Alternating pH landscapes shape epithelial cancer initiation and progression: Focus on pancreatic cancer

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We present here the hypothesis that the unique microenvironmental pH landscape of acid-base transporting epithelia is an important factor in development of epithelial cancers, by rendering the epithelial and stromal cells pre-adapted to the heterogeneous extracellular pH (pH$_e$) in the tumor microenvironment. Cells residing in organs with net acid-base transporting epithelia such as the pancreatic ductal and gastric epithelia are exposed to very different, temporally highly variable pH$_e$ values apically and basolaterally. This translates into spatially and temporally non-uniform intracellular pH (pH$_i$) patterns. Disturbed pH$_e$- and pH$_i$-homeostasis contributes to essentially all hallmarks of cancer. Our hypothesis, that the physiological pH$_e$ microenvironment in acid-base secreting epithelia shapes cancers arising in these tissues, can be tested using novel imaging tools. The acidic tumor pH$_e$ in turn might be exploited therapeutically. Pancreatic cancers are used as our prime example, but we propose that this concept is also relevant for other cancers of acid-base transporting epithelia.

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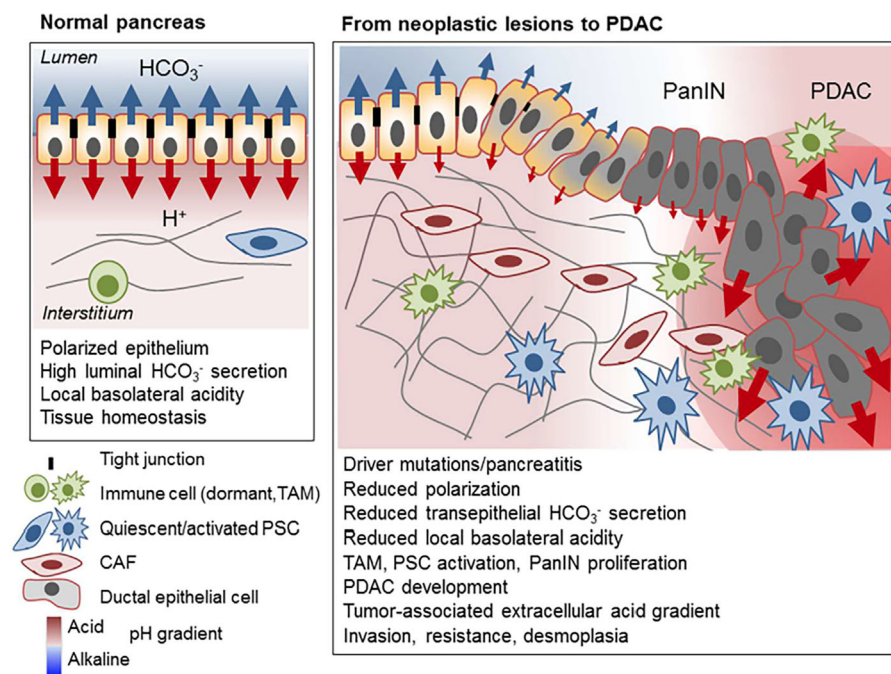Luis A. Pardo
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Abbreviations:
ADM, acino-ductal metaplasia; **ASIC,** acid-sensing ion channel; **CF,** cystic fibrosis; **CFTR,** cystic fibrosis transmembrane conductance regulator; **CP,** chronic pancreatitis; **ECM,** extracellular matrix; **EGFR,** epidermal growth factor receptor; **EMT,** epithelial to mesenchymal transition; **EPR,** enhanced permeability and retention; **GEMM,** genetically engineered mouse model; **MR,** magnetic resonance; **NIRF,** near-infrared fluorescence; **PDAC,** pancreatic ductal adenocarcinoma; **pH$_e$,** extracellular pH; **pH$_i$,** intracellular pH; **pHLIP,** pH (low) insertion peptide; **TGFβ,** transforming growth factor β; **V$_m$,** membrane potential.

Introduction and hypothesis

Epithelia performing net transepithelial acid-base transport share the remarkable physiological premise that the apical and basolateral surfaces of the epithelial cells generate – and are exposed to – very different extracellular pH (pH$_e$) values. In epithelia with periodic secretion or absorption patterns (as in the digestive system), these pH$_e$ values furthermore undergo temporal changes. Prominent examples include the gastric epithelium and the pancreatic ductal epithelium [1, 2]. Because changes in pH$_e$ elicit qualitatively similar changes in intracellular pH (pH$_i$) in most cells [3], and pH$_i$ gradients have been demonstrated in a range of cell types [4, 5], epithelial cells in such tissues can moreover be predicted to exhibit non-uniform spatial and temporal pH$_i$ patterns. Finally, basolateral pH$_e$ conditions will affect not only the epithelial cells, but all cell types residing in the interstitium, including fibroblasts, endothelial cells and immune cells. Here, we propose the hypothesis that this cyclically variable pH$_e$ microenvironment profoundly impacts the development of epithelial cancers (Fig. 1), and we present suggestions for how this hypothesis can be tested. We propose that this unique environment might be exploited therapeutically for cancers arising in these tissues, and we discuss avenues to explore this. Because of the

Hypotheses

Figure 1. Hypothesis. The figure illustrates the net transport of acid (red) and base (blue) across the normal (left, light grays) and transformed (right, dark gray) pancreatic ductal epithelium, and the uniquely acidic pancreatic interstitium. We propose that the acid milieu and its dynamic changes may act as a double-edged sword during PDAC development. On the one hand they retain premalignant lesions in a dormant state. On the other hand they promote rapid PDAC progression, once the tumor cells have acquired their fully transformed state. See text for details.

