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Pathogenesis of Pancreatic Cancer: Lessons from Animal Models

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Abstract

The past several decades have seen great effort devoted to mimicking the key features of pancreatic ductal adenocarcinoma (PDAC) in animals, and have produced two robust models of this deadly cancer. Carcinogen-treated Syrian hamsters develop PDAC with genetic lesions that reproduce those of human, including activation of the *Kras* oncogene, and early studies in this species validated non-genetic risk factors for PDAC including pancreatitis, obesity and diabetes. More recently, PDAC research has been invigorated by the development of genetically-engineered mouse models based on tissue-specific *Kras* activation and deletion of tumor suppressor genes. Surprisingly, mouse PDAC appears to arise from exocrine acinar rather than ductal cells, via a process of phenotypic reprogramming that is accelerated by inflammation. Studies in both models have uncovered molecular mechanisms by which inflammation promotes and sustains PDAC, and identified targets for chemoprevention to suppress PDAC in high-risk individuals. The mouse model, in particular, has also been instrumental in developing new approaches to early detection as well as treatment of advanced disease. Together, animal models enable diverse approaches to basic and preclinical research on pancreatic cancer, the results of which will accelerate progress against this currently intractable cancer.

Keywords

Pancreatic cancer; Animal Models; Gastrointestinal system; Genetically engineered mice

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is a relatively rare cancer: at approximately 45,000 new cases per year in the United States, it currently ranks as the 11th most common cancer overall. Despite this overall low incidence, pancreatic cancer is the 4th leading cause of cancer death in both men and women, with approximately 38,000 deaths per year and an overall 5-year survival rate of only 6% (Siegel *et al.* 2013). This rate increases only to 20% for the rare patients (approximately one in ten) who present with apparently local disease and thus qualify for surgical resection (Schneider *et al.* 2005). For those patients ineligible for surgery, conventional chemotherapy or radiotherapy approaches appear to extend lifespan by only a few months. The overall cure rate of PDAC has been estimated at less than 1%, and even this number is likely inflated by misdiagnosis.

The fact that PDAC commonly recurs even after total pancreatectomy indicates that it is diagnosed only after already having seeded metastases in the liver, lung and elsewhere. Other solid cancers are similarly deadly following metastasis; pancreatic cancer is

remarkable in never being caught early enough to cure. Nonetheless, a recent study estimates that >10 years pass between the first genetic “hit” of PDAC and the formation of invasive cancer, and >6 years more before metastatic lesions are established (Yachida *et al.* 2010). In theory, this provides time for intervention, yet the relative scarcity of PDAC patients – let alone those with precancerous lesions detected prior to death – hinders the study of disease initiation and progression. This motivates efforts to develop animal models of autochthonous (i.e. originating in the place where it is found) PDAC, which might also serve as preclinical models to test new therapies for advanced cancer. These are the focus of this review: what animal models exist for pancreatic cancer, how have they enhanced our understanding of PDAC biology, and how they inform our efforts to improve patient outcomes.

Evolving approaches to an animal model of pancreatic cancer

Two roughly parallel tracks have been pursued to mimic human PDAC in animals: mutagenesis and transgenesis. Chemical carcinogen treatment can induce a variety of tumors in rodents, including skin cancer in mice. In this so-called multistage carcinogenesis model, tumors are induced by exposure to the mutagen 9,10-dimethyl-1,2-benzanthracene (DMBA), followed by treatment with chemical agents, such as 12-O-tetradecanoylphorbol-13-acetate (TPA), that promote their growth into self-sustaining carcinomas (Zoumpourlis *et al.* 2003). As discussed later in this review, a multistage initiation-promotion paradigm is likely to apply in the pancreas as well, with inflammatory stimuli playing the role of tumor promoter. Efforts to model PDAC via carcinogen treatment, however, have produced robust success only in the Syrian hamster, where a single injection of the mutagen *N*-Nitrosobis(2-oxopropyl)amine (BOP) induces invasive PDAC in up to 90% of animals (Pour *et al.* 1977). Invasive tumors arise within 3–12 months, and exhibit close histological similarity to their human counterparts. This model has been used to validate and dissect numerous conditions thought to modulate human cancer risk (Takahashi *et al.* 2011).

Importantly, hamster PDAC accumulates many of the same genetic alterations as human. The defining mutational event of pancreatic cancer is activation of the *KRAS* proto-oncogene, which occurs in >90% of all human PDAC (Almoguera *et al.* 1988; Maitra and Hruban 2008). *KRAS* encodes a member of the RAS family of small GTPase signaling proteins, and mutational activation (most commonly affecting amino acid Gly12) disables GTPase activity and traps RAS in a GTP-bound “on” state that normally requires mitogen stimulation (Pasca di Magliano and Logsdon 2013). As depicted in Fig. 1, *KRAS* mutations are detected in the earliest precancerous lesions of the human pancreas, pancreatic intraepithelial neoplasia (PanIN)-1, while more advanced lesions accumulate additional mutations such as loss of the tumor suppressors *CDKN2A* (p16), *TP53* (p53), *SMAD4* and *BRCA2* (Maitra and Hruban 2008). The hamster *CDKN2A* homolog is also mutated in BOP-induced pancreatic cancer (Takahashi *et al.* 2011), further supporting the suitability of hamster as a model for human PDAC.

