Chapter 1

The Molecular Frame of Pancreatic Carcinogenesis

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1. Introduction

Annually, approximately 43,140 people are diagnosed (incidence 10-12/100000) with pancreatic ductal adenocarcinoma (PDAC) in the United States and the mortality rate of 36800 almost equals this number [1]. PDAC ranks fourth on the list of cancer-related causes of death and despite extensive scientific and clinical effort, the prognosis of this exceptionally lethal disease has not improved significantly over the past decades [2]. Surgical resection, for which only a minority (less than 20%) of the patients qualify due to late diagnosis in advanced stages, is currently the only chance of cure, improving 5-year survival rates from <4% if left untreated to 20-30% after resection [3]. Unresectable tumors are characterized by early invasion and metastases as well as by an extreme chemoresistance. Despite subtle progress over the years in terms of therapeutic strategies in many malignancies, no major conventional treatment options have come forward from numerous clinical trials in pancreatic cancer.

Considering its bad prognosis much effort was put into revealing the hidden secrets of pancreatic cancer that explain the severity of this disease. Among the different fields of tumor biology in pancreatic cancer research, this chapter will focus on the morphological and molecular features that cause and accompany pancreatic carcinogenesis.

2. Morphological features of pancreatic carcinogenesis

Although there was little improvement in pancreatic cancer treatment during the past decades, much effort has been made in understanding the pathogenesis of pancreatic cancer. In contrast to its rapid progress after diagnosis, recent published data clearly show that the clonal

evolution of the earliest alterations in cancer initiating cells towards frankly invasive and metastasized PDAC takes at least more than a decade [4, 5]. This creates an important window of opportunity for early detection and much effort is put into attempts to map the molecular and morphological changes resulting in pancreatic cancer formation.

The current model of pancreatic carcinogenesis describes a stepwise process from healthy acinar cells to frank pancreatic adenocarcinoma: Recent lineage-tracing studies have shown that acinar cells harboring molecular alterations are induced to transdifferentiate, generating duct-like cells through a process known as acinar-to-ductal metaplasia (ADM) [6]. ADM lesions then convert to precancerous pancreatic intraepithelial neoplasia (PanIN) that progress to PDAC over time [7]. PanIN lesions are found in the smaller pancreatic ducts and are classified in four grades based on the degree of dysplasia reflected in the cytonuclear atypia and architectural change of the epithelial cell: PanIN-1A, -1B, -2 and -3 [7]. The lowest grade PanIN lesions can be flat (-1A) or papillary (-1B), but are characterized by absence of nuclear atypia and retained nuclear polarity. PanIN-2 lesions show micropapillary features with evidence of nuclear atypia and infrequent mitoses. PanIN-3 lesions demonstrate all hallmarks of cancer, including a widespread loss of polarity, nuclear atypia and frequent mitoses and are considered as *Carcinoma in situ* [1, 8]. Yet, the lesion is confined within the basement membrane and no invasive growth is present. The increasing grades of dysplasia in the various PanIN lesions manifest the morphological steps of tumor progression that precede invasive PDAC. These consecutive steps of tumor progression are accompanied by a cumulative occurrence of molecular alterations.

3. Molecular characteristics of pancreatic carcinogenesis

3.1. Genetic alterations in pancreatic carcinogenesis

For many decades pancreatic cancer was described as an exclusively genetic disease. In 2008 Jones and colleagues discovered 1561 somatic gene mutations within more than 20000 analyzed genes, yielding an average rate of 63 genetic abnormalities per pancreatic cancer, emphasizing the extreme complexity of this disease [9]. These genetic alterations can be clustered in 12 partially overlapping signaling pathways (compare Fig. 1). Five of the pathways comprise specific cellular functions: apoptosis, DNA-damage repair, G1/S phase cell cycle progression, cell-cell adhesion and invasion.

Apoptosis or programmed cell death, plays an essential role in carcinogenesis since resistance to apoptosis is a key factor of the survival of a cancer cell [1]. In PDAC, genes implicated in the apoptosis pathway (Bcl2, Mcl-1, p53, NF-kB among others) were found altered in all tumors studied and many reports document impaired apoptotic signaling in this disease [10, 11]. For example, a high fraction of apoptotic cells has been correlated with longer overall survival as well as absence of nodal involvement [12]. Moreover, resistance to chemotherapeutics is mostly a result of defective apoptosis pathways.

DNA damage control genes code for proteins that repair any damage that occurs in the cell during its lifespan and thus are responsible for safeguarding the integrity of DNA [1]. For

instance, the BRCA2 protein is involved in DNA damage repair, especially after occurrence of interstrand brakes [13]. Germline BRCA2 gene mutations are responsible for approximately 10% of familial pancreatic cancers [14]. Mismatch repair family (MMR) genes target base substitution mismatches as well as intersection deletions that arise as a result of errors occurring regularly during replication. Alterations in these mismatch repair genes lead to genetic instability and make the genome vulnerable for additional, more severe genetic alterations [15].

One of the most important and best studied proteins involved in DNA damage repair is the tumor suppressor protein p53. p53 is responsible for the cellular response to genotoxic stress as it mediates apoptosis and cell cycle arrest [16]. p53 is frequently disrupted in many human malignancies and the tumor suppressor is lost in 50-75% of PDACs [17].

Cell cycle regulation and progression is affected in virtually all transformed pancreatic cells. Enhanced activation of genes promoting G1/S-phase transition or loss of cell cycle inhibitors results in uncontrolled cell division which facilitates tumor progression and unrestrained tumor growth [1].

In normal pancreatic tissue, cells are anchored to each other and their surroundings via multiple connections. A decrease in these interactions can allow cells to detach from their surroundings and allows transformation, migration and metastasis. As such, cell to cell adhesion and interaction plays an important role in carcinogenesis [18, 19].