Pancreatic cancer development involves the interplay between driver mutations and a complex tumor microenvironment

Pancreatic ductal adenocarcinoma (PDAC) has a 5-year mortality rate that nearly equals its incidence, and is predicted to be the second leading cause of cancer-related death in the US in a decade [9]. Current therapeutic schemes only marginally prolong survival. The major risk factors for development of PDAC are believed to be chronic pancreatitis (CP), obesity, smoking, diabetes, and aging [9, 10]. PDAC has recently been subclassified into three molecular subtypes [11, 12]. The prevalent model is that PDAC development occurs through a consecutive series of mutations, usually starting with *KRAS*, and later involving mutations in *TP53*, *SMAD4*, and *CDKN2A* [10, 13]. Recent work suggests that the disease may at least sometimes have a more catastrophic etiology, in which chromotripsis elicits multiple simultaneous mutations [14]. Considerable debate remains about the cellular origin and molecular subtypes of PDAC [11–13, 15]. However, it is widely held that early stages of pancreatic cancer, the so-called intraepithelial neoplasias (PanIN-1, -2, and -3) can be dormant for decades and may never develop to full-blown disease [10, 16]. An alternative view to the ductal origin is that acinar cells may give rise to pancreatic cancer through acinar-to-ductal metaplasia (ADM; [17]) or, rarely, through cystic malformations such as Intraductal Papillary Mucinous Neoplasms (IPMN) and mucinous cystic neoplasms (MCNs) [17, 18]. Here we will focus primarily on the "classical" model of progression from PanINs, from which the vast majority of PDACs emerge [17]. A characteristic feature of PDAC is a very dense, poorly vascularized microenvironment, with stromal cells such as pancreatic stellate cells (PSCs) and extracellular matrix (ECM) constituting the majority of the tumor mass [19]. It is extensively demonstrated for other cancers that tumors exhibit pronounced extracellular acidity [20–22]. The few available studies suggest a similar pattern for PDAC [23, 24].

unusual pH environment and strong susceptibility to mutations in the pancreas, pancreatic cancers are used as an example, but we are persuaded that the concept is relevant for most, if not all, cancers of acid-base transporting epithelia.
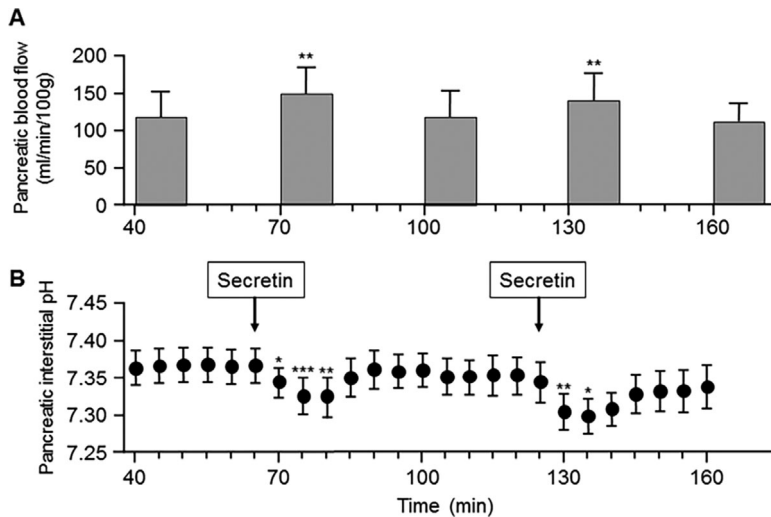
Cells in acid-base secreting epithelia exist in a challenging pH environment

Net HCO_3^--secreting epithelia such as pancreatic ducts will release acid basolaterally, and hence will create acidic conditions in the basolateral interstitium during active secretion, as demonstrated in elegant in vivo measurements in anesthetized cats [1, 2] (Fig. 2). Conversely, acid secreting epithelial cells such as the parietal cells of the gastric epithelium will extrude HCO_3^- basolaterally and thus,

create alkaline basolateral conditions [6]. The latter is reflected in an alkaline change in blood pH, termed the alkaline tide [6, 7]. For the pancreas, the opposite phenomenon, an acid tide reflecting pancreatic interstitial acidosis, has been proposed [8]. It is important to note that the secreted HCO_3^- ions and protons are generated in a 1:1 stoichiometry by the interconversion of CO_2 and water $(CO_2 + H_2O \Leftrightarrow H^+ + HCO_3^-)$ catalyzed by carbonic anhydrases. Thus, reciprocal apical and basolateral acid/base fluxes are of about equal magnitude. The extent and duration of the interstitial pH changes will depend on the one hand on the amount and duration of acid/base secretion by epithelia, which are stimulated by hormones, neurotransmitters, and local agonists following food intake. On the other hand, proton buffering capacity, local blood flow, diffusional fluxes, and the geometry of the interstitium also govern pH_e dynamics.