The major drawback of a mutagenesis-based model is that the investigator cannot control what genes are actually mutated in a given tumor: in addition to *KRAS* and *CDKN2A*, BOP-induced cancers likely harbor additional mutations of unknown relevance to human tumors. Hamsters also lack the extensive genome-manipulation toolkit of mice, which has facilitated the development of genetically engineered mouse (GEM) models of cancer (van Miltenburg and Jonkers 2012). Even studies of carcinogen-induced tumors in mice, such as the skin cancer model, have benefitted from genetic manipulation to address issues such as tumor suppressor gene function (Zoumpourlis *et al.* 2003).

A barrier to studying PDAC in mouse, however, is that it is difficult or impossible to induce with BOP or other mutagens (Takahashi *et al.* 2011). Instead, mice offer the approach of directly expressing mutant KRAS in susceptible cells of the pancreas. A “first generation” of GEM models employed transgenic mice driving activated human *KRAS* expression with promoters of the duct-specific *Cytokeratin-19* (*Krt19*) or acinar-specific *Elastase1* (*Ela1*) genes (Brembeck *et al.* 2003; Grippo *et al.* 2003). Acinar cells normally produce digestive enzymes, which are channeled to the gut via a network of ducts; together, acini and ducts comprise the exocrine pancreas, with anatomically scattered islets of Langerhans making up the endocrine pancreas. The resemblance of PDAC to ducts, in morphology and marker expression, has generally been taken to suggest an origin among normal duct cells. Surprisingly, however, *Krt19-KRAS^{G12V}* mice develop only mild inflammation without cancer or dysplasia (Brembeck *et al.* 2003), while *Ela1-KRAS^{G12D}* mice exhibit premalignant lesions of mixed ductal-acinar character (Grippo *et al.* 2003). Similar lesions were described more than 20 years ago, in mice overexpressing the oncogenic transcription factor *Myc* via the same *Ela1* promoter (Sandgren *et al.* 1991). Although mixed acinar-ductal lesions do not have a clear human counterpart (Hruban *et al.* 2006), the *Ela1-Myc* and *Ela1-KRAS^{G12D}* studies were among the first to indicate that ductlike tumor precursors might arise from acinar cells.

Two shortcomings affect conventional transgenic approaches: they require a promoter element that is active in a “competent” cell of origin for the cancer, and they inevitably drive oncogene expression at non-physiological levels. This is a particular concern for mutant RAS, which actually induces senescence when overexpressed (Serrano *et al.* 1997). The creation of conditional mutant alleles of the endogenous mouse *Kras* gene, 10 years ago, therefore constituted a major advance in GEM cancer models (Fig. 2A) (Guerra *et al.* 2003; Tuveson *et al.* 2004). (Here and elsewhere, we use all-capital letters for human gene symbols, while capitalizing only the first letter of mouse genes.) These were immediately used to establish a “second generation” of PDAC models that has since become the major animal model of pancreatic cancer (Aguirre *et al.* 2003; Hingorani *et al.* 2003; Perez-Mancera *et al.* 2012).

Two conditional mutant *Kras* alleles exist, with similar overall design: a transcriptional “STOP” sequence, consisting of multiple strong polyadenylation signals, is placed upstream of *Kras* exon 2, in which the Gly12 codon has been mutated to encode either aspartic acid (G12D) or valine (G12V), the two most prevalent mutations in human PDAC (Guerra *et al.* 2003; Tuveson *et al.* 2004). The STOP sequence prevents expression of mutant *Kras*, but it is flanked by loxP sites (floxed) so that it can be recognized and excised by Cre recombinase protein. Without Cre, mutant *Kras* is silent and the resulting mice, with one wild-type and one silent mutant allele, are phenotypically normal. When Cre is active in a specific mature or progenitor cell (i.e. by breeding conditional *Kras* to a Cre-expressing mouse), the STOP cassette is removed and that cell and its offspring will permanently express oncogenic *Kras*. Functionally, this should be identical to a spontaneous G12D or G12V point mutation, except that its prevalence potentially encompasses an entire organ rather than a single cell. Mutant *Kras* is expressed from its endogenous locus, and does not induce senescence associated with RAS overactivity (Guerra *et al.* 2003; Serrano *et al.* 1997; Tuveson *et al.* 2004). The conditional G12D and G12V alleles behave generally similarly, although G12D appears slightly more transforming in the pancreas. We will refer to both alleles as *Kras^{LSL}*, short for “loxP-STOP-loxP.”

