Opening new doors: Hedgehog signaling and the pancreatic cancer stroma
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CHAPTER 1
General Introduction
INTRODUCTION

Some of the most fascinating and complex problems in biology are the mechanisms that orchestrate the development of multi-cellular organisms from a single ancestor cell. Questions like how these complex organisms evolve from the moment of first cell division, and how genetic information is translated into a spatially organized body with intricate patterns and a multitude of specialized cells are still not truly answered. However, in the past three decades, many of the genes playing a role in tissue patterning and cell fate have been identified and our knowledge about developmental mechanisms has been expanded quite extensively by characterizing these genes and their interaction.

Decisions regarding cell fate are not taken at the level of one individual cell, but are orchestrated by extrinsic signals that are soluble or transmembrane factors, produced by one cell and received and converted into an intracellular signaling cascade by another. Soluble factors that act in a concentration-dependent manner to control cell fate have been termed morphogens. This concept was illustrated by Lewis Wolpert in the 1960s [1], who developed the so-called French flag model (Figure 1). In this model, a flag is used to represent the concentration-dependent effect of a morphogen on the differentiation state of receiving cells. These states are represented by the different colors of the French flag: high concentrations activate genes that results in a ‘blue’ cell state, lower concentrations lead to a ‘white’ phenotype, and ‘red’ serving as the default cell state below the necessary concentration threshold. Thus, the morphogen gradient confers positional information by specifying distinct phenotypes in a field of receiving cells depending on the distance to the source of the signal. It is important to realize that this model is an oversimplification of the mechanisms governing cell fate decisions, and in reality many morphogens (and

Figure 1. The French flag model of morphogen gradients and resulting cell fates. This model summarizes how a specific cell state is achieved by localized production of a morphogen. As a consequence, a gradient is established with differential local concentrations depending on the distance from the source of morphogen production (left panel). Different thresholds of the morphogen concentration determine the cellular phenotype of the receiving cell into A-blue, B-white or C-red (right panel), representing the French flag. (modelled after Wolpert et al. [246])
their antagonists) are involved in regulating a multitude of different genes leading to the generation of much more complex patterns. In addition, it has been shown that cells in tissue exposed to a gradient of morphogens migrate to form sharply demarcated domains after differentiating in response to the morphogen, essentially fine-tuning the differentiation pattern to eliminate signaling noise [2].

**Discovery and role of Hedgehog morphogen**

Most of the evolutionary conserved morphogens that are essential for cell fate decisions in complex multi-cellular organisms were initially discovered in genetic screens in the fruit fly *Drosophila melanogaster*. One of the molecules that emerged from a screen for mutations that affected the segmental patterning of the *Drosophila* larvae was Hedgehog (Hh) [3]. In wild type *Drosophila* larvae, a band of bristles called denticles runs across the anterior half of each segment, whereas the posterior half is smooth (the so-called naked cuticle) (Figure 2A). In the segment polarity mutant discovered by Nüsslein-Volhard and Wieschaus, the posterior half of each segment failed to develop, resulting in a larva entirely covered by denticles (Figure 2B). This ‘spiky’ phenotype gave the larva the aspect of a hedgehog, which inspired the name.

After the initial identification of the Hh mutant phenotype in 1980 and the discovery of the *Hh* gene more than a decade later by three independent groups [4-6], the vertebrate homologues were identified shortly after the cloning of the *Drosophila* gene [7]. Echelard and colleagues identified three murine homologues, which were named Sonic Hedgehog (Shh),

![Figure 2. The phenotype of the *Drosophila* hedgehog mutant. (A) Schematic representation of a wild type *Drosophila* larva. Each segment has denticles at the anterior end and a smooth posterior end, the so-called 'naked cuticle'. (B) Hedgehog signaling specifies the posterior end of each segment. As a result, the hedgehog mutant larva lacks the naked cuticles, giving it a 'spiky' appearance which inspired the name.](image-url)
after a popular Sega computer game character, *Indian Hedgehog* (*Ihh*), and *Desert Hedgehog* (*Dhh*) [8]. Whereas *Dhh* is the closest homologue to *Drosophila* Hh, Shh and *Ihh* are more closely related to one another than they are to the fly protein. All three *Hh* homologues are highly conserved between mouse and human [9]. Studies in knock-out animals have shown that *Dhh* is essential for testis development [10], and *Ihh* is best known for its critical role in bone morphogenesis [11, 12]. Shh, the best characterized *Hh* ligand, is involved in a broad spectrum of developmental processes. During early vertebrate embryogenesis Shh controls formation of the left-right axis of the heart and visceral organs such as the gut tube [13-15] as well as the dorso-ventral polarity of the developing midbrain [16]. Expression in the zone of polarizing activity (ZPA) of the limb bud directs digit formation along the anterior-posterior axis [17]. In neural tube development, a Shh morphogen gradient originating from the notochord and floor plate directs the formation of multiple neuronal subtypes at specific positions [18].

However, the role of hedgehog signaling goes beyond embryonic patterning and is important even in the adult organism. Shh has been shown to regulate proliferation and differentiation of adult stem cells from various tissues, including the mammary gland [19], brain [20], teeth [21], skin [22], and hematopoietic system [23]. In the small intestine, epithelial cell-derived *Ihh* seems to be the more important player in regulating homeostasis of the adult organ by signaling to the adjacent mesenchyme and this in turn limits the size of the precursor cell compartment in the crypt [24]. Given the importance of *Hh* signaling in regulation of adult stem cell compartments and tissue homeostasis, it is not surprising that deregulation of this pathway was found to be involved in promoting cancer development and progression (discussed below).