Figure 2. Effects of secretin on pancreatic blood flow and interstitial pH in anesthetized cats. Secretin (2 IU/kg) was given intravenously and pancreatic blood flow (A) and pancreatic interstitial pH (B) were recorded at 30 and 5 min intervals, respectively. As seen, secretin infusion elicited a transient increase in blood flow and a transient decrease in interstitial pH. $^{*}p < 0.05$; $^{**}p < 0.01$, and $^{***}p < 0.001$, compared to baseline. Redrawn from [1] with permission.

Luminal and basolateral pH_e are altered in the diseased pancreas

The normal healthy pancreas produces an alkaline juice because of the extraordinarily high ductal HCO_3^- secretion that is to some extent species-dependent [2]. One of the major tasks of the HCO_3^--rich pancreatic juice is the neutralization of the acid chyme arriving from the stomach. In humans the secretin-stimulated secretion can lead to luminal HCO_3^- concentrations of up to 150 mmol/l and pH values of 8–8.5. Alkaline secretion drives a parallel acidification of the interstitium as documented in several older studies [1, 25] (Fig. 2). Thus, under normal conditions (alternating between the resting and digestive stage) the pancreatic duct epithelial cells reside in a remarkable and changing pH_e gradient brought about by themselves through hormone/agonist-induced secretion: from modestly to very alkaline on the apical side, and from normal to acidic on the basolateral side. The smallest pancreatic ducts most proximal to acini possess ion transporters and enzymes necessary for HCO_3^- secretion, while larger more distal ducts have lower secretory function but can perform HCO_3^-/Cl^- exchange [2]. Thus, there is also pH_e heterogeneity between juxta-
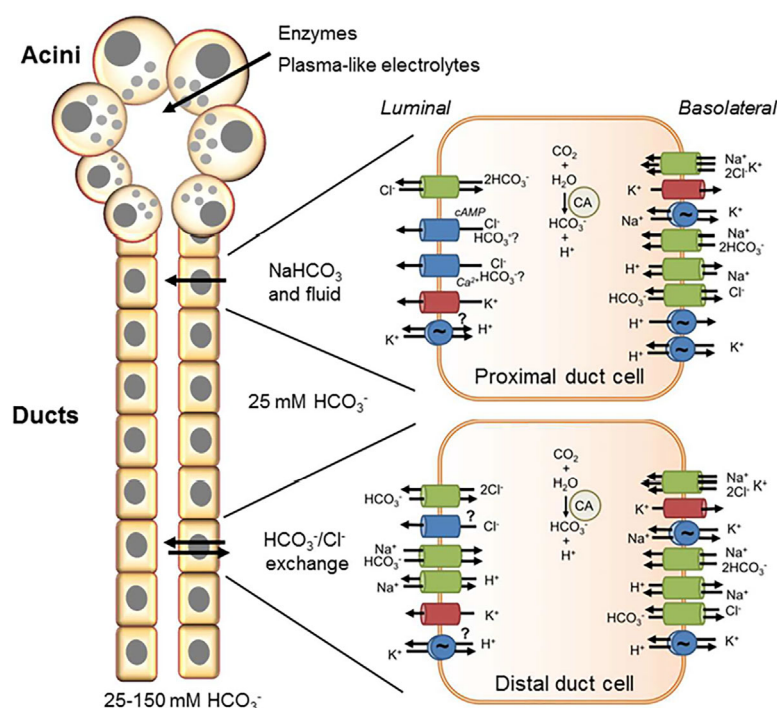
acinar and distal parts of the duct system. Figure 3 illustrates this difference and summarizes the current concept of ductal HCO_3^- secretion. Importantly, also the neighboring acini and stromal cells will experience variations in pH_e. In marked contrast to the ductal cells, the pancreatic acini produce an enzyme-rich fluid that is plasma-like in composition and relatively neutral in pH under physiological conditions (reviewed in [2]). Nonetheless, pH_i regulation of pancreatic acinar cells is crucial for avoiding a disruption of zymogen granules [26] and thus for cellular integrity. Taken together, the spatially and temporally highly heterogenous pH_e landscape in the pancreas will translate into a diverse and varying pH_i landscape, in a manner dependent on the interplay between local pH_e conditions and the specific pH_i-regulatory and -buffering capacity of the cells. Further, it can be predicted that these conditions will be most pronounced in humans and other meat-eaters that have well developed duct morphology and HCO_3^- secretory systems (reviewed in [2]; for further discussion of this topic, see Conclusions and outlook section).

