Published in final edited form as:

Expert Rev Anticancer Ther. 2017 January ; 17(1): 19–31. doi:10.1080/14737140.2017.1261017.

Targeting reactive oxygen species in development and progression of pancreatic cancer

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Abstract

Introduction—Pancreatic ductal adenocarcinoma (PDA) is characterized by expression of oncogenic KRas which drives all aspects of tumorigenesis. Oncogenic KRas induces the formation of reactive oxygen species (ROS) which have been implicated in initiation and progression of PDA. To facilitate tumor promoting levels and to avoid oncogene-induced senescence or cytotoxicity, ROS homeostasis in PDA cells is balanced by additional up-regulation of antioxidant systems.

Areas Covered—We examine the sources of ROS in PDA, the mechanisms by which ROS homeostasis is maintained, and the biological consequences of ROS in PDA. Additionally, we discuss the potential mechanisms for targeting ROS homeostasis as a point of therapeutic intervention. An extensive review of the relevant literature as it relates to the topic was conducted using PubMed.

Expert Commentary—Even though oncogenic mutations in the *KRAS* gene have been detected in over 95% of human pancreatic adenocarcinoma, targeting its gene product, KRas, has been difficult. The dependency of PDA cells on balancing ROS homeostasis could be an angle for new prevention or treatment strategies. These include use of antioxidants to prevent formation or progression of precancerous lesions, or methods to increase ROS in tumor cells to toxic levels.

Keywords

reactive oxygen species; pancreatic cancer; antioxidants; PanIN; KRas; NOX; mitochondria

1. Introduction

Pancreatic ductal adenocarcinoma (PDA) is the most lethal of all malignancies and has an overall 5-year survival rate of less than 5% [1,2]. In the United States, PDA is the fourth leading cause of cancer-associated deaths [3], and by the year 2030, it is expected to become the second leading cause of cancer-related deaths globally [4,5]. Late diagnosis and resistance to conventional therapy contributes to the lethality of this disease [6]. Genetic

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Declaration of Interest

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alterations are the major etiologic factor in the development of PDA and the disease is characterized by mutations in key oncogenes and tumor suppressor genes. Over 90% of all PDA patients have oncogenic mutations in the *KRAS* gene [7,8]. However, this by itself is not sufficient to drive the development of PDA, and additional external signaling is needed to raise oncogenic KRas activity to levels where it drives an inflammatory program that leads to a feed-forward loop [9,10]. Sustained activation of signaling pathways downstream of mutant KRas then drive cancer cell survival, proliferation, migration and invasion [11]. Inactivating mutations in the tumor suppressor genes *CDKN2A*, *TP53* and *SMAD4* also promote pancreatic carcinogenesis by facilitating enhanced tumor growth and survival and are associated with poor prognosis [6,12–14]. In addition to above genetic components, development of PDA is also driven by environmental factors. Such risk factors include smoking, obesity, diabetes and chronic pancreatitis [15,16].

Most cases of PDA develop from precursor lesions known as pancreatic intraepithelial neoplasia (PanIN) [17]. Besides PanIN, other precursor lesions that are present in the pancreas include mucinous cystic neoplasms (MCNs) and intraductal papillary mucinous neoplasms (IPMNs) [18]. PanIN lesions arise from pancreatic acinar cells when they undergo a transdifferentiation process into duct-like cells, a process termed acinar-to-ductal metaplasia (ADM) [19]. PanIN can be classified as PanIN1A/B, PanIN2, or PanIN3, with each stage exhibiting an increasing degree of cytologic atypia [20]. PanIN lesions harbor the same genetic changes that are present in PDA, and using this progression model, genetic alterations have been classified as early (*KRAS* mutation), intermediate (*CDKN2A* mutation) or late (*TP53* and *SMAD4* mutations) [21]. This suggests that these early genetic changes, like acquisition of activating mutations in *KRAS* are fundamental prerequisites for PDA initiation. Another defining feature of PDA is that it is highly desmoplastic, a term which describes the presence of a highly diffused fibrotic stroma. Present within the pancreatic stroma are fibroblasts, immune cells, blood vessels and matrix proteins such as collagen. The tumor stroma has been shown to mediate PDA growth, metastasis to distant organs, and drug resistance [22]. The dense stromal network hinders the delivery of nutrients and oxygen to pancreatic cancer cells and leads to a state of hypoxic stress [23]. In PDA, the hypoxic environment can induce several metabolic changes such as increased glycolytic activity and hexosamine biosynthesis, both of which facilitates PDA cell survival and aggressiveness [24,25].

The genetic alterations and metabolic changes occurring during development of PDA and in PDA creates an environment that fosters the increased production of ROS [26,27], a group of highly reactive biological molecules including hydroxyl ion (HO[•]), superoxide (O₂^{•-}), and hydrogen peroxide (H₂O₂) [28,29]. While ROS can damage macromolecules [28] and at high concentrations are toxic to cells [30], their DNA damaging function can also contribute to mutagenesis [29]. In addition, at low levels they can have *bona fide* second messenger functions [31] and contribute to all aspects of tumorigenesis. It should be noted that the diverse effects of ROS on cell behavior are not only mediated by their concentration or distinct reactive species, but also the local site of ROS generation (for further reading [29]).