How is mutant *Kras* targeted to the pancreas? Borrowing tools from pancreas developmental biology, the vast majority of published PDAC modeling experiments have used “deletor” strains in which Cre is expressed in multipotent progenitor cells of the early embryonic pancreas, under control of the *Pdx1* or *Ptf1a* promoters (Fig. 2B) (Murtaugh and Melton

2003). In the resulting bigenic mice (e.g. *Kras^{LSL-G12D/+}; Pdx1-Cre*), almost all pancreatic cells express oncogenic *Kras* from embryonic stages. (These mice are frequently referred to in the literature as “KC,” for “Kras/Cre”; when mutant *p53/Tp53* is also present, they are called “KPC.”) At birth, mutant *Kras*-expressing pancreata are relatively normal, but within 1–2 months they begin to develop lesions resembling human PanIN-1 (Fig. 1) (Aguirre *et al.* 2003; Hingorani *et al.* 2003). PanINs become more numerous and dysplastic with age, resembling human PanIN-2 and PanIN-3 (Hruban *et al.* 2006). [Table 1 summarizes the key histological features of both human and mouse PanINs, as defined by pathologist working groups (Hruban *et al.* 2006; Hruban *et al.* 2001).] At >1 year of age, a minority of these otherwise “wild-type” mice develop invasive PDAC, presumably due to additional somatic mutations. Mouse PDAC is dramatically accelerated when oncogenic *Kras* is compounded with heterozygous or homozygous mutations in tumor suppressor genes such as *p16/Cdkn2a*, *Tp53*, *Smad4* and *Brca2*, in some cases resulting in lethal, metastatic disease within three months of birth (Aguirre *et al.* 2003; Bardeesy *et al.* 2006a; Bardeesy *et al.* 2006b; Hingorani *et al.* 2005; Skoulidis *et al.* 2010). *Kras^{LSL}* mice thus directly validate the relevance of human PDAC mutations, and have become the major preclinical model in the field.

In the *Kras^{LSL}* models, activation by Cre leaves cells in an irreversibly KRAS-active state from embryogenesis onwards. Given that human PDAC affects older adults, activation in utero is probably nonphysiological. Furthermore, while the irreversibility of *Kras^{LSL}* activation mimics the situation of a human cancer cell, it precludes investigation of “oncogene addiction,” i.e. the hypothesis that maintenance of a cancer requires the same activated oncogenes that triggered its initiation. Although RAS molecules are considered challenging drug targets (Pasca di Magliano and Logsdon 2013), validating KRAS addiction in PDAC would provide a clinical rationale for pursuing this goal.

Two groups have recently developed “third generation” GEM models to address these issues (Collins *et al.* 2012; Ying *et al.* 2012). These models combine three genetically engineered components: a pancreatic Cre deleter (*Ptf1a^{Cre}*) activates expression throughout the organ of the synthetic transcription factor *rtTA*, which itself binds to and activates a *TetO-Kras^{G12D}* transgene in a doxycycline-dependent manner. The end result is that *Kras^{G12D}* can be turned on and off almost at will in these mice, which we refer to as *iKras* (inducible-Kras). Confirming that activated KRAS can induce PDAC in adulthood, *iKras* mice develop PanINs when doxycycline administration is delayed until 4–6 weeks of age (Collins *et al.* 2012). More importantly, if doxycycline is subsequently withdrawn to downregulate *Kras^{G12D}*, these PanINs disappear: early-stage PanINs appear to redifferentiate into normal exocrine pancreas tissue, while more advanced PanINs undergo apoptosis. And most important, when PDAC is induced by *Tp53* deletion in *iKras* mice, doxycycline withdrawal causes regression of both local and metastatic cancer (Collins *et al.* 2012; Ying *et al.* 2012). The dramatic oncogene addiction demonstrated in these studies provides a gold standard for preclinical studies aimed at blocking oncogenic KRAS.

Where and why does PDAC initiate: insights from animal models

Almost 200 papers have been published using the *Kras^{LSL}* models in the past ten years, and at least as many using the Syrian hamster model since its development in the mid-1970s. A comprehensive review of these models is therefore impossible, and we will instead focus on three areas in which they have proved particularly useful: dissecting the initial steps of tumorigenesis, providing insights into risk factors and prevention, and serving as a preclinical test platform for detection and treatment modalities.

The first of these issues, how PDAC initiates, may seem trivial given the abundant evidence that *KRAS* mutations induce pancreatic tumorigenesis. Yet a cursory examination of the *Kras^{LSL}* mouse model reveals that mutant *KRAS* is not sufficient for tumor initiation: almost all cells of the pancreas express activated *Kras*, but PanINs arise in isolation within an abundance of apparently normal tissue (Fig. 1) (Hingorani *et al.* 2003). Two questions arise from this observation: what cells within the pancreas actually give rise to PanINs, and what determines the ability of *KRAS* to transform these cells?

The question of the cell of origin for PDAC has been controversial at least as far back as the development of the Syrian hamster model, with opposing histological evidence marshaled in support of an origin among ductal or acinar cells (Pour 1984). An analogous controversy, of even longer standing, concerns the question of where new insulin-producing β -cells come from in the resting or injured pancreas (Granger and Kushner 2009). Only recently has any definitive answer been approached, using the technique of Cre-mediated lineage tracing (Kretzschmar and Watt 2012). This technique relies on Cre-activated reporter genes, such as green fluorescent protein (GFP) or beta-galactosidase, the activation of which will mark the descendants of a Cre-expressing progenitor cell. In the pancreas, this approach revealed that all pancreatic lineages arise from cells expressing the transcription factors *Pdx1* and *Ptf1a* (Murtaugh and Melton 2003); these same Cre lines are now used in the *Kras^{LSL}* PDAC model. Cre recombinase can be fused to a modified form of the estrogen receptor (ER) ligand binding domain, responsive to the drug tamoxifen; such an “inducible Cre,” referred to as CreERT, will activate recombination only after tamoxifen administration. CreERT lines have been used to follow the fates of islet, acinar and duct cells in the adult mouse, and to show that new β -cells arise from old β -cells, not via transdifferentiation of ducts or acini (Desai *et al.* 2007; Dor *et al.* 2004; Kopinke and Murtaugh 2010; Kopp *et al.* 2011; Solar *et al.* 2009).

