CHAPTER SIX

K-Ras-Driven Pancreatic Cancer Mouse Model for Anticancer Inhibitor Analyses

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Contents

1. Introduction 74
2. Pancreatic Ductal Adenocarcinoma Models 75
3. Imaging 76
   3.1. Imaging of *in situ* PDAs by high-resolution ultrasound 77
4. Drug Delivery 78
   4.1. Intraperitoneal injections 79
5. End Point Analysis 79
   5.1. Removal of blood from mice (Less than 0.1 ml) 80
   5.2. Necropsy protocol 80
   5.3. RNA isolation from pancreatic tissue 81
6. Model Fidelity for Preclinical Testing 82
7. Novel Therapeutics and Preclinical Trial Design 82
8. Conclusions 84
References 84

Abstract

Genetically engineered mouse (GEM) models of cancer have progressively improved in technical sophistication and accurately recapitulating the cognate human condition and have had a measurable impact upon our knowledge of tumorigenesis. However, the application of such models toward the development of innovative therapeutic and diagnostic approaches has lagged behind. Our laboratory has established accurate mouse models of early and advanced ductal pancreatic cancer by conditionally expressing mutant K-ras and Trp53

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alleles from their endogenous promoters in pancreatic progenitor cells. These K-Ras-dependent preclinical models provide valuable information on the cell types and pathways involved in the development of pancreatic cancer. Furthermore, they can be used to investigate the molecular, cellular, pharmacokinetic, and radiological characteristics of drug response to classical chemotherapeutics and to targeted agents. This chapter reviews the methods used to explore issues of drug delivery, imaging, and preclinical trial design in our GEM models for pancreatic cancer. We hypothesize that results of our preclinical studies will inform the design of clinical trials for pancreatic cancer patients.

1. Introduction

The primary purpose of drug efficacy testing is to predict, near the end of the preclinical development pipeline, whether a particular compound will be successful in the clinic. Two broad approaches are utilized in efficacy testing: cell-based *in vitro* systems and *in vivo* autochthonous animal models. At the interface between these two classes are tumor xenograft models in which cultured cells or tumor explants are grafted into immunodeficient mice. While such ''animal culture'' models are convenient to use, they generally behave differently than the corresponding human cancer. In fact, there is a poor correlation between the therapeutic activity of compounds tested in either xenografts or cell-based assays and their efficacy in humans (Johnson *et al.*, 2001). Xenograft models in particular have been used extensively in academic and industry research settings to prioritize compounds for clinical testing. Unfortunately, most drugs are found to be ineffective late in their development, with only a small percentage (<5%) of patients in phase 1 clinical trials responding to the therapies being tested (Roberts *et al.*, 2004). Such failures are costly to scientists and drug companies and are of great consequence to the patients that optimistically enroll in experimental clinical trials.

There are many reasons why preclinical studies fail to predict clinical activity, among them, differences in pharmacokinetics, pharmacodynamics, drug delivery, and metabolism. However, the basic problem is that neither cell-based studies nor xenograft models accurately reconstruct the complex interactions between tumor and host. Tumors are complicated entities, composed of mutated primary tumor cells, as well as recruited host cells that engineer a rich stromal environment. Indeed, in many tumor types, stromal cells outnumber tumor cells. This diversity is diminished and altered in xenograft systems.

In autochthonous genetically engineered mouse (GEM) models, tumor development occurs *in situ* in the appropriate tissue compartments and

complex processes can be modeled. Thus it is reasonable to expect that GEM models, carrying the genetic signature of the native malignancy, could recapitulate clinical behavior, offering an alternative to traditional preclinical assays. The utility of a GEM model is dependent on a number of issues, including fidelity of the genetic lesions, tumor penetrance, kinetics of tumor initiation/progression, and ability to detect disease and perform specific interventions. To date, very few accurate GEM models have been used in well-designed preclinical drug evaluation trials.

