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Histological complexities of pancreatic lesions from transgenic mouse models are consistent with biological and morphological heterogeneity of human pancreatic cancer

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Summary

Although pancreatic cancer is the fourth leading cause of cancer death, it has received much less attention compared to other malignancies. There are several transgenic animal models available for studies of pancreatic carcinogenesis, but most of them do not recapitulate, histologically, human pancratic cancer. Here we review some detailed molecular complexity of human pancreatic cancer and their reflection in histomorphological complexities of pancreatic lesions developed in various transgenic mouse models with a special concern for studying the effects of chemotherapeutic and chemopreventive agents. These studies usually require a large number of animals that are at the same age and gender and should be either homozygote or heterozygote but not a mixture of both. Only single-transgene models can meet these special requirements, but many currently available models require a mouse to simultaneously bear several transgene alleles. Thus it is imperative to identify new gene promoters or enhancers that are specific for the ductal cells of the pancreas and are highly active in vivo so as to establish new single-transgene models that yield pancreatic ductal adenocarcinomas for chemotherapeutic and chemopreventive studies.

Keywords

Carcinogenesis; Animal model; Molecular signaling

Introduction

Pancreatic cancer (PC) is the fourth leading cause of cancer death in the United States and many other western countries. According to 2006 statistics of American Cancer Society, more Americans die of pancreas cancer than prostate cancer (American Cancer Society, 2006; Jemal et al., 2006). Pancreatic cancer has dismal prognosis; most patients are dead within six months after diagnosis, and the five-year survival rate is less than 5% in most countries, mainly because the tumors are usually diagnosed only at an advanced stage and are resistant to various treatments (American Cancer Society, 2006; Jemal et al., 2006). The vast majority of human PCs are ductal adenocarcinomas, whereas acinar cell carcinomas and other histological types are much less common.

The cell origin of ductal adenocarcinomas is still under debate (Pour et al., 2003). Some studies have suggested that it arises from metaplasia (transdifferentiation) of acinar cells or even endocrine(islet) cells to ductal cells leading to ductal adenocarcinoma (Pour et al., 2002; Schmid, 2002; Miyatsuka et al., 2006). While this has been the impression based on

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cell-lines and animal studies, observations on human carcinomas, however, imply a different scenario. Hyperplastic and dysplastic epithelial lesions of the pancreatic ducts have been identified frequently in association with ductal adenocarcinomas (Cubilla and Fitzgerald, 1979; Andea et al., 2003), and these are now referred to as pancreatic intraepithelial neoplasia (PanINs) (Hruban et al., 2001, 2004). These evidences in human pancreas suggest that ductal adenocarcinomas may simply be originated from ductal cells, although it cannot be ruled out that ductal metaplasia of other cell types, especially acinar cells (transdifferentiation or formation of ductular structures) could also be involved in the development of ductal adenocarcinoma under different situations. It is certain that the etiology of pancreatic ductal adenocarcinomas is fairly complex and poorly understood. Here we provide an overview of the histological and molecular complexities of pancreatic neoplasia as they apply to and correlate with different animal models, with a focus on transgenic mice, and their potential usefulness for developing preventive and/or therapeutic strategies.

Histological and genetic complexities of PC

There are several chemical- or transgene-induced animal models of pancreatic carcinogenesis available. The morphologic traits of pancreatic lesions from most transgenic models have been reviewed by a bevy of pathologists in a workshop in 2004 (Hruban et al., 2006b) and further summarized in a recent review article (Hruban et al., 2006a). However, few of these transgenic models have really been studied in a systematic manner, and thus the morphologic details related to age, gender, genetic background, and hetero-/homo-zygous status are inadequate. Moreover, ultra-structural data are basically lacking as well. Human PC exhibits changes in a variety of genetic markers (Maitra et al., 2006). In order to understand the complexities of histological findings in humans and their complex reflection in different animal models of PC, one must also appreciate the molecular complexities of PC. Therefore, we shall first summarize what is known thus far regarding the role of specific genetic markers relevant to human and animal PC.

EGFR signaling with TGFα as the preferred ligand plays crucial roles in PC

It has been reported that 43%, 46% and 54% of human PC show over-expression of epidermal growth factor receptor (EGFR), EGF and TGFα, respectively (Yamanaka et al., 1993). A marked increase in EGFR expression (Friess et al., 1999; Tobita et al., 2003) or a concomitant expression of EGFR with its ligand EGF, TGFα or amphiregulin (Kleeff et al., 2000) has been shown to be associated with a poor prognosis of PC. Other reports also confirm high expression of both TGFα and EGFR in human PC (Korc et al., 1992; Srivastava et al., 2001). *In vitro* studies showed that EGF, TGFα and amphiregulin significantly enhanced the proliferation of human PC cell lines (Liu et al., 1998). TGFα expressed in human PC cells is secreted to the circulation and excreted into the urine, which leads to a suggestion that measurement of serum or urinary TGFα levels may have a prognostic value as tumor marker (Chuang et al., 1994; Moskal et al., 1995). Conversely, spontaneous or butyrate-induced differentiation of cultured human PC cells is associated with decrease in the expression of TGFα (Estival et al., 1992). TGFα has been shown to be expressed in ducts, acini and islets of the human fetal pancreas (Miettinen and Heikinheimo, 1992; Hormi and Lehy, 1994) and is considered to be important for development of the organs that undergo branching, such as the mammary gland, lung and pancreas. However, our own immunohistochemical staining (Liao et al., 2006) revealed that in addition to the cells of ductal adenocarcinomas (Fig. 1a), TGFα was expressed only in ductal cells, not acinar cells, in the normal tissue adjacent to the cancer. However, "ductalized" acini in the adjacent tissue also showed positive staining (Fig. 1b), suggesting that TGFα is also expressed in acinar-ductal metaplasia. These results indicate that in adult pancreas, probably

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only ductal cells retain the ability to express TGFα. Moreover, TGFα positivity in the lineage from normal ductal cells to acini-ductal metaplasia and ductal adenocarcinomas seems to support the notions that the cancer can originate from ductal cells and ductal-acinal metaplasia.

