Correction

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Correction for “Targeting reactive nitrogen species suppresses hereditary pancreatic cancer,” by Mo Li, Qian Chen, Teng Ma, and Xiaochun Yu, which was first published June 19, 2017; 10.1073/pnas.1702156114 (Proc Natl Acad Sci USA 114: 7106–7111).

The authors note that the author contributions footnote appeared incorrectly. Xiaochun Yu should be credited with designing research, analyzing data, and writing the paper. The corrected author contributions footnote appears below.

Author contributions: X.Y. designed research; M.L., Q.C., and T.M. performed research; M.L. and X.Y. analyzed data; and M.L. and X.Y. wrote the paper.

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Targeting reactive nitrogen species suppresses hereditary pancreatic cancer

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Germline mutation of BRCA2 induces hereditary pancreatic cancer. However, how BRCA2 mutation specifically induces pancreatic tumorigenesis remains elusive. Here, we have examined a mouse model of BRCA2-deficiency-induced pancreatic tumors and found that excessive reactive nitrogen species (RNS), such as nitrite, are generated in precancerous pancreases, which induce massive DNA damage, including DNA double-strand breaks. RNS-induced DNA lesions cause genomic instability in the absence of Brca2. Moreover, with the treatment of antioxidant tempol to suppress RNS, not only are DNA lesions significantly reduced, but also the onset of pancreatic cancer is delayed. Thus, this study demonstrates that excess RNS are a nongenetic driving force for Brca2-deficiency-induced pancreatic tumors. Suppression of RNS could be an important strategy for pancreatic cancer prevention.

DNA damage response | DNA damage repair | BRCA2 | RNS | pancreatic cancer

Pancreatic cancer is one of the deadliest human cancers (1, 2). The median survival of patients with pancreatic cancer is merely 6 mo (3, 4). Thus, it is crucial to understand the molecular mechanism of pancreatic cancer, which will facilitate cancer prevention, diagnosis, and therapy.

Accumulated evidence suggests that genetic alterations on activating oncogenes such as KRas, as well as inactivating tumor suppressor genes such as p53 and BRCA2, induce pancreatic tumorigenesis (1, 5, 6). Most of these genetic alterations are somatic mutations during tumorigenesis. However, BRCA2 is often mutated in germ cells (1, 7). BRCA2 mutation carriers are predisposed to familial pancreatic cancer (8). Moreover, PALB2, a functional partner of BRCA2 during homologous recombination (HR), is involved in familial pancreatic cancer suppression (9, 10). It suggests BRCA2 and its dependent pathway play key roles to suppress hereditary pancreatic cancer.

The BRCA2 gene product is a nuclear polypeptide with 3,418 residues and is involved in DNA damage repair (11–13). After DNA double-strand breaks (DSBs), BRCA2 is recruited to DNA lesions via PALB2 and facilitates the loading of RAD51, the key recombinase for HR (14–18). Loss of BRCA2 abolishes DSB repair, and thus induces genetic instability (19, 20). In addition to pancreatic cancer, mutations of BRCA2 are also associated with several other types of cancer, such as breast cancer, ovarian cancer, prostate cancer, and lymphoma (21–25). However, compared with the mutation of KRas or p53-induced tumor spectrum, the BRCA2 mutation-induced tumor spectrum is much narrower (26, 27). Because the major known function of BRCA2 is to repair DNA lesions, we hypothesize that a type of carcinogen is specifically accumulated in pancreas and induces massive DNA damage. Without BRCA2, DNA lesions are not repaired, which causes genomic instability and pancreatic tumorigenesis.

The specific carcinogen inducing pancreatic cancer has not been revealed yet. Interestingly, it has been shown that pancreatic, including both acute and chronic pancreatitis, is associated with pancreatic cancer, especially hereditary pancreatic cancer (28–30). Pancreatitis is an inflammation of the pancreas, which draws phagocytic macrophages and neutrophils to secrete a large amount of nitric oxide (NO) and superoxide (O\(_2^\cdot\)) for the eradication of microbial pathogens and processing dead cells (31, 32). NO and O\(_2^\cdot\) are unstable and react with each other to generate peroxynitrite (ONO\(_2^\cdot\)) at the site of inflammation (33, 34). ONO\(_2^\cdot\) itself is not a free radical. However, pancreatic acinar cells secrete high concentrations (up to 140 mM in pancreatic duct) of bicarbonate (HCO\(_3^-\)) for neutralizing acidic chyme from stomach (35, 36). ONO\(_2^\cdot\) can react with HCO\(_3^-\) from pancreatic acinar cells to generate nitrosoperoxycarbonate (ONOOCO\(_3^\cdot\)), a major reactive nitrogen species (RNS) in vivo that homolyses to form carbonate radical (CO\(_3^-\)) and nitrite (NO\(_2^-\)) (34, 37).

