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Crosstalk of Sp1 and Stat3 signaling in pancreatic cancer pathogenesis

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Abstract

Pancreatic cancer progression is attributed to genetic and epigenetic alterations and a chaotic tumor microenvironment. Those diverse “upstream signal” factors appear to converge on specific sets of central nuclear regulators, namely, transcription factors. Specificity Protein 1 (Sp1) and signal transducer and activator of transcription 3 (Stat3) are central transcription factors that regulate a number of pathways important to tumorigenesis, including tumor cell-cycle progression, apoptosis, angiogenesis, metastasis, and evasion of the immune system. Recently, researchers demonstrated many types of crosstalk of Sp1 and Stat3 in tumor signal transduction and that these factors function cooperatively to activate targeted genes and promote tumorigenesis in pancreatic cancer. Therefore, targeting both Sp1 and Stat3 is a potential preventive and therapeutic strategy for pancreatic cancer.

Keywords

Therapy; angiogenesis; metastasis

1. Introduction

Pancreatic cancer is a widespread disease that is difficult to treat, with a 5-year survival rate less than 5% [1,2]. Researchers estimated that about 43,140 new cases of pancreatic cancer were diagnosed and 36,800 pancreatic cancer deaths occurred in the United States in 2010 [3]. Thus, it is one of the leading causes of cancer deaths in industrialized countries [4]. Regarding the treatment of pancreatic cancer, surgery combined with adjuvant chemotherapy is the primary modality. However, most patients have local or metastatic spread at the time of presentation that precludes resection because of the insidious, aggressive natural history of the disease. Therefore, less than 10% of patients are candidates

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for surgical resection at the time of diagnosis. Even among patients who undergo potentially curative resection, long-term outcomes remain unsatisfactory owing to early recurrence and metastasis [5,6]. Effective systemic therapy capable of reversing the aggressive biology of this disease is currently unavailable. Therefore, the need for a better understanding of the mechanisms that contribute to pancreatic cancer progression and for the design of more effective targeted pancreatic cancer treatment strategies is an urgent one.

Specificity Protein 1 (Sp1) is a zinc finger transcription factor that is important to transcription of many cellular and viral genes that contain GC boxes in their promoters [7,8]. Because Sp1 is an essential transcription factor for many genes key to regulation of multiple aspects of tumor cell survival, growth, and angiogenesis, abnormal Sp1 expression and activation may contribute to human cancer development and progression [9]. Lines of evidence demonstrate that overactivation of Sp1 occurs frequently in a wide variety of human tumors, including pancreatic cancer, and that high Sp1 expression correlates with aggressive biology and poor clinical outcome of these tumors [10–14].

Signal transducer and activator of transcription 3 (Stat3) is a central cytoplasmic transcription factor that is activated by phosphorylation of a conserved tyrosine residue in response to oncogenic proteins and extracellular signals, such as cytokines and growth factors [15,16]. Stat3 regulates a number of genes that are critical to tumor cell survival and proliferation, angiogenesis, invasion, metastasis, and immune evasion [17,18]. Extensive studies have demonstrated that inappropriate activation of Stat3 occurs at a surprisingly high frequency in a wide variety of human cancers, including leukemias and lymphomas, as well as solid tumors, such as prostate, breast, and pancreatic cancer [19–23].

In this review, we provide a brief introduction to brief description of Sp1 and Stat3 expression and describe their roles in pancreatic cancer development and progression and the possibility of modulating Sp1 and Stat3 functions for therapeutic gain. Furthermore, we highlight the significance of Sp1 and Stat3 crosstalk in pancreatic cancer development and progression. Targeting both Sp1 and Stat3 may be a preventive and therapeutic strategy for pancreatic cancer.

2. Molecular pathogenesis of pancreatic cancer

Pancreatic cancer affects approximately 40,000 people in the United States every year, and almost an equivalent number die of it each year, making pancreatic cancer the fourth leading cause of cancer deaths in the United States [3]. Pancreatic ductal adenocarcinoma (PDA) is the most common and deadliest histological subtype of pancreatic cancer. Researchers have identified three different ductal precursor lesions that lead to invasive PDA: pancreatic intraepithelial neoplasia, intraductal papillary mucinous neoplasia, and mucinous cystic neoplasia [24–26]. Identification of these precursor lesions in pancreatic ducts has led to the formulation of a progression model of PDA: the pancreatic ductal epithelium progresses from normal epithelium to increasing grades of pancreatic intraepithelial neoplasia to invasive cancer [27].

Over the past few years, oncologists' knowledge of pancreatic cancer pathogenesis has advanced significantly because of an explosion in understanding of its molecular biology. Like many other malignant diseases, pancreatic cancer develops via accumulation of inherent and acquired genetic and epigenetic alterations. Both oncogene and tumor suppressor gene activity may change in pancreatic tumors in a combination of events. Oncogenes, which normally serve as accelerators to activate the cell cycle and promote cellular growth, may be activated by **their** mutation, amplification, translocation, or overexpression. For example, activating mutations of the KRAS oncogene are thought to be

the initiating mutations responsible for pancreatic cancer. More than 85% of pancreatic cancer cases have an activating point mutation of the KRAS gene at a very early stage of development [28].

Conversely, tumor suppressor genes, which serve as brakes to slow the growth of tumor cells under normal conditions, may be inactivated by homozygous deletion, single allele loss, or epigenetic events. These genetic modifications include inactivation of the p16, TP53, and SMAD4/DPC4 genes. Inactivation of these tumor suppressor genes is a late event in pancreatic carcinogenesis [29]. Recently, Jones et al. [30] reported on the sequencing of 23,219 transcripts representing 20,661 protein-coding genes in 24 PDAs. In their landmark study, they found that all of the tumors harbored mutations of the KRAS oncogene. The rates of mutation of the tumor suppressor genes KRAS, p16/CDKN2A, TP53, and SMAD4 were 100%, 80%, 80%, and 60%, respectively. Presumably, alterations in these oncogenes and tumor suppressor genes have pleiotropic effects that lead to deregulation of signaling pathways that control cell proliferation, survival, angiogenesis, invasion, and metastasis.