Early efforts to model pancreatic cancer failed to reproduce the dominant histological subtype of ductal adenocarcinoma. However, substantial efforts in the molecular genetics of human pancreatic ductal adenocarcinoma (PDA) have delineated a number of common genetic alterations. Among the most common alterations in PDA are activating mutations in the K-ras protooncogene. Such mutations are found in almost 95% of human cases and have the effect of locking the K-ras protein in an active signaling conformation (Smit *et al.*, 1998). Other common alterations include inactivation of a number of tumor suppressor genes: $p16^{INK4a}$ (90% of cases), p53 (approximately 75% of cases), SMAD4 (55%), and BRCA2 (10%) (Bardeesy *et al.*, 2006; Redston *et al.*, 1994). Moreover, these alterations accumulate with increasing frequency in a series of premalignant lesions associated with pancreatic cancer, most notably pancreatic intraepithelial neoplasia (PanIN) (Hruban *et al.*, 2000). These studies have led to the understanding that K-Ras and Ink4a alterations are early events in PDA development, whereas loss of SMAD4 and p53 tends to occur later (Maitra *et al.*, 2003). Additionally, advances have been made in the understanding of pancreatic developmental biology and in identifying signaling pathways that are important in PDA. These include the Notch and Hedgehog signaling pathways (Miyamoto *et al.*, 2003; Thayer *et al.*, 2003). Further understanding of how these pathways interact with oncogenic K-ras signaling should enable us to advance therapeutic options through the development of drugs targeting key pathways and molecules.

2. Pancreatic Ductal Adenocarcinoma Models

A number of models of pancreatic disease have now been developed in genetically engineered mice. A workshop took place in 2004 to establish an internationally accepted uniform nomenclature for the pathology of genetically engineered mouse models of exocrine pancreas neoplasia (Hruban *et al.*, 2006a,b). This resulted in the elaboration of specific proposals and goals that will allow uniform progression and hopefully success in this field.

Previous work in our laboratory has unveiled dramatic phenotypic differences between exogenous overexpression of mutant Kras and mutation of the endogenous gene at physiological levels. We therefore used homologous recombination in mouse embryonic stem cells to engineer a conditional mutant allele of Kras to enable the expression of endogenous mutant Kras exclusively in the pancreas. Mice harboring this conditional Kras mutant allele ($Kras^{LSL.G12D}$) in combination with a pancreas-specific Cre recombinase transgene (PdxCre or p48Cre) develop a full range of premalignant lesions in the pancreas, termed pancreatic intraepithelial neoplasia, before succumbing to invasive PDA and other tumors at late ages (Hingorani *et al.*, 2003). These mice are an excellent model of PanIN development and are useful for studying tumor progression. However, their use in therapeutic trials is limited because of the late onset of PDA.

For preclinical studies, a second model that also incorporates a conditional mutation in the endogenous p53 gene has proved to be more useful. These mice develop PDA with 100% penetrance and have a median survival of 4.5 months; approximately 70% of these mice present with gross metastasis (Hingorani *et al.*, 2005). Tissues not expressing Cre recombinase remain functionally hemizygous for these loci. Disease progression from preinvasive to invasive disease involved loss of the wild-type Trp53 allele, although it remains unclear at exactly what stage this occurs. The histopathology of pancreatic tumors from PDA mice is extremely similar to human PDA and also shows a high degree of chromosomal instability, another hallmark of human pancreatic cancer.

3. Imaging

Both PanIN and PDA mice develop tumors with variable latency. Therefore, noninvasive modalities that detect and quantify tumor size are needed to properly enroll such animals in preclinical trials. Subcutaneous xenografts can be monitored easily with calipers, but most GEM models require specialized imaging modalities to monitor tumor development. Several imaging techniques are available for mice, including magnetic resonance imaging (MRI), positron emission tomography, micro-X-ray computed tomography, and high-resolution ultrasound (US). All of these methods are also used in cancer patients. Another method used exclusively in genetically engineered mice is bioluminescent imaging, relying upon transgenic reporters engineered to reflect either anatomical or functional properties *in vivo*.

The main imaging technique currently used in our PDA mice is high-resolution US. A number of factors should be considered when deciding

which modality to use. These include the site of the tumor being imaged, whether the imaging modality is available within an animal barrier facility, the need for repeated imaging, session time, and invasiveness of technique. We chose to use US because it is noninvasive, readily available, requires a short session time, and is small enough to fit into our animal room. To detect tumors, mice had bimonthly US from 2 months of age and tumors were detected when they were as small as 1 mm in diameter. Animals with a defined tumor burden (3- to 5-mm-diameter tumor as measured by ultrasound) were enrolled into intervention studies along with matched littermate control mice that harbor K-ras and p53 alleles but lack Cre.

