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Emerging Drug Target In Pancreatic Cancer: Placing Sirtuin 1 on the Canvas

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Abstract: Sirtuin 1 is a protein deacetylase that regulates a large number of proteins often functionally implicated in tumor development and progression. Its pleiotropic function has turned SIRT1 into an attractive chemotherapeutic target, underscored by very promising preclinical results with SIRT1 inhibitors in the treatment of chronic myeloid leukemia. Here, we revisit the studies on SIRT1 as an emerging target for therapy in pancreatic cancer, a tumor with dismal outcomes for which currently few therapeutic options are available. We highlight those potential SIRT1 target genes that are commonly affected in pancreatic cancer according to recent genomic analyses.

Keywords: Chemotherapy, Deleted in breast cancer 1, genomic, mutation, pancreas, personalized medicine, Sirtuin 1

THERAPEUTIC STRATEGIES IN PANCREATIC DUCTAL ADENOCARCINOMA

Pancreatic ductal adenocarcinoma (PDAC, 95% of all pancreatic cancer cases), is a lethal tumor and is forecasted by the Pancreatic Cancer Action Network to become the second most common cause of cancer death in the US by 2030. The incidence and the death rate of PDAC are rising and patients are faced with an extremely poor 5-year survival rate below 10% [1]. The poor prognosis of this tumor is due to late diagnosis, lack of adequate therapeutic options and failure to stratify clinical trials according to patient heterogeneity.

Gemcitabine, and more recently FOLFIRINOX and nab-paclitaxel, are the commonly used chemotherapies for PDAC [2]. Only a proportion of patients respond to these drugs and improvement in overall outcome, despite being statistically significant, is disappointingly small. Since it has become clear that no drug suits all PDAC patients, therapies are sought for specific molecular signatures [3], hijacking successful approaches applied to other tumors, *e.g.* Epidermal Growth Factor Receptor (EGFR) inhibition of *KRAS* wild type tumors in colorectal cancer [4] and treatment of *HER2* amplified tumors with trastuzumab in breast cancer [5]. Unfortunately, similar drug approaches are applicable to relatively few cases of PDAC due to its mutational landscape being highly heterogeneous [6].

This heterogeneity demands research into drugs that can simultaneously target different proteins known to promote the tumor through mutation or through (epi)genetic changes in their coding genes.

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SIRTUIN 1, A PROTEIN DEACETYLASE WITH MANY TARGETS

Within the category of druggable proteins that have pleiotropic actions in cancer progression, we would like to highlight Sirtuin 1 (Sirt1), member of a family of 7 mammalian homologues (Sirt1-7). Sirtuins have different substrates and reside in different cellular compartments (nucleus, cytoplasm and/or mitochondria) affecting their activity as well as substrate selection [7, 8]. Sirt1 operates through protein deacetylation and (mono) ADP-ribosylation. The unique deacetylation reaction of Sirt1 acts on modified lysine residues and requires NAD+ cleavage with each reaction cycle. During this deacetylation reaction, *O*-acetyl-ADP ribose and nicotinamide are generated. Sirt1 mediated protein deacetylation modifies the activity and/or intracellular localization of a wide variety of proteins [8]. Consequently, many roles for Sirt1 have been reported, including repression of genome instability and extension of the organism's lifespan. Using various transgenic mouse models of Sirt1 overexpression or knock down, previous studies have asserted Sirt1 as a robust protector against oxidative stress, aging, inflammation and apoptosis in most organs including the pancreas [9], the liver [10, 11], the hearth [12, 13], the brain [14], the kidneys [15] and the immune system [16]. Sirt1 expression varies with the organ considered, and is altered by aging. Kwon and colleagues show that the expression of Sirt1 in 24-month-old rats was increased in kidney, lung, and spleen tissue, compared with that in younger rats [17].

