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Review

Molecular Biology of Pancreatic Cancer – New Aspects and Targets

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Abstract. *Pancreatic ductal adenocarcinoma is a dismal disease with a median survival of less than 6 months and an overall 5-year survival rate less than 1% . This bad prognosis is due to early lymphatic and hematogenic dissemination. Effective therapies for locally advanced or metastatic tumors are very limited and curatively resected patients experience relapse in over 80% of cases. Together, these findings reflect the aggressive biology of the disease. Here, we describe molecular mechanisms leading to unrestrained proliferation, insensitivity to growth inhibitory signals, evasion of apoptosis, limitless replicative potential, tissue invasion, metastasis and sustained angiogenesis. Potential therapeutic targets are highlighted.*

Pancreatic ductal adenocarcinoma (PDAC) is a chronic disease resulting from defective genome surveillance and signal transduction mechanisms. Key cellular processes, which were summarized by Hannahan and Weinberg as self-sufficiency in growth signals, insensitivity to growth inhibitory signals, evasion of apoptosis, limitless replicative potential, tissue invasion and metastasis and sustained angiogenesis contribute to the emergence of this neoplasia and its malignant progression (1). This review will cover important new aspects of the molecular biology of the disease. For a comprehensive overview of the molecular biology of pancreatic ductal adenocarcinoma, we refer the reader to recent excellent reviews (2-4).

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Growth Signals

Self-sufficiency in growth signals in PDAC is ensured on multiple levels, namely autocrine production of growth factors, overexpression of growth factor receptors, activation of oncogenes and inactivation of tumor suppressor genes. The epidermal growth factor (EGF) system is a good example of how autocrine stimulation contributes to the self-sufficiency in growth signals of PDAC cells. Overexpression of EGF receptors (ErbB1/EGFR and ErbB3) is found in the vast majority of PDAC. Together with the overexpressed ligands, EGF and TGFα, a signaling circuit is built, driving unrestrained cell cycle progression (5-8). In line with this fact, EGF receptor antagonists are now used for the treatment of pancreatic cancer patients. In addition to the EGF system, other growth factor systems such as the IGF, HGF or FGF system contribute to the carcinogenesis of pancreatic cancer (9).

Synergistic to the growth factor receptor/ligands circuits, oncogenes and inactivated tumor suppressor genes promote the proliferation of PDAC. The *K-RAS* oncogene is mutated in nearly all human PDACs. Since *K-RAS* codon 12 mutations are found in early pancreatic preneoplastic and neoplastic lesions and oncogenic *K-RAS* induces PDAC in mouse models, the *K-RAS* mutation is an initiating event in the pathogenesis of pancreatic cancer (10, 11). Oncogenic *K-RAS* is linked to the cell cycle machinery of PDAC cells by the activation of multiple mitogenic signaling pathways, notably the PI3K, RAF-mitogen-activated kinase (MAPK) and the NF-κB pathway. Therefore, targeting K-RAS expression was shown to reduce proliferation of PDAC cells (12).

PDAC cells are characterized by a profound acceleration of the G1-phase to S-phase progression due to a functionally

inactivated retinoblastoma (RB)-dependent G1-phase checkpoint (13). Multiple alterations contribute to RB inactivation in PDAC cells. Here, the frequent overexpression of cyclins, such as cyclin D1, cyclin D3, cyclin A and cyclin E, as well as cyclin-dependent kinases, such as CDK2 and CDK4, was recently described in PDAC (14, 15). High cyclin E expression, occurring in around one third of PDAC, is an independent predictor of patient outcome, pointing to the importance of this cell cycle regulator (16). At the genetic level, RB inactivation is explained by the frequent mutation of the tumor suppressors *INK4A* and *TP53*. INK4A blocks S-phase progression by inhibiting the cyclin-dependent kinases CDK4 and -6, leading to RB activation. Loss of INK4A expression, mediated by mutation, deletion or promoter hypermethylation is observed in up to 95% of sporadic PDAC (13). The sequence-specific transcription factor p53 is activated by γ-irradiation, DNA-damage or oncogene activation. Activated p53 is involved in cell cycle control, induction of apoptosis or the senescent program. The *TP53* gene is mutated in greater than 50% of PDACs (17). Since the pan-CDK-inhibitor $p21^{Cip1}$ is a p53 target gene, *TP53* mutations enable G1- to S-phase progression under conditions of oncogenic stress conditions.

