Murine models for familial pancreatic cancer: Histopathology, latency and drug sensitivity among cancers of Palb2, Brca1 and Brca2 mutant mouse strains

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Abstract

Alterations of the PALB2 tumor suppressor gene have been identified in familial breast, ovarian and pancreatic cancer cases. PALB2 cooperates with BRCA1/2 proteins through physical interaction in initiation of homologous recombination, in maintenance of genome integrity following DNA double-strand breaks. To determine if the role of PALB2 as a linker between BRCA1 and BRCA2 is critical for BRCA1/2-mediated tumor suppression, we generated Palb2 mouse pancreatic cancer models and compared tumor latencies, phenotypes and drug responses with previously generated Brca1/2 pancreatic cancer models. For development of Palb2 pancreatic cancer, we crossed conditional Palb2 null mouse with mice carrying the Kras<sup>G12D</sup>; p53<sup>R270H</sup>; Pdx1-Cre (KPC) constructs, and these animals were observed for pancreatic tumor development. Individual deletion of Palb2, Brca1 or Brca2 genes in pancreas per se using Pdx1-Cre was insufficient to cause tumors, but it reduced pancreata size. Concurrent expression of mutant Kras<sup>G12D</sup> and p53<sup>R270H</sup>, with tumor suppressor inactivated strains in Palb2-KPC, Brca1-KPC or Brca2-KPC, accelerated pancreatic ductal adenocarcinoma (PDAC) development. Moreover, most Brca1-KPC and some Palb2-KPC animals developed mucinous cystic neoplasms with PDAC, while Brca2-KPC and KPC animals did not. 26% of Palb2-KPC mice developed MCNs in pancreata, which resemble closely the Brca1 deficient tumors. However, the remaining 74% of Palb2-KPC animals developed PDACs without any cysts like Brca2 deficient tumors. In addition, the number of ADM lesions and immune cells infiltrations (CD3<sup>+</sup> and F4/80<sup>+</sup>) were significantly...
increased in Brca1-KPC tumors, but not in Brca2-KPC tumors. Interestingly, the level of ADM lesions and infiltration of CD3+ or F/480+ cells in Palb2-KPC tumors were intermediate between Brca1-KPC and Brca2-KPC tumors. As expected, disruption of Palb2 and Brca1/2 sensitized tumor cells to DNA damaging agents in vitro and in vivo. Altogether, Palb2-KPC PDAC exhibited features observed in both Brca1-KPC and Brca2-KPC tumors, which could be due to its role, as a linker between Brca1 and Brca2.

Introduction

Pancreatic cancer is one of the deadliest cancer types, with a 5 year survival rate of 8%, due to the lack of early detection, which limits treatment options [1]. Despite many research efforts, initiating factors for pancreatic cancer are not well defined. An estimated 5~10% of pancreatic cancer is familial, with breast cancer susceptibility genes 1/2 (BRCA1/2) and partner and localizer of BRCA2 (PALB2) among established pancreatic susceptibility genes [2–7]. In 2016, Bailey et al. reported 5% germline mutations and 12% somatic mutations in the BRCA pathway (BRCA1, BRCA2, ATM and PALB2) through whole genome sequencing of 456 pancreatic cancer [8]. Therefore, it is important to understand how these genes involved in BRCA pathway contribute to pancreatic cancer development.

After discovery of BRCA2 in 1995 [9, 10], when a homozygous deletion lying within 13q12.3 where the BRCA2 gene resides was identified in a human pancreatic cancer [11], more germline BRCA2 mutations were found in pancreatic cancer patients [6, 12–14]. Generation of Brca2 pancreatic cancer mice model by pancreas specific disruption of Brca2 gene with inactivation of p53 determined that BRCA2 is a bonifide pancreatic tumor suppressor gene, reflecting increased risk in BRCA2 mutation carriers for pancreatic cancer [15–17]. Several studies reported increased cancer risk in BRCA1 mutant carriers [5, 18, 19], although the association between BRCA1 and pancreatic cancer predisposition is not well-established [18]. Previously, we showed that Brca1 suppresses pancreatic tumor development by showing dramatically reduced tumor latency in Brca1 deleted triple mutant animals (Brca1flox/flox; KrasG12D; p53flox7/flox; Pdx1-Cre) [20]. More recently, the PALB2 gene was discovered [21] when researchers were looking for genes that confer susceptibility to pancreatic cancer, and Jones et al. reported inherited PALB2 mutations in familial pancreatic cancer [22]. Since then, more mutations in PALB2 gene have been identified in pancreatic cancer [8, 23], implying the urgent need of Palb2 pancreatic cancer mouse models to understand its role in pancreatic cancer development.

