neoplastic cells. In this report, we demonstrate that, first, c-myc is sufficient to induce acinar hyperplasia, though neoplastic lesions develop focally. Second, cell proliferation remains elevated in the neoplastic duct cell compartment of mixed neoplasms. Third, the proliferation/apoptosis ratio in cells from all lesion types remains constant, suggesting that differential regulation of these processes is not a feature of cancer progression in this model. Fourth, before the development of mixed neoplasms, there is transcriptional activation of the duct cell-specific cytokeratin-19 gene promoter in multicellular foci of amylase-positive acinar neoplasms. This observation provides direct evidence for metaplasia as the mechanism underlying development of ductal neoplastic cells within the context of an acinar neoplasm and suggests that the stimulus for this transformation acts over a multicellular domain or field within a neoplasm. Finally, focal ductal elements develop in some acinar cell carcinomas in Ela-c-myc transgenic rats, indicating that myc-associated acinar-to-ductal metaplasia is not restricted to the mouse.

The hallmark of cancer is excessive tissue growth, but neoplastic tissue also can differ from normal tissue in other ways. Anaplasia, or loss of cellular and tissue differentiation, is observed commonly in malignant neoplasms and their metastases. In other neoplasms, the pattern of differentiation may be redirected. The result is metaplasia, the replacement of one type of differentiated cell by another. Both anaplasia and metaplasia can obscure the identity of the neoplasm's cell of origin.

We and other workers have reported that c-myc-induced transformation of transgenic mouse acinar cells produces both acinar and mixed acinar/ductal neoplasms.[1–4] The latter were unexpected, given the transgene targeting strategy, which used the acinar cell-specific elastase (Ela) enhancer/promoter. Neoplastic duct-like cells first were identified in small foci associated with localized fibrosis within Ela-c-myc acinar cell carcinomas. Eventually, large neoplasms developed that were composed of cytokeratin-19 (CK19)-positive ductal epithelial cells embedded in a fibrous stroma and that contained focal collections of amylase-positive acinar-like neoplastic cells. These observations supported suggestions that acinar-to-ductal metaplasia could develop within acinar cell carcinomas.[5]

Subsequent to that study, we[6] and other workers[7,8] demonstrated that acinar cell targeted expression of mutant Kras caused acinar-to-ductal metaplasia in non-neoplastic pancreas, leading to preneoplastic pancreatic intraductal neoplastia. Cells lining these structures display morphological features of ductal epithelium. Similarly, transgenic mice overexpressing the epidermal growth factor receptor ligand transforming growth factor alpha (TGFα) in pancreatic acinar cells developed severe pancreatic fibrosis and displayed multifocal acinar-to-ductal metaplasia.[9–12] Note that metaplasia associated with Kras mutation and TGFα overexpression leads to preneoplastic lesions, whereas c-myc-associated metaplasia appears to develop within an existing neoplasm.

Several lines of evidence implicate c-myc in pancreatic cancer pathogenesis, including carcinogenic activity of c-myc in pancreatic cancer cell lines,[13–16] and amplification of the c-myc locus and/or frequent c-myc overexpression in

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pancreatic neoplasms.[17–20] In this report, we revisit the Ela-c-myc model to address several important unanswered questions about c-myc-induced pancreatic carcinogenesis. First, we evaluate cell turnover in lesion subtypes. Second, we describe a tumor cell phenotype that combines features of acinar cells and duct cells, providing direct evidence that mixed neoplasms in this model develop *via* intratumoral metaplasia. Finally, we examine tumor progression in Ela-c-myc rats, to identify whether metaplasia can accompany pancreatic c-myc-induced tumor development in a second species. Our findings indicate that metaplasia in pancreatic cancer is a complex phenomenon: it is expressed in different cellular contexts and at different stages of progression depending on the molecular etiology of the disease.