Disease conditions in the pancreas can profoundly alter this pH_e landscape. For instance, in cystic fibrosis (CF), luminal HCO_3^- secretion is reduced, at least in part due to the defective function of the apical cystic fibrosis

transmembrane conductance regulator (CFTR) channel [27, 28]. This would predictably lead to reduced acidification of the interstitium if the blood flow remained normal. To our knowledge, thorough measurements addressing this question have not been performed in models of the CF pancreas, and the net outcome in terms of interstitial pH is difficult to anticipate due to the additional impact of CF mutations on acinar and gastric secretion, inflammation, and other stressors (for a discussion, see [28]). During CP, HCO_3^- secretion is also reduced [29, 30]. It has been shown in a feline model of CP that interstitial pH still decreases with secretion during CP, but from a much more acidic baseline (around pH 7.25 compared to 7.4 in the healthy cat) [31, 32] (Fig. 4). The increased basal acidity at least in part reflects the inflammation and reduced perfusion associated with this condition [31, 32]. Similar to the acidification observed in the feline models, human CP patients exhibit an interstitial pH of about 7, compared to 7.25 in non-CP controls [32].

PDAC development is associated with early impairment of epithelial polarity (e.g. following stimulation with stromal transforming growth factor β [TGFβ], leading to epithelial to mesenchymal transition [EMT]) [33]. This is predicted to reduce the pH transients and render the interstitium less acidic due to deficiency of epithelial barrier function and reduced transepithelial HCO_3^- secretion. To our knowledge, however, these parameters have yet to be measured carefully under controlled conditions, so that pH_e and pH_i changes can be followed in real time (see section, How can the hypothesis be experimentally tested?). What is clear is that once the cancer has fully developed, it is characterized by the development of a dense, hypoxic, and, as far as available evidence suggests, acidic stroma [19, 23, 24]. As in other cancers [21, 22], the precise conditions will vary between regions of the tumor, and over time with tumor growth, but pH_e dynamics within the fully developed cancer are predicted to exhibit a much lower temporal variability than in the normal organ that is periodically alternating between digestive and interdigestive phases.

Hypotheses

Figure 3. Simplified model of pancreatic HCO_3^- secretion. The model shows a schematic of the pancreatic duct, and outlines the main acid/base transporters in the luminal and basolateral membranes of a proximal duct cell (mediating net HCO_3^- secretion) and a distal duct cell (mediating Cl^-/HCO_3^- exchange). For further details on the mechanism of secretion, see [2].

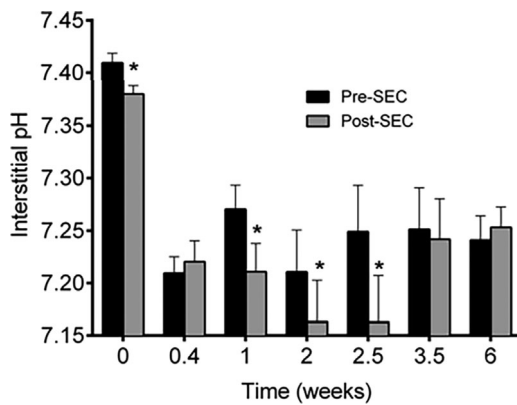## Numerous pH-dependent processes shape cell function and cancer development

On the basis of the knowledge summarized in the previous sections, it has become clear that pancreatic epithelial cells create and exist in a unique and dynamic pH environment that also affects the surrounding stromal cells. Pancreatic epithelial cell polarization, metabolism, and the expression pattern of plasma membrane ion transporters, channels, and receptors are tuned to this characteristic pH_e landscape. We propose that once one or several driver mutation(s) trigger(s) transformation of the ductal epithelial cells, the combination of the oncogenic mutation(s) with the inherent ability of these cells to withstand and even benefit from adverse and variable pH conditions makes the cancer cells extremely aggressive (Fig. 1). Thus, PDAC cells do not have to "learn" how to cope with the heterogeneous, acidic tumor microenvironment – this is their intrinsic capacity. Well-

established hallmark properties of cancer cells, such as increased proliferation and metabolism, invasiveness, and resistance to cell death, are all dependent on altered expression of ion channels and transporters, and are all sensitive to pH_e and pH_i. Only a few studies to date have directly addressed this in pancreatic cancers, but pertinent examples, drawn mostly from studies in other cancers, are numerous.