In one interesting study, a stronger immunohistochemical staining, relative to the adjacent normal tissue and chronic pancreatitis, was observed in 95% of the tumors for TGFα but only in 12% of the tumors for EGF (Barton et al., 1991). An *in vitro* study also showed that TGFα was more potent than EGF in enhancing the anchorage-independent growth of several human PC cell lines (Korc, 1991). It has been considered that PC cells may be more sensitive than normal pancreatic cells in response to TGFα (Kullenberg et al., 2000). Carcinogen-induced pancreatic cancer in the hamster and rat expressed only TGFα and EGFR, but not EGF (Visser et al., 1995, 1996). Administration of TGFα to the hamsters bearing xenograft tumors of hamster-derived ductal PC cells induced DNA synthesis, whereas treatment with EGF failed to do so (Morita et al., 1998). Collectively, these data suggest that TGFα may be the preferred growth factor over EGF or other EGFR ligands for normal ductal cells and cancer cells of the pancreas (Vaughan et al., 1992, 1993; Barnard et al., 1995; Giraud, 2000). TGFα/EGFR singling is well known to initiate Ras signaling (Harris et al., 2003) and induce transcription of cyclin D1 (Yan et al., 1997) and many other genes to effect cancer promotion. Thus the cross-talk between TGFα/EGFR singling with other cellular signaling pathways may play important roles in human PC and is thereby considered an important target for the development of animal models of PCs.

Human PC shows high frequencies of overexpression and amplification of c-myc

Voluminous studies have shown that c-*myc* is over-expressed at high frequencies in various human cancers because it is frequently amplified or it is a downstream effector of many growth signaling pathways such as Ras, Notch, TGFα/EGFR, NF-κB or PI3-Akt signaling that are known to be activated in various types of malignancies, including PC (Bachireddy et al., 2005; Mimeault et al., 2005). Overexpression of c-*myc* mRNA (Han et al., 2002) and protein (Schleger et al., 2002) has been found in 50% and 43.5% of human pancreatic adenocarcinomas, respectively. In our immunohistochemical study, 13 of 15 ductal adenocarcinomas showed strong nuclear staining of *c-myc* in about 20-70% of cancer cells (Fig 1c,d). Gene amplification occurs in about one-third of the human PC biopsies (Schleger et al., 2002), accounts for the aberrant expression in certain cases (Adsay et al., 1999), and is positively associated with the tumor grade (Nagy et al., 2001). In one report, 54% and 28% of 31 PC cell lines show c-*myc* and cyclin D1 gene amplification, respectively (Mahlamaki et al., 2002), whereas another study showed concomitant amplification of activated K-ras and c-*myc* in both primary tumor and lymph node metastasis (Yamada et al., 1986). These data suggest that *c-myc* may frequently collaborate with K-ras or its downstream effector cyclin D1 during formation or progression of human PC (Brackett et al., 2003; Asano et al., 2004; Holzmann et al., 2004). Animal studies have also revealed that PC induced by chemical carcinogen in the rat manifests increased c-*myc* overexpression (Silverman et al., 1990; Calvo et al., 1991). Schleger et al reported that *c-myc* overexpression was found in 43.5% of the primary pancreatic adenocarcinomas but only in 31.6% of the metastatic tumors (Schleger et al, 2002). A possible explanation for the slightly lower rate of c-*myc* overexpression in the metastases could be due in part to the fact that a lower c-*myc* level provides survival advantage, since *c-myc* expression can signal apoptotic cell death (Ponzielli et al., 2005).

Alterations in c-myc have dual prognostic values for various types of cancer

Numerous studies have been published addressing the prognostic values of overexpression and/or amplification of c-*myc* in various malignancies, but the data are still largely

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controversial and confusing. Positive, null and negative correlation of c-*myc* overexpression or amplification with prognosis or patient survival of various types of cancer has been reported (Sklar and Prochownik, 1991; Mizutani et al., 1994; Chana et al., 1999, 2001; Donzelli et al., 1999; Arango et al., 2001, 2003; Soh et al., 2002; Vijayalakshmi et al., 2002; Akervall et al., 2003; Chang et al., 2003; Grover et al., 2003; Nagy et al., 2003; Vora et al., 2003; Yu et al., 2003). Good prognostic value of c-*myc* is exemplified by the reports that colon cancer patients with amplified c-*myc* gene show improved disease-free and overall survival in response to 5-fluorouracil (5-FU) treatment (Donzelli et al., 1999) whereas down-regulation of c-*myc* using c-*myc* anti-sense decreases the sensitivity of colon cancer cells to 5-FU (Arango et al., 2001). The dual functions of c-*myc*, i.e. promotion of both cell proliferation and apoptosis (Liu and Levens, 2006), may be one of the main reasons for the controversial reports.

We have previously observed that in the mammary tumors from MMTV-c-*myc* transgenic mice, the c-*myc* transgene is silenced when some tumor cells progress to a more aggressive phenotype, coined as “tumor focus”, that is less apoptotic and more proliferative (Liao et al., 2000). It has also been reported that expression of c-*myc* transgene may not be needed for sustaining the mammary, pancreatic islet and skin tumors (Boxer et al., 2004; Pelengaris et al., 2004), although it may not be the case for *c-myc* transgenic tumors in other tissues (Jain et al., 2002; Shachaf et al., 2004). These data raise a question whether in certain types of cancer, *c-myc* plays its role in a “hit-and-run” fashion for some unique reason that tumor cells need to silence the c-*myc* to decrease its apoptotic potential when they advance to a certain stage (Liao and Dickson, 2000). If this is proved to be the case by more thorough studies, it may explain why roughly half of the cases of the human PCs and other types of cancer do not show elevated c-*myc* expression at diagnosis: in some of the *c-myc* negative cases, *c-myc* might have been elevated initially and then silenced to gain survival advantage. If some tumor cells somehow cannot silence the c-*myc* during advanced stages of carcinogenesis then *c-myc*-induced apoptosis may lead to a good prognosis.

A second reason for the controversial reports could be related to the interactions of *c-myc* with different protein factors under different situations. For instance, many studies have shown that concomitant expression of *c-myc* and TGFα in double transgenic mouse models makes mammary (Liao et al., 2000; Liao and Dickson, 2000), liver (Cavin et al., 2005) and pancreatic (Sandgren et al., 1993; Liao et al., 2006) tumors with less apoptotic and more proliferating cells relative to the tumors expressing *c-myc* alone. *C-myc* may suppress cyclin D1 expression and NF-κB activity whereas TGFα relieves the repression (Liao and Dickson, 2000; Cavin et al., 2005), which may be a mechanism behind the synergy between *c-myc* and TGFα. In one clinical study, the better prognosis was seen in c-*myc* overexpressing colorectal carcinomas but it was offset when there was concomitant p53 mutations (Smith and Goh, 1996). Collectively, these data suggest that c-*myc* expression may not always need to be silenced for cancer cells to acquire more survival ability in advanced stages. Concomitant expression of some survival genes such as TGFa or cyclin D1 or silencing of some pro-apoptotic genes such as p53 may also render a survival advantage via mechanisms that remain to be fully elucidated.