PALB2 was first identified as a binding partner of BRCA2 and shown to be required for the localization of BRCA2 to sites of DNA damage, and thus crucial for homologous recombination (HR) [21]. PALB2 harbors a series of C-terminal WD repeats that bind the N-terminus of BRCA2. In addition, the coiled-coil (CC) region at the N-terminus of PALB2 interacts with the CC domain of BRCA1. Down regulation of PALB2 by siRNA suppresses HR in a manner similar to BRCA1 and BRCA2 depletion [24]. Like BRCA1 (FANCS) [25] and BRCA2 (FANCD1) [26], monoallelic mutations in PALB2 confer familial susceptibility to breast, ovarian and pancreatic cancer [4, 7], while biallelic PALB2 lesions cause Fanconi anemia (FA) subtype N (FANCN) [27]. FA patients are highly prone to cancer due to their inherited defect in FA/HR DNA damage repair pathways [28]. The evidence that PALB2 is critical for HR and functions as a breast and pancreatic susceptibility gene suggest that the role of the adaptor protein, PALB2, may be critical for BRCA1/2- mediated tumor suppression by physically linking
BRCA1 to BRCA2. Since both germline and somatic mutations in PALB2 and BRCA1/2 genes were found in a significant proportion of pancreatic cancer cases [8], to understand those tumors better, it is also important to study whether tumors derived from defective function of PALB2, BRCA1 and BRCA2 are caused through a same mechanistic pathway by comparing similarities and differences between PALB2 and BRCA1/2 tumors. Thus, we generated mouse models of pancreatic cancer by inactivation of Palb2, Brca1 or Brca2 genes specifically in the pancreas and compared the resulting tumor latencies, histo-pathologies, anticancer drug responses and immune cell infiltration.

Materials and methods

Generation of murine models for pancreatic cancer

Brca1^{flox2/flox2} [29], Brca2^{flox3-4/flox3-4} [30], and Palb2^{flox2-3/flox2-3} (obtained from the laboratory of Dr. Bing Xia group, Cancer Institute of New Jersey) [31] were crossed to strains carrying Kras^{LSL-G12D/+}, Trp53^{LSL-R270H/+} and Pdx1-cre (Strain number 01XL5, 01XM3 and 01XJ6 respectively, National Cancer Institute Frederick Mouse Repository) alleles to generate all the genotypes in this study. All transgenic animals were maintained on a mixed genetic background (129/B6). Genotyping results and primers are shown in supporting information—S1 Supporting Information and S1 Table respectively (S1 Supporting Information and S1 Table).

Ethics statement

All animal studies were approved by the Ohio State University Institutional Animal Care and Use Committee (IACUC), and performed in compliance with the Guide for the Care and Use of Laboratory under protocols 2012A0000063 (PI-TL) and 2013A0000141 (PI-RS). Mice were housed under controlled conditions (12 hours light/dark cycle), given water and food ad libitum, and monitored every day by trained staff. When animals displayed any of early removal criteria or distress signs such as unresponsiveness, immobility, inability to feed or drink, excessive cachexia, dyspnea, and rough hair coat we consulted with veterinary staff and euthanized the animals by CO2 inhalation followed by cervical dislocation. Tumor tissues were collected from euthanized mice for histological analysis.

Histological analysis and immunohistochemistry (IHC)

Organs were fixed in a 10% formalin solution for 24-48hrs and stored in 70% ethanol. Formalin-fixed mouse pancreata and tumor tissue were embedded in paraffin, sectioned to obtain 4μm thick sections, and stained with hematoxylin and eosin (H&E) for histological analysis.

For immunohistochemistry, tissue sections were stained using a Bond Rx autostainer (Leica) [32]. Amylase (1:400, CST 3796), rat antibody-cytokeratin 19 (TROMA-III) (1:150, Developmental Studies Hybridoma Bank, University of Iowa), and CD3 (DAKO, A0452) were diluted in antibody diluent (Leica). Images were taken using the VECTRA® Automated Quantitative Pathology Imaging system (PerkinElmer, Hopkinton, MA, USA). For estrogen receptor alpha (ER) (1:500, Santa Cruz, SC-542) and progesterone receptor (PR) (1:200, DAKO, A0098) staining, the manufacturer’s recommended protocol (Vector laboratories, VECTASTAIN ABC HRP Kit, PK-6101) was followed. For F4/80 (1:500, Invitrogen MF48000) immunofluorescence staining, deparaffinized and rehydrated slides were microwaved in sodium citrate solution. After blocking, primary antibody was incubated at 4 degree overnight and secondary antibody (Alexa Fluor 594, 1:500) for 1 hour at room temperature. Washed slides were mounted with mounting solution with DAPI.
Establishment of primary pancreatic tumor cells

Isolated tumor tissue was cut into small pieces, trypsinized and neutralized. Cells were dispersed by passing through a syringe and needle several times and cultured until cell lines were established.

Karyotype analysis

Cells were incubated in medium with or without DNA damaging agents (Mitomycin C (MMC) 40ng/ml or Olaparib 1μM) for 16hrs and treated with 0.05μg/ml KaryoMax colcemid (GIBCO) for 2hrs. Cells were harvested, incubated in pre-warmed 0.56% KCl solution for 30 minutes at 37 degree and fixed in Carnoy’s solution (75% methanol: 25% acetic acid). Metaphase spreads were prepared and stained in 0.5% Giemsa solution and analyzed on a Zeiss Axioskop microscope with a 100X objective under oil.

Allograft assay

For subcutaneous injection of tumor cells into nude mice, cultured tumor cells (50~60% confluent) were harvested by trypsinization. After cell counting, cells were resuspended in 1% FBS in PBS solution. Mice were anaesthetized with 4% isoflurane, and 0.3×10^6 cells/100μl were injected subcutaneously in the dorsal side of the upper hind limb of nude mice. 2 tumor cells per genotype were used, and each tumor cell line was injected to 3 animals for each treatment group. After 10 to 14 days, when tumor size was approximately 100mm^3, MMC (5mg/kg, at day 1), Cisplatin (6mg/kg, at day 1 and 8) or vehicle control were injected to mice intraperitoneally. Tumor size was measured using calipers every 2–3 days after injection, and ulcerated tumors were excluded from data. When we observe weight loss exceeding >20% of body weight or allografted tumor diameter exceeding 1.5 cm we consulted with the veterinary staff and humanely euthanized the animals.

