Pathophysiological roles of Pim-3 kinase in pancreatic cancer development and progression

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The pathophysiological roles of Pim-3 kinase in pancreatic cancer development and progression

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Abstract (no less than 200 words)

Pim-3 is a member of Provirus integration site for Moloney murine leukemia virus (Pim) family proteins that exhibits serine/threonine kinase activity. Similar to other Pim kinases (Pim-1 and Pim-2), Pim-3 is involved in many cellular processes including cell proliferation, survival, and protein synthesis. Although Pim-3 is expressed in normal vital organs, it is overexpressed particularly in tumor tissues of endoderm-derived organs including the liver, pancreas, and colon. Silencing of Pim-3 expression can retard *in vitro* cell proliferation of hepatocellular, pancreatic, and colon carcinoma cell lines, by promoting cell apoptosis. Pim-3 lacks any regulatory domains similarly as Pim-1 and Pim-2 do, and therefore, Pim-3 can exhibit its kinase activity once it is expressed. Pim-3 expression is regulated at transcriptional and post-transcriptional levels, by transcription factors such as Ets-1 and post translational modifiers such as translationally controlled tumor protein (TCTP), respectively. Pim-3 could promote growth and angiogenesis of human pancreatic cancer cells *in vivo* in an orthotopic nude mouse model. Furthermore, a Pim-3 kinase inhibitor inhibited cell proliferation when human pancreatic cancer cells were injected into nude mice, without inducing any major adverse effects. Thus, Pim-3 kinase may serve as a novel molecular target for developing targeting drugs against pancreatic and other types of cancer.

Key words: Serine/threonine kinase; Pancreatic cancer; Ets-1; Translationally Controlled Tumor Protein; c-Myc; Vascular endothelium growth factor; Apoptosis; Cell cycle

Core tip: The present review describes the advanced knowledge on the roles of Pim-3 in pancreatic cancer development and progression, and provides a new idea for Pim-3 as a therapeutic target in human pancreatic cancer.

1. Introduction

Pancreatic cancer is the fourth leading cause of cancer related deaths in the USA [1], Patients usually suffer from non-specific abdominal discomforts in the primary stages, which often delay early diagnosis and treatment. Furthermore, even in the initial evolutionary phase of disease development, pancreatic cancer cells tend to undergo invasion and metastasis. Therefore, complete removal of tumors by surgical procedures is often impossible. Another major stumbling block in treating pancreatic cancer is its frequent resistance to the treatments of chemotherapy and radiotherapy. Consequently, pancreatic cancer has an exceptionally poor prognosis with an overall 5-year survival rate of less than 5% [2, 3]. Thus, a novel molecular targeted therapy will be a required therapeutic option for human pancreatic cancer treatment.

Malignant lesions of the pancreas show a ductal, an acinar, or an endocrine lineage. Nearly 80 % of pancreatic carcinomas are classified as pancreatic ductal adenocarcinoma (PDAC) [4]. An activating mutation in a key proto-oncogene, K-ras, has been observed in most PDACs and is presumed to be the first significant event involved in pancreatic carcinogenesis [4]. The development and progression of PDAC are associated with additional multiple genetic and epigenetic alterations in several proto-oncogenes, tumor-suppressor genes, and signaling pathways. Pim-3 kinase has essential roles in the regulation of signal transduction cascades. Moreover, its expression is enhanced in human pancreatic cancer cell lines and blocking of its expression induced apoptosis and decreased chemoresistance in human pancreatic cancer[5, 6].

Provirus integration site for Moloney murine leukemia virus (Pim) family is a proto-oncogene, which belongs to the group of calcium/calmodulin-regulated kinase and exhibits serine/threonine kinase activity [7]. The Pim family consists of three members, Pim-1, Pim-2, and Pim-3 [8]. The Pim-1 gene was first discovered as a proviral insertion site in Moloney murine leukemia virus [9]. A subsequent study demonstrated that Pim-1 transgenic mice are predisposed to the development of experimental T cell lymphoma in cooperation with c-Myc and N-Myc [10]. Pim-2 was similarly identified as a proviral integration site in Moloney murine leukemia virus-induced T cell lymphomas [11] and can

synergize with c-Myc-induced lymphomagenesis [8]. Pim-3 was first identified as a novel gene, which is induced by membrane depolarization or forskolin in rat PC12 pheochromocytoma cells, and was designated as kinase induced by depolarization (KID-1) [12]. Subsequently, KID-1 was renamed Pim-3 due to its high sequence similarity with other Pim family proteins, Pim-1 and Pim-2. Although Pim-3 can be detected in several normal tissues including those of the brain and heart, it is expressed in high levels in tumor tissues of various organs particularly those of endoderm-derived organs such as the pancreas, liver, colon and stomach [5, 13, 14].

In this review, we aim to highlight the pathophysiological roles of Pim-3 in the development and progression of cancer, particularly pancreatic cancer. Moreover, by considering the sequence similarity of Pim-3 with other Pim kinases, we were able to rationalize and predict the possible functions of Pim-3 by extrapolating from the data established for other Pim family members, particularly Pim-1. We further discuss the potential of Pim-3 as a novel molecular target for antineoplastic therapy.

2. Structure of Pim-3 protein

The open reading frame of human Pim-3 mRNA encodes a protein consisting of 326 amino acids with a calculated molecular weight of 35,861 (Figure 1) [13]. Human Pim-3 protein shares a high percentage of sequence homology with other members of the Pim family; Pim-3 and Pim-1 are 71% identical at the amino acid level, and Pim-3 and Pim-2 are 44.0% identical [14-17].

