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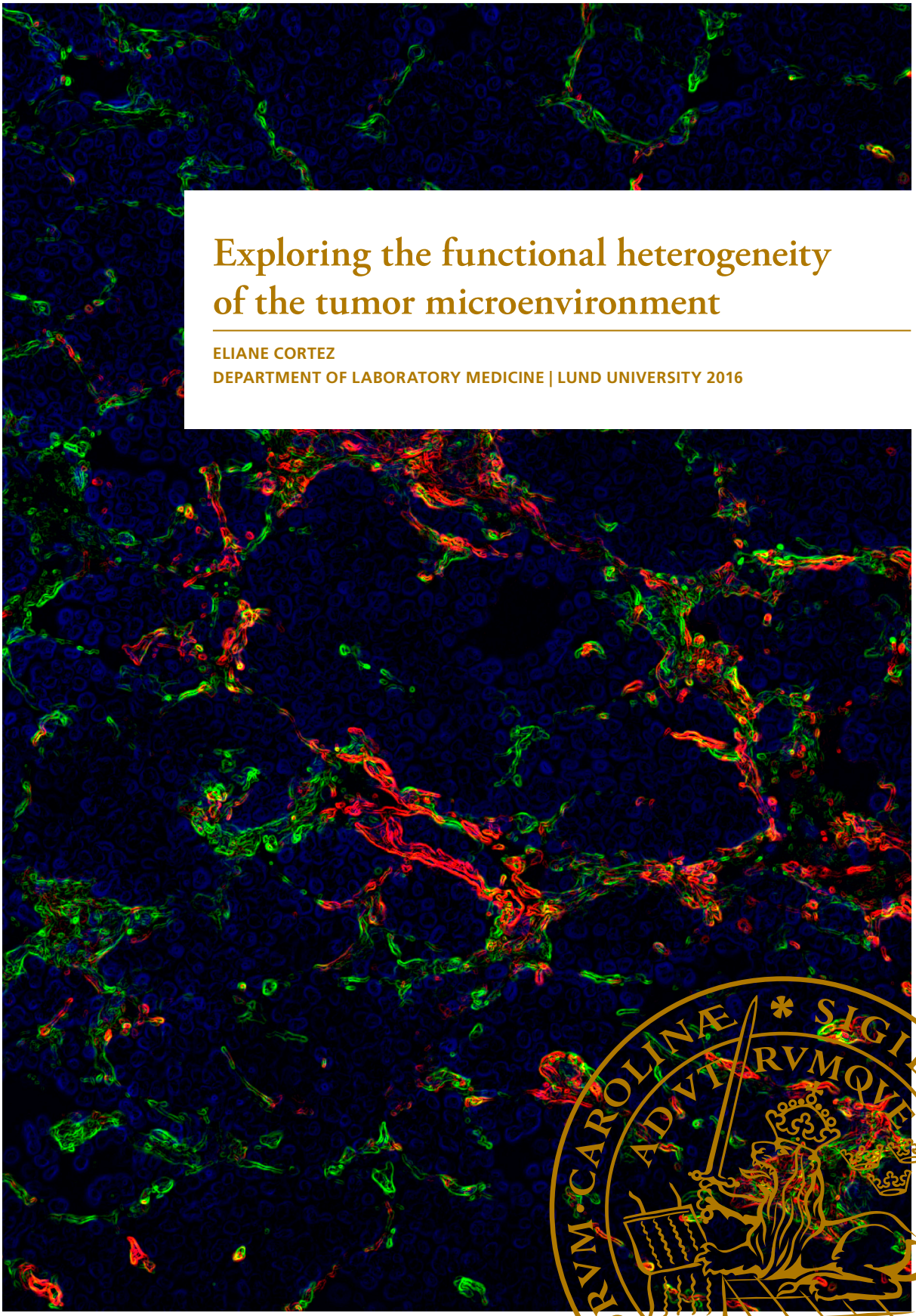

Exploring the functional heterogeneity of the tumor microenvironment

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UNIVERSITY

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Abstract

Tumors are complex entities, composed of different functional populations of malignant and stromal cells. There is an emerging importance to understand previously unmapped communication routes between different cell and non-cell compartments of the tumor microenvironment. Targeted drugs have been used for years in an attempt to block essential pathways for tumor growth. Specifically, drugs targeting tumor angiogenesis have shown significant success in different types of tumors. However, as effective as some of these compounds are, durable responses are rare and resistance does ultimately occur. Studies exploring therapeutic resistance have largely focused on endothelial cells (ECs). However, more recent reports suggest that development of resistance can be conferred by functional alterations of supporting cells like pericytes. Pericytes are a heterogeneous and highly mystifying population of mural cells and have been for years suggested to protect ECs from anti-angiogenic insults, promote vessel regrowth and tumor progression. However, most of the knowledge on pericytes in tumor growth is confounding and often conflicting.