Statistical analysis

Statistical analyses were performed using unpaired two-tailed Student’s t-test to compare sets of results from independent groups, and values of p < 0.05 was considered statistically significant. For Kaplan-Meier survival curves, significance was estimated with the log-rank test using Graph-Pad Prism 7 software.

Results

Pancreas-specific deletion of Palb2, Brca1 or Brca2 early in development results in smaller pancreata

Whole body deletion of the mouse Palb2 gene results in early embryonic lethality similarly to the Brca1 and Brca2 knock-out animals, indicating that all three tumor suppressor gene products, Palb2, Brca1 and Brca2 are essential for embryonic viability [33–35]. Hence, to circumvent the embryonic lethality and to study the role of these tumor suppressors in pancreatic development and malignant transformation, we specifically deleted Palb2, Brca1 or Brca2 in the pancreas only using the Cre-LoxP recombination technology. For this purpose, we used conditional null alleles of Palb2 (Palb2^flx2-3) [31], Brca1 (Brca1^flx2) [29] and Brca2 (Brca2^flx3-4) [30] in combination with the well characterized Pdx1-Cre transgene that has been extensively used for modeling pancreatic ductal adenocarcinoma (PDAC) in mice. The Pdx1-Cre transgene is expressed in the epithelial lineages of the embryonic pancreas (which includes both exocrine and endocrine lineages) and continues to be expressed throughout adulthood [36]. PALB2,
BRCA1 and BRCA2 genes are essential for viability of normal cells; when normal cells are depleted of any of these gene products, they fail to proliferate and rapidly undergo senescence/apoptosis [33, 37, 38]. Hence, we investigated whether pancreas-specific deletion of any of these genes would affect the pancreas development. Animals with conditional deletion of Palb2, Brca1 or Brca2 were born at expected Mendelian frequency and developed into healthy, fertile adults with overall body size and weight gain similar to the control littermates. In contrast, the pancreata among these animals of all three genotypes were significantly reduced in size versus the control littermate pancreata (Fig 1A and 1B). However, the overall histo-architecture of the pancreata was normal and not significantly different from those of control pancreata (Fig 1C). Next, we sought to confirm the recombination status of the conditional-null alleles of these genes in the affected pancreata by Southern blot analysis. For these recombination experiments, we utilized animals carrying only one floxed allele along with either one wild type for control animals, or one null allele for experimental animals, thus allowing comparison of recombination of equimolar amount of floxed allele between control vs experimental groups. In pancreata of heterozygous Brca1\(^{\text{flox/+;Pdx1-Cre}}\) or Brca2\(^{\text{flox/+;Pdx1-Cre}}\) mice, the “floxed” conditional allele were fully recombined. In contrast, in pancreata of Brca1\(^{\text{flox/-;Pdx1-Cre}}\) and Brca2\(^{\text{flox/-;Pdx1-Cre}}\) mice, the conditional allele remained unrecombined (Fig 1D). To verify that the lack of recombination of the floxed allele among the experimental animals is not due to the absence of Pdx1-Cre transgene expression in the affected pancreata, we further bred Brca1\(^{\text{flox/-;Pdx1-Cre}}\) animals with the conditional Rosa26R-LacZ reporter allele, and observed the affected pancreata showed robust expression of the reporter lacZ as evidenced by X-gal staining (Fig 1E). These results indicate that the viability of pancreatic progenitor cells lacking Brca1 or Brca2 expression during pancreas development is severely compromised which in turn results in smaller pancreas size among Brca1/2\(^{\text{flox/flox;Pdx1-Cre}}\) animals. We hypothesize that loss of these essential gene functions in normal pancreas cells likely causes either proliferative burst deficiency, senescence or apoptosis (or combination of these) which are likely dependent on intact p53 tumor suppressor pathway activation. We postulate that likely a similar scenario occurs among pancreata of Palb2\(^{\text{flox/-;Pdx1-Cre}}\) animals as well. Unfortunately, since we did not have access to mice carrying Palb2-null allele, we could not empirically test the recombination status of the Palb2-floxed allele in pancreata in a similar manner. Palb2\(^{\text{flox/flox;Pdx1-Cre}}, \) Brca1\(^{\text{flox/flox;Pdx1-Cre}}\) and Brca2\(^{\text{flox/flox;Pdx1-Cre}}\) animals did not develop pancreatic cancer during the lifespans of these mouse strains, which could be because these mice still express the “floxed” conditional allele. Consistent with our findings, other group reported that inactivation of the Brca2 gene alone without disruption of p53 is not sufficient to promote tumor development [16].