Downstream of oncogenic *KRAS*, elevated ROS levels promote several biological processes implicated in PDA development. Moreover, in PDA, elevated ROS levels induce hyper-

activation of signaling cascades that sustain enhanced tumor growth, survival and metastasis [32,33]. Modulation of ROS levels, either an inhibition to attenuate pro-tumorigenic signaling, or an increase to induce cell death, are possible therapeutic approaches for PDA.

2. ROS in PDA development

The ability of Ras molecules to induce ROS production was demonstrated almost two decades ago, when it was found that fibroblasts stably expressing a constitutively-active form of HRas produced increased amounts of superoxide [34]. Subsequently, it was shown that ROS generation is a critical requirement for Ras-mediated transformation *in vitro* [35], and that ROS production can also be induced by other Ras alleles such as KRas [36].

2.1. Generation of ROS downstream of oncogenic KRas

KRas-induced ROS production is primarily mediated by NADPH oxidase (NOX) enzymes and mitochondrial respiratory chain enzymatic complexes (Fig. 1).

KRas transformed PDA cells have increased NOX activity and superoxide levels, as compared to parental cells [37,38]. NOX is a multi-subunit enzyme which is activated through the small GTPase Rac1 [39,40]. Consequently, in PDA cell lines, presence of oncogenic KRas links to increased Rac1 activity and superoxide production [33]; and KRas-induced ROS production can be inhibited by downregulation of p47^{phox}, the cytosolic regulatory subunit of NOX [36]. Beyond studies conducted *in vitro* in cellular systems and *in vivo* in animal models which dissected the mechanistic details of KRas-NOX-mediated ROS production, several reports have indicated that in human PDA, expression of NOX family members is increased when compared to non-transformed pancreatic tissue [41–43].

Mitochondria are the other major source of ROS generated by oncogenic KRas. For example, acinar cells that express KRas^{G12D} showed a mitochondrial dysfunction that led to increased ROS production [27]. This is of importance for development of PDA, since gradually increased levels of ROS can be observed in regions of ADM and in different PanIN progression states [27]. Moreover, pancreatic cancer cells transformed with oncogenic KRas have also been shown to exhibit aberrant mitochondrial function [44]. KRas mediated disruption of mitochondrial metabolism is not limited to just PDA. Human embryonic kidney cells transformed with oncogenic KRas exhibit increased mitochondrial dysfunction with enhanced ROS production and elevated glycolytic activity [45]. Furthermore, in a mouse model of lung adenocarcinoma driven by oncogenic KRas^{G12D}, mitochondria-derived ROS was indispensable during tumorigenicity [32].

2.2. ROS as an inducer of PDA precursor lesions

Mitochondrial ROS produced in response to oncogenic KRas plays an essential role in inducing the formation of precancerous lesions in the pancreas (Fig. 2). Using pancreatic acini 3D explant (organoid) culture it was shown that ROS leads to ADM [27]. Moreover, KRas-induced ADM and PanIN formation in mice was blocked with mitochondrially-targeted antioxidants such as mitoQ, indicating that mitochondrial ROS production is a driving factor for tumor initiation [27]. Signaling downstream of mitochondrial ROS was mediated by the ROS-responsive transcription factor nuclear factor kappa-light-chain-

enhancer of activated B cells (NF- κ B) [27] and Protein Kinase D1 (PKD1), a sensor of oxidative stress [31], previously implicated in KRas-induced PanIN formation [46]. In addition, in mice expressing KRas^{G12D}, the loss of the stress response protein, tumor protein p53-induced nuclear protein 1 (Tp53INP1), accelerated the formation of cystic lesions resembling IPMNs. In this setting, the loss of Tp53INP1 was associated with increased oxidative stress [47].

2.3. Antioxidants and the Role of Nrf2 in Evading Senescence and Cell Death

Elevated ROS levels have emerged as a critical prerequisite for PDA initiation [27,47] (Fig. 2). However, as a mechanism for keeping ROS levels within narrow limits, where they drive hyper-activation of tumor-inducing signaling pathways, but do not trigger oncogene-induced senescence (OIS) or cell death, transformed cells often also up-regulate their antioxidant defense capacity [48,49]. Both, inactivation of genes like *CDKN2A*, which encodes tumor suppressor p16, as well as modulation of ROS levels allow PanIN to overcome Ras induced senescence [50].

One of the primary detoxification mechanisms employed by cells involves antioxidant enzymes like superoxide dismutase (SOD), glutathione (GSH) and peroxidases [28,51–53]. GSH functions as a primary defense mechanism against oxidative stress because of its ability to function as a free radical scavenger and its ability to reduce H₂O₂ in a chemical process which is catalyzed by glutathione peroxidases [54].

A key regulator of expression of multiple antioxidant proteins is the ROS-responsive transcriptional activator, nuclear factor erythroid-derived-2 (Nrf2). Under normal physiological conditions Nrf2 is bound by Kelch-like ECH-associated protein 1 (Keap1) in the cytoplasm and targeted for proteasomal degradation. In response to increasing ROS levels, Keap1 is inactivated and Nrf2 translocates to the nucleus and activates genes that mediate antioxidant responses [55]. In pancreatic cancer cell lines and in human PDA samples, Nrf2 expression is increased [56,57]. In addition, expression of Nrf2 is gradually increased during progression of mouse ADM and PanIN lesions [27]. Inhibition of Nrf2 expression in the pancreas of mice expressing oncogenic KRas not only decreased the number of PanIN lesions, but also decreased their ability to proliferate and upregulated markers for senescence such as SA β gal [58]. In a subsequent study from the same lab, in a mouse model of KRas^{G12D}-driven PDA, Nrf2 was shown to promote tumor growth and development by stimulating protein synthesis and mitogenic signaling. In this setting, oxidative damage of key components within the translational regulatory network was inhibited by Nrf2 [59].

