Abstract
In human studies and mouse models, the contributions of p53 and p16Ink4a/p19Arf loss are well established in pancreatic ductal adenocarcinoma (PDAC). Although loss of functional p53 pathway and loss of Ink4a/Arf in human pancreatic acinar cell carcinoma (PACC) and pancreatic neuroendocrine tumor (PanNET) are identified, their direct roles in tumorigenesis of PACC and PanNET remain to be determined. Using transgenic mouse models expressing the viral oncogene polyoma middle T antigen (PyMT), we demonstrate that p53 loss in pancreatic Pdx1+ progenitor cells results in aggressive PACC, whereas Ink4a/Arf loss results in PanNETs. Concurrent loss of p53 and Ink4a/Arf resembles loss of p53 alone, suggesting that Ink4a/Arf loss has no additive effect to PACC progression. Our results show that specific tumor suppressor genotypes provocatively influence the tumor biological phenotypes in pancreatic progenitor cells. Additionally, in a mouse model of β-cell hyperplasia, we demonstrate that p53 and Ink4a/Arf play cooperative roles in constraining the progression of PanNETs.

Introduction
Pancreatic acinar cell carcinomas (PACCs) and pancreatic neuroendocrine tumors (PanNETs) are neoplasms that each account for 1% to 2% of pancreatic cancers in adults [1,2]. PACC has a 25% to 50% 5-year survival rate, whereas PanNET survival is 100% for low-stage cancers and drops to 55% for stage IV cancers [2–4]. Both PACCs and PanNETs have a tremendous amount of genomic instability at the nucleotide and chromosomal level [2,5–7]. Complicating clinical diagnosis, PACC can also present as mixed acinar-neuroendocrine carcinomas (neuroendocrine differentiation in >30% of tumor), mixed acinar-ductal carcinomas, or mixed with both neuroendocrine and ductal components [8]. Because of the availability of clinical PanNET and PACC specimens, studies have been limited, and the pathogenesis of both cancers is poorly understood.

Alterations in PDAC are well-defined and commonly include activating mutations in KRAS as the driver mutations and alterations in the tumor suppressors p53 (50%-70% of cases) and p16Ink4a (80%-95% of cases) [9]. The loss of p53 and p16Ink4a in PDAC suggests important, nonredundant roles in tumorigenesis. The anticancer functions of p53 include activating DNA repair enzymes, stopping the G1 to S phase transition by inducing p21 expression, and initiating apoptosis [6]. The Ink4a/Arf (CDKN2A) locus encodes tumor suppressors, p16Ink4a and p14Arf (p19Arf in mouse). p16Ink4a is a cyclin-dependent kinase inhibitor that binds to CDK4/6 to prevent interaction with cyclin D, effectively stopping the G1 to S phase transition [10]. Arf stabilizes p53 by promoting MDM2 degradation [11,12]. In addition, Arf possesses p53-independent functions such as cell cycle arrest, apoptosis, angiogenesis, and ribosomal RNA processing [13–17]. It has also been shown that Arf inhibits tumor cell colonization independent of p53 in a mouse model of PDAC metastasis [18].

Abbreviations: PACC, pancreatic acinar cell carcinoma; PDAC, pancreatic ductal adenocarcinoma; PanNET, pancreatic neuroendocrine tumor; H&E, hematoxylin and eosin stain
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Early studies found no p53 mutations in PACCs by partial exon sequencing analysis [19,20], whereas p53 loss of heterozygosity was observed in 50% and 70% of PACCs [20,21]. Because of advances in sequencing technology, recent studies report that p53 loss by mutation and chromosomal loss is common in PACC [4,22–24]. Comprehensive genomic studies found that 13% to 23% of primary tumors had TP53 point mutations or truncations [4,22] and 39% to 53% of primary tumors had TP53 loss of heterozygosity [22,23]. In metastatic tumors, point mutations were identified in 31% and chromosomal deletions in 50%, suggesting that intact p53 is a barrier to metastatic potential [23].