Starting with membrane-localized proteins, the importance of pH in modifying electrical properties of cells is well established and in large part reflects a profound pH_i- and/or pH_e sensitivity of many K^+ channels [34, 35]. For instance, acidic pH_e slows the activation of $K_V10.1$ [36], and low pH_i reduces its current amplitude [37]. The two-pore K^+ channels TASK-2 ($K_{2P}5.1$), TALK-1 ($K_{2P}16.1$), and TALK-2 ($K_{2P}17.1$) are activated by extracellular alkalinization, and have reduced activity in acidic pH_e [38, 39]. Another family member, TREK-1 ($K_{2P}2.1$) is inhibited by extracellular acidity in PDAC cells, leading to significant changes of the membrane potential (V_m) [40], in turn

regulating Ca^{2+} influx and hence proliferation [41]. Several TRP channels, including TRPV1 and TRPA1, are activated by extracellular acid (for a review, see [42]) and their activation has been implicated in the pain associated with development of CP [43]. Acid-sensing ion channels (ASICs) and related channels are activated by extracellular acidity [44, 45]. Hormone- and growth factor receptor signaling is pH_e sensitive, including that of the important PDAC oncogene, the epidermal growth factor receptor (EGFR) [46]. Last but not least are, the bona fide acid-sensing receptors, that is, the G-protein-coupled receptors OGR1, GPR4, G2A, and TDAG8, which link pH_e to cellular cAMP and Ca^{2+} signaling [47, 48]. Also CO_2/HCO_3^- was recently reported to be directly sensed, via the transmembrane protein RPTPγ [49]. Acidic pH_e is important for driving the processing of cytokines and other signaling ligands in the tumor microenvironment, including the release of the major PDAC cytokine, TGFβ, from its latent form [50]. Finally, expression of the major candidate driver for ADM (*SOX9*) [51] has been described as pH_e-dependent in some cell types [52].

In turn therefore, pH_e will profoundly impact numerous cellular processes, either via the mechanisms listed above, or via corresponding changes in pH_i [3, 53]. These changes can be rapid, as the acidosis-mediated Ca^{2+} signaling [54] or V_m changes, or can involve slower adaptive events. In most cases, it has not been mechanistically dissected whether the trigger of a given downstream event was pH_e or pH_i (or both) and the signaling mechanisms involved are generally incompletely understood. However, numerous events that potentially favor cancer development are affected by pH_i/pH_e. For example, low pH_e: (i) leads to overexpression of LAMP2 in the plasma membrane, serving to protect the cells from acidotic stress [55]; (ii) favors histone deacetylation [56]; (iii) induces preferential extension of short telomeres [57]; (iv) induces a reprogramming of cellular metabolism that reduces oxidative stress [58]; (v) induces chronic autophagy [59]; (vi) favors uptake of nutrients such as lipids from the microenvironment [60], (vii) favors EMT [61]; and

Figure 4. Consequences of chronic pancreatitis for pancreatic interstitial pH and pancreatic microvascular blood flow. Pancreatic interstitial pH was followed in a feline model of chronic pancreatitis (CP) induced by duct narrowing. Time 0 is prior to operation and week 6 is full-blown CP. Secretin (SEC, 2 U/kg, intravenously) infusion lowers interstitial pH both in the normal pancreas and after CP induction (until severe stages), but from a much more acidic baseline. Redrawn from [32] with permission.

(viii) invasiveness [62]. Long-term exposure to acidic pH_e/pH_i also increases the capacity of the cells for acid extrusion [63], as shown for the Na^+/H^+ exchanger NHE1 and the Na^+, HCO_3^- cotransporter NBCn1 in other contexts than cancer [64, 65]. An acute reduction of pH_e is on the other hand, inhibitory for both of these transporters due to binding site competition, which would be expected to negatively regulate their activity in very acidic tumor regions [63, 66]. Acidic pH_e has also been reported to be clastogenic and cause double-strand breaks, conceivably further favoring genetic instability [67–69]. Conversely, an increased pH_i [70, 71] or exposure to increased alkalinity [72, 73] is sufficient to elicit transformation, and alkaline pH_i is a well-accepted growth stimulus [74–76]. While the above cited studies were largely performed with tumor cells, there is increasing evidence that stromal cells also respond to the ambient acid-base homeostasis. Thus, NHE1 is involved in activating hepatic stellate cells and its inhibition or genetic deletion attenuates liver fibrosis [77]. Unpublished work from the authors' laboratories on PSCs revealed that metabolic reprogramming by hypoxia is followed by altered carbonic anhydrase expression and increased H^+ production leading to an acidification of pH_e. Similarly, immune cell function critically depends on pH. However, due to space limitations we refer the reader to recent reviews on the pH sensitivity of the immune response (see e.g. [78]).

Temporal and spatial dynamics of pH shape pancreatic cancer initiation and progression

Collectively, thus, a plethora of studies on various cancer models seems to support the notion that the acid-base challenges in the epithelial microenvironment of the pancreas can facilitate/are linked with cancer development. Additionally, and less studied in the cancer setting, these challenging conditions encountered during the normal function of the organ are likely to function in a manner analogous to the acid preconditioning phenomenon known to reduce damage to cardiac and endothelial cells under reperfusion after ischemic conditions, by upregulation of proteins and signaling pathways contributing to increased net survival [79, 80]. With the important difference of course that, in the context of pancreatic cancer, such preconditioning would increase the survival potential (the evolutionary fitness) of the cancer cells [13, 81].