The other pathways discovered by Jones and colleagues which proofed to be frequently affected by genetic alterations in pancreatic cancer are signaling cascades that can be divided into three groups: embryonic signaling pathways, MAPKinase signaling pathways and TGFß-signaling pathways [9]. The transforming growth factor ß (TGFß) pathway has been linked to PDAC for many years. TGFß signaling is involved in a wide range of cellular processes including differentiation, proliferation, apoptosis and angiogenesis [20]. As discussed in detail later in this chapter, TGFß signaling functions as a double-edged sword as it comprises tumor-suppressive as well as oncogenic qualities.

All MAPK signaling pathways consist of the same basic kinase components. Stimulation of an upstream MAP2K kinase by growth factors, stress or other extracellular signals leads to phosphorylation of one of the terminal MAPK: extracellular signal-regulated kinase (Erk), c-Jun N-terminal kinase (JNK) or p38 [8]. These signaling cascades result in the activation of multiple oncogenic cellular functions.

One growth factor receptor responsible for many signaling events in early carcinogenesis is the Epidermal Growth Factor Receptor (EGFR). EGFR is located in the cell membrane and is activated by binding of its specific ligands, including epidermal growth factor (EGF) and Transforming Growth Factor alpha (TGFα) [6, 21]. Upon activation, EGFR undergoes dimerization, thus stimulating its intrinsic intracellular protein-tyrosine kinase activity resulting in autophosphorylation of several tyrosine residues in the C-terminal region of the receptor. This autophosphorylation elicits activation of numerous downstream kinases and signal transduction cascades that modulate cancer associated phenotypes as cell proliferation, migration and adhesion [6]. Recent work has proven a high impact of EGFR signaling on induction of

pancreatic metaplasia, and overexpression of the receptor already occurs in early pancreatic precursor lesions [6, 21]. The relevance of EGFR dependent signal cascades was emphasized by a therapeutic beneficial effect of the EGFR inhibitor Erlotinib in a subgroup of pancreatic cancer patients [22].

Since embryogenesis shares many characteristics with carcinogenesis, not surprisingly many embryonic pathways are involved in tumor development. The three embryonic pathways operative in pancreatic carcinogenesis are the Hedgehog-, Notch- and Wnt-signaling cascades [9]. Several studies have shown upregulation of these pathways during pancreatic carcinogenesis and in invasive pancreatic cancer and their inhibition results in decreased tumor proliferation and enhanced apoptosis [1]. For instance, activation of the Notch signaling pathway is involved in cell proliferation and angiogenesis in a variety of human cancers, including pancreatic cancer [23]. Notch signaling is initiated when Notch ligand binds to its receptor between adjacent cells. Upon activation, Notch is cleaved and releases the Notch intracellular domain (NICD) via a cascade of proteolytic enzymes including Y-secretase. Finally, NICD translocates into the nucleus and activates its target genes such as Hes-1, Hey-1, Cyclin D1 and cMyc [24]. Additional to its growth promoting functions accumulating evidence shows a molecular link between Notch and epithelial-to-mesenchymal transition (EMT) in pancreatic cancer [25]. During the EMT process, epithelial cells gain a mesenchymal phenotype accompanied by the cumulative expression of the mesenchymal markers Vimentin, Slug, Snail and ZEB1 and reduced expression of the epithelial marker E-cadherin. EMT-type cells harbor an increased migratory and invasive capacity resulting in invasion and spread of tumor cells even during early carcinogenesis [26]. Inhibition of Notch-signaling leads to reduction of EMT resulting in a better clinical outcome [25].

Similar to Notch-signaling, the hedgehog pathway belongs to the developmental programs of pancreatogenesis. The hedgehog gene was originally identified in Drosophila when a large-scale screening for mutations revealed an altered segmentation pattern of larvae, resulting in a short, fat larva covered in a “lawn” of denticles resembling a hedgehog [27]. Early in development, around embryonic day 8.5-9.0, the hedgehog ligands Indian Hedgehog (Ihh) and Sonic hedgehog (Shh) are expressed throughout the endodermal epithelium of the primitive gut but are noticeably absent in the developed organ [28]. Sonic hedgehog signaling is reactivated in the case of pancreatic regeneration, for example in response to inflammation-associated pancreatic injury [29]. Through inappropriate activation of these pathways, chronic injury might contribute to misdirection of tissue repair, ultimately resulting in neoplasia. Aberrant expression of members of the hedgehog-pathway in chronic pancreatitis and pancreatic carcinogenesis was first noted by Kayed and colleagues [30]. Subsequent research proved that the ligand Shh is expressed aberrantly in pancreatic cancer and its precursor lesions and that Shh functions as a mediator of cancer initiation and growth [31]. Mice with transgenic misexpression of Shh in the pancreatic endoderm develop lesions resembling PanIN, and hedgehog inhibition induces apoptosis and blockes proliferation in pancreatic cancer cells *in vivo* and *in vitro* [31]. Thus, hedgehog signaling can be described as an early and late mediator of pancreatic ductal adenocarcinoma.

Conceptually, these data suggest that pancreatic cancer is substantially a disease of pathways. But research into these pathways rendered clearly that these cascades must ultimately engage the function of epigenetic regulators to influence gene expression in a heritable manner. Thus studies into epigenetics in pancreatic cancer demonstrate a logical extension to the genetic paradigm of this malignant disease.

Signaling pathway	Affected genes
Apoptosis	p53, NF-ĸB, PI3K/Akt
DNA damage repair	p53, BRCA2, MMR-genes
G1/S transition	$p16^{Ink4a}$, $p14^{arf}$, $p15^{Ink4b}$, Cyclin D
Regulation of invasion	TGFß, Integrin signaling
Embryonic signaling	Notch, Hedgehog, Wnt
MAPK signaling	Erk, Jnk, p38
TGFß signaling	TGFß, Smad-proteins

Figure 1. The commonly altered signaling pathways in PDAC accompanied by affected genes from these pathways (adopted from [1]).