The crystal structure of the Pim-3 protein has not yet been established, but several research groups have independently reported the crystal structure of Pim-1 and Pim-2 in the free form as well as in complex with their inhibitors [18-22]. The Pim-1 kinase adopts a two-lobe kinase fold connected by a hinge region (residues 121–126) [18]. The N-terminal lobe is composed of antiparallel β-sheets while the C-terminal lobe is composed mainly of α-helices (Figure 1). The adenosine triphosphate (ATP)-binding site is located in a deep intervening cleft between the two lobes and the hinge region. The Pim family proteins have no regulatory domains. Moreover, the ATP binding pocket in Pim-1

remains open irrespective of the presence or the absence of ATP [18], indicating a continuous maintenance of an active state conformation. Similar findings have been reported for the structure of Pim-2 kinase [20]. Thus, this may account for a good correlation between protein expression levels and overall kinase activity in the case of Pim-1 and Pim-2 [15]. Given a high sequence similarity (Figure 1 and NCBI Reference Sequence: NP_001001852.2), it is highly likely that Pim-3 kinase can adopt a similar three dimensional active conformation. Importantly, several residues believed to confer specificity in Pim-1 kinase are also conserved within Pim-2 and Pim-3 proteins.

3. Mechanisms underlying control of Pim-3 expression

Pim-3 mRNA is detected in several normal human tissues including the heart, brain, lung, kidney, spleen, placenta, skeletal muscle, and peripheral blood leukocytes, but not in colon, thymus, liver, and small intestine [13]. Pim-3 is expressed in endothelial cells [23]. Focal cerebral ischemia enhances Pim-3 mRNA expression in the peri-infarction cortex at early time points [24]. Similarly, ischemia reperfusion injury enhances intra-cardiac Pim-3 expression through p38-mediated signaling pathway [25]. In the mouse embryo, *Pim-3* gene expression is detected in the liver, kidneys, lungs, thymus, central nervous system, periphery of the pancreas, secretory epithelium of the stomach and intestinal epithelium [26].

Pim-3 mRNA is found to be expressed in a panel of human Ewing's family tumor cell lines [27] and nasopharyngeal carcinoma cell lines [28]. Likewise, we revealed that Pim-3 protein is scarcely detected in adult normal endoderm-derived organs such as liver, pancreas, colon, and stomach, but its expression is augmented in premalignant and malignant lesions of these organs (Table 1) [5, 13, 29, 30]. Pim-3 protein is mostly detected in the cytoplasm of these tumors. In the liver, aberrant expression of Pim-3 protein is also observed in the precancerous lesions such as regenerative nodules and adenomatous hyperplasia [13]. Similarly, in the colon and the stomach, Pim-3 protein is detected at higher levels in adenoma tissues compared with adenocarcinoma tissues [29, 30]. These observations suggest that Pim-3 plays a crucial role in the initial phase of carcinogenesis.

Pim-3 expression is regulated mainly at transcriptional and post-transcriptional levels. We will discuss the regulatory mechanisms at these two levels in detail.

3.1. Transcriptional regulation of Pim-3 expression

The EWS/Ets fusion proteins are pathognomonic for Ewing's sarcoma (EWS). These fusion proteins arise from the chromosomal translocations that combine a portion of the amino-terminal region of EWS to one of the five members of Ets family transcription factors, friend leukemia integration transcription factor (FLI), Ets-related gene (ERG), FEV, Ets translocation variant 1 (ETV1), Ets translocation variant 4 (ETV4/E1AF) [31]. Deneen and colleagues demonstrated that EWS/Ets fusion proteins can enhance Pim-3 gene transcription [27] in NIH 3T3 cells.

We have determined the 5′-flanking region of the human *Pim-3* gene, in order to elucidate the molecular mechanisms underlying constitutive Pim-3 expression in human pancreatic cancer cells. The human Pim-3 gene contains a canonical TATA box and putative binding sites for several known transcription factors, such as signal transducer and activator of transcription (Stat)3, Sp1, Ets-1, and nuclear factor NF-κB and NF-1 [32]. Pim-3 expression is enhanced in murine embryonic stem cells by leukemia inhibitory factor (LIF)/gp130-dependent signaling and the Stat3 transcription factor [33]. In contrast, the transfection of dominant negative form of Stat3 failed to inhibit the promoter activity of *Pim-3* gene in human pancreatic cancer cells [32]. We further demonstrated that the region between −264 and −164 bp is essential for constitutive *Pim-3* gene expression. This region contains one NF-κB, two Sp1, and two Ets-1 binding sites. *Pim-1* gene can be induced by CD40-mediated signaling in an NF-κB-dependent manner [34]. However, the mutation in NF-κB binding site of the *Pim-3* gene failed to reduce promoter activities in human pancreatic cancer cells [32]. Further examination has revealed that two Sp1 binding sites and the distal Ets binding site are crucial for the constitutive *Pim-3* gene expression in human pancreatic cancer cells. The crucial roles of Ets-1 in constitutive *Pim-3* gene expression are further supported by our observations that the overexpression of Ets-1 enhances Pim-3 expression, whereas the transfection of dominant negative form of Ets-1

or Ets-1 small interfering RNA (siRNA) reduces Pim-3 expression in human pancreatic cancer cells [32]. As the expression of both Ets-1 and Sp1 is enhanced in various types of cancer, including pancreatic cancer [35, 36], Ets-1 and Sp1 may act cooperatively to induce constitutive *Pim-3* gene expression as observed with their other target genes [37].