**HEDGEHOG PATHWAY**

*Hedgehog production and processing*

One of the very unique features of the *Hh* protein family members is their unusual posttranslational modification. Following cleavage of an amino-terminal (N-terminal) signal sequence upon entry into the secretory pathway, the ~45 kDa precursor *Hh* protein undergoes an autocatalytic processing reaction, yielding a 19 kDa N-terminal fragment (*Hh*-N) containing all the signaling functions and a 26 kDa C-terminal fragment (*Hh*-C) that catalyzes the cleavage [25-27]. *Hh* cleavage appears to be essential for the signaling function, as mutations in human Shh that block this processing of full-length protein cause loss of Shh function, resulting in holoprosencephaly [28, 29], a condition in which the forebrain of the embryo fails to develop into two hemispheres. During the autocatalytic cleavage a cholesterol moiety is covalently attached to the C-terminal part of *Hh*-N [30], making Hedgehogs the only proteins described so far with this very unique lipid modification. After cleavage, *Hh* is additionally modified by the attachment of a palmitic acid group at the N-terminal Cys24 site, catalyzed by hedgehog acyltransferase *Hhat* [31, 32], which greatly increases the activity of the protein in cell-based assays [33, 34] (Figure 3). Both hydrophobic modifications are generally thought to be essential for the activity of the protein as well.
as for regulating the spatial distribution of the signaling molecule, by anchoring it to membranes and thus hampering diffusion [35, 36]. However, if decreased signaling potency of Hh molecules lacking lipid modifications is a result of defective intracellular trafficking and release, rather than the biological activity of the protein itself, is still under debate.

**Spread of Hh from producing cells**

Several mechanisms have been proposed to explain how the hydrophobic, dually lipid modified Hh ligand can spread through the aqueous environment of the extracellular space and activate long-range target gene expression over at least 8-10 cell diameters away from the source of ligand [37]. One such release mechanism requires the function of Dispatched (Disp), a multipass transmembrane protein from the resistance-nodulation-division (RND) transporter family. Disp binds directly to the cholesterol modification of Hh proteins and acts in synergy with the vertebrate-specific Scube2, a secreted glycoprotein binding to a different part of the cholesterol moiety, to promote the release of lipid modified Hh from the surface [38-40]. This release mechanism was first described to be crucial for long range signaling of lipidated Hh in the fruit fly [35], and later on in vertebrates as well [38, 41]. However, even in the absence of Disp function, juxtacrine Hedgehog signaling is still observed and cholesterol-free Hh is constitutively released [42, 43]. This suggests diverse cell exit mechanisms to exist for differentially lipidated proteins.

**Figure 3.** Posttranslational processing of Hedgehog proteins. The 45 kDa precursor protein is processed, after removal of the signal sequence, to a 19 kDa signaling protein. The C-terminal half catalyzes this reaction and attaches a cholesterol moiety in the process. Hh-N is further lipid modified with a palmitate on the N-terminus by HHAT, resulting in the fully processed Hh-Np protein. SS, signal sequence, Hh-N, N-terminal cleavage product after autocatalytic processing and cholesterol modification; Hh-C, C-terminal cleavage product; HHAT, hedgehog acyltransferase
Another concept of Hh spreading in regard to the lipidated nature of the morphogen is that both cholesterol and palmitate are required to mediate the formation of multimeric complexes of Hh proteins, in which the lipid moieties are thought to be embedded in the core, similarly to micelles. Biochemical analysis of Hh produced in vitro and in vivo revealed that the ligand is mainly found in large multimers and these complexes facilitate long-range dispersal [44-46]. Additionally, work by Susan Eaton and colleagues has shown that Hedgehog proteins can also associate with lipoproteins in flies and mammals and can even be transported systemically via the Drosophila hemolymph system [47, 48]. Also in human plasma samples, Ihh was found on lipoprotein particles and can increase the survival of endothelial cells [49], showing that systemic lipoprotein associated Hh proteins are not restricted to the fly and can potentially even have systemic effects in human. In addition to the soluble multimers and lipoproteins, there is evidence that Hh can be transported in extracellular vesicular particles (exovesicles, exosomes) [50, 51]. How these multimers, lipoprotein particles or exosomes carrying Hh proteins are formed and released remains an unanswered question.

Members of the A disintegrin and metalloprotease (ADAM) family of proteases have also been implicated in the formation of soluble Hh proteins. In this model, Hh complexes are generated by limited proteolysis, leading to the deletion of N-terminal residues and the truncated protein may have greater solubility due to lack of palmitate [52]. Interestingly, ADAMs are frequently found to be upregulated in different malignancies such as prostate, lung and pancreatic cancer [53], in which Hh ligands are also aberrantly expressed by tumor cells and reported to aid in tumor progression [54]. In chapter 4 of this thesis the role of ADAM mediated processing of endogenous Hh ligands produced by pancreatic cancer cells is investigated. We show that Hh release can be mediated by ADAM10, ADAM12, and ADAM17. Intriguingly, blockage of Hh release from pancreatic cancer cells by knockdown of ADAMs resulted in an increased juxtacrine signaling potency as well as expansion of the signaling range, challenging the dogma that Hh signaling molecules need to be freely diffusible to establish a signaling gradient.

There is also evidence that the composition of the extracellular environment is critical to Hh dispersal and signaling. In Drosophila wing disc, cholesterol-modified Hh is not able to move or signal across cells that lack heparan sulfate proteoglycans (HSPGs) [55, 56], adding another layer of complexity to the mechanisms of Hh distribution in tissues.

Finally, modes of transportation that do not depend on the release of Hh from producing cells to travel to a responsive compartment have been recently reported. In Drosophila, Hh has been found on long basal filopodia, dubbed cytonemes that can deliver Hh at some distance from the producing to the receiving cell [57-59]. Hh was shown to travel along these cytonemes in the form of exovesicles [60]. Similar cytoneme-like extensions carrying particles containing Shh have been reported in the chicken limb bud [61].

Taken together, the impressive complexity of different release and dispersal mechanisms for Hh proteins may be bewildering, especially since it is still very unclear how these different mechanisms relate to each other, if different pools of Hh proteins such as lipidated and lipid-free proteins, are released via independent mechanisms and if all
Hh-producing cell types use only one method or the whole possible spectrum described. Answering these questions about the control of Hh transport mechanisms especially in vertebrate tissue or human disease will represent a major challenge in the next few years.

