Functional malignant cell heterogeneity in pancreatic neuroendocrine tumors revealed by targeting of PDGF-DD


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Intratumoral heterogeneity is an inherent feature of most human cancers and has profound implications for cancer therapy. As a result, there is an emergent need to explore previously unmapped mechanisms regulating distinct subpopulations of tumor cells and to understand their contribution to tumor progression and treatment response. Aberrant platelet-derived growth factor receptor beta (PDGFRβ) signaling in cancer has motivated the development of several antagonists currently in clinical use, including imatinib, sunitinib, and sorafenib. The discovery of a novel ligand for PDGFRβ, platelet-derived growth factor (PDGF)-DD, opened the possibility of a previously unidentified signaling pathway involved in tumor development. However, the precise function of PDGF-DD in tumor growth and invasion remains elusive. Here, making use of a newly generated Pdgfd knockout mouse, we reveal a functionally important malignant cell expression of PDGF-DD signaling in pancreatic neuroendocrine tumors (PanNET). Our analyses demonstrate that tumor growth was delayed in the absence of signaling by PDGF-DD. Surprisingly, ablation of PDGF-DD did not affect the vasculature or stroma of PanNET; instead, we found that PDGF-DD stimulated bulk tumor cell proliferation by induction of paracrine mitogenic signaling between heterogeneous malignant cell clones, some of which expressed PDGFRβ. The presence of a subclonal population of tumor cells characterized by PDGFRβ expression was further validated in a cohort of human PanNET. In conclusion, we demonstrate a previously unrecognized heterogeneity in PanNET characterized by the PDGF-DD/PDGFRβ axis.

Significance

Emerging evidence suggest that the cellular composition of tumors is highly heterogeneous. Subclonal species of malignant cells may account for variability in therapeutic responses and for relapse following treatments. However, little is known about the molecular drivers of specific subsets of cancer cells. Herein, we identify expression of platelet-derived growth factor receptor beta (PDGFRβ) as a previously unrecognized feature of a minor malignant cell population in pancreatic neuroendocrine tumors. By the use of mice genetically deficient for Pdgfd, we reveal a crucial and non-redundant function for signaling by platelet-derived growth factor (PDGF)-DD in promoting functional tumor heterogeneity by providing growth-stimulatory cues. Taken together, the use of drugs targeting PDGFRβ signaling, such as the approved targeted therapy sunitinib, may affect the functional intratumoral cross talk in pancreatic neuroendocrine tumors.


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Undeniably, cancer progression is the consequence of dynamic, and yet poorly understood, cell-cell interactions driven by frequently deregulated signaling pathways (1). Further complexity arises from the notion that tumors are composed of phenotypically and functionally distinct subsets of both malignant and stromal cells (2, 3). Therefore, accounting for intratumoral heterogeneity poses an additional challenge when designing therapies that can efficiently control or eliminate tumors. An improved understanding of the functional contribution of different signaling pathways to genetic and phenotypic variation within tumors is therefore highly warranted.

Members of the platelet-derived growth factor (PDGF) family and their receptors (PDGFRs) have been extensively investigated and shown to be critical for cellular processes such as proliferation, survival, and motility during tumor growth and invasion (4). The roles of PDGF isoforms and their target cells in tumor development have been charted in different tumor types (5), and as a result, pharmacological blockade of PDGF signaling is now routinely used for the treatment of diverse malignancies, such as gastrointestinal stromal tumors and chronic myelomonocytic leukemia, among others (6, 7). The PDGF family is composed of four polypeptide chains that assemble into five dimeric isoforms (PDGF-AA, PDGF-BB, PDGF-AB, PDGF-CC, and PDGF-DD) that bind and activate two receptor tyrosine kinases (PDGFRα and PDGFRβ) expressed mainly by cells of mesenchymal origin (8). PDGF-DD is the most recently identified member of the family (9, 10), and unlike the other ligands, the role of PDGF-DD in normal development and pathology is largely a conundrum.

