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Relevance of the stroma in pancreatic ductal adenocarcinoma and its challenges for translational research

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ABSTRACT

Pancreatic ductal adenocarcinoma (PDAC) is the fourth most lethal tumor around the world and stands out from many other cancers. Despite increasing research for better diagnostic and treatment strategies, there has hardly been a substantial improvement over the last decades in the 5-year survival. This is mainly because, at the time of diagnosis, the cancer cells have already migrated and invaded distant organs and tissues. At this stage, patients in most cases are ineligible for surgery and their response to conventional therapies, such as radiotherapy and chemotherapy, is also very poor.

Researchers have mainly been targeting the genetic alterations of the cancer cells, and these genetic therapies in pancreatic cancer have significantly failed to improve patient survival. It is true that the early stages of tumor formation are based on a combination of genetic and epigenetic alterations that activate oncogenes and/or inhibit tumor suppressor genes, but progress in PDAC is mainly orchestrated by microenvironmental factors. Recently, targeting tumor microenvironment components that play a prominent role in tumor progression (fibrous tissue, angiogenesis, hypoxia, tumor-associated macrophages, etc.) have begun to attract the focus of cancer researchers. In addition, immunotherapy, which has shown some success in other types of cancer, is now also emerging for pancreatic cancer for which the microenvironment possess specific challenges.

This review highlights current obstacles and opportunities in pancreatic cancer research and treatment (in vitro and in vivo (patient-derived tumor xenografts and genetic engineered mouse models)) and also indicates future directions to be explored as a potential strategy to improve patient outcomes.

Introduction: pancreatic cancer stands out from most other major forms of cancer

Pancreatic ductal adenocarcinoma (PDAC) is the most common (90%) among the tumors that originate from the pancreas[1,2] and remains one of the main causes of cancer-related death in the Western world. At this moment, the highest incidence of pancreatic cancer is in Northern America and Europe, and the lowest incidence is found in Africa and Asia[3]. In the Western world, pancreatic cancer ranks as the fourth most important cause of cancer-related death (3th in the USA). The variation in the incidence of pancreatic cancer in parts of the world is attributed to differences in exposure to known or suspected risk factors or is indirectly related to life style or socioeconomic values. There are several factors that can increase the risk of acquiring pancreatic cancer: obesity, diabetes,

high fat-content of the diet, smoking, heavy consumption of alcohol, low fruit, and folate intake, long-term red meat consumption, Helicobacter pylori infection, Non-O blood groups[1,4].

In contrast to most of the other major cancers (breast, colon, etc.), the 5-year relative survival rate has over the past decades not improved and is currently still less than 8%. This low survival figure is partly because more than 50% of cases are diagnosed at the late stage of the disease and furthermore the poor response of PDAC to the conventional therapy[5,6]. The only potential curative treatment for PDAC is local surgical resection, unfortunately, at the time of diagnosis; more than 85% of patients have already developed a metastatic tumor which is not eligible for resection[7,8]. In the coming few years there is no substantial improvement to be expected. Rather, before 2025, the number of deaths from pancreatic cancer is predicted to become 25% higher than those from breast cancer and the rate keeps increasing. Reports further predict that by 2030, the total number of deaths due to pancreas cancer is expected to increase dramatically making it the second leading cause of cancer-related deaths in the Western world[2,9-12]. In contrast, the overall cancer-related deaths for most other major cancer types are expected to decrease significantly due to changes in screening and prevention programs and/or improved treatment strategies[9,13].

Pancreatic ductal adenocarcinoma arises from the malignant transformation of cells in the exocrine part of the pancreas. Clinical and histopathological studies have identified three morphologically distinct noninvasive precursor lesions of invasive human PDAC: pancreatic intraepithelial neoplasms (PanIN), intraductal papillary mucinous neoplasms (IPMN), and mucinous cystic neoplasms (MCN). Of these the PanIN lesions, which are found in the smaller-caliber pancreatic ducts, have been hypothesized as the major pre-invasive precursor lesions from which invasive PDAC will arise (85%) and up to 15% are thought to arise from IPMN/cystic neoplasms[14].

PDAC has a unique tumor microenvironment that is important for its specific behavior with an abundance of inflammatory cells, limited effector immune cells, fibroblast cells and cancer cells[15]. Here the interaction of the tumor cells with the surrounding stroma plays a major role (with both suppressive and pro-tumoral effects) for therapeutic resistance.

Microenvironment of PDAC

Pancreatic ductal adenocarcinoma as mentioned above, has a unique microenvironment and represents the most stroma-rich type of cancer. This stromal microenvironment in pancreatic cancer can comprise up to 90% of the tumor mass and is not only a passive scaffold for the tumor cells but it can also nurture tumor cells and shield these cells from chemo and immune therapy by forming thick connective tissue layers around them[16,17]. In addition, the tumor microenvironment provides the malignant cancer cells with all necessary signals that helps them to migrate and invade the surrounding tissues demonstrating a phenotypic shift from epithelial to mesenchymal cell phenotype through the process of Epithelial-to-Mesenchymal Transition (EMT) which is a another major challenge in PDAC treatment. This transition involves extensive remodeling and activation of several cell signaling pathways and differentiation programs in the cancer cells that accelerate tumor progression and drug resistance[18-22].

The stroma is very heterogeneous and consists of cellular and acellular components, such as fibroblasts, myofibroblasts, immune cells, blood vessels, extracellular matrix and soluble proteins such as cytokines and growth factors[15]; together forming a comfortable microenvironment for the pancreatic cancer cells growth, proliferation and progression (Figure.1). The tumor microenvironment is not a static entity but is constantly changing in its composition and size during progression of the tumor from pre-neoplastic lesion to invasive PDAC[16]. Under normal conditions, the cellular microenvironment is able to suppress the growth of the tumor cells restricting their numbers while tumor-stroma interactions can modulate the microenvironment to be more permissive for malignant cell proliferation, motility and adhesion[23].

PDAC microenvironment is further characterized by low oxygen and comparably low microvessel density. Other features of PDAC microenvironment, also seen in other tumors, are: - low pH, due to lactic acid accumulation caused by increased glycolysis and - formation of new blood vessels (angiogenesis) to supply growing tumors with sufficient oxygen and nutrients that additionally allows the cancer cells to migrate to nearby and distant tissues and invade[24]. In addition, for further tumor growth and progression, additional physiological changes in the tumor microenvironment are also necessary including self-sufficient growth signals, evasion of programmed cells death (apoptosis), limitless replicative potential, insensitivity to growth-inhibitors (antigrowth) signals and phenotypic shift from Epithelial-to-Mesenchymal to facilitate tissue invasion and metastasis[25].