However, the molecular mechanisms that link these genetic changes with the aggressive nature of pancreatic cancer remain poorly understood. Generally, these genetic alterations are perceived to regulate numerous signal transduction pathways that affect their downstream signal genes involved in the control of cell proliferation and differentiation [31]. Numerous aberrant activating signal transduction pathways are involved in the initiation and progression of pancreatic tumors [32–34]. Ultimately, transcription factors participate at the ends of all aberrant activation signal transduction pathways by causing upregulation or downregulation of expression of specific genes. Therefore, recent research efforts have focused on studying this activation mechanism and targeting the activity of transcription factors, which ultimately control the expression of genes involved in almost all aspects of cancer cell biology. Because Sp1 and Stat3 are important transcription factors in pancreatic cancer initiation and progression [10,23], we focused on their roles in pancreatic cancer pathogenesis and their crosstalk in pancreatic cancer progression.

3. Sp1 and pancreatic cancer biology

Sp1 is an essential transcription factor for many genes in regulation of cell growth, angiogenesis, and survival. Sp1 overexpression and abnormal activation are frequently detected in a wide variety of human cancer cell lines and tumors including pancreatic cancer (Table 1) [10,35]. Studies have demonstrated that abnormal Sp1 expression and activation may promote the growth, angiogenesis, and metastatic potential of tumor cells by inducing overexpression of many genes downstream from Sp1. The effects of Sp1 expression and activation on pancreatic cancer biology are mediated, at least in part, by regulation of the expression of these target genes (Fig. 1).

3.1. Roles of Sp1 in pancreatic tumor angiogenesis

Angiogenesis, which is the formation of new vessels via budding of existing ones, is essential for tumor growth and metastasis. Without angiogenesis, a solid tumor rarely grows larger than 2 mm in diameter [36]. Angiogenesis depends on the presence of angiogenic factors with direct action, such as endothelial growth factor, placental growth factor, and angiopoietins, as well as angiogenic factors with indirect action, such as fibroblast growth factor (FGF), platelet-derived endothelial growth factor, and interleukins (ILs) [37]. Although the numerous angiogenic factors may affect the angiogenic phenotype, only a small number of transcription factors may actually regulate expression of these angiogenic factors. For example, Sp1 is a key transcription factor in tumor angiogenesis in that it regulates angiogenic gene expression [38,39].

Vascular endothelial growth factor (VEGF) is a key angiogenic molecule that plays an important role in the growth, angiogenesis, and metastasis of many human cancers, including pancreatic cancer. VEGF recently proved to be a target gene of Sp1, and Sp1 can promote tumor angiogenesis and metastasis by regulating VEGF expression. Consistent with these results, our laboratory personnel demonstrated that human pancreatic tumors and most pancreatic cancer cell lines had high levels of Sp1 and VEGF expression. In addition, Sp1 expression and activity were directly correlated with VEGF expression according to immunostaining, Western blot analysis, and electrophoretic mobility shift assay (EMSA) [10]. Furthermore, our *in vitro* and animal experiments have shown that inhibition of Sp1 activity and expression by celecoxib could inhibit VEGF expression in pancreatic cancer cells and microvessel formation in pancreatic tumors [40].

More recently, Abdelrahim et al [41] found that not only Sp1 but also Sp3 and Sp4 could regulate VEGF expression. In that interesting study, the researchers used RNA interference (RNAi) to investigate the role of Sp family proteins in regulation of VEGF expression in pancreatic cancer cells. They found that Sp1 and Sp3 were required for transactivation of VEGF promoter constructs. In addition, they also showed that Sp4 was expressed in pancreatic cancer cells and cooperatively interacted with Sp1 and Sp3 to activate VEGF promoter constructs in pancreatic cancer cells..

In addition to VEGF, vascular endothelial growth factor receptor 2 (VEGFR2/KDR) is an important mediator of angiogenesis in pancreatic tumor progression. Recently, Higgins et al [42] reported that the pancreatic cancer cell lines Panc-1, AsPC-1, and MiaPaCa-2 expressed VEGFR2 mRNA as well as Sp1, Sp3, and Sp4 proteins, which can bind directly to the GC-rich region of the VEGFR2 promoter. Moreover, RNAi with small inhibitory RNAs for Sp1, Sp3, and Sp4 decreased VEGFR2 mRNA and reporter gene activity. These results suggest that Sp proteins can regulate not only VEGF but also VEGFR2 expression to promote pancreatic tumor angiogenesis.

3.2. Roles of Sp1 in pancreatic cancer metastasis

Metastasis plays a major role in the morbidity and mortality of most cancer patients. Metastasis is most often described as a series of sequential processes that involve processes consisting of the following steps: local invasion of tumor cells into tissue surrounding the primary tumor, growth of new blood or lymphatic vessels into the primary tumor, intravasation of components of the blood vessel wall via proteolysis and survival of tumor cells during transport in blood and/or lymphatic vessels. After reaching a new organ, tumor cells undergo adhesion to the endothelial lining followed by extravasation through the vessel wall and subsequent proliferation at the secondary site [43].

Recent studies showed that each step of the metastatic process—from the initial epithelial-mesenchymal transition (EMT) to the ultimate organotropic colonization—can be regulated by Sp1, suggesting a master regulator role for Sp1 in metastasis. During EMT, tumor cells undergo a developmental switch, changing from a polarized epithelial phenotype to a highly motile fibroblastoid or mesenchymal phenotype [44], which is often accompanied by dissolution of tight epithelial junctions, loss of cell adhesion, downregulated expression of some epithelial markers, and acquisition of migratory and invasive properties. Recently, Jungert et al [45] analyzed the role of the transcription factor Sp1 in EMT and migration of pancreatic cancer cells. They showed that Sp1 expression and activity is required for transforming growth factor- β -induced EMT and migration and that this function is mediated especially via transcriptional induction of vimentin expression. These results thus demonstrate that Sp1 may play an important role in pancreatic cancer metastasis via regulation of EMT and migration of pancreatic cancer cells.