**Hedgehog signal transduction**

Like the transport mechanisms underlying distribution of its ligands, signal transduction of the Hh pathway is highly unusual as well. The pathway's activating receptor Smoothened (Smo), which belongs to the family of G-protein-coupled receptors (GPCR), is not bound directly by Hh ligands as opposed to conventional signal transduction routes of other signaling pathways [62]. Instead, the activity of Smo is controlled indirectly by the 12-transmembrane receptor called Patched (Ptc), which contains two large extracellular loops that mediate Hh ligand binding [63, 64]. Two Ptc genes exist in vertebrates Ptc1 [65] and Ptc2 [66], but Ptc1 is thought to mediate most effects of Hh signaling. Nevertheless, a recent report revealed that Ptc2 can respond to Shh ligand and take over certain signaling functions in the absence of Ptc1, especially during early developmental stages [67].

In the absence of ligand, Ptc represses the activity of Smo catalytically, very likely by the release of small inhibitory molecules such as vitamin D3 [68], or depletion of Smo activating substances like oxysterols [69, 70] which in turn are controlling the subcellular localization of Smo [71]. However, the exact mechanism by which Ptc prevents Smo from activating the pathway in the absence of ligand is still only poorly understood. Other cell-surface molecules exist that bind Hh proteins, functioning to fine-tune perception and distribution of Hh signal. The coreceptors Growth-arrest-specific 1 (Gas1) [72], low-density lipoprotein receptor-related protein 2 (Lrp2) [73], as well as CAM-related/downregulated by oncogenes (Cdo) and brother of Cdo (Boc) function as positive regulators by enhancing binding of Hh to Ptc and therefore increase signaling output [74]. Glypican 3 (Gpc3) and hedgehog interacting protein (Hhip1) compete with Ptc for Hh-binding and thus act as negative regulators of the pathway [75, 76] adding another layer of complexity to the interpretation of extracellular Hh signal by responsive cells.

In vertebrates the first and best characterized, and therefore considered ‘canonical’, Hh pathway downstream of Smo acts through three zinc finger transcription factors, termed glioma-associated oncogene homolog Gli1, Gli2 and Gli3 [77]. Gli proteins can act as transcriptional activators or repressors depending on their processing, and were shown to bind with similar affinity to the consensus sequence GACCACCCA [78]. When Smo is inactive, the Gli transcription factors Gli2 and Gli3 are sequentially phosphorylated by protein kinase A (PKA), glycogen synthase kinase-3 β (GSK3β), and casein kinase 1 (CK1), which recruits the E3 ubiquitin ligase β-TrCP resulting in formation of proteolytically processed N-terminal repressors by the proteasome [79-81], preventing activation of Hh pathway target genes. Gli2 has both activator and repressor functions and Gli3 is mostly a repressor, although it can also have positive effects [82-84]. Gli1 lacks an N-terminal repressor domain and only occurs as transcriptional activator [85-87]) which is directly upregulated by Gli2 in response to an active Hh pathway [88, 89]. Another crucial negative
regulator of Gli transcriptional activity in vertebrate Hh signaling is Suppressor of Fused (Sufu), in contrast to its subtle role in Drosophila [90, 91]. On the one hand, Sufu sequesters the Gli proteins in the cytoplasm and prevents their entry into the nucleus; on the other hand Sufu can repress transcription by recruiting the histone deacetylase complex SAP18-mSin3 [92, 93]. Mice lacking the Sufu allele die at mid gestation and show a strong Hh gain-of-function phenotype, similar to Ptch1 null mutants [90, 94]. (Figure 4A)

In the presence of Hh ligand, the repression of Smo by Ptch1 is relieved [64], activated Smo accumulates in the primary cilium and Hh signaling is initiated [71]. Active Hh signaling inhibits Gli repressor formation and promotes the conversion of full-length forms of Gli2 and Gli3 into transcriptional activators, leading to activation of Hh target gene expression (Figure 4B). Genes induced by Hh, such as Ptch1, Hhip1 and Gli1, which are most widely used as a readout for pathway activity, can modify the strength and duration of the signal by triggering positive or negative feedbacks on the pathway [88, 95]. The expression levels of Hh coreceptors Cdo and Boc are down-regulated in response to Hh signaling, resulting in yet another negative feedback that limits pathway activity [74]. Additional Gli targets include genes regulating proliferation, differentiation and survival such as CyclinD1 and D2, N-Myc, Wnts, FoxM1, Hes1, Bcl2 [96-101], genes for self-renewal (Bmi1, Nanog) [102, 103] and genes contributing to epithelial-mesenchymal transition (Snail1) [104] or invasiveness (Osteopontin) [105].

**Figure 4.** The Hedgehog signal transduction pathway. Diagram of the vertebrate Hh signaling pathway. (A) In the absence of Hh ligand, the Ptc receptor suppresses the function of Smo by preventing its entry into the primary cilium. The Gli proteins (yellow) are sequentially phosphorylated by GSK3β, PKA, and CKI, which creates binding sites for the adaptor protein β-TrCP, leading to the formation of a C-terminally truncated repressor form (GliR, red). GliR mediates transcriptional repression of Hh target genes. (B) Binding of the Hh ligand inhibits the function of Ptc, which results in the movement of Smo to the primary cilium. The Hh/Ptc complex becomes internalized and degraded in endosomes. Smo in the cilium promotes the activation of full-length Gli proteins (GliA, green), which enter the nucleus and turn on transcription of target genes. IFT, intraflagellar transport along the primary cilium.
Increasing evidence suggests that the subcellular localization of vertebrate Hh pathway components is a major regulator of their activity. The examination of developmental defects arising in mice showed that mutations within the intraflagellar transport proteins Kif3a and Ift88 produce patterning defects that mimic Hh loss-of-function mutations [106]. These proteins are required for the assembly and maintenance of primary cilia which are present on most mammalian cells and involved in a wide variety of cellular processes including mechanosensation and the transduction of several signaling pathways [107]. Activated mammalian Smo accumulates in the primary cilium in response to Shh treatment [108] and Ptch suppresses the function of Smo by preventing its entry into the cilium in the absence of ligand [71]. Other signaling components like Sufu and the unprocessed Gli proteins also localize to the primary cilium [109]. Interestingly, although Sufu co-localizes together with Smo and Gli to the primary cilium, its inhibitory function on Gli activity is independent of this organelle [110, 111].

