

Constitutively Active Akt1 Cooperates with KRas^{G12D} to Accelerate *In Vivo* Pancreatic Tumor Onset and Progression

Toya M. Albury^{*,1}, Veethika Pandey^{*,1}, Sarah B. Gitto^{*,1}, Lisette Dominguez^{*}, Lina P. Spinel^{*}, Jacqueline Talarchek[†], Andres J. Klein-Szanto[‡], Joseph R. Testa[†] and Deborah A. Altomare^{*}

^{*}Burnett School of Biomedical Sciences, University of Central Florida, Orlando, FL, USA; [†]Cancer Biology Program, Fox Chase Cancer Center, Philadelphia, PA, USA; [‡]Department of Pathology, Fox Chase Cancer Center, Philadelphia, PA, USA

Abstract

BACKGROUND AND AIMS: Pancreatic adenocarcinoma is a deadly disease characterized by metastatic progression and resistance to conventional therapeutics. Mutation of *KRAS* is the most frequent early event in pancreatic tumor progression. AKT isoforms are frequently activated in pancreatic cancer, and reports have implicated hyperactivation of AKT1, as well as AKT2, in pancreatic tumor formation. The objective here is to delineate the role of AKT in facilitating *in vivo* pancreatic tumor progression in the context of *KRAS* mutation and predisposition to pancreatic cancer. **METHODS:** Mice with *Akt1* and *KRas* mutant alleles expressed using the pancreas *Pdx* promoter were mated to characterize the incidence and frequency of histologic and genetic alterations known to occur commonly in human pancreatic ductal adenocarcinoma. **RESULTS:** Active Akt1 (Akt1^{Myr}, containing a myristoylation sequence) cooperated with active mutant KRas^{G12D} to accelerate pancreatic carcinoma onset and progression and increase phosphorylation of downstream effectors in the Akt pathway. Mucin and smooth muscle actin expression was found in and around pancreatic intraepithelial neoplasms (PanINs), and accelerated time to metastasis was found in Akt1^{Myr}/KRas^{G12D} mice. **CONCLUSIONS:** In contrast to prior reports of pancreatic KRas mutant mice mated with mice deficient for various tumor suppressor genes, which resulted in aggressive disease within a few months of age, Akt1^{Myr}/KRas^{G12D} mice enabled the study of PanINs and spontaneous pancreatic transformation more characteristic of human pancreatic progression in elderly individuals. The Akt1^{Myr}/KRas^{G12D} model holds promise for delineating the tumor biology and biomarkers critical for understanding their cooperation in cancer oncogenesis and future targeting in therapeutic strategies.

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Introduction

Activating *KRAS* mutations are present in virtually all human pancreatic adenocarcinomas and occur with increasing frequency in later stage pancreatic intraepithelial neoplasia (PanIN) lesions [1,2]. To date, the model that most faithfully recapitulates human PanIN development and its early progression is a mouse model that expresses a *Cre*-activated *KRAS* allele knocked into the endogenous *KRAS* locus [3] when crossed with mice expressing a *Pdx1*-*Cre* recombinase transgene promoter [4]. Oncogenic *KRAS*^{G12D} in the progeny of the *Cre* matings developed PanINs within a few months, with activation of the Notch pathway and overexpression of COX-2 and MMP-7 [5]. This model thus offered the first recapitulation of human PanIN lesions, with low incidence of progression to pancreatic adenocarcinoma [5].

Address all correspondence to: Deborah A. Altomare, PhD, University of Central Florida, College of Medicine, Burnett School of Biomedical Sciences, 6900 Lake Nona Blvd., Orlando, FL 32827, USA.

E-mail: Deborah.Altomare@ucf.edu

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¹These authors contributed equally to this work.

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In comparison, *Pdx-Cre;Pten^{lox/lox}* mice with pancreatic knockout of *Pten* display rapid progression of pancreatic ductal metaplasia, development of PanINs, and low frequency of malignant transformation [6]. Under normal conditions, mouse and human PTEN functions as a dual-specificity protein phosphatase and a lipid phosphatase. Because PTEN modulates phosphatidylinositol 3-kinase (PI3K)–AKT signaling, loss of PTEN tumor suppressor function in pancreatic tumor progression further implicates the importance of AKT signaling in pancreatic pathogenesis.

Other mouse models of pancreatic cancer have been developed to study components of the PI3K/PTEN/AKT signaling pathway. A previous study analyzed constitutively active mutant AKT1 under control of *Pdx-Cre*, elastase-Cre, and rat insulin promoter-2–Cre expression [7]. Interestingly, premalignant lesions and acinar tumors were observed when expression was driven by *Pdx-Cre*. Constitutively active myristoylated (Myr) Akt1 under rat insulin promoter-2 was also shown to result in malignant transformation of islet cells to develop islet cell carcinomas [8]. Most recently, a mouse model with a constitutively activated catalytic subunit of PI3K was used to study the importance of PI3K signaling in *Kras*-driven pancreatic ductal adenocarcinoma (PDAC) initiation and maintenance [9] and the requirement of 3-phosphoinositide dependent protein kinase 1 (PDK1) signaling for *KRas* pancreatic cell plasticity and cancer. Of relevance to human pancreatic cancer, earlier reports showed amplification and protein overexpression of AKT2 in human pancreatic cell lines [10], and both AKT1 and AKT2 were found in human PDACs and in metastases [11]. AKT alterations are among the most commonly activated oncogenic changes in solid tumors and activation of AKT isoforms is frequently attributed to down-regulation of PTEN tumor suppressor or activation of upstream signaling components (activating PI3K mutations or activating growth factor receptors). A prior study, using immunohistochemistry and tissue microarrays, revealed that 34 of 133 (~25%) stage II PDACs exhibited loss of PTEN expression [12]. Haplo-insufficiency and occasional homozygous loss of *PTEN* have also been found in human PDACs [11]. Overall, the data support a dosage-dependent role for mouse and human PTEN and in the activation of downstream AKT [13].