Herein, we report the use of a Pdgfd knockout mouse to explore the specific role of PDGF-DD in malignant growth. By monitoring tumorogenesis in the RIP1-TAg2 transgenic mouse model of pancreatic neuroendocrine tumors (PanNET), we found that disruption of PDGF-DD signaling significantly delayed tumor growth. In the absence of PDGF-DD, functional compensation by PDGF-BB was apparent in the stromal compartment. Unexpectedly, however, we identified a subpopulation of malignant cells expressing PDGFRβ with accompanying responsiveness to PDGF-DD. By modulating PDGFRβ+ malignant cells, PDGF-DD contributes to the maintenance of functional malignant cell heterogeneity in experimental PanNET.

Results

Pdgfd Is Predominantly Expressed in the Endothelial Cell Compartment of Tumors from RIP1-TAg2 Mice. To study the effect of Pdgfd depletion in tumor development, we made use of the RIP1-TAg2 transgenic mouse model of multistage PanNET (11). Briefly, pancreatic β-cells...
observations suggest that in the RIP1-TAg2 tumor model, ECs are the predominant source for PDGF-DD.

**Pdgfd Deficiency Delays Tumor Growth, Leading to Prolonged Survival.** Mice homozygous for the inactivated *Pdgfd* allele (*Pdgfd−/−* mice) are viable and fertile and do not display any obvious discrepancies in the histology or insulin secretion of the islets of Langerhans, compared with *Pdgfd+/−* littermates (Fig. 2A). Next we examined the tumorigenic progression of RIP1-TAg2:*Pdgfd−/−* and RIP1-TAg2:*Pdgfd+/−* mice to that of RIP1-TAg2:*Pdgfd−/+* littermates. First, we examined the effect of impaired *Pdgfd* expression on the activation of the angiogenic switch by quantifying the number of angiogenic islets and tumors present in the pancreas of 12-wk-old RIP1-TAg2 mice. Our analysis revealed a similar number of both angiogenic islets and tumors regardless of genotype (Fig. 2B and C), suggesting that PDGF-DD does not affect the progression of tumors from premalignant angiogenic lesions into overt tumors. In sharp contrast, both RIP1-TAg2:*Pdgfd−/−* and RIP1-TAg2:*Pdgfd+/−* mice presented with a significantly reduced total tumor burden (29.5 ± 18 mm³ and 25.7 ± 21.1 mm³, respectively) compared with RIP1-TAg2:*Pdgfd−/+* mice (71.9 ± 68 mm³) (Fig. 2D). Consistent with the decrease in tumor burden, RIP1-TAg2:*Pdgfd−/−* and RIP1-TAg2:*Pdgfd−/+* mice also showed significantly prolonged median survival (15.9 wk and 15.4 wk, respectively) compared with RIP1-TAg2:*Pdgfd−/+* littermates (13.7 wk) (Fig. 2E).

**Pdgfd Ablation Reduces Tumor Cell Proliferation but Does Not Affect the Invasive or Metastatic Properties of Tumors from RIP1-TAg2 Mice.** To investigate whether the diminished tumor size in tumors from RIP1-TAg2:*Pdgfd−/−* mice was due to an increase in apoptosis or a decrease in proliferation, we stained tumor tissue sections for cleaved caspase3, an apoptotic cell marker (Fig. 3A and B), and assessed the proliferative rate by injecting mice with BrdU (Fig. 3C and D). No change was observed when we quantified apoptotic cells in tumors from RIP1-TAg2:*Pdgfd−/−* mice compared with RIP1-TAg2:*Pdgfd−/+* controls (Fig. 3B). In contrast, we detected a considerable decrease of 69% in proliferating BrdU+ cells in tumors from RIP1-TAg2:*Pdgfd−/−* compared with RIP1-TAg2:*Pdgfd−/+* littermates (Fig. 3D). An increasing number of studies propose that PDGF-DD regulates the process of epithelial-to-mesenchymal transition (EMT), an event preceding metastatic spread (14). Hematoxylin/eosin (H&E) staining of liver tissue sections (Fig. S1A) revealed that the number of hepatic micrometastatic lesions was not different in RIP1-TAg2:*Pdgfd−/−* mice compared with RIP1-TAg2:*Pdgfd−/+* mice (Fig. S1B). Furthermore, visualization of local tumor invasion, as determined by the border of the pancreatic exocrine lesion (assessed by immunostaining for insulin) with the surrounding exocrine tissue (assessed by immunostaining for α-amylase) demonstrated that tumors invaded the adjacent exocrine tissue to the same extent, regardless of *Pdgfd* expression (Fig. S1C).