In the first part of this thesis, we investigated several aspects of pericyte function during tumor progression. Using an experimental model of pancreatic neuroendocrine tumors (PanNETs), we characterized the nature of signals exchanged between pericytes and ECs in tumor vessels. We showed that pericytes, when in the vicinity of ECs, promote the upregulation of genes involved in cell survival like *Bcl-w*. We further demonstrated that upregulation of survival genes is dependent on autocrine vascular endothelial growth factor A (VEGF-A) signaling in ECs. Moreover, we showed that the amount of pericytes expressing alpha-smooth muscle actin (α-SMA) is increased in the tumor parenchyma upon anti-VEGF-A therapy. This highlights the potential value of using pericyte marker expression to predict clinical response to anti-vascular therapy. Finally, we characterized a novel imaging tool, the PDGFRβ-Affibody, a small molecule showing specific binding to platelet derived growth factor receptor beta (PDGFRβ) in tumors. This Affibody molecule holds the potential of being used to identify pericytes in tumors and to deliver cytotoxic compounds directly to the tumor microvasculature.

In the second part of this thesis, we investigated the biological function of the latest identified ligand for PDGFRβ, PDGF-DD, in tumor progression. Making use of a newly generated *Pdgfd* knockout mouse, we demonstrated that growth of PanNET in the RIP1-TAg2 model is significantly impaired in the absence of PDGF-DD. Deficient PDGF-DD signaling did not affect angiogenesis or pericyte recruitment to blood vessels. Instead, we found that PDGF-DD stimulated the proliferation of the bulk tumor cell population by inducing expression of mitogenic factors by a rare population of malignant cells expressing PDGFRβ. The existence of a heterogeneous population of tumor cells, marked by rare expression of PDGFRβ in malignant clones, was further confirmed in a cohort of human primary and metastatic PanNET.

Our studies emphasize the prevalent theory that targeting multiple compartments of the tumor microenvironment may represent a viable alternative to prevent resistance and achieve durable responses in patients. However, elucidating the relationship between the heterogeneous composition of tumors and the therapeutic outcome is still a significant challenge.

Key words: Pancreatic neuroendocrine tumors, pericytes, PDGF-DD, tumor heterogeneity.

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The image shows a tissue section of a pancreatic neuroendocrine tumor in the RIP1-TAg2 mouse, displaying pericytes staining positive for NG2 (green), α-SMA (red) or both markers. Cell nuclei are stained with DAPI (blue).

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Para ti avó

"O que dá o verdadeiro sentido ao encontro é a busca, e é preciso andar muito para se alcançar o que está perto" - José Saramago

Content

List of original papers

The thesis is based on the following papers, referred to in the text by their Roman numerals:

I. Pericytes promote endothelial cell survival through induction of autocrine VEGF-A signaling and Bcl-w expression.
Franco M, Roswall P, **Cortez E**, Hanahan D, Pietras K.
Blood. 118(10):2906-17. 2011

II. Use of a mouse model of pancreatic neuroendocrine tumors to find pericyte biomarkers of resistance to anti-angiogenic therapy.
Franco M, Pàez-Ribes M, **Cortez E**, Casanovas O, Pietras K.
Horm Metab Res. 43(12):884-9. 2011

III. Engineered high-affinity affibody molecules targeting platelet-derived growth factor receptor β in vivo.
Lindborg M, **Cortez E**, Höidén-Guthenberg I, Gunneriusson E, von Hage E, Syud F, Morrison M, Abrahmsén L, Herne N, Pietras K, Frejd FY.
J Mol Biol. 407(2):298-315. 2011