Pancreatic stellate cells and desmoplastic reaction

Generation of massive desmoplastic tissue is the main characteristics of pancreatic ductal adenocarcinoma and evidence from both in vitro and in vivo data confirmed that this strong desmoplastic reaction surrounding PDAC tissues was established by the continuous interaction of cancer cells with pancreatic stellate cells (PSCs)[26]. In a normal pancreas, stellate cells with the fat-storing phenotype are quiescent and they are present in low

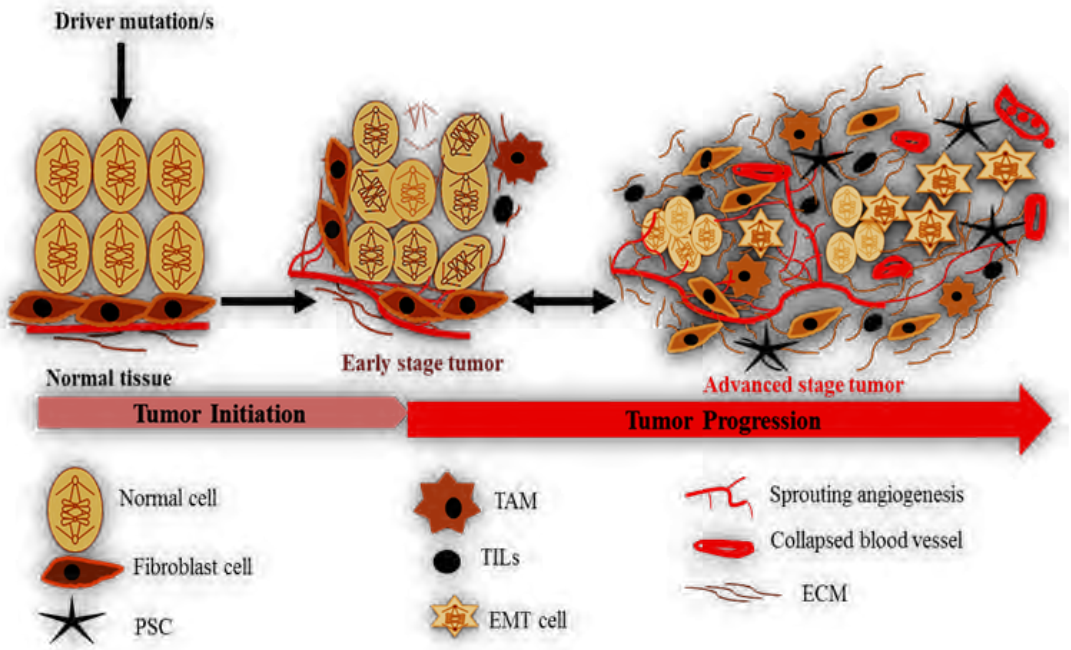

Figure.1. Tumor initiation and progression: Genetic alteration drive tumor initiation and the progression of the tumor is mainly by the continuous interaction of the tumor cells with the components of the surrounding microenvironment. The tumor microenvironment is composed of tumor cells at different stage of invasiveness, fibroblasts, pancreatic stellate cells (PSCs), extracellular matrix (ECM), vascular and lymphatic endothelial cells and variety of bone marrow derived cells (tumor infiltrated lymphatic cells (TILs), Tumor associated macrophages (TAM) etc.).

numbers in the periacinar and interlobular space of exocrine pancreas. In PDAC, the stellate cells change their phenotype from a quiescent fat-storing cell to highly active myofibroblast-like cell (activated PSC) upon induction by extra- and intra-cellular effector molecules; including inflammatory cytokines like IL-1 and IL-6, growth factors like Transforming Growth Factor beta1 (TGFβ1), Vascular Endothelial Growth Factor (VEGF), Tumor Necrosis Factor alpha (TNFα), Platelet-Derived Growth Factor (PDGF), Connective Tissue Growth Factor (CTGF), ethanol, acetaldehyde, and oxidative stress[23,27,28]. Activated PSCs are highly proliferating cells that secrete elevated levels of extracellular matrix (ECM), in particular collagens I, III and fibronectin together with matrix metalloproteinases (MMPs) that remodel the matrix which eventually lead to prominent fibrosis[29-33]. In addition, activated PSCs themselves also synthesize multiple cytokines and growth factors including PDGF, FGF, TGFβ1, CTGF, IL-1β, IL-6, IL-8, and TNFα[27,28].

Blood vessel compression and angiogenesis

Accumulating evidence has shown that tumor cannot grow beyond 1-2 mm^3 in size without sufficient supply of oxygen and nutrients and the removal of waste products. When a tumor expands rapidly, the distance for oxygen diffusion (and other products) from the existing normal blood vessels to the tumor cells increases, resulting in a condition of low oxygen tension for the tumor cells[34]. Similarly, with the replacement of the normal parenchyma by excessive dense desmoplastic tissue there are highly compressed blood vessels, which result in excessive reduction in functional microvascular density compared to the normal pancreas. As a consequence, the tumor tissue is hypoxic and hypo vascular. To meet this demand, the tissue responds by forming sustainable new blood vessels or sprouting angiogenesis[22,34-36].

Sprouting angiogenesis plays a fundamental role in tumor growth and metastasis. In PDAC, as in other tumor types, blood vessels are formed as a result of dynamic neovascularization and vascular remodeling, in which the density and architecture of neo-plastic blood vessels depend on angiogenic factors. The angiogenic switch is promoted by hypoxia, which induces an increased expression of pro-angiogenic factors, among which VEGF and Angiopoietin (Ang) are the most important ones for sustainable tumor growth. High expression of VEGF, which is secreted by both inflammatory and cancer cells, has been shown to be associated with poor prognosis for patients with PDAC. It has directly been demonstrated that the VEGF/VEGF-RII pathway regulates angiogenesis, local cancer growth, and cancer spread in PDAC[24]. The VEGF angiogenic growth factor plays a central role, as revealed by gene knockout and pharmacological inhibition. VEGF stimulates endothelial cells to sprout and proliferate to form new vessel structures. VEGF-driven tumor vasculature has been revealed to be maintained in significant part by

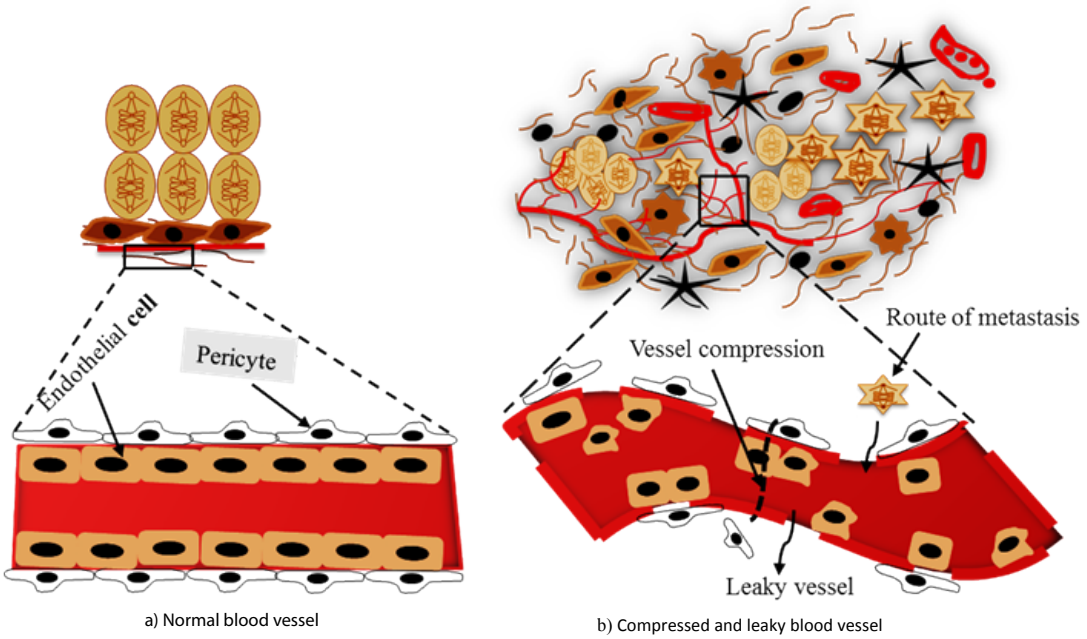

Figure 2. In normal pancreatic tissue, the integrity of the components of the vessels are intact (a); whereas in PDAC the blood vessels are compressed and leaky which enormously limits oxygen, nutrient and therapeutic delivery. The leaky vessels are also a route for cancer cells to invade and form metastasis in nearby tissue and distant organ (b)