Concomitant expression of mutant Kras\(^{\text{G12D}}\) and p53\(^{\text{R270H}}\) cooperate with Palb2, Brca1 or Brca2 loss in the pancreas to promote PDAC tumorigenesis

PALB2, BRCA1 and BRCA2 proteins are involved in DNA damage repair, primarily through their roles in homologous recombination (HR). PALB2 is a linker protein physically and functionally connecting BRCA1 and BRCA2 during HR [24]. In the context of an intact p53-induced DNA damage checkpoint, the accumulation of chromosomal abnormalities as a result of loss of PALB2, BRCA1 and BRCA2 culminates in cell death. Hence, for cells that have lost PALB2, BRCA1 or BRCA2 to undergo neoplastic transformation, first, they would have to overcome the DNA damage induced checkpoint by inactivation of p53. As mentioned above, pancreatic progenitor cells that lack Palb2, Brca1 or Brca2 functions are eliminated during embryonic development, presumably via p53-induced apoptosis. Most p53 mutations identified from tumors are missense and typically affect the DNA binding domain. The R273H mutation (R270H in the mouse) is one of the hot-spot mutations in the human p53 gene [39].
Previous reports showed that the mutant p53 R270H protein has a dominant-negative inhibition effect of wild type p53. More specifically, heterozygous mutant allele of p53 R270H delayed transcriptional activation of its downstream target genes and inhibited p53 dependent...
apoptosis [40]. Therefore, we chose the “conditional knock-in” LSL-\(p53^{R270H}\) mutant allele, LSL-\(p53^{R270H}\), for our pancreatic tumor mouse models.

Activating mutations in the \(Kras\) gene (e.g., \(Kras^{G12D}\)) are the most frequent mutations found in human PDACs with some studies reporting a prevalence rate as high as 90% [41, 42]. Also, in agreement with the hypothesis that \(Kras^{G12D}\) mutations are likely to be involved in PDAC initiation, these mutations are frequently found in early precursor lesions of PDAC, such as pancreatic intraepithelial lesions (PanINs) [42]. Hence, we decided to delete \(Palb2\), \(Brca1\) or \(Brca2\) concomitant with mutant \(Kras^{G12D}\) expression—using a “conditional knock-in” mutant allele, LSL-\(Kras^{G12D}\), and henceforth simply referred as \(Kras^{G12D}\) [43].

It is known that the Palb2, Brca1, or Brca2 proteins, with p53, synergistically suppresses tumor development, and mutations in the \(p53\) gene are common in pancreatic cancer [16, 20, 31]. Therefore, we generated the \(Palb2-KPC\) (\(Palb2^{flox/flox}; Kras^{G12D}; p53^{R270H+}; Pdx1-Cre\)), \(Brca1-KPC\) (\(Brca1^{flox/flox}; Kras^{G12D}; p53^{R270H+}; Pdx1-Cre\)), \(Brca2-KPC\) (\(Brca2^{flox/flox}; Kras^{G12D}; p53^{R270H+}; Pdx1-Cre\)), or \(KPC\) (\(Kras^{G12D}; p53^{R270H+}; Pdx1-Cre\)) animals, and monitored tumor development. In contrast to \(KPC\) animals (\(n = 28, T_{50} = 24.6\) weeks), \(Palb2-KPC\), \(Brca1-KPC\) or \(Brca2-KPC\) respectively developed PDAC with a much shorter pancreatic tumor-free median survival of 10.1, 11.9 and 13.7 weeks, respectively (Fig 2A). Like \(KPC\) animals, these animals when moribund presented with swollen abdomen (upon necropsy, we often found hemorrhagic ascitic fluid within the peritoneal cavity) and severe lack of body fat. \(Palb2-KPC\) mice became moribund slightly sooner than \(Brca1-KPC\) or \(Brca2-KPC\) animals. Most of the \(Palb2-KPC\) mice have tumor developing in the head of the pancreas where they are more likely to grow into the bile-duct (Fig 2B). 21 of 23 \(Palb2-KPC\) animals had a solid tumor in the head of the pancreas and in 14 cases, the tumor caused blockage of the bile-duct causing jaundice. Based on the survival data, we could conclude that concomitant loss of \(p53\) and \(Palb2\), \(Brca1\) or \(Brca2\) tumor-suppressor functions cooperate to dramatically augment tumorigenic potential of oncogenic mutations, such as \(Kras^{G12D}\).

We compared the histo-pathology of the pancreatic tumors that developed among \(Palb2-KPC\), \(Brca1-KPC\) and \(Brca2-KPC\) to those of \(KPC\) animals. Among all four genotypes, we found precursor pancreatic intraepithelial neoplasia (PanIN) lesions at various stages of atypia that progressed into full-blown PDAC (Fig 2C). With the progression of the disease, these PDACs eventually engulfed the entire pancreas and began to invade the nearby organs within the peritoneal cavity, such as the duodenum, spleen and kidney (Fig 2C). Besides classical PanINs that are thought to originate within the epithelial lining of the pancreatic ductules, Acinar-to-ductal metaplasia (ADM) within the acinar population in the pancreas can also result in PDAC precursor lesions [42, 44]. We observed ADM among all four genotypes as determined by immuno-histochemical (IHC) analysis of amylase and cytokeratin 19 (CK19) (Fig 2D). ADM incidence was significantly elevated in \(Brca1-KPC\) tumors compared to \(KPC\) tumors, but not in \(Brca2-KPC\) tumors. \(Palb2-KPC\) showed intermediate levels of ADM lesions between those of \(Brca1-KPC\) and \(Brca2-KPC\) tumors (Fig 2E).