Herein, we report the first evidence describing the contribution of constitutively active myristoylated Akt1 *in vivo* to pancreatic ductal tumor progression using genetically defined transgenic models to delineate potential cooperation with *Pdx*-regulated expression of *KRas^{G12D}*. Collectively, these studies provide insights regarding the pathogenic implication of Akt perturbations in combination with *KRas* oncogenic mutations to accelerate pancreatic progression toward the development of invasive PDAC. In addition, we propose that this dual oncogene model may offer long-term preclinical utility for testing of novel therapeutic strategies against pancreatic tumor progression and an opportunity to intervene before extensive desmoplastic fibrosis and irreversible tissue remodeling, which is a confounding problem in the treatment of late-stage pancreatic disease.

Material and Methods

Genetically Engineered Mice

Animal care and use was at Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC)-accredited facilities. Protocols were approved by Institutional Animal Care and Use Committees at each respective institution and were compliant with NIH guidelines. *Pdx-tTA* mice in an FVB/n background [14] were mated with *TetO-MyrAkt1* mice in a C57Bl/6 to generate *Pdx-tTA;TetO-MyrAkt1* mice (Figure S1). *LSL-KRas^{G12D}* [3] in a 129SvJ background and *Pdx-Cre* [4] mice in a C57Bl/6 were mated to generate

Pdx-Cre;LSL-KRas^{G12D} mice. The progeny were mated to generate litters that were genotyped using DNA extracted from tail snips (Wizard Genomic DNA Kit; Promega, Madison, WI) and were monitored for the tumor latency study. In total, 29 *Pdx-Cre;LSL-KRas^{G12D}* (designated *KRas^{G12D}* mice) and 30 *Pdx-tTA;TetO-MyrAkt1* (designated *Akt1^{Myr}/KRas^{G12D}* mice) were followed. Animals were housed until times outlined and then killed in accordance with American Veterinary Medical Association guidelines. Single Nucleotide Polymorphism (SNP) analysis performed by Charles River Laboratories International (Wilmington, MA) on randomly selected mice that were re-derived for the < 1 year analysis revealed that the background of *Akt1^{Myr}/KRas^{G12D}* mutant mice was ~60% C57Bl/6N.

Genotype Analysis

Reactions were assembled using JumpStart REDTaq ReadyMix (Sigma, St Louis, MO). Primers for polymerase chain reaction (PCR) detected presence of *Pdx-Cre* (5'-ATCGCTGATTTGTGTAGTCGGT-3'; 5'-CAACAGTTGCGCAGCCTGAATG-3'), mutant or non-recombined *LSL-KRas^{G12D}* (5'-GTCGACAAGCTCATGCGGGTG-3'; 5'-AGCTAGCC-ACCATGGCTTGAGTACGTCTGCA-3'; 5'-CCTTTACAAGCGCACGCA-GACTGTAGA-3'), heterozygous knock-in for *tTA* into the endogenous *Pdx* gene (5'-ACCATGAACAGTGAGGAGCAGTAC-3'; 5'-GCGGGTTTCA-GAGGAATTTGT-3'; 5'-TAGAAGGGGAAAGCTGGCAAG-3'; 5'-TCCAGATCGAAATCGTCTAGCG-3'), or presence of *TetO-MyrAkt1* (5'-CTGGACTACTTGCCTCCGAGAAG-3'; 5'-CTGTGTAGGGTCCTTCTTGAGCAG-3').

Histologic Analysis

Specimens were fixed in 10% neutral buffered formalin (Surgipath Leica, Buffalo Grove, IL) and paraffin embedded. Five-micrometer sections were cut with a rotary microtome (Leica). Histologic staining used SelecTech hematoxylin and eosin (H&E) reagents (Surgipath). Staining with Alcian Blue or Trichrome (both from American MasterTech, Lodi, CA) was performed as per manufacturers' instructions.

Antigen retrieval for immunohistochemistry was optimized with sodium citrate (pH 6.0) or EDTA (pH 9.0; Leica). Primary antibodies were against phospho-Akt Ser473 (GeneTex, Irvine, CA), phospho-mTOR Ser2448 (Cell Signaling Technology, Danvers, MA), phospho-p70S6K Thr389 (Upstate Cell Signaling, Temecula, CA or LifeSpan BioSciences, Seattle, WA), and phospho-p70S6K Thr389 (Cell Signaling Technology), mucin-4 (Muc-4; LifeSpan BioSciences), α -2 smooth muscle actin (α -SMA; Novus, Littleton, CO), cytokeratin 17/19 (Cell Signaling Technology), and Ki67 (Abcam, Cambridge, MA). Detection used Polymer Refine Detection reagents (Leica). A Bond-Max Immunostainer and Polymer Refine Detection reagents (Leica) were used.