3.1. Imaging of *in situ* PDAs by high-resolution ultrasound

1. All imaging modalities require the mice to be anesthetized. Although injectable anesthetics are available, inhalation anesthetics such as isofluorane are better tolerated and therefore preferable for studies in which mice are imaged repeatedly.
2. Mice are anesthetized with a 3% isofluorane/oxygen mixture in an induction chamber and then transferred to a platform heated to 40° (VisualSonics, Inc.).
3. Core body temperature is monitored continuously using a digital rectal thermometer. Long fur is shaved from the abdomen and flanks of the mice, and a depilatory cream is applied to remove the remaining stubble. Ample washing with sterile water is used to remove the cream and prevent skin irritation.
4. We found that intraperitoneal (IP) injection of 3 to 4 ml of normal saline shortly before imaging results in a substantially improved image of the pancreas and other abdominal organs. This treatment is well tolerated, as normal saline is eliminated rapidly by diuresis.
5. Ultrasound gel, preheated to 37°, is applied to the abdomen of the mice, and scanning is performed with a 35-MHz ultrasound probe. This frequency allows for a 1-cm focal depth, which is ideal for abdominal imaging in mice. Care must be taken to minimize the introduction of bubbles into the ultrasound gel.
6. Optimal viewing of the head of pancreas is achieved through axial imaging of mice positioned supine and using the pyloris and proximal duodenum as landmarks (Figs. 6.1A and B). The tail of the pancreas is imaged axially with both supine and lateral positioning using the left kidney and the inner curvature of the spleen as landmarks (see Fig. 6.1C and D).
7. Using this protocol, mice are evaluated rapidly for the presence of pancreatic tumors with a session time of 10 to 15 min per mouse. Tumor volume is quantified by using the 3D motor (VisualSonics, Inc.)

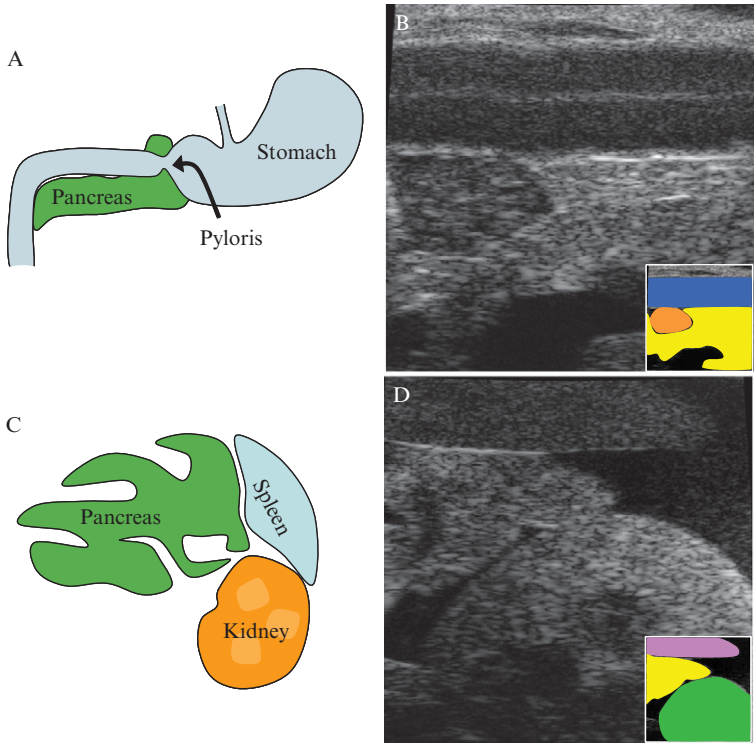

Figure 6.1 (A) Diagram showing landmarks used to locate the head of the pancreas by ultrasound. (B) Ultrasound image of the head of the pancreas showing a longitudinal section of the proximal duodenum (coded blue in the inset key), pancreas (yellow), and a axial section of a more distal segment of intestine (orange). (C) Diagram of the landmarks used to locate the tail of the pancreas by ultrasound. (D). Ultrasound image of the tail of the pancreas showing the proximity of the pancreas (coded yellow in the inset key) to the left kidney (green) and spleen (lavender).

to acquire volume data and then manually outlining the tumor on frames spaced 0.25 mm apart.