3.2. Post-transcriptional regulation of Pim-3 expression

Pim kinase mRNAs have multiple copies of AUUUA sequences in their 3′ untranslated regions (UTR), a typical characteristic sequence of mRNA with a short half-life. Moreover, GC-rich sequences are present in the 5′ UTR of Pim mRNAs and frequently require cap-dependent translation. Indeed, the overexpression of eukaryotic translation initiation factor 4E (eIF4E) leads to an increase in Pim-1 protein levels, indicating that Pim-1 mRNA is translated in a cap-dependent manner [38]. Moreover, eukaryotic translation initiation factor, eIF4E, can bind a stem-loop-pair sequence present in the 3′ UTR of Pim-1 mRNA, which allows nuclear export and translation of Pim-1 transcript [39]. Since Pim-3 mRNA shows analogous sequences as Pim-1 mRNA, the translation of Pim-3 mRNA can be regulated in a similar way.

Similar to Pim-1, Pim-3 can autophosphorylate some of their serine residues but whether this has any functional significance is yet to be elusive [19]. Moreover, Pim-1 and Pim-3 have been shown to bind to the serine/threonine protein phosphatase 2A (PP2A), resulting in their dephosphorylation, ubiquitination, and proteasomal degradation[40, 41].

3′UTR of Pim-1 harbors multiple binding sites for miRNAs including miRNA-33 [42], miRNA-16 [43], miRNA-1 [44], miRNA-328 [45], and miRNA-210 [46]. The miRNAs are generally highly conserved evolutionarily. [42] They can bind to the putative target sites present in 3′UTR of *Pim-1* gene and can directly inhibit its expression at the post-transcriptional level, thereby blocking proliferation and growth of cancer cells and smooth muscle cells. The relevant analysis for the structure of human *Pim-3* mRNA indicates that 3’ UTR of *Pim-3* gene harbors multiple binding sites for miRNAs (www.ebi.ac.uk; www.microrna.org). It will be interesting to know whether Pim-3 translation can be regulated in a similar manner.

We have identified a translationally controlled tumor protein (TCTP/TPT1) that interacts with Pim-3 by using yeast two-hybrid screening [47]. TCTP was aberrantly expressed and co-localized with Pim-3 in human pancreatic cancer cells. Mapping studies have confirmed that the co-localization is due to the interaction between the amino acids in the C-terminal fold of Pim-3 and the amino acids in the N-terminal sequence of TCTP. Pim-3 had no effect on TCTP expression or phosphorylation; however, overexpression of TCTP increased Pim-3 expression in a dose-dependent manner. Moreover, RNAi-mediated ablation of TCTP expression reduced Pim-3 protein but not mRNA via ubiquitin-proteasome degradation pathway. The resultant reduced Pim-3 expression eventually inhibited tumor growth *in vitro* and *in vivo* by arresting cell cycle progression and enhancing apoptosis. Furthermore, TCTP and Pim-3 expression were significantly correlated in pancreatic adenocarcinoma specimens, and in tumors from patients showing high expression levels of TCTP and Pim-3 obtained at an advanced stage of cancer. Thus, TCTP-mediated enhancement of Pim-3 protein expression may be involved in the regulation of cell cycle progression and apoptosis in pancreatic carcinogenesis [47].

4. Biological functions of Pim-3

Treatment with Pim-3 shRNA can decrease *in vitro* proliferation of various types of cancer cells by inducing apoptosis [5, 13, 29]. The major function of Bad, a pro-apoptotic BH3-only protein, is to regulate apoptosis. Unphosphorylated Bad binds and eventually inactivates anti-apoptotic family members, primarily Bcl-X_L and also Bcl-2. Phosphorylation of Bad at Ser^{112}, Ser^{136}, and Ser^{155} impairs its binding to Bcl-X_L and Bc1-2 and the translocation of Bad from the surface of mitochondria to the cytosol is guided by the protein 14-3-3. The presence of unbound Bc1-X_L maintains a mitochondrial membrane potential and inhibits apoptosis [48, 49]. Pim-1 and Pim-2 can phosphorylate Bad at Ser^{112}, while Akt phosphorylates Ser^{136} and Ser^{155}. The phosphorylation of Bad can result in its inactivation and subsequent inhibition of apoptosis [50, 51]. Elevated levels of Pim-3 increases the amount of Bad phosphorylated at

Ser^{112} and inhibits apoptosis, while Pim-3 shRNA treatment dephosphorylates Ser^{112} and promotes apoptosis (Figure 2) [5, 29]. Therefore, similar to Akt and other Pim kinases, Pim-3 can modulate apoptosis by phosphorylating the pro-apoptotic molecule, Bad. Moreover, *Pim-3* gene transduction increased Bcl-2 expression, suppressed apoptosis as evidenced by reduced caspase-3 activation, and eventually protected against hepatic failure in D-galactosamine-sensitized rats, which received lipopolysaccharide [52]. Similarly, the transfection of *Pim-3* gene into cardiomyocytes attenuated ischemia/reperfusion injury-induced cell death through a p38 mediated MAPK signaling pathway [25]. Erythropoietin can protect renal cells from apoptosis by activating Stat5 and this anti-apoptotic effect is also mediated by Pim-3 [53].