In addition to $p21^{Cip1}$, another pan-CDK inhibitor, $p27^{Kip1}$, is critically involved in controlling the G1- to S-phase progression of PDAC cells. Pancreatic cancer cells express little or no $p27^{Kip1}$, suggesting contribution of this pan-CDK-inhibitor to pancreatic carcinogenesis (18-20). Regulation of $p27^{Kip1}$ is complex and although $p27^{Kip1}$ is controlled at the level of synthesis, the protein abundance is regulated mainly by posttranslational modifications, affecting $p27^{Kip1}$ protein turnover. Phosphorylation of $p27^{Kip1}$ on Thr-187 by the cyclin E-CDK2 complex tags the protein for recognition by S-phase kinase associated protein 2 (SKP2), an F-box protein that functions as a receptor component of the SCF ubiquitin ligase complex, resulting in p27Kip1 ubiquitination and degradation (21). High level SKP2 expression was observed in about one third of pancreatic cancer specimens and is an independent predictor of patient outcome (22). Even though SKP2 protein stability is regulated, recent work demonstrate that transcriptional control is important for SKP2 regulation in PDAC. Two important signaling pathways, namely the NF-κB and the PI3K pathway, are integrated at the *SKP2* gene promoter and both pathways are known to lower $p27^{Kip1}$ expression in PDAC cells (23-25). In addition to NF-κB and PI3K signaling, $p27^{Kip1}$ protein abundance is lowered by the jun activation domain-binding protein (JAB1), an AP1 co-activator. JAB1 is overexpressed in 100% of pancreatic cancers and lowers $p27^{Kip1}$ expression in a SKP2-independent fashion (26). Whether the miRNAs miR-221 and miR-222, shown to be highly expressed in pancreatic cancer and potentially targeting $p27^{Kip1}$ mRNA, contribute to low $p27^{Kip1}$ expression awaits further functional investigations (27). Together, these data show that PDAC cells use different pathways to assure low $p27^{Kip1}$ expression. Therefore, increasing $p27^{Kip1}$ is a promising approach for new therapeutic strategies.

Recently the transcription factor "nuclear factor of activated T-cells", NF-ATc1, commonly overexpressed in PDAC and controlled by the Ca^{2+}-sensing phosphatase calcineurin, was shown to contribute to growth control by inducing the transcription of oncogenic c-myc (28). Therefore, targeting calcineurin may offer therapeutic intervention in the future.

In addition to the mentioned genetic alterations, proliferation of pancreatic cancer cells is controlled by the epigenetic machinery. In particular, histone deacetylases (HDACs) promote proliferation of pancreatic cancer cells. HDACs catalyze the removal of acetyl groups from histones which results in chromatin condensation and transcriptional repression (29). Treatment of pancreatic cancer cells with HDAC inhibitors induces a cell cycle arrest in the G1- or G2/M-phases in pancreatic cancer cells (30-32). Notably, the HDAC inhibitor suberoylanilide hydroxamic acid (SAHA), inhibiting HDAC 1, 2, 3 and 6, was shown to cease proliferation of pancreatic cancer cells by down-regulating the cell cycle-promoting proteins cyclin B1, cyclin D1 and c-myc (33, 34). Since HDAC 3 was recently shown to accelerate the proliferation of the pancreatic cancer cell line MiaPaCa2, HDAC 3 might be the target for the growth inhibition of pancreatic cancer cells induced by HDAC inhibitors (35).