Emphasizing the clinical significance of p53 status in PACC, copy number alterations and mutation with loss of heterozygosity were associated with shorter patient survival [23,24]. Although p53 mutations are rare in panNETs (~3%) [20], the p53 pathway is altered in approximately 70% of panNETs through aberrant activation of its negative regulators, including MDM2, MDM4, and WIP1 [6].

Loss of p16\(^{\text{flabab}}\) in PACC occurs in lower frequency than loss of p53. Genomewide analyses identified homozygous deletion of p16\(^{\text{flabab}}\) in 14% to 17% of PACCs [4,22]. In PanNETs, p16\(^{\text{flabab}}\) is recognized as commonly altered and of prognostic value [10,25–27]. For PanNETs, Muscarella and colleagues analyzed 5 primary tumors and 5 metastatic PanNETs for genetic alteration of p16\(^{\text{flabab}}\) and found that 9 of these 10 tumor specimens contain inactivating alteration of p16\(^{\text{flabab}}\) [26]. With regard to clinical significance, low p16\(^{\text{flabab}}\) levels in tumor tissue and promoter hypermethylation are negative prognostic factors of poor survival [10,27].

Although mouse model studies demonstrated that alterations in p53 and p16/p19 enable the malignant progression of PDAC [9,18,28], their contributions to PACC and PanNET progression were not still determined. To address the roles of p53 and p16/p19 in PACC and PanNET progression, we used mouse models that produce PACC and β-cell hyperplasia [29]. We previously showed that PyMT induction in Pdx1+ pancreatic progenitor cells results in β-cell hyperplasia in the majority of the mice and lethal PACC in <10% of mice, whereas PyMT induction in β cells results only in β-cell hyperplasia [29]. Although PyMT is not implicated in human cancer, it remains an important experimental tool because it stimulates inactivation of p53 and p16/p19 in pancreatic β-cells, synaptophysin to identify neuroendocrine cells, and keratin 17/μ\text{m} slides and stained for hematoxylin and eosin (H&E) at Histoserv Inc.

Histology and Immunohistochemistry

Mice were sacrificed based on \textit{in vivo} bioluminescent imaging (PyMT is linked to a luciferase reporter) when values in the region of pancreas exceed 16 photons/sec, outward symptoms, and survival predictions. Mice found dead before sacrifice were dissected, and when possible, tumor burden, metastasis burden, and histology were examined. The tumor types were confirmed with immunohistochemistry using antibodies against chymotrypsin to identify acinar cells, synaptophysin to identify neuroendocrine cells, and keratin 17/19 to identify ductal cells.

Tissues from dissected mice were fixed in 10% buffered formalin overnight at room temperature and transferred to 70% ethanol for long-term storage. Tissues were sectioned into 5-μm slides and stained for hematoxylin and eosin (H&E) at Histoserv Inc. (Germantown, MD). Unstained sections were deparaffinized and rehydrated through a Clear-Rite/ethanol series. Histologic assessment was confirmed by immunohistochemistry with VECTASTAIN Elite ABC Kit (Vector Laboratories) performed based on the manufacturer’s instructions. Primary antibodies used were anti-synaptophysin (1:100, Vector Labs, VP-S284) as a neuroendocrine marker and anti-keratin 17/19 (1:300, Cell Signaling, 3984) as a ductal marker. For automated staining of chymotrypsin, slides were pretreated with Protease 1 (Ventana) for 16 minutes and primary antibody, anti-human chymotrypsin antibody (Biodesign International, 1:300), at 4°C overnight on the Ventana Benchmark platform according to manufacturer’s instructions. The cutoff size used to differentiate between β-cell hyperplasia and PanNET is 1 mm in diameter [38].
Later than Pdx1 and is required to commit cells to a pancreatic fate. The tumor incidence of this genotype was 5% (5/100), and tumors were PACC (Table 1), in agreement with the previously reported tumor characteristics of the PyMT model in pure C57/BL6 genetic background [29]. Majority of the mice (95%) did not develop tumors, and only 2 of 100 mice (2%) had liver metastasis (Table 1). The survival of mice in this genotype, including those found dead, with or without tumors, is shown in Figure 2A. The phenotype of a normal pancreas and liver, commonly found in this control group, is shown in Figure 3A. Representative images of H&E staining of normal pancreatic tissue, β-cell hyperplasia, and PACC are presented in Figure 4, A–C, and normal liver histology is presented in Figure 4D.