Angiogenesis also plays an important role in cancer metastasis. Researchers have well established that angiogenesis is required for the acquisition of nutrients by tumors and the spread of tumor cells in the initial step in the metastatic process. After reaching a new organ, tumor cells also need angiogenesis to acquire nutrients and proliferate. Recently, Sp1 has proven to play a key role in tumor angiogenesis, and this role is especially mediated by transcriptional regulation of VEGF. Sp1 overexpression and activity correlate with VEGF expression and tumor microvessel formation in the pancreas not only in humans but also in orthotopic animal models of pancreatic cancer. Moreover, Sp1 can induce VEGF expression by directly binding to the VEGF promoter. Most importantly, inhibition of Sp1 expression may significantly suppress the invasion and metastatic ability of pancreatic cancer cells in vitro and in vivo by downregulating VEGF expression. Thus, these data suggest that Sp1 plays an important role in the metastasis of pancreatic cancer and that inhibition of Sp1 may be a useful therapeutic option for metastatic pancreatic cancer [40].

The mechanism of Sp1 in pancreatic cancer metastasis is also considered to be a regulator of proteolysis-related genes. During tumor invasion, the synthesis, secretion, and activation of proteases and their degradation of various components of the basement membrane and extracellular matrix are believed to be indispensable aspects of the invasive phenotype [46,47]. According to studies using pancreatic tumor samples and pancreatic cancer cell lines, the urokinase receptor (uPAR) plays important roles in tumor cell invasion and metastasis by degrading components of the basement membranes and extracellular matrix [48–50]. Recently, a study demonstrated the involvement of Sp1 in upregulation of uPAR expression in breast carcinoma [51]. The researchers in that study also found that downregulation of Sp1 activity by a novel PNA-DNA chimera could inhibit uPAR expression in and the migratory ability of breast cancer cells [52]. To confirm these results, our laboratory personnel are investigating whether Sp1 regulates uPAR expression at the transcriptional level and whether downregulation of Sp1 activity inhibits uPAR expression in and the metastatic ability of pancreatic cancer cells.

3.3. Sp1 expression in, and prognosis and targeted therapy for pancreatic cancer

Pancreatic cancer is one of the leading causes of cancer deaths. Surgery is the primary treatment of pancreatic cancer, and patients who can undergo curative tumor resection may have better outcomes than those who cannot. However, most patients are not eligible for surgical resection, and conventional chemotherapy and radiation therapy for this cancer remain largely ineffective. Even in patients undergoing potentially curative resection, long-term outcomes remain unsatisfactory owing to early recurrence and metastasis. Biomarkers have attracted much attention with the hope that they can predict the behavior of and serve as therapeutic targets for pancreatic tumors, thus providing tools for the individualized treatment of pancreatic cancer.

Numerous reports over the past several decades have described a variety of molecular markers that may have prognostic significance for pancreatic cancer. Although no single marker currently is perfect in predicting outcome of this cancer, a profile based on Sp1 may be helpful in directing therapy for it. Specifically, pancreatic cancer cells and tumors have aberrant overexpression of Sp1, whereas normal pancreatic tissue has very low expression of Sp1 [10,40]. In addition, multiple studies have demonstrated the prognostic importance of Sp1 expression at both the mRNA and protein level. High Sp1 expression in pancreatic neoplasms correlates with aggressive behavior, invasive clinical phenotypes, increased relapse rates, and decreased survival durations. For example, in a study of 42 patients with pancreatic tumors, Sp1 protein expression in the tumors was associated with lymph node metastasis and advanced disease stages (specifically, stage IIB-IV), and patients with Sp1-positive cancer had much shorter overall survival durations than did those with Sp1-negative cancer [53]. In addition, all patients with Sp1 expression had lymph node metastasis,

whereas none of those without this expression had lymph node metastasis. In the patients with metastasis, 77% of the primary tumors were positive for Sp1. Therefore, Sp1 may act as a marker of lymph node metastasis of pancreatic cancer. Moreover, the ability to identify pancreatic cancer patients with lymph node metastasis according to Sp1 expression can assist in individualized therapy decision making and treatment options.

Because Sp1 is preferentially expressed in pancreatic cancer cells and plays important roles in pancreatic cancer growth, angiogenesis, and metastasis, several approaches to targeting Sp1 in cancer therapy are being studied (Table 2). Many compounds and drugs used for this purpose have exhibited anti-pancreatic cancer characteristics by downregulating Sp1 activity and expression in pancreatic cancer. Of these drugs, nonsteroidal anti-inflammatory drugs (NSAIDs) are the most popular. Specifically, celecoxib is the first NSAID proven to have an anti-pancreatic cancer function by reducing both Sp1 DNA-binding activity and Sp1 transactivating activity. In a previous study, we found that celecoxib significantly inhibits pancreatic tumor growth, angiogenesis, and metastasis in vitro and in vivo. The molecular mechanism of the antitumor activity of celecoxib is suppression of the expression and phosphorylation of Sp1 protein and, in turn, its DNA-binding and transcriptional activity, which leads to inhibition of VEGF gene expression [40].

Recently, another NSAID, tolfenamic acid, also exhibited anti-pancreatic cancer activity. In a study in which researchers screened NSAIDs and cyclooxygenase inhibitors for their ability to reduce the expression of Sp proteins in pancreatic cancer cells, Abdelrahim et al [54] found that treatment with tolfenamic acid and structurally related biaryl derivatives significantly induced degradation of Sp1, Sp3, and Sp4, which led to inhibition of VEGF mRNA and protein expression in pancreatic cancer cells. Furthermore, they observed that treatment with tolfenamic acid significantly decreased tumor growth and weight, liver metastasis, and Sp1, Sp3, Sp4, and VEGF protein expression levels in tumors but that control treatment did not in a mouse model of pancreatic cancer.

Like some NSAIDs, the antibiotic mithramycin A (MIT) has anti-pancreatic cancer functions, as it downregulates Sp1 activity and expression in cancer cells. MIT can bind to GC-rich regions in chromatin and interfere with the transcription of genes bearing GC-rich motifs in their promoters. In a previous study, we demonstrated that MIT can inhibit pancreatic tumor angiogenesis, growth, and metastasis in vivo. Moreover, the anti-pancreatic cancer activity of MIT correlates directly with its suppression of the expression of Sp1 and its downstream molecules VEGF, platelet-derived growth factor, and epidermal growth factor. Thus, the anti-pancreatic cancer activity of MIT is a result, at least in part, of suppression of Sp1 expression and consequent downregulation of Sp1 downstream target expression [55].