PDGF signaling from endothelial cells to PDGF receptors on pericytes[29]. Unfortunately, these newly formed blood vessels are leaky, and the impaired lymphatics fail to drain fluid leaking from these blood vessels; as a consequence, there is an increase in interstitial fluid pressure (IFP) (Figure.2)[35].

Hypoxia

Hypoxia is generally referred to as the status when the oxygen level in a tissue is below the physiological level that is caused by an imbalance in oxygen delivery and oxygen consumption[37]. In normal tissue, the oxygen pressure varies from 1.3 to 11%, while in advanced solid tumors the oxygen tension can go below 1%, which is referred to as hypoxic region[38]. Direct intraoperative measurements of tumor oxygenation in patients with pancreatic adenocarcinoma have demonstrated significant hypoxia in the tumor compared to the adjacent normal tissue. This intratumoral hypoxia is an important component of the PDAC microenvironment that plays an active role in promoting tumor progression, malignancy, and resistance to chemotherapy and radiation[39,40]. Further, in vitro experiments in different pancreatic adenocarcinoma cell lines and in vivo in an orthotopic murine model have shown that tumor hypoxia is actively involved in pancreatic cancer progression[41,42]. In order to adapt to the hypoxic microenvironment, cancer cells co-opt physiological responses that are mediated by hypoxia-inducible factors (HIFs)[40]. HIFs are heterodimers consisting of an O_2-sensitive α subunit and constitutively expressed β subunit. There are three HIF α-subunits, HIF-1α (HIF1A), HIF-2α (EPAS) and HIF-3α (HIF3A) of which HIF-1α and HIF-2α are the most structurally similar and best characterized. HIF-1α expressed ubiquitously in all cells, whereas HIF-2α and HIF-3α selectively expressed in certain tissues; including vascular endothelial cells, type II pneumocytes, renal interstitial cells, liver parenchymal cells, and cells of the myeloid lineage[43]. HIF-1α is well-studied hypoxia-associated transcription factors in pancreatic cancer. Under normoxia, HIF-1α subunits are subjected to rapid ubiquitination and proteasome degradation mediated by the von Hippel–Lindau (VHL) protein in the cytoplasm. Under hypoxic conditions, the HIF-α subunits become stabilized and translocate to the nuclei where they dimerize with the HIF-1β subunit (ARNT), to form a functional transcription factor capable of binding to hypoxia response elements (HRE) on the DNA and transcriptionally activating target genes[44,45]. Clinically, tumor hypoxia is a therapeutic challenge because it renders solid tumors to be more resistant to sparsely ionizing radiation (IR) and chemotherapeutic drugs. Therefore, HIF-1α plays a central role in tumor survival and tumor progression and thus is an attractive anticancer target. A number of molecules have been reported to inhibit HIF activity through a wide variety of molecular mechanisms, including decreased HIF-1α mRNA levels, decreased HIF-1α protein synthesis, increased HIF-1α degradation, decreased HIF subunit hetero-dimerization, decreased HIF binding to DNA, and decreased HIF transcriptional activity[40,46].

Epithelial-to-Mesenchymal Transition in PDAC

Metastatic disease remains the major cause of mortality in PDAC patients, and there is currently no curative treatment for a tumor in this stage. Although there is often a large mass of highly heterogeneous cancer cells in the primary tumor, the molecular events leading to metastasis at distant sites most likely only involves a few tumor cells that can accomplish a series of sequential transforming events[47]. Many reports underline that induction of Epithelial-to-Mesenchymal transition (EMT) endows invasive and metastatic properties upon cancer cells that favor successful colonization of distal target tissues and organs[48].

EMT is described as, the switch from non-motile, polarized epithelial cells to motile, non-polarized mesenchymal cells with the potential to migrate from the primary tumor site to distant organs. Subsequently, the disseminated mesenchymal tumor cells undergo a reverse transition at the site of metastases; Mesenchymal-to-Epithelial transition (MET), in order to seed and re-grow thereby recapitulating the characteristics of their corresponding primary tumors[49]. Tumor cells exhibit EMT/MET plasticity that enables them to adapt the change in microenvironment they encounter both at the primary and at distant sites[48,50]. The interactions of cancer cells with their tumor microenvironment are important determinants of cancer progression toward metastasis. Various biochemical and biophysical factors in the tumor microenvironment can induce an EMT program; specifically, hypoxia and cytokines such as TGFβ, TNFα and IL-6 are capable of inducing EMT in various tumors. During the process of EMT, cancer cells loss epithelial markers such as E-cadherin, certain cytokeratins, occludin, and claudin and gain mesenchymal markers such as N-cadherin, vimentin, and fibronectin[51,52]. The changes in gene expression that contribute to the repression of the epithelial phenotype and activation of the mesenchymal phenotype are mediated, directly or indirectly, by key transcription factors including SNAI1 (Snail), SNAI2 (Slug), TWIST, zinc-finger E-box binding (ZEB), SMAD's, BMP and HIF-1α (Figure.3). Their expression is activated early in EMT, and they have central roles in the development of fibrosis and cancer progression[50,53-55].