**Figure 1.** Analysis of primary tumor specimens from mice with p53 and p16/p19 mutants. Genotyping confirmed Cre-mediated recombination of the p53lox/lox and p16/p19lox/lox loci in tumor tissues. Genomic DNA from ears is used as control for unrecombined alleles.

**Table 1.** Phenotypes of p53 and p16/p19 Loss in PACC and PanNET Mouse Models

| Genotype                          | Tumor Incidence (%) | P Value (χ²) Tumor Incidence | PACC | PanNET | Metastasis (%) | P Value Survival |
|-----------------------------------|---------------------|------------------------------|------|--------|----------------|-----------------|
| Pdx1-tTA; tet-o-PyMT-ires-Luc     | 5/100 (5%)          |                              | 5 pure | 0 | 2/5 (40%)   |                 |
| p53lox/lox; p16/p19lox/lox        | 4/20 (20%)          | .020074                       | 3A, 1 A/N | 0 | 4/4 (100%)  | .0002           |
| p16/p19lox/lox                   | 4/35 (11%)          | .07102                        | 1 pure | 3 | 0/4 (0%)    | .0025           |
| p53lox/lox; p16/p19lox/lox        | 4/12 (33%)          | .000646                       | 2A, 2U | 0 | 4/4 (100%)  | <.0001          |
| RIP7-retTA; tet-o-MT; p48-cre     | 0/50 (0%)           |                              | 0     | 0     | 0 (0%)       |                 |
| p53lox/lox; p16/p19lox/lox        | 0/2 (17%)           | .003341                       | 0     | 0     | 2/2 (100%)  | .0129           |
| p16/p19lox/lox                   | 12/60 (20%)         | .000807                       | 0     | 12    | 5/12 (42%)  | .3951           |
| p53lox/lox; p16/p19lox/lox        | 12/30 (40%)         | .000002                       | 0     | 12    | 4/12 (33%)  | <.0001          |

A, mixed acinar ductal carcinoma; A/N, mixed acinar ductal neuroendocrine carcinoma; U, undetermined because of inability to stain necrotic tissue.
survival compared with Pdx1-tTA; tet-o-MT mice (P < .0001) (Table 1, Figure 2A). The tumor incidence was significantly higher than control mice (33% vs 5%, P = .0006) (Table 1). Survival was comparable to Pdx1-tTA; tet-o-MT; p53lox/lox mice but was significantly shorter than Pdx1-tTA; tet-o-MT; p16/p19lox/lox mice (P = .0449) (Figure 2A). PACC with liver metastasis occurred in four mice, but there was no PanNET incidence in this genotype (Table 1). The incidence of metastasis is similar to Pdx1-tTA; tet-o-MT; p53lox/lox but differed significantly from Pdx1-tTA; tet-o-MT; p16/p19lox/lox (P = .0289). The tumor latency of Pdx1-tTA; tet-o-MT; p53lox/lox; p16/p19lox/lox closely resembled Pdx1-tTA; tet-o-MT; p53lox/lox (114 vs 119 days). Based on immunohistochemistry, two tumors were mixed acinar-ductal carcinomas and two were not determined because of the inability to stain necrotic tissue (Table 1). The average PACC tumor burden was 4447 cm³ (range: 196-6240 cm³). The tumor measuring 196 cm³ was an acinar nodule in an 84-day-old mouse in early PACC tumor development. Taken together, the Pdx1-tTA; tet-o-MT; p53lox/lox; p16/p19lox/lox mice show similar survival, tumor incidence, phenotype, and metastasis to the Pdx1-tTA; tet-o-MT; p53lox/lox mice, implying that p16/p19 loss has no additive effect to p53 loss.