IV. Functional malignant cell heterogeneity in pancreatic neuroendocrine tumors revealed by targeting of PDGF-DD.
Cortez E, Gladh H, Braun S, Bocci M, Cordero, Björkström NK, Miyazaki H, Michael IP, Eriksson U, Folestad E, Pietras K.
Proc Natl Acad Sci USA. 113(7):E864-73. 2016

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Papers not included in this thesis:

Functional subsets of mesenchymal cell types in the tumor microenvironment.
Cortez E, Roswall P, Pietras K.
Semin Cancer Biol. 25:3-9. 2014

Deficiency for endoglin in tumor vasculature weakens the endothelial barrier to metastatic dissemination.
Anderberg C*, Cunha SI*, Zhai Z*, **Cortez E**, Pardali E, Johnson JR, Franco M, Páez-Ribes M, Cordiner R, Fuxe J, Johansson BR, Goumans MJ, Casanovas O, ten Dijke P, Arthur HM, Pietras K.
J Exp Med. 210(3):563-79. 2013 (* Equal contribution)

Image-based 3D modeling study of the influence of vessel density and blood hemoglobin concentration on tumor oxygenation and response to irradiation.
Lagerlöf JH, Kindblom J, **Cortez E**, Pietras K, Bernhardt P.
Med Phys. 40(2):024101. 2013

Abbreviations

ANG	Angiopoietin
ABD	Albumin binding domain
ATRX	alpha thalassemia/mental retardation syndrome X-linked
BM	Basement membrane
CML	Chronic myeloid leukemia
CMML	Chronic myelomonocytic leukemia
CNS	Central nervous system
CRC	Colorectal cancer
CSC	Cancer stem cell
CT	Computed tomography
CTLA-4	Cytotoxic T-lymphocyte-associated protein-4
DAXX	Death domain associated protein
DFSP	Dermatofibrosarcoma protuberans
EC	Endothelial cell
ECM	Extracellular matrix
EGF	Epidermal growth factor
EMT	Epithelial-to-mesenchymal transition
FDA	Food and drug administration
FACS	Fluorescent activated cell sorting
FGF	Fibroblast growth factor
GBM	Glioblastoma multiforme
GIST	Gastrointestinal stromal tumor
HCC	Hepatocellular carcinoma
HGF	Hepatocyte growth factor
HER2	Human epidermal growth factor receptor 2
IFP	Interstitial fluid pressure
IGF1	Insulin growth factor 1
ITH	Intratumor heterogeneity
LLC	Lewis lung carcinoma
MEN1	Multiple endocrine neoplasia type 1
Met CTX	Metronomic cyclophosphamide
MSC	Mesenchymal stem cell
mTOR	Mammalian target of rapamycin
NG2	Neural glial antigen 2
NSCLC	Non-small cell lung carcinoma
OS	Overall survival
PanNET	Pancreatic neuroendocrine tumor
PDGF/R	Platelet derived growth factor/receptor
PD-1/-L1	Programmed death 1/ligand 1
PDX	Patient derived xenograft
PET	Positron emission tomography
PFS	Progression-free survival
Rb	Retinoblastoma
RCC	Renal cell carcinoma
Ret	Retention motif
RGS5	Regulator of G protein signaling 5
RIP1-TAg2	Rat Insulin Promoter 1 - T antigen 2
RTK/I	Receptor tyrosine kinase/inhibitor
TCGA	The cancer genome atlas
TGF-β	Transforming growth factor beta
tPA	Tissue plasminogen activator
uPA	Urokinase plasminogen activator
VEGF/R	Vascular endothelial growth factor/receptor
vSMC	Vascular smooth muscle cell
WHO	World health organization
α-SMA	Alpha-smooth muscle actin

Abstract

Tumors are complex entities, composed of different functional populations of malignant and stromal cells. There is an emerging importance to understand previously unmapped communication routes between different cell and non-cell compartments of the tumor microenvironment. Targeted drugs have been used for years in an attempt to block essential pathways for tumor growth. Specifically, drugs targeting tumor angiogenesis have shown significant success in different types of tumors. However, as effective as some of these compounds are, durable responses are rare and resistance does ultimately occur. Studies exploring therapeutic resistance have largely focused on endothelial cells (ECs). However, more recent reports suggest that development of resistance can be conferred by functional alterations of supporting cells like pericytes. Pericytes are a heterogeneous and highly mystifying population of mural cells and have been for years suggested to protect ECs from anti-angiogenic insults, promote vessel regrowth and tumor progression. However, most of the knowledge on pericytes in tumor growth is confounding and often conflicting.