Tolfenamic acid and MIT have distinct mechanisms of regulating Sp1 activity: the former facilitates Sp1 protein degradation [54,56], whereas the latter inhibits Sp1 activity via reversible interaction with double-stranded DNA with GC base specificity and selective regulation of transcription of genes having GC-rich promoter sequences [57]. Therefore, we sought to determine whether treatment with a combination of these two compounds had a synergistic effect on Sp1 activity and tumor growth in an animal model of pancreatic cancer. In that interesting study, we demonstrated that use of low-dose MIT in combination with low-dose tolfenamic acid is an important novel strategy of targeting the angiogenic molecule Sp1 at both the transcriptional and protein degradation level and that treatment with this combination in clinical studies is a rational step forward in the development of effective targeted therapies for pancreatic cancer [58].

Moreover, a recent study showed that treatment with betulinic acid (BA), a naturally occurring pentacyclic triterpene, inhibits prostate tumor growth by reducing the specificity protein transcription factors [59]. More importantly, MIT, bevacizumab, and BA have distinct mechanisms of inhibiting Sp1 activity [57,59]. In our previous studies, we demonstrated that treatment with a combination of MIT and bevacizumab or BA has a synergistic effect on Sp1 activity in and growth of, angiogenesis in, invasion of, migration of, and metastasis of pancreatic tumors [60,61].

4. Stat3 and pancreatic cancer biology

Constitutive activation of Stat3 in tumor cells is sufficient to induce development of a variety of cancers [62,63]. Constitutive activation of Stat3 is frequently detected in pancreatic tumor specimens obtained from patients with advanced disease and in pancreatic carcinoma cell lines (Table 3) [23,64]. Recently, researches reported on a number of genes downstream from Stat3 [17,18]. Altered expression and regulation of these genes play important roles in the pathogenesis of pancreatic cancer [23,64–65]. The effects of Stat3 on pancreatic cancer biology are mediated, at least in part, by regulation of these target genes, which participate in oncogenesis by stimulating cell proliferation, promoting angiogenesis and metastasis, mediating immune evasion, and conferring resistance to apoptosis induced by conventional therapies (Fig. 2).

4.1. Roles of Stat3 in pancreatic cancer proliferation

In addition to being a point of convergence for numerous oncogenic signaling pathways, studies have shown that Stat3 protects pancreatic tumor cells against apoptosis and promotes cell proliferation by regulating the expression of genes encoding antiapoptotic and proliferation-associated proteins, such as Bcl-xL, Bcl-2, Fas, MCL1, survivin, and cyclin D1 [66–68].

Regulation of genes in the BCL-2 family is the first indication that Stat3 signaling contributes to pancreatic malignancy by preventing apoptosis. Stat3 can upregulate transcription of the antiapoptotic BCL-2 family gene BCL-XL via direct interaction with the BCL-XL promoter. BCL-XL expression is dependent on constitutively activated STAT3 in multiple myeloma cells, and investigators showed that inhibition of STAT3 signaling by various means blocked the expression of BCL-XL in these tumor cells and sensitized them to FAS-mediated apoptosis [69]. Consistent with these findings, STAT3 has regulated BCL-XL expression and apoptosis in human pancreatic cancer cells [23,64].

MCL1 is another antiapoptotic gene in the BCL-2 family that is a target of Stat3. Blockade of Stat3 proteins in human tumor cells can downregulate MCL1 expression and induce tumor cell apoptosis [70]. Recently, Huang and Sinicrope [71] reported that knockdown of Stat3 or dominant-negative Stat3 expression downregulated Mcl-1 expression and sensitized pancreatic cancer cells to tumor necrosis factor-related apoptosis-inducing ligand-mediated apoptosis.

Activated Stat3 also induces expression of the antiapoptotic protein survivin, which is a member of the inhibitor of apoptosis family [72]. Moreover, treatment of pancreatic cancer cells with curcumin may inhibit constitutive STAT3 phosphorylation and result in induction of apoptosis by downregulating survivin gene expression [73].

In addition, Stat3 signaling pathways are known to regulate cancer cell proliferation by enhancing the activity of cyclins, contributing to accelerated cell-cycle progression [74,75]. Constitutive activation of STAT3 can promote expression of cyclin D1 by binding to the cyclin D1 promoter and subsequently affecting cell-cycle progression in tumor cells [76,77].

Moreover, Stat3 can regulate cyclin D1 expression and cell-cycle progression in human pancreatic cancer cells [64].

The extent to which Stat3 is needed to regulate the expression of genes that control pancreatic cancer cell proliferation and survival varies among different cell lines. Recently, Glienke et al [78,79] found that downregulation of Stat3 expression resulted in decreased cell viability in PANC-1 and BxPC-3 cell lines and induced apoptosis in BxPC-3 cells. However, they did not observe subsequent downregulation of expression of the antiapoptotic genes survivin or BCL-xL despite significant inhibition of Stat3. Furthermore, BxPC-3 cells exhibited significant increases in survivin and BCL-xL expression after downregulation of Stat3 expression.

4.2. Roles of Stat3 in pancreatic tumor angiogenesis

Angiogenesis plays important roles in many developmental and pathological processes, including tumor growth and metastasis. Researchers have well established that all tumors must undergo angiogenesis, also known as neovascularization, to acquire the nutrients needed for continued growth and metastatic spread [80]. An abundance of angiogenesis regulators and activation of oncogenes are essential for the maintenance of an angiogenic phenotype that supports tumorigenicity [81]. Although numerous angiogenesis regulators and oncogenes are involved in tumor angiogenesis, Stat3 in particular plays a pivotal role in it [82]. VEGF is one of the most important of the known angiogenic mitogens [83]. The role of Stat3 in angiogenesis was first evidenced when researchers found that VEGF was a direct target of Stat3 in murine melanoma cells and that transfection of these cells with the constitutively activated Stat3 mutant STAT3C was sufficient to increase VEGF expression and induce angiogenesis in vivo [84]. Also, blocking Stat3 signaling in cervical tumor can inhibit IL-6-induced upregulation of VEGF expression [85]. In addition, Stat3 indirectly regulates the transcription of VEGF by controlling the expression of hypoxia-inducible factor-1 α , a key inducible transcription factor for the VEGF gene [86].