Cell Culture

Primary cells were collected following the killing of mice, and cells were derived from phosphate-buffered saline-washed peritoneum and cultured using Dulbecco's modified Eagle's medium (Cellgro Mediatech, Manassas, VA) supplemented with 15% FBS, 2 mM L-glutamine, and 2 mM penicillin-streptomycin. Murine pancreatic cell cultures were maintained in Dulbecco's modified Eagle's medium/10% FBS supplemented with 2 mM L-glutamine and penicillin-streptomycin. Human cell lines were from American Type Culture Collection (Manassas, VA) and grown as recommended.

Genomic PCR

Genomic DNA was extracted (Wizard Genomic DNA Isolation Kit; Promega), and PCRs were performed with GoTaq Green Master

Mix (Promega). Primers for PCR detected presence of *Tp53* (5'-CTTGACACCTGATCGTTACTC-3' and 5'-CAGTCCTAACCC CACAGGCGG-3'), *p16Ink4a* (5'-TGGTCACACGAC TGGGCGATTG-3' and 5'-GAATCGGGGTACGACCGAAAG-3'), *p19Arf* (5'-AGCATGGGTGCGAGTTCTTGG-3' and 5'-TTGAGGAGGACCGTGAAGCCGA-3'), and control glyceraldehyde 3-phosphate dehydrogenase or *Gapdh* (5'-AGGCCGGTGCTGAG-TATGTC-3' and 5'-TGCCTGCTTACCACCTTCT-3').

Western Blots

Whole-cell extracts were harvested from low passage cell cultures with 1 × cell lysis buffer (Cell Signaling Technology) for protein, 1 mM phenylmethylsulfonyl fluoride (Sigma), and 2 mM Halt protease and phosphatase inhibitor cocktail (Thermo, Waltham, MA). Protein was quantified using the Bradford method. For Western blot analysis, 60 μg of each protein extract was combined with Laemmli's sodium dodecyl sulfate sample buffer (final 1 ×) and denatured in a boiling water bath for 5 minutes. Precision Plus (Bio-Rad Laboratories, Hercules, CA) protein standard and total protein were separated on 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels using a Mini-Protein Tetra Cell (Bio-Rad) unit set for 15 minutes at 20 mA and then 1 hour at 40 mA constant current. Proteins were transferred to Hybond ECL Nitrocellulose (GE Healthcare Amersham, Pittsburgh, PA) at 25 V at 4°C for 2 hours using an XCell II Blot Module (Invitrogen Life Technologies, Grand Island, NY). Antibodies for Western blots were against total Akt, phospho-Akt Ser473, and p53 (all from Cell Signaling Technology), mouse p16Ink4a (Santa Cruz Biotechnology, Santa Cruz, CA), mouse p19Arf (Abcam), and actin (Millipore, Billerica, MA). Secondary antibodies were anti-mouse (DyLight, Thermo) and anti-rabbit (IR Dye; LI-COR Biosciences, Lincoln, NE), and signals were visualized using an Odyssey Infrared Imaging System (LI-COR).

Results

Accelerated Frequency of PDACs in Double Mutant Mice Compared to Single Mutant Mice

The pancreatic tumor model uses the *Pdx1* pancreas promoter to drive expression of myristoylated, membrane-targeted, and therefore activated Akt. Specifically, it uses a dual transgenic system with tetracycline operator (TetO) sequences fused to MyrAkt1 [15–17], and then the progeny were crossed with *Pdx-TetA* (*Pdx-tTA*) knock-in mice expressing tTA in the pancreas [14]. Resultant *Pdx-tTA;TetO-MyrAkt1* transgenic mice were identified by genotyping (Figure S1).

Transgenic Lox-Stop-Lox (LSL) *KRas*^{G12D} [3] and *Pdx-Cre* [4] mice were expanded, and these parental mice were mated to obtain compound mice expressing *Pdx-Cre*-activated *KRas*^{G12D}. Resultant *Pdx-Cre;LSL-KRas*^{G12D} were then mated to *Pdx-tTA;TetO-MyrAkt1* mice to generate pancreatic-specific active mutant *KRas*^{G12D}, active MyrAkt1, compound mutant Akt1^{Myr}/*KRas*^{G12D} mice or non-mutant littermates. The progeny were genotyped and littermates were followed phenotypically to determine if there is cooperation between mutant active *KRas*^{G12D} and MyrAkt1 to accelerate malignancy and/or metastasis and to establish a potentially clinically relevant pancreatic tumor model useful for future preclinical studies to test novel targeted therapies.

Figure 1 shows age in weeks when a carcinoma was detected in mice with compound Akt1^{Myr}/*KRas*^{G12D} relative to mice with *KRas*^{G12D} mutant alone. Kaplan-Meier curves (GraphPad Prism 5, San Diego, CA) were used to calculate median tumor detection at 54 weeks in Akt1^{Myr}/*KRas*^{G12D} mice compared to 74 weeks in *KRas*^{G12D} mice. At the median 54-week time point for Akt1^{Myr}/*KRas*^{G12D} mice, 11 of

22 mice examined had developed early to full pancreatic carcinoma. In comparison, only one early carcinoma and one PDAC were found in seven age-matched *KRas*^{G12D} mice, and tumor progression to PDAC was significantly delayed in a subset of *KRas*^{G12D} mice until approximately 15 months of age. Overall, the number of mice found with early carcinoma to PDAC was 14 of 30 Akt1^{Myr}/*KRas*^{G12D} and 13 of 29 *KRas*^{G12D} mice, even though Akt1/*KRas*^{G12D} mice declined in health earlier and could not be aged as far as *KRas*^{G12D} mice. Four metastatic tumors were found in Akt1^{Myr}/*KRas*^{G12D} mice at 8 to 12 months of age compared to 0 metastatic tumors in *KRas*^{G12D} (Table 1). In contrast, littermates that were Akt1^{Myr} mice had a tendency to develop islet carcinomas (Figure S2), rather than ductal carcinomas, at a mean latency of 75 weeks in 6 of 24 mice. Because of the predominant islet carcinoma lineage in the Akt1^{Myr} subset, these mice were not characterized further in the context of this PDAC study.