Accumulating evidence supports that EMT is actively involves in tumor resistance to treatment, invasion, and metastasis, thereby suggesting that targeting this process could be a promising therapeutic approach[56]. However, recently contradicting ideas have emerged from a few groups. Their observations raised the possibility that tumor cells may disseminate without switching to a mesenchymal phenotype thereby creating dilemma on the importance of EMT for cancer metastasis[54,57]. These studies underlined that SNAI1- or TWIST-induced EMT is not rate limiting for invasion and metastasis, but acknowledged involvement of EMT in drug resistance. Using the same pancreatic cancer model, another group showed that the EMT transcription factor ZEB1 is a key factor for the formation of precursor lesions, invasion and metastasis and that depletion of ZEB1 suppressed stemness, colonization capacity and phenotypic/metabolic plasticity of tumor cells. This group argued that different EMT-transcription factors may have

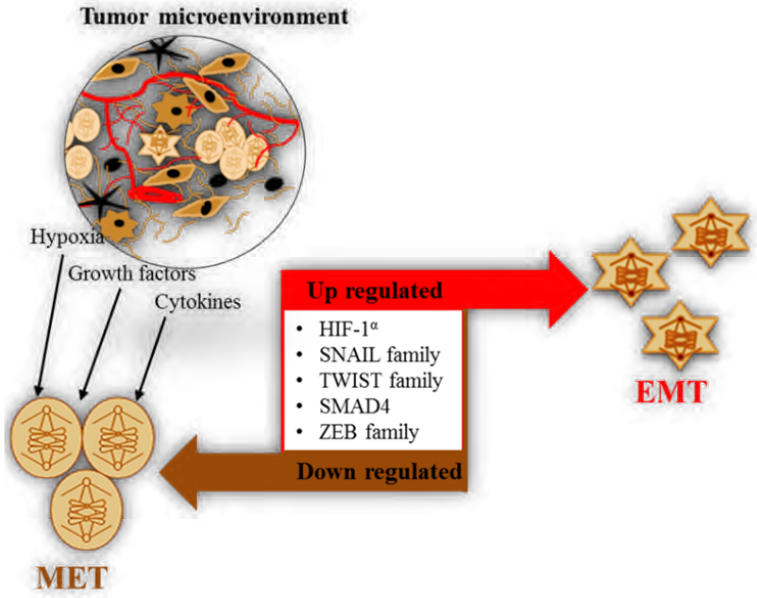

Figure 3. Epithelial-to-Mesenchymal transition and Mesenchymal-to-Epithelial transition are regulated by different transcription factors. EMT is induced by signals that the cancer cells receives from the tumor microenvironment and by which the cells acquire the capacity to migrate, invade and resistance to drug treatments. At the metastatic site, the tumor cells shift to epithelial phenotype (MET) in order to establish themselves for re-growth and proliferation.

complementary subfunctions in driving pancreatic tumor metastasis and suggested therapeutic strategies should be considered accordingly[58]. Further studies are required to clarify for which tumor processes EMT is responsible and in which it is a bystander. Important to note that not only tumor cells but also PSC undergo EMT with decreased expression of markers like E-cadherin and BMP7 and increased expression of Vimentin, N-cadherin and collagen1a1[32,59].

Hypoxia mediated Epithelial–to-Mesenchymal transition

Both EMT and hypoxia are considered as crucial events facilitating invasion and metastasis of cancer cells through activation and sustained accumulation of HIF-1α in the cells. Previous studies have shown that hypoxia-induced EMT program occurs in a biphasic mode. The very early stage, EMT is triggered by a transient, increased generation of intracellular reactive oxygen species (ROS) dependent inhibition of GSK-3β, followed by early SNAI1 nuclear translocation and E-cadherin down-regulation that can switch on EMT program. The later events involve nuclear translocation of β-catenin, which is regulated by HIF-1α-dependent autocrine–paracrine release of VEGF, which effectively sustain EMT program[42,44,45].

TGFβ Epithelial–to-Mesenchymal transition

Different growth factors can also play a role in the EMT process including TGFβ, HGF/MET, EGF, IGF, CTGF, TNFα, and FGF. TGFβ is one of the most important cytokines that can induce EMT and has also implications for wound healing[51]. TGFβ signaling can be pro-tumorigenic or tumor suppressive. In pancreatic ductal adenocarcinoma, TGFβ induces SMAD4 dependent EMT, which is considered as pro-tumorigenic event[60].

Extracellular matrix and Epithelial–to-Mesenchymal transition

PDAC is associated with an intense desmoplastic reaction involving pathological deposition of altered extracellular matrix that contains high levels of fibrous collagen, proteoglycans, and glycosaminoglycans including hyaluronic acid (HA, hyaluronan). The extracellular matrix deposited by pancreatic fibroblasts and cancer cells modulates the behavior of pancreatic ductal cells by inducing a more scattered phenotype and enhancing cell motility[61]. Among the components of ECM, hyaluronic acid has attracted considerable attention of scientists as a therapeutic target for aggressive PDAC. Hyaluronic acid, which is secreted from the tumor cells as well as the stromal cells[62], accumulates in the ECM in many solid tumors, in the case of PDAC with a very high frequency (87%). The accumulated HA molecules can absorb a substantial amount of water molecules causing the ECM to swell, resulting in high tumor interstitial pressure, the collapse of the tumor vasculature, and tumor hypoxia. Many reports from mouse models and cancer patients have confirmed that tumors that accumulate high amount of HA are aggressive and have acquired EMT phenotype[63,64]. Also, in a recent study by Laklai et al [65], they found that the mechanical properties of the PDAC stroma (stiffness) plays a role in regulating aggressiveness, influencing TGFb and STAT3 expression, an additional argument for anti-stromal therapy in PDAC.

On the other hand, ECM proteins are also implicated in the regulation of the EMT process[19]. Stroma and EMT cells synthesize and secrete large quantities of ECM protein that promote tumorigenesis. Among these proteins are integrins, matrix metalloproteinases, enzymes such as lysyl oxidase (LOX) and growth factors like the connective tissue growth factor (CTGF). Recent studies revealed that due to high secretory load of ECM proteins, the EMT cells activates PERK-elF2α-ATF4 signaling, one branch of the unfolded protein response, to maintain the endoplasmic reticulum (ER) function. Maintaining this homeostasis is indispensable for cell survival, invasion and metastasis of EMT cells. Therefore, disrupting this homeostatic process and augmenting ER stress can be an option to kill malignant cells[23,55,66].

Tumor-infiltrating immune cells and immune evasion

The formation of pancreatic ductal adenocarcinoma precursor lesions (PanIN) is followed by the establishment of a more immune-tolerant microenvironment. The role of the immune cells during further progression of PDAC is poorly understood[67]. Despite the existence of a functional cancer immune surveillance in PDAC, apparently immunocompetent individuals can develop cancer.

Pro-tumoral activity of immune cells

For the growth and progression of PDAC, evading antitumor immune responses is critical. The tumor microenvironment is profoundly immunosuppressive with regulatory T cells, regulatory B cells, myeloid-derived suppressor cells, tumor-associated macrophages, and other stromal elements that interact with the tumor and/or secrete suppressive factors. Despite the number of immune cells that surround and infiltrate the tumor, the tumor cells have several techniques to escape or overcome the immune response of the host[68,69].