While the increased antioxidant activity and pro-tumorigenic effects of Nrf2 observed during PDA carcinogenesis might seem counterintuitive to other publications indicating an increase in ROS as driver of tumor development, there are plausible explanations for these observations. One is that ROS need to be kept at a threshold level that promotes proliferation, but prevents senescence and cell death. Therefore, up-regulation of antioxidant systems need to occur when ROS increase, and oncogenic KRas seems to be capable of facilitating both [60].

3. ROS in PDA Progression

Besides their role in regulating formation of early pancreatic lesions and tumor initiation, ROS also have been implicated in all aspects of tumor progression (Fig. 2). In addition, ROS have emerged as key regulators of the reciprocal signaling between PDA and the surrounding stromal microenvironment.

3.1. ROS-induced Genomic Instability

DNA damage and genomic instability is pertinent to all facets of tumorigenesis, and alterations in key genetic sequences can facilitate the acquisition of a malignant phenotype. Multiple factors have been implicated in the induction of DNA damage and genomic instability, including increased oxidative stress [61]. In acinar cells isolated from rat pancreas, glucose oxidase-induced production of hydrogen peroxide induced DNA fragmentation [62]. In pancreatic cancer cells, hydrogen peroxide can induce the formation of DNA double-strand breaks (DSB) [63]. Data obtained from PDA patients support these *in vitro* findings. According to several reports, there are elevated levels of DNA adducts [64] and oxidative DNA damage [65] in PDA tissue when compared to normal pancreatic tissue. In human PDA, it is likely that NOX-induced ROS production mediates oxidative DNA damage, since the expression of NOX4 in PanIN and invasive PDA followed a very similar staining pattern to that of γ H2AX, a marker for DSB [66].

3.2. Promotion of PDA Proliferation, Growth and Survival

Elevated ROS levels are mediators of enhanced tumor cell proliferation [67–69]. The requirement for ROS in PDA growth has been demonstrated in multiple studies. In pancreatic cancer cells, inhibition of Rac1-mediated superoxide production attenuated cell growth *in vitro* [33]. Since Rac1 also regulates cytoskeletal reorganization [70] and functions in signal transduction at multiple levels [71], it is possible that proliferation defects produced in response to loss of Rac1 are not solely dependent on reduced superoxide production. In another study it was shown that reduced superoxide levels mediated by ectopic expression of SOD isoenzymes (extracellular, manganese, and copper-zinc) inhibited the growth of MiaPaca2 pancreatic cancer cells and diminished their ability to form tumors *in vivo* [72]. In addition, pancreatic cancer cell growth is also promoted by the transferrin receptor (TfR1) in a manner which is dependent on the receptors' ability to support mitochondrial respiration and the accompanying ROS production [73].

In pancreatic acinar cells, mitochondrial ROS generated downstream of oncogenic KRas induced the activation of NF- κ B, driving the expression of the epidermal growth factor receptor (EGFR) and its ligands [27]. EGFR signaling is essential for KRas-mediated pancreatic tumor growth via Erk1/2 both *in vitro* and *in vivo* [32,74–76]. Not only has the growth and development of PDA shown to be dependent on Erk1/2, but PDA initiation mediated by PanIN development is also facilitated by this signaling pathway [77–79]. In addition to Erk1/2, downstream of KRas^{G12D}, EGFR can also drive pancreatic epithelial cell proliferation via activation of c-MYC [80]. However, the ability of ROS to directly activate signaling molecules like c-MYC in an EGFR independent manner warrants further investigation.

ROS induced activation of NF- κ B not only promotes cell proliferation and tumor growth, but also mediates potent survival responses [81,82]. In pancreatic cancer cells, activated NF- κ B incorporates signals to and from multiple molecules such as receptor for advanced glycation end products (RAGE), and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) to enhance cell survival [83,84]. Activation of NF- κ B in response to mitochondrial ROS is mediated by PKD1 [27,85] and in response to NOX-induced ROS most likely via PI3K/Akt [40,86,87]. Finally, other signaling molecules, such as MUC4 [88], JAK2 [41] and 5-lipoxygenase (5-LO) [89] have been shown to coordinate survival responses in pancreatic cancer cells in conjunction with the ROS-generating machinery.

3.3. ROS in PDA Invasion and Metastasis

PDA is a highly metastatic disease [6], and several steps in this process are under the influence of ROS and its regulators (Fig. 2). Epithelial to mesenchymal transition (EMT) is usually a precursor to metastasis; and in response to hypoxia-induced oxidative stress, the EMT program is activated in pancreatic cancer cells [90]. It has also been shown that stimulation of pancreatic cancer cells with transforming growth factor β (TGF β) induces EMT through NOX/ROS-dependent mechanisms [91,92]. Degradation of the extracellular matrix (ECM) is mediated by matrix metalloproteinases (MMPs), which also are regulated by ROS. For example, increased invasion of pancreatic cancer cells is facilitated by MMP-2 which is dependent on NADPH oxidase-induced ROS production [91,93,94]. ROS mediated activation of MMPs is a likely mechanism utilized by cancer cells to escape from the primary pancreatic tumor [94].