4. Epigenetic mechanisms in pancreatic carcinogenesis

Epigenetics are defined as any heritable genomic mechanism unrelated to changes in the DNA sequence [32]. Epigenetic modifications are involved in normal cellular development and maintenance, but they are also responsible for deregulation of gene expression, resulting in diseased cellular phenotypes. Most notably, deregulation of epigenetic mechanisms can contribute to cancer development [33-38]. The past years have witnessed an explosive increase in our knowledge about epigenetic features in pancreatic carcinogenesis. Several well-known epigenetic mechanisms are active in pancreatic cancer, sub-divided into DNA methylation, histone modification and microRNAs, all of them affecting the cell by induction or suppression of gene expression [39-42]. For instance, the introduction of genome-wide screening techniques has accelerated the discovery of a growing list of genes with abnormal methylation patterns in the transforming pancreatic epithelial cell that play a role in the neoplastic process [43]. Hypermethylation of promoter cytosine-phospho-guanine (CpG) islands is closely linked to gene silencing and loss of tumor suppressor function in many cancer entities [44]. Since the first detailed analysis of DNA hypermethylation in pancreatic cancer was reported in 1997 by Schutte et al., many tumor-suppressor or cancer-related genes that undergo aberrant methylation during pancreatic cancer development have been identified, including APC, RUNX3, SOCS-1, $p16^{Ink4a}$, Cyclin D2 and CHD13 [44, 45].

By influencing the structure of chromatin, in addition to DNA methylation, posttranslational modifications of histone tail residues highly affect the transcriptional activity of genes. While acetylation of histones is primarily associated with transcriptional activation, methylation of histones can lead to both, activation and repression, depending on the modified residue [46,

47]. For instance, Polycomb proteins, which are known for their crucial role in induction of repressive histone modifications, embody oncogenic properties in many human cancers. Polycomb proteins can be divided into two functional biochemical categories, Polycomb repressive complexes (PRC) 1 and 2. While members of the PRC 2 complex initiate gene repression by catalysation of H3K27 trimethylation, proteins belonging to PRC1 maintain the repressive state [48, 49]. Under physiological conditions, the activity of Polycomb proteins is crucial in development as well as in maintenance and proliferation of pluripotent progenitor cells in a variety of tissues. Overexpression of these proteins may promote tumorigenesis by fostering a self-renewing population of cells [50, 51]. Indeed, overexpression of Polycomb proteins is responsible for malignant progression and poor prognosis in breast [52], bladder [53] and prostate [54] cancer and shows strong association with hallmarks of cancer, including induced cellular proliferation [55], angiogenesis [56], survival [57] and migration [58]. Enhancer of Zeste Homolog 2 (EZH2) is the only PRC2 protein member thus far studied in pancreatic cancer. Strong nuclear accumulation of EZH2 was found in 55% of well differentiated tumors and 98% of poorly differentiated samples in a comprehensive immunohistochemical analysis of PDACs, indicating a significant correlation between EZH2 expression and dedifferentiation in pancreatic cancer [59]. Additionally, EZH2 overexpression participates in epithelial-to-mesenchymal transition (EMT) and invasion through repression of epithelial proteins like E-cadherin [60].

The third group among the epigenetic players in pancreatic carcinogenesis comprises the MicroRNA (miRNA) family, a class of small non-protein coding RNAs which participate in post-transcriptional control of gene expression in eukaryotic organisms [61]. In the last years, advanced global screening technologies have enabled large scale analyses of miRNA profiles in diverse tissue samples, indicating that miRNAs can function as either oncogenes or tumor suppressors in the development of various cancer types, including pancreatic cancer [62, 63]. The analysis of miRNA expression patterns has let to completely novel insights into pancreatic cancer biology. Specific miRNAs, such as the miR-200 family, miR-34a and miR-155 are involved in PDAC-biology by regulating genes associated with metastasis and cell stemness [64, 65].

The era of epigenetics in pancreatic cancer has emerged strongly within the last years and deepened our understanding of pancreatic cancer biology. One of the most important characteristics of epigenetic mechanisms which clearly demarcates them from genetics is their reversibility. This feature provides new targets for novel therapeutic interventions in pancreatic cancer and other epithelial tumors.

The manifold genetic and epigenetic events observed in pancreatic carcinogenesis mirror the complexity of this malignancy and lead to the assumption that targeting one molecular feature of pancreatic carcinogenesis is not sufficient for successful pancreatic cancer treatment. Though inaccessible for therapeutic options, there exists at least one molecular event found in virtually all invasively growing pancreatic tumors and their precursor lesions: The constitutive activation of oncogenic Kras probably demonstrates the most important and best studied event in pancreatic carcinogenesis.

5. Impact of Kras activation on pancreatic carcinogenesis

The mutation of Kras belongs to the earliest events in pancreatic carcinogenesis. Kras proteins comprise a family of signal-transducing GTPases that mediate a wide variety of cellular functions including proliferation, differentiation and survival and are frequently mutated in human cancers [66]. Although Kras is a GTPase, its intrinsic activity is inefficient and requires GTPase activating proteins to promote GTP hydrolysis and attenuate downstream signaling [1]. Oncogenic mutation of Kras ($Kras^{G12D}$) is generally accepted to represent the initial key event in pancreatic carcinogenesis and found in virtually all invasively growing pancreatic tumors [7]. Due to its prominent role in pancreatic carcinogenesis Kras is considered to be an attractive therapeutic target of PDAC-treatment, but specific biochemical properties of the protein have made this an elusive goal [67]. Activating Kras point mutations at codon 12 (from GGT to GAT or GTT and more rarely CGT) result in substitution of glycine with aspartate, valine or arginine. Oncogenic Kras mutations lock the protein in its GTP-bound form thus permitting its constitutive interaction with and activation of multiple effectors, independent on growth factor stimulation [67].