Another important aspect of regulation of angiogenesis in tumors involves endothelial cell proliferation, migration, and microvascular tube formation. Several studies have shown that Stat3 activation is required for VEGF receptor signaling in endothelial cells and that blocking Stat3 in endothelial cells inhibits their migration and vessel formation [87,88]. A recent study also found that Stat3 regulates the expression of basic FGF (bFGF), another potent angiogenic factor, contributing to endothelial cell proliferation and migration [89].

Consistent with these findings, our studies indicated that Stat3 activation in human pancreatic cancer cell lines correlated with VEGF expression and that blockade of activated Stat3 via ectopic expression of dominant-negative Stat3 significantly suppressed VEGF expression, angiogenesis, and tumor growth in vivo. Furthermore, constitutively activated Stat3 directly activated the VEGF promoter in pancreatic cancer cells, whereas dominant-negative Stat3 inhibited the activity of the VEGF promoter. These results indicated that Stat3 directly regulates VEGF expression and hence angiogenesis in, growth of, and metastasis of human pancreatic tumors [65]. In addition, in a recent study, we found that RNAi for STAT3 inhibited STAT3-induced human umbilical vein endothelial cell migration and proliferation, significantly suppressing the expression of secreted VEGF in pancreatic cancer cells. Furthermore, blockade of activated Stat3 via RNAi could suppress pancreatic tumor growth and angiogenesis in vivo via downregulation of VEGF expression [90]. Collectively, these results demonstrated that Stat3 plays important roles in pancreatic tumor angiogenesis by directly regulating VEGF expression.

4.3. Roles of Stat3 in pancreatic cancer metastasis

Metastasis is the leading cause of pancreatic cancer-related deaths. However, the specific molecular circuitry that governs metastasis is not completely understood. In general, the molecular mechanisms that control the steps in metastasis are related to alterations of various oncogenes, tumor suppressor genes, metastasis suppressor genes, and growth factors and their receptors [91,92].

Stat3 is known to be activated by numerous cytokines, growth factors, and oncogenic proteins, suggesting that Stat3 signaling is one of the pathways commonly involved in regulation of cancer metastasis. The reported evidence of Stat3 involvement in tumor migration, invasion, and metastasis is substantial. Specifically, studies demonstrated that the expression of activated Stat3 protein is associated with metastasis of various types of tumors, including thymic epithelial tumors, colorectal adenocarcinoma, renal cell carcinoma, and cutaneous squamous cell carcinoma [93–96]. Recently, investigators showed that Stat3 transcriptionally activates the expression of genes that promote tumor invasion and metastasis. Specifically, activated Stat3 regulated invasion of melanoma cells by regulating the gene transcription of matrix metalloproteinase (MMP)-2 [89,97]. Also, Stat3 activation regulated invasion and metastasis of breast and bladder cancer cells by inducing MMP-1 and MMP-10 activation [98,99]. Furthermore, Stat3 may contribute to tumor cell migration and invasion via transcription-independent pathways, such as by modulating the invasiveness of ovarian cancer cells by interacting with phosphorylated focal adhesion kinase and paxillin [100].

More recently, we analyzed Stat3 activity in human pancreatic cancer cell lines and found that phosphorylated STAT3 protein expression and STAT3 DNA-binding activity were positively correlated with metastatic potential [101]. Additionally, blockade of activated Stat3 with AG-490 or RNAi significantly inhibited the activity of Stat3 in and subsequently reduced the invasion and adhesion potential of pancreatic cancer cells in vitro [101,102]. Moreover, researchers demonstrated the roles and mechanisms of activated Stat3 in pancreatic cancer metastasis using in vivo models of liver and lung metastasis. For example, in a study using an orthotopic animal model of pancreatic tumors, we showed that blockade of activated Stat3 via ectopic expression of dominant-negative Stat3 significantly suppressed VEGF expression in, angiogenesis of, and liver metastasis of pancreatic tumor cells [65]. In addition, blockade of Stat3 signaling in highly metastatic pancreatic cancer cells significantly suppressed the expression of the MMP-2 and VEGF genes in and invasiveness of the tumor cells and prevented lung metastasis in a mouse model [102]. The mechanisms of promotion of liver and lung metastasis of pancreatic cancer by Stat3 may be attributable to overexpression of MMP-2 and VEGF caused by Stat3 activation, which increases tumor invasion and angiogenesis.

Further implicating Stat3 in pancreatic tumor metastasis is the finding that STAT3 activity is required for transforming growth factor- β -mediated EMT in, invasion of, and metastasis of pancreatic cancer cells [103]. Recently, we also observed that activation of the Stat3 signaling pathway plays an important role in EMT in pancreatic cancer cells by regulating Snail gene expression and subsequently promoting the invasion potential of pancreatic cancer cells in vitro [104]. Collectively, these findings provide evidence that Stat3 affects pancreatic cancer metastasis via regulation of multiple steps in the process.

4.4. Modulation of Stat3 functions for therapeutic for pancreatic cancer

Because Stat3 is preferentially expressed in cancer cells and plays important roles in cancer proliferation, angiogenesis, and metastasis, it is an attractive therapeutic target for pancreatic cancer (Table 4). Efforts to develop inhibitors of Stat3 are under way. Approaches to

inhibition of Stat3 activity in human cancer cells include the use of tyrosine kinase inhibitors, RNAi, small-molecule compounds, antisense oligonucleotides (ASONs), decoy oligonucleotides, and phosphopeptides [105–109]. Although researchers have made progress in pursuing each of these strategies, no antitumor drug whose main mode of action is targeting of Stat3 has yet to reach the clinic.

4.4.1. Tyrosine kinase inhibitors—As described below, Stat3 can be targeted at one or more steps in tumor progression. Specifically, tyrosine kinase inhibitors can reduce tyrosine phosphorylation of Stat3 and receptor complexes. The most common tyrosine kinase inhibitors that inhibit Stat3 activity are AG-490 and cucurbitacin.