In cancer, depending on the tissue and the context, Sirt1 has so far been found to have oncogenic as well as tumor suppressive functions [18]. Sirt1 can be classified as an epigenetic modulator because it regulates histone acetylation and heterochromatin formation. As opposed to other histone deacetylases (HDACs), sirtuins are not inhibited by trichostatin A and do not depend on Zn^{2+} as a cofactor. As such, Sirt1 is classified as a class III HDAC. However, Sirt1 contains a Zn^{2+} atom coordinated by four cysteines which is

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essential for its activity [19, 20]. With regards to the effects on histones, the typical H4K16 hypoacetylation in cancer has been linked to SIRT1 [21]. In addition, H3K9 methylation subsequent to its deacetylation [22], has been found to be inhibited by Sirt1's endogenous inhibitor Deleted in breast cancer 1 (Dbc1, also named KiAA1967 or Ccar2) [23]. Sirt1 also deacetylates H3K56Ac upon DNA damage [24] and deacetylates the H1 isoform, believed to be involved in heterochromatin formation [22]. Profiling of the acetylome in Sirt1 knock out cells highlighted that Sirt1 indirectly affects histone acetylation by targeting a number of histone acetyl transferases [25]. In summary, Sirt1 directly and indirectly affects histone acetylation and chromatin compaction, as extensively reviewed by Martinez-Redondo and Vaquero [26].

Sirt1 targets a wider range of non-histone proteins. We refer the readers to several excellent reviews on the different histone and non-histone substrates [7, 8, 26, 27]. From here onwards, we will mainly focus on the role of Sirt1 and its non-histone targets that may be particularly relevant in the normal pancreas, pancreatic carcinogenesis and progression.

SIRT1'S EMERGING ROLE IN THE DEVELOPMENT AND PROGRESSION OF PDAC

In contrast to multiple studies on the function of Sirt1 in pancreatic endocrine insulin producing cells [28], very few studies have explored the role of Sirt1 in the normal exocrine pancreas. The exocrine tissue compartment comprises the digestive enzyme secreting acinar cells and the duct cells that align tubular structures to channel the secretory products into the duodenum [29]. In the present review we focus on the exocrine compartment since it is here that PDAC originates [30].

We have shown that Sirt1 and its endogenous inhibitor Dbc1 are co-expressed in the nuclei of exocrine pancreatic cells [31]. During acinar to ductal metaplasia, a change in exocrine cell differentiation that predisposes to PDAC development, Sirt1 translocates from the nucleus to the cytoplasm. This likely leads to increased Sirt1 activity in the acinar cells as a result of loss of Sirt1 inhibition by the nuclear Dbc1. Our findings showed concomitantly altered acetylation of beta-catenin and of the acinar transcription factor Ptf1a (Pancreatic transcription factor-1a), underscoring a role of Sirt1 in the regulation of acinar cell differentiation [31]. This may have implications for enhanced predisposition to tumor development known to be facilitated by altered acinar cell differentiation [30]. However, one study on calorie restriction where neoplasia in the exocrine pancreatic cell compartment is delayed whilst levels of Sirt1 are increased [32], indirectly suggests that Sirt1 could be tumor suppressive in the pre-cancer stage. The role of Sirt1 during PDAC development thus needs to be further explored.

An epigenetic signature for SIRT1 was tested on a cancer cell line panel representing different tumor types where pancreatic cancer demonstrated high SIRT1 activation [33]. Expression patterns and levels of SIRT1 have been further studied in cell lines and clinical samples of PDAC [31, 34-36]. We reported that SIRT1 expression was high in early stage I and II resected PDAC samples but we did not find any relation between the intracellular expression patterns or the expression levels and clinico-pathological features [31]. Stenzinger *et al*. reported on nuclear SIRT1 levels that were high in 30% of the PDAC samples and correlated with poor histological differentiation and poor postsurgical survival [35]. Zhao *et al*. reported that SIRT1 expression was increased in pancreatic tumors of patients >60 yr old at diagnosis, and larger than 4cm, and that this was associated with a higher lymph node burden and the development of hepatic metastases [34]. Knight and colleagues were the first to report the role of Active regulator of SIRT1 (AROS) in the regulation of survival in a panel of human cancer cell lines [37]. A recent paper [38] also highlights the roles of SIRT1 and SIRT2 in positively regulating the levels of Rac1-GTP and the activity of TIAM 1 (T-cell lymphoma invasion and metastasis) that confer motility to cancer cells, including PDAC, and thereby may be stimulating metastasis.