The invasive potential of pancreatic cancer cells can be enhanced by nitric oxide through activation of PI3K/AKT and RhoA signaling [95]. The adhesion of pancreatic tumor cells to the microvasculature, a process required for disseminated tumor cells to colonize novel sites can be induced by superoxide [96]. Lastly, the importance of superoxide in the invasive capacity of PDA was demonstrated by overexpression of EcSOD (superoxide scavenger) in BxPC3 pancreatic cancer cells which inhibited their ability to form tumors and to disseminate *in vivo* [97].

3.4. Roles of ROS in Maintenance of the PDA Microenvironment

The fibrotic stroma which encompasses PDA cells serves as a reservoir of molecular and cellular agents that not only support PDA development, but also functions as a barrier to therapeutic intervention [98]. Some of the key components present in the stroma include pancreatic stellate cells (PSCs), fibroblasts, immune cells, and matrix proteins like collagen and fibronectin [98].

Increased ROS production induced fibrosis in mouse pancreas [99] and the activation of pancreatic stellate cells in the PDA microenvironment can be induced by ROS [100,101]. PSCs once active produce chemokines, α -smooth muscle actin (α -SMA) and collagen [102]. Pancreatic cancer cell growth, long-term survival and invasive potential are positively regulated by collagen [103,104]; and in PSCs, collagen production is induced by 4-HNE-dependent activation of activator protein-1 (AP-1) [105]. Not only can ROS and the associated oxidative stress induce fibrosis, but they can also be generated downstream of

fibrotic signaling [106]. Downstream of 5-LO, ECM components like fibronectin can induce NOX-dependent ROS production in PDA cells prolonging their survival.

4. Targeting ROS in PDA Development and Progression

Multiple lines of evidence implicate ROS in PDA carcinogenesis [64,67,81,90]. Therefore, one possibility for therapeutic intervention is to curtail ROS production and maintain them at levels where they do not facilitate their pro-tumorigenic effects (Fig. 3). Another possibility is to increase ROS to levels where they are toxic for cancer cells [30,107,108].

4.1. Strategy I: Reducing ROS to Non-Oncogenic Levels

Since elevated ROS levels promote several aspects of PDA carcinogenesis, the use of antioxidant therapy to reduce ROS levels has been investigated extensively. Natural antioxidants like vitamins C and E, and curcumin have been implicated in the prevention and development of multiple types of cancers [109,110]. However, the manner in which some of the epidemiological studies evaluating the correlation between antioxidants and cancer development were conducted is not without limitations [111]. Nonetheless, data obtained from *in vitro* and *in vivo* studies in which ROS levels have been lowered via antioxidant-dependent or independent mechanisms have produced anti-cancer effects (Fig. 3).

The effect of exogenous antioxidants has been evaluated in pancreatic cancer cells and PDA mouse models. Curcumin, a natural polyphenol with potent antioxidant activity, inhibits the proliferation of MiaPaca2 pancreatic cancer cells *in vitro*. Furthermore, treatment with curcumin decreased MiaPaca2 orthotopic tumor growth in mice by attenuating the redox-responsive transcription factor NF- κ B and its downstream targets [112]. Resveratrol, a natural plant antioxidant, has been shown to reduce proliferation and induce apoptosis in pancreatic cancer cells [113,114]. Moreover, in KRas^{G12D} mice, administration of resveratrol inhibited tumor growth and development [115], and in mice orthotopically-implanted with Panc1 cells, treatment with resveratrol inhibited their ability to form tumors [116]. Mechanistically, resveratrol was shown to elicit its anti-tumorigenic effects by stimulating pro-apoptotic proteins [114], inhibition of PKD1/NF- κ B signaling [117], and activating redox-sensitive transcription factors like FOXO3a [116]. In the previously discussed studies involving curcumin and resveratrol it is important to note that though these agents can exhibit antioxidant activity, it is likely that their anti-cancer effects are mediated through other modes of action in addition to their antioxidant properties. Vitamin E, another well-characterized antioxidant, suppresses Panc1, MiaPaca2 and BxPC3 pancreatic cancer cell growth *in vitro* [118,119]. In addition, mice expressing oncogenic KRas^{G12D} in their pancreas survived longer when they were treated with vitamin E, and their PanIN failed to progress [120]. In chemically-induced PDA, administration of vitamins A, C and E decreased the incidence of PDA, and this was mediated by increased levels of SOD [121]. The efficacy of Vitamin C has also been demonstrated in a mouse model of KRas^{G12D} driven colorectal cancer, where it was shown to inhibit tumor growth *in vivo* [122]. In contrast to studies where application of Vitamin E was shown to produce anti-proliferative effects in PDA cells [118,119], in lung cancer cells with oncogenic KRas^{G12D} mutation, vitamin E application actually enhanced cell growth [123]. The varied outcomes

between these studies could be explained by differences in the concentration of Vitamin E used, duration of Vitamin E application, variations in basal ROS levels or genetic variations in key tumor suppressor or oncogenes within cells.