4.4.2. AG-490—AG-490 exhibits specificity for Janus kinase 2 (JAK2). Investigators found that AG-490 reduced Stat3 DNA-binding activity in U266 myeloma and mycosis fungoides cells, resulting in inhibition of their growth [110]. Administration of AG-490 also has reduced expression of activated Stat3, causing transient regression of murine myeloma and/or plasmacytoma tumors and inducing apoptosis of tumor cells in vivo. Moreover, combination therapy with AG-490 and IL-12 induced greater antitumor effects than did therapy with either agent alone [111]. Consistent with these results, we found that AG-490 markedly inhibited Stat3 activation in pancreatic cancer cells. In addition, inhibition of activated STAT3 by AG-490 not only highly suppressed MMP-2 and VEGF expression in but also reduced the invasiveness of human pancreatic cancer cells with high metastatic potential [101].

4.4.3. Cucurbitacin—Cucurbitacin is a natural product isolated from plants in the Cucurbitaceae and Cruciferae families that has been used as a folk remedy for some diseases in China and India for centuries. In the first reported study of cucurbitacin I (JSI-124), it reduced the expression of constitutively activated Stat3 in many cancer cells as well as reduce Stat3 DNA-binding activity and Stat3-mediated gene transcription. Also, JSI-124 has inhibited proliferation and induced apoptosis of multiple cancer cell lines in vitro as well as growth of A549 lung adenocarcinoma and MDA-MB-468 breast cancer cells in nude mice [112]. A recent study demonstrated that cucurbitacin B induced apoptosis of and had profound antiproliferative effects on human pancreatic cancer cells in vitro and in vivo by inhibiting the JAK/STAT pathway [113]. Furthermore, cucurbitacin B and cucurbitacin E have inhibited the growth of pancreatic cell cells in a dose- and time-dependent manner and caused accumulation of cells at the G2/M phase as well as apoptosis by downregulating Stat3 signaling [114,115].

4.4.4. Stat3-targeting RNAi—RNAi is a sequence-specific, posttranscriptional gene-silencing mechanism. Currently, RNAi is a powerful, widely used method of silencing mammalian gene expression in gene function studies [116]. Two procedures can effectively inhibit expression of targeted genes in mammalian cells without activation of the nonspecific interferon response: in vitro synthesized 21- to 25-nucleotide double-stranded RNAs (small interfering RNA [siRNA]) and 21- to 29-nucleotide double-stranded short hairpin RNA (shRNA) [117,118]. Several studies using experimental human tumor models have demonstrated the feasibility of RNAi for inhibition of expression of cancer-related genes, including Stat3. For example, a study demonstrated that blockade of Stat3 expression by an siRNA specific for Stat3 inhibited the growth of human prostate cancer cells and induced apoptosis in them [119]. Another study demonstrated that blockade of Stat3 expression using an shRNA downregulated the expression of Bcl-2, cyclin D1, and c-Myc in prostate cancer cells [105]. Moreover, inhibition of Stat3 and its related genes was accompanied by growth suppression and apoptosis induction in prostate cancer cells in vitro and in prostate tumors implanted in nude mice [105].

To investigate the use of RNAi to block Stat3 expression in and its effect on the biology of human pancreatic cancer cells, we have employed shRNA expression vector systems to knock down Stat3 gene expression in pancreatic cancer cells. We found that RNAi silencing of the Stat3 gene significantly suppressed the expression of MMP-2, VEGF, cyclin D1, and Bcl-xL, which was accompanied by marked inhibition of tumor-cell proliferation, angiogenesis, invasion, and metastasis in vitro and in vivo [90,102,120].

Recently, authors have reported that shRNA delivery systems using retroviral vectors, adenoviral vectors [121], and lentiviral vectors [122] can be used and are safe for humans. Of these vectors, the lentiviral ones are the most popular. Lin et al [123] used lentiviral infection to deliver a specially designed siRNA for murine STAT3 into the murine breast cancer cell line 4T1 to inhibit STAT3 protein expression. They found that elimination of Stat3 protein by the vector mediating RNAi in these cells eliminated the tumorigenicity of these cells in immunocompetent mice. To assess the application of a lentiviral vector mediating RNAi for Stat3 in pancreatic cancer cells, Yang et al [124] constructed such a vector mediating RNAi targeting of STAT3 (LV-STAT3siRNA) and proved that it could downregulate expression of the STAT3 gene in pancreatic cancer cells. In addition, LV-STAT3siRNA suppressed the proliferation and growth of pancreatic cancer cells. Moreover, the vector significantly inhibited the invasive ability of pancreatic cancer cells by decreasing their expression of VEGF and MMP-2. All of these results demonstrate that RNAi is capable of specific, highly stable functional silencing of Stat3 gene expression in pancreatic cancer cells and that silencing of the Stat3 gene by RNAi may be a novel strategy for pancreatic cancer treatment.

4.4.5. Small-molecule compounds—The small-molecule compounds that potentially inhibit Stat3 activity include those that either transcriptionally or posttranscriptionally inhibit Stat3 expression or abrogate Stat3 function. For example, a novel platinum(IV) compound (IS3 295) from the 2000-compound National Cancer Institute diversity set attenuated Stat3 activity by interacting directly with Stat3 and inhibiting its binding to specific DNA-response elements. In human and murine tumor cell lines with constitutively active Stat3, IS3 295 has functioned by directly suppressing expression of cyclin D1 and Bcl-xL, two of the known Stat3-regulated genes, resulting in cell growth arrest at G0/G1 phase and apoptosis [125]. Also, several small-molecule compounds directly or indirectly affect Stat3 levels. These compounds, including AUH-6-96, STA-21, and LLL-3, reduce Stat3 expression via different mechanisms [126–128].

With regard to pancreatic cancer, Lin et al [129] recently developed two novel small-molecule Stat3 inhibitors: FLLL31 and FLLL32. These compounds can inhibit Stat3 phosphorylation, DNA-binding activity, and transactivation in vitro, leading to impediment of multiple oncogenic processes and induction of apoptosis in pancreatic cancer cell lines. In addition, FLLL32 can inhibit pancreatic tumor growth and vascularity in chicken embryo xenografts as well as substantially reduce tumor volumes in mouse xenografts. These effects highlight the potential and efficacy of these new compounds in targeting pancreatic cancer cells that exhibit constitutive Stat3 signaling. In the future, initial use of molecules that target Stat3 as single agents or in combination with low-dose chemotherapy in pancreatic cancer patients with relapsed or refractory disease is expected. As experience using them increases, oncologists will administer these targeted therapies in treatment of pancreatic cancer in combination with standard chemotherapy or other small-molecule compounds, such as Sp1-specific inhibitors.