The PI3K pathway is crucial to many aspects of cell growth and survival and is active in around 60% of pancreatic cancer cases (36, 37). PI3K/AKT signaling regulates chemotherapeutic resistance and proliferation of pancreatic cancer cells (23, 25, 38-43). Although PI3K signaling induces proliferation of pancreatic cancer cells, the mode of activation of the PI3K pathway is not entirely clear. Here, the tumor suppressor phosphatase and tensin homolog deleted in chromosome ten (PTEN) and the insulin receptor substrate 1 (IRS-1) are known to contribute to PI3K activation (44, 45). Although oncogenic *K-RAS* activates PI3K-AKT signaling, a novel and unexpected pathway was recently shown to mediate the activation of PI3K-AKT signaling in pancreatic cancer cells (Figure 1). This pathway is regulated by the architectural transcription factor HMGA1. HMGA1 is highly overexpressed in pancreatic cancer and known to account for the unrestrained proliferation of pancreatic cancer cells (46-48). Recently, it was shown that HMGA1 activates PI3K-AKT signaling of pancreatic cancer cells (49, 50). Activation of PI3K-AKT signaling is likely downstream of the insulin receptor, whose transcription is controlled by an HMGA1 and C/EBPβ containing complex in pancreatic cancer cells (51). Finally, the HMGA1-insulin receptor pathway increases cyclin D1 translation to promote proliferation (51). The new aspects of PI3K-AKT signaling in pancreatic cancer cells are summarized in Figure 1.

A further pathway clearly increasing proliferation of pancreatic cancer cells is that of sonic hedgehog signaling (52). This pathway is activated by the NF-κB-dependent aberrant expression of the ligand sonic hedgehog and acts at multiple stages during pancreatic carcinogenesis (53-55). Interfering with sonic hedgehog signaling ceases proliferation of pancreatic cancer cells by the induction of a G1-phase arrest (56). At the molecular level, sonic hedgehog signaling transcriptionally induces cyclin D1 expression and reduces $p21^{Cip1}$ expression, explaining the effect of this pathway towards the G1-phase of the cell cycle (57). Since small molecules exists which interfere with sonic hedgehog signaling, this pathway is a suitable target for future therapies (58).

Insensitivity to Growth Inhibitory Signals

The best-documented signaling molecule with antigrowth properties is transforming growth factor (TGF) β. During carcinogenesis, TGFβ displays functional duality: it inhibits the growth of early malignant lesions, whereas proliferation of advanced tumors is promoted (59). TGFβ inhibits proliferation of normal epithelial cells by inducing the expression of the cyclin-dependent kinase inhibitors $p15^{INK4b}$ and $p21^{Cip1}$, or by repression of the *c-myc* gene. The SMAD transcription factor family transfers TGFβ signaling to the nucleus (60). In pancreatic cancer, the TGFβ-mediated growth inhibitory signal is frequently lost (61). In part, this loss is due to the mutation of the *MADH4/SMAD4/DPC4* gene. Loss of heterozygosity for the *SMAD4* gene locus is found in up to 90% of pancreatic cancers while no SMAD4 expression is found in about 50% (62, 63). In addition to *SMAD4* mutation, PDAC cells underexpress the type 1 TGFβ receptor and overexpress the inhibitory SMADs, SMAD6 or SMAD7 (64-67). These alterations result in increased mitogenic signaling of the TGFβ pathway together with loss of growth inhibitory function. Furthermore, the TGFβ signaling pathway, activated by autocrine production of TGFβ, induces invasiveness of PDACs, mediated by target genes such as *MMP-2, uPA* or *CUTL1* (68-70). Moreover, angiogenesis is controlled by the TGFβ-SMAD pathway, since SMAD4 represses the vascular endothelial growth factor (VEGF) and induces the angiogenesis inhibitor thrombospondin 1 (TSP1) in PDAC cells (71).