Double Akt1^{Myr}/*KRas*^{G12D} Mice at ≤ 1 Year of Age Exhibit PanINs and PDACs

In a separate experiment, compound mutant Akt1^{Myr}/*KRas*^{G12D} mice were then studied within 12 months for the frequency of PanINs and metastatic PDACs. Nearly 77% of compound Akt1^{Myr}/*KRas*^{G12D} mice aged ≤ 1 year exhibited high-grade PanINs and or PDACs (Table 2). Four of 17 mice developed PDACs. This is consistent with the results from the Kaplan-Meier study, which showed median histologic detection of malignant tumor progression at 54 weeks in Akt1^{Myr}/*KRas*^{G12D} mice (Figure 1).

Akt Pathway Effector Proteins Are Activated in Early PanINs and Metastatic PDACs

Immunohistochemistry using phosphorylation-specific antibodies depicted abundant Akt pathway signaling in PanINs and metastatic PDACs (Figure 2). Pathway markers, including phospho-Akt, phospho-mTOR, and phospho-p70S6 kinase were found in the pancreas of both Akt1^{Myr}/*KRas*^{G12D} (Figure 2A) and *KRas*^{G12D} mice (Figure 2B). A possible mechanism may be increased proliferation, as detected by Ki67 staining, in the PanINs shown in Figure 2A (see Figure S3). Metastatic PDACs in Akt1^{Myr}/*KRas*^{G12D} mice exhibited ascites and metastasis to liver or other abdominal sites, with abundant levels of phospho-Akt and elevated levels of phospho-mTOR and phospho-p70S6 kinase at the metastatic sites (Figure 2C).

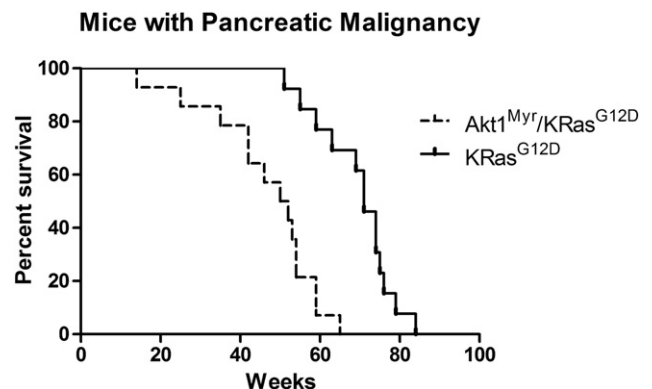


Figure 1. Tumor latency in Akt1^{Myr}/*KRas*^{G12D} versus *KRas*^{G12D} mice. Akt1^{Myr}/*KRas*^{G12D} mice (broken line) developed pancreatic tumors (PDACs) at a faster rate than *KRas*^{G12D} mice (solid line). Curves were significantly different with a *P* value < .0001 by log rank (Mantel-Cox) or Gehan-Breslow-Wilcoxon tests (GraphPad Prism 5).

Table 1. Mice with Pancreatic Carcinomas for Kaplan-Meier Analysis

	<8 Months	8 to16 Months	>16 Months	Total
Akt1 ^{Myr} /KRas ^{G12D}	1/6 mice	12/23 mice (four metastases)	1/1 mouse (0 metastasis)	14/30
KRAS ^{G12D}		5/14 mice (0 metastasis)	8/15 mice (two metastases)	13/29 [†]

Akt1^{Myr}/KRas^{G12D} mice were collected at <8 months primarily because of weight loss, although KRas^{G12D} mice did not exhibit comparable issues.

[†] Other pathologies in aged KRas^{G12D} mice ≥12 months of age included lymphomas, hepatocellular carcinoma, and lung adenocarcinoma; a lung adenocarcinoma was found in an aged-matched Akt1^{Myr}/KRas^{G12D} mouse.

Markers of Tissue Remodeling in the Pancreas of Mice Undergoing Progression to PDAC

Alcian Blue and Muc-4 staining, markers of mucin expression, were evident in the pancreatic ducts found in proximity to PDACs. Importantly, Muc-4 is a mucin that has been implicated as a marker of pancreatic ductal tissue transformation in human PanINs and PDACs [18]. In mice, Muc-4 has also been shown to correlate with the progression of pancreatic cancer from PanIN lesions to PDAC [19]. Staining for Muc-4 was found in ductal regions, as well as some acinar regions of Akt1^{Myr}/KRas^{G12D} mice with PDAC (Figure 3).