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PDGFRβ. The existence of a heterogeneous population of tumor cells, marked by rare expression of PDGFRβ in malignant clones, was further confirmed in a cohort of human primary and metastatic PanNET.

Our studies emphasize the prevalent theory that targeting multiple compartments of the tumor microenvironment may represent a viable alternative to prevent resistance and achieve durable responses in patients. However, elucidating the relationship between the heterogeneous composition of tumors and the therapeutic outcome is still a significant challenge.

Introduction

Recent decades were marked by significant advances in the identification of different regulatory mechanisms involved in tumor development. As a consequence, there has been considerable investment in cancer prevention and early detection, and massive efforts to develop therapeutic tools to control and treat several types of cancer. Nonetheless, 15 million people worldwide are diagnosed annually with cancer and up to 9 million people still die from it (1). Notable progress has been achieved, as the survival rates of patients diagnosed with certain forms of cancer including prostate cancer, malignant melanoma, non-Hodgkin Lymphoma and leukemia, has doubled in the last 40 years (2). Unfortunately, this increment has not been uniform across all types of cancer. Resistance to therapy, and ultimately tumor recurrence leading to high mortality rates, are still posing a major challenge in the clinical management of cancer.

Cancer results from the failure of our body to contain the proliferation of defective cells, often carrying mutations that allow them to thrive in an inadequate environment, which is modified along time to become supportive rather than suppressive. The traits necessary for malignant cells to survive and proliferate have been extensively described, and over recent decades tumor biologists and clinicians have incessantly attempted to treat and cure cancer by modulating and interfering with the so-called hallmarks of cancer (3, 4). One feature of tumor cells that has posed the biggest challenge in the clinic, is their capacity to evade the primary tumor, metastasize into distant organs and compromise their normal function. Metastatic disease is the leading cause of death for patients (5, 6) and the stage of disease most frequently treated in the clinic. Surgery with curative intent is the first line of intervention for most well confined solid tumors, however, metastatic disease is largely incurable due to its systemic nature. Inhibiting the initial steps of metastatic dissemination has led to many efforts exploring the ability of tumor cells and ECs to develop mesenchymal-like features (epithelial- and endothelial-to-mesenchymal transition; End-MT and EMT) (7-10). Understanding these events, and how the interaction between the different players in the supporting microenvironment aid tumor spread, hold the prospect for the development of more powerful tools to prevent or control metastatic disease. However, increasing difficulties arise from the realization that the molecular profile of metastatic lesions does not always mimic the primary tumor from where they originate (11).

Based on histological and molecular assessment, for years now we have referred to breast cancer not as a single disease but as a group of at least five malignancies exhibiting different progression patterns and each associated with different prognosis given the available treatments (12). The establishment of databases like The Cancer Genome Atlas (TCGA),

cataloguing genomic and transcriptomic data including the mutational landscape of thousands of tumors with paired normal-tumor samples, has allowed the subdivision of major cancers such as colorectal cancer (CRC) (13) and pancreatic adenocarcinomas (14) into distinct molecular subtypes. Inter-patient tumor heterogeneity might explain why individuals with the same type of tumor respond so differently to the same line of therapy. Hopefully in the near future, as it is now e.g. for breast cancer, it will be possible to design therapies for other cancer types taking their molecular profile into account and further define subgroups of patients to improve clinical response rates.

The complexity of the tumor microenvironment

Tumors are complex entities, harboring not only malignant cells but also multiple interacting cell types such as mesenchymal cells, i.e. pericytes and fibroblasts, ECs composing the inner lining of tumor blood vessels, immune cells and also non-cell constituents such as the extracellular matrix (ECM) (Figure 1) (15). It is believed that stromal components, recruited from local and distant organs, e.g. bone marrow, can be reprogrammed and co-evolve with tumor cells to become tumor promoting (16-18). However, preclinical observations suggest that the tumor stroma can also restrain tumors from metastasizing (19-21). Therefore, understanding the contribution of stromal cells to tumorigenesis when designing new therapies is fundamental. Breaking down the tumor to its smallest components has given us a better picture of the hierarchical organization and the many allies tumor cells rely on in order to survive in a specific environment, proliferate and metastasize.