Tumor suppressive function of the TGFβ pathway in the carcinogenesis of the pancreas is best proven in the conditional $K\text{-}Ras^{G12D}$ mouse model. As well as the conditional deletion of *MADH4/SMAD4/DPC4* in the pancreas as the blockage of TGFβ signaling by conditional deletion of the type II *TGFβ receptor* synergizes with $K\text{-}Ras^{G12D}$ to induce pancreatic tumors (72-74).

Evasion of Apoptosis

The apoptosis sensing, inducing and executing machinery is regulated at multiple levels, whereby every level can be disturbed in cancer cells, leading to an apoptosis resistant phenotype (75). In pancreatic cancer cells, the executing machinery is intact and resistance mechanisms have evolved working especially at the death receptor, mitochondrial and caspase inhibitor levels (76, 77). Most pancreatic cancer cells are resistant towards CD95L- or TRAIL-mediated apoptosis, although they express the corresponding death receptors. Several strategies are exploited in pancreatic cancer cells to evade death receptor-induced apoptosis, ranging from the overexpression of the decoy receptor 3 (DcR3), a soluble receptor for the Fas-ligand, to the overexpression of c-Flip, which is a potent inhibitor of caspase 8 activation (78, 79). Additionally, the death receptor system is not only blocked, but also abused by the pancreatic tumor cells to switch the death signal towards an invasion signal. Recently, the death receptor signaling intermediate TRAF2 was shown to be overexpressed, switching the CD95 signal from apoptosis to the induction of invasiveness in pancreatic cancer cells (80).

At the mitochondrial level, the fine-tuned expression of pro-death and pro-survival Bcl-2 family members sense and decide about live or death of a cell (81). In contrast to Bcl2, whose expression is not significantly altered, Bcl_{xL} is overexpressed in pancreatic cancer cells, suggesting an important role for this pro-survival Bcl2-family member in apoptosis-resistance of pancreatic cancer cells (82, 83). A second pro-survival Bcl-2 family member, Mcl-1, is overexpressed and regulates survival of pancreatic cancer cells (84, 85). Functionally, Mcl-1 was recently linked to the integrative stress response (86). Therefore, high Mcl-1 might protect pancreatic cancer cells from hypoxia and oxidative stress, conditions frequently found in the tumor microenvironment.

The third level of apoptosis resistance is conferred by the IAP protein family, including cIAP1, cIAP2, XIAP and survivin. One molecular action of this protein family is to inhibit executioner caspase activity. At least for the family members XIAP and survivin, increased protein abundance was demonstrated in pancreatic cancer cells (87-90). Interestingly, survivin expression is cell cycle-dependent, being low in the G1-phase in PDAC cells. In line with this, a sensitization towards TRAIL-induced apoptosis by the arrest of pancreatic cancer cells in the G1-phase of the cell cycle was observed (91). Furthermore, TRAIL resistance of pancreatic cancer cells is known to be regulated at the level of XIAP (92). In addition to TRAIL, targeting XIAP was shown to sensitize pancreatic cancer cells for gamma-irradiation and gemcitabine (90, 93). Therefore, a combined sensitizer (G1-phase arrest or XIAP targeting)/inducer (TRAIL/gamma-irradiation/ chemotherapy) strategy may be a therapeutic approach for the future. While TRAIL treatment is attractive due to its tumor selective mode of action, recent experiments demonstrate a protumoral and metastasis-inducing function of TRAIL for PDAC cells *in vivo*, which might limit clinical applications (94).