These same lineage-specific CreERT lines have recently been used to test the susceptibility of adult cells to mutant *KRAS* (Fig. 2B). Working independently, several groups demonstrated that activating *Kras^{LSL}* in adult acinar cells produces PanIN lesions indistinguishable from those obtained in the conventional *Pdx1-Cre/Ptf1a^{Cre}*-based models (De La O *et al.* 2008; Guerra *et al.* 2007; Habbe *et al.* 2008). Acinar-derived PanINs formed more quickly and more abundantly when the mice were subjected to acute or chronic pancreatitis via treatment with the cholecystokinin analog caerulein (De La O and Murtaugh 2009; Guerra *et al.* 2007; Morris *et al.* 2010), and progressed to invasive PDAC in the context of either chronic pancreatitis or *Tp53* deletion (Guerra *et al.* 2007; Ji *et al.* 2009). Remarkably, targeting *Kras^{LSL}* to adult duct cells via *Sox9-CreERT* produced almost no PanINs, even after pancreatitis (Kopp *et al.* 2012). By carefully comparing the efficiency of Cre deletion between acini and ducts, it was concluded that the latter cell type was >100-fold less susceptible to *KRAS* transformation than the former. These results lead to the provocative conclusion that pancreatic ductal cancers originate from differentiated, non-ductal cells, and they highlight the unique power of mice for understanding basic tumor biology.

Three caveats apply to these studies. First, they do not exclude the existence of *KRAS*-sensitive duct cells that are *Sox9*-negative and are thus inaccessible to *Sox9-CreERT*. Centroacinar cells (CACs), for example, a subset of duct cells that lie at the junction of ductal and acinar epithelium, have been ascribed both progenitor-like and tumor-initiating potential (Cleveland *et al.* 2012; Ekholm *et al.* 1962; Pour 1984). Although *Sox9-CreERT* appears to be active in most CACs (Kopp *et al.* 2011; Kopp *et al.* 2012), it is conceivable that specialized, *Sox9*-negative CACs were missed in these experiments. Second, while these findings indicate that adult acinar cells can give rise to PanINs and PDAC, they do not directly address the origin of lesions that arise with embryonic *Kras^{LSL}* activation (i.e. using

Pdx1-Cre or *Ptf1a^{Cre}*). Third, these experiments were performed in mice and, like any result obtained in an animal model, may not apply to humans. Interestingly, expression profiling defines up to a third of human PDACs as “exocrine-like,” in which ductal tumor cells express detectable levels of acinar enzymes (Collisson *et al.* 2011). It is tempting to speculate that these tumors retain an epigenetic memory of their origin.

Although similar profiling has not been performed in mice, it is safe to say that acinar-derived PanINs in the *Kras^{LSL}* model do not betray obvious acinar characteristics, implying that lesion formation requires extensive phenotypic reprogramming (De La O *et al.* 2008; Kopp *et al.* 2012). Rare but apparently sustained acinar-ductal reprogramming (ADR) also occurs in vivo after chronic pancreatitis (Strobel *et al.* 2007), and can be induced both in vitro and in vivo by exposure to the growth factor TGF α (Blaine *et al.* 2010; Means *et al.* 2005), which itself activates endogenous KRAS. Recent studies address mechanisms controlling KRAS-induced reprogramming: it requires *Sox9* (Kopp *et al.* 2012), which is upregulated at an early stage of the process, and it is inhibited by Wnt/ β -catenin signaling and the acinar transcription factor *Mist1*, both of which are downregulated during reprogramming (Morris *et al.* 2010; Shi *et al.* 2013). In these respects, it is distinct from “acinar-ductal metaplasia” (ADM), the transient upregulation of duct markers by wild-type acinar cells in response to injury: ADM does not absolutely require *Sox9*, nor does the resolution of ADM absolutely require β -catenin or *Mist1* activity (Keefe *et al.* 2012; Kopp *et al.* 2012; Kowalik *et al.* 2007). It should be noted that the distinction between ADR and ADM has only recently been drawn, with both phenomena previously termed “metaplasia” (Kopp *et al.* 2012). We reserve the term reprogramming for the complete conversion of acinar cells to a duct-like phenotype, which we propose represents a rate-limiting step of PDAC initiation (Fig. 3).

The earliest lesions of the *Kras^{LSL}* model contain abnormal cells, co-expressing acinar and duct markers, that have upregulated the epidermal growth factor receptor (EGFR) but are negative for phospho-ERK (pERK), a downstream target of the EGFR/RAS/RAF/MAPK pathway (Zhu *et al.* 2007). These cells co-mingle with pERK-positive cells that express only duct markers and have PanIN-like morphology, presumably further along the reprogramming pathway. Remarkably, genetic or pharmacologic inhibition of EGFR blocks acinar reprogramming and abolishes ERK activation in cells expressing oncogenic KRAS (Ardito *et al.* 2012; Navas *et al.* 2012). Together, these results suggest that EGFR upregulation is a proximal target of mutant KRAS, but that full activation of the “RAS pathway,” including ERK phosphorylation, requires autocrine EGFR stimulation. Most acinar cells do not experience autocrine EGFR signals, and therefore remain normal despite expressing mutant KRAS.