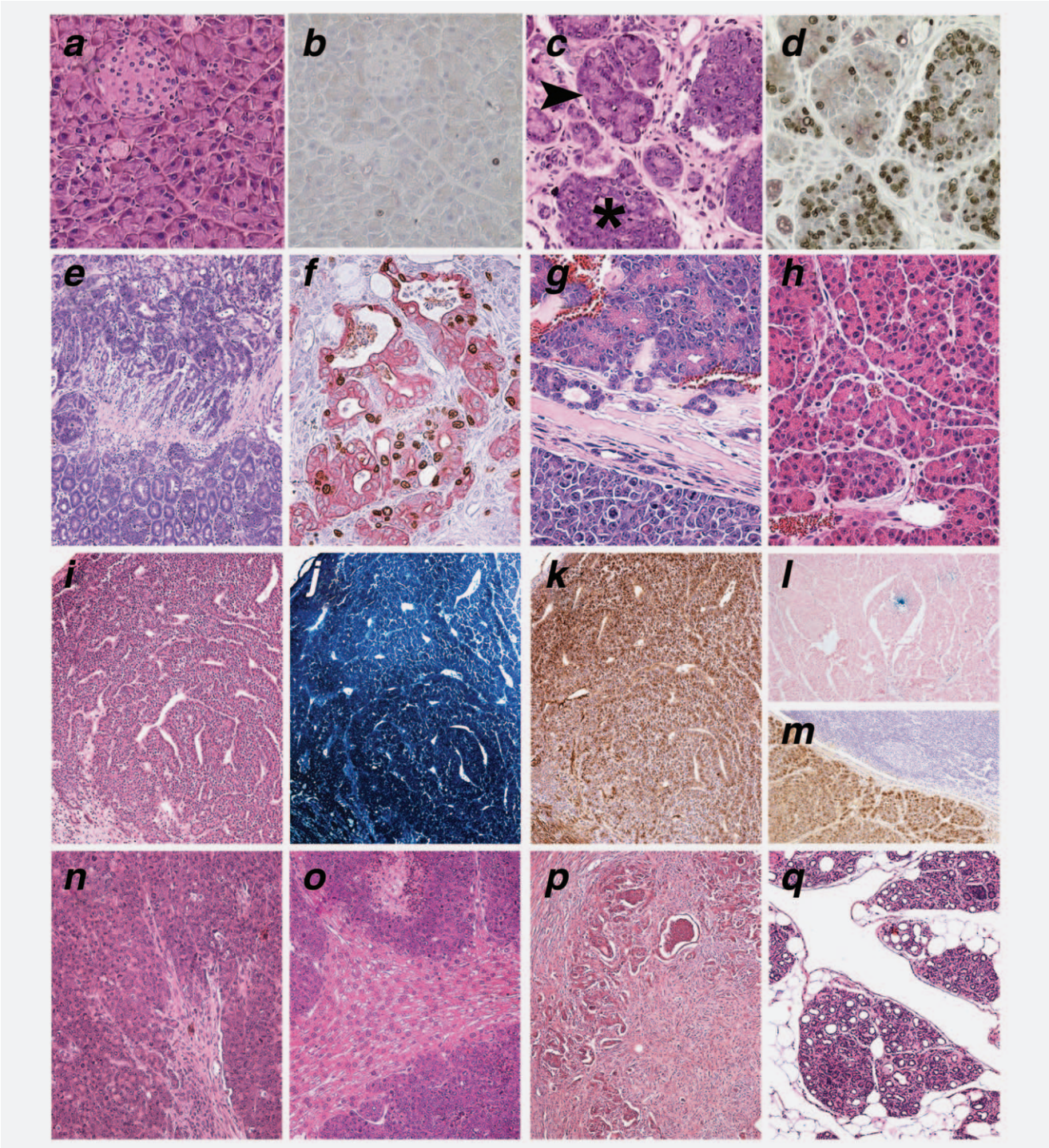

Figure 1.

Material and Methods

Experimental animals

Animals were housed in AAALAC-accredited facilities and provided with standard rodent chow and water *ad libitum*. Experimental procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee. The Ela-c-myc transgenic mouse line 1195-3 has been described[2–4] and assigned the genetic nomenclature TgN(Ela1Myc)159Bri. It is congenic in the C57BL/6J strain. The 30-1 line of Ela-c-myc rats was generated using the same transgene and has been assigned the genetic nomenclature TgN(Ela1Myc)22EPS. FVB/N strain transgenic mice expressing the CK19-hPAP transgene, which combines CK19 gene regulatory sequences with DNA encoding human placental alkaline phosphatase (hPAP), have been assigned the genetic nomenclature TgN(CK19ALPP)6EPS.[21] These mice express the hPAP marker gene in cells that transcribe endogenous CK19. In normal pancreas, expression is limited to ductal epithelia and occasional centroacinar cells. All transgenic animals were identified *via* PCR analysis of tissue DNA.