With the Sirt1 protein known to shuttle between the nucleus and the cytoplasm and the effect of Sirt1-Dbc1 interaction in regulating Sirt1 activity [39], caution is needed when comparing the different studies. Nevertheless, a consensus is building that SIRT1 expression is high in PDAC and is potentially associated with a more aggressive phenotype and metastasis. Validation in independent and comprehensively clinically annotated cohorts of patients is warranted, together with the inclusion of the intracellular localization patterns of SIRT1 and its interaction with DBC1 as important parameters.

THE GENOMIC LANDSCAPE OF PDAC AND SIRT1'S PLEIOTROPIC FIELD OF ACTION

The genomic landscape sequencing study of PDAC [6] revealed 16 significantly mutated genes. Some of these were previously known mutations in PDAC (*KRAS*, *TP53*, *CDKN2A, SMAD4*, *MLL3*, *TGFBR2, ARID1A* and *SF3B1)* but other were novel genes, such as chromatin modifiers (*EPC1* and *ARID2*) and DNA damage repair genes (*ATM*). We explored whether SIRT1 could have relevant actions on these.

With constitutively activating mutations in >90% of PDAC samples, KRAS activation is an essential oncogenic driver of pancreatic cancer. It is thought to be the first significant step in the development of pancreatic lesions. While Kriegl *et al*. found that up-regulation of SIRT1 expression correlates with the presence of *KRAS* mutations in the serrated route to colorectal cancer [40], such correlation remains more elusive in PDAC. This is likely due to the fact that *KRAS* is almost uniformly mutated in this tumor [6]. Interestingly, KRAS is tightly regulated by post-translational modifications and acetylation of KRAS attenuates its activity. A role in the deacetylation of KRAS has been demonstrated for SIRT2 but not for SIRT1 [41].

About 50% of resected PDAC cases have a mutation or loss of heterozygosity (LOH) in the tumor suppressor gene *TP53* [6]. It remains to be seen if PDAC cases with wild type *TP53* would have inactivation of p53 through SIRT1, since p53 is SIRT1's best-characterized target for deacetylation and inactivation in other tissues [42, 43]. Previous studies have shown that SIRT1 deacetylates p53 and thereby blocks stress induced apoptosis [42-44]. Increased p53 acetylation is

found in PDAC cells when treated with SIRT1 RNAi or with SIRT1 inhibitors, conferring enhanced apoptosis and cytostasis [31, 45]. We note that mutations in *TP53* are known to confer a gain of function to mutant p53, and others have demonstrated that mutant p53 drives PDAC metastasis [46]. In this regard, activating mutant p53 through increased acetylation might promote metastasis. Different studies have dealt with the effects of SIRT1 in the context of *TP53* mutations [27, 47-49], but it has not yet been addressed for PDAC.

In addition to direct deacetylation of p53, Sirt1 can also act indirectly, through deacetylation of the histone acetyltransferase kat2b/p300, with comparable outcomes (the more Sirt1, the less active p53) [50]. Interestingly, Enhancer of PolyComb homolog 1 (EPC1), approximately affected by mutation in 3% of PDAC cases [6]), and a component of the NuA4 histone acetyltransferase (HAT) complex, binds directly to p300 [51]. Additionally, acetylation of Epc1 was reported to be significantly higher on *Sirt1* knockout cells [25], suggesting that Epc1 could be a direct target of Sirt1. In conclusion, there is plenty of evidence to support an oncogenic activity of SIRT1 in wild type *TP53* cases of PDAC but further research is required to fully understand how SIRT1 regulates p53 if it is mutant, and how this contributes to PDAC development and progression.