The net effect of EGFR stimulation is to amplify signaling through mutant KRAS itself, which at physiological levels has only modest effects on downstream signaling components (Guerra *et al.* 2007; Huang *et al.* 2013; Tuveson *et al.* 2004). Once tumors reach the invasive PDAC stage, their RAS activity becomes elevated and independent of EGFR. Mimicking this process by *Kras^{G12V}* overexpression in acinar cells is sufficient to induce widespread ADR and rapid PanIN/PDAC progression (Ardito *et al.* 2012; Ji *et al.* 2009). Thus, acinar reprogramming both unleashes and is induced by high RAS activity, creating a positive feedback loop that drives tumor initiation. Although EGFR is a major component of this loop, there is also a key requirement for inflammatory stimuli, which themselves both feed into and are upregulated by oncogenic RAS (Daniluk *et al.* 2012; Ji *et al.* 2009). As we will discuss in the next section, a variety of inflammatory conditions have been linked to human pancreatic cancer risk (Greer and Whitcomb 2009), and we hypothesize that a major impact of inflammation is to enhance ductal reprogramming of *KRAS* mutant acinar cells (Fig. 3).

Actionable insights into pancreatic cancer risk factors from animal models

Mice provide an ideal model to test genetic risk factors, of particular relevance to PDAC given that up to 10% of cases occur in familial clusters (Maitra and Hruban 2008; Petersen *et al.* 2006). A few major PDAC risk genes have been identified, including the tumor suppressors *CDKN2A* and *BRCA2* that are frequently disrupted during “sporadic” PDAC progression. As described above, heterozygous mutations in these genes increases PDAC incidence in mice, validating them as familial PDAC susceptibility genes (Bardeesy *et al.* 2006a; Skoulidis *et al.* 2010).

These relatively high-impact risk alleles account for only a minority of familial PDAC cases, and genome-wide association studies (GWAS) have recently been used to identify additional risk alleles more widespread in the population, albeit of lower penetrance. These include common non-coding SNPs in the orphan nuclear hormone receptor *NR5A2*, which are associated with approximately 1.3-fold increased PDAC risk (Li *et al.* 2012; Petersen *et al.* 2010). Mouse *NR5A2*, also called Liver receptor homolog-1 (LRH1), normally promotes acinar-specific gene expression in collaboration with the PTF1 transcription factor complex (Holmstrom *et al.* 2011). In the *Kras^{LSL}* model, heterozygous loss of *Nr5a2* dramatically increases PanIN formation while cell-autonomous deletion of *Nr5a2* sensitizes acinar cells to KRAS-induced reprogramming (Flandez *et al.* 2013; von Figura *et al.* 2013). While these manipulations are more severe than the presumably subtle effects of human *NR5A2* SNPs, they provide the first experimental assessment of a GWAS-identified PDAC risk factor, and implicate acinar reprogramming as a key step of human PDAC initiation.

In addition to sensitizing acinar cells to mutant KRAS, loss of *Nr5a2* also reduces their capacity for regeneration after experimentally-induced pancreatitis (Flandez *et al.* 2013; von Figura *et al.* 2013). These are the latest in an abundance of studies linking pancreatitis and pancreatic inflammation to PDAC risk, including epidemiological research (Duell *et al.* 2012; Greer and Whitcomb 2009). In fact, one of the most penetrant PDAC risk alleles in humans is a gain-of-function mutation in the cationic trypsinogen gene, *PRSS1*, which causes hereditary chronic pancreatitis and increases PDAC risk >50-fold (Maitra and Hruban 2008). Studies have found that acute pancreatitis accelerates ADR and PanIN formation in *Kras^{LSL}* mice, while chronic pancreatitis promotes progression to PDAC (Carriere *et al.* 2009; De La O and Murtaugh 2009; Guerra *et al.* 2007; Morris *et al.* 2010). Of note, the PDAC-promoting role of pancreatitis was first demonstrated in the Syrian hamster, a decade before strong epidemiological results in humans and almost 25 years before similar findings in mouse (Pour *et al.* 1983).

Indeed, the Syrian hamster provides independent validation of additional non-genetic risk factors for PDAC, and ought not be neglected in the GEM model era (Takahashi *et al.* 2011). Among these epidemiologically-identified risk factors are obesity and type-2 diabetes, which are themselves linked and which may promote PDAC through elevated systemic inflammation (Greer and Whitcomb 2009). Feeding *Kras^{LSL}* mice a high-fat diet accelerates PanIN formation (Khasawneh *et al.* 2009), a result anticipated in hamster by almost three decades (Birt *et al.* 1981). Interestingly, hamsters exhibit constitutive hyperlipidemia, and treatment with the PPAR γ ligand pioglitazone both lowers circulating lipids and reduces PDAC incidence after exposure to BOP (Takeuchi *et al.* 2007). Pioglitazone and other PPAR γ ligands have both anti-diabetic and anti-inflammatory effects, and have been shown to inhibit pancreatitis in mice (Konturek *et al.* 2005; van Westerloo *et al.* 2005). Together, these studies highlight the intimate link between inflammation and PDAC, and raise the question of whether anti-inflammatory drugs could serve as chemopreventive agents.