Over the years, hundreds of studies have explored the role of the different cells in the stroma and how they provide cues that affect tumor cells, contribute to tumor progression and response to therapy. Given the importance of the tumor stroma, different therapeutic drugs currently used in the clinic aim at targeting signaling pathways involved in crosstalk between the different cells of the tumor microenvironment.

As the focus of this thesis, the interaction between components of the tumor microenvironment including pericytes, ECs and tumor cells, will be discussed in more detail in the section "PDGF signaling and pericytes".

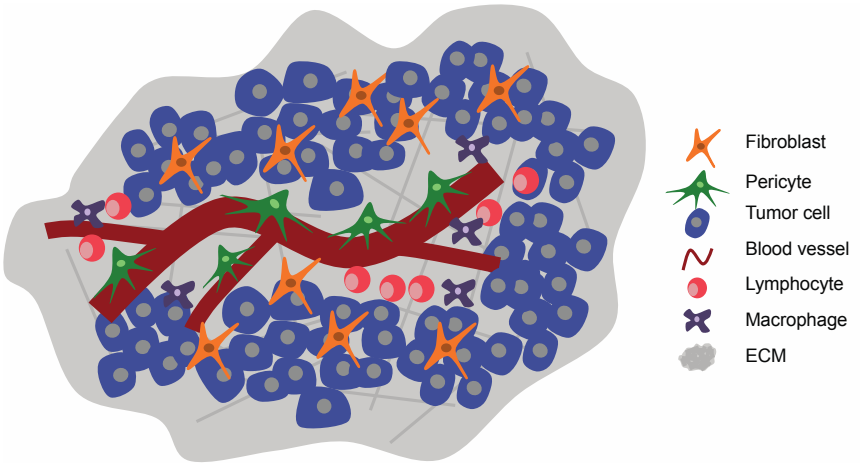

Figure 1.
Schematic representation of the tumor microenvironment. Solid tumors are composed of malignant tumor cells, blood vessels formed by an inner lining of endothelial cells and surrounded by pericytes; fibroblasts, immune cells and the extracellular matrix.

Molecular targeted therapy

Chemotherapy has been the standard-of-care for cancer therapy and in most cases the first line of pharmacologic treatment for various types of cancer before and/or after tumor resection. Chemotherapeutic agents kill tumor cells by damaging the DNA or disrupting pathways involved in cell division. They are aimed at rapidly proliferating cells, a key trait of tumor cells. However, this class of drugs is particularly toxic to cells and targets actively dividing non-cancer cells, producing numerous undesirable side effects. At the same time, although effective in some types of tumors, the high doses normally used in the clinic require resting periods (22). It is believed that malignant cells, given their high genetic instability, can recover between therapy cycles, and tumors that initially respond often become drug resistant and relapse (23).

To counteract adverse side effects and development of resistance, researchers have been trying instead to define and target signal transduction pathways essential to tumor and stromal cells, as well as specific oncogenic defects in malignant cells in order to prevent harm to healthy cells. Our growing knowledge on the key molecular changes between normal and malignant cells has allowed for the development of several targeted drugs that have been approved for use in the clinic (24). Different types of targeted therapies include hormone therapies, signal transduction inhibitors, gene expression modulators, apoptosis inducers, angiogenesis inhibitors and immune modulatory therapies.

The potential of molecular targeted therapy was first recognized in 1998 with the approval by the U.S. Food and Drug Administration (FDA) of trastuzumab, a monoclonal antibody against the human epidermal growth factor receptor 2 (HER2), a known proto-oncogene. Trastuzumab is used to treat patients with HER2-positive metastatic breast cancer (25) and also HER2 positive gastric cancer (26). In 2001, imatinib, a small molecule receptor tyrosine kinase inhibitor (RTKI) targeting the constitutively active BCR-ABL fusion protein was introduced with great success as a therapy for patients with chronic myeloid leukemia (CML) (27) and later on for gastrointestinal stromal tumors (GIST) with *c-Kit* or *PDGFRα* mutations (28). Imatinib is currently used also to interfere with signaling via PDGFRβ in chronic myelomonocytic leukemia (CMML) (29) and dermatofibrosarcoma protuberans (DFSP) (30).