**Non-Gli mediated Hh signaling**

Beside the canonical transcriptional Hh pathway, several non-canonical downstream effects in cells stimulated with Hh ligand have been reported in recent years. For instance, biochemical studies revealed that the Hh receptor Ptch1 can directly interact with both Cyclin B1[112] and caspases [113], to inhibit cell proliferation and promote apoptosis, respectively, independent of Smo function. These functions of Ptch1 as a dependence receptor, meaning that cell survival is dependent on the present of ligand, was found to be inhibited in the presence of Hh morphogen [113].

It is now well established that Shh can also act as an guidance cue for axons of commissural neurons and retinal axons during embryonic development [114-118], with Src family kinase members Src and Fyn being the downstream mediators of this response [114]. Smo activation by Shh can additionally stimulate calcium release from the endoplasmatic reticulum in a dose-dependent manner in neural tube explants through opening of IP$_3$-dependent channels via G$_{i}$ and PLC-γ [119], which occurred within a few seconds after exposure to ligand. Other work demonstrated that Hh proteins can regulate cytoskeletal rearrangements and migration in a Smo-dependent manner via coupling to G$_{i}$-proteins and activation of the small GTPases RhoA and Rac1 in different cell systems [120-122]. This regulation seems to occur in a context-dependent manner, as neurons engage Tiam1 or Src kinase family members for cytoskeletal remodeling [123], whereas fibroblasts are thought to signal to RhoA/Rac1 via Gαi proteins and PI3K [124]. The rapid time course of the above mentioned Smo-dependent responses, lack of detectable Gli-dependent transcription, as well as the inability of Gli3 repressor protein to prevent these responses suggests a Gli-independent nature of these Smo-mediated signaling pathways [125, 126]. A major difficulty in studying non-canonical Hh signaling is that the canonical signaling pathway is activated in parallel, which influences the cellular response after ligand stimulation and complicates interpretation of experimental findings.

Despite the very prominent role of the primary cilium in canonical Hh signaling, its impact on the non-transcriptional responses has not been investigated in detail previously.
In chapter 2 of this thesis we investigate changes in chemotactic Hh signaling by modulating the location of Smo and the ability of cells to form primary cilia and unravel a negative correlation between ciliary localization and chemotactic signaling capacity. This indicates the use of a different pool of Smo protein to mediate either cilia-dependent transcriptional responses or cilia-independent migratory responses.

**Hedgehog signal pathway in malignancies**

The link between Hh pathway activity and cancer was initially established by the identification of inactivating mutations in the Hh receptor Patched as the cause of Gorlin syndrome [127-129]. This syndrome is associated with an increased incidence of basal cell carcinoma (BCC), medulloblastoma, and rhabdomyosarcoma [130]. Further evidence that aberrant pathway activity may play a causal role in human malignancies was provided by the identification of inactivating mutations in PTCH1 or gain-of-function mutations in SMO in medulloblastoma and sporadic BCC patients [131-133], but also other pathway components can be genetically altered in human cancers including SUFU mutations in medulloblastoma, GLI1 gene amplification in glioblastoma, GLI2 amplification in squamous cell carcinoma, as well as GLI1 and GLI3 mutations - though of unknown functional significance - in pancreatic adenocarcinoma [134-136].

Several cancers that rely on the overexpression of Hh ligands, as opposed to pathway-activating mutations, have been identified in the past few years. These include lung, pancreatic, colorectal, prostate, breast, upper gastrointestinal tract, and melanoma tumors [137-145], and often these cancers rely on Hh signaling at different stages in tumor progression.

The underlying mechanism of tumor development, maintenance and progression by deregulated Hh signaling could be stimulation of proliferation, inhibition of apoptosis by induction of BCL2 expression, enhancement of invasiveness and metastasis by activation of epithelial-to-mesenchymal transition (EMT)-promoting factors such as Snail and osteopontin, and possibly even activation of (cancer) stem cells [54, 146]. As a consequence, several pharmacological inhibitors of Hh signaling are currently investigated as new therapeutic approaches in a wide range of human malignancies, with very promising results in cancers that harbor activating mutations of Hh pathway components, such as BCC and medulloblastoma. Drug discovery efforts on small molecule pathway inhibitors have been focused predominantly on targeting the activating receptor Smo. The frontrunner, Vismodegib (GDC0449, Curis/Roche), was approved in 2012 by the US Food and Drug Administrations (FDA) for treating locally advanced and metastatic BCC [147, 148], and also effects seen in medulloblastoma patients treated with sonidegib (LDE225, Novartis) are encouraging [149, 150]. However, for many cancers in which Hh ligand overexpression is thought to drive tumor growth, and preclinical studies showed great potential, results in clinical trials have been largely disappointing. This highlights a need to better understand the complex biology underlying Hh signaling in these cancer types [151].
Chapter 1

**PANCREATIC CANCER**

At present, pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer related deaths in the Western world. In Europe, approximately 104,000 patients are diagnosed with PDAC annually, and nearly an equal number will die from this disease [152]. Treatment of this highly lethal malignancy has proven to be difficult and is not successful in most cases. This poor outcome is mainly due to the inability to diagnose pancreatic adenocarcinoma at an early stage because of the lack of specific symptoms and diagnostic markers, but also the inaccessible location of the pancreas, its high resistance to all forms of conventional chemotherapy and radiotherapy, and the early occurrence of metastatic spread [153, 154]. The most common site of distant metastases is the liver, followed by the peritoneum, lung, bones, and adrenal glands [155]. Even small (<2cm) tumors are often accompanied by metastases [156].