Apoptosis may be an important part of the mechanism for c-myc-induced carcinogenesis

Most proto-oncogenes have a function to drive cell proliferation, but few, if any, of them are as potent as *c-myc* in induction of apoptosis, although some of them (such as Ras and E2F1) have been shown to have certain ability of inducing apoptosis as well (Cox and Der, 2003; Bell and Ryan, 2004). A second trait that distinguishes *c-myc* from most other proto-oncogenes is its potent carcinogenicity in transgenic mouse models. For most proto-oncogenes (not viral oncogenes), each alone either fails to induce cancer in transgenic

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models or induces cancer only at a very low frequency with a long latent period, which is likely because of the need of alterations in a second gene. Besides the *K-ras* gene, which can also induce apoptosis, c-*myc* seems to be the only proto-oncogene that can induce cancer formation in most transgenic mouse models established to date, some of which yield tumors at 100% penetrance with a relatively short latent period. Tumors from most, if not all, of these transgenic models show pronounced apoptosis with distinctive morphology that is coined by us as "dead cell island"(Liao and Dickson, 2003). Therefore, it is possible that *c-myc* can efficiently induce carcinogenesis not only because it can drive cell proliferation, which many other proto-oncogenes can do as well, but also because it is the most potent apoptosis inducer among known proto-oncogenes.

There are two major types of cell proliferation, i.e. compensatory and hyperplastic proliferation, in solid organs such as the liver, pancreas and kidney (Ledda-Columbano et al., 1993; Columbano and Shinozuka, 1996). Compensatory proliferation is actually regeneration, usually triggered by tissue loss from surgical removal of part of the organ, chemical- or physical-induced cell death, etc. The proliferation ceases when the organ has restored its physiological size and number of cells. If the cell loss continues, such as in a situation of continuous exposure to chemical hazard or constant expression of apoptosis-inducing genes like *c-myc*, the regenerative proliferation also becomes constant. In contrast, hyperplastic proliferation is usually trigged by direct growth stimuli, such as treatment with or expression of growth factors (Ledda-Columbano et al., 1993; Columbano and Shinozuka, 1996). This type of cell proliferation results in redundancy of the cells and enlargement of the organ; the proliferating cells usually undergo apoptosis, presumably because the organ attempts to eliminate the redundant cells and retain its physiologic size. While initiation of carcinogenesis requires at least one round of cell replication to fix mutations of critical genes into the daughter cells before the mutations are repaired, promotion step of carcinogenesis requires many rounds of cell replication to propagate the initiated cells and to undergo malignant transformation. Ample animal studies have demonstrated that only compensatory proliferation can efficiently facilitate initiation and promotion of cancer formation, whereas hyperplastic proliferation is a very poor stimulus for these steps of carcinogenesis (Ledda-Columbano et al., 1993; Farber, 1995, 1996a,b; Columbano and Shinozuka, 1996; Laconi et al., 2001), presumably because the proliferating cells will undergo apoptosis (Laconi et al., 2001). It is likely that *c-myc*-induced apoptosis provides the organ or tissue a constant need for compensatory proliferation, which in turn drives those non-apoptotic cells to proliferate continuously while undergoing malignant transformation.

It is completely unknown that in the target organ of a c-*myc* transgenic mouse model, which c-*myc* expressing cells will undergo apoptosis and which cells will undergo proliferation and malignant transformation. Recent studies with transgenic drosophila have shown that this selection may not be a random event. Neighboring cells are shown to compete with each other on their c-*myc* levels, and the losers, i.e. the cells with lower *c-myc* activity, are actively eliminated via apoptotic death, while the winners in this so-called "cell competition" undergo compensatory proliferation (de la et al., 2004; Donaldson and Duronio, 2004; Moreno and Basler, 2004; Gallant, 2005). These results again suggest that *c-myc*-induced apoptosis and ensuing compensatory proliferation are of importance for carcinogenesis (Donaldson and Duronio, 2004; Gallant, 2005), and thus the *c-myc* transgenic animal model of PC is a good model but has certain limited value as discussed below.

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Different animal models of pancreatic carcinogenesis have different strengths and weaknesses

Pancreatic carcinogenesis can be induced by chemical carcinogens in animals, as summarized in several reviews (Hotz et al., 2000; Standop et al., 2001; Wei et al., 2003; Leach, 2004). Of the animal models, hamster PCs induced by N-nitros-bis-(2oxopropyl) amine most resemble human PC (Schneider et al., 2001; Wei et al., 2003) because the tumors are ductal phenotypes with obvious fibrosis, progress rapidly with strong invasion potential, and metastasize to the liver (Hotz et al., 2000; Wei et al., 2003). Moreover, the hamster tumors also show high frequency of K-ras mutations as seen in human PC (Konishi et al., 1998; Hotz et al., 2000; Wei et al., 2003). In contrast, pancreatic tumors induced by the same chemical or other carcinogens in rats and mice are usually acinar cell carcinomas (Hotz et al., 2000; Wei et al., 2003) and rarely show K-ras mutations (Hotz et al., 2000; Wei et al., 2003). These species differences become the first intrinsic weakness of chemical-induced models in rodents since it raises a concern on the human relevance of these models and chemicals. Second, the carcinogens are used at high doses and for long periods of time, which does not occur in humans and thus has little relevance to humans in the context of etiology of human PC. Third, the chemicals cause complex biochemical, genetic and toxic alterations, and therefore it is almost impossible to pinpoint the critical molecular and cellular events that are crucial for cancer formation.

As reviewed recently by Hruban et al. (2006a), several transgenic mouse models have been established to study the excocrine pancreatic cancers (Ornitz et al., 1987; Quaife et al., 1987; Jhappan et al., 1990; Hotz et al., 2000; Bardeesy et al., 2002; Aguirre et al., 2003; Brembeck et al., 2003; Hingorani et al., 2003; Lewis et al., 2003; Lowy, 2003; Wei et al., 2003; Grippo and Sandgren, 2004; Leach, 2004; Hingorani et al., 2005; Maitra and Hruban, 2005; Tuveson and Hingorani, 2005; Tuveson et al., 2006) in addition to several other transgenic models of endocrine pancreatic tumors (Pelengaris and Khan, 2001). Because the majority of PC in humans are ductal adenocarcinomas but currently no gene promoter/enhancer has been known to be specific for pancreatic ductal cells (Grippo and Sandgren, 2004), all these transgenic mouse models share a common deficiency: If the transgene is controlled by a pancreas-specific promoter, such as *elastase-1* gene (Ela) promoter, it is dominantly expressed in the acinar cells. Conversely, if the transgene is driven by a promoter specific for ductal cells, its expression is not pancreas-specific. For instance, the *metallothionin-1* gene (MT) promoter targets the transgene to the mammary gland, liver and pancreas (Jhappan et al., 1990), while *cytokeratin 19* gene promoter targets the transgene to the stomach, pancreas and other organs (Brembeck et al., 2003); both promoters are much weaker than the Ela-promoter.