Pim-3 shows a high sequence identity with Pim-1 even at their kinase domains (Figure 1). Both Pim-1 and Pim-3 bind to a consensus peptide substrate (AKRRRRHPSGPPTA) with a remarkable high affinity (*Kd* = 40–60 nM), whereas the binding affinity of this peptide for Pim-2 is relatively weak (640 nM) [19]. Therefore, Pim-1 and Pim-3 can phosphorylate the same or a similar set of substrates, and the evaluation of Pim-1 characteristics and functions can provide useful insights to decipher the major biological functions of Pim-3. In addition to Bad, Pim kinases can phosphorylate a wide range of cellular proteins. These include transcription factors (Stat [54], c-Myc [55], Myb [56], runt-related transcription factor (Runx) 1 and Runx3), cell cycle regulators (p^{21CIP} and p^{27KIP1}, Cdc25A, and Cdc25C), signaling pathway intermediates (suppressor of cytokine signaling 1 (SOCS1) [57], SOCS3 [58], and MAP3K5 [59]), and regulators of protein synthesis (eukaryotic translation initiation factor 4B (eIF4B))[60].

Pim-1 can phosphorylate Cdc25A, thereby increasing its phosphatase activity and the activity of cyclin D1-associated kinases, which can result in cell cycle progression [61]. Pim-1 phosphorylates Cdc25C-associated kinase 1 (C-TAK1), which can potently inhibit Cdc25C and can promote cell cycle progression at the G_2/M phase [62]. Pim-1 can phosphorylate the threonine residue of p21, another molecule involved in cell cycle progression. Its phosphorylation leads to its relocation to the cytoplasm resulting in enhanced protein stability and eventually increased cell proliferation [63, 64]. All Pim

kinases including Pim-3 can phosphorylate CDK inhibitor, p27 at its threonine residues, thereby inducing the binding of p27 to 14-3-3 protein, resulting in its nuclear export and proteasome-dependent degradation [65]. Moreover, Pim-1 phosphorylates and inactivates forkhead transcription factors, FoxO1a and FoxO3a, resulting in depressed *p27* gene transcription, which leads to cell cycle progression (Figure 2) [65]. Similarly, the transfection with Pim-3 shRNA reduced G1 population of human pancreatic cancer cells compared with the cells transfected with scramble shRNA[5]. Moreover, a small-molecule Pim-3 kinase inhibitor markedly retarded *in vitro* growth of human pancreatic cancer cell lines by inducing G2/M arrest[66], suggesting a potential role for Pim-3 in cell cycle progression. Consistently, cell cycle progression is accelerated in hepatocytes of transgenic mice, which express human Pim-3 cDNA selectively in hepatocytes[67] and downregulation of Pim-3 decreased the amounts of Cdc25C, cyclin B1, and phospho-p21 (Our unpublished data). Thus, Pim-3 can promote cell cycle progression and eventually contribute to carcinogenesis by modulating the functions of these regulatory molecules involved in cell cycle progression.

Mice deficient in all three Pim kinases are designated as triple knockout (TKO) mice. TKO mice have reduced body size at birth and throughout the postnatal period of their life and they are viable and fertile [68]. However, TKO mouse-derived embryonic fibroblasts (MEFs) show depressed AMP-dependent protein kinase (AMPK) activity, grow slowly in culture medium, and have decreased rates of 5′-cap-dependent protein synthesis [69]. Transduction of *Pim-3* gene alone into these MEFs can reverse AMPK activation, increase protein synthesis, and drive the growth to a similar level as wild-type MEFs. Moreover, Pim-3 expression can markedly increase the levels of c-Myc and the peroxisome proliferation-activated receptor γ co-activator 1α (PGC-1α), enzymes capable of regulating glycolysis and mitochondrial biogenesis [69]. Similarly, Pim-1 and Pim-2 phosphorylate serine and threonine residues of c-Myc protein [55]. Furthermore, Pim-1 can act as a co-activator of Myc by phosphorylating Ser^{10} of histone H3 on the nucleosome at the Myc-binding sites [70]. Thus, Pim-3 can augment the rate of protein synthesis by modulating AMPK, c-Myc and PGC-1α (Figure 2).

Pim-1 and Pim-3 together play a significant role in maintaining the self-renewal capacity of mouse embryonic stem (ES) cells *in vitro* [33]. ES cells overexpressing Pim-1 and Pim-3 have a greater capacity to self-renew and display a greater resistance to LIF deprivation as evidenced by a clonal assay. On the contrary, ablation of Pim-1 and Pim-3 genes increases the rate of spontaneous differentiation in a self-renewal assay and impairs the growth of undifferentiated ES cell colonies with increased rate of apoptosis [33].

Pim-3 is highly expressed at the cellular lamellipodia in endothelial cells and is co-localized with focal adhesion kinase (FAK). In addition, Pim-3 shRNA treatment impairs endothelial cell spreading, migration, and proliferation, leading to a reduction in tube-like structure development in a Matrigel assay [23]. However, TKO mice did not display any apparent abnormal phenotypes in embryogenesis and vascular development [68].

Pim-3 expression is detected in the β cells located in the pancreatic islets [71]. Pim-3-deficient mice exhibit an increased glucose tolerance and insulin sensitivity. Moreover, Pim-3 can negatively regulate insulin secretion by inhibiting the activation of Erk1/2 via SOCS6 [71]. In contrast, the inhibition of another survival kinase, Akt, can induce hyperglycemia [72, 73].

The switch from latent phase to productive viral reactivation (lytic phase) is crucial for sustaining viral multiplication in infected host cells. Findings from recent clinico-epidemiological study indicated the importance of lytic reactivation in the development and progression of Kaposi's sarcoma (KS) [74]. Latency-associated nuclear antigen (LANA) is presumed to be a novel regulator of the life cycle of γ herpes virus including Kaposi's sarcoma herpes virus (KSHV). Pim-1 and Pim-3 contribute to the viral reactivation of KSHV by phosphorylating LANA, and thereby promote KS progression [74].