**Pancreatic cystic lesions resembling MCNs are common to \(Palb2\) and \(Brca1\)-mutant animals**

As described above, \(Brca1-KPC\) animals developed classical PanINs and ADMs that eventually progressed to PDAC. In addition, the majority of \(Brca1-KPC\) presented with cysts that were grossly visible upon dissection (Fig 3A). These cystic lesions were frequently numerous and multi-lobular, some as large as 2 to 3 cm in size and yielded as much as a few milliliters of serous fluid with hemorrhagic components and cellular debris. The epithelial lining of some of these cysts displayed atypia. These cystic lesions often resembled mucinous cystic neoplasms...
A

![Graph showing survival rates and tumor progression in different murine models.](image)

B

*Palb2-KPC*

C

|     | KPC | Brca1-KPC | Palb2-KPC | Brca2-KPC |
|-----|-----|-----------|-----------|-----------|
| PanIN | ![Image of PanIN](image) | ![Image of Brca1-KPC PanIN](image) | ![Image of Palb2-KPC PanIN](image) | ![Image of Brca2-KPC PanIN](image) |
| PDAC  | ![Image of PDAC](image) | ![Image of Brca1-KPC PDAC](image) | ![Image of Palb2-KPC PDAC](image) | ![Image of Brca2-KPC PDAC](image) |
| Duodenal invasion | ![Image of Duodenal invasion](image) | ![Image of Brca1-KPC Duodenal invasion](image) | ![Image of Palb2-KPC Duodenal invasion](image) | ![Image of Brca2-KPC Duodenal invasion](image) |

D

![Image of Amylase and CK19 expression in different murine models.](image)

E

![Bar graph showing number of ADM lesions/field.](image)
(MCN) (Fig 3B); they were often found in the tail and body of pancreas and were circumscribed by ovarian-like stroma with wavy nuclei and expressed steroid hormone receptors, namely estrogen receptor (ER) and progesterone receptor (PR) [45, 46]. Among Palb2-KPC animals, 6 of 23 (26%) animals presented these cystic lesions that were surrounded by ER and PR positive ovarian-like stroma (Fig 3B).

Unlike Brca1-KPC and Palb2-KPC animals, we did not observe clearly visible cystic lesions in the KPC and Brca2-KPC animals. Therefore, Palb2-KPC animals present an intermediate
phenotype regarding the pancreatic neoplasia spectrum, some presenting a phenotype similar to *Brca1-KPC* (presence of large cystic lesions), while others are more similar to *Brca2-KPC* animals.

**Primary tumor cells derived from Palb2-KPC, Brca1-KPC and Brca2-KPC pancreatic tumors exhibit hypersensitivity to DNA damaging agents**

As noted above, PALB2, BRCA1 and BRCA2 play important roles in DNA damage repair, mainly in the HR pathway [47]. Therefore, deletions or hypomorphic mutations in these genes cause genome instability often characterized by gross chromosomal abnormalities, and thus resulting in cells with hypersensitivity to DNA damaging drugs such as interstrand cross-linking agents (ICL agents) (e.g., Mitomycin C and Cisplatin) and poly ADP ribose polymerase inhibitors (PARP inhibitors) (e.g., Olaparib) [48–52]. Thus, we expected chromosomal instability and hypersensitivity of primary tumor cells isolated from *Palb2-KPC, Brca1-KPC, or Brca2-KPC* pancreatic tumors to DNA damaging agents. To verify this, we generated multiple cell lines from tumors of each mouse model (*KPC, Palb2-KPC, Brca1-KPC, Brca2-KPC*) and performed metaphase karyotype analyses after confirmed recombination of “floxed” alleles (S1 Supporting Information). All primary pancreatic tumor cell lines expressed CK19, indicating they were originated from ductal cells (S2 Supporting Information). As expected, metaphases of *Palb2-, Brca1- or Brca2- deleted cells showed significantly increased numbers of chromosomal aberrations even without treatment with DNA damaging drugs compared to *KPC* control cells. After Mitomycin C (MMC) or Olaparib treatment, the numbers of chromosomal abnormalities of *Palb2-KPC, Brca1-KPC, or Brca2-KPC* cells were dramatically increased compared to metaphases of *KPC* control cells. Metaphases from these cells had various types of chromosomal aberrations including breaks, gaps and exchanges (Fig 4A). We also compared drug IC50s of tumor cells from each group. For the IC50 measurement, *Palb2^{flox/+}-KPC* cells were included to assess haploinsufficiency in DNA damaging drug sensitivity. Consistent with karyotype results, IC50 values for DNA damaging agents are much lower in *Palb2-KPC, Brca1-KPC, and Brca2-KPC* cells, vs *KPC* cells, whereas *Palb2^{flox/+-} KPC* cells behave like *KPC* cells, indicating that one wild type copy of *Palb2* is sufficient for DNA repair caused by MMC, Cisplatin and Olaparib. In contrast to the response to DNA damaging agents, we did not observe differences of sensitivity to other classes of chemotherapy drugs such as Paclitaxel, Fluorouracil (5-FU) and Gemcitabine among the 5 different genotypes (*KPC, Palb2^{flox/+}-KPC, Palb2-KPC, Brca1-KPC, and Brca2-KPC*) (Fig 4B and Table 1).