The Ela-SV40TAg, Ela-PyMT, and Ela-H-*ras* mutant transgenic mice develop mainly acinar cell carcinomas (Ornitz et al., 1987; Quaife et al., 1987; Lewis et al., 2003). However, Ueda et al. recently established a transgenic rat in which human *H-ras* mutant (G12V) is regulated by the Cre/lox system (Ueda et al., 2006). In these rats, injection of Cre carrying adenovirus into the pancreatic ducts and acini through the common bile duct can target expression of *H-ras* mutant to pancreatic ducts and acini and induce PanIN-like lesions and carcinomas. In contrast, *K-ras*-mutant mice with Ela- or cytokeratin-19 gene promoter develop only hyperplasia (early mouse PanIN lesions) without formation of frank adenocarcinomas (Brembeck et al., 2003; Wei et al., 2003; Grippo and Sandgren, 2004; Leach, 2004). *K-ras* mutant targeted to the pancreas using Pdx1-Cre system induces proliferating ductal lesions that histologically closely resemble human PanIN lesions. While this model appears to be the best thus far in creating mPanINs, unfortunately, progression to frank tumor occurs at very low frequency (2 out of 29 animals) (Hingorani et al., 2003) thus rendering it highly impractical for the purpose of cancer research in chemotherapy and

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chemoprevention. The time point for activation of the *K-ras* mutant is probably also important since Mist1-driven expression of the K-rasG12D mutant has recently been shown to induce invasive and metastatic hepatocellular carcinomas and pancreatic tumors of mixed cell types (Tuveson et al., 2006). Because Mist1 is a transcription factor controlling pancreatic development at an early stage, this may be a model of embryonic carcinogenesis that recapitulates childhood tumors in humans, such as retinoblastoma. If so, it may have little relevance to human PC.

It is likely that *K-ras* mutation alone is not sufficient to complete the whole carcinogenic process and alterations in other genes are required. Consistent with this thought, *K-ras* mutation in combination with deficiency of the p16-Ink4α, p19-Arf, or *p53* tumor suppressor gene, achieved by crossing different transgenic lines, led to ductal adenocarcinomas that show propensity to metastasize to the liver (Bardeesy et al., 2002; Aguirre et al., 2003; Hingorani et al., 2005). While the *Pdx1-Cre/LSL- K-ras*^{G12D} model (Hingorani et al., 2003; Tuveson and Hingorani, 2005) mostly recapitulate human PanIN lesions, the one simultaneously deficient with p53 (Hingorani et al., 2005; Tuveson and Hingorani, 2005) recapitulates human PC and its liver metastasis; both models are thus excellent for studying different stages of pancreatic carcinogenesis. Deficiency in PTEN tumor suppressor gene alone also induces ductal metaplasia, but the lesions progress to invasive ductal adenocarcinomas only at a low frequency (Maitra and Hruban, 2005). A weakness of those models that involve both transgenic and knockout systems is the requirement of a mouse concomitantly to bear four transgene alleles. For instance, the K-ras mutant/Ink4a/Arf deficient mouse needs to bear Pdx1-Cre, LSL- K-rasG12D and homozygous Ink4a/Arf lox/lox (Aguirre et al., 2003). Thus, this type of animal models involve huge amount of work for animal breeding and genotyping because only a small percent (12.5%) of the total pups will bear all four transgene alleles according to Mendelian inheritance. A few pre-bred animals may be obtained as breeders from the establishers of these models to help decrease the huge animal work. However, it will only help a little because one round of breeding procedure will segregate the transgenic alleles and the second round of breeding needs to start from heterozygous breeders again, which limits the broader usefulness of these animal models for prevention and/or therapeutic studies.

Female MT-TGFα transgenic mice of the MT100 line develop ductal adenocarcinomas at low frequency

Besides the above-described transgenic models, several lines of TGFα transgenic mice have been generated using Ela- or MT-promoter. The Ela-TGFα transgenic mice, which were generated by Drs. E. Sandgren and D.C. Lee et al (Sandgren et al., 1990) but studied extensively by Schmid and Wagner et al. (Schmid et al., 1999; Greten et al., 2001; Schmid, 2002), develop prominent fibrosis at an early age and show acinar-ductal transformation at four months of age (Wagner et al., 1998, 2001). At the age of one year or older, about one-fourth to one-third of the animals develop pancreatic tumors, mainly acinar cell carcinomas. Pronounced metaplasia from acinar to ductal cells appears in this model before tumor formation, but the meaning of this morphological trait is unclear since the majority of the tumors are acinar cell type. Genetic alterations of these Ela-TGFα transgenic pancreatic tumors resemble those seen in human PC (Wagner et al., 1998, 2001). Recently, Garbe et al. reported that the Ela-TGFα mice with deficiency of p53 gene (p53-/-), created by crossing Ela-TGFα mouse with p53 knockout mouse, developed growing tumors within 120 days of age (Garbe et al., 2006), although the morphological details of the tumors were not described. The shorter latent period indicate a synergistic effect of TGFα overexpression and p53 deficiency in pancreatic carcinogenesis.

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Of the MT-TGFα mice available, the MT100 and MT42 lines developed by Drs. C. Jhappan and G. T. Merlino et al. (Jhappan et al., 1990) and the line developed by Drs. E. Sandgren and David C. Lee et al. (Sandgren et al., 1990), thus coined herein as MT-TGFα-SE, are widely used by many investigators. However, these mice are mainly studied for carcinogenesis of the mammary gland (Liao et al., 2000; Liao and Dickson, 2000) and liver (Thorgeirsson et al., 2000) but rarely for the pancreas. During our studies of mammary carcinogenesis with the MT100 line, we accidentally found that the female mice live only for 6-8 months of age whereas males live well up to 14 months of age (males older than this age were not followed). This female predominance may be one of the reasons why it is less studied for pancreatic lesions by other investigators who are engaged in using male animals. Currently, our laboratory is the only one that still keeps live animals of MT100 line (although Jackson Lab has cryo-preserved stock) and uses it to address pancreatic carcinogenesis in a systematic manner.