PDA tumor growth can also be inhibited by specifically targeting ROS-generating entities. In pancreatic cancer cells, stimulation with diphenylene iodonium (DPI), a NADPH inhibitor, blocked ROS production and induced apoptosis [87]. Treatment of pancreatic cancer cells with Lipoxin A4 (LXA4), a metabolite of arachidonic acid, decreased ROS levels and subsequently inhibited Erk1/2 activity and MMP expression [124]. Metformin, a drug used in the treatment of diabetes, inhibits pancreatic cancer cell proliferation by decreasing the expression of NOX2 and NOX4, and increasing the expression of ROS scavengers like MnSOD and catalase [125]. Stable expression of extracellular SOD (EcSOD) inhibited the ability of BxPC3 pancreatic cancer cells to form tumor xenografts in athymic nude mice and also prolonged their survival [97]. N-acetylcysteine (NAC), an antioxidant which is a precursor of glutathione, blocks KRas-induced ADM [27], but also inhibits the growth and invasiveness of pancreatic cancer cells [126,127]. MitoQ, a mitochondria-targeted antioxidant can block ADM and PanIN formation [27].

The effect of using antioxidants in conjunction with chemotherapeutic agents like Gemcitabine has also been investigated in PDA. Combination treatment with curcumin augmented gemcitabine-induced apoptosis in various pancreatic cancer cells and inhibited the ability of MiaPaca2 cells to form orthotopic tumors in nude mice [128]. Combination of gemcitabine and curcumin nanoparticles (NanoCurc) attenuated the growth of Pa03C orthotopic xenografts, in comparison to the use of each drug as a single agent [129]. The anti-cancer effects of curcumin and gemcitabine were mediated by inactivation of NF- κ B [128,129]. While single dose administration of NAC or gemcitabine failed to inhibit the growth of MiaPaca2 xenograft tumors in male athymic mice, combination of both agents reduced tumor growth by about 50%. Again, in this setting, activation of NF- κ B was attenuated [130]. Finally, both vitamin E and resveratrol have been shown to amplify the anti-tumorigenic effects of gemcitabine in pancreatic cancer cells and in both xenograft and orthotopic MiaPaca2 mouse models of PDA in a manner that was dependent on the inhibition of NF- κ B activity [131,132].

Although data from the studies discussed above indicate that inhibition of ROS production might be a possible mechanism by which PDA carcinogenesis can be antagonized, with respect to the use of antioxidants at the organismal level, several questions with regard to their bioavailability, toxicity, molecular targets and interactions still remain unanswered [133,134]. Moreover, most of the compounds described above are still in the pre-clinical phase of development (Table 1).

In addition, there are studies which indicate that antioxidants like Nrf2 can elicit pro-tumorigenic effects during PDA development. Loss of Nrf2 and the accompanying oxidative damage can compromise mRNA translation and ultimately mediators of oncogenic signaling like EGFR [59]. Acting in a similar fashion to Nrf2, GSH can also promote cellular transformation during the initiation of carcinogenesis. This was demonstrated by Harris *et al.* who showed that loss of GSH delayed the onset of mammary tumor formation in mice.

Interestingly, in the case of established tumors, depleting GSH levels did not produce any anti-cancer effects [135].

Unlike the dietary antioxidants like Vitamins C and E, and curcumin, increased expression of endogenous antioxidants like Nrf2 or GSH might not produce any clinical benefit for PDA patients or for those at risk for developing the disease. However, the effect of specific inhibition of Nrf2 or GSH still requires extensive evaluation at the clinical level, with specific emphasis on the timing of treatment. This is an important parameter to take into consideration since the pre-clinical data suggests that the efficacy of antioxidants targeting strategies can be influenced by multiple factors for example stage of disease development.

Finally, the effect of antioxidants on ROS homeostasis and function in non-transformed cells have been largely unexplored and may produce unintended effects, and this another important factor that should not be overlooked [69].

4.2. Strategy II: Increasing ROS to Toxic Levels

Increasing the already elevated ROS levels in transformed cells to induce cytotoxicity provides another point of therapeutic invention. This could be potentiated in combination with first line chemotherapeutic agents which elicit anti-tumorigenic effects by inducing permanent oxidative damage. Induction of ROS levels beyond the threshold needed to trigger cell death both *in vitro* and *in vivo* models of PDA is an active area of ongoing research (Fig. 3).

In pancreatic cancer cells treatment with dicumarol, a natural anticoagulating agent, inhibited proliferation and induced apoptosis. These effects were mediated by dicumarol-dependent inhibition of NADPH:quinone oxidoreductase (NQO₁) and the subsequent increase in superoxide production [136,137]. The efficacy of dicumarol was also evaluated *in vivo* in nude mice where it was shown to inhibit MiaPaca2 xenograft tumor growth in a dose-dependent manner [137]. Benzyl isothiocyanate (BITC), a constituent of cruciferous vegetables, when introduced into pancreatic cancer cells induced G₂/M arrest and apoptosis. In pancreatic cancer cells, stimulation with BITC increased ROS production, activated MAPK signaling and reduced GSH levels. Most importantly, in this study, treatment of normal pancreatic epithelial cells with BITC produced no effects [138]. Increased ROS production, and the ensuing DNA damage mediated by piperlongumine (PPLGM), a biochemical derivative from long peppers, suppressed pancreatic cancer cell growth *in vitro*, and Panc1 tumor growth in a xenograft mouse model of human PDA [139].