**Interstrand crosslinking agents inhibit Palb2-KPC, Brca1-KPC and Brca2-KPC tumor growth in vivo**

As described above, *Palb2-, Brca1- or Brca2- deleted tumor cells exhibit dramatically elevated sensitivity to DNA damaging drugs compared to *KPC* cells in *vitro*. Next, we tested whether this *in vitro* hypersensitivity to DNA damaging drugs can translate into tumor growth retardation *in vivo*. For this purpose, we generated subcutaneous allografts in immune-compromised athymic nude mice with above mentioned *Palb2-KPC, Brca2-KPC* and *KPC* pancreatic tumor cells and treated tumor-bearing mice with either MMC, or Cisplatin, or vehicle. Regardless of genotype, there was no significant difference in tumor growth among vehicle treated groups. Consistent with the *in vitro* results, MMC or Cisplatin treatment dramatically retarded growth of *Palb2-KPC* and *Brca2-KPC* tumors vs vehicle-treatment (Fig 5A and 5B). In contrast, at the same drug dose and regimen, *KPC* tumor growth was indistinguishable from vehicle-treated tumors. We further confirmed these *in vivo* drug-efficacy observations by treating *Palb2-KPC* animals that spontaneously develop PDAC with MMC on a weekly basis. Starting at 3 weeks of
when these animals were treated with MMC for three weeks, the MMC-treatment significantly increased ($P < 0.0001$) median survival to 14.4 weeks (MMC, $T_{50} = 14.4$ weeks vs untreated, $T_{50} = 10.1$ weeks) compared to vehicle-treated animals (Fig 5C).

Immune cell infiltration increase in *Palb2-KPC* and *Brca1-KPC* tumors but not in *Brca2-KPC* tumors

Although immunotherapy has emerged as a powerful cancer-targeting therapy, it has met with limited success in PDAC because of its immune suppressive features [53, 54]. Infiltration of immune cells such as effector T cells is necessary for the response to immune therapy. To determine the level of tumor-infiltrating immune cells, we examined CD3$^+$ T cells and F4/80$^+$ macrophages in tumors from *KPC*, *Palb2-KPC*, *Brca1-KPC*, and *Brca2-KPC* animals. All four groups of tumors showed increased T cells in PDAC as well as macrophages compared to normal pancreata. *Brca1-KPC* and *Palb2-KPC* tumors were highly infiltrated with CD3$^+$ T cells and F4/80$^+$ macrophages, although the difference of immune cells recruitments between groups was not significant (Fig 6A). Previous data from Mace *et al.* have shown that *Brca2-KPC* mice are resistant to single agent PD-L1 antibody blockade immunotherapy [55]. This data suggests that *Brca1* - and *Palb2* - deleted tumors might show different levels of resistance for immunotherapy.

Discussion

It is well-known that germline mutations in *BRCA1* or *BRCA2* genes increase risks of breast and ovarian cancer, as well as pancreatic cancer [5, 6, 56, 57]. As *PALB2* mutations also have been observed in families with breast, ovarian and pancreatic cancer like *BRCA1/2*, the *PALB2* gene has also been recognized as a tumor susceptibility gene [4, 7]. Proteins encoded by these

### Table 1. IC$_{50}$ values.

|          | Olaparib [95% CI] (μM) | Cisplatin [95% CI] (μM) | MMC [95% CI] (ng/ml) | Gemcitabine [95% CI] (μM) | 5-FU [95% CI] (μM) | Paclitaxel [95% CI] (mM) |
|----------|------------------------|-------------------------|----------------------|--------------------------|------------------|------------------------|
| *KPC*    | 5.221 [3.439–7.978]    | 0.775 [0.657–0.908]     | 158.0 [115.3–224.6]  | 0.1705 [0.132–0.218]     | 0.441 [0.376–0.514] | 0.041 [0.027–0.063]   |
| *Brca1-KPC* | 0.170 [0.100–0.341] | 0.103 [0.068–0.158] | 10.71 [7.721–15.14] | 0.150 [0.113–0.196] | 0.561 [0.451–0.689] | 0.044 [0.031–0.064] |
| *Palb2-KPC* | 0.067 [0.035–0.122] | 0.098 [0.046–0.273] | 7.837 [6.018–10.34] | 0.175 [0.135–0.225] | 0.574 [0.473–0.691] | 0.037 [0.025–0.055] |
| *Brca2-KPC* | 0.061 [0.036–0.104] | 0.016 [0.009–0.029] | 15.70 [11.69–21.17] | 0.129 [0.108–0.153] | 0.428 [0.363–0.511] | 0.042 [0.028–0.063] |
| *Palb2*<sup>flx/+</sup>-KPC | 8.783 [6.161–12.64] | 0.700 [0.522–0.981] | 78.36 [65.20–94.60] | 0.339 [0.244–0.463] | 0.734 [0.655–0.814] | 0.035 [0.027–0.044] |

IC$_{50}$ values were determined using MTT assay after 72 h drug treatment of each indicated drug. Experiments were done in triplicate or quadruplicate ± SEM, and all values are averages of replicates from two different cell lines relative to cell viability values without treatment. IC$_{50}$ values were calculated by nonlinear regression using Graph-Pad Prism 7 software.