Thus, again using PDAC as our example for epithelial cancer, we envision the following sequence of interstitial pH_e patterns during the transition from a normal pancreatic epithelium to full-blown PDAC (Fig. 1). Under normal conditions with alternating phases of digestive stimulation of secretion and resting phases, the interstitial pH_e varies in a cyclic manner, reaching relatively acidic values, especially close to the basolateral epithelial cell membranes. At onset of PDAC either (i) a reduced extracellular acidosis develops as transepithelial HCO_3^- secretion is presumably reduced with reduced epithelial integrity; or (ii) if induced by CP or other inflammatory conditions, a phase of moderately increased and sustained extracellular acidity will prevail, still with cyclic changes until very severe CP is reached. Finally, a strongly increased and sustained extracellular acidosis, with attenuated feeding-associated fluctuations, will dominate with progressing dysplasia and eventually tumor growth. The precise pattern and magnitude of these changes are currently unknown, and will need to be carefully assessed using for example intravital fluorescence imaging in combination with pH sensing probes (see How can the hypothesis be experimentally tested?).

We propose that the growth, survival, invasive, and metastatic properties of PDAC derive from the interplay between this dynamic pH_e landscape and its disruption during pre-neoplastic stages. The developing hypoxia in the tumor region combined with the onset of PDAC-associated mutations can further increment acid extrusion capacity and facilitate growth in the increasingly hostile tumor microenvironment. The intermittent acidosis in the normal pancreatic interstitium may contribute to the dormancy of the PanIN lesions, but at the same time "precondition" the cells to favor proliferation, survival, migration, formation of metastasis, and thereby malignancy in later stages. PDAC driver mutations, which increase net acid extrusion capacity and/or alter the pH threshold for proliferation and migration, will increase the risk that the pre-neoplastic lesions progress to malignant cancer, rather than remaining less aggressive or even going into oncogene-induced senescence or dying.

Thus, the intermittent interstitial acidity surrounding the normal pancreatic epithelium with early neoplastic lesions and the interstitial acidity within the PDAC tumor microenvironment may

be viewed as a double-edged sword. On the one hand, it prevents the progression of pre-neoplastic lesions to fully developed PDAC, but on the other hand, it accelerates the progression of a fully developed PDAC by providing an environment that favors the aggressive traits of cancer cells. Finally, the prediction will also be that cells exposed to different pH values in the developing tumor may develop different properties, with for example, highly acid-adapted cells being the main culprits of local invasiveness.

How can the hypothesis be experimentally tested?

Can pancreatic cancer pH dynamics be detected in vivo?

In vivo measurement of the precise pH values in and surrounding the pancreatic epithelial cells is challenging and requires high spatial resolution, and therefore, cutting edge in vivo imaging technology. Local pH_e at the basolateral side of the epithelium is likely to change much more dramatically than that measured in bulk interstitium and in blood [1, 25, 32] (Figs. 1 and 2). Thus, testing the hypothesis requires direct measurements of the local and global interstitial pH_e, as well as pH_i, in the normal pancreas and during disease development. The testing of the hypothesis therefore, necessitates advanced intravital fluorescence imaging combined with expressed or injected pH sensing probes and targeting strategies for delivery to the desired compartment (basolateral or luminal membrane, intra- or, extracellular compartment). Numerous imaging modalities have been tested in the context of pancreatic cancer [82, 83]. For well-developed tumors, non-invasive magnetic resonance (MR) based techniques are well suited [22]. However, for the best resolution of the pH landscape during normal to PDAC development, fluorescence-based intravital imaging methods are preferred. In terminal acute experiments, the pancreas can be exposed and imaged with multiphoton microscopy. However, the pancreas is not easily amenable to non-invasive observations. A common approach is to implant the cells in more easily accessible places such as subcutaneously, in a kidney

capsule or in the eyes and observe through an imaging window – this can be done longitudinally up to 2 weeks by repeated multiphoton microscopy [84, 85]. However, procedures for abdominal chambers have been published and long-term observation of pancreas and cancer development is likely to be possible [86].

Combining mouse PDAC models with genetically engineered pH sensor proteins

Murine PDAC models will be central to the testing of the hypothesis. However, it has to be kept in mind that because of marked differences in feeding patterns and the organ physiology of the two species, including major differences in secretion rates, the mouse pancreas is not an ideal model for that of humans. Both transgenic PDAC models [87] and orthotopic PDAC models will be useful. The latter should preferably be syngeneic with an intact immune system or, ideally, humanized by using highly immunodeficient mice, in which various types of human tumor cells and the human immune system are engrafted [88, 89]. The ultimate aim is to be able to monitor pH in the normal pancreas and during the transition to fully developed PDAC. One approach to this would be to generate a transgenic mouse with pancreas epithelial cell-specific expression of SE-pHluorin-mCherry [90] that allows ratiometric determination of pH_i in combination with Hoechst 33342, and should be compatible with two-photon imaging [91]. This mouse could be crossed with a genetically engineered PDAC mouse model (GEMM) to allow ratiometric pH_i imaging before, during and after PDAC development, or used for orthotopic murine PDAC cell injection. Analogously, in vivo analysis of pH_e could involve the transgenic expression of a pH_e reporter under a pancreatic epithelial cell promoter, although a suitable reporter is, to our knowledge, not yet available. It would be ideal to specifically monitor pH_e immediately exterior to the luminal and basolateral membranes. This could conceivably be done by making a chimeric protein in which the sensor contains luminal or basolateral targeting signals [92, 93], or is coupled to a basolaterally or luminally

located transmembrane protein. It will furthermore be advantageous if such probes are as narrow in spectrum as possible, to be compatible with simultaneous assessment of, for example, hypoxia.