The activation of Kras engaged effector pathways, like the RAF-mitogen-activated kinase (MAPK)-cascade, phosphoinositide-3-kinase- (PI3K) signaling and the Ral GDS pathway results in stimulation of proliferation, invasion, metastases and survival thus enabling pancreatic cancer progression [3]. Given the aforementioned limitations in Kras inhibition, these downstream targets may provide alternative effective points of therapeutic intervention and thus are the focus of ongoing studies in pancreatic specific systems.

The impact of constitutive Kras activation is not limited on the epithelial cell but also participates in the modulation of the tumor environment. One hallmark of PDAC is an extensive stromal remodeling, the most prominent features of which are the recruitment of inflammatory and mesenchymal cells as well as fibrotic replacement of pancreatic parenchyma [68]. Recent studies revealed that even early stages of PanIN development are associated with a stromal reaction, which is characterized by a robust desmoplastic response and recruitment of immune cells. Subclasses of these immune cells, immature myeloid cells, suppress infiltrating T cells and thus establish an immune privilege in the tumor microenvironment promoting pancreatic carcinogenesis [69, 70]. Mechanistically, constitutive activation of Kras in pancreatic ductal cells triggers the production of the cytokine GM-CSF, which, in turn, promotes the expansion of immunosuppressive myeloid cells, leading to the evasion of $CD8^+$ T-cell-driven-antitumor immunity [69, 70].

Due to its high biological relevance for pancreatic carcinogenesis, a genetically engineered mouse model (GEMM) with pancreas specific Kras mutation was created, allowing detailed investigations of morphological as well as molecular features of this disease [71]. This transgenic mouse model bares a mutation of the endogenous murine Kras gene specifically in pancreatic progenitor cells by crossing mice with a conditionally activated Kras allel (LSL-$Kras^{G12D}$) to transgenic strains that express Cre recombinase in pancreatic lineages (PdxCre or p48Cre). These “KC” mice develop low and high grade PanIN lesions recapitulating pancreatic carcinogenesis in the human situation but only slowly progress

to PDAC at an advanced age [71]. This mouse model taught us that in spite of the requirement of Kras-activation for pancreatic cancer development oncogenic Kras mutation alone fails to transform precursor lesions into invasive cancer due to activation of powerful fail-safe mechanisms (compare Fig. 2).

Counteracting transformation and growth, cellular senescence, a permanent cell growth arrest, is increasingly recognized as one of the most critical fail-safe programs in pancreatic carcinogenesis [72]. A major cause of this permanent growth arrest was found in telomeres, which are non-coding nucleoprotein complexes positioned in the extremes of chromosomes [73]. During successive cellular divisions, telomeres in normal human cells shorten progressively and, when telomeres erode down below a threshold length, the cell ceases to divide itself and becomes senescent. Importantly, senescence can also be observed in the absence of any detectable telomere shortening or dysfunction in numerous conditions such as cellular stress or oncogene activation. Oncogene induced senescence (OIS) has emerged as a powerful tumor suppressor mechanism protecting cells from unrestrained proliferation imposed by oncogenic signaling [74]. It has been proven that normal cells, when forced to express high levels of oncogenic Ras, undergo a permanent and irreversible cell cycle arrest [75]. OIS is frequently found in premalignant lesions but is essentially absent in advanced cancers, suggesting that malignant tumor cells can find ways to bypass or escape senescence [76].

Pancreas specific expression of oncogenic $Kras^{G12D}$ promotes an initial burst of proliferation accompanied by the development of PanIN precursor lesions before cells stop dividing. These precursor lesions then exhibit many features of senescence including positive senescence-associated ß-galactosidase staining and induction of cell cycle inhibitors [77]. Successful progression of PanIN lesions towards frank adenocarcinoma requires evasion from senescence. This can result from additional genetic or epigenetic events concerning major tumor suppressor pathways, namely the $p19^{Arf}$-p53 pathway and the $p16^{Ink4a}$-Rb cascade [74].

6. Role of tumor suppressor inactivation in pancreatic carcinogenesis

The p53 protein plays a central role in modulating cellular responses to cytotoxic stress by contributing to both, cell cycle arrest and programmed cell death [3]. Signals of mitogenic oncogenes, such as cMyc or Kras lead to activation of p53, which depending on cell type and stimulus induces either apoptosis or senescence and consequently leads to the elimination of cells with oncogenic activation. p53 is integrated in a complex network of upstream sensors and downstream effectors. An important sensor of oncogenic signals for p53 is $p19^{Arf}$, which is encoded in an alternative reading frame (ARF) by the tumor suppressor locus CDKN2A [78]. Activation of $p19^{Arf}$ antagonizes the effect of the E3 ubiquitin ligase MDM2 that acts upon p53 to initiate its proteasomal degradation, thereby contributing to the stabilization of the tumor suppressor gene [74]. In the nucleus, stabilized p53 binds to promoters of more than 300 target genes with implications for cell growth control. One such important p53 downstream target is p21. p21 binds to and inhibits the activity of Cyclin-CDK2 and Cyclin-CDK1 complexes and thus functions as a negative regulator of cell cycle progression at the G1 phase [79].

In agreement with its key role in senescence and tumor suppression, mutational p53 inactivation is associated with accelerated carcinogenesis in many tumor entities [80]. In the pancreas, p53 inactivation on chromosome 17 has been reported in 50-75% of carcinomas [1]. In the murine pancreas carcinoma model, genetic loss of p53 allows Kras to bypass senescence resulting in 100% penetrance at an early age, thus recapitulating human PDAC including histopathological similarities in neoplastic cells, desmoplasia and occurrence of liver and lung metastases [81].