Gross examination of dead or moribund females at 6-8 months of age revealed that the pancreas in most (about 70%) of the females became severely atrophic, weighing only 10-20% of the pancreas from age-matched wild type littermates. Histological examinations of the pancreas from females showed that at two months of age the pancreatic lesions (Liao et al., 2006) were characterized by ductal proliferation with acinar-to-ductal metaplasia (Fig. 2c,d), acinar cell death, and obvious fibrosis. In proliferating ducts, the mucin was predominantly blue with both alcian blue and high iron diamine (acidic sialomucin, also regarded as the "neoplastic" mucin in the pancreas) (Fig. 2e) and, in rare foci, it was black with high iron diamine. All lesions became severe with increasing age. At four months of age, the mucinous ducts began to efface the pancreas, separated by a subtle fibrous stroma, and acini became scattered due to cell death and fibrosis. Severely proliferated ducts showed focally positive staining for CA19-9 and CEA, which are known to be more commonly expressed in neoplatically transformed ductal cells. At about five months of age, the proliferating ducts changed their characteristics, becoming haphazardly distributed and exhibited irregular contours. The cells became less mucinous and more cuboidal, suggestive of an evolving adenocarcinoma. In the areas without proliferating ducts, acini disappeared and were replaced by stroma (Fig. 2f). The dramatic disappearance of acinar cells may be the reason for the smaller pancreas and the death of the animals.

In the remaining 30% of the female mice, the pancreas was dramatically enlarged, as opposed to the shrinking pancreas described above, due to the development of multi-locular cystic neoplasms. Histologically, the pancreas manifested all of the above described ductal proliferation, acinar-to-ductal metaplasia, acinar cell death and fibrosis. In addition, however, there were multilocular cysts (Fig. 2g), which became evident at two months of age and developed to multi-locular cystic masses (1.2 and 1.0 cm) with 11 to 16 loculi in each cystic neoplasm (Fig. 2h) at the age of 4-5 months. Size of the loculi ranged from 0.5 to 5 mm. The largest cystic neoplasm with colloidal liquid obtained was 2.2 cm in diameter from a mouse at 6 months of age. The septae were thin and contained loose mesenchymal tissue reminiscent of ovarian stroma, manifested by clusters of epithelioid cells with features suggestive of luteal cells (Fig. 2i). The lining epithelium was mucinous and focally positive for B72.3, CA19-9 and CEA. The septae sometimes contained small foci of ductal proliferation with all the morphologic features of an adenocarcinoma, including severe cytological atypia, marked nuclear pleomorphism and mitotic figures (Fig. 2j). Ductal adenocarcinomas (Fig. 2k,l) were found in roughly 10% of the total females.

Bardeesy et al. also observed similar cystic lesions in MT100 mice that were *Ink4a* and/or *p53* heterozygous, but not in MT100 mice with *Ink4a* or *p53* wild type (Bardeesy et al., 2002). The reason for this slight discrepancy is unknown, but it could be partly because their study involved only 25 mice without information of animal gender, since multi-locular

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neoplasms appeared in only 30% of the females in our laboratory. Moreover, Muller-Decker et al. also reported recently similar cystic neoplasms in association with serous cystadenomas and dysplasia in cyclooxygenase-2 (COX2) transgenic mice driven by keratin-5 promoter (Muller-Decker et al., 2006). In our laboratory, pancreatic lesions in male MT100 mice were much less severe and do not progress to cystic lesions and cancer. The reason for the female predilection is unknown. The MT promoter is probably more potent in female pancreas, like in the female mammary gland. The preferential occurrence in female mice also raised the analogy with mucinous cystic neoplasm of the pancreas in humans, which occur almost exclusively in females and is characterized by ovarian like stroma. It was difficult to ascertain the nature of the hypercellular mesenchyme in these female mice; however, the similarities were striking.

Immunohistochemical staining showed that the *TGFα* transgene was mainly expressed in the proliferating ductal cells of MT100 mice. Even in the same acinar-to-ductal glandular loop (acinar transdifferentiation), the ductal cells showed much stronger staining for TGFα than the neighboring acinar cells (Liao et al., 2006). This feature indicates that the MT-promoter is active mainly in the ductal epithelial cells, which makes the MT100 mouse a good model for studies of pancreatic carcinogenesis of ductal cell origin. At this moment when the morphology of ductal lesions from the aforementioned kartin-5 driven COX2 mice have not yet been described detailed enough (Muller-Decker et al., 2006), the MT100 line may be the only single-transgene model established to date that produces a series of premalignant and malignant ductal lesions. Although in only 10% of the females there were frank adenocarcinomas, the MT100 line may still be a cost-effective model for ductal adenocarcinomas compared with the aforementioned models that involve expression of K-ras mutant and simultaneous knockout of a tumor suppressor gene because the MT100 line requires less animal work, animal housing and genotyping. On the other hand, the model itself may have some imperfections such as lack of typical mPanINs.

As indicated earlier that there may be a cross-talk between *c-myc*, TGFα/EGFR and cyclin D1, cyclin D1 expression was found to be colocalized with TGFα to the proliferating ducts in this animal model (Fig. 3a), together with the expression of proliferating cell nuclear antigen (PCNA) (Fig. 3b) and Ki67 staining. Therefore, TGFα-induced proliferation of ductal cells may be mediated by cyclin D1, a well known downstream effector of TGFα/EGFR signaling pathway. On the other hand, immunohistochemical staining for fibroblast growth factor-2 (FGF2) showed that proliferating ductal cells were strongly positive in the cytoplasm, whereas fibroblasts in the adjacent stroma were positive in the nuclei (Fig. 3c,d). A logical explanation for these results is that TGFα may induce expression of FGF2 in ductal cells, which is later secreted and taken up by stromal cells leading to the induction of proliferation of stromal fibroblasts resulting in fibrosis as frequently seen in human PC. Interestingly, Ela-Smad7 transgenic mice, in which the TGFß inhibitor Smad7 was presumably targeted to acinar cells by Ela-promoter, developed ductal change (acinar to ductal metaplasia) and fibrosis at 6 months of age (Kuang et al., 2006). It deserves further investigation whether the Smad7 transgene is mainly expressed in acini in this model and, if so, whether inhibition of TGFß in acini causes the ductal proliferation and fibrosis via an epithelial-stromal interaction.