Both CO\(_3^-\) and NO\(_2^-\) are free radicals and extremely dangerous for the integrity of genomic DNA (37, 38). Among four different DNA bases, deoxyguanine (dG) has the least reduction potential (37, 39), which makes it the most easily oxidized base by CO\(_3^-\) and NO\(_2^-\). Although most DNA oxidation damage is quickly repaired by the base excision repair (BER) pathway, a small amount of lesions may escape from surveillance and induce DNA single-strand breaks or DSBs (40, 41). In the absence of DSB repair, such as BRCA2-dependent homologous recombination, these lesions will ultimately lead to genomic instability in pancreas and then induce pancreatic tumorigenesis. Here, we have tested our hypothesis in vivo and found that excessive RNS induces massive DNA damage in the absence of BRCA2, which may lead to genomic instability and tumorigenesis. Suppression of RNS by antioxidant reduces DNA lesions and delays the onset of pancreatic tumorigenesis. Thus, this study reveals a molecular mechanism of pancreatic tumorigenesis and provides a potential approach to suppressing tumor development.

Significance

Germline mutations of BRCA2 associate with tissue-specific cancers, such as familial pancreatic cancer. However, it is unclear why BRCA2 mutation causes tissue-specific cancer. Here, we show that pancreas-specific reactive nitrogen species (RNS) causes oxidative damage and DNA double-strand breaks. Lacking BRCA2, cells could not repair DNA lesions, which led to genomic instability and pancreatic tumorigenesis. On the basis of the mechanistic analysis, we treated Brca2-deficient mice with antioxidant. Interestingly, the antioxidant treatment suppressed RNS-induced lesions and delayed the onset of pancreatic tumorigenesis. Thus, our study reveals a potential approach for the eradication of BRCA2 mutation-associated pancreatic cancer.

Author contributions: X.Y. designed research; M.L., Q.C., and T.M. performed research; M.L. analyzed data; and M.L. wrote the paper.

The authors declare no conflict of interest.

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Results

Development of Pancreatic Ductal Adenocarcinoma in the KPB Mouse Model. To examine the molecular mechanism of pancreatic cancer, we set up a pancreatic ductal adenocarcinoma (PDAC) mouse model, as PDAC is the most common type of pancreatic cancer. On the basis of previous animal studies (27, 43), we generated the Ptf1a-Cre; Kras<sup>G12D</sup>, p53<sup>flox/+</sup>, Brca2<sup>γ</sup>, mouse, in which the Ptf1a-Cre allele drives Cre recombinase expression to conditionally activate endogenous oncogenic Kras<sup>G12D</sup> (K) and knockout one p53 allele (P) and the exon 11 of both p53 and Brca2 alleles (B) in pancreas (Fig. S1A), also named the KPB mice. The exon 11 of Brca2 encodes the Rad51-binding region, and loss of this region abolishes the function of Brca2 in DNA damage repair (43, 44). During the generation of the KPB mice, we also maintained the littermates (43, 44). During the generation of the KPB mice, we also maintained the littermates and found DNA breaks did exist in these pancreatic tumors examined the mitotic spreads of tumor samples from the KPB mice, further indicating that DSBs exist in these mice. In addition, we examined the expression of 53BP1, another important DSB repair machinery, in pancreases from the WT mice. Moreover, we also found that the expression of 53BP1 is elevated in the precancerous and tumor tissues of pancreases from the KP mice, or in the normal pancreases at pre- cancerous or tumor stages from the KP mice, and the normal pancreases from WT mice, followed by the examination of endogenous nitrite based on an established protocol (46). Precancerous pancreases from KPB mice and normal pancreas from WT mouse were harvested, followed by the detection of endogenous nitrite. Compared with that in pancreases of the WT mice, the level of nitrite was around sixfold and 10-fold higher in pancreases of the KP and KPB mice, respectively (Fig. 2D). Moreover, the RNS react with dG in vivo and form two major DNA adducts: 8-oxo-guanine (8-oxo-G) and 8-nitro-guanine (8-nitro-G) (33, 47). Because 8-oxo-G can also be generated by other free radicals such as reactive oxidative species, we only focused on 8-nitro-G. The relative level of 8-nitro-G increased 22.4-fold and 29-fold in the pancreases of the KP and KPB mice compared with that in the pancreases of WT mice (Fig. 2C). 8-nitro-G is not stable and is quickly depurinated from the genomic DNA, during which abasic sites, aka AP (apurinic/apyrimidinic) sites, are generated (38). Thus, we examined AP sites by fluorescence staining and found that AP sites remarkably increased in both the pre- cancerous and tumor tissues of pancreases from the KP and KPB mice (Fig. 2D). We also examined AP endonuclease 1 (APE1), the major enzyme to repair AP sites, by immunostaining. The expression level of APE1 is elevated in pancreatic tissues from the KP and KPB mice. (Fig. 2E). Collectively, these results suggest that a high level of RNS causes extensive oxidative damage in the pancreases of the KP and KPB mice.
Antioxidant Treatment Suppresses RNS-Induced DNA Damage and Tumorigenesis. If RNS induce DNA damage and genomic instability in pancreatic cells, treatment with antioxidants should be able to suppress DNA lesions and tumorigenesis. To test our hypothesis, we treated the primary pancreatic acinar cells from the KPB mice with tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl), a membrane-permeable free radical scavenger. To confirm the antioxidative efficiency of tempol, we first examined the nitrite level of the KPB cells in the presence of ONOOO⁻₂⁻. As expected, tempol decreased the cellular level of nitrite significantly, and this effect was shown in a dose-dependent manner (Fig. 4A). Pretreatment of tempol remarkably reduced RNS-induced 8-nitro-G and the AP sites in these cells (Fig. 4B and C). Moreover, ONOOO⁻₂⁻-induced foci of γH2AX and 53BP1 were repressed in the KPB cells in the presence of tempol (Fig. 4D). Consistently, with the increased level of tempol, DSBs were also suppressed (Fig. 4E).