Autocrine and paracrine signals between epithelium, fibroblast and immune cells establish an inflammatory microenvironment from early stages of PanIN progression (Clark *et al.* 2007; Perez-Mancera *et al.* 2012). Mechanistically, pancreatic inflammation activates STAT3 and NFκB signaling, both of which are required for tumorigenesis (Daniluk *et al.* 2012; Fukuda *et al.* 2011; Ji *et al.* 2009; Lesina *et al.* 2011; Maniati *et al.* 2011). As inflammation does not cause cancer in the absence of mutant KRAS, it appears to represent a “tumor promoter” analogous to TPA treatment in mouse skin cancer (Zoumpourlis *et al.* 2003): unable to induce tumorigenesis itself, but required to expand and sustain mutant cells. NFκB appears particularly critical in early tumorigenesis, as it participates in the positive feedback loop that amplifies KRAS signaling to the levels required for acinar-ductal reprogramming (Daniluk *et al.* 2012). Insofar as anti-inflammatory agents could inhibit this loop, they should prevent pancreatic cancer. Indeed, several such agents have proven effective at delaying PanIN progression and PDAC initiation in the hamster and *Kras^{LSL}* mouse models, including cyclooxygenase inhibitors (Daniluk *et al.* 2012; Funahashi *et al.* 2007; Takahashi *et al.* 1990), dexamethasone (Rhim *et al.* 2012), and the diabetes drug metformin (Schneider *et al.* 2001), the last of which exhibits anti-inflammatory effects due to inhibition of NFκB (Isoda *et al.* 2006). Interestingly, metformin use in human diabetics has been associated with decreased risk of PDAC (Li *et al.* 2009), suggesting that the drug has chemopreventive properties within this patient population.

Additional PDAC risk factors remain to be assessed in animal models. For example, cigarette smoking imposes at least a 2-fold increased risk in humans (Bosetti *et al.* 2012), comparable to that associated with nonhereditary chronic pancreatitis (Duell *et al.* 2012). The fact that this not been pursued in animals may reflect the already high emphasis placed on anti-smoking efforts. For more innocuous-seeming risk factors, however, animal studies could establish causal relationships with pancreatic cancer. For example, individuals with periodontal disease have similarly elevated PDAC risk to smokers (Michaud *et al.* 2012); this could indicate a tumor-promoting role for infectious bacteria, or it could mean that both PDAC and periodontal disease occur secondary to immune system defects.

We expect that animal PDAC models will continue to validate human risk factors, identify their mechanisms of action and help develop counteracting therapeutics. Prospective testing of chemoprevention in humans will be a challenge, however, given that only a small minority of individuals subject to any of the risk factors discussed here will ultimately develop PDAC. Chemopreventive trials will likely have to focus on high-risk patients defined by a combination of risk factors, and perhaps also exhibiting signs of incipient cancer. In the next section, we will discuss the use of animal models in developing new methods for early PDAC detection, as well as efforts to improve treatment of the advanced cancers that represent most newly diagnosed cases.

Before ending this discussion of risk factors, it is important to consider the possibility that animal models are too cancer-susceptible, and thus affected by risk factors of minor or no human relevance. For example, although deletion of the *Rb1* (retinoblastoma) tumor suppressor accelerates *Kras^{LSL}* tumorigenesis (Carriere *et al.* 2011), the human homolog of this gene is not mutated in familial or sporadic PDAC. Similarly, studies in hamster indicate increased susceptibility to BOP-induced tumors on a diet supplemented with heterocyclic amines, byproducts of meat cooking (Yoshimoto *et al.* 1999), yet there is scant epidemiological evidence that meat consumption increases human PDAC risk independent of its contribution to obesity (Nitsche *et al.* 2011).

It is with this context in mind that one must consider the recent controversy over whether glucagon-like peptide-1 (GLP-1)-based diabetes drugs, such as sitagliptin and exenatide, increase the risk of pancreatic cancer (Gale 2012). Following up on preliminary

epidemiological evidence for excessive pancreatitis and PDAC cases among patients on these drugs (Elashoff *et al.* 2011), a preclinical study indicated that exenatide treatment increased PanIN burden and organ-wide inflammation in *Kras^{LSL}* mice (Gier *et al.* 2012). However, the interaction of *Kras^{LSL}* and exenatide appears less severe than that of *Kras^{LSL}* and caerulein-induced pancreatitis (Carriere *et al.* 2009; Guerra *et al.* 2007; Morris *et al.* 2010). Given that only a small minority of pancreatitis patients develops PDAC, the risk associated with GLP-1 drugs may be quite low. On the other hand, there is little comfort in the fact that PanINs and PDAC do not arise in wild-type animals treated with GLP-1 drugs (Nyborg *et al.* 2012), given that rodents rarely if ever develop these lesions without induced or engineered *Kras* mutations. If further epidemiological studies confirm a link between GLP-1 drugs and human PDAC risk, animal models will undoubtedly help dissect the basis of that link.