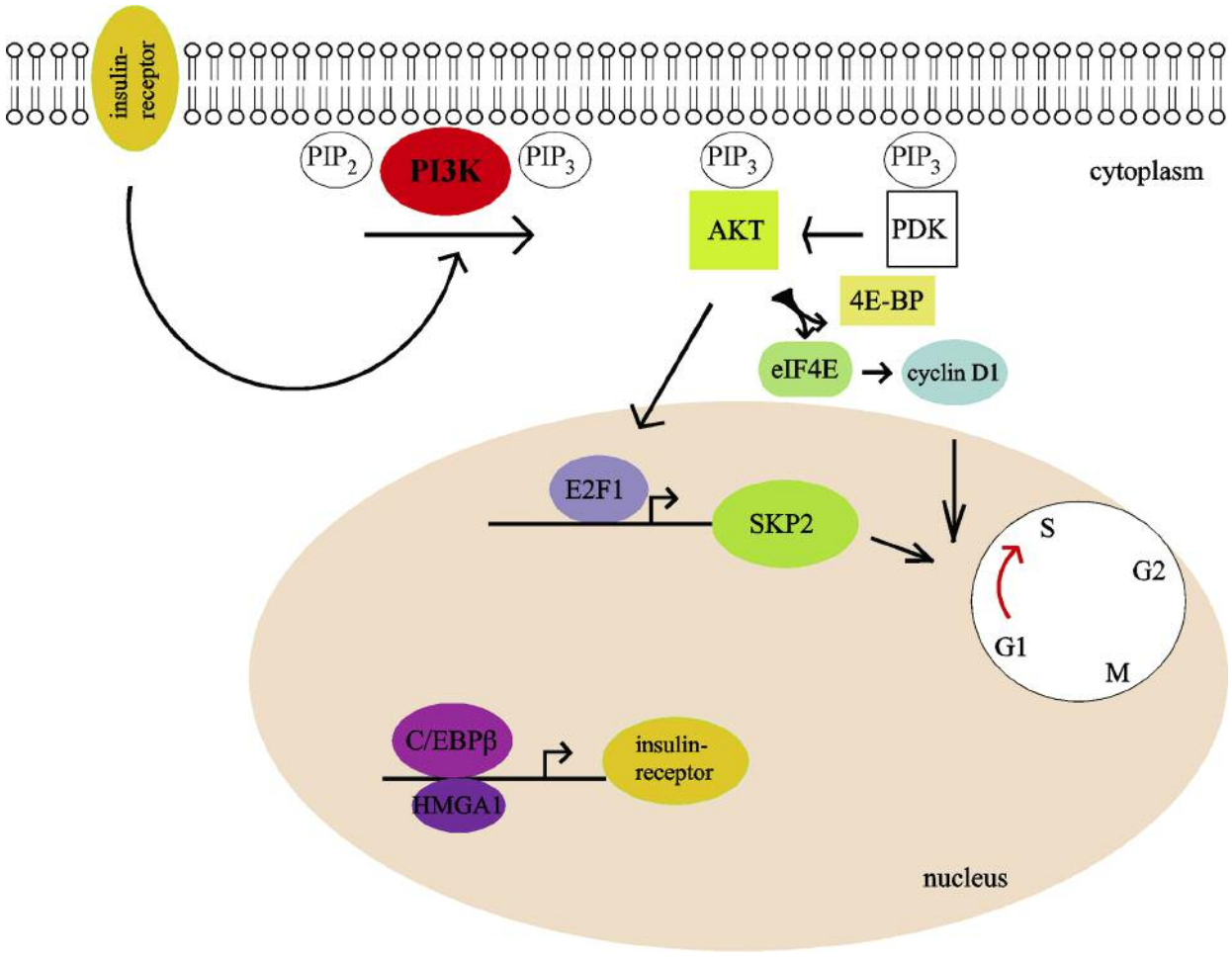

Figure 1. *New aspects of PI3K-AKT signaling in pancreatic cancer cells. An HMGA1 and C/EBPβ-containing complex activates the insulin receptor gene in pancreatic cancer cells. Insulin receptor signaling activates PI3K-AKT signaling and augments cyclin D1 translation by influencing the binding of translation inhibiting factor eIF4E binding protein, 4E-BP, to the translation initiation factor eIF4E. In addition, PI3K-AKT signaling is linked to the cell cycle by the transcription factor E2F1, controlling S-phase-promoting genes such as SKP2.*

