Protein Kinase D family kinases

Roads start to segregate

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Highly invasive pancreatic tumors are often recognized in late stages due to a lack of clear symptoms and pose major challenges for treatment and disease management. Broad-band Protein Kinase D (PKD) inhibitors have recently been proposed as additional treatment option for this disease. PKDs are implicated in the control of cancer cell motility, angiogenesis, proliferation and metastasis. In particular, PKD2 expression is elevated in pancreatic cancer, whereas PKD1 expression is comparably lower. In our recent study we report that both kinases control PDAC cell invasive properties in an isoform-specific, but opposing manner. PKD1 selectively mediates anti-migratory/anti-invasive features by preferential regulation of the actin-regulatory Cofilin-phosphatase Slingshot1L (SSH1L). PKD2, on the other hand enhances invasion and angiogenesis of PDAC cells in 3D-ECM cultures and chorioallantois tumor models by stimulating expression and secretion of matrix-metalloproteinase 7 and 9 (MMP7/9). MMP9 also enhances PKD2-mediated tumor angiogenesis releasing extracellular matrix-bound VEGF-A. We thus suggest high PKD2 expression and loss of PKD1 may be beneficial for tumor cells to enhance their matrix-invading abilities. In our recent study we demonstrate for the first time PKD1 and 2 isoform-selective effects on pancreatic cancer cell invasion, *in-vitro* and *in-vivo*, defining isoform-specific regulation of PKDs as a major future issue.

Introduction

Extensive interactions with the extracellular matrix and tumor stroma as well as perineural and retroperitoneal invasion are hallmarks of pancreatic ductal adenocarcinoma (PDAC).¹⁻³ These features are also responsible for the severely impaired treatment options of PDACs.^{2,4-6} Development of strategies to control invasive properties of PDAC thus could be crucial to improve treatment options. Recently small molecule inhibitors against Protein Kinase D (PKD) family kinases have been suggested for the treatment of pancreatic tumors.⁷⁻⁹ PKD isoforms, PKD1 (PKC μ), PKD2 and PKD3 have been described as vital regulators of diverse pathways controlling cancer-relevant target genes, actin-regulatory proteins, tumor proliferation, angiogenesis and tumor cell motility.^{3,10-14} However data addressing the distinct roles of PKDs and even of one particular PKD isoform during tumor progression, invasion or metastasis are rare and sometimes contradictory. For example, some studies have shown that PKDs inhibit cancer cell motility or invasion in different cancer cells^{10,11,15-18} while others primarily using broad-band inhibitors describe pro-invasive functions for PKDs.^{7,9} It gets even more complicated, when studies generated by the use of different PKD isoforms are directly compared. In particular, PKD1 and PKD2 isoforms display very similar structural properties and phenotypes.^{12,19} However, PKD isoforms are not uniformly expressed in cancer cells. PKD1 is downregulated in invasive breast

cancer cell lines and tumor samples by promoter methylation.^{17,20} These data support a role of PKD1 in the inhibition of tumor cell motility in breast cancer and various other cancer cell lines including pancreatic cancer cells by phosphorylation of actin regulatory substrates such as Slingshot1L (SSH1L) and Cortactin.^{10,11,16} In our recent study we have now identified PKD2 as a selective, key upstream regulator of MMPs 7 and 9 thereby promoting invasion and angiogenesis *in-vitro* and *in-vivo*.³ We further demonstrate that, in marked contrast to PKD2, PKD1 impairs motility/invasion of pancreatic cancer cells by selective phosphorylation of actin regulatory proteins such as SSH1L.³

PKD1 and 2 Isoforms Are Differentially Expressed in PDAC Cell Lines and Tumors

We performed a meta-analysis of micro array data utilizing the R2 micro-array analysis and visualization platform (<http://r2.amc.nl>). We were able to support findings that PKD1 mRNA was comparably lower expressed in various pancreatic tumor and cancer cell lines, whereas PKD2 expression was elevated (Wang, Maupin data sets^{21,22}). On the other hand, PKD isoforms seem to fulfill a variety of overlapping functions in different cancers types including control of proliferation, angiogenesis or cell motility.^{11-15,19,23} Yet, differential expression of PKD1 and 2 isoforms in tumors suggest that vital tumor-relevant parameters could be controlled in an isoform-selective manner by specific PKDs.