Because tempol is a powerful antioxidant in vitro, we next examined whether tempol could suppress oxidative damage and pancreatic tumorigenesis in vivo. The pancreatic cancer developed very early in the KPB mice and induced the lethality as

RNS Induce DSBs. Next, we examined whether massive oxidative lesions can be converted to DSBs. Primary pancreatic acinar cells were isolated from the KP and KPB mice and treated with ONOOO⁻₂⁻. As expected, we found that the level of 8-nitro-G was significantly increased in the cells after the treatment with ONOOO⁻₂⁻ (Fig. 3A). Moreover, the AP sites were induced in both the KP and KPB cells (Fig. 3B). Most of the oxidative lesions were quickly repaired, as we could not detect the obvious AP sites after 24 h recovery from the treatment with ONOOO⁻₂⁻. However, if many oxidative lesions occur simultaneously, some lesions may not be timely repaired, which can be converted into DSBs. Thus, we examined DSBs by immunostaining γH2AX. Immediately after the treatment with ONOOO⁻₂⁻, we found that DSBs occurred in both the KP and KPB cells. After 24 h recovery from the RNS insults, most DSBs in the KP cells were repaired, as we could only find sporadic and weak γH2AX foci. However, in the KPB cells, strong and big γH2AX foci were observed under the same assay condition, suggesting DSBs still exist in the KPB cells (Fig. 3C). Moreover, we performed the neutral comet assay to examine DSB repair after the treatment with ONOOO⁻₂⁻. With the increased dose of ONOOO⁻₂⁻, DNA breaks were clearly detected in the KPB cells (Fig. 3D). Taken together, these results show that RNS treatment could induce DSBs in pancreatic cells. In the absence of Brca2, the cells lose the last protection system to correct DSBs, which may induce genomic instability and tumorigenesis.

**Fig. 2.** RNS and RNS-induced DNA adduct in the KPB pancreas. (A) Schematic diagram of RNS and RNS-induced DNA adducts. (B) The level of nitrite in the pancreases of the WT, KP, and KPB mice was measured. (C) The relative level of 8-nitro-G in pancreases of the WT and KPB mice was examined by mass spectrometry. The molecular weight of the reference of 8-nitro-G and 8-nitro-G isolated from pancreases of the WT and KPB mice was determined by Q-TOF mass spectrometry (Left). Peak value of 8-nitro-G from different samples in the mass spectrometry is shown (Right). (D and E) AP sites (D) and APE1 (E) in the pancreatic tissues of the WT, KP, and KPB mice were immunostained. (Scale bars, 50 μm.) Means and SDs were plotted. ***P < 0.001.