4.4.6. Other therapies—Many other Stat3-inhibiting therapies for human cancer are in use, for example, ASONDs, decoy oligonucleotides, and phosphopeptides. Use of ASONDs and decoy oligonucleotides is a simple, efficient, targeted approach to inhibiting specific

genes. In the laboratory, introduction of Stat3 ASONDs or decoy oligonucleotides into human cancer cells to cause selective degradation of Stat3 mRNA has resulted in decreased constitutive Stat3 activity, targeted-gene expression, and tumor growth [108,130]. Additionally, researchers have used phosphopeptides to effectively block Stat3-mediated DNA-binding activity, gene regulation, and cell transformation [109]. Although, to our knowledge, the literature contains little research on the use of ASONDs, decoy oligonucleotides, and phosphopeptides against Stat3 in pancreatic cancer cells, it makes a compelling argument for examining the therapeutic benefits of these methods for this purpose in the future.

5. Sp1 and Stat3 crosstalk in pancreatic cancer cells

Transcriptional factors in the Sp1 and Stat families are expressed ubiquitously and control numerous physiological processes, including differentiation, development, and cancer progression. Sp1 and Stat3 interact in many ways. For example, both Sp1 and Stat3 are rapidly activated in response to various stimuli, including growth factors and cytokines, although their expression is regulated by different molecular mechanisms. Moreover, once activated, Sp1 and Stat3 control the expression of proliferative, antiapoptotic, proangiogenic, and prometastatic genes. Some of these genes overlap and require cooperatively transcriptional regulation by Sp1 and Stat3 (Fig. 3).

5.1. Interactions between Sp1 and Stat3

Global chromatin binding surveys have revealed that Stat3 binds to at least 3000 different gene promoters and that the number of genes targeted by Sp1 family members is even larger [131,132]. Importantly, Sp1 and Stat3 regulate both distinct and overlapping groups of genes during tumorigenesis, such as cell growth, apoptosis, differentiation, angiogenesis, and metastasis. This can be explained in part by the distribution of Sp1 and Stat3 binding sites in the regulatory regions of such genes. For instance, a gene that contains both Sp1 and Stat3 binding sites may be regulated by both factors in a cooperative manner. Indeed, Gibadulinova et al [133] showed that adjacent Stat3 and Sp1 binding sites in the S100P promoter are critical for activation of S100P gene transcription in cancer cells, demonstrating that Stat3 and Sp1 function cooperatively to activate targeted genes in cancer progression. Furthermore, recent studies demonstrated that adjacent Sp1 and Stat3 binding sites in the C/EBPdelta and the Jak3 promoter are critical for activation of C/EBPdelta and Jak3 transcription in M1 murine myeloid leukemia cells [134,135].

In addition to binding to adjacent sites in the control regions of shared target genes, several Stat family members, particularly Stat1 and Stat3, physically interact with Sp1 and Sp3. Such interactions may result in either specific transcriptional synergy or repression of Stat3- and/or Sp1-regulated genes. Stat3 and Sp1 interact under several different scenarios. For example, a study demonstrated that Stat1 (as a phosphorylated homodimer) could bind to the inverted repeat and interact with Sp1 to cause maximal interferon- γ -dependent intercellular adhesion molecule-1 gene activation [136]. In another study, Stat1/3 directly controls promoter P1, and the Stats indirectly regulate promoter P2 via Sp1/3 in response to IL-6 [137].

In addition, certain Sp1-induced inhibitory proteins can bind to Stat3 and inhibit its binding to DNA. However, the precise mechanism of Stat3-Sp1 interaction in the nuclei of cancer cells remains to be defined. Moreover, Sp1 is not the only transcription factor that cooperates with Stat3. Other Stat3 partners include nuclear factor- κ B, hypoxia-inducible factor-1 α , and other factors [131,138]. These factors may further modulate Sp1-Stat3 interaction. Therefore, further exploration of the collaboration and crosstalk between Sp1 and Stat3 in pancreatic cancer is tempting.

Functional interaction or antagonism between Sp1 and Stat3 can also occur at the level of signal transduction regulators, such as suppressor of cytokine signaling (SOCS) proteins, whose expression is controlled by Sp family members. Particularly, Sp3 is important for IL-6-induced transcriptional activation of the SOCS3 gene promoter and acts as an enhancer of basal as well as induced transcriptional activity, resulting in enhanced SOCS3 mRNA and protein expression [139]. Likewise, Sp2 is required for interferon- γ -induced regulation of SOCS1 gene expression [140]. Moreover, prolactin-induced SOCS3 promoter activation requires Sp1 binding to the GC-rich region of the promoter, and overexpression of SOCS3 markedly suppresses STAT3 expression and abrogates STAT5 phosphorylation in breast cancer cells, resulting in decreased proliferation of them [141]. Considering that SOCS proteins play important roles in negative regulatory control of signal transduction of the STAT cascade, Sp family members may inhibit the STAT signaling pathway in cancer signal transduction by downregulating SOCS gene expression.

With regard to pancreatic cancer, Stat3 has proven to regulate the expression of bFGF, hypoxia inducible factor-1 α , and VEGF, all of which are important mediators in pancreatic cancer angiogenesis [84,89]. Also, Sp1 is an important regulator of VEGF gene transcription and potential regulator of bFGF gene expression [10,142]. However, whether Stat3 and Sp1 can function cooperatively to activate VEGF and bFGF gene expression in tumor angiogenesis remains to be proven. Therefore, further study of the collaboration and crosstalk between Sp1 and Stat3 in pancreatic cancer cells may not only shed more light on pancreatic tumorigenesis but also help improve understanding of and aid in the development of effective therapies that target Sp1 and Stat3.