8. During image acquisition, it is important to avoid obstructions in the image, such as fecal matter in the intestines and stomach.

4. Drug Delivery

There are various different methods of delivering drugs in mouse models. Common routes of administration include subcutaneous (SC), IP, and oral gavage routes. Brief intravenous (IV) infusions and long-term

catheterization are also possible, although the risk of infection and thrombosis can be problematic. The effect of the chosen delivery route on drug metabolism should always be taken into account.

4.1. Intraperitoneal injections

1. First sterilize the skin using an aseptic technique.
2. The needle route is usually through the abdominal wall into the peritoneal cavity. An alternative approach is to enter via the flank but this can cause retroperitoneal organ laceration.
3. The needle enters bevel up into the ventral abdominal midline with the syringe held parallel to the hind leg.
4. Initially insert the needle subcutaneously, but change the angle to almost vertical as the needle passes through the peritoneum. Take care to avoid entering the bladder and liver.
5. Repeated injections may cause adhesions between abdominal organs. The maximum amount allowed via an IP injection is usually 20 ml/kg. The frequency of injections depends on the protocol being used, for example, minimum 24 h between injections/maximum two injections within 24 h.

Another delivery method is the use of a microosmotic pump (e.g., Alzet Inc.). These have the potential to ensure long-term, continuous exposure to therapies, reduce handling and stress to laboratory animals, and can be used via IP, SC, or IV routes. This approach may have particular relevance in delivering drugs with a short half-life. Microosmotic pumps can be used with a number of different solvents, but the solubility of the drug can become an issue as the total volume of the pump is often limiting.

5. End Point Analysis

The use of GEM models for PDA to evaluate antineoplastic therapeutics requires the development of methods to measure pertinent pharmacokinetic and pharmacodynamic characteristics. This includes the availability of molecular diagnostics, including cytogenetics, genomics, and proteomics, as well as the rapid assessment of drug levels from the mouse tissues. Standard methods of tissue procurement and processing need to be established for the assessment of target inhibition in pancreatic tumors because acinar cells that occupy the majority of pancreatic tissue contain high levels of enzymes that degrade nucleic acids, proteins, carbohydrates, and fats. As high-quality human pancreatic tumor tissue is very difficult to obtain, it is likely that methods developed with GEM models may be applicable to clinical trials.

Another use of preclinical models is for the identification of biomarkers of disease progression and response to intervention. Serum proteomic screens and RNA transcriptional analyses are examples of unbiased methods used to identify correlative biomarkers for therapeutic responsiveness or resistance. Results from our PanIN mice have already shown a unique proteomic signature for mice with preinvasive pancreatic cancer (Hingorani *et al.*, 2003).

Methods for blood removal, necropsy, and RNA isolation are described next.

5.1. Removal of blood from mice (Less than 0.1 ml)

1. Asepsis should be maintained throughout so hair and superficial debris must be removed. Anesthetic is not normally needed for superficial vein bleeds.
2. The most common site for venipuncture on a mouse (and the method we use) is by puncturing the tail vein.
3. The animal should be gently restrained by an experienced animal handler and the vein raised, that is, some pressure may be needed proximal to the vein to occlude venous return and allow adequate venous sampling.
4. A sterile needle or lancet can be used to puncture the skin and underlying blood vessel.
5. Blood can be removed by a capillary tube or micropipette and plastic tip.
6. A maximum of 10% of circulating blood volume can be removed on a single occasion and a maximum of 15% of circulating blood volume in any 28-day period.
7. After blood removal, maintain firm pressure on the site for 30 s to prevent any further bleeding.
8. Possible complications associated with venipuncture include bleeding, bruising, thrombosis, and stress to the animal.