Many of the gene products mediating apoptosis resistance are regulated by the NF-κB transcription factor family in PDACs, pointing to an important contribution of NF-κB towards apoptosis and chemotherapeutic resistance (95, 96). Recently, the *GADD45α* gene was shown to be downstream of an IκBα-regulated pathway in PDAC. GADD45α acts in a non-cancerous environment as a pro-apoptotic protein and was surprisingly found to be overexpressed at the mRNA and protein levels in PDAC, mediating proliferation and apoptosis resistance in a so far unknown molecular pathway (97).

Limitless Replicative Potential

Mammalian cells carry an intrinsic, cell autonomous program which limits the possible number of mitoses. The deactivation of these failsafe programs is a prerequisite of cancer and senescence is an important mechanism for restricting the replicative potential (98). Senescence, a permanent growth/cell cycle arrest that occurs after extended periods of cell division, oxidative stress or activated oncogenes, is clearly induced by *K-RAS* in non-immortal human and mouse cells. Senescent cells are characterized by an active metabolic state and altered morphology, physiology and gene signature. These cells typically show a senescence-associated β-galactosidase activity and are unable to express genes needed for cell cycle progression, even in a mitogenic environment (99). The ARF-p53 and the $p16^{INK4A}$-RB tumor suppressor systems are critically involved in the molecular regulation of oncogene-induced premature senescence; the relative contribution of each system differs significantly among species and tissues (99). In recent years, the definitions of markers for senescence, such as $p15^{INK4b}$, DCR2 and DEC1, have helped to demonstrate the senescence concept *in vivo* (100). Since senescence markers are found predominantly in premalignant lesion (PanINs) in a