Ela-myc transgenic mice develop mixed acinar and ductal adenocarcinomas

Ela-*myc* transgenic mice were generated by Drs. E. Sandgren and Brinster et al. (Sandgren et al., 1991). These mice develop pancreatic tumors at 100% penetrance at 2-7 months of age without obvious sex predilection. One-half of the tumors are acinar cell carcinomas, while the remaining one-half are mixed ductal adenocarcinomas and acinar carcinomas (Sandgren et al., 1991; Schaeffer et al., 1994; Aguilar et al., 2004; Liao et al., 2006). The mixture of

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ductal and acinar tumors manifests in different ways. It can be pure acinar cell tumors in some areas whereas pure ductal adenocarcinomas in other areas (Fig. 4a,b). It is also observed that acinar tumor cells are mingled with ductal tumor cells in the same tumor area and even in the same glandular loops (Fig. 4c), which is probably related to the continuation of acinar-to-ductal metaplasia (acinar cell transdifferentiation). Ductal tumor areas usually show obvious fibrosis, a morphologic trait of human PC. According to our observation (Liao et al., 2006), the acinar tumors manifested many "dead cell islands", a feature coined by us to define *c-myc*-induced programmed cell death in tumors from *c-myc* transgenic mice (Liao and Dickson, 2000, 2003). Many tumor cells express cyclin D1 (Fig. 4e,f), which is somewhat inconsistent with the observations that *c-myc* suppresses cyclin D1 expression in fibroblasts, MMTV-c-*myc* transgenic mammary tumors and mouse pancreatic cancer cell lines (Jansen-Durr et al., 1993; Philipp et al., 1994; Marhin et al., 1996; Liao et al., 2000). It remains to be determined whether those cyclin D1 positive cells have lost expression of the c-*myc* transgene, as seen in some focal lesions in MMTVc-*myc* mammary tumors (Liao et al., 2000) or show activation of some growth factor signaling pathways such as Ras or TGFα that activate cyclin D1 signaling.

In about 20% of the Ela-*myc* mice, the pancreatic tumors metastasize to the liver (Fig. 4d) (Liao et al., 2006), which was not reported in the original study by Sandgren et al. (1991). The reason for the metastasis uniquely observed in our animals is unclear, but it is probably because we changed the genetic background of the mice from C57BL/6xSJL to a mixture of FVBxC57BL/6xSJL after we received a breeder mouse from Dr. Sandgren. To our knowledge, this Ela-*myc* mouse is the first and the only single-transgene model that yields the highest frequency of malignant ductal lesions, although associated with acinar cell lesions, and develop frank tumors in the shortest latency period compared with other single-transgene models (Ornitz et al., 1987; Quaife et al., 1987; Lowy, 2003; Wei et al., 2003; Grippo and Sandgren, 2004; Leach, 2004; Hruban et al., 2006a). This transgenic line is also one of the very few transgenic models that produce liver metastasis.

MT-TGFα/Ela-myc double transgenic mice develop various ductal lesions and tumors with a higher frequency of liver metastasis

Sandgren et al. reported that Ela-*TGFα*/Ela-*myc* or MT-TGFα-ES/Ela-*myc* double transgenic mice, generated by crossing an Ela-*myc* mouse with an Ela-*TGFα* or MT-*TGFα*-ES mouse, developed pancreatic tumors at an earlier age than Ela-*myc* mice, and the tumors manifested more malignant histology, i.e. less differentiation than Ela-*myc* tumors (Sandgren et al., 1993). We crossed MT100 with Ela-*myc* mice to create MT-*TGFα*/El*a-myc* mice, considering that the appearance of various ductal lesions in the Ela-*myc* pancreas indicates that the Ela-*myc* transgene is also expressed in the ductal cells, although at a lower level than in the acinar cells, and thus may synergize with the MT-TGFα transgene to induce ductal tumors. We found that the pancreatic tumors from MT100/Ela-*myc* mice were more malignant than the Ela-*myc* tumors, especially in females (Liao et al., 2006). Apoptotic and necrotic cell death is common in the double transgenic tumors (Fig. 4g). We also observed various ductal lesions, cysts and adenocarcinomas (Fig. 4g,h) in the double transgenic pancreas (Liao et al., 2006), in addition to mixed tumors (Fig. 4i) and pure acinar cell tumors as seen in Ela-*myc* mice. These findings are surprising because Sandgren et al. did not observe ductal elements in the pancreas of their Ela-TGFα/Ela-*myc* or MT-*TGFα*-ES/Ela-*myc* double transgenic mice (Sandgren et al., 1993). Another of our novel findings was that the MT100/Ela-*myc* tumors metastasized to the liver at a slightly higher frequency (30%) than the Ela-myc pancreatic tumors (Liao et al., 2006), which was, again, not observed by Sandgren et al (Sandgren et al., 1991, 1993). A possible explanation for these discrepancies is that their double transgenic mice were probably mainly Ela-TGFα/Ela-*myc* and less MT-TGFα-ES/Ela-*myc*. Moreover, according to our observation, the ductal lesions

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in MT-TGFα-ES mice are less severe and progressed much slower than those in the MT100 line we used (Liao et al., 2006). In addition, Sandgren et al. found primary liver tumors in their MT-TGFα-ES mice with C57BL/6xSJL background at a high frequency (16 of 27 animals) and in their wild type mice at a low frequency (1 of 20 animals) (Sandgren et al., 1993), whereas we did not find any primary liver tumor in any of our mice with FVBxC57BL/6xSJL background (Liao et al., 2006). Therefore, different genetic backgrounds may partly explain the discrepancy.

Different animal models bear different disadvantages with regard to their use for testing drug efficacy

Xenograft models, in which human cancer cell lines are inoculated at a subcutaneous site of immunodeficient mice, are frequently used in studies of cancer chemotherapy. Sometimes, the human cancer cells are not implanted at a subcutaneous site but at a specific organ of the mouse, such as the pancreas; in this case it is called orthotopic model. Both xenograft and orthotopic models are the most commonly used tools in studies of testing new therapeutic or preventive approaches for cancer. However, these *in vivo* methods have several weaknesses: 1) Cancer cells retain certain features of the adjacent normal cells, which make it difficult for the tested agent to kill just the cancer cells without hurting normal tissue. Human cells differ from mouse cells in genome and thus in many parameters; the sharp contrast between the two species makes it easier for a drug to differentially kill cancer cells in these models. 2) Many human cancer cell lines cannot grow in immunodeficient mice, usually because they are not malignant enough. Thus, these models actually favor those that are more malignant, faster growing cells, which are more sensitive to chemotherapy. 3) Because in xenograft or orthotopic models the tumors do not actually develop from host (recipient) mice in a chronic process, the host mice are in a better health condition than most patients with advanced cancer and, thus, better tolerate the treatment. 4) The deficiency of immune function in the host mouse may affect the efficacy of the tested agent, especially considering that inflammatory response is common in human cancer. In addition to these weaknesses, xenograft and orthotopic models cannot be used in the studies of cancer prevention since the implanted cells are already malignant.