Isoform-Selective Regulation of PDAC Cell Invasion

We therefore initiated our study to investigate how PKD1 and 2 isoforms selectively affect vital PDAC cell invasive properties, such as extracellular matrix (ECM) degradation, angiogenesis, and directed cell motility in an identical PDAC model system.³ Thus, utilizing stable Panc89 PDAC cell lines with specific knockdown of PKD1 and 2 or vice versa expression of the respective isoforms, we

compared invasive features in 3D-ECM culture. The ectopic expression of PKD2-GFP significantly enhanced invasion of cells in the surrounding ECM matrix as compared with vector and PKD1-GFP expressing cells, while knockdown of PKD2 inhibited invasion. Conversely, in line with data obtained from breast cancer cells,^{10,17,20} expression of PKD1-GFP indeed inhibited invasion into the extracellular matrix while stable knockdown of PKD1 strongly enhanced invasive outgrowth from tumor clusters.³

PKD2 Drives Invasion and Angiogenesis by Secretion of MMPs 7 and 9

ECM and cancer cells show an intimate interaction:²⁴ On one the hand, changes within the ECM can influence cellular behavior. On the other hand, cancer cells are able to directly affect ECM composition or cause ECM breakdown, by enhanced secretion of ECM remodeling enzymes, such as matrix-metalloproteinases (MMPs).²⁴ Degradation of the ECM by MMPs constitutes a key step during cancer cell invasion and metastasis.⁶ PKDs are involved in the regulation of MMPs,^{17,25-27} but isoform-selective regulation by PKD1 and 2 has never been investigated. Another important role of MMPs is the control of tumor angiogenesis.²⁸ We have previously shown that PKD2 is also implicated in the control of tumor angiogenesis and growth by transcriptional upregulation and secretion of vascular endothelial growth factor A (VEGF-A).^{13,14} Interestingly, secreted MMPs, in particular MMP9 are responsible for the mobilization of growth factors such as VEGF-A from the ECM²⁸⁻³⁰ vitally affecting tumor vascularization. MMP9 is a member of the gelatinase MMP sub-family³¹ and MMP9 knockout mice display alterations during physiological and tumor angiogenesis.^{28,30-32} MMP7 (Matrilysin) is expressed in 98% of well-differentiated PDACs and in 100% of metaplastic duct epithelia. MMP7 is also associated with the formation of pre-neoplastic pancreatic lesions.^{4,6} Its substrates are both components of the extracellular matrix and cell surface proteins.⁴ MMP7

may also process the secreted MMP9 pro-enzyme (zymogen) to its active state.³³

By performing an initial limited screening approach with Panc89 cell lines expressing PKD1 and 2 isoforms in 3D-ECM culture with the broad-band MMP-inhibitor Marimastat and by gathering MMP mRNA and protein expression data we identified a strong upregulation of MMPs 7 and 9 selectively by PKD2. Since PKDs were also shown to be involved in fission of transport carriers from the *trans*-Golgi network (TGN),³⁴⁻³⁷ release of MMP carriers from the TGN and regulation of constitutive MMP secretion was further investigated. Interestingly, secretion of tagged-MMP7 and 9 into cell culture supernatants and at the level of carrier release from the TGN were predominantly regulated by PKD2 and not by PKD1.³ This was also confirmed for endogenous MMPs. How this novel preferential regulation of constitutive MMP secretion at the TGN is controlled by the PKD2 isoform at the molecular level is currently still under investigation.

Involvement of MMP7 and 9 secretion in PKD2-driven invasion was shown utilizing fibroblast-overlay invasion assays with the respective PKD isoforms in Panc1 cells combined with MMP-inhibition as well as by specific knockdown of MMPs 7 and 9 in PKD2-expressing Panc89 cells and 3D-ECM culture.

Tumor angiogenesis promotes tumor invasion and metastasis. One reason is an insufficient formation of pericytes around newly formed tumor vessels. Therefore, tumor vessels are more leaky and prone toward penetration by tumor cells. VEGF-A is an important mediator of tumor vascularization. Following secretion, VEGF-A is not per-se active, but sequestered in the ECM and therefore not available to endothelial cells. A potent liberator of VEGF-A from the ECM is MMP9.²⁸ PKD2 and the other PKD isoforms are also able to regulate VEGF-A transcription and secretion in pancreatic cancer cells.¹⁴ Interestingly, we now were able to demonstrate an isoform-selective role for PKD2 in the liberation of VEGF-A by MMP9 from the ECM utilizing 3D-ECM assays.³ This finding is of interest because it adds another selective regulatory role for PKD2 in pancreatic

Figure 1. Differential control of pancreatic cancer cell invasive properties by PKD1 and PKD2 isoforms. Novel isoform-selective pro-invasive role for PKD2 in the regulation of PDAC cell invasion and tumor angiogenesis: (1) PKD2 enhances expression of MMPs 7 and 9 as well as VEGF-A. (2) PKD2 enhances secretion of MMPs 7 and 9 from pancreatic cancer cells at the TGN. (3) MMPs 7/9 drive PKD2-mediated invasion, VEGF-A bio-release and tumor angiogenesis. Conversely, PKD1 conveys anti-migratory phenotypes via preferential interaction and phosphorylation of SSH1L.