**Angiogenesis, Pericyte Recruitment, and Immune Cell Infiltration Are Not Affected by PDGF-DD Inhibition.** Given the reported effect of PDGF ligands on tumor angiogenesis in general and pericyte recruitment in particular (15), we characterized the vascular phenotype of tumors in RIP1-TAg2 mice following *Pdgfd* disruption. The vascular density, as shown by immunostaining for the luminal vessel marker podocalyxin (Fig. S2A), was unchanged upon blinded *Pdgfd* expression (Fig. S2B). Similarly, tumor vessel perfusion, measured in mice that were systemically administered with fluorescein-coupled tomato lectin before sacrifice, was not significantly affected by the absence of PDGF-DD (Fig. S2 C and D). The role of PDGF-ββ, the prototypical ligand for PDGFRβ, in recruitment of pericytes to the tumor vasculature in RIP1-TAg2 mice has been previously described (16). Therefore, we asked whether PDGF-DD would have a similar effect on pericyte recruitment to tumor blood vessels. By immunostaining, we analyzed tumor sections from RIP1-TAg2 mice in the islets of Langerhans of RIP1-TAg2 mice are engineered to express the oncogenic SV40 T antigens, under the control of the rat insulin promoter, leading to the formation of hyperproliferative islets (12). Consistent with these results, we observed a significant enrichment of *Pdgf*B, in recruitment of pericytes to the tumor vasculature in RIP1-TAg2 mice has been previously described (16). Therefore, we asked whether PDGF-DD would have a similar effect on pericyte recruitment to tumor blood vessels. By immunostaining, we analyzed tumor sections from RIP1-TAg2 mice...
**Fig. 2.** *Pdgfd* deficiency delays tumor growth, leading to prolonged survival. (A) Representative images of islets of Langerhans from *Pdgfd*+/+ and *Pdgfd*−/− mice used for assessment of histology, demonstrated by H&E staining, and functionality, shown by immunostaining for insulin (red) to visualize secretion and distribution. Nuclei were stained with DAPI in (B) and (C) the number of angiogenic islets, (D) total tumor burden in 12-wk-old *RIP1-TAg2; Pdgfd*+/+ and *RIP1-TAg2; Pdgfd*−/− mice compared with *RIP1-TAg2; Pdgfd*+/+ mice (Fig. 4B). However, we observed in rare malignant lesions that *PDGFRB*+ peri-vascular cells in tumors from *RIP1-TAg2; Pdgfd*−/− mice appeared more detached from the abluminal endothelial surface in blood vessels, compared with tumors from *RIP1-TAg2; Pdgfd*+/+ mice (Fig. 4, arrows). Given that the increased detachment did not correlate with changes in vessel density or functionality (Fig. S2A–D), we concluded that pericyte detachment was most likely not sufficient to account for the differences in tumor size observed upon impairment of *Pdgfd* expression.

Additionally, in a mouse model of wound healing, *Pdgfd* over-expression was accompanied by an increased recruitment of macrophages (19), a cell type associated with the angiogenic phenotype in tumors from *RIP1-TAg2* mice (20). However, the immune profile of *RIP1-TAg2* mice characterized by immunostaining of tumor tissue sections for a general leukocyte marker (CD45) and a macrophage marker (F4/80) did not give any evidence for gross differences in the infiltration of inflammatory cells upon *Pdgfd* deletion (Fig. S3A–D).