The etiology of pancreatic cancer, similar to other malignancies, is not well understood. Only a few known demographic and environmental risk factors and a handful of genetic conditions are associated with the development of PDAC, like germ-line mutations in tumor suppressor genes *BRCA2*, *INK4A*, or *LKB1*. Multiple studies have established advanced age, cigarette smoking, and chronic pancreatitis as clear risk factors; diabetes and obesity appear to additionally confer increased risk, as well as an inherited genetic predisposition as part of a family history of pancreatic cancer [157].

A variety of precursor lesions have been described in the pancreas. These include pancreatic intraepithelial neoplasms (PanINs), intraductal papillary mucinous neoplasms (IPMNs), and mucinous cystadenomas (MCNs) [158]. Although adenomas can arise from any of these precursor lesions, adenocarcinomas arising from PanINs are most commonly observed and best characterized [159]. PanINs are grouped into three histologic stages based on the increasing degrees of architectural and nuclear atypia [160]. These lesions are thought to sequentially acquire specific genetic alterations during progression towards malignancy, including activation of KRAS oncogene and loss of CDKN2A in early stages of tumor development (PanIN-1 and PanIN-2), accompanied by loss tumor suppressor genes TP53 and SMAD4 in later stages (PanIN-3 and PDAC) [161]. In particular, activating mutations of KRAS are a prominent feature in pancreatic cancer. They can be found in up to 95% of cancer patients and are thought to represent the initiating event of pancreatic carcinogenesis, but have proven difficult to target therapeutically [157, 162]. However, in addition to these hallmark genetic changes, large-scale analysis of the pancreatic cancer genomes has revealed a remarkable intratumor heterogeneity and complexity, revealing an average of 63 genetic alterations per tumor [136] and most of these alterations are unique to one patient tumor [163]. This enormous complexity on the genetic level alone makes it difficult to generalize treatments for patient cohorts whose tumors display such high diversity.

To date, the radical surgical resection of PDAC remains the only curative treatment option after which median survival is only 15-20 months, while five-year survival ranges from 8-25% [164], but unfortunately in approximately 80% of the cases, the disease has progressed to a stage too advanced for surgical intervention at time of diagnosis [165].
Europe, patients are usually treated with adjuvant 5FU/folonic acid or gemcitabine after resection, based on the improved (disease free) survival shown in different trials [166-168]. However, median overall survival, even after surgery and adjuvant chemotherapy, still does not exceed two years. Treatment options for the majority of PDAC patients that are diagnosed with locally advanced or metastatic disease, and therefore not eligible for resection, are rather limited. The current first-line standard of care therapy using gemcitabine extends patient survival only modestly by a few months [169]. However, since the introduction of gemcitabine, therapeutic progress has been extremely slow. One phase III study showed a small survival benefit (0.33 months) with the addition of the EGFR tyrosine kinase inhibitor erlotinib, which led to the approval by the US FDA, but was also accompanied by a substantial increase in toxicity [170]. Recently, the combination of oxaliplatin/irinotecan/5-FU/leucovorin (FOLFIRINOX) showed a significant increase in overall survival compared to gemcitabine [171]. These therapies typically prolong survival by only a few months and all patients ultimately succumb to progressive disease [172].

Despite these modest successes, a long list of phase III clinical trials with compounds that initially did show activity in other malignancies, and were successful in pre-clinical studies, were not effective in unselected PDAC patient populations [173, 174]. Factors that are believed to contribute the poor response to chemotherapy are, among others, the intra- and inter-tumoral heterogeneity, a lack of predictive biomarkers and the particular characteristics of the tumor microenvironment. The lack of efficient therapeutic options currently available underlines the importance of identifying new targeting strategies for treating aggressive and metastatic PDAC, as well as the need to develop novel pre-clinical model systems to improve the predictive value of therapeutic regimens and to gain more insight into the complex pathobiology of this malignancy.

**Mouse models of pancreatic cancer**

Since knowledge about the genetic basis of PDAC was revealed, several genetically engineered mouse models (GEMMs) have been developed to mimic the pathobiology of this aggressive malignancy. The most commonly used strategy is to introduce activating mutations of Kras specifically in the pancreas by using mouse strains expressing a Cre-recombinase under the control of a promoter for key regulators of early pancreatic development such as Pdx1 or Ptf1a-p48 [175]. The mutated Kras gene (Kras<sup>G12D</sup>) in this case is kept inactive by a stop codon preceding the gene, flanked by two LoxP sites (Lox-Stop-Lox cassette; LSL). Only cells expressing Cre will recombine the LoxP sites, and remove the stop codon on the allele to allow expression of mutated Kras in the pancreas [176]. The presence of a Kras mutation alone, although leading to the development of PanIN precursor lesions, was found to be insufficient to induce progression to the invasive stage of pancreatic cancer. Therefore, additional deletions of tumor suppressors such as Ink4a [177], p53 [178], Smad4 [179], or Tgfbr2 [180] were introduced to generate more aggressive models. Most of these GEMMs faithfully recapitulate the progression from the development of PanIN precursor lesions to carcinoma and finally metastatic disease, as well
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as clinical manifestations such as jaundice, cachexia and ascites, closely resembling what is observed in patients. However, activation of oncogenes and deletion of tumor suppressor genes in GEMMs takes place simultaneously during early development in the whole organ instead of a few selected cells, whereas in patients mutations are likely acquired in the adult organ over a period of several years in consecutive steps [181]. Furthermore, as these models are based on the most commonly found mutations in pancreatic cancer, they still are not able to span the genetic and epigenetic heterogeneity found in patient tumors, which limits their predictive value in drug testing.