These include Tumor-associated macrophages (TAMs), either derived from recruited blood monocytes or resident tissue macrophages; their phagocytic activity remains one of the most important immune anti-tumor functions[70]. Macrophages are classified according to the type of response in which they participate. Classically activated (M1) macrophages are activated in response to a microenvironment enriched with Th1 cytokines (IFN-γ, GM-

CSF, IL-12, ROI, RNI, iNOS, and CXCL10). These M1-polarized macrophages are primarily considered as anti-tumorigenic. In contrast, alternatively activated (M2) macrophages are formed in response to Th2 cytokines (IL-4, IL-10, IL-13) and HIF-1α. M2 macrophages are characterized by their secretion of anti-inflammatory mediators such as transforming growth factor β (TGFβ1). M2-polarized macrophages can promote proliferation, angiogenesis, invasion, metastasis of tumor cells and they also express the program cell death ligand (PD-L1) which is involved in immune suppression and T-cell apoptosis that in turn can promote tumor progression. Also in the PDAC, tumor-infiltrating lymphocyte B cells (TIL-Bs) can be found which are fore mostly known as part of the antigen-driven humoral immune response. Recent studies have provided compelling evidence that TIL-Bs are involved in the initiation and progression of PDAC through a subset of B cells that inhibit the antitumor immune responses[71]. Pylayeva-Gupta et al.[72] showed that the growth of orthotopic pancreatic neoplasms harboring oncogenic K-RAS was significantly compromised in B cell-deficient mice and this growth deficiency was rescued by the reconstitution of a $CD1d^{high}CD5+$ B cell subset. B-cells are activated in response to Th2 cells that secrete interleukins (IL-4, IL-10 and IL-13).

In vitro and in vivo models to study PDAC microenvironment

In vitro models

Established cell lines for pancreatic cancer are a useful research tool and convenient starting point for industry and academia and have been applied in many studies[73]. Nevertheless, they carry several drawbacks and are often poor in mimicking the complexity of tumor microenvironment and predicting therapeutic response in humans. In many instances, they have been successfully exploited to generate hypothesis and identify molecular targets, but these observations need subsequently further in-depth characterization using more advanced in vivo model systems.

In vivo models

So far, several mouse models have been developed that mimic to a degree the tumor's environment as can be found in the clinical situation in patients. These models, bearing mouse pancreatic tumors, were used to study the pathology, investigate tumor growth, invasion, metastasis, biomarker identification as well as used to evaluate therapeutic responses[47]. Nevertheless, an ideal model that can mimic closely a real human PDAC with corresponding tumor microenvironment and its progression is still lacking.

Xenograft mouse models

The next best option and one of the now most widely used models is the human tumor xenograft. In this type of model, human tumor cells or tissues are transplanted, either under the skin or into the organ from which the tumor originated, of immunocompromised mice that do not reject human cells. The mice most frequently used as xenograft recipients are athymic nude mice, severely compromised immunodeficient (NOD/SCID) mice, or NOG/SCID mice with an additional loss of NK-cell function[47]. A human tumor xenograft model has several advantages: 1) at early passages it still closely represent the complexity of genetic and epigenetic variations of the original human tumor, 2) the model creates a suitable platform to identify novel therapeutic approaches, 3) in a condition of scarce tumor tissue samples, after expansion in vivo multiple experiments can be carried out on the same tissue. To this point, recently, we successfully established pancreatic patient-derived tumor xenografts (PDTXs) by using endoscopic ultrasound (EUS) guided fine needle biopsies in the subcutaneous tissue of nude mice[74]. In this model, after expansion in three generation, the tumor showed histological and genetic resemblance to the original tumor[74]. 4) PDTX is a useful tool to develop personalized drug screening program as results from xenografting studies can be obtained within a few weeks, 5) More importantly, even though orthotopic tumor models are more time consuming and can sometimes be technically challenging, orthotopic implantations should be preferred whenever possible, since the tumor can then be investigated within its relatively normal (micro-) environment. For therapeutic studies, this represents a great advantage, since questions of drug delivery and biodistribution can be assessed in a more relevant setting. Orthotopic xenograft mouse models of human pancreatic cancer exhibited greater tumor growth and metastasis than the subcutaneous xenograft mouse models[47,75].

The remaining drawbacks of xenograft models include the impaired immune response owing to the need to use immunocompromised mice as hosts, the inability to perpetuate in full the human tumor microenvironment and the profound differences in tumor structure and vasculature compared with endogenous human PDACs. Accordingly, results obtained from a number of xenograft studies have not translated well into the clinic yet. For instance, PDAC xenografts often respond well to anti-angiogenic agents, but these same agents often fail to show any clinical benefit in the corresponding human tumor in vivo[47].

Genetically engineered mouse models

Another important tool in PDAC research is the development of genetically engineered mouse models (GEMM). GEMM are mutant mice that have been engineered to express oncogenes and/or lose the expression of tumor suppressor genes from their native promoters by using knockout or knock-in technologies. In these models, important genes that are frequently mutated in human PDAC

(e.g., KRAS, p53) can be reproduced, and their role in tumor initiation, progression and tumor response to different pre-clinical treatment trial can be studied. In contrast to xenograft model, GEMM bear immune competence that could make them more suited to elucidate certain aspects of the tumor-microenvironment interactions in PDAC initiation and progression. For example, both Dawson et al.[76] and Philip et al.[77] showed dense fibrotic stroma surrounding tumor cells, which is one of the most interesting characteristics of PDAC, in their different GEMM model of high-fat diet-induced pancreatic cancer development. When carcinoma-associated fibroblast were depleted in a transgenic mouse model this accelerated pancreas cancer and reduced survival[78]. Combination treatment in which reduction of the stroma could transiently stabilize the disease in a mouse model[79]. Furthermore, Smith et al. [80], showed that blocking hypertrophy and hyperplasia of the pancreas and reducing fibrosis in transgenic mice could arrest PanIN progression to advanced lesions when using cholecystokinin as inducer. These are important examples to show the contributions made through GEMM models. However, the disadvantage of the GEMM models is that the heterogeneity and the complexity of PDAC as seen in humans is not yet well addressed. For example, although desmoplastic stroma arises in some GEMM models of PDAC, it never develops to the extreme levels as seen human pancreatic tumors. This maybe because in these mice the tumor develops over short period compared to in humans where this process from initiation mostly takes a longer period (years even decades). Another limitation of the GEMM model is the way of acquisition of genetic alterations during tumor development. In most human pancreatic cancers this is a stepwise process, genetic alteration begins with the activation of oncogenes followed by inactivation of tumor suppressors, while in the current mouse models multiple genetic insults are introduced at the same time[81].

Pancreatic ductal adenocarcinoma in the clinic and the existing bottle necks

Current screening, diagnostic and prognostic approaches of PDAC

Pancreatic ductal adenocarcinoma is a silent disease at its early stages of development. It is only apparent after the tumor has invaded the surrounding tissues or forms metastasis in distant organs. The diagnosis is thus usually made at a late stage when the only curative treatment of surgical resection, is less effective or not possible[82,83]. Tumors located in the pancreatic body or tail are more likely to be diagnosed at a more advanced stage than those located in the head where the symptoms are frequently related to obstruction of the common bile duct and/or pancreatic duct[82]. An ideal screening test for early pancreatic cancer would be a highly accurate blood marker that can be measured minimal invasively. Unfortunately, to date, none has proven sufficiently specific for diagnosis.

Up to now, the focus of screening efforts has been to detect preinvasive lesions rather than early pancreatic cancers, since resection of preinvasive lesions will prevent the development of an invasive pancreatic cancer. Once an invasive pancreatic cancer has developed, its spread beyond the pancreas is probably rapid thereby restricting the usefulness of markers for invasive pancreatic cancer. The screening and diagnostic approach of pancreatic masses is primarily by endoscopic ultrasound-fine needle aspiration (EUS-FNA) and histological or cytological analysis, which cannot be applied at the population level[82].