On the never-ending quest to find new targets for therapy, major progress has been achieved with compounds targeting the immune compartment of the tumor microenvironment. These drugs block pathways involved in preventing cancer cells from being targeted and killed by T cells. As such, in 2010 FDA approved a monoclonal antibody, ipilimumab, that targets cytotoxic T-lymphocyte-associated antigen 4 (CTLA4), for advanced melanoma. More recently, other monoclonal antibodies targeting the programmed death 1 (PD-1) (pembrolizumab and nivolumab) or its ligand PD-L1 (atezolizumab), have been approved or are currently waiting for approval, respectively, to treat e.g. advanced renal cell carcinoma (RCC), unresectable melanoma, and non-small cell lung carcinoma (NSCLC) (31).

Anti-angiogenic therapy

The formation of the human vascular network involves two distinct processes: vasculogenesis and angiogenesis. Vasculogenesis is characterized by the development of blood vessels from endothelial progenitor cells while angiogenesis is the generation of new blood vessels from pre-existing ones, via sprouting and branching (32). Angiogenesis involves a multi-step process, tightly regulated by a balance between stimulatory and inhibitory factors. In adults, blood vessels normally remain quiescent with the exception of transient phases during wound healing or during the female reproductive cycle. Abnormal and persistent angiogenesis in adults is often associated with pathological conditions such as diabetic retinopathy, rheumatoid arthritis and cancer (33).

Tumor angiogenesis has been recognized as a critical event for solid tumors to secure a sufficient supply of nutrients (i.e. growth factors, cytokines, glucose) and oxygen, and to grow beyond a certain size (34). Based on this notion, in 1971, Judah Folkman proposed that targeting such a pivotal process for tumor growth might improve cancer treatment and possibly cause regression of already established tumors. Indeed, Folkman suggested that blocking angiogenesis could be achieved by neutralizing soluble factors that regulate the process (35).

During tumor growth, there is usually an excess of production of pro-angiogenic signals. Due to this imbalance, tumor vessels display several functional and structural abnormalities, i.e. lack of hierarchical organization, irregular shape, poor coverage by supportive cells like pericytes and smooth muscle cells (SMC), resulting in poor perfusion and increased leakiness (36). These abnormalities make blood flow and oxygen distribution often inefficient, leading to the development of areas of tumor necrosis. Importantly, the tumor vasculature is the major route used by tumor cells to escape the primary site and metastasize in distant tissues (6). These notions, together with the belief that ECs are genetically more stable than malignant cells and thus development of resistance would be prevented (37), shaped the rationale to develop drugs to starve tumors and prevent metastasis. Following these predictions, several signaling pathways involving activation of RTKs by different growth factors have been associated with EC proliferation, migration and vessel growth. As such, several factors have been identified and shown to drive angiogenesis in tumors, including members of the VEGF (38, 39) and PDGF families, basic fibroblast growth factor (bFGF) (40), transforming growth factor (TGF)-β (41) and angiopoietins, among others.

Clinical anti-angiogenic therapy

The identification of the VEGF/VEGFR axis as the major signaling pathway for EC proliferation and migration led to the development of therapeutic tools including neutralizing antibodies (42), small molecule antagonists of VEGFRs (43) and DNA aptamers (44). As a result, several of these agents as well as drugs targeting other pro-angiogenic signaling pathways are currently being tested in preclinical and clinical trials or already in use in the clinic. For example, bevacizumab (Avastin, Genentech/Roche), a humanized monoclonal antibody against VEGF-A is approved for treatment of metastatic CRC, glioblastoma multiforme (GBM), metastatic kidney cancer and NSCLC (45).

Other drugs affect tumor angiogenesis by interfering not only with ECs but also with supporting cells such as fibroblasts and pericytes, by inhibiting signaling via e.g. PDGF-BB/PDGFRβ, essential for the proper recruitment and function of these cells (see section "Pericytes"). As such, small molecule RTKIs like sunitinib (Sutent/Pfizer) and sorafenib (Nexavar/Bayer), that inhibit among other RTKs, VEGFR and PDGFR, are currently used to treat advanced RCC and unresectable PanNET, as well as imatinib-resistant GIST and unresectable hepatocellular carcinoma (HCC), respectively.