To more closely recapitulate the tumor heterogeneity observed in patients, many researchers are turning to patient-derived xenograft models (PDXs) for pre-clinical studies. For these models, pieces of, or freshly isolated cell suspensions from, patient tumors are transplanted into immunocompromised host animals, either subcutaneously into the flank or orthotopically into the pancreas of mice. Orthotopically grafted tumors are considered the superior model, as they preserve the native anatomical microenvironment of the organ, are more prone to show invasive features including formation of metastasis, and mimic resistance to drugs such as gemcitabine [182, 183]. However, they are also more challenging technically and monitoring of tumor growth requires manipulation of tumor cells to allow bioluminescence/fluorescence imaging, or alternatively the access to sophisticated imaging methods including computed tomography (CT), magnetic resonance imaging (MRI), or positron-emission tomography (PET). After successful engraftment, tumor material can be propagated by passing into new recipient animals and expanded for desired studies. PDXs generally resemble very closely the original patient tumor histologically but also on a molecular basis, and they have been found to be genetically stable over several passages [184, 185] and were shown to be predictive for response rates of the donor patient to different drug regimens tested [186].

One of the caveats of PDXs that still needs improvement is the fact that not all tumors from patients grow out in animals and the time to engraftment, which on average is 4-8 months, precludes the use of PDXs as a patient ‘avatar’ for personalized drug testing. Furthermore, the human stroma in PDXs is rapidly replaced by murine stromal cells within the first passages, which could influence paracrine signaling: Some growth factors and cytokines are species-specific and therefore not able to bind and/or activate the receptors in the other compartment [187, 188]. The lack of a functional immune system, necessary to prevent rejection of patient tissue by the host organism, can also impact on the response to drugs and testing of immune-modulating therapies is currently not possible in these models. One strategy to improve the PDXs is therefore to humanize the model by reconstituting the mice with a human immune system [189], which should further improve the predictive value of these models. In chapter 3 of this thesis we report the generation of several new PDX and primary cell line models for pancreatic and esophagogastric cancer and show that these models can be used to study paracrine Hh signaling. These PDXs present a valuable addition to GEMM models in studying therapeutic interventions and to facilitate the identification of potential drug susceptibility and resistance biomarkers for pancreatic cancer patients.
The tumor microenvironment in PDAC

The dense stroma surrounding malignant epithelial cells is considered one of the histological hallmarks of PDAC. The stromal microenvironment, also often referred to as desmoplastic reaction, consists of numerous cellular and extracellular constituents that often make up the majority of the tumor bulk. Prominent cell types in the pancreatic cancer stroma include fibroblasts, stellate cells, immune cells, endothelial cells and nerve cells. The acellular stromal fraction is composed of a variety of fibrous proteins (e.g. collagen), polysaccharides (e.g. hyaluronan), glycoproteins (e.g. fibronectin), and a diversity of growth factors and proteases that form together a very dense extracellular matrix (ECM) [190] (Figure 5). Due to the relative abundance of ECM proteins and fibroblasts, the stroma is considered to function as a mechanical barrier to the tumor. A further consequence of this desmoplasia is the hypovascular nature of PDAC tumors. Increased interstitial pressure, caused by the dense matrix, leads to compression of existing capillaries and restricts formation of new vasculature [191], limiting the effective delivery of anticancer agents to pancreatic cancer cells [192].

In addition, other components of the pancreatic stroma ECM have been shown not only to impair vascular function, but also to have tumorigenic properties, by increase pancreatic cancer cell chemoresistance against gemcitabine and enhancing cancer cell proliferation [193-195]. Furthermore, it was shown that the stroma produces signals to affect epithelial stemness, induce EMT, and consequently aid in invasiveness and metastasis formation [196]. At the molecular level, stroma production is promoted by multiple cancer cell-derived signals such as transforming growth factor beta (TGF-β), hepatocyte growth factor (HGF), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), and Shh [197].

Pancreatic stellate cells (PSCs) play a central role in the formation of the fibrotic tumor stroma. They reside, in their quiescent state, in the space between acini and endothelial cells [198]. Upon activation by growth factors, cytokines or oxidant stress, PSCs transform into a myofibroblast-like phenotype characterized by the expression of alpha smooth

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**Figure 5.** Histology of pancreatic ductal adenocarcinoma. (A) Haematoxylin & eosin (H&E) staining of representative pancreatic ductal adenocarcinoma patient tissue shows abundance of stromal infiltration in the tissue (black arrows). White arrow indicates epithelial cancer cells. Insert shows higher magnification of selected area. (B) Picosirius red staining shows extensive collagen deposition (red) in the stroma surrounding the tumor cells. (C) Immunohistochemistry for stromal activation marker α-smooth muscle actin (brown). Nuclei are counterstained with haematoxylin. Scale bar in panels A, B indicates 1 mm, and 500 µm in C. Images courtesy of A.Steins.
Chapter 1

muscle actin (αSMA) and secrete excessive amounts of ECM components like collagen I, III, fibronectin and matrix remodeling enzymes such as matrix metalloproteases (MMPs) [199]. Several studies have shown that PSCs support tumor growth and metastatic spread, increase chemoresistance and impact on tumor vascularity [191, 200].

Taken together, all these observations have underlined the impact of tumor-stroma interactions on therapy-resistance and the key features of malignant progression in pancreatic cancer. In turn, this has led to the development of stromal targeting therapeutic approaches. Since stromal cells maintain intact intracellular signaling without oncogenic mutations, these cells are assumed to show better responses to therapeutic interventions compared to cancer cells. The combination of novel strategies targeting the stroma with conventional chemotherapy directed at cancer cells is therefore a promising rational to increase efficacy of therapeutic interventions.

**Stromal targeting therapies**

In recent years, the development of agents capable of targeting both the tumor and stroma in pancreatic cancer has been subject to intense research efforts. A number of agents directed against stromal proteins or signaling pathways have been tested, and these will be discussed in the following section.