5. Roles of Pim-3 in cancer development and progression, particularly in pancreas

Pim-3 can contribute to cancer development and progression by acting on tumor

cells and tumor microenvironments. The primary activities of Pim-3 on tumor cells include the delivery of survival signaling, the regulation of cell cycle progression, protein synthesis, and Myc activation (Figure 3). In addition to its effects on tumor cells, Pim-3 can have profound impacts on tumor microenvironments, especially neovascularization process (Figure 3). In the following sections, we will discuss the roles of Pim-3 in carcinogenesis, with a focus on these two aspects.

5.1. Effects of Pim-3 on tumor cells

Forced expression of Pim-3 can promote anchorage-independent growth whereas co-expression of a kinase-dead Pim-3 mutant can attenuate EWS/FLI-mediated NIH 3T3 tumorigenesis in immunodeficient mice [27]. These observations suggest the involvement of Pim-3 in cancer development and progression.

Pim-3 can prevent apoptosis in pancreatic cancer cells by phosphorylating Bad a pro-apoptotic molecule on the serine residues (Ser^{112}, Ser^{136}, or Ser^{155}), which in turn prevents Bcl-X_L binding and promotes Bad translocation from the surface of the mitochondria to the cytosol by the protein 14-3-3 [48, 49]. Among the serine residues present in Bad, Ser^{112}, but not Ser^{136} and Ser^{155}, is abundantly phosphorylated in human pancreatic cancer cell lines. Moreover, the ablation of endogenous Pim-3 reduces the population of phosphorylated Bad followed by an enhancement of apoptosis, whereas Pim-3 overexpression produces exactly the opposite phenotypes. These observations suggest that Pim-3 has a crucial role in preventing apoptosis of human pancreatic cancer cells.

Cell survival can be regulated by Wnt/β-catenin and Stat3 signaling pathways. An integrative molecular screening by using siRNA identified Pim-3 as a new regulator of Wnt/β-catenin signaling[75]. Thus, Pim-3 can positively regulate the Wnt/β-catenin signaling pathway in the colorectal cancer cell lines (DLD-1 and SW480) [75]. Moreover, Pim-3 is a positive regulator of Stat3 signaling in the prostate cancer cell line (DU-145) and in the pancreatic cancer derived cell line (MiaPaCa2) [56]. Thus, Pim-3 can promote cancer cell survival by modulating Wnt/β-catenin and/or Stat3 signaling pathways.

Pm-1 can promote cell cycle progression by phosphorylating and modulating the functions of molecules involved in cell cycle progression. Moreover, Pim kinases positively regulate transcription factors controlling the expression of genes implicated in cell cycle progression [65]. Since Pim-3 shares a high sequence identity with Pim-1, it is possible that Pim-3 can perform similar regulatory functions as Pim-1. Treatment with Pim-3 shRNA showed a marked reduction in G1 population of human pancreatic cancer cells while scramble shRNA had few effects [5]. Furthermore, a small-molecule Pim-3 kinase inhibitor markedly retarded the *in vitro* growth of human pancreatic cancer cell lines by inducing G2/M arrest [66]. These findings indicate that Pim-3 may have a major influence in cell cycle progression of cancer cells.

Pim-1 and Pim-2 help in cell survival by suppressing myc-induced apoptosis [10, 11]. Transgenic mice expressing *Eμ*(immunoglobulin heavy-chain enhancer)-*Pim-1* and *Eμ-Myc* succumb to lymphoma in utero or around birth [76]. On the contrary, *Eμ-Myc* transgenic mice that are deficient in *Pim-1* and *Pim-2* genes develop lymphoma slowly with time [8]. Thus, Myc-driven tumorigenesis depends on physiological levels of Pim-1 and Pim-2 expression. Several mechanisms have been proposed to explain the cooperation between Myc and Pim kinases. Myc recruits Pim-1 to the E-boxes of the Myc target genes such as Fos-related antigen 1*(FOSL1* (*Fra-1))* and DNA-binding protein inhibitor *ID2*, and Pim-1 phosphorylates Ser^{10} of histone H3 on the nucleosome at the Myc-binding sites thereby, acting as a co-activator of Myc [70]. An expression profile analysis demonstrated that about 20 % of the Myc-regulated genes are also under the control of Pim-1 [70]. Moreover, Pim-1 and Pim-2 phosphorylate c-Myc protein at its serine and threonine residues [55]. This results in stabilization and subsequent enhancement of the transcription activities of c-Myc protein. Furthermore, Pim-3 can enhance c-Myc mRNA expression through the activation of PGC-1α [69]. The enhanced expression of c-Myc and PGC-1α may account for enhanced glycolysis. Thus, Pim kinases can promote tumorigenesis by modulating the activities of c-Myc and promoting Warburg effects [10, 11].

5.2 Roles of Pim-3 in tumor microenvironments

One of the basic characteristic features of tumor tissues is the abundance of newly formed vasculature for supply of nutrients and oxygen to the growing tumor cells and elimination of metabolic wastes and carbon dioxide. Pim-3 is abundantly expressed at mRNA and protein levels at the cellular lamellipodia and is co-localized with FAK in endothelial cells [23]. Pim-3 shRNA treatment impaired endothelial cell spreading, migration, and proliferation, leading to a reduction in tube-like structure formation in a Matrigel assay [23]. Moreover, tumor necrosis factor (TNF)-α transiently increases Pim-3 mRNA expression through TNF receptor-1 (TNFR1) pathway in endothelial cells (ECs) and eventually promotes EC spreading and migration [77]. Constitutive Pim-3 overexpression in gastric cancer tissues can induce angiogenesis [30].