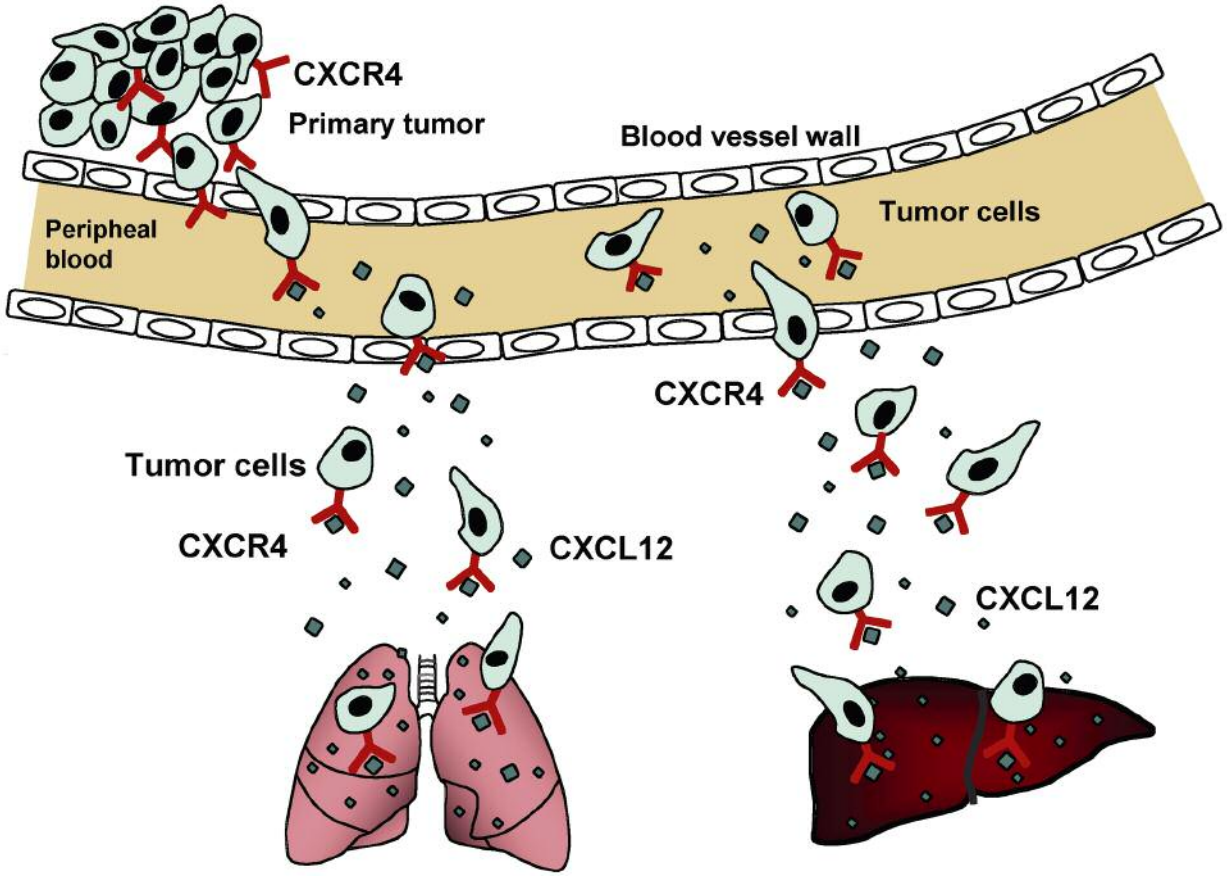

Figure 2. *Targeted metastasis of primary pancreatic cancer cells to the liver and lung. Metastasis is a non-random, highly organ-specific multistep process that requires distinct interactions between tumor cells and the host. First, pancreatic cancer cells of the primary tumor proliferate and induce angiogenesis. Tumor cells then detach from the primary tumor, invade into the host stroma, intravasate into lymph and blood vessels thus entering the systemic circulation. The chemokine CXCL12 that recognizes the chemokine receptor CXCR4 expressed on the surface of pancreatic cancer cells is released in high quantities only by certain organs, such as the liver and lung. Other organs, such as the brain contain only low amounts of CXCL12. Binding of CXCL12 to CXCR4 induces the migration of cancer cells into normal tissues such as the liver and lung, where the cells form metastatic tumors.*

mouse tumor model that targets the *K-RasV12* oncogene to the pancreas, senescence is an early event during carcinogenesis of the pancreas (101). This might explain the frequent alterations found in the *CDKN2A* locus, the *TP53* locus and the functionally inactivated RB in PDAC cells.

Invasion and Metastasis

Metastasis plays a major role in pancreatic cancer morbidity and the vast majority of human pancreatic cancer deaths are caused by the formation of metastases. Metastasis is a nonrandom, highly organ-specific carcinogenic process that requires multiple steps and interactions between tumor cells and the host, such as detachment of the tumor cells from the primary tumor, invasion into the host stroma, intravasation into lymph and blood vessels, survival in the circulation, extravasation into target organs and subsequent proliferation and induction of angiogenesis (102). A central mechanism of metastasis is the change from a highly differentiated epithelial cell morphology to a mesenchymal phenotype. This process is called epithelial-mesenchymal transition (EMT). During EMT, an epithelial cell loses polarity and intercellular adhesions and acquires a fibroblastoid phenotype. Furthermore, the transcriptome and proteome is changed from epithelial towards a mesenchymal profile, leading to expression of mesenchymal markers such as vimentin, N-cadherin, Snail and Twist, whereas epithelial markers, such as E-Cadherin, are lost (103). EMT leads to a more invasive, migratory phenotype, a prerequisite of metastasis. The molecular mechanisms of EMT have not been investigated in detail for PDACs. At least in cell lines with an intact TGFβ pathway, TGFβ signaling is known to induce a mesenchymal phenotype (104). Furthermore, signaling induced by the extracellular matrix is important for the down-regulation of E-cadherin, a central player in the