One of the most promising new agents is nab-paclitaxel (Abraxane), an albumin-bound nanoparticle form of paclitaxel that needs no solvent for resuspension, and as a consequence of this has demonstrated an enhanced uptake and delivery to the tumor compared to classical taxanes [201, 202]. The proposed mechanisms by which this is achieved are active transport across endothelial cells via albumin receptor gp60/caveolin and active binding to secreted protein acidic and rich in cysteine (SPARC) [203, 204]. SPARC, also known as osteonectin, is highly expressed and secreted by peritumoral fibroblasts [205] and was proposed to serve as an albumin-binding protein that sequesters nab-paclitaxel to concentrate the drug intratumorally [206]. Interestingly, elevated expression of SPARC has been shown to correlate with poor prognosis in pancreatic cancer patients [205] and patients with high SPARC in the stroma, but not in the tumor epithelium, showed an increased overall survival after nab-paclitaxel treatment [207]. Additionally, preoperative nab-paclitaxel administration decreased collagen deposition and cancer-associated fibroblasts in resected specimens [208], highlighting the relevance of the stroma in general and stromal SPARC expression in particular for this therapeutic approach. Despite a recent mouse study arguing against a specific action of nab-paclitaxel on and through the stroma, nab-paclitaxel remains a potent and promising chemotherapeutic agent [209]. Following the very promising outcome of a big phase III trial in 861 pancreatic cancer patients, demonstrating that nab-paclitaxel and gemcitabine regimen improved overall survival from 6.7 months to 8.5 months when compared to singlet agent gemcitabine alone [210], nab-paclitaxel in combination with gemcitabine was approved by the FDA in September 2013 for first line treatment of metastatic pancreatic cancer patients [211] and will very likely replace gemcitabine monotherapy as a standard of care in Europe as well [212].
To target the stiff extracellular matrix in tumors containing hyaluronan, researchers have employed the enzyme hyaluronidase or the hyaluronan synthesis inhibitor 4-methylesculetin. Upon treatment, decreased rigidity in tumors was observed, which was accompanied by increased vascularization and sensitivity to gemcitabine in different animal models. Although not reported in all conditions, tumor growth decreased upon this stromal-targeting approach and survival of animals was extended [195, 213-215]. Also in a phase Ib clinical trial in advanced disease (stage IV) PDAC patients, administration of PEGPH20 (PEGylated recombinant human hyaluronidase) showed promising results with partial response in 43% and stable disease in additional 30% of the patients, with partial response rate even higher in patients with high level of hyaluronan [216]. This encouraging response rates led to further testing of PEGPH20 in combination with gemcitabine and nab-paclitaxel in a randomized phase II clinical trial.

A potential immune modulatory target in the tumor microenvironment is CD40, a costimulatory molecule found on antigen presenting cells (APCs) that is required for their activation by CD4+ T-helper cells and consecutive activation of cytotoxic effector T cells. Key studies showing the use of CD40 antibody to effectively stimulate APCs even in the absence of CD4+ helper cells, and successfully prime and activate CD8+ T cells [217]. Those promising preclinical studies have led to development of activating CD40 antibodies, which have been used in clinical trials. One study showed that combination of CD40 agonist with gemcitabine resulted in tumor regression in PDAC patients and mouse models. Interestingly, this effect was mediated by activated macrophages rather than CD8+ T cells and was accompanied by marked depletion of the cancer stroma [218]. In a small clinical phase I trial in advance PDAC patients testing CD40 antibody plus gemcitabine, 24% of patients had a partial response and 52% patients showed stable disease. Additionally overall survival was increased (7.4 months vs 5.7 months with gemcitabine alone) [219], warranting testing in phase II studies.

However, it is difficult to monitor these stromal targeting therapies in a non-invasive manner as no good stromal biomarkers exist at the moment. In chapter 6 of this thesis we describe ADAM12 as a stromal derived protein in pancreatic cancer that correlates with stromal content of the tumor. Furthermore we found that ADAM12 levels were elevated in the serum of PDAC patients and predicted poor clinical outcome, making it an interesting new biomarker for therapies aiming to ablate the tumor stroma.

**Hedgehog signaling in pancreatic cancer**

Multiple lines of evidence support the idea that Hh signaling functions in the maintenance and progression of pancreatic cancer. The Hh signaling pathway was shown to belong to one of the “core” signaling pathways that undergo somatic alterations in pancreatic cancers [136]. This is intriguing as Shh expression is markedly excluded from the developing pancreas, as well as the healthy organ in adults [220]. However, analysis of pancreata from patients with adenocarcinoma revealed that Shh is aberrantly expressed in 70% of the specimens and Hh signaling increases dramatically during progression of PanIN
lesions to metastatic tumors [141]. Further, ectopic expression of Shh under the control of the pancreatic and duodenal homeobox factor 1 (Pdx1) promoter, active in pancreatic progenitor cells, induces intestinal metaplasia in the pancreas accompanied by mutations in Kras [141, 221]. Activating the Hh pathway in pancreatic progenitor cells by using a constitutive active Gli2 (CLEG) is sufficient to drive early-stage pancreatic neoplasia, but simultaneous activation of Ras signaling accelerates PDAC development with several characteristics reminiscent of human pancreatic cancer [222].

Human pancreatic cancer cell lines also produce Shh and Ihh as well as target genes such as Gli1 and Ptch1, which was initially thought to be indicative for ongoing pathway activity. Proliferation of some of these cell lines can be blocked by the Smo antagonist cyclopamine both in vivo and in vitro [138, 141, 223, 224]. Furthermore, inhibition of Hedgehog signaling with the Smo antagonist cyclopamine or the Hh-blocking antibody 5E1 can reduce the incidence of systemic metastasis, reduce desmoplasia and prolong survival in a pancreatic adenocarcinoma xenograft model [223, 225, 226]. These data suggest that active Hh signaling is a critical mediator of pancreatic cancer development in both early and later stages.

However, many PDAC cell lines were found to be unresponsive to treatment with Smo inhibitors, and typically very high doses of inhibitor were needed to achieve some cytotoxic effects, although most cell lines did show expression of Gli target genes suggesting activation of the pathway downstream of Smo [227, 228]. With respect to this observation, activation of TGF-β signaling via Smad3 has been shown to be a Smo-independent inducer of Gli activity and pharmacologic blockage of TGF-β signaling decreases cell proliferation in pancreatic adenocarcinoma cell lines resistant to Hh pathway inhibition [229, 230]. Similar results were found for oncogenic KRAS, which can also activate Hh signaling in the absence of additional Hh ligand during pancreatic tumorigenesis [231].