Unfortunately, there are no specific predictive biomarkers that can be used to guide treatment decisions in clinical practice. Carbohydrate antigen (CA) 19-9 is the most useful tumor marker in pancreatic cancer so far. Although it has no utility in the primary diagnosis, CA 19-9 has significant value as prognostic factor and can be used to assess disease burden and potentially guide treatment decision. A pre-operative serum CA 19-9 level ≥ 500 UI/ML indicates a poor post-surgery prognosis. Imaging work-up must be used to determine tumor size and burden and arterial and venous local involvements[83]. Elevated CA 19-9 by itself is insufficient to differentiate pancreatic carcinoma from chronic pancreatitis however, it increases the suspicion of pancreatic carcinoma and may complement with other clinical findings to improve diagnostic accuracy[83]. Furthermore, microRNAs (miRNA) have a potential as diagnostic and prognostic biomarkers and as therapeutic targets in cancer. In PDAC, expression patterns of miRNAs are significantly altered. Aberrant expression of several miRNAs has independently been associated with reduced survival, including the overexpression of miR-21 or under expression of miR-124[84-86].

Most biomarkers that are currently used are substances expressed by the cancer cells themselves. In PDAC, in which the stromal content dominate the tumor microenvironment, identification of biomarkers that arise from its abundant stroma could greatly expand the possibilities and benefit therapeutics. Particularly, as stroma in the tumor is continuously remodeling during cancer progression and many biological factors from the tumor stroma are released into the surrounding microenvironment; this could give the chance to detect the tumor stage and phenotype with specific biomarkers in the blood and/or tissue of the patients. These markers could involve markers related to carcinoma-associated fibroblast and the EMT conversion (e.g. fibroblast-specific protein-1 (FSP1/S100A4) and CD31/PECAM)[69]. Therefore, the potential use of the stroma as a source of biomarkers in combination with those derived from the cancer cells needs to be evaluated.

Treatment and therapeutic resistance

Surgery, radiation therapy and chemotherapy are treatment approaches that may prolong survival and/or relieve symptoms in many PDAC patients, but they seldom result in a cure.

Surgery

Surgical resection is the only potentially curative treatment for pancreatic adenocarcinomas, but only approximately 15% to 20% of patients at time of diagnosis have a resectable disease. The decision on the resectability of a tumor requires a multidisciplinary consultation and distinction should be made between tumors that are resectable, borderline resectable or unresectable (locally advanced and/or metastatic). Locally advanced cancers of the pancreas, in which the tumor has grown into the nearby blood vessels and other tissues but has not spread to the liver or distant organs or tissues, cannot be removed completely by surgery. Several studies have shown that removing only a part of the cancer does not help the patient to live longer. Surgery in the case of locally advanced cancer is therefore mainly used to relieve bile duct blockage or to bypass a blocked intestine caused by a pressing tumor. The standard treatment options for locally advanced cancers are chemo and/or chemo-radiation[87,88]. The most advanced form of pancreatic cancer is the metastatic form, whereby their spread renders them impossible to be removed by surgery or treated by radiation therapy alone. Currently, a combination of chemo and/or chemo-radiation to increase patient survival together with palliative therapy to relieve symptoms and improve quality is the recommended therapeutic approach for advanced metastatic disease[89].

Radiotherapy

Radiotherapy is an option for PDAC; however, the cytotoxic effect is highly affected by low oxygen tension, making hypoxia a critical limitation for the success of this treatment. Molecular oxygen (O_2) is a potent chemical radiosensitizer. Oxygen has the highest affinity for electrons of any molecule in the cell; it reacts rapidly with unpaired electrons of free radicals formed when DNA is irradiated, thereby aggravating radiation damage. Pancreatic cancer is among the most hypoxic solid tumors as a result of extensive stromal reactions[37]. Experimental evidence has shown that in response to the hypoxic stress in the tumor microenvironment, the nuclear HIF-1α expression was increased in 88% of human pancreatic ductal carcinoma compared to only 16% in the normal pancreas. Stroma adjacent to the pancreatic ductal carcinoma also showed nuclear HIF-1α expression in 43% of cases. Increased expression of the HIF-1α transcription factor results in an adaptive switch to glycolytic metabolism, angiogenic signaling, increased survival and metastasis and also very importantly is associated with tumor resistance to chemo-radiotherapy and poor patient outcomes. In pre-clinical study several HIF-inhibitory drugs, alone or in combination with conventional PDAC therapy, showed remarkable improvement in in vitro and in vivo models[55,90]. Therefore, drug development research has to take into account the hypoxic PDAC microenvironment to overcome HIF-1 expression or explore ways to improve oxygen supply or reduce oxygen consumption of tumor cells, which may help to restore sensitivity to radiation or drugs.

Chemotherapy

According to the American Cancer society[91], many different chemo drugs can be used to treat pancreatic cancer, including gemcitabine, 5-fluorouracil (5-FU) and irinotecan. Pancreatic Cancer UK list as the main chemotherapy drugs and drug combinations for pancreatic cancer: gemcitabine, FOLFIRINOX – a combination of oxaliplatin, leucovirin, irinotecan and 5-FU, GemCap - gemcitabine and capecitabine, FOLFOX - oxaliplatin with 5-FU and folinic acid or Nab-paclitaxel (Abraxane®) with gemcitabine. None of the current drugs however is very successful, at least for large groups of patients.

Gemcitabine has long been the standard chemotherapy for pancreatic cancer patients, but most patients do not respond well and end up with gemcitabine resistance and disease progression. Hence, the overall survival of this cancer remains poor and few other options are available for patients that fail gemcitabine-based therapy[92,93]. Gemcitabine in combinations with cytotoxic agents and targeted therapies have been disappointing. The EGFR/ tyrosine kinase inhibitor erlotinib, has regulatory approval for use in combination with Gemcitabine but due to a small expected gain-in-survival, is not widely used. Gemcitabine and nab-paclitaxel can be considered only for a selected group of patients[83].

FOLFIRINOX, the combination of 3 chemotherapeutic agents (fluorouracil [5-FU], leucovorin, irinotecan and oxaliplatin) can now be used as second-line treatment for selected patients with metastatic or locally advanced pancreatic cancer. However, the FOLFIRINOX regime is very toxic and the side effects can be high[94].

There are other promising targeted therapies under pre-clinical investigations. Genetic targeting of VEGF-RII, has been shown to inhibit local growth and meta-static spread of pancreatic cancer cells[24]. Hyaluronan (HA) depletion strategies accomplished antitumor effects by multiple mechanisms that include targeting both biophysical and molecular signaling pathways. The potential effects of HA accumulation include shielding cancer cells from immune cell attack and from antineoplastic therapies through a variety of mechanisms. Intravenous hyaluronidase treatment together with conventional chemotherapy considerably improved survival in HA-

rich PDAC patients[64,95]. Pegylated recombinant human hyaluronidase (PEGPH20) is a novel agent that degrades HA and normalizes interstitial fluid pressure to enhance the delivery of cytotoxic agents. Ongoing clinical trials have demonstrated the benefits of adding PEGPH20 to chemotherapy for advanced pancreatic cancer. Results from phase 1b clinical trials of PEGPH20 together with gemcitabine in patients with pancreatic cancer have shown promising signs of efficacy and acceptable tolerability. Phase 2 and 3 trials of PEGPH20 plus chemotherapy are ongoing in metastatic PDAC, and it is also being evaluated in other malignancies in combination with radiation and immunotherapy. Moreover, intratumoral HA content appears to be a predictive biomarker of response[63,95-97].