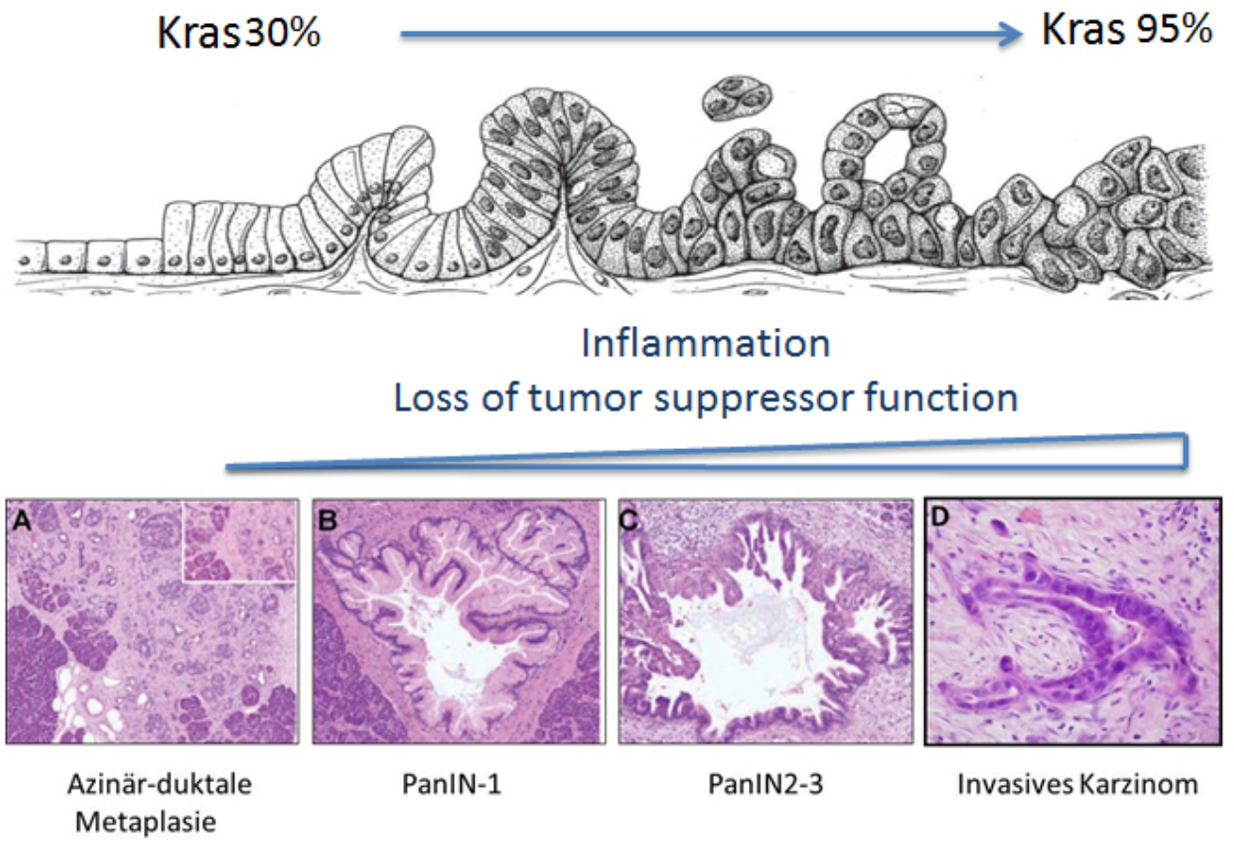

Figure 2. Current model of pancreatic carcinogenesis: on the background of Kras mutation ADM lesions progress to PanIN-precursor lesions and invasive carcinoma depending on additional signals as loss of tumor suppressor function or activation of inflammatory pathways. A: Acinar-ductal metaplasia, B: PanIN-1, C: PanIN-2-3, D: Invasive pancreatic cancer.

The $p16^{Ink4a}$ gene, located on the short arm of chromosome 9, is one of the most frequently inactivated tumor suppressor genes in pancreatic cancer [1, 2]. Remarkably, virtually all pancreatic carcinomas bare loss of $p16^{Ink4a}$ function, in 40% of pancreatic cancer through homozygous deletion, in 40% by intragenic mutation coupled with loss of the second allele, and in 15% by hypermethylation of the $p16^{Ink4a}$ gene promoter [8].

The protein $p16^{Ink4a}$ belongs to the cyclin D-dependent kinase (CDK) inhibitor family and functions to prevent the phosphorylation of Rb-1 by CDK 4 and 6, resulting in a blockage of G1/S-phase transition of the cell cycle [82]. This event is a decisive step in the inhibition of cell

cycle progression and also in senescence initiation. In contrast to that, loss of $p16^{Ink4a}$ results in inappropriate phosphorylation of Rb-1, thereby facilitating progression of the cell cycle through enhanced G1/S transition [1-3, 74].

Additional to inactivation of tumor suppressor genes, Kras-initiated pancreatic carcinogenesis can be promoted by signals from the inflammatory environment [69, 70]. This type of proinflammatory environment can be provided by chronic pancreatitis, the most relevant risk factor for PDAC development in human [83]. Chronic pancreatitis supports the initiation and progression of this malignancy by direct modification of gene expression networks in pancreatic epithelial cells. For instance, pancreatitis contributes to tumor progression by abrogating the senescence barrier characteristic of low-grade PanIN lesions [84]. Most importantly, chronic pancreatitis induces a wide range of proteins, predominantly inflammatory transcription factors. The majority of these inflammatory transcription factors inhabits oncogenic potential, mediated by inhibition of tumor suppressor genes or synergism with $Kras^{G12D}$ signaling to promote pancreatic carcinogenesis.

By introducing the inflammatory family of Nuclear factor of activated T cells (NFAT) proteins, the following part of the chapter will cite an example how deregulated oncogenes participate in and cooperate with $Kras^{G12D}$ mediated signaling in every single step of pancreatic carcinogenesis, beginning from induction of ADM over progression of pancreatic precursor lesions to frank invasive pancreatic ductal adenocarcinoma.