Similarly, activation of pancreatic stellate cells near centroacinar cells has been implicated as contributing to desmoplastic or fibrotic areas in PDACs and frequently expresses α-SMA [20]. Recurrent staining for trichrome and α-SMA was found around the acinar regions of pancreatic tissue with PDAC and PanIN lesions in Akt1^{Myr}/KRas^{G12D} mice (Figure 3A, representative age 7 months) and KRas^{G12D} mice (Figure 3B, representative age 12 months). Trichrome stain revealed mild to moderate fibrosis, reminiscent of desmoplasia. Intensity of trichrome stain was variable, as depicted by green-blue staining (Figure S4) in a 12-month-old Akt1^{Myr}/KRas^{G12D} mouse. α-SMA staining was frequently detected in pancreatic acini exhibiting a myoepithelial distribution pattern.

Table 2. Representative Histology of Akt1^{Myr}/KRas^{G12D} Mice up to 1 Year of Age

Mouse ID	Genotype	Age (Weeks)	PanIN Low Grade	PanIN High Grade	PDAC
173	Akt1 ^{Myr} /KRas ^{G12D}	13.6	X	X	
524 [†]	Akt1 ^{Myr} /KRas ^{G12D}	20.6	X		
179	Akt1 ^{Myr} /KRas ^{G12D}	27.7	X	X	X (papillary)
505	Akt1 ^{Myr} /KRas ^{G12D}	29.1	X		
547	Akt1 ^{Myr} /KRas ^{G12D}	29.4	X	X	
192	Akt1 ^{Myr} /KRas ^{G12D}	31.1	X	X	
195 (562)	Akt1 ^{Myr} /KRas ^{G12D}	31.1	X	X	X (papillary)
160	Akt1 ^{Myr} /KRas ^{G12D}	31.9	X		
161	Akt1 ^{Myr} /KRas ^{G12D}	31.9	X	X	
165	Akt1 ^{Myr} /KRas ^{G12D}	31.9	X	X	
139	Akt1 ^{Myr} /KRas ^{G12D}	38.4	X		
157	Akt1 ^{Myr} /KRas ^{G12D}	46.4		X	
533	Akt1 ^{Myr} /KRas ^{G12D}	43.1		X	X (metastasis, carcinomatosis)
9C	Akt1 ^{Myr} /KRas ^{G12D}	46.4		X	X (metastasis)
167	Akt1 ^{Myr} /KRas ^{G12D}	48.4		X	
106	Akt1 ^{Myr} /KRas ^{G12D}	50.9	X	X	
177-2	Akt1 ^{Myr} /KRas ^{G12D}	52.9		X	
			12 of 17	13 of 17	4 of 17

Fibroadenomatous lesions are also found in 173, 524, 547, 160, 161, 165, 157, 106, and 177-2.

[†] Cystic papillary lesion, early cystadenoma, and intraductal papillary tumor are found in 524, 192, and 167, respectively.

Tumor Cells from Double Mutant Mice Exhibit High Akt Phosphorylation and Loss of Tumor Suppressors Known to be Important in Human Pancreatic Tumor Progression

Cell cultures were derived from three KRas^{G12D} mice diagnosed with PanINs at age 10 to 11 months and also three Akt1^{Myr}/KRas^{G12D} mice with preneoplastic lesions and/or PDACs at age 7 to 11 months of age. Western blot analysis revealed high levels of phosphorylated Akt in low passage cell cultures from Akt1^{Myr}/KRas^{G12D} mice, relative to cells from KRas^{G12D} mice and two human PDAC cell lines (Figure 4A). For mouse tumor cells, p53 tumor suppressor protein was retained in all cases. p16Ink4a protein expression was frequently downregulated in the cell cultures, with complete loss of p16Ink4a in one of the Akt1^{Myr}/KRas^{G12D} PDAC cell cultures. p19Arf protein expression was also difficult to detect, and p19Arf protein was absent in the same PDAC cell culture with loss of p16Ink4a. Genomic PCR confirmed biallelic loss of the overlapping genes *p16Ink4a* and *p19Arf*, which reside at the *Cdk2na* locus, in tumor cells from an Akt1^{Myr}/KRas^{G12D} mouse that developed PDAC (Figure 4B). Such homozygous losses of *CDKN2A* are common in human PDACs. Moreover, a tumorigenicity study with this same PDAC tumor cell culture revealed that it was capable of forming tumors in syngeneic mice without the mutant alleles at ~3 weeks after the orthotopic injection of 1 × 10⁶ cells into mouse pancreas (Figure S5).

Discussion

Pancreatic mouse models targeting genes known to be mapped to the histologic and genetic profile of PDACs have been used to test cooperativity with *KRAS* mutations, as reviewed elsewhere [21]. In most cases, the combination of the predisposing *KRAS* mutation with loss of tumor suppressors, such as p16Ink4a or p53, greatly accelerates malignancy such that mice frequently die within a few months. In humans, according to 2014 American Cancer Society projections, the median age of pancreatic cancer detection is 71 years old and increases with age (<http://www.cancer.org/acs/groups/content/@research/documents/document/acspc-038828.pdf>). Moreover, in humans, the progression of PanINs to PDAC probably takes more than a decade to develop [22]. Thus, the rapid onset of pancreatic disease in compound *KRAS*/tumor suppressor knockout mice has limited utility for studies relevant to disease in the elderly and with other non-genetic factors that contribute to disease and treatment. The overall objective of the current *in vivo* study was to combine two oncogenic changes important for cancer progression to accelerate tumorigenesis, while maintaining a time frame that would align more closely with the physiological progression observed in the human disease.