Tumor-associated neovasculature formation is regulated by various angiogenic factors. Notably, vascular endothelial growth factor (VEGF) has an important role in tumor-associated vasculature formation [78, 79]. Although most pancreatic cancer tissues are hypovascular, elevated levels of VEGF are sometimes detected in pancreatic cancer cells[80]. Earlier studies have demonstrated that Pim-3 overexpression was responsible for increased VEGF expression and the growth of pancreatic cancer *in vivo* in an orthotopic nude mouse model [81]. The lack of any vascular phenotypes in Pim-3-deficient mice indicates that Pim-3 is dispensable for normal vasculature formation. However, given distinctive gene expression profiles of tumor-associated endothelial cells (ECs) [82], Pim-3 may have distinct roles in the tumor-associated endothelial cells.

6. Pharmacological characterization of Pim-3 Inhibitors

It is obvious from our discussions that aberrant activation and expression of Pim kinases are associated with various types of cancer. Enhanced expression of Pim-2 kinase is detected in hematologic malignancies and prostate cancer. Additionally increased Pim-1 expression is observed in pancreatic cancer, squamous cell carcinoma, gastric cancer, colorectal cancer, hepatocellular carcinoma [83-85], bladder carcinoma [86], and liposarcoma [87]. In contrast, Pim-3 expression is selectively overexpressed in malignant

lesions of endoderm-derived organs such as the liver [13], pancreas [5], colon [29], and stomach [30]. Furthermore, lack of apparent phenotypes in TKO mice suggests that Pim kinases are dispensable for the maintenance of normal functions of vital organs. Collectively, Pim kinases can be good candidate molecules for targeted cancer therapy. Examples of Pim-1 inhibitors include an anti-Pim-1 antibody and a cell penetrating peptide, both of which suppresses tumor growth *in vivo* in xenograft mouse models transplanted with human cancer cell lines [88, 89].

The crystal structure of Pim-3 has not yet been reported. However, the crystal structure of Pim-1 and Pim-2 has been resolved and revealed the presence of a unique hinge region that connects the two lobes of the protein kinase domain [18-20]. As a result, ATP binds to Pim kinases in a fundamentally different way from how it binds to other protein kinases [18, 19]. Thus, it may be possible to design compounds, which will selectively inhibit Pim kinases but no other serine/threonine kinases [16].

Several independent research groups have developed small-molecule inhibitors against Pim kinases including flavonol quercetargetin [90], imidazole[1,2-b]pyridazines [91, 92], bezylindene-thiazolidine-2,4-dione [93-95], 3,5-disubstituted indole derivatives [96], pyrazolo[3,4-g]quinoxaline derivatives [97], 1,6-dihydropyrazolo[4,3-c]carbazoles and 3,6-dihydropyrazolo[3,4-c]carbazole derivatives [98], and pyrrolo[2,3-a]carbazole and pyrrolo[2,3-g]indazole derivatives [99-101]. Among them, 1,6-dihydropyrazolo[4,3-c]carbazoles, 3,6-dihydropyrazolo[3,4-c]carbazoles, and pyrrolo[2,3-g]indazoles can inhibit Pim-3 activities [98, 100]. In our previous studies we have demonstrated that derivatives of stemonamide synthetic intermediates can inhibit Pim-3 as well as Pim-1 and Pim-2 activities and can reduce tumor growth *in vivo* in mouse xenograft models using human pancreatic cancer cell line without causing major adverse side-effects [102, 103].

The substrates preferred by Pim-1 and Pim-3 [19] are very similar in identity. Therefore, designing of isoform specific inhibitors that will differentiate and preferentially bind to one Pim member over the other is extremely challenging. Indeed, pyrrolo[2,3-*a*]carbazole has low nano molar binding affinity for Pim-1 and Pim-3 kinases

but only weakly inhibits Pim-2 (IC_{50} for Pim-1, 0.57 $\pm$ 0.04 μM; IC_{50} for Pim-2, > 10 μM; IC_{50} for Pim-3, 0.04 $\pm$ 0.01 μM) [104]. Similar pharmacological observations have been recorded with phenanthrene derivatives [77]. However, it will be interesting to find out if an inhibitor which specifically inhibits the action of one Pim member will provide any additional advantage over a multi-Pim kinase inhibitor.

Akt, similar to Pim kinases can phosphorylate a similar set of substrates, such as Bad, thereby initiating the proliferation of cancer cells [105]. Akt is aberrantly activated in various types of tumors and Akt inhibitors have been extensively investigated [72]. An Akt inhibitor, "GSK690693," has exhibited potent antitumor activity in pre-clinical trials on animals [105]. Akt is a key signaling protein and Akt-2 is directly involved in insulin receptor signaling pathway. Consequently, the genetic disruption of Akt kinase genes results in severe phenotypic changes, such as neonatal mortality, severe growth retardation and reduced brain size [106-108] and Akt-2 inhibition induces severe hyperglycemia [105]. The use of Akt inhibitors for anticancer treatment is seriously limited because of these shortcomings. In contrast, Pim kinases including Pim-3 are not involved in insulin receptor signaling pathway, and the inhibition of Pim kinases hardly shows any detrimental effects on normal glucose metabolism. Thus, Pim kinases are more effective molecular targets than Akt for targeted cancer therapy, and are particularly useful for treating pancreatic cancer which gets frequently complicated by hyperglycemia.