**Fig. 3.** RNS induce DNA lesions in vitro. (A) ONOOO⁻₂⁻ treatment induces 8-nitro-G. Primary pancreatic acinar cells from the KP and KPB mice were treated with or without ONOOO⁻₂⁻. Mass spectrometry analysis shows the molecular weight of 8-nitro-G isolated from the KPB cells (Left). Peak value of 8-nitro-G in mass spectrometry is shown. ND, nondetectable (Right). (B) KPB cells were treated with ONOOO⁻₂⁻ (4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl), a membrane-permeable free radical scavenger. To confirm the oxidative efficiency of tempol, we first examined the nitrite level of the KPB cells in the presence of ONOOO⁻₂⁻. As expected, tempol decreased the cellular level of nitrite significantly, and this effect was shown in a dose-dependent manner (Fig. 4A). Pretreatment of tempol remarkably reduced RNS-induced 8-nitro-G and the AP sites in these cells (Fig. 4B and C). Moreover, ONOOO⁻₂⁻-induced foci of γH2AX and 53BP1 were repressed in the KPB cells in the presence of tempol (Fig. 4D). Consistently, with the increased level of tempol, DSBs were also suppressed (Fig. 4E).

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early as 5 wk. Tempol treatment after weaning only slightly suppressed the tumor onset of the KPB mice (Fig. 5A). Thus, we treated pregnant female mice with tempol and kept administering tempol to the newborn mice after weaning. Interestingly, tempol treatment significantly delayed onset of pancreatic tumor and increased the life span of the KPB mice (Fig. S3 and Fig. 5A). The median survival of the KPB mice was increased from 62 to 114 d by tempol treatment. Moreover, the level of nitrite and 8-nitro-G were significantly reduced in vivo (Fig. 5 B and C). Along with the reduction of RNS, both AP sites and DSBs were dramatically decreased in the precancerous pancreases or pancreatic tumors after tempol treatment (Fig. 5 D and E). In addition, the tempol treatment for pregnant female mice and the newborn mice showed a trend toward increased survival of the KP mice, but there is no statistical significance (Fig. 5F). Thus, tempol can be used as a potent suppressor of RNS-induced DNA damage and tumorigenesis in pancreas.

Discussion
In this study, we demonstrate that RNS is a critical nongenetic factor for BRCA2 deficiency-induced pancreatic tumorigenesis. The RNS is likely to be generated by pancreatic acinar cells, macrophages, and neutrophils. Acinar cells uniquely secrete a large amount of HCO$_3^-$ into pancreatic ducts, which protects the surrounding cells from acid chyme (35, 36). However, this physiological process is a double-edged sword, as HCO$_3^-$ can react with ONOO$^-$ from macrophages and neutrophils and generate ONOO$_2^-$, a dangerous RNS that releases free radicals to damage genomic DNA (34, 37). Both macrophages and neutrophils are recruited by inflammation (31, 32). It has been shown that oncogenic Kras regulates and facilitates inflammation in pancreas (48–50). Consistently, we found that in the Ptf1a-Cre;
Unlike other oncogenic mutations, it is rare to find the somatic mutations of BRCA2 in spontaneous tumors. Most BRCA2 mutations are germline mutations that mainly induce familial breast, ovarian, and pancreatic cancers (21, 22, 53). A high level of HCO₂⁻-induced RNS in pancreas could be the unique factor for BRCA2 mutation carriers to predispose to tumorigenesis in pancreas. With similar mechanisms, other tissue-specific carcinogens such as oxidized estrogen could induce DSBs that lead to breast and ovarian tumorigenesis in BRCA2 mutation carriers.

Our study also provides a feasible chemoprevention approach for familial pancreatic cancer. The antioxidant treatment significantly reduces the RNS, DNA oxidative adds, and DNA lesions. More important, the antioxidant treatment significantly delays tumor onset and nearly doubles the life span of the KPB mice. To our knowledge, this chemoprevention strategy is one of the most effective approaches to extend life span in a mouse pancreatic cancer model. Moreover, for BRCA2 mutation or other familial pancreatic tumor suppressor gene (such as PALB2) mutation carriers, our study has provided the molecular mechanism for a possible chemoprevention trial. In addition, although there is no statistical significance, the antioxidant treatment also showed a trend to extend the life span of the KP mice. However, because of the limited animal strains examined in this study, it is unclear whether the Kras and p53 mutations induce oxidative damage in other tissues. Additional studies on other tumor model animals will reveal whether the antioxidant treatment could be an effective chemoprevention approach for other Kras and p53 mutation-induced tumors.

Methods

Chemicals and Antibodies. All chemicals were purchased from Sigma except those specifically mentioned. Anti-γH2AX antibody was purchased from Cell Signaling Technology; Anti-S3BP1 and APE1 antibodies were purchased from Novus Biologicals.