Tissue procedures

Animals were administered 200 mg/kg bromodeoxyuridine (BrdU; Sigma Chemical, St. Louis, MO) intraperitoneally, then euthanized 1–2 hr later. BrdU is a nucleotide analog that is incorporated into DNA during S-phase of the cell cycle. For immunohistochemistry, fixed and sectioned tissues were treated with rat monoclonal anti-BrdU (Accurate Chemical & Scientific, Westbury, NY) at 1:40; rat monoclonal anti-CK-19 (TROMA3, gift of Rolf Kemler, Max Planck Institute, Freiburg, Germany) at 1:100; rabbit anti-pancreatic amylase (Sigma) at 1:500.

BrdU labeling index, proportional to S-phase index, was determined by counting BrdU-labeled nuclei within a lesion and dividing by the total number of nuclei identified in the section. For most structures, all nuclei (up to 500) were counted. Apoptotic index was determined by morphology. An apoptotic cell was identified using the following criteria: darkly basophilic and/or fragmented nuclei, loss of cytoplasmic detail and/or cytoplasmic blebbing and cell shrinkage relative to surrounding cells. Apoptotic index was calculating by dividing the number of apoptotic cells by the total number of nuclei identified in the section. Again, for most structures, all nuclei (up to 500) were counted. All apoptotic counts were performed in non-necrotic areas of lesions.

To detect hPAP, unstained sections of tissue fixed in 4°C Carnoy's fixative were incubated with the hPAP substrate BCIP (5-bromo-4-chloro-3-indolylphosphate; Sigma) as described.[21]

Results

Cell turnover in Ela-c-myc transgenic mouse pancreas

By 5 months of age, all Ela-c-myc transgenic mice require euthanasia due to development of exocrine pancreatic neoplasms. For this report, we classified lesion histotypes as a function of age and quantified BrdU labeling and apoptotic indices. At all ages, areas of exocrine pancreas with normal architecture were present (compare Fig. 1*a* with Fig. 1*c*). However, acini were hyperplastic compared to nontransgenic mouse pancreas, and both BrdU labeling index and apoptotic index were increased dramatically in acinar cells (Table 1 and Figs. 1*b* and 1*d*). At 7 weeks, all mice also displayed small (<0.2 mm diameter) to medium (0.2–1.0 mm diameter) focal lesions composed of moderately to poorly differentiated acinar-like cells that were smaller and more basophilic than normal acinar cells and that did not retain typical acinar architecture. These lesions were solid to glandular (Fig. 1*c*) and displayed a 3-fold increase in both BrdU and apoptotic indices relative to the more normal-appearing adjacent acinar tissue (Fig. 1*d* and Table 1). Between 10 and 14 weeks of age, all mice developed basophilic acinar neoplasms that differed from basophilic acinar hyperplasias only by size (>1.0 mm) and their frequent ability to invade into surrounding tissues