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three tumor susceptibility genes (PALB2, BRCA1, BRCA2) cooperate in the BRCA pathway by forming a complex, which is essential for HR repair. In the complex, PALB2 physically interacts with and connects BRCA1 and BRCA2 [24]. The fact that mutations in those genes predispose to particular types of cancer and the BRCA1-PALB2-BRCA2 complex plays an important role in HR, suggest that they likely act together for their tumor suppression functions. In

![Fig 5. Interstrand crosslinking agents inhibit Palb2-KPC and Brca2-KPC tumor growth in vivo.](https://doi.org/10.1371/journal.pone.0226714.g005)

(A, B) Growth curves of allograft tumors in nude mouse models. MMC and Cisplatin treatment inhibited growth of Palb2-KPC, Brca1-KPC and Brca2-KPC tumors (Means ± SEM; ***P<0.0005, ****P<0.0001 vs vehicle treated group using). (C) Kaplan-Meier survival curve of Palb2-KPC with or without MMC. MMC treatment prolonged survival of Palb2-KPC animals (5mg/kg, every 3 weeks injection from 3~6 weeks of age) (****P<0.0001 compared with the untreated group).

![Fig 6. Infiltrating immune cells are increased in Palb2-KPC and Brca1-KPC tumors but not in Brca2-KPC.](https://doi.org/10.1371/journal.pone.0226714.g006)

(A) Representative images of immunohistochemistry and immunofluorescence staining of CD3 and F4/80 in KPC, Brca1-KPC, Palb2-KPC and Brca2-KPC tumors. (B) Quantification of CD3 and F4/80 staining per field showed elevated CD3 positive T cells and F4/80 positive macrophages observed in Brca1-KPC and Palb2-KPC pancreatic tumors compared with KPC and Brca2-KPC (Means ± SEM; *P<0.05, **P = 0.0005, ***P = 0.0003 vs. wild type (WT) pancreata).
support of this thought, mutations that disrupt formation of the BRCA1-PALB2-BRCA2 complex have been found in breast, ovarian and pancreatic cancers [58, 59]. Thus, we hypothesized that the role of the PALB2 linker protein may be critical for BRCA1/2-mediated tumor suppression. To test this hypothesis, we generated Palb2-, Brca1- and Brca2- deficient pancreatic tumor mouse models and have shown that loss of Palb2 cooperates with mutant p53R270H and KrasG12D in the development of PDACs, similarly to the Brca1- and Brca2-KPC mouse pancreatic tumor models, but Palb2-, Brca1- and Brca2-KPC tumors were not identical.

As for Brca1 and Brca2, whole-body knock out of Palb2 in mice leads to embryonic lethality, and the growth arrested Palb2-, Brca1- or Brca2- embryos commonly showed activation of p21 [33, 37, 38], indicating that the full-length gene products are essential for cellular viability and proliferation in the early embryo. In this study, we observed that pancreas specific deletion of Palb2, Brca1 or Brca2 results in decreased organ size. More importantly, recombination of the floxed alleles was not detectable in Brca1/2fl/o;Pdx1-Cre animals, indicating that Palb2, Brca1 or Brca2 deficient pancreatic progenitor cells during early embryonic development could not survive long term either due to proliferation defect, senescence or apoptosis or combination of these among them. Thus, for these defective cells to survive and proliferate, there is immense pressure on these cells to acquire secondary mutations in other genes that aid in overcoming proliferative arrest, senescence and apoptosis, e.g., p53 which can lead to uncontrolled cell growth and proliferation and thus eventually cancer. We also questioned how pancreas could be developed if all progenitors that are null for one of these genes are not present in the pancreas during early development. It has been reported that Pdx1-Cre mediated gene recombination is variable because of mosaic Cre recombinase activity [60]. Therefore, it is likely that the organ will repopulate from stem cells in which recombination of some conditional alleles has not occurred.

Rowley et al. has reported that deletion of Brca2 in pancreas that concomitantly expresses mutant KrasG12D inhibits tumor development, and the few pancreatic tumors that form spontaneously acquire p53 mutations [16]. Indeed the few animals in our study with deletion of Palb2, Brca1 or Brca2 with concomitant KrasG12D mutation (i.e., Palb2-, Brca1- or Brca2-KC animals) showed accumulation of p53 protein in the pancreas consistent with Rowley et al observations (unpublished observations). Unfortunately, the numbers of animals of these genotypes were insufficient for us to either support or refute Rowley et al. study conclusions regarding tumor latency and prevalence. Next, we hypothesized that preventing growth inhibition and apoptosis induced by p53 activation upon loss of Palb2, Brca1 or Brca2 functions may allow resulting genomic instability to accumulate in KrasG12D mutant pancreata. This, in turn, could help to promote and accelerate PDAC progression in these triple-mutant animals. In this study, we showed that concomitant expression of KrasG12D and p53R270H mutations with Palb2, Brca1 or Brca2 deletion accelerated pancreatic tumor development. These results are consistent with Rowley et al. data for Brca2 mutation in mouse model of pancreatic cancer in which Brca2 and p53 mutations synergize to accelerate pancreatic tumor development. Palb2-KPC mice developed PDAC and became moribund with shorter latency than Brca1-KPC or Brca2-KPC mice, possibly because >90% of Palb2-KPC tumors originated in the head of the pancreas obstructing the bile duct. We have seen a similar phenotype with Brca2Δex3-KPC mice (unpublished observations) where at least 70% of animals presented with jaundice due to bile-duct blockage by tumors in the head of the pancreas. It has been reported that patient derived missense mutations located in Exon3 of BRCA2 abolished or dramatically reduced the PALB2-BRCA2 interaction [21]. Therefore, the specific tumor location in the head of the pancreas in Palb2-KPC and Brca2Δex3-KPC mice might be due to ablation of PALB2-BRCA2 interaction. Further study is required to prove this hypothesis.
Unexpectedly, Brca1-KPC and Brca2-KPC tumors were quite different in terms of formation of cystic lesions. While almost all Brca1-KPC animals developed large cysts with PanIN derived PDAC, no apparent cystic lesions were observed in Brca2-KPC and KPC mice. Similar to our finding, a study has shown increased incidence of abnormal pancreatic imaging findings in BRCA1 and BRCA2 mutation carriers, in which pancreatic cysts were found only in BRCA1 mutation carriers (2 out of 14), not BRCA2 mutation carriers (0 out of 6), although the number of patients was limited [61]. Interestingly, Palb2-KPC tumors showed a mixture of Brca1-KPC and Brca2-KPC tumor phenotypes regarding the presence of cysts. 6 of 23 Palb2-KPC tumors had large cysts with PDAC, which closely resembled the Brca1-KPC tumors. However, the remaining tumors showed no large cystic lesions similarly to Brca2-KPC or KPC tumors. These results suggest that pancreatic cysts in patients with BRCA1 or PALB2 mutations require a detailed autopsy and analysis. The large size of cystic lesions observed in BRCA1 and some PALB2-KPC animals might be easily detected by abdominal imaging. The observed dichotomy in precursor lesions between the Brca1-deficient and Brca2-deficient tumors and the mixture of both in Palb2 deficient tumors might provide insights into mechanisms underlying the higher pancreatic cancer incidence and aggressiveness in BRCA2 vs BRCA1 mutation carriers. Moreover, we observed increased ADM lesions and immune cells infiltrations such as CD3+ and F/480+ cells in Brca1-KPC, but not in Brca2-KPC, compared to KPC tumors. Palb2-KPC tumors showed intermediate phenotypes between Brca1-KPC and Brca2-KPC. The mixed phenotype of Palb2-KPC tumors could be due to the role of PALB2, as a linker protein between BRCA1 and BRCA2, in BRCA1/2 mediated tumor suppression (Fig 7).