6.1. NFAT proteins and their role in pancreatic carcinogenesis

6.1.1. The family of NFAT transcription factors and their cellular regulation

The NFAT family, first described as a regulator of T cell activation and differentiation, comprises four calcium-responsive isoforms named NFATc1, NFATc2, NFATc3 and NFATc4 as well as a more distant relative, NFAT5 [85]. In resting cells, NFAT factors are located in the cytoplasm in a highly phosphorylated, inactive state [85, 86]. Ligand binding to many receptors results in the activation of phospholipase C (PLC), the release of IP_3 and in a transient release of Ca^{2+} from intracellular stores through IP_3 receptors. This initial release of Ca^{2+} demonstrates the prerequisite for increased influx of Ca^{2+} through specialized Ca^{2+} released activated channels (termed CRAC) [86]. CRACs provide the persistent Ca^{2+} signal that is necessary for sufficient activation of the phosphatase calcineurin that targets and dephosphorylates moderately conserved serine rich motifs in the N-terminal homology region of NFAT proteins to unmask its nuclear localization signals [87]. Subsequently, NFAT proteins shuttle into the nucleus where they are either ubiquitinated for HDM2-dependent proteasomal degradation or stabilized by GSK3ß-mediated phosphorylation (compare Fig. 3) [88]. Upon stabilization the transcription factor recognizes its GGAAA consensus sequence within target gene elements and binds DNA either as homodimer or heterodimer [85-88]. In fact, NFAT proteins frequently cooperate with other transcription factors to elicit high-affinity binding on common target genes. GATA Proteins, FoxP3 and members of the MEF family are only few among a wide range of NFAT partner proteins [89]. Additionally, NFAT recruits other signaling regulated transcription factors (e.g. Smad3 and NKkB) to integrate pathway specific signals to

Ca^{2+}/calcineurin regulated transcription [90]. Thus, NFAT transcription complexes function as signal integrators and detectors. One signal has to be Ca^{2+}/calcineurin, while the second one can have developmental origin or can embody oncogenic qualities as the Ras-MAP kinase pathway [89, 90]. Doing so, the cooperation between NFAT and its partners helps controlling the specificity of NFAT target gene binding and the resulting mode of action.

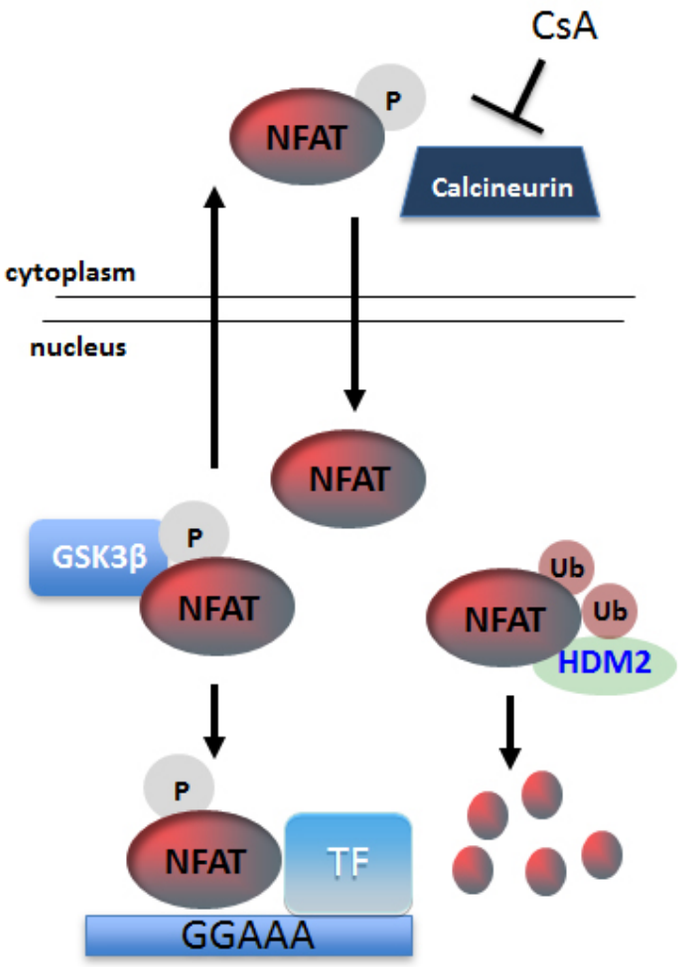

Figure 3. NFAT signaling in pancreatic cancer. Upon Ca^{2+} - dependent activation of Calcineurin, NFAT becomes dephosphorylated and shuttles into the nucleus. The calcineurin-inhibitor Cycosporin A (CsA) prevents NFAT activation. In the nucleus GSK3ß-dependent phosphorylation of NFAT either leads to its nuclear export or allows binding to target genes in association with partner transcription factors. Ubiquitination of NFAT proteins labels them for proteosomal degradation by HDM2.

7. Oncogenic potential of NFAT signaling

The NFAT family of transcription factors was originally identified as a group of inducible nuclear proteins which regulate transcription during T lymphocyte activation [91]. Following

their initial discovery, a multitude of studies quickly established that NFAT proteins are also expressed outside the immune system where they participate in the regulation of the expression of genes influencing cell growth and differentiation [86]. One of the first studies implicating NFAT factors in cell proliferation was performed in fibroblasts, in which constitutively active NFATc1 induces cell transformation and colony formation [92]. Similarly, in pancreatic tumor cells proliferation and anchorage-independent growth is - at least in part - dependent on calcineurin activity and nuclear translocation of NFAT proteins [93]. This is consistent with high levels of nuclear NFAT in pancreatic cancer cells and in particular in those cells with accelerated growth. Nowadays, ectopic activation of NFAT members is recognized as an important aspect of oncogenic transformation in several human malignancies, most notably in pancreatic cancer [88, 93]. Proliferation and anchorage-independent growth of cultured pancreatic cancer cells is significantly attenuated by inhibition of Ca^{2+}/Calcineurin signaling with Cyclosporin A or siRNA-technology-mediated depletion of NFATc1 [94]. Besides proliferation and growth, NFAT proteins incorporate additional features of tumor biology. Being downstream mediators of α6ß4 integrin signaling NFATc2 and NFAT5 promote cancer invasion in breast and colon cancer [95]. Stimulation of angiogenesis through upregulation of VEGF and enhancement of tumor cell migration via transcriptional activation of Cox2 are additional oncogenic features of NFAT proteins [86, 96].