In contrast to the previously proposed cell-autonomous role for Hh-mediated tumorigenesis, recent studies provided compelling evidence supporting a paracrine signaling model, where Hh ligands produced by tumor cells act on the stromal compartment to support tumor growth indirectly [227, 228]. Supportive of this paracrine model is the observation that transformed epithelial cells in the pancreas are devoid of primary cilia, the organelle necessary to transduce the Hh signal to the downstream effectors, and therefore are unable to respond to Hh ligands by activating the canonical pathway [232]. Additionally, mutant KRAS was found to induce Shh expression in pancreatic cancer epithelium, likely in cooperation with NFκB signaling [233, 234], and at the same time represses autocrine signaling suggesting that KRAS activation is involved in shifting Hh signaling from an autocrine to a paracrine mode leading to stroma activation [235]. After paracrine activation of the Hh signaling pathway in the stromal compartment by tumor cell derived Hh ligands, the tumor microenvironment is thought to signal back to the tumor cells, positively influencing tumor growth, perineural invasion, angiogenesis and metastasis formation [225, 227, 228, 236, 237]. However, the nature of the paracrine signals produced by stromal cells in response to Hh pathway activation is largely unknown. In chapter 5 of this thesis we employed next-generation sequencing in combination with a new co-culture method to identify several
of these stromal derived factors. In our screen and further validation we show that *PLAUR*, *SPOCK1*, *EDIL3*, and *CDA* are promising Hh-regulated factors that are expressed in the stroma of patient tumor and associate with poor clinical outcome.

**Hh targeted therapy in PDAC**

Olive and colleagues demonstrated that depletion of the stroma by administration of the Smo-inhibitor saridegib (IPI-926, Infinity), improved delivery of gemcitabine by increasing intratumoral vasculature density and therefore increased anti-tumor effects as well as survival with the combination treatment in a genetically engineered mouse model of PDAC [238]. Based on these promising pre-clinical results, phase II clinical trials were performed evaluating saridegib or alternatively vismodegib (GDC0449) in combination with gemcitabine in pancreatic cancer patients. Unexpectedly, vismodegib combination failed to show any improvement in progression-free or overall survival compared to gemcitabine alone, and in the saridegib trial even higher rates of progressive disease were observed in the Hh inhibitor treated patient cohort compared to placebo, leading to the discontinuation of the trial after interim analysis [239, 240]. Although Hh inhibitors have been successfully used for treating BCC and medulloblastoma, results in pancreatic cancer patients were disappointing and did not meet the high expectations placed on these drugs. However, a more recent trial combining vismodegib with gemcitabine and nab-paclitaxel was potentially positive, possibly due to greater potency of cytotoxic regimen and several other trials using Hh inhibitors in pancreatic cancer patients are still ongoing [241].

One explanation for the discrepancy between pre-clinical mouse models and the failure of Hh inhibitors in pancreatic cancer patients may be the short duration of treatment (3 weeks) in the experimental mouse models, which may have missed long-term effects and disease progression. Two more recent studies addressed the effects of extended Hh blockage by either genetic deletion of Shh in the epithelial compartment of genetically engineered mouse models of pancreatic cancer or long-term treatment with Hh inhibitors in these animal models [242, 243]. In both studies, the authors found smaller, but more aggressive and poorly differentiated tumors with increased vascularity and as a result faster disease progression in the Hh deficient animals, reflecting the outcome of the phase II clinical trials. Another important conclusion from these two studies was that the desmoplastic stroma, that was found to be reduced after Hh blockage, has a restraining rather than supportive function and prevents progression of disease, shaking up the paradigm of the tumor promoting role of the PDAC stroma. Corroborating these finding about the role of the stroma in PDAC, Özdemir and colleagues deleted stromal fibroblast by genetically targeting αSMA positive cells in a mouse model of PDAC, and also found more invasive, undifferentiated tumors and reduced survival of animals after stromal depletion [244]. On the other hand, ablation of a subpopulation of stromal cells expressing fibroblast activation protein (FAP) permitted immune control of tumor growth and revealed the efficacy of immunotherapeutic antibodies (anti-CTLA-4 or anti-PD-L1), which resulted in acute tumor regression in the same mouse models [245].
The complexity of these findings reflects our incomplete understanding of the role of the tumor microenvironment in disease progression in general and more specifically the impact of Hh signaling on the pathobiology of pancreatic cancer. Work provided in this thesis will aid future research efforts to gain more detailed insight into tumor-stroma crosstalk and the consequence thereof on tumor biology.

REFERENCES


69 Dwyer JR, Sever N, Carlson M, Nelson SF, Beachy PA, Parhami F: Oxysterols are novel activators of the hedgehog signaling pathway.


89 Cheng SY, Bishop JM: Suppressor of Fused represses Gli-mediated transcription by recruiting


140 Yuan Z, Goetz JA, Singh S, Ogden SK, Petty WJ, Black CC, Memoli VA, Dmitrovsky E, Robbins DJ:


161 Iacobuzio-Donahue CA: Genetic evolution of pancreatic cancer: lessons learnt from the
pancreatic cancer genome sequencing project. 


211 Yao S: FDA approves Abraxane for late-stage pancreatic cancer. In *Book FDA approves Abraxane for late-stage pancreatic cancer* (Editor ed.^eds.). City; 2013.

1


239 Madden JJ: Infinity Reports Update from Phase 2 Study of Saridegib Plus Gemcitabine in Patients with Metastatic Pancreatic Cancer. In Book Infinity Reports Update from Phase 2 Study of Saridegib Plus Gemcitabine in Patients with Metastatic Pancreatic Cancer (Editor ed.^eds.). City; 2012.