Figure 1. Microscopic characteristics of Ela-c-myc-induced exocrine pancreatic lesions. (*a*) Nontransgenic adult mouse pancreas. H&E. (*b*) Adjacent section to *a*, treated immunohistochemically to detect BrdU. (*c*) Ela-c-myc 9–week-old transgenic mouse pancreas, showing hyperplastic acini (arrowhead) and small basophilic acinar hyperplasias (asterisk). H&E. (*d*) Adjacent section to *c*, treated immunohistochemically to detect BrdU. (*e*) Acinar cell carcinoma invading into intestinal stroma and epithelium (center and bottom). H&E. (*f*) CK19-positive duct-like neoplastic cells showing frequent nuclear labeling with BrdU (dark brown); treated immunohistochemically to detect CK19 and BrdU. (*g*) Well-differentiated basophilic acinar neoplasm (top) adjacent to basophilic acinar neoplasm (bottom). H&E. (*h*) Well-differentiated eosinophilic acinar neoplasm. H&E. (*i*) Acinar cell carcinoma in 17-week-old Ela-c-myc/CK19-hPAP bitransgenic mouse. H&E. (*j*) Adjacent section to *i*, treated with BCIP to detect hPAP (blue reaction product). (*k*) Adjacent section to *i* and *j*, treated immunohistochemically to detect amylase (brown). (*l*) Section of a different acinar cell carcinoma from the Ela-c-myc/CK19-hPAP bitransgenic mouse used for *i–k*, treated with BCIP to detect hPAP. Note the lack of blue reaction product, indicating lack of transcription from CK19 gene regulatory elements. Nuclear fast red counterstain. (*m*) Section of acinar cell carcinoma (bottom) and spleen (top) from the Ela-c-myc/CK19-hPAP bitransgenic mouse used for *i–l*, treated immunohistochemically to detect amylase. Amylase immunoreactivity is present in the acinar cell carcinoma but absent from spleen. Hematoxylin counterstain. (*n*) Acinar cell carcinoma from a 10-month-old Ela-c-myc transgenic rat. H&E. (*o*) Liver metastases present in the rat used for *n*; note compression of and invasion into hepatic parenchyma (center) by neoplastic cells (top and lower right). H&E. (*p*) Nests of duct-like neoplastic pancreatic epithelial cells surrounded by extensive fibrosis in an 8-month-old Ela-c-myc transgenic rat. H&E. (*q*) Exocrine pancreatic atrophy and tubular complex formation in a 21-month-old Ela-c-myc transgenic rat. H&E. Original magnifications: *a–d*, *f–h*, 400×; *e*, *i–m*, *p*, *q*, 100×; *n*, *o*, 200×.

Table 1. Lesion incidence and cell turnover in Ela-c-myc transgenic mice

Lesion type[1]	Incidence at >10 weeks	BrdU index $X \pm$ SD (n)	Apop. Index $X \pm$ SD (n)	Index ratio
Nontransgenic control				
Acinar	–	0.46 ± 0.17 (4)	0 ± 0 (4)	–
Ductal[2]	–	0 ± 0 (4)	0 ± 0 (4)	–
Transgenic, normal architecture				
Acinar	–	17 ± 8.8(17)	3.5 ± 2.1 (14)	4.9
Ductal[2]	–	0.52 ± 0.47 (17)	0.13 ± 0.52 (16)	4.0
Basophilic acinar hyperplasia	17/17 (100%)			
<0.2 mm	–	49 ± 12 (16)	10 ± 4.6 (12)	4.9
0.2–1.0 mm	–	48 ± 14 (12)	10 ± 4.7 (10)	4.8
Basophilic acinar neoplasm	17/17 (100%)	49 ± 16 (8)	9.0 ± 0.1 (7)	5.4
Mixed neoplasm	13/17 (77%)			
Acinar component	–	41 ± 10 (13)	8.8 ± 4.2 (13)	4.7
Ductal component[2]	–	19 ± 6 (13)	3.7 ± 3.7 (13)	5.1
Well-differentiated basophilic acinar neoplasia	4/17 (24%)	6.4 ± 1.1 (4)	1.6 ± 1.4 (4)	4.0
Well-differentiated eosinophilic acinar neoplasia	3/17 (18%)	17 ± 17 (3)	3.1 ± 3.1 (3)	5.4

[1]Refer to corresponding photomicrographs in Figure 1. [2]Defined as ductal morphology and CK19 positive on immunohistochemistry.

(Table 1, Fig. 1*e*). Mice also displayed mixed neoplasms, composed of diffusely intermingled acinar (identified using morphology and amylase immunoreactivity) and ductal (identified using morphology and CK19 immunoreactivity) elements (Fig. 1*f*). In this series of mice, 13 of 28 neoplasms examined (46%) were classified as mixed. Two additional lesion histotypes were identified less frequently in older mice (Table 1, Figs. 1*g* and 1*h*).

We made two additional observations. First, primary ductal lesions of any size were not present in the tissue sections examined, ruling out ductal epithelium as a common source of duct-like neoplastic cells in this model. Second, the ratios of BrdU labeling index to apoptotic index for all lesion types in transgenic mice (including hyperplastic exocrine pancreas that retained normal architecture) were similar (range 4.0–5.4; Table 1). This finding indicates that lesion progression did not involve a marked shift in the relative rates of cell birth and death.