Recently, E17K mutations in the AKT1 pleckstrin homology domain have been found in human pancreatic intraductal papillary mucinous neoplasms (3 of 36), along with activating mutations in PI3K or loss of PTEN [23]. AKT1 E17K mutations were not found in a previous study of PDACs [24], although the number of cases examined (12) was small. Thus, the role of the AKT1 E17K mutation is still being defined and may be context dependent. As proof of principle, we used an Akt1 construct with an N-terminal myristoylation sequence (MyrAkt1), one of the oldest known constitutively active mutants of Akt1 [25], to directly test the experimental *in vivo* role of constitutively active Akt1^{Myr} in the progression from PanINs to PDAC. In contrast to the E17K mutation, the myristoylation sequence is well documented as directing Akt to the plasma membrane and facilitating constitutive activation [26], which in turn has been shown to be important for oncogenic transformation [27].

In addition to active mutant AKT1, loss of the PTEN tumor suppressor protein or constitutive activation of receptor-mediated or

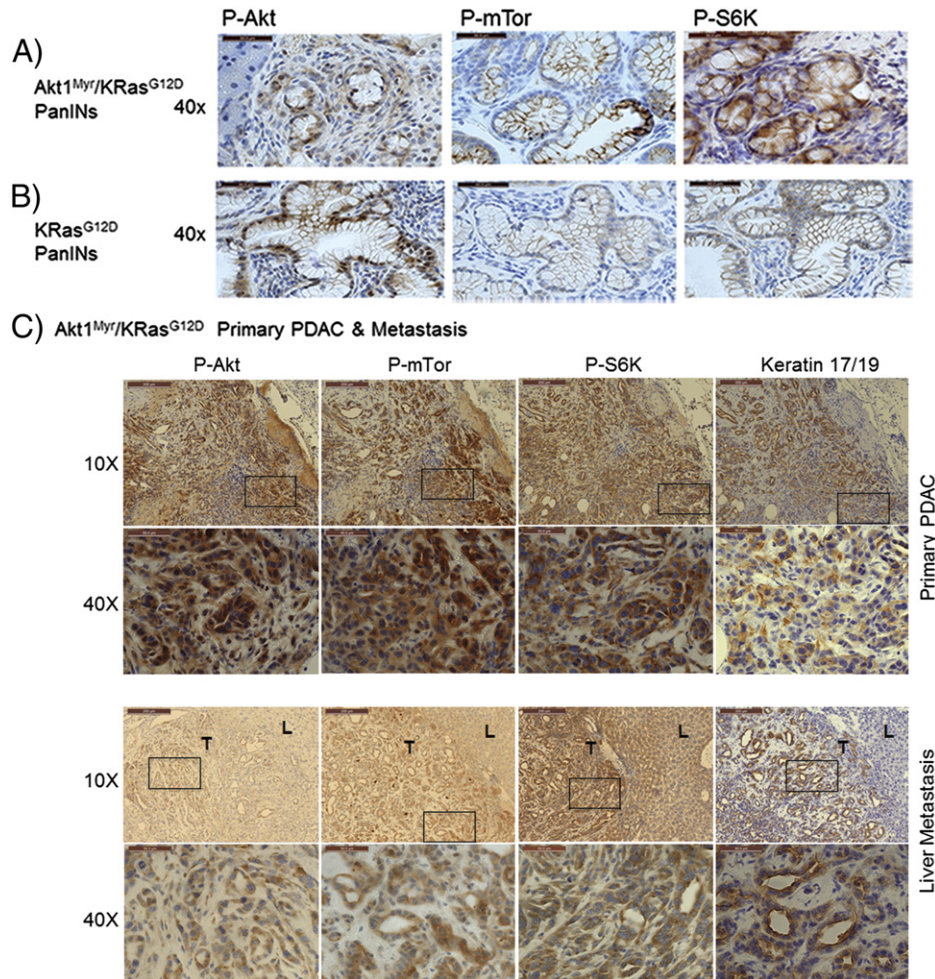


Figure 2. Activation of the Akt/mTor/S6K pathway in pancreatic tumor progression. The panels show representative early ductal pancreatic lesions, similar to human low-grade PanINs, with strong activation (brown DAB stain) for phospho-Akt, phospho-mTor, and phospho-p70S6 kinase in PanINs of (A) Akt1^{Myr}/KRas^{G12D} and (B) KRas^{G12D} mice (40× objective). (C) Immunohistochemical staining of primary PDAC and metastatic specimens from a ~43-week-old Akt1^{Myr}/KRas^{G12D} mouse for phospho-Akt, phospho-mTor, and phospho-p70S6 kinase and cytokeratin 17/19; a set of panels corresponding to PDAC metastasis to liver (10× objective and a scale bar corresponding to 200 μm, with boxed-in close ups from the 40× objective and a scale bar of 50 μm). In the metastasis panels, L = liver and T = tumor. Images were acquired using a Leica DM 2000 microscope with a digital DFC 295 camera.