Preclinical anti-angiogenic therapy

The number of experimental studies exploring the effects of genetic or pharmacologic inhibition of VEGF/VEGFR signaling is vast. Despite affecting tumor vessel density and leading to tumor shrinkage, resistance to prolonged VEGF-A inhibition seen by re-vascularization, tumor growth and increased metastatic dissemination has been documented in mouse models of PanNET and metastatic breast cancer (46-48). Other studies have revealed that targeting ECs and supporting pericytes has a synergistic inhibitory effect on tumor growth (49-54). Similarly, combining anti-angiogenic drugs with chemotherapeutic

agents appears to further enhance cytotoxic effects (55-57). The enhanced effect of chemotherapeutic drugs used in combination with anti-VEGF therapies has been suggested to result from a transient normalization of blood vessels, associated with a decrease in interstitial fluid pressure (IFP) and increased oxygenation, which would allow for better drug delivery and uptake, however this mechanism remains to be clinically confirmed (58).

Despite the initial tumor response to anti-angiogenic drugs in experimental therapeutic studies, tumor regression is often followed by disease progression (46-48). Similarly, the outcome of anti-angiogenic therapies in the clinic has been limited to a transient effect in tumor shrinkage with mild to no improvement in the overall survival (OS) and progression-free survival (PFS) of patients with advanced solid tumors, that ultimately succumb to the disease (59). The limited efficacy of anti-angiogenic drugs, especially when used as single agents, has been attributed to either intrinsic or acquired resistance to therapy following an initial response phase. Several mechanisms have been proposed to explain the development of acquired resistance to anti-vascular therapies as e.g. development of mutations by ECs, alternative pro-angiogenic signals, recruitment of bone-marrow derived cells, increased pericyte coverage, increased tumor dissemination (60-63), induction of EMT, or even activation of cancer stem cells (CSC) (64). Alternatively, it has been speculated that tumors can also acquire oxygen via vessel co-option (the use of pre-existent tissue vessels), vasculogenesis, vascular mimicry (the formation of microvascular channels by tumor cells) or trans-differentiation of tumor cells into ECs (65). Some of these mechanisms, however, have not been properly confirmed and are still controversial.

The failure of bevacizumab in a phase III clinical trial for patients with metastatic breast cancer and following revocation of the drug by FDA does not completely show inefficiency of anti-angiogenic drugs, but instead points towards the need for screening patients to identify those that have VEGF-driven angiogenesis and might benefit from the treatment (66, 67). There is an imperative need to determine biomarkers for both prediction of response and monitoring of efficacy of therapy and ultimately select patients that can benefit from specific treatments and at the same time validate alternative drivers of angiogenesis (68).

Therapeutic combinations

Alone, targeted therapies might only prevent tumor cell proliferation and not kill cancer cells, thus not circumventing resistance. The conflicting results obtained with anti-VEGFR/PDGFR therapies in different preclinical and clinical settings strengthen the prevalent idea that very few cells in a tumor depend on a single pathway to survive and proliferate (69, 70). In the tumor biology field, the pursuit for alternative pathways has led to the identification of new potential targets for angiogenesis, including TGF-β signaling through the EC receptor Activin receptor-like kinase 1 (ALK1) (71, 72), fibroblast growth factors (FGFs) (73, 74) and Angiopoietin 2 (ANG2) (75, 76). Also, given the increasing availability of agents targeting different tumor compartments and pathways, and together with our growing understanding of the interplay between different cellular mediators in the

tumor stroma, there is a strong motivation to use combination of anti-angiogenic therapies with other targeted therapies. Such clinical trials are currently undergoing and results are much anticipated.

Tumor heterogeneity

The assumption that ECs were genetically stable compared to malignant tumor cells steered the belief that targeted therapies against the tumor vasculature would circumvent acquired resistance observed upon standard chemotherapy. However, as mentioned before, ECs in tumor vessels display a range of morphological and molecular abnormalities compared to their normal counterparts. How these alterations impact the limited outcome seen with anti-angiogenic drugs is the scope of several new studies (77). Recent progress in next-generation sequencing methodologies is revealing the hitherto unrecognized complex and dynamic heterogeneity of the entire tumor milieu (78, 79). The existence of distinct cellular clones in tumors has long been documented and individual subpopulations of tumor cells have been proposed to have the ability to interact with each other (80, 81). Nowadays, data from advanced methodologies continuously attest that tumors can be composed of multiple clones of malignant cells, tumor-initiating cells and stromal cells with genetic, phenotypic and often functional disparities between and within individual tumors (82-86).