Chemical-induced models of carcinogenesis are better than xenograft or orthotopic models because the tumor develops spontaneously in the animals and thus are superior for studies of new therapeutic approaches for cancer. In addition, these models can be used for studies of cancer prevention. However, in most of these models, chemical carcinogens are used at high doses for a relatively long period which cause toxicity in the liver, kidney, immune system and probably other organs. These toxic effects likely affect the metabolism of the tested drugs and weaken the general health of the animals. Therefore, the animals actually receive double drugs, i.e. the chemical carcinogen and the tested agent, although the two agents may not be administered simultaneously. Moreover, chemical-induced carcinogenesis is a long-time process, usually in months, which makes it difficult to determine when the treatment with the to-be-tested agent should be started.

Transgenic models eliminate the deficiencies of xenograft and orthotopic models when used for studies of new therapeutic or preventive approaches. However, when transgenic mice are used for these purposes, special concerns should be considered regarding the homozygous or heterozygous status of transgene carriers. Researchers frequently breed animals by mating a heterozygous male with a heterozygous female to increase the frequency of transgenic pups. This breeding procedure produces a mixture of homozygous and heterozygous pups. Technically, southern blot with radioactive probe is the common method to distinguish homozygous from heterozygous pups, although it still has certain difficulties because the homozygote shows only one-fold higher signal. It is not practical, although possible, to

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perform Southern blot to genotype a large number of pups. Because the mice bearing two alleles of the transgene may show different sensitivity to the agent to be tested, compared with those bearing only one allele, animals bred this way cannot be used for the purpose of testing drug efficacy, although they may still be used for studies of carcinogenic mechanisms.

Theoretically, one can use homozygous male and homozygous female as breeders because the pups bred in this way will be 100% homozygote. If homozygous animals are not overly sensitive to the to-be-tested agent, they can be used in chemotherapeutic or chemopreventive studies. Unfortunately, this breeding method is usually not an option because homozygous transgene carriers are often infertile or do not nurse the pup. Because many publications of transgenic models do not provide information on the animal's fertility, it is difficult to assess which of the currently available transgenic models can be propagated this way. Ideally, transgenic mice to be used for drug testing should be bred by mating a heterozygous mouse with a wild type animal. This procedure should produce 50% of heterozygous pups according to Mendelian inheritance, but the actual frequency may be lower, especially when the phenotype is severe, probably because of the natural selection of relatively healthy embryos.

The above-described special concern of the breeding method further increases the difficulty in animal propagation and limits the use of transgenic models that require a single mouse bear several transgene alleles in the studies of chemotherapy and chemoprevention. After taking all the aforementioned limitations of transgenic models, it seems that the MT100 and MT100/Ela-*myc* mice may be superior to other currently available transgenic models for testing the effects of chemopreventive and/or chemotherapeutic agents and will likely provide data that could be easily translated for the prevention and/or treatment of human PC.

Perspectives

Compared with other types of malignancies, pancreatic cancer has hitherto received much less attention with studies on its mechanism, treatment and prevention, although it is the 4th leading cause of cancer deaths, now higher than prostate cancer. Several transgenic mouse models have been established thus far. While some of these models are excellent for mechanistic studies of pancreatic carcinogenesis, none of them are ideal for therapeutic studies. Studies of cancer therapeutics usually require a large number of animals that are the same gender and age because gender and age affect drug metabolism. Moreover, either heterozygous or homozygous, but not both, transgenic animals should be used in therapeutic studies. Single-transgene models are the best to meet these special requirements, but, unfortunately, no one single-transgene model established hitherto produces mainly ductal adenocarcinomas at a high penetrance. Pertaining to the studies of cancer prevention, the MT100 line of TGFα transgenic mice may be one of the best alternatives available thus far since the females yield ductal proliferation with 100% penetrance at several months of age. In future studies, more efforts should be assigned to identification of gene promoters or enhancers that are specific for ductal cells of the pancreas and are highly active in vivo, and also practical with high-yield so that the model can be utilized in testing potential molecules for chemoprevention and chemotherapy.

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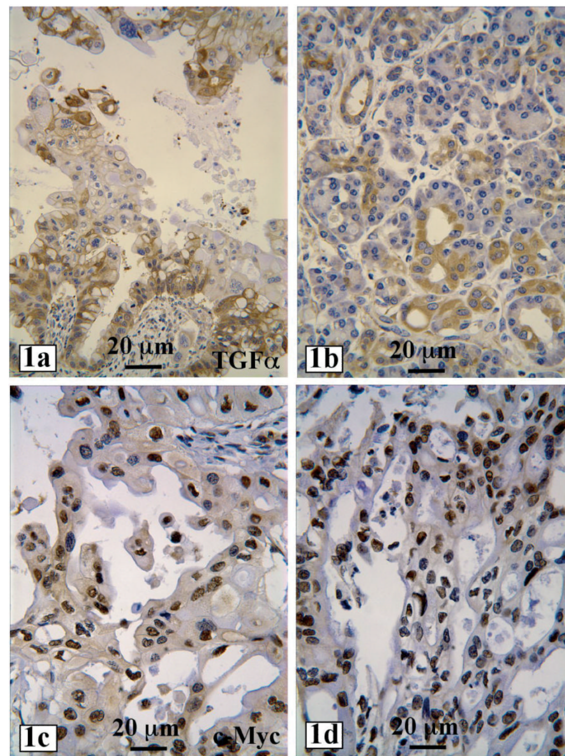

Fig. 1.

Immunohistochemical staining shows TGFα expression in human ductal adenocarcinoma and its adjacent normal ductal cells as well as ductalized acini. c-myc shows positive nuclear staining in cancer cells of two human ductal adenocarcinomas. (see Liao, et al., 2006 for detail).