**Identification of a Subset of Malignant Pancreatic β-Cells Expressing PDGFRB in RIP1-TAg2 Mice.** Because we did not detect major alterations in the tumor stroma of PanNET lesions from *RIP1-TAg2* mice following *Pdgfd* depletion, we profiled the expression of PDGF family members by quantitative RT-PCR (qRT-PCR). Notably, we found that the level of *Pdgfb* transcript was significantly increased in tumors from *RIP1-TAg2; Pdgfd*−/− mice compared with *RIP1-TAg2; Pdgfd*+/+ mice (Fig. 5), suggesting a compensatory effect due to the lack of PDGF-DD. Nevertheless, the up-regulation for the expression of well-characterized markers denoting different subsets of pericytes, i.e., PDGFRB, NG2, and desmin (2, 17, 18) (Fig. 4A). Unexpectedly, pericyte coverage was unchanged in the tumor vasculature of *RIP1-TAg2; Pdgfd*+/+ and *RIP1-TAg2; Pdgfd*−/− mice compared with *RIP1-TAg2; Pdgfd*+/+ mice (Fig. 4B). However, we observed in rare malignant lesions that *PDGFRB*+ peri-vascular cells in tumors from *RIP1-TAg2; Pdgfd*−/− mice appeared more detached from the abluminal endothelial surface in blood vessels, compared with tumors from *RIP1-TAg2; Pdgfd*+/+ mice (Fig. 4, arrows). Given that the increased detachment did not correlate with changes in vessel density or functionality (Fig. S2A–D), we concluded that pericyte detachment was most likely not sufficient to account for the differences in tumor size observed upon impairment of *Pdgfd* expression.

*Fig. 3.** *Pdgfd* deficiency does not affect cell apoptosis, but reduces tumor cell proliferation in tumors from *RIP1-TAg2* mice. (A) Apoptotic index assessed by cleaved-caspase 3 immunostaining and (C) proliferation assessed by BrdU immunostaining of tumor sections from *RIP1-TAg2; Pdgfd*−/− (n = 4) and *RIP1-TAg2; Pdgfd*+/+ (n = 6). Seven to twelve islet lesions were assessed for each mouse. The number of apoptotic and proliferating cells was determined by quantification of the positively stained cells in relation to the total tumor lesion area (mm²) or total number of cellvlesions, respectively. Error bars show the mean ± SD. ***P < 0.001. (Scale bars, 50 μm.)
of Pdgfb was still unable to rescue the observed reduction in overall tumor size, indicative of a specific role for PDGF-DD signaling during PanNET development. Therefore, we explored alternative mechanisms that could account for the significant reduction in tumor size. We speculated that there might exist cell types outside of the perivascular compartment responsive to PDGF-DD by expression of PDGFRβ. Consequently, we performed immunostaining of tumor and liver tissue sections from RIP1-TAg2 mice for PDGFRβ and insulin or T-Antigen to label malignant cells. We identified rare cells coexpressing PDGFRβ and insulin in primary tumors (Fig. 6A, Right), consistent with previous reports of activated PDGFRβ in whole tumor lysates of human PanNET lesions (21). Malignant cells expressing PDGFRβ were more prevalent in micrometastatic lesions in the liver (Fig. 6B and C). To further confirm the observation of PDGFRβ malignant β-cells, we prepared a single-cell suspension from tumors of RIP1-TAg2 mice and ex vivo labeled the cells with fluorescently coupled Exendin 4, a peptide ligand for the glucagon-like peptide 1 receptor (GLP1R), which is selectively expressed by β-cells in the endocrine pancreas (22). By analyzing the cells using fluorescence activated cell sorting (FACS), we detected a subpopulation of cells (∼0.3–0.8% depending on the tumor) coexpressing GLP1R and PDGFRβ (Fig. 6D). We also made use of double transgenic RIP1-TAg2;PDGFRβ-EGFP mice (23), which faithfully produce the fluorescent marker in cells expressing PDGFRβ (Fig. 6E). In parallel with the in vivo characterization, we validated our findings using various pancreatic β-tumor cell lines. First, we performed qRT-PCR analysis and detected Pdgfrß transcripts in malignant βTC3 cells (24), premalignant βHClII cells, and additional cell lines established from tumors of RIP1-TAg2 mice (βTC PO, βTC-99-3o, and βTC-1710-1) (Fig. 7A). Furthermore, to confirm the expression of PDGFRβ, we immunostained βTC3 cells and detected strongly positive staining on rare cells, indicating high expression of PDGFRβ by a subpopulation of cells, rather than a widespread low expression (Fig. 7B). Finally, we established the coexistence of PDGFRβ+ and PDGFRβ− cells in βTC3 cultures by FACS (Fig. 7C). In parallel analyses, no cells expressing PDGFRα were detected.