Eicosapentaenoic acid (EPA), an omega-3 fatty acid, present in high quantities in fish oil also elicits anti-tumorigenic effects by inducing ROS production. Administration of EPA attenuated pancreatic cell proliferation and blocked MiaPaca2 xenograft tumor growth in athymic nude mice [140]. When pancreatic cancer cells were stimulated with nimbolide, a natural plant extract, they failed to proliferate and their ability to migrate and invade was severely compromised. In a mouse xenograft model of HPAC pancreatic cancer cells, treatment with nimbolide blocked tumor growth *in vivo* and metastasis to distant organs. In this setting, the anti-oncogenic effects of nimbolide were mediated through the generation of excessive ROS and induction of autophagy and apoptosis [141]. Other naturally derived compounds like berberine and isoalantolactone have also demonstrated anti-cancer activity

in PDA cells *in vitro* in a manner which is dependent on enhanced ROS production [142,143].

In addition to the plethora of natural-based compounds that have been shown to induce excessive ROS production, pharmacologically-derived agents also have potent ROS-inducing capacities. The inhibition of thioredoxin reductase with gold (I) NHC complexes increased ROS levels and reduced mitochondrial membrane potentials in pancreatic cancer cells, which resulted in decreased growth and enhanced apoptosis [144]. The induction of oxidative stress in the endoplasmic reticulum (ER) with the thiol-reactive small molecule imexon can also suppress pancreatic cancer cell growth *in vitro* [145]. The ability of BML-275, a small molecule inhibitor of adenosine monophosphate-activated protein kinase (AMPK) to induce apoptosis in human pancreatic cancer cells is also dependent on ROS-mediated DNA damage [146].

The efficacy of ROS-generating small molecule compounds has also been evaluated in animal models. The small-molecule inhibitor, YLT256 blocked BxPC3 growth in a xenograft mouse model of PDA. Mechanistically, YLT256 induced mitochondrial dysfunction, ROS production, and stimulated pro-apoptotic signaling [147]. Pharmacological inhibition of CDK4/6 with the use of PD-0332991 suppressed the growth of PL5 pancreatic cancer xenografts and reduced tumor volume of orthotopically-implanted PL45 pancreatic cancer cells. In this setting, PD-0332991 was shown to induce ROS production and the application of a PI3K/MTOR inhibitor was shown to potentiate the anti-cancer effects of PD-0332991 *in vivo* [148]. Finally, the results of a synthetic lethality screen identified lanperisone (LP) family compounds as agents that selectively target MEFs expressing oncogenic KRas. Stimulation of oncogenic KRas expressing MEFs with LP induced ROS production, apoptosis, and inhibited the ability of these cells to form tumors *in vivo* in nude mice [149]. Given the pivotal role of oncogenic KRas in PDA carcinogenesis, specifically as it relates to the generation of ROS, this makes ROS an even more attractive therapeutic target in malignancies driven by active KRas.

Elevated ROS production has been shown to synergize with currently-used chemotherapeutic agents such as gemcitabine. Gemcitabine by itself induces ROS production, and addition of either inhibitors of antioxidants or inducers of additional ROS production can drive cytotoxicity. For example, β -Phenylethyl isothiocyanate (PEITC) inhibits endogenous antioxidants to augment the efficacy of gemcitabine. Treatment of gemcitabine-resistant pancreatic cancer cells with PEITC led to a decrease in ROS scavenging and increased ROS production and apoptosis. Most importantly, combined treatment with gemcitabine and PEITC had profound anti-cancer effects *in vivo* in an orthotopic mouse model of AsPc1 pancreatic cancer cells [150]. Cannabinoids also can enhance the anti-cancer effects of gemcitabine in PDA. Pancreatic cancer cells treated with gemcitabine and cannabinoids were less proliferative than cells treated with gemcitabine only, and PaCa44 pancreatic cancer cells stimulated with both agents had less tumor-forming capacity *in vivo*. These anti-tumorigenic effects of the gemcitabine/cannabinoid treatment were mediated by increased ROS production, and ROS-induced autophagic cell death [151].

Evidence from other studies also demonstrate the effectiveness of specific induction of ROS as it relates to gemcitabine efficacy; Donadelli *et al.* showed that trichostatin A (TSA), a histone deacetylase (HDAC) inhibitor enhanced gemcitabine-mediated cytotoxicity *in vitro* and blocked the growth of T3M4 human pancreatic cancer cells *in vivo* [152]. Additionally, genetic inhibition of Bmi1 was shown to augment gemcitabine sensitivity in pancreatic cancer cells [153], and in both of these cases, the enhanced effectiveness of gemcitabine was dependent on increased ROS production.

Finally, since KRas^{G12D} expression links to increased mitochondrial dysfunction and elevated ROS production [27], pancreatic cancer cells could potentially be sensitive to agents that alter mitophagy, a process whereby dysfunctional and damaged mitochondria are specifically eliminated from cells [154]. The efficacy of ceramide, a bioactive phospholipid which can induce mitophagy, has already been demonstrated in an AsPc1 xenograft mouse model of pancreatic cancer, where it was shown to stimulate mitochondrial depolarization and inhibit tumor growth *in vivo* [155]. However, many questions with regard to the role of mitophagy in PDA remain unanswered, and additional studies to determine the specific effects mediated by mitophagy during PDA initiation and development are required.