5.2. Necropsy protocol

Materials: neutral-buffered formalin, 1000 U/ml heparin solution, cryogenic freezing tubes, microcentrifuge tubes, RNALater (Qiagen), and dissection instruments

1. Euthanize the mouse using standard approaches.
2. Serum/plasma: Immediately perform cardiac puncture to collect blood.
3. Use a 21-gauge needle with a 1-ml syringe.
4. For plasma, precoat the needle and syringe with heparin by drawing a small amount into the needle and expelling, leaving some heparin in the void volume of the syringe. Collect blood into a microcentrifuge tube. For plasma, store the tube on wet ice for no more than 15 min.

Spin blood at 8000 rpm for 10 min at 4°. For serum, allow blood to clot at room temperature for 1 h. Spin at 8000 rpm for 10 min at 4°. Collect plasma or serum and usually store at −80°.

5. Following blood draw, open the abdominal cavity and immediately collect pancreatic samples, beginning with RNA samples.
6. RNA samples: Collect 50 to 250 mg of tissue into 500 μl of RNALater (Qiagen). Incubate at 4° over night. Decant RNALater and process immediately (see additional information provided later).
7. Photograph each animal with a digital camera to record anatomical position of tumors. Include the animal's identification in the photograph.
8. Snap frozen tissues: Depending on the availability of tissues, collect up to five snap frozen samples of tumor and other tissues (approximately 100 mg) into cryogenic screw-top tubes and snap freeze in liquid nitrogen.
9. Frozen sections: Collect tissue into a cryogenic mold filled with O.C.T. compound (Tissue-Tek). Place mold on dry ice and store at −80°. Do not immerse in liquid nitrogen.
10. Paraffin sections: Collect tissues into 10% formalin solution and fix for up to 24 h. An alternative is zinc-buffered formalin (Z-Fix, Anatech Ltd.). Following fixation, decant formalin and transfer tissue to 70% ethanol. Cassette and process tissue by standard protocols.
11. DNA: Collect several centimeters of tail into a microcentrifuge tube and store at −20° as an archival DNA sample from each mouse.

5.3. RNA isolation from pancreatic tissue

Note: This procedure is only used to isolate mRNA (Qiagen). Another class of RNAs that may prove diagnostically useful is micro-RNAs.

1. Always determine the correct amount of starting material to obtain optimal RNA yield and purity. In general 10 to 100 mg of fresh or frozen tissue can be processed. If there is no information about the nature of your starting material, start with 10 mg or less. For RNeasy mini-columns, use 10 mg of normal pancreatic tissue, as more than 10 mg seems to decrease the quality of the isolated RNA. Notably, Qiagen provides no information about mRNA preparation from pancreatic tissue.
2. We use the RNAeasy Protect Mini procedure (Qiagen). Always follow aseptic techniques and use sterile, RNase-free pipette tips and disposable gloves. Reagents from Qiagen are all RNase free and therefore do not require diethyl pyrocarbonate treatment.
3. Excise the tissue sample from the animal. Some method of RNA stabilization is absolutely required when isolating RNA from normal and PanIN pancreatic tissue. This is less important for tumor tissue, presumably

because there are few acinar cells present and therefore less RNase; however, if you want to compare tumor tissue to normal or PanIN tissue, they should all be treated similarly.

4. We currently use RNALater (Qiagen) for stabilization. It should be noted that RNALater-treated tissue is difficult to process for histological and immunohistochemical evaluation. We place approximately 50 to 100 mg tissue in a 1.5-m Eppendorf tube that has 10 volumes of cold RNALater and incubate overnight at 4°. The next day, RNALater can be drained and the sample is stored at −80°. For further information about RNA stabilization, please refer to the Qiagen manual

6. Model Fidelity for Preclinical Testing

The following points should be considered for pancreatic cancer GEM models used in preclinical studies.

- Mutant mice should accurately reflect the genetics and pathology of human pancreatic cancer and pathophysiological processes such as cachexia.
- The molecular alterations in murine PDA tumors should reflect those observed in human PDA, including biochemical pathways, gene expression patterns, and genomic alterations. As described previously, our PDA model accurately reflects these points, further validating our mouse model.
- Before assessing new therapies, GEM models of PDA should be treated with drugs normally used to treat patients. We refer to this as "credentialing"—the model should respond to drugs in a manner comparable to human patients (Olive and Tureson, 2006). Because the current standard therapy for pancreatic cancer, gemcitabine, has a relatively low response rate in patients with advanced disease, we would therefore predict a similar ineffectiveness in our model of PDA. Surprisingly, relatively few studies of conventional cytotoxic agents have been reported to date in GEM models.