To confirm HR deficiency in tumor cells deficient Palb2, Brca1 or Brca2 genes, drug sensitivity was determined by karyotype analysis and IC50 determination. In this study, using primary tumor cell lines, we measured the degree of sensitivity to different chemotherapeutic drugs. Palb2-, Brca1- or Brca2- deficient tumor cells in comparison to KPC or Palb2flox/+ -KPC tumor cells are exquisitely sensitive to DNA damage inducing agents, including Olaparib, MMC and Cisplatin. However, the cells from the 5 different tumor genotypes did not show differential sensitivity to non-DNA damage-inducing drugs (5-Fluorouracil, Paclitaxel or Gemcitabine) that are routinely used in PDAC therapy regimens. Based on the in-vitro experiments, we also confirmed efficacy of MMC and cisplatin treatment in vivo. The DNA damaging drugs also inhibited tumor growth in vivo, resulting in decreased tumor burden and prolonged survival of treated animals. These preclinical results indicate that DNA damaging agents are effective and could be particularly useful in the treatment of PALB2-, BRCA1- or BRCA2- deficient pancreatic tumors, which is reminiscent of human cancers defective for ‘BRCAness’ [62, 63]. Although the DNA damaging agents have shown clinical efficacy in PALB2-, BRCA1- or BRCA2- cancers, tumor cells often develop resistance to these drugs, with tumor recurrence and progression. Therefore, it may be especially useful to study the mechanisms of acquired resistance using these mouse pancreatic cancer models.

Loss of heterozygosity (LOH) is often detected in tumors developing in BRCA1/2 mutant carriers, indicating loss of the wild type allele is a critical step in initiation of carcinogenesis [64]. However, we did not observe LOH in heterozygous Brca1flox/+, or Brca2flox/+, KPC tumors. Among pancreatic tumors of patients with PALB2, as for BRCA1/2 mutation carriers, LOH has been reported [65]. Therefore, using our Palb2-KPC mouse model, we tested whether LOH is required for pancreatic tumor development in Palb2flox/+; KPC animals. Like Brca1flox/+; KPC or Brca2flox/+; KPC mice, tumor latency of Palb2flox/+; KPC animals was similar to KPC animals and the tumors maintained the intact wild type allele as well as functional HR (Fig 5B), which is consistent with previous reports showing that deletion one allele of Palb2, Brca1 or Brca2 in mice did not affect tumor frequency or genome stability [16, 66].

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In summary, we have generated *Palb2*, *Brca1* and *Brca2* deleted mouse models for pancreatic cancer development and compared their tumor phenotypes, as summarized in Fig 7.

Improved preclinical models such as our triple mutant pancreatic tumor models, recapitulating the pathogenesis of human pancreatic cancer, combined with powerful high-throughput screening techniques will help to identify novel diagnostic/therapeutic targets for familial pancreatic cancer and overcome mechanisms of drug resistance. Moreover, these animal models will be useful to test new therapeutic regimens, drug combination therapies and immune therapy for both familial and sporadic pancreatic cancer patients having mutations in BRCA pathway genes.

**Supporting information**

S1 Table. List of PCR genotyping primers.

(TIF)

S1 Supporting Information. Genotype confirmation of mouse pancreatic tumor cells.

(TIF)
S2 Supporting Information. Immunofluorescence staining for CK19 on pancreatic tumor cells. (TIF)

S3 Supporting Information. Uncropped row images of gels and films. (TIF)

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