Fig. 4. Pericyte recruitment is not affected by PDGF-D ablation in tumors of RIP1-TAg2 mice. (A and B) Pericyte coverage quantification in tumor sections from RIP1-TAg2;Pdgfd+/+, RIP1-TAg2;Pdgfd+/−, and RIP1-TAg2;Pdgfd−/− mice based on immunostaining for pericyte markers NG2, PDGFRβ, and desmin (red) in relation to the endothelial cell marker podocalyxin (green). Cell nuclei were visualized using DAPI (blue) (n = 3 mice per group). Error bars show the mean ± SD. (Scale bars, 50 μm.)

Fig. 5. Pdgfb is up-regulated in tumors of RIP1-TAg2;Pdgfd−/− mice. Analysis of Pdgfa, Pdgfb, Pdgfc, Pdgfrα, and Pdgfrβ mRNA expression by qRT-PCR in tumors of RIP1-TAg2;Pdgfd+/+ and RIP1-TAg2;Pdgfd−/− mice (n = 3 mice per group). Error bars show the mean ± SD. *P < 0.05.

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βTC3 Cells Respond to PDGF-DD, but Not PDGF-BB, Stimulation with Increased Proliferation. Next, we tested whether PDGFRβ− βTC3 cells responded to PDGF-DD. We treated βTC3 cells with recombinant PDGF-DD protein and assessed cell proliferation by phosopho-Histone H3 immunostaining (Fig. 7 D and E). We found that PDGF-DD induced a significant increase in the total number of cells by 37%, and the number of cells positively stained for phospho-Histone H3 was 2.4-fold higher with control cultures (Fig. 7E). Surprisingly, the prototypical ligand for PDGFRβ, i.e., PDGF-BB, did not augment the proliferation of βTC3 cells (Fig. 7E).

Because obtaining for PDGFRβ and phospho-Histone H3 demonstrated that it was predominantly PDGFRβ− cells in the vicinity of quiescent PDGFRβ+ cells that were engaged in mitosis (Fig. 7F), we reasoned that the effect may be indirect through paracrine secretion of mitogenic factors by PDGFRβ+ cells in response to PDGF-DD stimulation. To test this proposition, we assessed the expression of candidate known mitogens for malignant PanNET cells from RIP1-TAg2 mice following stimulation with PDGF-DD. Indeed, the expression of Igf1 and Hgf, but not of Igf2 or Egf, was significantly induced in βTC3 cells stimulated with PDGF-DD (Fig. 7G). Subsequently, to define the characteristics of PDGFRβ− expressing malignant β-cells, we tested whether PDGF-DD induced tumor-initiating capacity. We found that βTC3 cells stimulated by PDGF-DD, but not by PDGF-BB, in anchorage-independent conditions formed a significantly higher number of tumor spheroids, compared with untreated βTC3 control cells (Fig. 7H). Additionally, to investigate the tumorigenic properties of PDGFRβ− βTC3 cells, we sorted cells by FACS based on their expression of PDGFRβ and transplanted subcutaneously (sc) into NOD-SCID mice. Injection of as few as 200 PDGFRβ− or PDGFRβ+ βTC3 cells resulted in tumor establishment and progressive growth in three out of four mice and two out of four mice, respectively. The resulting histology of tumors from PDGFRβ− cells was indistinguishable from that of tumors established from PDGFRβ+ βTC3 cells (Fig. 7I, Upper).

In addition, the expression of PDGFRβ in the parenchyma of tumors was similar regardless of cell of origin, indicating interconversion between the different subsets of βTC3 cells (Fig. 7I, Lower). Furthermore, FACS analysis revealed that tumors established from PDGFRβ− cells reestablished the original relationship between PDGFRβ− and PDGFRβ+ malignant cells (Fig. 8A).

To corroborate this finding, we investigated the prevalence of PDGFRβ− and PDGFRβ+ malignant cells (Fig. 8A). We isolated PDGFRβ− PanNET cells from βTC3 cultures. We found that PDGFRβ− PanNET cells from βTC3 cultures, and after immediately verifying the purity of the cell suspension, were propagated for 7 d. Flow cytometric analysis of the resulting culture for PDGFRβ+ demonstrated the occurrence of a mixed population of cells with the original frequencies, indicative of rapid conversion of PDGFRβ− malignant cells into PDGFRβ+ (Fig. 8B).