Use of animal models for improved PDAC diagnosis and treatment

There are currently no diagnostic tools with the specificity and sensitivity required for PDAC screening of the general population. Even restricted to high-risk individuals, current screening modalities may be of limited clinical impact (Canto *et al.* 2013; Poruk *et al.* 2013). A key advantage of animal models is that they represent the ultimate “high-risk” patients: all affected individuals will develop PanINs or PDAC, depending on the model, while all untreated or wild-type littermates will remain completely cancer-free. The *Kras^{LSL}* mouse has been particularly useful in this regard: one of the first studies of this model included serum proteomic analysis to detect potential circulating biomarkers of PanIN burden (Hingorani *et al.* 2003), and subsequent work has correlated specific circulating protein markers between PanIN/PDAC-bearing mice and human PDAC patients (Faca *et al.* 2008). A recent study of the *Kras^{LSL}* model indicates that mutant cells themselves are shed into the bloodstream from PanIN formation onwards, providing a potentially powerful mechanism to detect tumor-prone cells before they become invasive (Rhim *et al.* 2012).

New imaging modalities are also emerging from studies of the *Kras^{LSL}* model, including targeted nanoparticles detectable by MRI imaging (Kelly *et al.* 2008), and optical imaging based on tumor-specific enzymatic activity (Cruz-Monserrate *et al.* 2012; Eser *et al.* 2011). While these have yet to be scaled up to the human organ, they also provide facile methods to monitor the evolution of tumor burden and metastasis in living mice. Such an approach is essential if we are to realize the most pressing goal of pancreatic cancer research, which is improving the treatment of advanced disease. A disadvantage of autochthonous cancer models, as opposed to traditional models based on xenografts or transplanted cell lines, is that tumor progression is inevitably asynchronous between individual animals. Simultaneously treating age-matched GEM mice with a particular drug can therefore yield incoherent results: some mice will be too far along to respond, while others may not have invasive disease at the onset of treatment. By applying *in vivo* imaging, whether traditional methods such as ultrasound or newer methods discussed above, a clinical trial-like study design can be implemented in which individual mice are “enrolled” only once they develop detectable cancer (Cook *et al.* 2008).

Such approaches continue to be refined, but have already confirmed the particular utility of *Kras^{LSL}* mice as a model to improve existing drugs and test new ones. For example, the standard-of-care chemotherapy drug gemcitabine was found to almost cure transplantation models of human and mouse PDAC, but had practically no effect on tumors arising *in situ* in *Kras^{LSL}* mice (Olive *et al.* 2009). In this respect, the GEM model is much closer to the human situation, where gemcitabine barely extends the lifespan of PDAC patients. A potential reason for the inefficacy of chemotherapy is that tumor cells are separated from blood vessels by abundant stromal cells, limiting access to circulating drugs. Inhibiting the

Hedgehog signaling pathway in *Kras^{LSL}* mice caused stromal regression and enhanced gemcitabine killing of tumor cells, thus extending survival (Olive *et al.* 2009). Further supporting the importance of the stroma is the recent finding that increasing its physical permeability, by hyaluronidase treatment, also increases gemcitabine efficacy in *Kras^{LSL}* mice (Provenzano *et al.* 2012).

A central goal of cancer research, in the pancreas and elsewhere, is development of targeted therapies that kill cancer cells based on their unique molecular properties. EGF receptor inhibitors, for example, are known to inhibit the growth of human lung cancers carrying activating *EGFR* mutations (Laurent-Puig *et al.* 2009). These drugs are effective at inhibiting PDAC growth in transplantation models, but are generally unhelpful in human PDAC patients. Importantly, this lack of effect is reproduced in *Kras^{LSL}* mice (Singh *et al.* 2010), providing an example of the GEM model better predicting clinical outcome than traditional xenograft methods. The inefficacy of EGFR inhibitors may reflect the fact that this pathway acts only during PDAC initiation (Ardito *et al.* 2012), as well as the fact that mutant KRAS signaling is thought to bypass any need for upstream EGFR activation. Interventions targeted downstream of mutant KRAS might be more effective, and a recent study indicates that genetic or pharmacological inhibition of the RAS effector phosphoinositide (PI)-3-kinase inhibits both initiation and maintenance of *Kras^{LSL}*-induced PDAC (Eser *et al.* 2013). Clinical trials of PI-3-kinase inhibitors in PDAC are ongoing (www.clinicaltrials.gov), and should indicate whether the GEM model can predict positive as well as negative patient outcomes.

Conclusions and future directions

Studies of Syrian hamster and mouse have converged on a model for PDAC initiation and progression that begins with activation of the *KRAS* oncogene in differentiated acinar cells, establishment of a positive feedback loop to increase KRAS signaling and induce acinar reprogramming into ductal tumor precursors, and subsequent loss of tumor suppressor genes to facilitate invasion and metastasis. Several of the key risk factors for PDAC have been reproduced in these models, and collectively indicate an important role for inflammation in enhancing KRAS activity and promoting acinar reprogramming. Animal models are well suited for validation and dissection of known and emerging human risk factors, as well as for developing preventive interventions targeting those factors. The *Kras^{LSL}* mouse is particularly suited for studies of pancreatic cancer genetics, and it has emerged in the last decade as the model of choice for most researchers. We encourage the field not to neglect the hamster model, however, if only because it provides independent validation of many results recently obtained in mouse.