7. Future Perspectives

Pim-3 kinase is aberrantly expressed in malignant lesions but not in normal tissues of endoderm-derived organs such as liver, pancreas, colon, and stomach [5, 13, 29, 30], and contributes to tumorigenesis by inhibiting apoptosis of tumor cells and promoting cell cycle progression. Moreover, genetic deficiency of *Pim-3* gene does not result in apparent changes in phenotypes, suggesting that Pim-3 may be physiologically dispensable. Unlike Akt kinases [72], Pim kinases are not involved in insulin receptor signaling pathway; therefore, the inhibition of Pim kinases has very little influence on

glucose metabolism. Indeed, inhibition of Pim-3 kinase activities slows the growth or even causes regression of pancreatic tumors in mice without causing hypoglycemia [66, 102, 103]. Since Pim-3 kinase is constitutively active, once it is expressed aberrantly, inhibition of Pim-3 can be used for inhibiting cancer progression. Furthermore, there is accumulating evidence to suggest that Pim-3 plays a vital role in the interaction between tumor cells and their surrounding stroma. Further studies on these aspects will unravel the novel pathophysiological roles of Pim-3. Nevertheless, strategies to inhibit Pim-3 activity warrant an intensive investigation for the discovery, and development of new targeted anti-cancer therapeutics.

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Table 1. Increased expression patterns for Pim kinases in various types of malignancies

Tumor subtype	Pim-1	Pim-2	Pim-3
Solid tumor			
Pancreatic cancer	+	ND	+
Colon carcinoma	+	ND	+
Gastric cancer	+	ND	+
Hepatocellular carcinoma	+	+	+
Prostate adenocarcinoma	+	+	+
Bladder carcinoma	+	ND	ND
Squamous cell carcinoma of head and neck	+	ND	ND
Nasopharyngeal carcinoma	ND	ND	+
Oral squamous cell carcinoma	+	ND	ND
Liposarcoma	+	ND	ND
Ewing's sarcoma	ND	ND	+
Hematological malignancies			
Acute myeloid leukemia	ND	+	ND
B Cell chronic lymphocytic leukemia	ND	+	ND
Primary mediastinal large B cell lymphoma	+	ND	ND
Mantle cell lymphoma	+	+	ND
Diffuse large B cell lymphoma	+	+	ND
Burkitt's lymphoma	+	ND	ND

ND: not determined.

Figure Legends:

Figure 1. Amino acid alignment of human Pim family proteins [13]. The amino acid sequences of human Pim family kinases are aligned and common residues shared with Pim-3 are highlighted. The box indicates the hinge region. Residues marked with white and red color are important for ATP binding and substrate selectivity, respectively.

Figure 2. Presumed biological functions of Pim-3. Pim-3 can interact with various target molecules, and thereby regulates various biological pathways including apoptosis, cell cycle, protein synthesis, and transcription.

Figure 3. Presumed roles of Pim-3 in pancreatic carcinogenesis. Pim-3 expression is regulated at transcriptional and post-transcriptional levels, by transcription factors such as Ets-1 and post-translational controllers such as TCTP respectively. Pim-3 kinase activation contributes to pancreatic carcinogenesis by inducing cell survival, cell cycle progression, gene transcription, protein synthesis in tumor cells, and angiogenesis.