p16/p19 loss, But Not p53 Loss, Results in Metastatic PanNETs in β-Cells

In addition to exploring the role of tumor suppressor loss in the Pdx1-tTA; tet-o-MT mouse model, we determined the contribution of p53 and p16/p19 loss to PanNET tumorigenesis in PyMT-expressing β-cells in the RIP7-rtTA; tet-o-MT mouse model. This model allows for elucidating a role for p53 and p16/p19 in PanNET tumorigenesis directly from pancreatic β cells.

The rat insulin promoter (RIP) is activated at around day E9.5 [41], and doxycycline is able to cross the placenta and to accumulate in the milk of lactating females [42] to interact with rtTA for PyMT induction. Mice with the control RIP7-rtTA; tet-o-MT; p48-cre genotype (n = 50) developed β-cell hyperplasia and had no PanNET development. However, PanNETs occurred in 17% of RIP7-rtTA; tet-o-MT; p48-cre; p53lox/lox mice, 20% of RIP7-rtTA; tet-o-MT; p48-cre; p16/p19lox/lox mice, and 40% of RIP7-rtTA; tet-o-MT; p48-cre; p53lox/lox; p16/p19lox/lox mice (Table 1). Liver metastases were not present in RIP7-rtTA; tet-o-MT; p53lox/lox mice but occurred in 5 of 60 (8%) of RIP7-rtTA; tet-o-MT; p16/p19lox/lox mice and 4 of 30 (13%) of RIP7-rtTA; tet-o-MT; p53lox/lox; p16/p19lox/lox mice (Table 1). The absence of metastases from RIP7-rtTA; tet-o-MT; p53lox/lox further supports that p16/p19 loss is more relevant in PanNET progression than p53 loss. Figure 3C represents a PanNET and liver metastases that developed in RIP7-rtTA; tet-o-MT; p16/p19lox/lox. Figure 5, E–H presents the H&E and immunohistochemical staining of a representative PanNET from the RIP7-rtTA; tet-o-MT; p16/p19lox/lox genotype. Figure 6C displays the histology of a typical PanNET liver metastasis.

Discussion

We investigate the functions of p53 and p16/p19 tumor suppressor loss in PACC and PanNET in mouse models. This is the first study, to our knowledge, that shows a causal role of p53 loss in producing short-latency, metastatic PACC without neuroendocrine feature. We found that Pdx1-tTA; tet-o-MT; p48-cre; p53lox/lox mice demonstrated shorter survival, increased tumor incidence, and increased metastasis as compared with the control Pdx1-tTA; tet-o-MT; p48-cre mice, emphasizing that p53 plays an important role in PACC progression. On the other hand, p16/p19 loss is not sufficient to advance PACC progression. Pdx1-tTA; tet-o-MT; p48-cre; p16/p19lox/lox had no
increase in PACC incidence over the control characteristics of the Pdx1-tTA; tet-o-MT; p48-cre; p53lox/lox genotype. Additionally, there were no differences in tumor incidence, burden, metastasis, or survival between concurrent loss of both p53 and p16/p19 and loss of p53 alone in the Pdx1-tTA; tet-o-MT model, suggesting that there is no additive effect of p16/p19 loss to PACC promoted by p53 loss.