Altogether, our comprehensive analyses of the first Pdgfd-deficient mouse model of cancer to our knowledge demonstrate that functional malignant cell heterogeneity in experimental PanNET is reinforced by PDGF-DD by stimulation of a subset of tumor cells expressing PDGFRβ that engages in paracrine cross talk with neighboring malignant cells. However, although PDGF-DD stimulation some features of cancer stem cells in PDGFRβ− PanNET cells, the tumor-initiating capacity is not exclusive to this subset of malignant cells.

**Fig. 6.** Identification of a subset of malignant cells expressing PDGFRβ in RIP1-TAg2 tumors. (A) Immunostaining of tumor from RIP1-TAg2 mouse for malignant tumor cells (insulin; green) and PDGFRβ (red). (B and C) Expression of PDGFRβ by malignant cells in liver metastatic lesions of RIP1-TAg2 mice by immunohistochemistry (arrow) and immunofluorescence by staining with T-Antigen (T-Ag). (D) Quantification of PDGFRβ/×GLP1R+ cell populations in RIP1-TAg2 pancreatic tumors. Tumors were dispersed into single cells, incubated with fluorescently labeled antibody for PDGFRβ (APC) and peptide-ll-gand, Exendin 4 (FAM), and analyzed by FACS. (E) Immunostaining of tumors from RIP1-TAg2/PDGFRβ−EGFP compound mice with PDGFRβ antibody (red) to determine colocalization with EGFP (green) expressed by PDGFRβ cells. (F) Flow cytometry gating strategy to analyze double positive Exendin 47/PDGFRβ−EGFP+ by flow cytometry or immunostaining, illustrating the specific expression of PDGFRβ (Fig. S4 A–C). Similarly, at the mRNA level, βTC3 express very low levels of Pdgfra, compared with PDgfrβ (Fig. S4D). Taken together, both in vivo and in vitro analyses indicate that although most malignant β-cells are PDGFRβ−, a subset of pancreatic β-tumor cells readily expresses PDGFRβ+.
Human PanNET Harbor a Subset of PDGFRβ+ Malignant Cells. Finally, we analyzed the expression of PDGFRβ in human PanNET and their hepatic metastases by immunostaining. The normal pancreatic islet and liver parenchyma displayed expression of PDGFRβ exclusively in perivascular locations (Fig. 9A and B). In contrast, all primary PanNET and hepatic metastases analyzed (n = 50 each) exhibited a subset of malignant cells readily expressing PDGFRβ evenly distributed in the tissue (Fig. 9C–F). Although all malignant lesions harbored PDGFRβ+ tumor cells, the relative abundance was variable with some lesions containing more (Fig. 9E and F) and others fewer (Fig. 9C and D). The PDGFRβ+ tumor cells were distinguishable from pericytes (Fig. 9G, arrowheads) by the coexpression of chromogranin A, a widely used marker for malignant cells of neuroendocrine origin (Fig. 9G, arrows) (25).

Discussion

An emergent number of preclinical reports suggest that PDGF-DD is a key player in tumor formation by regulating various cellular processes, such as macrophage and stromal cell recruitment (19, 26), EMT (14, 27, 28), tumor cell proliferation,
and invasion (26, 29, 30). In human cancers, PDGFD up-regulation has been documented for prostate, lung, renal, ovarian, brain, breast, and pancreatic cancers (31, 32). However, the mechanisms underlying the effect of PDGF-DD on tumor growth are still largely unknown.

Our study revealed that disruption of PDGF-DD signaling greatly delayed PanNET development in RIP1-TAg2 mice. Genetic loss of a single copy of the gene encoding Pdgfd was sufficient to significantly retard tumor growth. The reduction in tumor burden was associated with decreased tumor cell proliferation and prolonged survival of Pdgfd-deficient RIP1-TAg2 mice. As previously reported for PDGFB (33, 34), the other known ligand for PDGFRβ, ECs in tumors from RIP1-TAg2 mice appear to be the major source for PDGF-DD. The effect of EC-secreted factors on the regulation of tissue and tumor growth has recently been highlighted (35–37), emphasizing the potential benefit of targeting the cross talk between ECs and tumor cells to supplement conventional antiangiogenic therapies. However, we do not exclude that there may be additional cells in the tumor microenvironment that express low levels of Pdgfd. Indeed, PDGFD expression has also been documented by malignant cells in ovarian, lung, breast, prostate, renal, and brain tumor-derived cell lines (10, 29, 38, 39).