Use of injected imaging probes for in vivo measurements of pancreatic and PDAC pH_i and pH_e

A number of injectable imaging probes are available that could conceivably be employed for in vivo pH_i and pH_e measurements in the mouse pancreas. One option would be to target a suitable pH_e sensor, such as the phospholipid N-(Fluorescein-5-Thiocarbamoyl)-1,2-Dihexadecanoyl-sn-Glycero-3-Phospho ethanol-amine (Fluorescein-DHPE) [94], to either an endogenous receptor in the basolateral membrane of pancreatic epithelial cells, or to an engineered, transgenetically expressed receptor in this location. The tumor stroma could be targeted by linking a pH_e sensor to antibodies that specifically recognize characteristic constituents of its extracellular matrix. A promising example is an antibody against a tumor-specific splice variant of fibronectin [95] that has been tested successfully for site-directed pharmacodelivery [96]. Pericellular pH under very acidic conditions can be imaged in vivo using, for example, the novel pH (Low) Insertion Peptide (pHLIP) probes, which can be conjugated to near-infrared fluorescent (NIRF) dyes and injected via the tail vein. The pHLIP peptides are inserted into cell membranes, allowing the formation of a transmembrane helix, only in an acidic microenvironment [97]. These probes have successfully been implemented in a variety of preclinical cancer studies, including PDAC mouse models [23, 24]. Recently, novel pH sensing probes have been reported, one hemicyanine based high resolution ratiometric NIRF probe [98] and nanoparticle-based activable photoacoustic probes with amplified brightness for in vivo imaging of pH [99].

Measurements such as those described above will establish the pH_i/pH_e landscape in the pancreas and during PDAC development. Given that secretin is a major agonist for HCO_3^- secretion, it

will also be relevant to test the prediction that reduced gastric acidity, and thus, reduced secretin release and pancreatic HCO_3^- secretion, will lead to reduced basolateral acidity and reduced PDAC development. This could be done in mouse models by knockout of the secretin receptor or reducing secretin release by limiting gastric acidity by cimetidine, since, for example, in humans, no secretin is released at duodenal pH values above 5 [100].

In vitro studies can complement in vivo observations

While the pivotal evidence for this hypothesis will come from in vivo studies, several aspects can be addressed ex vivo or in vitro (and in some cases can still only be addressed in vitro). As supplement to the in vivo pancreas studies, it is possible to excise pancreas lobules from normal and cancer model mice and perform two-photon imaging in suitable chambers for a limited time period. The hypothesis that intermittent acidic exposure of the normal pancreatic epithelial (and stromal) cells on the one hand reduces their proliferative capacity, but on the other hand increases the "fitness" of these cells during subsequent transformed growth, can be tested by exposing them to cyclic changes in pH corresponding to those measured in vivo. This could in principle be done in a simple monolayer system grown on filter support as a proxy for the ductal epithelium. Then the cells could be exposed to media of the relevant alkaline (apical compartment) and acidic (basolateral compartment) pH values, respectively. Furthermore, PDAC driver mutations could be added to assess the collective impact of acid preconditioning and these mutations. However, a constraint is the lack of ideal models for normal human pancreatic epithelial cells which can grow under such conditions. The most widely used cell line, the H6c7 also called HPDE, is HPV-16 E6/E7 transformed and consequently lacks p53; for a discussion and references see [101]). Thus, these experiments may be best done in an organotypic setting, such as the progressive organotypic models recently developed by the Tuveson group, which grow in 3D culture [102]. One can subject such "preconditioned" cells to a series of analyses. Functional measurements could

assess proliferation, invasiveness, and chemotherapy resistance or tumorigenic potential in xenograft models. Accompanying transcriptomic/proteomic analyses could evaluate expression changes induced by these conditions. Furthermore, one could exploit the progressive nature of the Tuveson series of pancreatic epithelial cells from the normal to the malignant stage [102] to make precise measurements of the pH_e microenvironment created by the cells, using well-established pH probes for this purpose and models of interstitial space/confined microenvironment, in order to validate and contrast/compare with the more complex system of intravital measurements.

Perspectives for management of PDAC and other epithelial cancers

The knowledge about pH dynamics in the development and progression of PDAC opens a window of opportunity to improve its management. Manipulation of the ionic environment in PDAC, for instance by interfering with specific acid-base transporters, may restore the malignancy-inhibitory milieu and fight immunologic escape in this particular tumor type in early stages. On the other hand, it also raises alarms for more advanced tumors. If intermittent acidity of interstitial pH [103] is an important factor for PDAC development and progression, many therapeutic paradigms currently under development must be adapted and modified to tackle this feature.