7. Novel Therapeutics and Preclinical Trial Design

Novel therapeutics for PDA can be evaluated in the PDA GEM model once the appropriate biochemical and analytical chemical assays are established to measure pertinent pharmacokinetic and pharmacodynamic parameters. Although it is currently not possible to inhibit the K-Ras oncoprotein directly, downstream Ras effector pathways, such as RAF/MEK and PI3K/AKT, and cooperating signaling networks, including Notch, Hedgehog, and various

mitogenic growth factor receptors (e.g., EGFR, Her2/neu, Met, IGFR), are potential candidates worthy of therapeutic investigation. Combinations of inhibitors blocking these pathways may be required for future effective therapies, and GEM models can be used to test synergy versus antagonism. Importantly, several candidate compounds targeting the aforementioned signaling cascades are currently undergoing early phase clinical evaluation.

Our PanIN and PDA GEM models will be used to evaluate potential therapies in a chemoprevention or intervention setting, respectively. Two study structures will be used to assess the effects of therapeutic agents on our murine PDAs (please also see Fig. 6.2).

Short-term intervention studies will be used to establish basal parameters such as serum and tumor pharmacokinetics (PK), pathway inhibition, and effects on basic tumor cell properties such as proliferation rate and apoptosis.

1. Mice are monitored by high-resolution ultrasound, MRI, or optical imaging techniques.
2. Animals with a defined tumor burden (a 3- to 5-mm-diameter tumor as measured by ultrasound) are enrolled into intervention studies.
3. Blood is collected by tail vein bleeding in order to assay serum PK levels.
4. Biweekly imaging is used to track tumor progression during the study, and daily weights are recorded.
5. Following brief pilot experiments, usually less than 2 weeks of duration, animals are euthanized and tissues harvested for tumor PK and tissue PD evaluation, including analysis of bromodeoxyuridine incorporation (injected 24 h prior to sacrifice), apoptosis, and biochemical signaling and gene expression alterations.

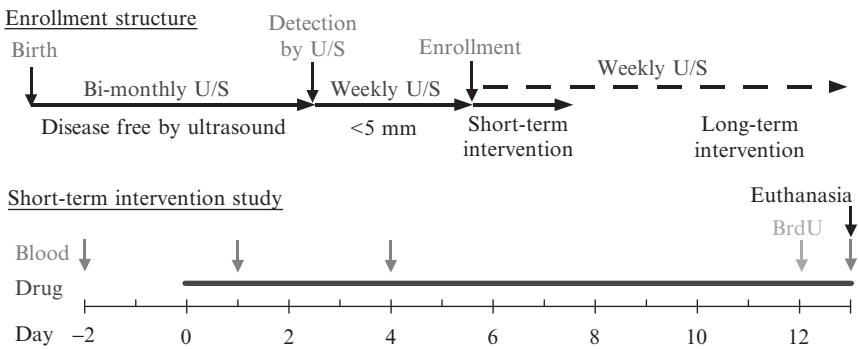

Figure 6.2 Enrollment and treatment algorithm: Mice are monitored by weekly ultrasound until the detection of measurable disease, after which they can be enrolled in a short- or long-term intervention study. Details of an example short-term intervention are provided.

Data from these studies will also advise the design of an intervention and prevention study with regards to drug dosing and schedule.

Long-term studies will directly examine the effects of drugs on survival. For these studies, drug dosages and schedules must be first evaluated for tolerability. Animals will remain on the drug treatment regimen until they require sacrifice due to demonstration of significant morbidity from pancreatic cancer. Methods of assessing the health of the mice include daily weight measurements and direct behavioral observation.

8. Conclusions

Limited progress has been made in treating pancreatic cancer over the recent years, and it is often challenging to evaluate new agents in patients due to the rapid progression of disease and the difficulty in monitoring the response to therapies. We suggest that GEM models of PDA will exceed the performance of xenograft models in predicting response to therapy due to the more accurate representation of various cell autonomous and tumor microenvironmental features in GEM models. This harbors great promise for accelerating the drug discovery process to markedly improve the success of pancreatic cancer drug development.

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