4.3. Clinical Implications for ROS in PDA

One of the initial wide scale assessments of antioxidant supplementation on PDA incidence and mortality was conducted in a population of male Finnish smokers. In this study, their diets were supplemented with β -carotene only, Vitamin E (α -Tocopherol) only, both compounds and placebo for a period of 5–10 years. At the end of the study, consumption of dietary antioxidants did not have any effect on PDA incidence nor mortality [156]. However, data obtained from other cohort studies showed that increased Vitamin E intake was associated with reduced risk of PDA development [157,158]. In a phase I clinical trial, administration of vitamin E (δ -tocotrienol) to 25 patients with PDA and PDA precursor lesions prior to surgery, showed that the compound was well-tolerated, and that apoptotic cells could be detected in the resected tumors of patients [159]. In another phase I trial, for eight weeks, patients with metastatic stage IV PDA were administered vitamin C (ascorbic acid) along with gemcitabine and erlotinib, and post vitamin C treatment, 8 out of the 9 patients had smaller primary tumors [160]. Finally, another phase I trial also showed that ascorbate when administered concurrently with gemcitabine was well-tolerated by node-positive PDA patients and produced moderate effects [161].

The applicability of ROS-generating compounds targeting PDA has also been evaluated in the clinic. As discussed above, inhibition of thioredoxin reductase using gold (I) NHC complexes induced ROS production and inhibited pancreatic cancer growth *in vitro* [144]. In a Phase II clinical trial using PX-12, a thioredoxin inhibitor, in patients with metastatic PDA who had been previously treated with gemcitabine, no anti-tumor effects were observed [162]. Anthracyclines such as doxorubicin and epirubicin, are the chemotherapeutic agents associated with the highest level of oxidative stress [163], and the significance of these drugs in PDA have been the basis of multiple clinical trials. However, in a phase II trial using liposomal doxorubicin in PDA patients with unresectable tumors, the data with regard to

clinical benefit was inconclusive [164], whereas co-administration of epirubicin and gemcitabine to PDA patients produced clinical benefit response [165].

5. Expert Commentary

To date, PDA still remains a highly devastating disease despite efforts on multiple fronts to mitigate its development. Current standard of care involves administration of Gemcitabine or FOLFIRINOX as single agents, or in combination with other drugs or radiation therapy, and even then, median survival rarely extends beyond 12 months [166]. Over 90% of all patients have oncogenic mutations in *KRAS* resulting in constitutive activation of its gene product KRas, but attempts made at targeting KRas so far have been unsuccessful [167,168]. Therefore, other targeting strategies are under intense investigation. These include the development of microRNA gene therapy [169] and immunotherapy [170]. Another option is to hit key signaling events downstream of KRas. There is a growing body of evidence showing that ROS and the associated oxidative stress is a product of active KRas signaling (Fig. 1) [27,33]. Such elevated ROS levels as seen in PDA, facilitate cellular transformation and promote key aspects of tumorigenesis and progression.

PDA develops from specific precursor lesions, and in concert with other cellular components, ROS can induce the formation of these lesions (Fig. 2) [27,47]. A possibility of intervention is to prevent the development of cancerous lesions by inhibiting either elevated ROS levels in precancerous PanIN cells or by preventing pro-tumorigenic signaling downstream of ROS. Modulating ROS levels as a mechanism to counteract disease initiation (Fig. 3) could include the use of antioxidants such as mitoQ or dietary antioxidants, or inhibitors for ROS activated molecules such as Erk1/2, PKD1 or NF- κ B [60]. This preventive strategy, however, requires an early detection method for pancreatic precancerous lesions.

A therapeutic strategy for established PDA involves the use of agents that further increase the already elevated ROS levels beyond the threshold needed for cytotoxicity (Fig. 3). This may be obtained by inhibition of antioxidant systems or co-treatment with inducers of oxidative stress. Data obtained from *in vitro* and *in vivo* models in which ROS levels have been modulated have produced potent anti-tumorigenic responses [141], however, application of these intervention strategies have been difficult to translate at the clinical level [165]. This can be attributed to many factors among which include cohort composition, drug dosage and timing, and the inability to replicate exact experimental conditions from animal models in human cohorts.

6. Five-year View

Data obtained from pancreatic cancer cells lines, mouse models of PDA and PDA patient samples show that elevated ROS levels facilitate PDA development and progression. Furthermore, multiple studies have demonstrated the beneficial effects of modulating ROS levels in pre-clinical models of PDA. Since administration of compounds that can alter ROS levels in human cohorts have not always produced the potent anti-cancer effects observed in the pre-clinical models, the applicability of ROS-based therapies in the clinic requires

additional studies to delineate in further detail the specific mechanisms by which ROS promote PDA carcinogenesis. Within the next five years, it is therefore imperative for investigators to focus on the parameters that can potentially bridge the gap between the outcomes observed at the pre-clinical and clinical levels. Special emphasis should be placed on the development of pre-clinical models that mimics human PDA more precisely, and on the identification of more effective strategies for modulating ROS levels to counteract the malignant phenotype.

Acknowledgments

Funding

This work was supported by grants from the National Institutes of Health (CA200572 and CA140182) to P Storz.