5.2. Modulation of both Sp1 and Stat3 function for therapy for pancreatic cancer

Given that Sp1 and Stat3 play key roles in pancreatic cancer progression via collaboration and crosstalk, they both may be preventive and therapeutic targets for pancreatic cancer. Efforts to develop inhibitors of Sp1 and Stat3 are under way. For example, a recent study demonstrated that 1,2,3,4,6-penta-O-galloyl-beta-D-glucose, a polyphenolic compound, could selectively inhibit malignancy without any toxic effects. The potential mechanisms of the polyphenolic compound included antiangiogenesis, antiproliferative action, and induction of apoptosis by regulation of Sp1 and Stat3 expression [143]. Furthermore, researchers have developed various methods of specific targeting of Sp1 and Stat3 to retard tumor progression. Of these methods, RNAi holds great therapeutic potential for cancer and is now being used to target Sp1 or Stat3 for this purpose. Recent studies have shown that simultaneous downregulation of the expression of two genes using RNAi had a synergistic beneficial effect in the treatment of cancer [144,145]. These results highlight the potential of using double gene targeting via RNAi in the treatment of cancer. However, until now, whether simultaneous inhibition of Sp1 and Stat3 expression via RNAi had a synergistic inhibitory effect on pancreatic cancer progression was unknown. Interestingly, we found that silencing both the Stat3 and Sp1 genes using RNAi significantly suppressed the growth and angiogenesis of pancreatic tumors in vitro more so than did silencing either gene alone (data not shown). Therefore, targeting both Sp1 and Stat3 via RNAi may have additive or synergistic benefits in the treatment of pancreatic cancer.

In addition, many compounds and drugs have exhibited anti-pancreatic cancer effects by downregulating Sp1 and/or Stat3 activity and expression in cancer cells. Of these agents, NSAIDs and tyrosine kinase inhibitors are the most popular. Therefore, we are seeking to determine whether treatment with a combination of NSAIDs and tyrosine kinase inhibitors has a synergistic effect on pancreatic cancer growth, angiogenesis, and metastasis. Studies have indicated that specifically targeting Sp1 and Stat3 together may be effective in treating pancreatic cancer.

6. Conclusions and future directions

Numerous lines of experimental and clinical evidence demonstrate that Sp1 and Stat3 play key roles in pancreatic cancer progression. Also, many Sp1 and Stat3 family members are cross-linked in tumor signal transduction, and Sp1 and Stat3 can function cooperatively to induce target gene expression in tumorigenesis. Therefore, targeting both Sp1 and Stat3 may be a preventive and therapeutic strategy for pancreatic cancer. Furthermore, the reported observations of the roles and crosstalk of Sp1 and Stat3 in pancreatic tumorigenesis have strong implications regarding tumorigenesis in other cancers, as overexpression of Sp1 and Stat3 is common in cancer patients. Future studies of the crosstalk between Sp1 and Stat3 will provide valuable insight into the mechanisms of Sp1 and Stat3 in pancreatic cancer progression, providing new therapeutic opportunities.

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Biography

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Dr. Xie is currently a professor of medicine at the Department of Gastrointestinal Medical Oncology of the University of Texas MD Anderson Cancer Center in Houston, Texas. His research interest has been cancer biology with focus on cancer progression and metastasis and translational oncology with focus on prognostic and therapeutic molecular biomarkers of gastrointestinal cancer, e.g., pancreatic cancer, gastric cancer and liver cancer. Using molecular and cell biology techniques coupled with human specimens and animal models, Dr. Xie has continued to investigate the molecular mechanisms governing pancreatic cancer development and progression, with a particular interest in the regulation and interplay of multiple signaling pathways such as Sp1, Stat3, KLF4 and FoxM1.

Fig. 1.
Function of Sp1 in pancreatic cancer cells.

Fig. 2.
Function of Stat3 in pancreatic cancer cells.

Fig. 3.
Sp1 and Stat3 collaborations in gene regulation.

Table 1

Expression of Sp1 in pancreatic cancer cell lines and tumor specimens

Author/Year	<u>Expression of Sp1</u>		Methods	References
	Cells	Tumors		
Shi Q/2001	↑	↑	Western blot/IHC	[10]
Abdelrahim M/2004	↑		Western blot	[41]
Higgins KJ/2006	↑		Western blot	[42]
Jungert K./2007	↑		Western blot	[45]
Jiang NY/2008		↑	IHC	[53]

Table 2

Modulation of Sp1 function in therapy for pancreatic cancer

Author/Year	Methods	Targeted Tumor Biology	References
Wei D/2004	Celecoxib	Tumor angiogenesis and metastasis	[40]
Higgins KJ/2006	RNAi	Tumor angiogenesis	[42]
Jungert K./2007	RNAi	Tumor migration and EMT	[45]
Abdelrahim M/2006	Tolfenamic Acid	Tumor growth, angiogenesis	[54]
Yuan P/2007	Tolfenamic Acid	Tumor growth, angiogenesis and metastasis	[55]
Jia Z/2007	Mithramycin A and Bevacizumab	Tumor growth and metastasis	[60]
Jia Z/2010	Mithramycin A and Tolfenamic Acid	Tumor angiogenesis and growth	[58]
Gao Y/2011	Mithramycin A and Betulinic Acid	Tumor growth, angiogenesis, invasion, migration and metastasis	[61]

Table 3

Expression of Stat3 in pancreatic cancer cell lines and tumor specimens

Author/Year	<u>Expression of Stat3</u>		Methods	References
	Cells	Tumors		
Scholz A/2003	↑	↑	Western blot/IHC	[23]
Toyonaga T/2003	↑	↑	EMSA/IHC	[64]
Wei D/2003	↑	↑	Western blot/IHC/EMSA	[65]
Huang S/2010	↑		Western blot	[71]
Huang C/2011	↑	↑	Western blot/IHC	[120]

Table 4

Modulation of Stat3 function in therapy for pancreatic cancer

Author/Year	Methods	Targeted Tumor Biology	References
Toyonaga T/2003	AG-490	Tumor growth	[64]
Huang C/2006	AG-490	Tumor invasion	[101]
Zhang M/2010	Cucurbitacin B	Tumor growth	[114]
Sun C/2010	Cucurbitacin E	Tumor growth	[115]
Huang C/2007	RNAi by a plasmid vector	Tumor invasion and metastasis	[102]
Yang G/2009	RNAi by a lentiviral vector	Tumor invasion and growth	[124]
Huang C/2011	RNAi by a plasmid vector	Tumor angiogenesis	[90]
Huang C/2011	RNAi by a plasmid vector	Tumor growth	[120]
Lin L/2010	Small-molecule Stat3 inhibitors	Tumor growth and angiogenesis	[129]