Acquisition of ductal characteristics during progression of acinar lesions

The morphological appearance of tumors suggested metaplasia rather than biclonality as the origin of lesions with both ductal and acinar character. In particular, the presence of discrete structures within tumors that contained both acinar-like and duct-like cells supported this conclusion.[3] Therefore, we examined the pattern of gene expression to determine whether transitional cells could be identified in Ela-c-myc induced tumors. Ela-c-myc transgenic mice were mated with CK19-hPAP transgenic mice, and bitransgenic offspring were examined to identify marker transgene activation. Our objective was to determine if CK19 gene transcription was activated in a subset of acinar-like cells in Ela-c-myc-induced neoplasms. This finding would provide molecular support for the conclusion that pathogenesis of mixed neoplasms involved an acinar-to-ductal cell transition, *i.e.*, metaplasia. Architecturally normal acini in transgenic mice displayed CK19 expression only in centroacinar cells. As expected, CK19-hPAP was expressed strongly in ductal elements of each of seven mixed Ela-c-myc neoplasms examined (see Fig. 1*f*). Remarkably, we also observed activation of the marker transgene in large regions of acinar-like tissue in 24 of 41 (59%) basophilic acinar neoplasms from 17 bitransgenic mice examined (Figs. 1*i* and 1*j*). hPAP expression usually was multifocal within each affected neoplasm. Presence of immunohistochemically detectable amylase, an acinar cell marker, confirmed the acinar character of the cells (Fig. 1*k*; for controls see Figs. 1*l* and 1*m*). Only six of 42 (14%) basophilic hyperplastic lesions (<1 mm in diameter) displayed faint hPAP expression, indicating that activation of CK19 expression was more frequent in large lesions and thus was not predominantly an early event in progression. In general, both molecular and morphological markers of metaplasia (CK19 gene activation and presence of neoplastic duct cells, respectively) were restricted to poorly differentiated basophilic neoplasms.

Ela-c-myc transgenic rats display a related lesion phenotype

To determine whether development of c-myc-induced mixed pancreatic neoplasms was specific to the mouse, we generated transgenic rats carrying the Ela-c-myc transgene. One line (30-1) was examined in detail. Rats in this line survived for 15 ± 3.7 months ($X \pm$ SD; 16 examined), then developed rapid weight loss. At necropsy, 13 of 16 displayed one or two reddish, friable pancreatic masses up to 3 cm in diameter. In

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nine of these 13 rats (69%), multiple masses also were present in liver and/or diffusely throughout peritoneal surfaces (Figs. 1*n* and 1*o*), indicating highly efficient tumor metastasis. Microscopically, lesions were composed of moderately to poorly differentiated acinar cells, arranged in solid to glandular patterns, consistent with the diagnosis of acinar cell carcinoma, but tumors in three mice displayed focal regions of increased stroma associated with duct-like epithelial elements (Fig. 1*p*). Other lesions identified in rats were lobular acinar cell atrophy and tubular complex development (Fig. 1*q*) and occasional focal acinar hyperplasia. Exocrine pancreatic atrophy also is a feature of Ela-c-myc transgenic mice, though usually it does not involve whole lobes.

Discussion

We have demonstrated transcriptional activation of the duct-specific CK19 gene in neoplastic acinar-like (amylase-positive) cells in Ela-c-myc transgenic mice. This finding identifies a class of intermediate cells within neoplasms with features of both acinar and ductal cells, implicating acinar-to-ductal metaplasia as a prominent and frequent feature of c-myc-induced exocrine pancreatic carcinogenesis. This process also can occur in Ela-c-myc transgenic rats. Furthermore, CK19 transcription significantly precedes morphological evidence of ductal metaplasia within neoplasms. The latter observation indicates that "transcriptional metaplasia" occurs before development of the fibrosis that always accompanies the ductal component of tumors in this model.[3] Therefore, the focal changes in tumor stroma must not initiate metaplasia. We also observed activation of CK19 transcription in metaplastic lesions that are precursors to ductal carcinoma *in situ* in Ela-mutant Kras transgenic mice.[6] However, in that Kras model, metaplasia produced early, preneoplastic lesions, whereas Ela-c-myc-associated metaplasia occurs within tumors.