upstream PI3K signaling is another means for constitutive activation of AKT isoforms [28,29]. Moreover, it has been suggested that loss of PTEN function and active mutations of *KRas* may converge to facilitate tumor growth [30]. In terms of previous mouse models of pancreatic cancer, it has been suggested that variations in phenotypes between *Pdx-Cre*-activated *KRas*^{G12D} and *Pdx-Cre*;*Pten*^{lox/lox} mice may be attributed to differences in the relative expression of *KRas* and *Pten* within centroacinar and duct cells. Previously, it was shown that all mice with *KRas*^{G12D} activation and *Pten* homozygous deletion succumb to cancer by 3 weeks of age, and compound mutant mice for *KRas*^{G12D} and heterozygous for *Pten*^{lox/+} show accelerated acinar-to-ductal metaplasia, PanINs, and PDAC within a year [13]. The high levels of phosphorylated effectors downstream in the *Pten*/Akt signaling cascade may be a mechanism to facilitate the ductal pancreatic tumor progression. In addition, *Pdx-Cre*;*Pten*^{-/-} pancreatic knockout mice were shown to display progressive replacement of the acinar cells, with ductal structures that expressed mucins.⁶

In the Akt1^{Myr}/KRas^{G12D} model presented here, tumor onset was accelerated compared to that observed in the KRas^{G12D} model. The first Kaplan-Meier analysis showed that Akt1^{Myr}/KRas^{G12D} mice aged 8 to 16 months had the greatest incidence of early or late carcinomas (12/23 or 52% of the mice in this age group), with 4 metastatic tumors, compared to only 5 of 14 (35%) of the KRas^{G12D} mice (Figure 1 and Table 1). Overall, only two KRas^{G12D} mice with metastasis were found at >16 months of age when more tumors were found in the KRas^{G12D} group and at an age when only one Akt1^{Myr}/KRas^{G12D} could be analyzed. The second Akt1^{Myr}/KRas^{G12D} study used re-derived mice when the colony was transferred to a new institution. It focused on mice aged to 1 year (Table 2) and was consistent with the Kaplan-Meier analysis in finding PanINs and PDAC, some with metastasis at less than 1 year of age. We cannot rule out other factors that may contribute to the decline of health in the Akt1^{Myr}/KRas^{G12D} mice, and these factors may come to light as we start analyzing the role of the MyrAkt1 construct in facilitating tissue

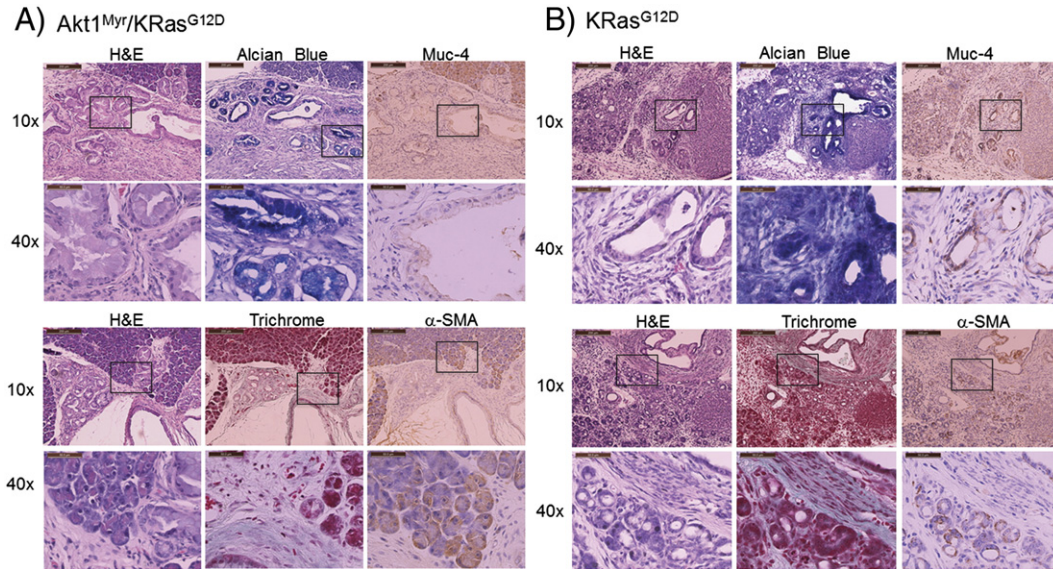


Figure 3. Pancreatic histologic alterations in Akt1^{Myr}/KRas^{G12D} and KRas^{G12D} mice. The panels from (A) Akt1^{Myr}/KRas^{G12D} and (B) KRas^{G12D} mice show staining of representative pancreatic tissues. Sections showed staining for H&E, Alcian Blue staining of ducts for detection of mucin (dark blue), Muc-4 (brown color) in areas of ducts, trichrome stain of red acinar cells, and green-blue collagen-rich fibrotic areas of the PDAC tumor and α -SMA marker (brown color) in areas of acinar cells near fibrotic regions. Boxed-in highlighted areas (10 \times objective, scale bar of 200 μ m) were magnified for a focal view with the 40 \times objective (scale bar of 50 μ m).

changes by using the doxycycline-off inducible MyrAkt1 construct in future studies.