GEMM with constitutive activation of NFATc1 revealed increased cellular proliferation in pancreata of young mice but mice baring a constitutive activation of NFATc1 failed to develop advanced PanIN lesions within a one-year observations span. In contrast to mice bearing an isolated transgenic induction of NFATc1, mice carrying combined constitutive activation of Kras and NFATc1, a situation found in 70% of human PDACs, surprise with a dramatically shortened survival compared to the $Kras^{G12D}$ animals [Baumgart et al., unpublished data]. Further resembling human PDAC, $Kras^{G12D}$;NFATc1 mice develop severe cachexia and abdominal distension caused by the accumulation of sanguineous ascites and bile duct obstruction. At necropsy, the pancreata from $Kras^{G12D}$;NFATc1 mice are enlarged by tumor mass, which contains both solid and cystic regions. Notably, pancreata from $Kras^{G12D}$;NFATc1 mice express nuclear NFATc1 throughout carcinogenesis and at equivalent levels to those observed in human PDAC.

Beyond doubt, the experience with the described transgenic mouse model which recapitulates human PDAC disease in a very accurate manner clearly shows that activation of NFAT proteins works synergistically with Kras signaling and leads to acceleration of pancreatic carcinogenesis. Further investigations shot light on the NFAT dependent mechanisms facilitating and hastening pancreatic carcinogenesis.

8. NFATc1 function in ADM

The cellular origin of PDAC has been a controversial topic for many decades. PDAC has long been considered to be a disease of pancreatic ducts. However, early efforts to model the disease by forcing Kras expression in pancreatic duct cells did not yield discernable pathology [97]. In

recent years, increasing evidence arised that PanIN precursor lesions and invasive PDAC originate from differentiated acinar cells. The development of duct-like PanIN lesions from acinar cells requires massive remodeling of these cells, both morphologically and with respect to gene expression profiles. The transition from acinar to ductal cell properties has been termed acinar-to-ductal metaplasia (ADM) and lineage tracing experiments have confirmed that this process is a result of direct transdifferentiation from adult acinar cells that convert to a ductal phenotype upon expression of constitutive active Kras [97, 98]. In murine and in human samples, ADM development has been shown to precede PanIN formation, suggesting that ADM represents the first step of pancreatic carcinogenesis.

Appreciating the relevance of ADM for pancreatic cancer development, much effort was put into research on the molecular mechanisms facilitating ADM. As a transcription factor that is involved in differentiation processes in many tissues NFAT constitutes a promising candidate to mediate ADM. Indeed, NFATc1 is highly operative in pancreatic ADM, while only rare expression of the transcription factor can be found in acinar cells. *In vitro* and *in vivo* studies have revealed that $Kras^{G12D}$ driven ADM requires ligand-dependent activation of the Epidermal growth factor receptor [6, 21]. Careful molecular studies have proven that EGFR signaling – at least in part – is mediated via NFATc1. Most importantly, in spite of active EGFR signaling, pharmacological or genetic inactivation of NFATc1 in acinar cell explants extracted from $Kras^{G12D}$ mice reduces duct formation *in vitro*. Furthermore, $Kras^{G12D}$ mice harboring a pancreas specific transgenic inactivation of NFATc1 are less susceptible to inflammation induced ADM and show a significant delay of pancreatic carcinogenesis [unpublished data]. These findings clearly indicate a key role of NFAT signaling in the initial steps of pancreatic carcinogenesis.

9. NFATc1 and STAT3 cooperation in pancreatic carcinogenesis

Recent investigations established that NFATc1 cooperates with the signal transducer and activator of transcription-3 (STAT3) [Baumgart et al., unpublished data]. Like NFAT proteins, STAT3 is also regulated primarily at the level of its subcellular localization [90]. In resting cells, STAT3 resides in a non-phosphorylated version in the cytoplasm. However, following cytokine or growth factor stimulation, STAT3 proteins are inducibly phosphorylated on critical regulatory tyrosine residues promoting their homodimerization and subsequent translocation into the nucleus where they control gene transcription [99]. Interestingly, genetic depletion of STAT3 attenuates the transformation capacity of NFATc1, suggesting a cooperative function of both transcription factors in pancreatic cancer. From the mechanistic point of view, NFATc1 interacts with STAT3 to form enhancer-promoter communications at jointly regulated genes involved in inflammation and oncogenesis, e.g. EGFR and Wnt-family members. The NFATc1-STAT3 transcription pathway is operative in pancreatitis-mediated carcinogenesis as well as in established human pancreatic cancer [Baumgart et al., under review].

10. Impact of NFAT proteins on the inflammatory tumor environment

Cancer-associated inflammation plays an important role in restraining anti-tumor immunity, particularly in pancreatic cancer for which a massive infiltration of immunosuppressive leukocytes into the tumor stroma is an early and consistent event in carcinogenesis [84]. In contrast to many other solid tumors, intratumoral T cells are rare in pancreatic cancer, which is associated with an immune escape and bad prognosis [70]. In PDAC, increasing evidence suggests, that oncogenic Kras drives an inflammatory program that establishes immune privilege in the tumor microenvironment [69, 70]. The immune surveillance of pancreatic cancer demonstrates the response to signals from the transformed epithelial pancreatic cell. Cytokines like GM-CSF are secreted by ductal pancreatic cells to modulate the inflammatory tumor environment. Recent work suggests an essential role of NFAT proteins in the transcriptional induction of a core of cytokines associated with encapsulation of the transformed cell from physiological immune response [100, unpublished data]. Thus, NFAT inactivation might represent a promising possibility to restore pancreatic cancer response to tumor suppressive immune signals.