It is well established that the interstitial pH of the tumor microenvironment shapes the behavior of the tumor and thereby plays a relevant role in therapeutic response [104]. In the absence of any other considerations, an acidic environment will affect the behavior of weakly basic drugs, which undergo ionization so that cellular drug uptake and therapeutic efficacy may be reduced. This is the case for doxorubicin and mitoxantrone, and for the zwitterionic paclitaxel. Additionally, low pH can also reduce therapy efficacy through less direct mechanisms, such as by impacting the activity of p-glycoprotein resulting in a multi-drug resistance phenotype [105]. It also affects the behavior of cellular elements

of the tumor stroma, such as lymphocytes (participating in immunological anergy, [106]) and macrophages (that acquire a "maintenance" M2 phenotype) [107], enhances resistance to radiation [108], and can induce stemness maintenance at least in some tumor types [109]. A widely studied approach to altering the tumor acidity is to interfere with acid extrusion from tumor cells (see e.g. [110]). More recently, it has been proposed that manipulating tumor pH by administering systemic buffers such as HCO_3^- could have therapeutic effects [111]. However, studies scrutinizing such treatments for potential toxicity issues in patients, such as metabolic alkalosis and other electrolyte imbalances are needed [112].

Another active research area focuses on design of nanocarriers that exploit pH at the tumor site to target the tumor and release its chemotherapeutic cargo. Modern nanotechnology allows the production of highly sophisticated nanocarriers that undergo several steps of change on their route to the tumor cell cytoplasm, as they progress from the interstitium to the endosome and the lysosome with the progressive change in pH. Nanoparticles are enriched in the tumor area through a combination of increased extravasation due to enhanced vascular permeability and reduced clearance through the loss of lymphoid filtering (Enhanced Permeability and Retention effect, EPR) [113]. Once in the vicinity of the tumor cells, the acidic pH is used to trigger release of a cytotoxic drug. The simplest approach to achieve local release is the direct binding of a drug to a polymeric nanocarrier through acid-cleavable bonds or groups responsive to protonation. More elaborate formulations include stimuli responsive nanocarriers [114] that release their cargo in response to acidification while being susceptible to functionalization through for example, specific ligands to target the desired site of action, or agents aimed to reduce vascular permeability and improve retention [115]. Examples are synthetic polymer or chitosan-based nanogels [116], which swell in an acidic environment and release the cytotoxic compound [115]. pH sensitive polymers have also been used to direct oncolytic viruses [117] to the tumor site. In

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addition to polymeric carriers, inorganic nanomaterials that undergo acidic dissolution have been utilized for pH responsive drug delivery (e.g. zinc oxide and calcium carbonate [118]).

From this summary it is evident that multiple current strategies rely on an acidic environment to enhance therapeutic response. However, for acid secreting epithelia such as the stomach this would be hampered during the active digestive secreting periods because of the alkaline basolateral efflux. Conversely, in the particular case of the pancreas, the advantage of targeting acidic extracellular pH would be lower in interdigestive or fasting periods, with the concomitant loss of selectivity and risk of undesired toxicity. Thus, such therapeutics would have to be given in conjunction with a meal to trigger the basolateral acid efflux and acidification of the pancreatic stroma. In addition, some selectivity would be retained through EPR, for example, by pH-dependent size switching to trap the nanocarrier at the tumor site. A reversible delivery mechanism should be included to limit the release in the case the carrier leaves the tumor.

Conclusions and outlook

We have presented here a concept that aims to understand the characteristic development and features of PDAC, including long-dormant precursor lesions and sudden, aggressive disease progression, from the unique physiology of the host organ. The interstitium of the pancreas is exposed to highly dynamic temporal and spatial changes of acidity. Normal epithelial cells are trained to cope with acid challenge. Possibly, this unique interstitial acidity acts as a "brake" on the precursor lesions and also prevents the over-activation of pancreatic stellate cells in the absence of factors secreted by the tumor cells. However, once the balance has been tipped by carcinogen exposure or other triggers of PDAC driver mutation(s), PDAC cells are acid-adapted cells in an acidic environment that offers them a growth advantage and further triggers their aggressive behavior. Thus, the physiological intermittent acidity of the normal pancreas stroma "paves the way"

for PDAC growth and promotes its progression. The link between CP and PDAC development is clearly consistent with the hypothesis. This is also the case for the fact that a vegetarian diet, that can be expected to lead to less gastric acid and thereby less pancreatic HCO_3^- secretion, is associated with a reduced risk of developing pancreatic cancer [119], and that an increased risk of PDAC has been linked to increased gastric/pancreatic secretion [120]. Of course, these observations are only consistent with the pH-centered concept proposed in this review, and not unique to it, and future studies must provide the experimental evidence, for example, by directly showing the anticipated temporal and local pH_e dynamics and its effect on PDAC development. It is our firm expectation that the consideration of the normal organ physiology of the pancreas with its extraordinary pH dynamics will not only deepen the understanding of PDAC but also lead to the development of new or refined therapeutic strategies.

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