The next genomic alteration to be discussed is *CDKN2A* encoding for p16. With greater than 95% of PDACs demonstrating a loss of *CDK2NA*, it is the most frequently inactivated tumor suppressor gene in this cancer [6, 52]. Its gene disruption occurs through multiple mechanisms including nearly half by homozygous deletion, more than a third by intragenic mutations with loss of heterozygosity, and the remainder by epigenetic promoter silencing. Its product p16 regulates the cell cycle by inhibiting cyclinD-CDK4/6, a kinase complex responsible for initiating the G1/S phase transition. SIRT1 was shown to down-regulate p16 expression in various *in vitro* models [53, 54] through direct deacetylation effects and indirect regulation of Akt/p70S6K1 signaling. If a SIRT1-mediated suppression of p16 that favors proliferation were to be confirmed in PDAC, again, it would underscore SIRT1's oncogenic effect in this tumor type.

Next, we consider *SMAD4* of which expression is lost in about 45% of PDAC (approximately 30% homozygous deletion and 15% mutation with loss of heterozygosity). *SMAD4* was originally identified as a tumor suppressor gene for pancreatic cancer [55] and is a key member of the Transforming Growth Factor-beta (TGF-beta) pathway. This pathway is involved in the regulation of a variety of cellular processes such as cell growth, differentiation, migration and apoptosis. It is a major contributor to PDAC but has a paradoxical role with both tumor suppressor and oncogenic effects in PDAC, and these effects vary with staging and molecular context. In short, prior to tumor initiation and early during progression, TGF-beta acts as a tumor suppressor; however, at later stages, it is often a tumor promoter [56, 57]. SMAD4 was identified as a direct target for SIRT1 with deacetylation leading to inhibition of TGF-beta signaling [58, 59]. *In vitro*, Sirt1 also reverts p300 acetylation of SMAD7, an inhibitory SMAD, promoting its ubiquitination and degradation, and thereby inhibiting TGF-beta mediated apoptosis [60]. Interestingly, wild-type phosphorylated p53 and Smads 2 and 3 interact and, after interaction with Smad4, coordinately induce transcription of a number of tumor suppressive genes [61]. Conversely, mutant p53 generally subverts tumor suppressive TGF-beta responses leading to a diminished transcriptional activation of key TGF-beta target genes [62]. Mutant p53 can also interact with SMADs and this enables formation of a complex with the p53-related tumor suppressor p63 eventually favoring tumor progression [63]. In conclusion, the effect of SIRT1 on SMAD4/TGF-beta can depend on the mutational status of *TP53*, reiterating what we discussed above.

We also draw the attention to *ATM* (Ataxia Telangiectasia Mutated), which is mutated in about 5% of PDAC. The ATM protein kinase is a master sensor of DNA damage [64]. ATM, activated by autophosphorylation through mutation or during DNA damage repair, phosphorylates multiple cellular substrates including DBC1. DBC1 phosphorylation results in binding to and inhibition of SIRT1 [65]. Activated ATM also phosphorylates NBS1 (Nijmegen Breakage Syndrome), an essential player in DNA damage repair signaling and a direct target of SIRT1. The deacetylation of NBS1 by SIRT1 is required for ATM-mediated NBS1 phosphorylation and efficient DNA damage repair [66]. In conclusion, SIRT1 plays an important role in genomic stability and repairing DNA damage. These are beneficial events in normal cells exposed to low levels of DNA damage and may seem paradoxical to SIRT1's oncogenic effects on the other target genes. In tumor cells, however, these interactions may be changed or outweighed by effects that promote the tumor, depending on the context.

Apart from *SF3B1* of which the acetylation was significantly increased in *SIRT1* knockout cells [25] with a potential role of Sirt1 in the RNA splicing pathway, there is little evidence that the other genes that were found significantly mutated in PDAC are connected with SIRT1.

Together these findings highlight the complex contextual mutational landscape of pancreatic cancer and the multifaceted (inter)actions that SIRT1 may play herein. Overall, SIRT1 seems to have pro-oncogenic effects although the context seems of major importance.