Various factors can contribute to chemo-resistance of tumors, such as the fibroblastic/stromal shielding, cellular microenvironment and some of the molecules synthesized by the cells in the stroma (cytokines, miRNAs, and extracellular vesicles). EMT has been shown to contribute significantly to chemo-resistance in several types of cancers, including in pancreatic cancer. Gene expression profiling of chemo-resistant cells showed a strong association between expression of the EMT transcription factors ZEB1, SNAI1, and TWIST and decreased expression of E-cadherin. Interestingly, maintaining of chemo-resistance in cell lines that have undergone EMT is dependent on Notch and NF-kB signaling. Moreover, induction of gemcitabine-resistance in previously sensitive cell lines resulted in the development of an EMT phenotype and was associated with an increased migratory and invasive ability compared to gemcitabine-sensitive cells[55,92]. Furthermore, the ATP binding cassette (ABC) protein superfamily plays an important role in the distribution of molecules (intrinsic and extrinsic) across the cellular membrane. Internalization of their substrates (molecules, drugs) occurs by active transport. Members of this superfamily of proteins are expressed in many tissues and their isoforms are widely studied. Among them, we should mention the group of MDR (multiple drug resistance) proteins. Cancer cells frequently show changed expression of MDR proteins and this also can contribute highly to chemo-resistance of tumors[98].

A recent study in genetically engineered mouse models (GEMMs) with deleted SNAI1 or TWIST, two key transcription factors in aggressive PDAC and other cancers, has shown resensitization of PDAC to gemcitabine[57]. In addition, TGFβ gene silencing with activation of retinoic acid-inducible gene I overcame tumor-induced CD8+ T cell suppression leading to prolonged survival in a PC mouse model[99].

Immunotherapy

Immunotherapy has shown positive results in many cancer types including, lymphoma, melanoma, renal cell carcinoma, and lung adenocarcinoma. However, immune therapy for PDAC is still a challenging issue. Although inflammatory cells have been shown to infiltrate the tumor microenvironment in pancreatic cancer, these cells promote tumor rather than inhibit PDAC growth. As mentioned already, several immune cells of the tumor microenvironment play a suppressive role. These suppressive immune cells have emerged as excellent therapeutic targets, and have already shown remarkable outcomes in patients with melanoma and lung cancer[71]. In many researchers, these outcomes have raised expectations for the application of these therapies in PDAC as well.

Immune therapy trials in PDAC includes passive immunotherapeutic approach using monoclonal antibodies or effector cells generated in vitro and active immunotherapeutic approach using vaccination to stimulate antitumor response[71] (Table 1). Cellular therapies with genetically engineered T cells (CAR-T-cells or antigen-specific T cell receptors) or tumor infiltrating lymphocytes have also proven to be efficacious in certain hematopoietic malignancies and solid tumors[100]. Monoclonal antibodies employed in passive immunotherapeutic approaches block ligand-receptor signaling for growth, thus leading to tumor cell death. They target tumor-associated antigens, such as mucin 1, Wilms tumor gene 1, human telomerase reverse transcriptase, mutated K-RAS, CEA, survivin, p53, HER-2/neu, VEGFR or EGFR. Vaccination therapy for active immunotherapeutic approaches involves administering tumor-associated antigens to activate tumor-specific T cells. Immune checkpoint inhibition to activate effector T cells is one of the most actively studied areas. The most well described therapeutically targeted immune checkpoint pathways that negatively regulate T cell function are those of PD-1 and CTLA-4. PD-L1 inhibitors (durvalumab, atezolizumab), PD-1 inhibitors (nivolumab, pembrolizumab, pidilizumab), and CTLA-4 inhibitors (ipilimumab, tremelimumab) have all been employed in clinical trials of various cancers and have produced long-term survival in approximately 20% of patients; these findings led to FDA approval of the above agents[69,101]. Nevertheless, Kunk et al.[69] listed many forms of pancreatic cancer immunotherapy trials, none of which have shown meaningful clinical benefit. In genetically engineered mouse models, pancreatic ductal adenocarcinoma was fully refractory to an approach with only monoclonal antibodies that block PD-1 or CTLA-4, which is in line with what was seen in patients[100].

Immunotherapy, in many cases, provides an alternative to conventional therapies, and has many advantages, including the potential to generate lifelong immune responses with less severe side effects. To date immunotherapy with immune cell targeting therapy for PDAC is at its early stage and the initial results suggests that a combined approach will be required for PDAC.

Table-1. Selected stromal components targeted therapeutics at different phase of clinical trials.

Targets	Therapeutics	Conditions	Phase of Clinical trials	N	Outcome	References
PD-L1	MEDI4736 monotherapy	Advanced Solid Tumors	I/II	1022	Study on going with evidence of antitumour activity	O'Reilly *et al*., 2016[103] & ClinicalTrials.gov., Identifier: NCT01693562[104]
PD-L1 &/or CTLA-4	MEDI4736 monotherapy or Tremelimumab+MEDI4736	Metastatic Pancreatic Ductal Adenocarcinoma	II	65	On going	O'Reilly et al., 2016[103] & ClinicalTrials.gov., Identifier: NCT02558894[105]
CSF1R blockade (deplete M2 macrophage)	anti-CSF1R (PEXIDARTINIB) + an anti-PD-L1 (DURVALUMAB)	Advanced/metastatic colorectal or pancreatic cancers	I	58	On going	ClinicalTrials.gov., Identifier: NCT02777710[106]
T-effector/T-reg ratios	GVAX pancreatic cancer vaccine + Cyclophosphamide	Stage I or stage II pancreatic cancer	I/II	59	Increased intratumoral Teffector/Treg ratios	Lutz *et al*., 2014[107]
			I/II	87	On going	ClinicalTrials. gov., Identifier: NCT00727441[108]
Hyaluronic acid (HA)	PEGPH20 + gem	Stage IV pancreatic ductal adenocarcinoma (PDA)	Ib	28	Well tolerated & PDAC patients with high HA tumors	Hingorani *et al*., 2016[96]
	PEGPH20+nab-paclitaxel+gemcitabine vs nab-paclitaxel + gemcitabine	Stage IV pancreatic ductal adenocarcinoma (PDA)	II	237	Well tolerated	Hingorani *et al*., 2015[109] & ClinicalTrials. gov., Identifier: NCT01839487
	PEGPH20 + nab-paclitaxel + gem vs Placebo + nab-paclitaxel + gem	Stage IV pancreatic ductal adenocarcinoma (PDA)	III	420	On going	ClinicalTrials. gov., Identifier NCT02715804[110]
Secreted protein rich in cysteine (SPARC)	Gemcitabine plus nab-paclitaxel	Advanced pancreatic cancer	I/II	67	Tolerable adverse effects with substantial antitumor activity	Von Hoff *et al*., 2011[111]
			III	861	Significantly improved overall survival	Von Hoff *et al*., 2013[112]

Granulocyte-macrophage colony-stimulating factor (GM-CSF)-secreting, allogeneic PDAC vaccine (GVAX), PEGPH20 (PEGylated human recombinant hyaluronidase), gem (Gemcitabine), HA (hyaluronic acid), programmed cell death ligand-1 (PD-L1) and cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4)

Drugs or drug-combinations targeting stromal components in ongoing clinical trials

Targeted therapies and immunotherapy have changed the survival of patients with many solid malignancies, including metastatic melanoma and lung cancer, but no such therapies exist at present for pancreatic ductal adenocarcinoma[102]. However, there are some ongoing clinical trials targeting tumor stromal components, one of the most interesting characteristics of PDAC, as emerging strategic approach to aid pancreatic cancer treatment (Table 1).