In tumors, PDGFRβ is expressed mainly by mesenchymal cells, i.e., fibroblasts and pericytes (2, 40). The role of PDGF-BB in the recruitment of pericytes to blood vessels has been well established in tumors from RIP1-TAg2 mice (16). Also, Pdgfd overexpression in an orthotopic model of renal cell carcinoma resulted in increased perivascular cell coverage (26). In our model, however, there was no measurable effect on stromal-cell (pericyte) recruitment upon disruption of PDGF-DD signaling. Up-regulation of Pdgfd in tumors from RIP1-TAg2/Pdgfd−/− mice compared with the wild-type littermates suggests that PDGF-BB exerts a compensatory effect for the loss of PDGF-DD in the present context. The apparent need for compensation by PDGF-BB implies a yet uncharted functional role for PDGF-DD in the tumor stroma. In addition, the sharp decrease in tumor burden caused by Pdgfd deficiency strongly supports that compensation by PDGF-BB is only partially attained and that the functions of PDGF-BB and PDGF-DD within the context of tumorigenesis are only partly overlapping. Indeed, stimulation of a PanNET cell line with PDGF-DD, but not with PDGF-BB, increased proliferation and improved sphere-forming capacity. Given the fact that genetic studies have demonstrated closely related phenotypes of mice deficient for Pdgfd and Pdgfrβ (41, 42), it is tempting to speculate that the unique functions of PDGF-DD are the result of binding to a distinct (co)-receptor that modulates the response of PDGFRβ. PDGF-DD and PDGF-BB share a conserved growth factor core domain motif, but contrary to PDGF-DD, PDGF-BB carries a basic retention motif domain, allowing binding to heparan sulfate proteoglycans present in the extracellular matrix (15). In contrast, the latent full-length form of PDGF-DD has been suggested to be freely diffusible (43). It is thus reasonable to assume that PDGF-DD has a distinct distribution in the tumor stroma, being able to reach non-vessel-associated PDGFRβ-expressing cells. Therefore, we explored the possibility that nonvascular cell types in the tumor parenchyma of RIP1-TAg2 mice expressed PDGFRβ and constituted potential targets for PDGF-DD. A study of pancreatic islet regeneration demonstrated that immature mouse pancreatic β-cells express PDGFRα and PDGFRβ, signaling by which is required for sustained cell proliferation and islet expansion (44). In agreement, we identified a rare population of malignant cells expressing PDGFRβ in primary tumors and metastatic lesions of RIP1-TAg2 mice. Notably, PDGFRβ tumor cells were more abundant in early metastatic lesions in the liver, suggesting that this subset of malignant cells may be involved in establishing distant metastases. However, PDGF-DD evidently did not contribute to dissemination as such, because we did not detect any difference in the incidence of hepatic metastatic lesions.

When sorted and transplanted into NOD-SCID mice at quantities down to 200 cells, βTC3 cells were able to recapitulate the morphology and heterogeneity found in tumors from RIP1-TAg2 mice, regardless of the expression of PDGFRβ, evidencing the conserved malignant phenotype of all subsets of cancer cells within this cell line. Although PDGFRβ+ malignant cells held some features of tumor-initiating cells, we could not demonstrate that this was an exclusive or general trait of the subpopulation expressing PDGFRβ. The fact that we were able to identify minor subpopulations of β-cells even in late passages of βTC cell lines led us to consider that the presence of PDGFRβ-expressing βTC3 cells is necessary for the maintenance of the bulk tumor cell population. The coexistence of subpopulations of tumor cells with distinct phenotypic and functional properties within the same tumor points to the existence of uncharted cellular interactions or pathways that contribute to tumor growth and dissemination. Studies in renal cell carcinoma (45) and lung adenocarcinoma (46) have revealed substantial intratumoral heterogeneity as a result of subclonal driver events. Additional studies establish that interactions between genetically distinct subclones of tumor cells are necessary for maintaining tumor heterogeneity (47) and that even minor subpopulations of tumor cells may promote bulk tumor growth.