Ultimately, progress toward the goal of reducing PDAC deaths will require improved prediction based on genetic and non-genetic risk factors, improved biomarker and imaging modalities to screen potential high-risk individuals for incipient cancer, and pharmacological interventions to prevent PDAC progression as well as limit the growth of advanced disease. The refinement of animal models in the past several decades has driven progress in each of these areas, and these models will continue to make major contributions in the years ahead.

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Abbreviations

ADR	acinar-ductal reprogramming
ADM	acinar-ductal metaplasia
BOP	<i>N</i> -Nitrosobis(2-oxopropyl)amine
CAC	centroacinar cell
DBMA	9,10-dimethyl-1,2-benzanthracene
EGFR	epidermal growth factor receptor
ER	estrogen receptor
GEM	genetically engineered mouse
GFP	green fluorescent protein
GWAS	genome-wide association studies
LRH1	liver receptor homolog 1
LSL	loxP-STOP-loxP
PanIN	pancreatic intraepithelial neoplasia
PDAC	pancreatic ductal adenocarcinoma
PI	phosphoinositide
SNP	single nucleotide polymorphism
TPA	12-O-tetradecanoylphorbol-13-acetate

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Figure 1. Pancreatic cancer progression in human and mouse

Top: schematic representation of normal exocrine pancreas cells, transitioning from left to right into increasingly dysplastic PanIN and PDAC. In addition to visible abnormalities including papillary morphology, loss of polarity, nuclear atypia, intraluminal budding and stromal invasion (see Table 1 for additional details), PanIN-PDAC progression is associated with increasing accumulation of genetic lesions including activation of *KRAS* and loss of tumor suppressor genes including *CDKN2A*, *TP53*, *SMAD4* and *BRCA2*. Bottom: H&E-stained sections of mouse pancreata of indicated genotypes, illustrating normal acinar (ac) and duct (du) cells as well as an early-stage PanIN lesion and advanced PDAC.

Figure 2. Inducible *Kras* mouse model of pancreatic cancer

(A) Exon-intron structure of mouse *Kras* locus, comparing the wild-type allele to the *LSL-G12D* targeted allele, before and after Cre-mediated recombination. Gray boxes indicate 5' and 3' UTRs, black boxes indicate coding regions. The oncogenic G12D mutation is targeted to exon 2, preceded by a floxed STOP cassette that prevents expression prior to Cre recombination. (B) Tissue-specific Cre lines used for PDAC modeling. *Pdx1-Cre* and *Ptf1a^{Cre}* induce widespread recombination in multipotent progenitors of the embryonic pancreas, which give rise to all three mature pancreatic lineages. Adult ductal recombination is achieved with *Sox9-CreERT*, while acinar cells are labeled with *Ela-CreERT* and *Ptf1a^{CreERT}*.

Figure 3. Acinar reprogramming in PDAC initiation

PDAC originates from differentiated acinar cells, in which *KRAS* mutation alone does not elevate total RAS activity to the levels required for transformation. Acinar-ductal reprogramming occurs when *KRAS* signaling increases in response to exogenous inflammatory stimuli and/or EGFR agonists; as *KRAS* activity increases, mutant cells produce endogenous signals that establish a positive feedback loop between *KRAS* and inflammation. Several genes counteract acinar reprogramming, including Nr5a2, Mist1 and β-catenin, but are themselves inhibited by high levels of *KRAS* signaling. We hypothesize that acinar reprogramming represents a key step of PDAC initiation, which might be inhibited by anti-inflammatory agents in a chemopreventive setting.

Table 1

Classification of human and mouse PanIN progression

Normal duct	Flat, cuboidal or low-columnar epithelium, without mucin accumulation, nuclear crowing or atypia
PanIN-1A	Tall columnar cells organized in flat epithelium, with basal nuclear localization and mucin-rich cytoplasm.
PanIN-1B	Identical cellular morphology to PanIN-1A, but organized in papillary, micropapillary or basally pseudostratified arrangements.
PanIN-2	Flat or (more commonly) papillary architecture, with nuclear abnormalities that may include loss of polarity, crowding, enlargement, pseudostratification and hyperchromatism.
PanIN-3	Papillary or (less commonly) flat architecture, with loss of nuclear polarity, dystrophic goblet cells, occasionally abnormal mitoses and prominent nuclear abnormalities. Cribiform achitecture or luminal “shedding” of viable or necrotic cells frequently observed.

Key morphological characteristics of preinvasive pancreatic ductal lesions in the human and mouse, as defined by pathologist working groups of the Pancreatic Cancer Think Tank and Pancreatic Cancer in Mice and Man conferences, respectively (Hruban *et al.* 2006; Hruban *et al.* 2001). Progression to pancreatic ductal adenocarcinoma (PDAC) is defined by invasion of tumor epithelial cells through the basement membrane. Additional diagnostic information is available in the original publications, and online at the Sol Goldman Pancreatic Cancer Research Center of Johns Hopkins Medicine: <http://pathology.jhu.edu/pc/professionals/DuctLesions.php>