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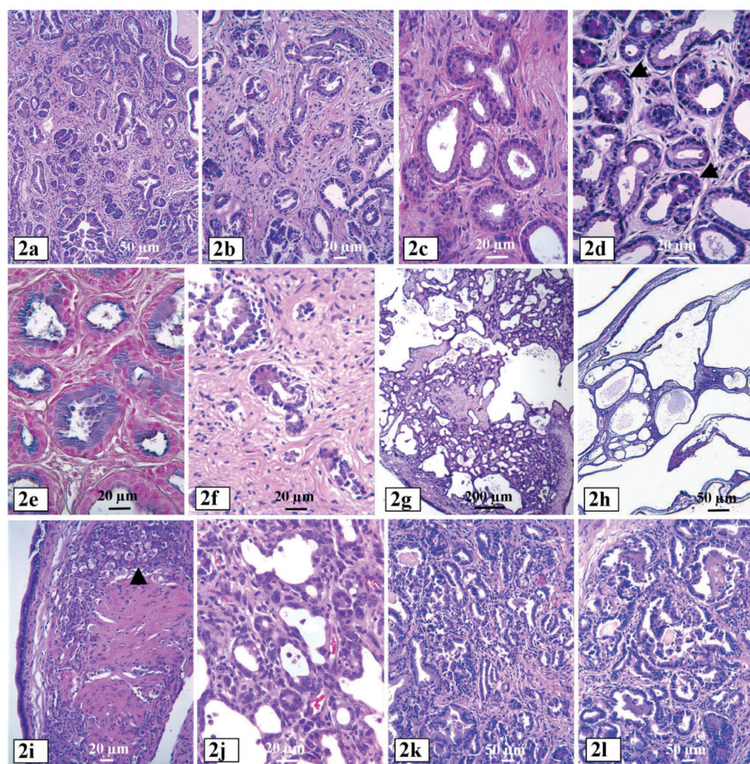

Fig. 2.

Pancreatic lesions from female MT100 mice. **a and b:** Ductal proliferation and fibrosis from mice at two months of age. **c:** Ducts lined by tall columnar mucinous cells. Some of the ducts are lined by acinar cells, an evidence of acinar to duct transformation (transdifferentiation). **d:** acinar cells in ductal-acinar glandular loops, indicating acinar-ductal metaplasia. **e:** High iron diamine stains blue mucin in the ducts. **f:** Prominent fibrosis and cell death of acinar-ductal cells. **g and h:** Cystic ductal lesions. **i:** A focus of epithelial cells with features resembling luteal cells (arrow) on the wall of a multilocular cystic tumor in a female mouse. **j:** The septae of cystic tumor show a small ductular proliferation with morphologic features of ductal adenocarcinoma with cytological atypia, marked nuclear pleomorphism and mitotic figures. **k and l:** ductal adenocarcinomas.

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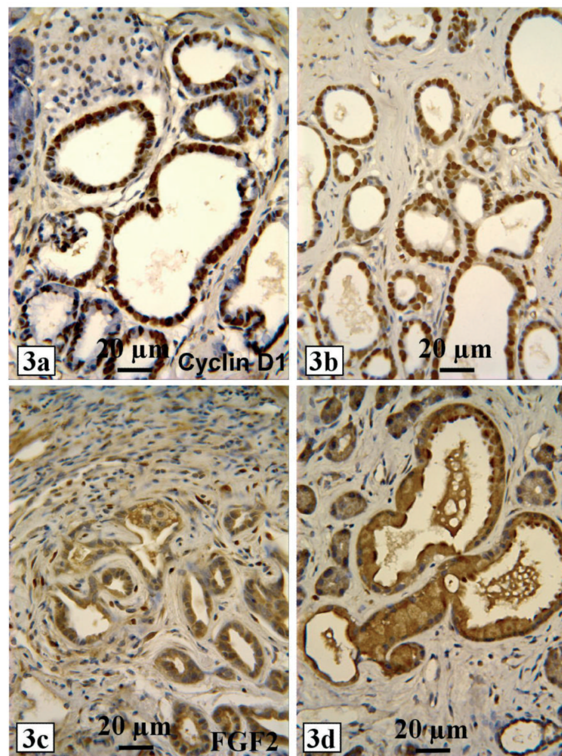

Fig. 3.

Proliferating ducts from female MT100 mice at two months of age show strong cyclin D1 immunohistochemical staining **(a)**, whereas many proliferating ductal cells and some stromal cells show strong PCNA staining **(b)**. FGF2 immunohistochemical staining is mainly localized in the cytoplasm of proliferating ductal cells but in the nuclei of stromal fibroblasts **(c and d)**.

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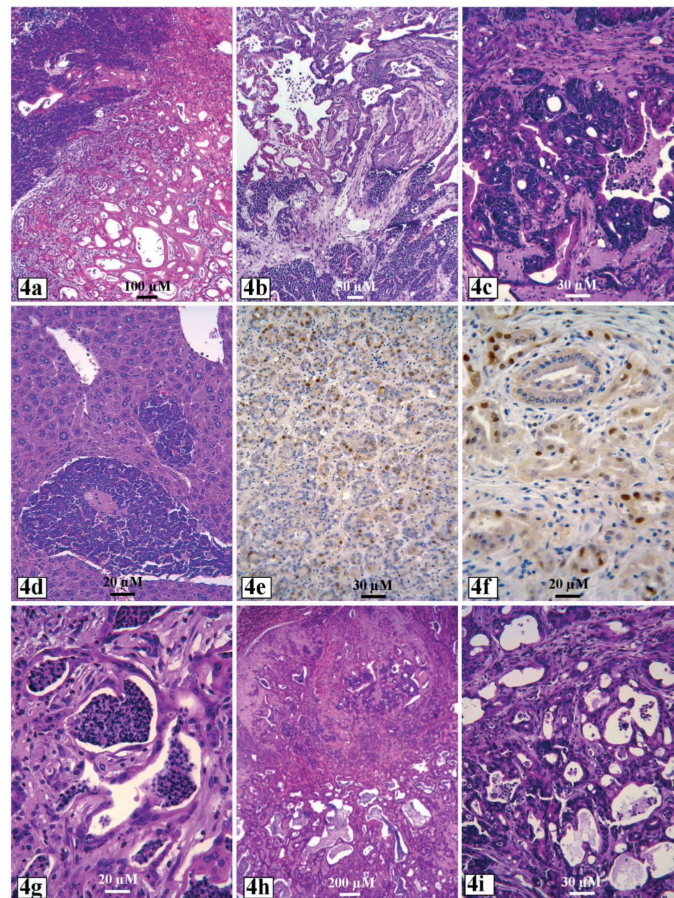

Fig. 4.

Ela-myc pancreatic tumors contain acinar cells in one area but ductal cells in another area **(a and b)**, or show mixture of acinar and ductal tumor cells in the same area or even the same glandular loops **(c)**. A liver shows metastatic acinar cell carcinoma **(d)**. Acinar **(e)** and ductal **(f)** tumor cells show positive cyclin D1 immunohistochemical staining. MT100/Ela-myc double transgenic pancreatic tumors show ductal elements, prominent cell death **(g)**, cystic alteration **(h)** and area of mixed ductal and acinar tumor cells **(i)**.