Fig. 8. Rapid conversion of PDGFRα− cells to PDGFRβ+ cells occurs in vitro and in vivo. (A) Flow cytometry analysis of tumor cells coexpressing GLP1R and PDGFRβ+ from tumors originated from transplanted PDGFRα−βTC3 cells. (B) Flow cytometry-gating strategy for sorting PDGFRβ− tumor cells from parental βTC3 cells. The proportion of PDGFRβ− βTC3 was analyzed immediately after sorting (0 d, dot plot) and after 7 d of culture (dot plot and column chart). Error bars show the mean ± SD.
via mechanisms of non-cell–autonomous stimulation (48). Thus, one might hypothesize that the cross talk between PDGFRβ⁺ and PDGFRβ⁻ malignant clones is necessary for more efficient tumor propagation. Indeed, we found that stimulation of PanNET cells with PDGF-DD in vitro engaged PDGFRβ⁺ cells in paracrine cross talk with neighboring PDGFRβ⁻ cells through the induction of mitogenic factors, the identity of which should be corroborated in vivo. A recent study using βTC cells from RIP1-TAg2 mice as a model demonstrates that artificial tumor heterogeneity is the result of stable coexistence of different clones, even without a strict interdependence between subclones (49). We should also consider the prospect that expression of PDGFRβ in malignant cells is due to interconversion between malignant cell populations, e.g., as a result of PDGF-DD-stimulated EMT. In support of this train of
thought, maintenance of a pure PDGFRβ- cell culture led to rapid reestablishment of the original frequencies of PDGFRα- and PDGFRβ- subclones, in close agreement with studies of subsets of breast cancer cells that hold the ability to spontaneously interconvert (50).

Consistently, we were able to confirm the presence of a rare population of tumor cells expressing PDGFRα in human primary and metastatic PanNET. Our findings are supported by previous studies showing that besides stromal cells, human primary PanNET and metastatic cells express high levels of PDGFRα compared with normal tissue (51, 52). Moreover, clinical reports show evidence of PDGFRα activation and copy number alteration in small intestine, gastroenteropancreatic, and pancreatic NET, although the use of whole tumor cell lysates precludes identification of the cell type expressing PDGFRα (21, 53). The identification of PDGFRα+ tumor cells in human PanNET has important clinical implications, considering that sunnithin, a PDGFRα/VEGFR small molecule inhibitor, was recently approved to treat patients with progressive well-differentiated PanNET (54). Preclinical studies suggest that the therapeutic efficacy of sunnithin is derivied from dual targeting of endothelial cells and pericytes (12, 55), but given our finding of the functional importance of PDGF-DD/PDGFRα for maintaining a functional malignant cell heterogeneity, direct inhibitory effects on tumor cells cannot be excluded.

Altogether, we provide strong evidence for the importance of PDGF-DD for PanNET growth. Moreover, we have identified a subpopulation of PDGFRα- malignant β-cells responsive to PDGF-DD in PanNET from an instructive mouse model, and in human primary and hepatic metastatic lesions. Our study thus provides evidence for a functional heterogeneity that needs to be explored to fully understand the tumorigenic process in PanNET.

Materials and Methods

A detailed description of additional procedures can be found in Supporting Information.

Animal Care.

All experimental procedures involving mice were approved by the Stockholm North and Malmö/Lund committees for animal care (permits N96/11 and M142/13). RIP1-TAg2 transgenic mice were crossed with RIP1-TAg2; Betsholtz, Uppsala University, Uppsala, Sweden) on the C57BL/6 background. All experimental procedures involving mice were approved by the Stockholm North and Malmö/Lund committees for animal care (permits N96/11 and M142/13). RIP1-TAg2 transgenic mice were crossed with RIP1-TAg2; Betsholtz, Uppsala University, Uppsala, Sweden) on the C57BL/6 background.

RIP1-TAg2 mice received 5% (wt/vol) sugar water to counteract symptoms of hypoglycemia.