SIRT1 INHIBITORS IN PDAC–ACCUMULATING EVIDENCE OF CLINICAL POTENTIAL

SIRT1 inhibitors are either substances that inhibit NAD^+ dependent activities (*e.g.* Nicotinamide = Vitamin B3), or Sirtuin-specific inhibitors (*e.g.* Tenovin-6). Nicotinamide can attenuate the incidence of hepatocellular carcinoma in a mouse model, without any evidence of toxic effects for the mice [67]. Nicotinamide is in a clinical trial as a preventive agent for skin cancer [68] and has recently been suggested for a therapeutic trial in Polycystic Kidney Disease [69]. Other SIRT1 inhibitors are already in clinical trials for Huntington's disease [70]. This can facilitate the consideration of testing SIRT1 inhibitors in a clinical trial for treating PDAC.

In respect to the strong protective effect of SIRT1 at so many levels and in so many organs, a systemic inhibition of

SIRT1 should be considered cautiously. A number of studies have already examined the effects of genetic and drug based inhibition of SIRT1 in PDAC cell lines. SIRT1 inhibition results in decreased tumor cell growth, induction of apoptosis, reversion of TGF-beta-induced epithelial-mesenchymal transition (EMT) and impairment of invasive capacity [31, 34, 71-73]. The role of SIRT1 in EMT regulation was linked to SIRT1 partnering with TWIST and MBD1 forming a complex on the E-Cadherin promoter and reducing E-Cadherin transcription [74].

Treatment with chemotherapeutic agents by itself increases SIRT1 expression in PDAC cells, contributing to chemoresistance [45]. It was shown that ectopic SIRT1 over-expression in cancer cells induces chemoresistance due to P-glycoprotein expression [75] which could potentially be a mechanism for the SIRT1-mediated drug resistance in pancreatic cancer. SIRT1 inhibitors sensitize the PDAC cells to the commonly used drug gemcitabine [45, 71, 76] and to the EGFR inhibitor gefitinib [35]. The fact that a variety of SIRT1 inhibitors have been found to be effective in PDAC cells, *e.g.* EX527 / Selisistat [45], Cambinol [35], Tenovin-6 [31], Sirtinol [76], Capsaine [77] and the natural inhibitor Nicotinamide [71] strengthens the observations of its potential as a druggable target. As an alternative to the small molecule inhibitors, natural inhibition of SIRT1 expression by restoration of miRNAs is also pursued for therapy [72]. Deng *et al*. showed that over-expression of TGF-beta1 in PDAC cell lines triggers the deregulation of the miR-217–SIRT1 pathway, which promotes EMT [72]. Applications of this miR-217 and a series of other miRNAs that have been reported to regulate SIRT1 expression in non-pancreatic cancer cell types (for instance miR204 in gastric cells [78], miR34a in meduloblastoma [79]) can be further investigated.

The above studies testing SIRT1 inhibitors were limited to *in vitro* assessments or to examinations of xenografts of PDAC cell lines [34, 76]. Validation of the effects in genetically engineered models of PDAC and in patient-derived xenografts is the next step for clinical translation. Interestingly Selisistat, a SIRT1 inhibitor, showed absence of overt toxicity in the mouse at dose levels up to 100 mg/kg [80]. In humans, very recent results of clinical trials have shown that Selisistat was safe and well tolerated by healthy subjects [81, 82]. It could eventually be evaluated whether the expression of SIRT1 on its own, in combination with DBC1 and/or the mutational status of *TP53* are useful biomarkers of response. Further evaluation of the safety of SIRT1 inhibitors is warranted, especially since SIRT1 affects such a large variety of substrates and may have opposite effects in wild type versus mutant *TP53* tumors. Together this knowledge will inform whether patients can be treated in a personalized fashion with the promising SIRT1 inhibitors that can tackle several tumor-promoting mechanisms at once.

CONFLICT OF INTEREST

The authors do not have any conflict of interest to declare. AP and IR are fellows of the Cancer Institute NSW.

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