cancer angiogenesis and defines PKD2 as vital upstream regulator of tumor angiogenesis and tumor cell invasion. *In-vitro* experiments were also supported by *in-vivo* data from Panc89 tumors transplanted on fertilized chicken eggs (chorioallantois membrane, CAM assays). In comparison to controls, tumors expressing PKD2 indeed displayed higher invasive capacities (penetrated tumor-CAM borders) and showed formation of secondary structures resembling early stage metastasis.³ These phenotypes were significantly impaired by combined knockdown of MMP7/9. In addition to impaired invasion, blood vessel formation within the tumor area was also significantly reduced, corroborating our data on MMP9-mediated VEGF-A release from *in-vitro* experiments.

PKD1 Conveys Anti-Migratory/ Anti-Invasive Effects by Preferential Interaction and Phosphorylation of Slingshot1L (SSH1L)

PKD2 regulated pro-invasive phenotypes in an isoform-specific manner. However, the role of PKD1 in PDAC cell invasion still needed to be clarified. Ectopic expression of PKD1 impaired invasive outgrowth from Panc89 tumors

in 3D-ECM culture.³ We therefore addressed this issue by depletion of PKD1 in Panc89 PKD2-GFP cells to simulate high PKD2 expression and loss of PKD1 found in PDAC tumors. These modifications resulted in a strongly magnified invasive phenotype.³

But how can loss of PKD1 contribute to an enhancement of invasive properties? One known substrate of PKD1 is the Cofilin-phosphatase Slingshot-1L (SSH1L). Phosphorylation of Ser-978 by PKDs impairs F-actin binding and thereby the activity state of the SSH1L phosphatase, which dephosphorylates Cofilin at Ser-3 and is critically involved in the control of cell motility by generating 'barbed ends' to drive early stage actin polymerization and membrane protrusion.¹⁰ We used Foerster energy transfer (FRET) to study different interaction patterns of PKD1 and 2 with SSH1L in cells. Quantitative analysis of FRET data clearly indicated that PKD1 as compared with PKD2 preferentially interacted with SSH1L at the cell periphery and in dynamic membrane protrusions, suggesting an isoform-specific, migratory-relevant regulation of SSH1L by PKD1.³ These data were further corroborated by cell migration assays with Panc1 cells following expression of PKD1- and PKD2-GFP constructs.

Opposing Roles for PKD1 and 2 Isoforms in PDAC Cell Invasion

In summary, with our study we define for the first time specific phenotypes of PKD1 and 2 isoforms controlling defined invasive properties of pancreatic cancer cells in an opposing fashion. We show that PKD2 regulates pro-invasive features at multiple levels whereas PKD1 seems to mediate an anti-migratory/anti-invasive function (Fig. 1). We have previously shown that PKD1 also enhances tumor cell proliferation via phosphorylation of the zinc-finger transcription factor Snail1 in an isoform-specific manner.²³ Thus, we suggest that PKD1 could be crucial for primary tumors to support the initial stages of tumor growth via signaling through the transcription factor Snail1.²³ Driving Snail-associated HDAC activity, tumor growth and potentially, in combination with additional factors, epithelial-to-mesenchymal transition (EMT)^{38,39} then fosters tumor progression. However, during later stages following progression or EMT, loss of PKD1 expression might even be of advantage for tumor cells to generate a high-migratory phenotype via its regulation of actin-regulatory proteins such as SSH1L promoting invasion and metastasis.^{3,11,23}

Concluding Remarks

In our recent study we have addressed for the first time PKD isoform-selective features of tumor cell invasion in pancreatic cancer cells and show in an identical model system opposing effects by PKD1 and PKD2 kinases. PKD1 and 2 are not the first kinase isoforms to mediate these “Ying and Yang”-effects on tumor cell invasion. AKT1 and 2 have been previously shown to differentially modulate motility/invasion of different cancer cells.^{40,41} Recently broad-band PKD inhibitors have been proposed as a treatment option for pancreatic cancer.⁷ Our data implies that broad-band inhibition of PKDs may not be the best strategy to control tumor progression, invasion and metastasis. Although it might be technically challenging to develop isoform-selective inhibitors for a kinase family, we suggest that this could be crucial to circumvent unwanted side-effects and enhance effectiveness of future therapeutic strategies. Our data further indicate PKD2-specific inhibitors might be a way to block pro-invasive phenotypes mediated by PKD2 and avoid potential compensation or avert negative effects by inhibition of the anti-invasive kinase PKD1.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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