11. NFAT mediated TGFß switch from tumor suppressor to oncogene in pancreatic carcinogenesis

As mentioned above, an emerging model in cancer biology supports a dual role for TGFß signaling in tumorigenesis, acting as a tumor suppressor in early carcinogenesis and as a strong promoter of cell proliferation, migration and invasion in advanced tumor stages [101, 102]. TGFß blocks cell proliferation in untransformed cells through the induction of a cell cycle arrest at late G1 phase. Two critical molecular events underlie TGFß anti-proliferative response: the transcriptional repression of cMyc and subsequent induction of cell cycle inhibitors like p21 and $p15^{Ink4b}$ [102, 103]. As an immediate early transcription factor proto-oncogenic cMyc functions as a master regulator of G1-S-cell cycle progression and growth promotion in pancreatic cancer [93, 103]. cMyc repression by TGFß requires the activation of a Smad3-4 complex to transduce its stimulus into the nucleus. Here, Smad proteins complex with the transcription factors E2F4/5 and DP1 and corepressor p107 to repress cMyc promoter via binding to its TGFß-inhibitory element (TIE) [104].

During pancreatic carcinogenesis, tumor cells change their transcriptional responsiveness to TGFß and become resistant to the growth inhibitory effects due to functional inactivation of the TGFß-Smad pathway [103]. Depending on the cell type and the activation status of a cell, TGFß then signals through Smad-independent pathways (e.g. PI3K and MAPK pathways) to promote the acquisition of a mesenchymal phenotype and stimulate tumor cell migration [102, 103].

TGFß induces expression of NFATc1 and c2, which accumulate in the nucleus and displace pre-existing Smad3 repressor complexes from the cMyc TIE element. Mechanistically, NFATc1 binding to the serum responsive element within the proximal cMyc promoter initiates p300-

dependent histone acetylation rendering the promoter transcriptionally active. Hyperacetylation of the cMyc promoter is required for recruitment of the Ets-like gene 1 (ELK-1), a protein signaling downstream of Kras, responsible for maximal activation of cMyc [94]. The functional significance of this pathway is emphasized by restoration of TGFß growth suppressor function in cancer cells and impaired cMyc expression indicated by reduced tumor growth and G1-arrest following the pharmacological or genetic inactivation of NFAT proteins [94, 102].

12. NFAT dependent silencing of tumor suppressor genes by formation of heterochromatin complexes

Activation of NFAT proteins does not only lead to target gene activation in pancreatic cancer, but also contributes to gene silencing. Being a member of the Ink4 family, $p15^{Ink4b}$ impedes the activation and function of Cyclin dependent kinases (CDK) 4 and 6 which leads to cell cycle inhibition and diminished G1-S phase transition [105]. Therefore, $p15^{Ink4b}$ incorporates important functions as a tumor suppressor in numerous malignancies, most importantly in pancreatic cancer, where $p15^{Ink4b}$ inactivation by genetic or epigenetic events occurs in over 90% of all tumors [9]. NFATc2 targets $p15^{Ink4b}$ for inducible and sequential heterochromatin formation and gene silencing. Sequential Chromatinimmunprecipitation revealed that NFATc2 binding to its putative binding side on the $p15^{Ink4b}$ promoter leads to recruitment of the histone methyltransferase Suv39H1. Local trimethylation of Lysine 9 on histone 3 (H3K9trime) allows docking of heterochromatin protein 1 y (HP1y) which results in stabilization of the heterochromatin complex on the $p15^{Ink4b}$ promoter. Conflicting with that, inactivation of NFATc2 disrupts the repressor complex and results in restoration of $p15^{Ink4b}$ expression and function [106].

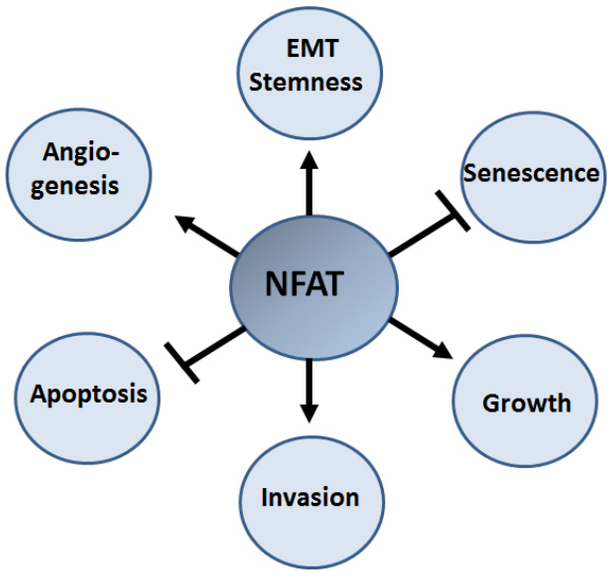

Figure 4. NFAT transcription factors and their impact on hallmarks of cancer

13. Perspective

These examples of NFAT dependent alterations in signaling pathways and transcriptional processes promoting pancreatic carcinogenesis only demonstrate a small insight into how oncogenic transcription factors contribute to pancreatic cancer development. Via transduction of EGFR signaling to downstream targets, by cooperation with other pre inflammatory oncogenes, by modulation of the tumor microenvironment, induction of cell cycle promoting genes as well as via silencing of important tumor suppressor genes, NFAT proteins are highly involved in all phases of pancreatic carcinogenesis reaching from early acinar-to-ductal-metaplasia over establishment of precursor lesions to frank invasive pancreatic adenocarcinoma.

As dismal as pancreatic cancer presents itself clinically, as complex and multi-layered are the histopathological and molecular mechanisms responsible for pancreatic carcinogenesis. As the molecular main reason for pancreatic cancer development - the constitutive activation of Kras - evades any pharmacological approach, targeting oncogenic factors like NFAT proteins represents a promising option approaching success in pancreatic cancer treatment.

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