Abbreviations

4HNE	4-hydroxy-2-nonenal
5-LO	5-lipoxygenase
α-SMA	α -smooth muscle actin
ADM	acinar-to-ductal metaplasia
AMPK	adenosine monophosphate-activated protein kinase
AP-1	activator protein-1
BITC	benzyl isothiocyanate
DPI	diphenylene iodonium
DSB	DNA double-strand breaks
ECM	extracellular matrix
EGFR	epidermal growth factor receptor
Erk1/2	extracellular signal-regulated kinase 1/2
EPA	eicosapentaenoic acid
GSH	glutathione
IL-6	Interleukin-6
IPMN	intraductal papillary mucinous neoplasm
Keap1	Kelch-like ECH-associated protein 1
LP	lanperisone
LXA4	lipoxin A4
MAPK	mitogen activated protein kinase

MCN	mucinous cystic neoplasm
MEF	mouse embryonic fibroblast
MMP	matrix metalloproteinase
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NOX	NADPH oxidase
NQO1	NADPH:quinone oxidoreductase
Nrf2	nuclear factor erythroid-derived-2
OIS	oncogene-induced senescence
PanIN	pancreatic intraepithelial neoplasia
PDA	pancreatic ductal adenocarcinoma
PEITC	β -phenylethyl isothiocyanate
PKD1	Protein Kinase D1
PPLGM	piperlongumine
PSC	pancreatic stellate cell
RAGE	receptor for advanced glycation end products
ROS	reactive oxygen species
SAβgal	senescence-associated β -galactosidase
SOD	superoxide dismutase
TfR1	transferrin receptor
Tp53INP1	tumor protein p53-induced nuclear protein 1
TGFβ	transforming growth factor β
TRAIL	tumor necrosis factor-related apoptosis-inducing ligand
TSA	trichostatin A

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Key Issues

- Oncogenic KRas stimulates the formation of ROS
- In PDA and its precursor lesions ROS is generated by NOX and mitochondria
- Increased ROS production in PDA is accompanied by increased antioxidant capacity; this serves as a protective mechanism to prevent OIS or cell death
- ROS facilitates the development of PDA precursor lesions and supports all facets of PDA carcinogenesis
- Decreasing ROS to non-oncogenic levels in pre-clinical models of PDA have produced anti-cancer effects
- Increasing the already elevated ROS levels to induce cytotoxicity has also produced anti-tumorigenic effects
- The applicability of modulating ROS at clinical levels stills requires extensive investigation

Fig. 1. Mechanisms of ROS production in pancreatic ductal adenocarcinoma

Oncogenic KRas or activation of wildtype Kras downstream of growth factor receptors induces generation of ROS via NADPH oxidases and mitochondrial respiratory chain complexes.

Fig. 2. ROS promote pancreatic ductal adenocarcinoma initiation and progression

Increased ROS can induce acinar cell metaplasia (ADM) and formation of precancerous PanIN lesions. Additional increases in ROS levels have been associated with progression of PanIN to PDA and all aspects of tumorigenesis. These include tumor cell growth and proliferation, tumor cell survival, genomic instability, invasion and metastasis, but also crosstalk with components of the tumor microenvironment.

Fig. 3. Modulation of ROS levels as a therapeutic strategy for pancreatic ductal adenocarcinoma

A decrease of ROS levels may be a valuable strategy to prevent the formation of precancerous lesions or to prevent their progression. This may be achieved using natural or chemical antioxidants, but requires an early detection method for precancerous lesions. Another option is to increase ROS levels in established tumors to levels that are toxic. This may be achieved by using natural or chemical inducers of ROS either alone or in combination with currently-used chemotherapeutic drugs.

Table 1

ROS regulating compounds evaluated in PDA carcinogenesis

Compound	Effects on ROS levels	Reported Molecular Targets	Developmental Phase	Reference
diphenylene iodonium (DPI)	Decrease	↑ cleaved PARP ↓ p-AKT ↓ p-ASK1	Pre-clinical	[87]
Lipoxin A ₄ (LXA ₄)	Decrease	↓ MMP-2, MMP-9 ↓ p-ERK	Pre-clinical	[124]
Metformin	Decrease	↓NOX2, NOX4 ↑MnSOD	Pre-clinical	[125]
Dicumarol	Increase	↓ NQO1	Pre-clinical	[136]
Benzyl isothiocyanate (BITC)	Increase	↓ GSH	Pre-clinical	[138]
piperlongumine (PPLGM)	Increase	↑8-OHdG	Pre-clinical	[139]
eicosapentaenoic acid (EPA)	Increase	↑ cleaved Capase-8 ↑ cleaved PARP	Pre-clinical	[140]
Nimbolide	Increase	↑ cleaved PARP ↑ BAX ↑ cleaved Capase-3 ↓ Bcl-2	Pre-clinical	[141]
Gold(I)NHC complex (MC3)	Increase	↓TrxR ↑ cleaved PARP	Pre-clinical	[144]
BML-275	Increase	↓ p-AMPKα	Pre-clinical	[146]
YLT256	Increase	↑ cleaved Caspase-3 ↑ cleaved Capase-9 ↑ BAK ↓ Bcl-2	Pre-Clinical	[147]
PD-0332991	Increase	↑ pS6	Pre-clinical	[148]
Ianperisone	Increase	↑ HIF-1	Pre-clinical	[149]