Here, we report that PDAC formation in the compound transgenic Akt1^{Myr}/KRas^{G12D} mice mimicked a subset of histologic alterations

found in human pancreatic tumor progression, KRas^{G12D} and perhaps KRas^{G12D}/Pten^{lox/+} deficient mice. Consistently, we found phosphorylation of Akt and downstream mTor kinase and p70S6 kinase in Akt1^{Myr}Ras^{G12D} mice, both in early lesions and in metastatic

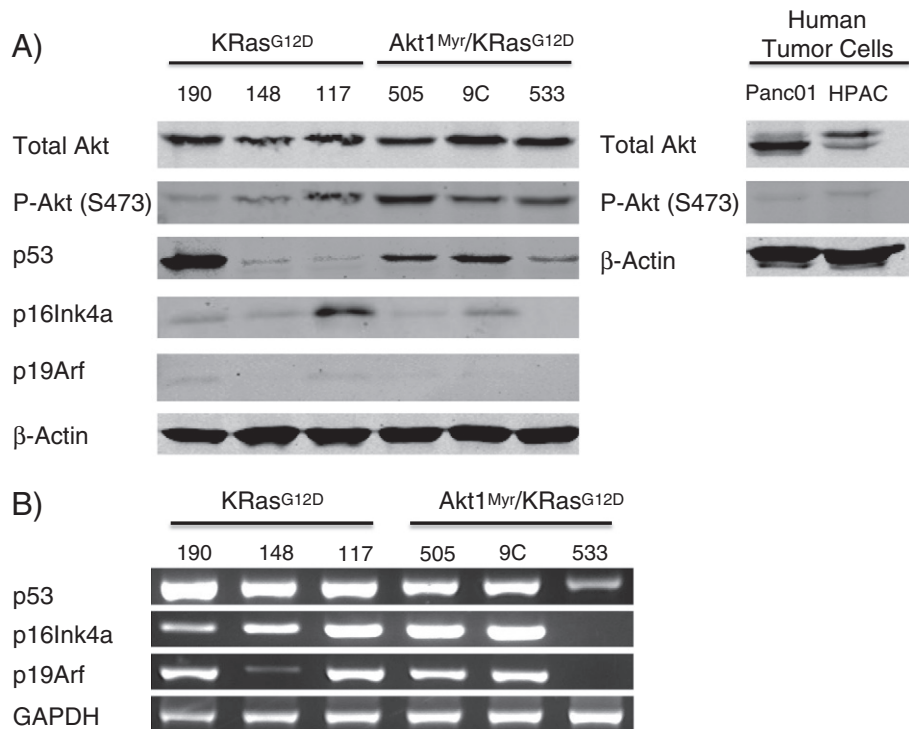


Figure 4. Phospho-Akt and tumor suppressors in mouse and human pancreatic tumor cells. (A) (Left) Representative Western blots from each of three KRas^{G12D} (mouse numbers 190, 148, and 117) and three Akt1^{Myr}/KRas^{G12D} (mouse numbers 505, 9C, and 533) tumor cell cultures analyzed for expression of total Akt, phospho-Akt (Ser473), and tumor suppressor genes *p53*, *p16Ink4a*, and *p19Arf*. Actin is a loading control. (Right) Representative human pancreatic tumor cell lines run adjacent to mouse tumor cells showing relative amount of total Akt, phospho-Akt (Ser473), and actin. (B) Genomic DNA PCR showing retention or loss of *Tp53*, *p16Ink4a*, or *p19Arf*.

PDACs (Figure 2). There also was extensive remodeling of both ductal and acinar components, as evident by increased mucin, α -SMA, and nearby fibrosis. Similar to other reports implicating Muc-4 as a marker of pancreatic ductal tissue transformation in human PanINs and PDACs [18], Muc-4 expression and overall Alcian Blue for both neutral and acidic mucins was increased in the ductal components in PanINs and in focal regions of the PDACs in these mice (Figure 3). Similarly, moderate to abundant collagen in the stroma was evident in disrupted acinar regions and around abnormal ducts.

To examine common genetic changes that are known to be important in the pancreatic tumor progression cascade, tumor cells were derived from the mice predisposed to pancreatic tumor progression and examined for down-regulation or occasional biallelic loss of tumor suppressor genes commonly implicated in PDAC. Overall, the establishment of cell cultures from the KRas^{G12D} mice was challenging, perhaps in part due to the inefficiency of developing full PDACs until mice had reached an advanced age. A limited number of primary cultures from Akt1^{Myr}/KRas^{G12D} PDACs were established. Similar to human pancreatic tumors, genomic PCR and Western blot analysis confirmed biallelic loss of p16Ink4a and p19Arf tumor suppressor gene expression in representative PDAC cells from an Akt1^{Myr}/KRas^{G12D} mouse (Figure 4). Moreover, staining for H&E and immunohistochemistry against cytokeratin 17/19 detected tumors from Akt1^{Myr}/KRas^{G12D} PDAC cells when they were orthotopically re-injected into the pancreas of a syngeneic mouse to show tumorigenic potential (Figure S5).

Collectively, compound Akt1^{Myr}/KRas^{G12D} mice exhibited accelerated PDAC development compared with KRas^{G12D} mice, and the tumors in Akt1^{Myr}/KRas^{G12D} mice showed histologic and genetic alterations that recapitulate those found in human pancreatic progression. Thus, this mouse model is likely to be of importance for preclinical testing of novel therapeutics targeting KRas and/or PI3K/Akt signaling in pancreatic cancer. Future analysis of the Akt1^{Myr}/KRas^{G12D} mouse model is expected to elucidate *in vivo* contexts in which Akt1 and KRas oncogenes interact in the pancreatic microenvironment to better facilitate treatment and overcome poor patient prognosis currently associated with this deadly disease. In particular, we suggest that the model may have added value for chemoprevention studies to block tumor progression at the PanIN or early carcinoma stage, perhaps before a stage where there is excessive desmoplastic damage and fibrosis.

Supplementary Materials

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.neo.2014.12.006>.

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