We can describe several characteristics of the signals that induce metaplasia. First, they appear only in response to activation of specific signaling pathways, including c-myc, as acinar cell carcinomas induced by the simian virus 40 T-antigens do not display metaplasia.[22] Nevertheless, c-myc overexpression alone is not sufficient to induce this effect, since it occurs in a subset of neoplasms: in the context of cancer, c-myc acts as a predisposing agent for metaplasia. Overexpression of TGFα also induces acinar metaplasia, although in the context of both non-neoplastic acini[9,10,12] and pancreatic neoplasms.[11] Interestingly, TGFα overexpression also causes pancreatic fibrosis. Second, induction of metaplasia appears to involve a "field effect," as CK19 activation occurs in multiple, often large regions within Ela-c-myc-induced acinar neoplasms. A field effect can be defined as a measurable change in phenotype (here, the pattern of gene expression) that is present throughout a spatially continuous region of tissue, rather than localized to individual cells. This field effect could be caused by (i) an environmental cue, such as hypoxia, that affected a large portion of a tumor, (ii) cell-to-cell transport (*via* gap junctions) of a signal originating focally, although cell–cell communication typically is reduced in neoplasms and/or (iii) metaplasia occurring in a predisposed neoplastic clone of cells, but this latter mechanism still would require a signal to initiate the process. Finally, although acinar-to-ductal metaplasia must start *via* activation of duct-specific genes in acinar-like cells, the sequence of subsequent molecular and morphological changes (and the role of c-myc in these changes) remains to be defined. Fibrosis still may regulate final stages of the transition if early metaplastic cells produce a signal that increases stroma, which could signal back to epithelium to complete the morphological transition to duct-like cells. Interestingly, Ge *et al*. recently have proposed a mechanistic link between stroma and epithelium during field cancerization in oral cancer.[23] However, the Ela-c-myc neoplasm "field metaplasia" differs from the classical field cancerization concept, in that the latter refers to preneoplastic lesions.[24]

The cell turnover data address four important aspects of c-myc-induced pancreatic carcinogenesis. First, c-myc overexpression is sufficient to induce increased DNA synthesis and apoptosis. Second, focal lesion development is accompanied by additional 3-fold increases in BrdU labeling and apoptotic indices, indicating that the additional cellular change(s) that accompany tumor progression involve coordinated, proportional changes in both aspects of cell turnover. Third, basophilic acinar lesions, regardless of size, display nearly identical BrdU labeling and apoptotic indices, in addition to their morphological similarity, suggesting that they may represent different sizes of a single class of lesion rather than a series progressing from preneoplasia to neoplasia. If so, then this class of neoplasm may arise directly from the hyperplastic but architecturally normal acinar tissue without a morphologically distinct intermediate. Fourth, the process of acinar-to-ductal metaplasia does not extinguish proliferation and apoptosis in ductal cancer cells. Thus, decreased Ela gene transcription and corresponding decreased Ela-c-myc transgene expression associated with loss of acinar differentiation[3] did not eliminate the transforming stimulus in these ductal cells: both acinar and ductal elements in the mixed neoplasms maintain cell turnover characteristics of neoplastic cells.

Finally, the 30-1 line of transgenic rats differed from line 1195-3 transgenic mice in several ways: exocrine pancreatic tumor latency was increased, tumor multiplicity was decreased, incidence of metastasis was increased and a much smaller fraction of acinar neoplasms displayed ductal metaplasia. Nevertheless, the appearance of several mixed neoplasms indicates that overexpression of c-myc can orchestrate ductal metaplasia in rat as well as mouse.

In summary, the Ela-c-myc transgenic models demonstrate that acinar-to-ductal metaplasia can occur within neoplasms during mouse and rat exocrine pancreatic carcinogenesis initiated by c-myc, likely through a mechanism involving localized signals within the tumor

microenvironment. In view of the strong carcinogenic reaction to overexpression of c-myc in exocrine pancreas of these species, even slight chronic elevation of c-myc in a subset of pancreatic epithelial cells may be sufficient to initiate carcinogenesis in humans.

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