Two elegant studies by Lewis et al. [43] and Morton et al. [44] used an RCASBP-tva system to deliver PyMT into elastase-tva mice postnatally. The elastase promoter has been used in transgenic mice with the intent of expressing genes specifically in the acinar cells of the pancreas. In this elastase-tva mouse model, RCAS-PyMT induces only microscopic pancreatic intraepithelial neoplasia in wild-type tumor suppressor background [43]. RCAS-PyMT–induced acinar cell carcinomas in p53 null background [44] are uniformly positive for both chymotrypsin (an acinar marker) and synaptophysin (a neuroendocrine marker). The feature is similar to human mixed acinar neuroendocrine carcinoma [45]. In contrast, the majority of acinar tumors in our Pdx1-tTA; tet-o-MT; p48-cre mice or Pdx1-tTA; tet-o-MT; p48-cre; p16/p19lox/lox mice expressed chymotrypsin but did not express synaptophysin, and therefore, this PACC tumor type described here is different from RCAS-PyMT tumors in elastase-tva mice. We did not see an additive effect of combined p53 and p16/p19 deletion for this acinar tumor type in our mouse model, which is different from the RCAS-PyMT mouse model [44]. Moreover, RCAS-PyMT–induced acinar cell carcinomas in p16/p19 null
are also uniformly positive for both chymotrypsin and synaptophysin. However, loss of p16/p19 in Pdx1-tTA; tet-o-MT; p48-cre; p16/p19$^{lox/lox}$ mice promoted PanNET, which is negative for chymotrypsin (Figure 5F). Altogether, the differences in tumor types may reflect cell of origin differences in our tet-inducible PyMT model and the RCAS-PyMT/elastase-tva model.

In addition, the tumor formation is slower and tumor incidence is lower in Pdx1-tTA; tet-o-MT; p48-cre; p53$^{lox/lox}$ mice than in RCAS-PyMT infected elastase-tva mice in p53 null background [44]. This could be a consequence of the low Pdx1 expression in acinar cells in adult pancreas and the timing of PyMT expression during pancreatic development in Pdx1-tTA; tet-o-MT; p48-cre; p53$^{lox/lox}$ mice.

We did not observe PanNETs in Pdx1-tTA; tet-o-MT; p48-cre; p53$^{lox/lox}$ mice. This is likely due to the rapidity of PACC tumorigenesis, which caused the lethality of the mice. By using another mouse model, RIP7-rtTA; tet-o-MT, which developed β-cell hyperplasia, p53 loss resulted in small nodular PanNETs without metastases, indicating that p53 loss can promote neuroendocrine tumorigenesis in β-cells but is not a barrier to metastatic potential.

Our study also establishes the causal role of p16/p19 loss in PanNET tumorigenesis but not in PACC tumorigenesis as evidenced by the development of only PanNETs in Pdx1-tTA; tet-o-MT; p48-cre; p16/p19$^{lox/lox}$ mice. Loss of p16/p19 in Pdx1+ pancreatic progenitor cells enhanced the tumorigenesis of specifically the β-cell lineage and not the acinar lineage. Interestingly, p16/p19 loss in the RIP7-rtTA model promoted liver metastases of PanNET, whereas its loss in the Pdx1-tTA model did not result in metastasis of PanNET. The differences could be due to 1) the expression levels of rtTA and tTA driven by RIP promoter and Pdx1 promoter or 2) the order of PyMT expression and the p16/p19 loss.

In this study, we demonstrate that with PyMT expression starting from pancreatic Pdx1+ progenitor cells, p53 loss has a causal role in PACC tumorigenesis and metastasis, whereas p16/p19 loss promotes long-latency, nonmetastatic PanNETs. p53 loss and p16/p19 loss do not have cooperative roles in PACC tumorigenesis. In PyMT-expressing β cells, both tumor suppressors play critical and cooperative roles in PanNET tumorigenesis.

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Figure 6. Microscopic phenotypic differences in liver histology. H&E staining of (A) normal liver from the Pdx1-tTA; tet-o-MT; p48-cre genotype. In the upper panel (4× magnification), bars indicate 500 μm. In the lower panel (60× magnification), bars indicate 50, 20, and 20 μm (left to right).
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