Granulocyte-macrophage colony-stimulating factor (GM-CSF)-secreting, allogeneic PDAC vaccine (GVAX), PEGPH20 (PEGylated human recombinant hyaluronidase), gem (Gemcitabine), HA (hyaluronic acid), programmed cell death ligand-1 (PD-L1) and cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4)

Conclusion and future directions

For decades, most studies in pancreas cancer have focused on PDAC tumor cells as potential diagnostic and therapeutic targets and have neglected the complex and heterogeneous tumor microenvironment. Despite extensive knowledge of oncogenic mechanisms, tumor suppressor genes and their signaling networks, effective strategies to stop PDAC could not be developed until now.

For PDAC we still face many challenges, the conventional therapies that mainly targets the driver mutations in the DNA have only given marginal improvement on the survival and quality of life. Genetic alteration might be the primary driver to induce in a small number of cells the tumor, further tumor initiation, progression and resistance to treatment is profoundly driven by the surrounding microenvironment. Another challenge comes from the absence of symptoms generally at the early stage of cancer and lack of non-

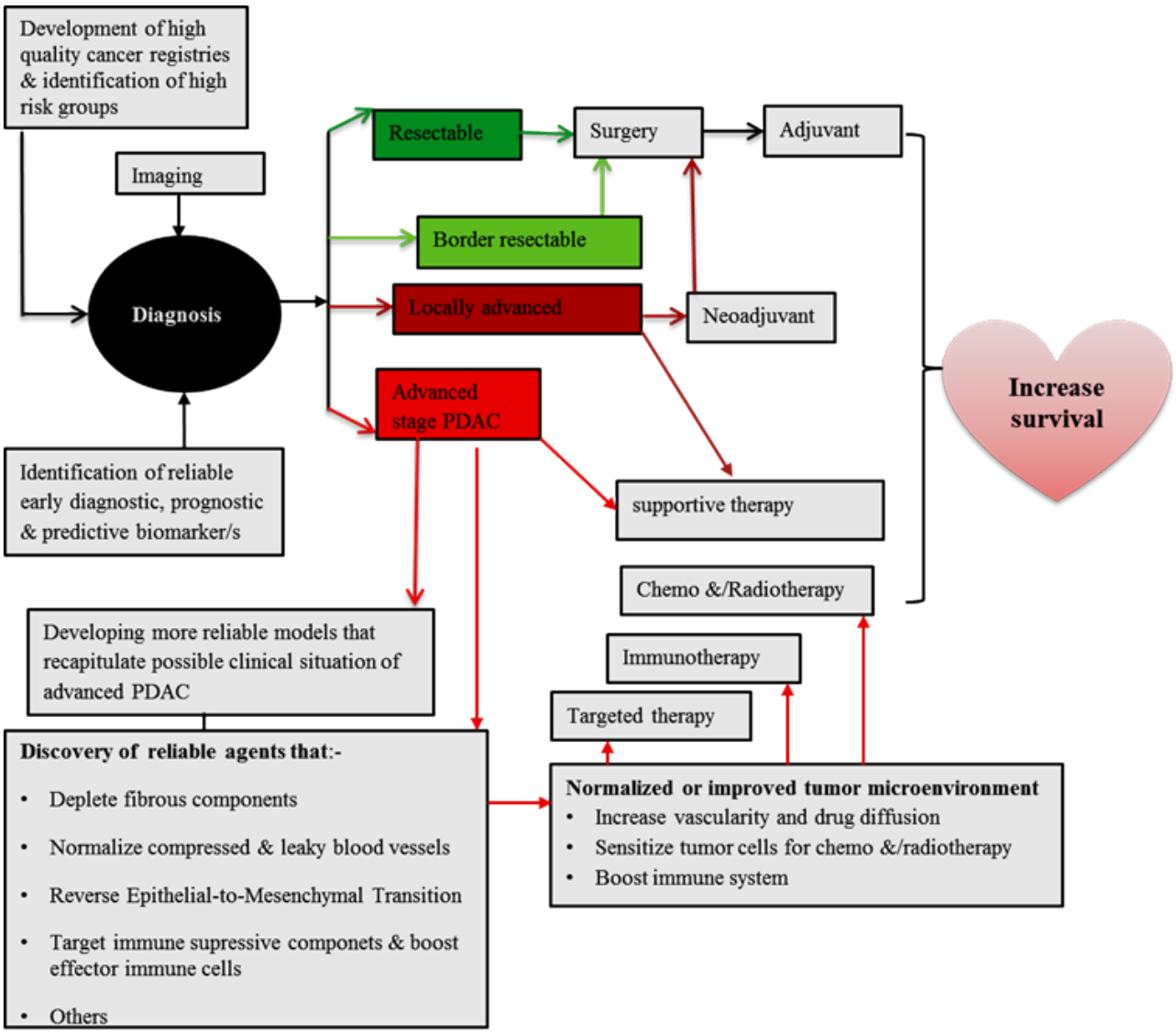

Figure 4. Summary of the current, stage dependent, treatment approaches of pancreatic ductal adenocarcinoma with critical areas that need improvement in the future. Focus on tumor-stroma interactions at two main areas: 1) looking for reliable diagnostic, prognostic and predictive biomarkers and 2) finding of reliable targets that help to normalize different aspects of the microenvironment in advanced stage PDAC, these have the potential to increase therapy effect and better patient survival.

invasive and low-cost screening tools for the general population that stands in the way to avoid the insidious clinical syndrome of PDAC at late stage. Furthermore, the discrepancy between experimental data and the clinical reality that result mostly from the inefficiency of our current models in recapitulating the clinical tumor microenvironment are among the main bottlenecks that hamper the finding of novel treatment strategies for PDAC. The above facts underline the importance of developing well-organized cancer registry data, identifying novel screening, diagnostic and predictive markers for early detection, treatment or counseling of the patient with PDAC. On the other hand, while the use of current models remains the basics, developing more reliable models, which mimic possible clinical scenarios, are crucial. Novel therapeutic development and an approach that normalize the homeostasis of the abnormal highly heterogeneous and networked tumor microenvironment is desperately needed (Figure 4).

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Conflict of interest statement

None of the authors report a potential conflict of interest regarding this work.

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