Animal Strains and Maintenance. The Ptfla-Cre, LSL-KrasG12D mice were kindly provided by Dr. Magliano at the University of Michigan Medical School, Ann Arbor, MI. The p53lox/lox; Brca2fl/− mice were provided by Dr. Yuan at the University of Michigan Medical School, Ann Arbor, MI. The Brca2fl/− mice were obtained from NCI Mouse Repository. The p53lox/lox; KrasG12D mice were intercrossed with Brca2fl/− mice to create the p53lox/lox; Brca2fl/− mice, which were further crossed with the Ptfla-Cre, LSL-KrasG12D mice to generate Ptfla-Cre; KrasG12D, p53lox/lox; Brca2fl/− mice (KPB mice) and Ptfla-Cre; KrasG12D, p53lox/lox; Brca2fl/− mice (KP mice). All experiments were performed using the littersmates from a mixed but uniform genetic background. The animals were maintained in a specific pathogen-free environment under a 12-h light/dark cycle. All experiments were performed in accordance with national and University of Michigan institutional guidelines. The study was approved by the ethical review committee of the University of Michigan.

Genotyping. Genomic DNA was isolated from mouse tails using the DNeasy Blood and Tissue Kit (Qiagen) and genotyped by PCR. Reaction conditions for Cre, Kras, p53, and Brca2 were 40 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min. PCR products were then run in the 1.5% agarose gel.

Histological Staining and Immunofluorescence Staining. Precancerous pancreatic tissues and pancreatic cancer tissues were harvested from the KP or KPB mice at the age of 50 d. Normal pancreatic tissues were harvest from the WT mice at the age of 50 d as well. Explanted tissues were fixed in 10% neutral-buffered formalin solution for at least 16 h and gradually transferred to 70% ethanol. Then the tissues were embedded in paraffin at room temperature. After washing and staining with DAPI, tissues were visualized by the Olympus IX 71 microscope.

Isolation and Culture of Mouse Primary Pancreatic Acinar Cells. Isolation and culture of mouse primary pancreatic acinar cells were performed according to the previous report (55). Briefly, mouse pancreas was mechanically cut into pieces and digested in 1x HBSS containing 10 mM Hepes, 200 μM collagenase IA, and 0.25 mg/ml trypsin inhibitor for around 30 min at 37 °C. The enzymatic reaction was stopped by adding 10 mL cold washing solution (HBSS 1x containing 5% FBS and 10 mM Hepes). After washing and suspension, cell mixture was filtered by a 100-pm cell strainer and then cultured in DPBS with 5% FBS, 50% growth factor-reduced type I collagen, and 50% Matrigel. After 24 h, the cells were trypsinized in 0.5 mL of 0.5 mg/ml trypsin and then incubated at 37 °C for 5 min before counting.

Culture Medium. For the ONOO⁻ treatment, 50 μM ONOO⁻ was mixed with 1 mM sodium bicarbonate in cell culture medium according to the previous studies (56, 57). ONOO⁻ treated cells were then subjected for other assays. For the recovery from ONOO⁻ treatment, cells were washed with fresh culture medium and cultured for 24 h. For the tempol treatment,
primary pancreatic acinar cells were incubated in the presence of 100 μM temop or temopat with the indicated concentrations during the culture.

Comet Assays. Single-cell gel electrophoretic comet assay was performed under normal condition to detect DSBs. Cells were collected and rinsed twice with ice-cold PBS; 2 × 10^5 cells were combined with 1% LMAgarose at 40 °C at a ratio of 1:3 (vol/vol) and immediately pipetted onto slides. For cellular lysis, the slides were immersed in the neutral lysis solution (2% sarkosyl, 0.5M Na2EDTA, 0.5 mg/mL proteinase K at pH 8.0) overnight at 37 °C in the dark, followed by washing in the rinse buffer (90 mM Tris buffer, 90 mM boric acid, 2mM Na2EDTA at pH 8.5) for 30 min with two repetitions. The slides were then subjected to electrophoresis at 20 V (0.6 V/cm) for 25 min and stained with 2.5 μg/mL propidium iodide for 20 min. All images were taken with a fluorescence microscope and analyzed by Comet Assay IV software.

In Vivo Tempol Treatment. Tempol treatment after weaning: tempol was added in the drinking water [0.05% (wt/vol)] for the newborn mice after weaning. Tempol treatment before birth: tempol was added in the drinking water [0.05% (wt/vol)] for the pregnant female mice until the pups were weaning. After weaning, tempol was directly administered to the pups through drinking water.

Statistical Analyses. All experiments were performed in triplicate unless indicated otherwise. Means and SDs were plotted. Student’s t test was used for statistical analyses. The log-rank test was performed on the Kaplan-Meier survival curves.

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