Figure 1 Li et al

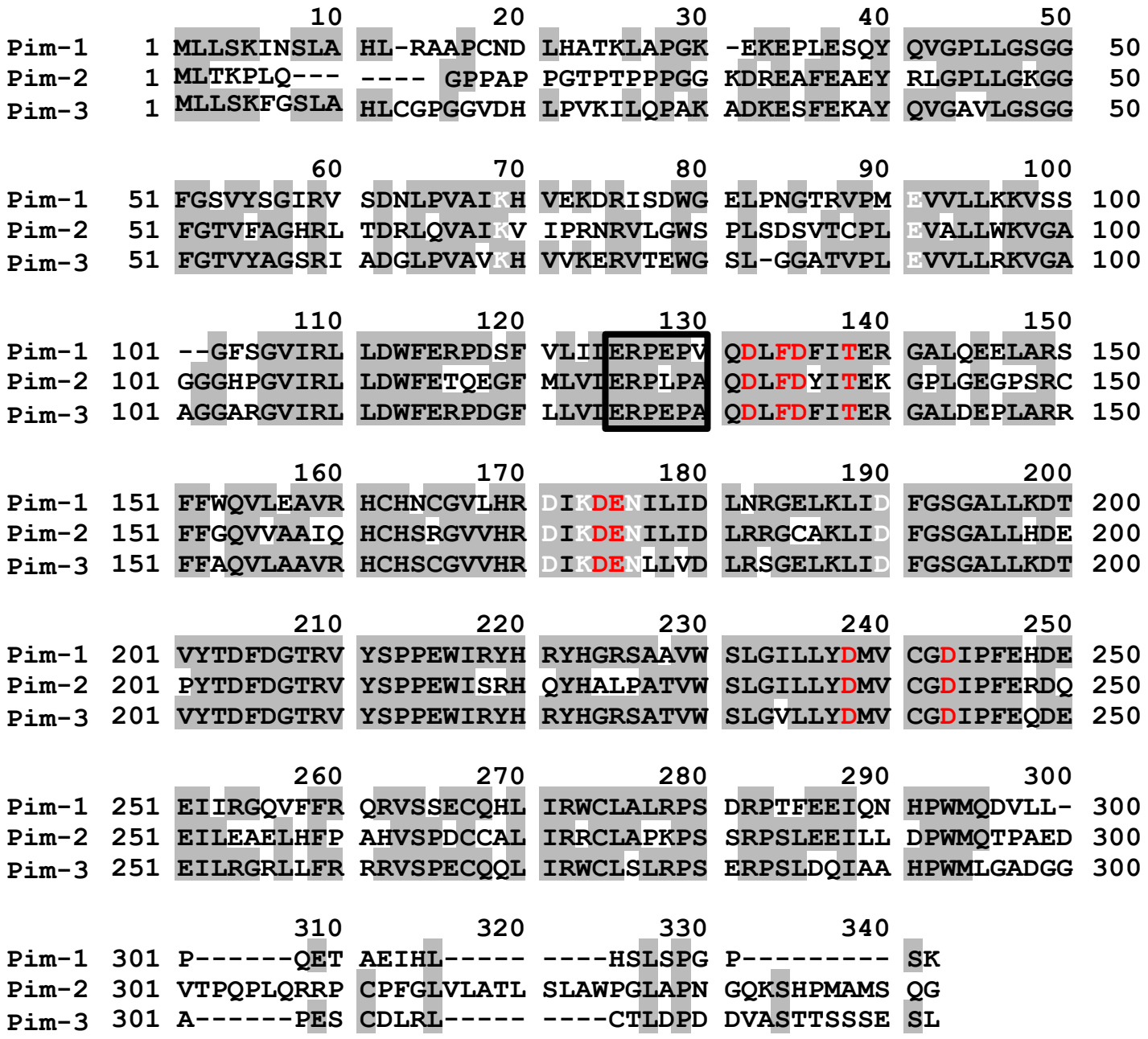

Figure 2. Li et al.

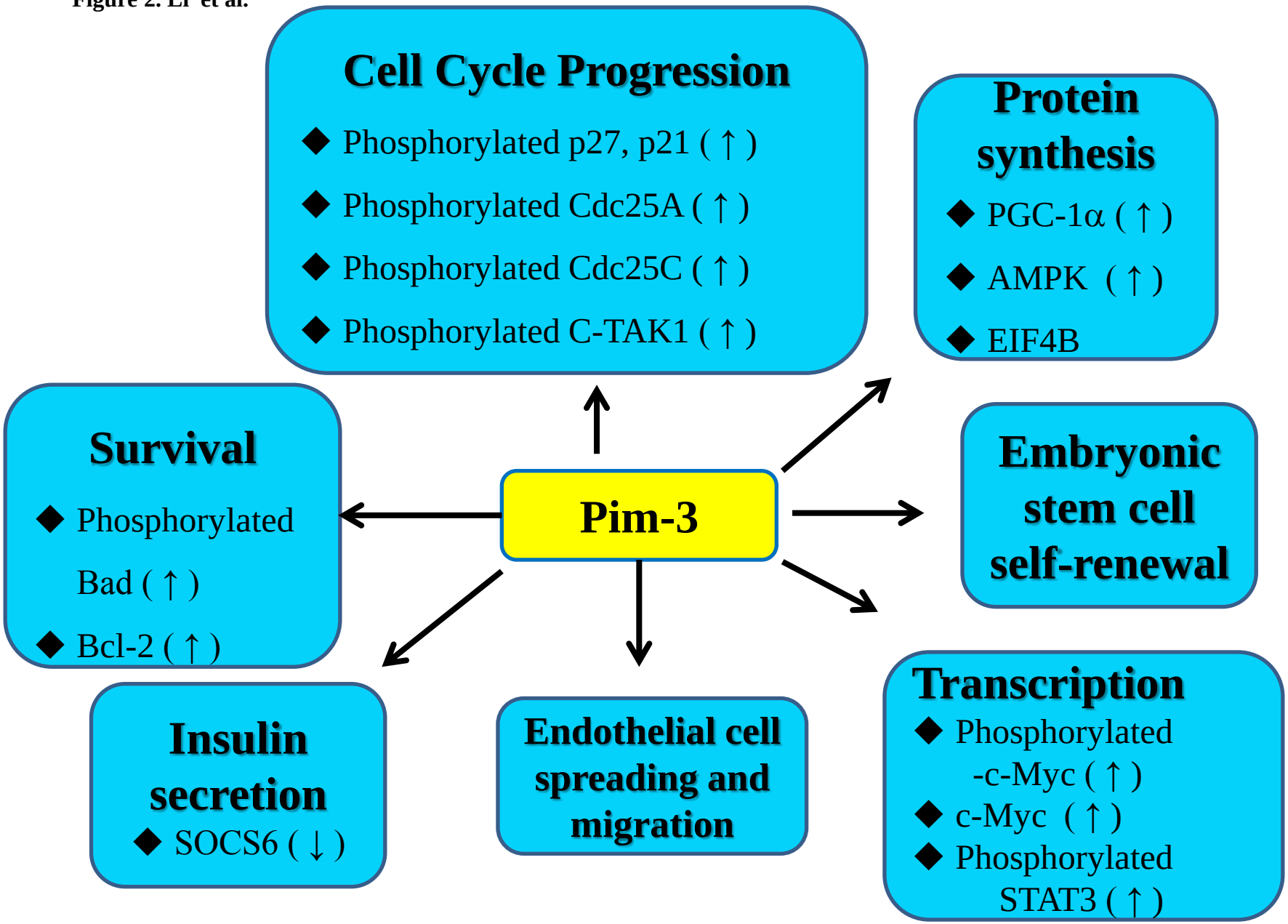

Figure 3. Li et al.