formation of epithelial polarity and organization, and increased invasion and proliferation of PDAC cells (105, 106). E-cadherin mutations or loss of E-cadherin expression is observed in 50-60% of primary sporadic PDAC and is significantly associated with lymph node and liver metastasis (107, 108). In contrast to E-cadherin, N-cadherin is up-regulated by oncogenic K-RAS and functions as an important modulator of migration and invasion of PDAC cells (109). The transcription factors involved in EMT of pancreatic cancer cells are mainly unknown, but recent evidence demonstrates the contribution of SP1 and Snail (110, 111).

Although liver and lung metastasis are key prognostic markers of PDAC, mechanisms leading to the homing of pancreatic cancer cells are largely unknown. In a recent report, the influence of the chemokine receptor CXCR4 for targeting pancreatic cancer to the liver and lung was demonstrated. Furthermore, a small molecule antagonist of CXCR4, AMD 3100, blocked targeted metastasis, offering the opportunity for a novel antimetastatic therapy (112). Figure 2 illustrates CXCR4-mediated metastasis to the liver and lung.

Angiogenesis

The process by which a new blood supply is built from existing vessels is termed angiogenesis. This process is important for tumors to grow greater than 1 to 2 mm, the distance oxygen is supplied by diffusion. Vessel density is increased in PDAC compared to normal pancreas (113). High vessel density is associated with a poor prognosis after curative resection and represents a risk factor for metastasis (113-116).

Angiogenesis is regulated by secreted pro- and antiangiogenic factors. Tumor cells as well as tumor stroma cells secrete proangiogenic factors, such as growth factors, cytokines or chemokines. The VEGF system plays a central role in regulating tumor angiogenesis (117). This system is regulated by hypoxia, a characteristic feature of the tumor microenvironment. Hypoxia is sensed by the transcription factor HIF1, containing a constitutive nuclear subunit HIF1β and hypoxia-regulated subunit HIF1α. HIF1 targets genes important in increasing oxygen tissue levels, such as erythropoietin, glycolytic pathway enzymes, carbonic anhydrase, heme oxygenase and VEGF-A. In addition, HIF1 upregulates CXCR4 expression, thereby linking angiogenesis and tumor metastasis. Under normoxia, HIF1α is ubiquitinated by the E3 ubiquitin ligase and tumor suppressor von Hippel-Lindau (pVHL) and proteasomal degraded. HIF1α mRNA level is upregulated in pancreatic ductal adenocarcinoma and positively correlates with VEGF-A mRNA expression (118, 119). Although it has been demonstrated that targeting the VEGF system in preclinical models suppresses the tumorigenic growth in xenografts, a phase III study of the Cancer and Leukemia Group B 80303 trail failed to demonstrate significant differences regarding overall survival using the recombinant, humanized anti-VEGF monoclonal antibody bevacizumab combined with gemcitabine (120-122). This points to a complex regulation of tumor angiogenesis in PDAC. Correspondingly, the overexpression of potential angiogenic growth factors EGF, TGFα, FGF-2, PDGFβ and chemokines IL-8 and CCL20 has been demonstrated in PDACs (123, 124).

Conclusion

Although our knowledge of the molecular basis of PDAC has increased during the past decade, an effective treatment is still missing. It is not time to raise the white flag, it is time to develop novel therapeutic strategies which should focus on signaling circuits which lead to unrestrained proliferation, insensitivity to growth inhibitory signals, evasion of apoptosis, limitless replicative potential, tissue invasion, metastasis and sustained angiogenesis. The FDA approval of Sunitinib, an oral small molecular tyrosine kinase inhibitor, for the treatment of advanced renal cell cancer demonstrates: (i) that such an approach is feasible and (ii) that therapy refractory solid cancers are treatable.

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