Intratumor heterogeneity (ITH) represents a major challenge to cancer diagnosis and treatment choices, and is thought to contribute to limited treatment efficacy and disease recurrence. It is becoming increasingly apparent that single biopsy samples for molecular analysis might underestimate the genetic make-up of a tumor and not instruct properly on the most appropriate therapeutic decision. Additional concerns arise with the notion that not all identified driver mutations can be easily druggable.

However, the cumulative knowledge on mutations and phenotypical variations within a cell population in the same tumor also provides unprecedented new therapeutic opportunities (87, 88). Nonetheless, we are far from understanding the dynamics involved in crosstalk between subclones of malignant cells. Studies in GBM show that a small subset of tumor cells expressing mutant epidermal growth factor receptor (EGFR) promotes expression of cytokines that in turn affect wild-type EGFR clones to proliferate and drive tumor growth (89). Similarly, Marusyk and colleagues showed that a minor subclone of tumor cells overexpressing IL-11 promoted expression of VEGF-D on a different clone that became highly proliferative and outgrew IL-11 clones in a non-cell autonomous way (90). More recently, in a p53-null mouse model of breast cancer, $CD29^{high}CD24^{high}$ clones with tumor initiating properties were shown to give rise to a $CD29^{high}CD24^{low}$ population that in turn secreted several growth factors and stimulated the self-renewal and tumor-initiating capacity of the $CD29^{high}CD24^{high}$ population (91). Further evidences start to point to a spatial and temporal ITH, suggesting that cells might exhibit distinct genetic make-ups depending on

the organ where tumors arise, the surrounding environment and throughout the disease course as a response to different lines of therapy (92, 93). Although some studies indicate that ITH correlates with a poor prognosis across a number of cancers (86, 94), others also suggest that the extent of ITH can work as a potential prognostic biomarker, although not always in a linear way, i.e. high ITH might not always work for the benefit of the tumor since a large number of clones can also attract more immune cells (95).

Evidently, there is an urgent need to develop genetically engineered models that more faithfully recapitulate the ITH both in the tumor and stroma cell compartments. At the same time, mouse models should help predict clinical outcomes by allowing for biomarker discovery that might, ultimately, guide patient selection at a very early stage. The use of patient derived xenografts (PDX), engrafted into immune-compromised rodents and derived 3D organoid systems, is becoming increasingly popular for preclinical modeling. PDX models appear to recapitulate the human tumor cell heterogeneity. However, components of the immune system are missing in PDX models and the stroma is derived from the mouse. Nonetheless, PDX are a valuable tool to test drug responsiveness given the specific molecular categorization of the patient (96). On the other hand, engineered mouse models that develop spontaneous tumors in the equivalent organ of origin in humans, incorporate the tumor, immune and stroma cell compartments but appear to display a reduced tumor mutational range (84). These limitations indicate that often more than one model (*in vivo* and *in vitro*) is necessary for proper preclinical evaluation.

As precision oncology develops, in the near future most likely a therapy regime will imply that disease progression is more closely monitored. As such, therapies could potentially be designed along the way, according to the genomic alterations of the tumor, during the course of a therapy or as a response to prior treatments (97, 98). Also, the hope is that, based on predictive and response biomarker validation, combining parallel pre-clinical and clinical studies will allow more reliable identification of patients that are most likely to benefit from a particular therapy, exclude non-responders from clinical trials and improve clinical response rates (99).

In the following sections, the role of different subpopulations of pericytes and tumor cells and the dynamic signaling by members of the PDGF family for tumor progression and metastatic dissemination will be further discussed. As previously mentioned, the use of predictive mouse models in preclinical studies is of key importance, allowing us to better mimic, and translate our findings to the clinical setting. During our studies, we made use of the RIP1-TAg2, a mouse engineered to develop spontaneous multifocal pancreatic neuroendocrine tumors (discussed in detail in the section "The RIP1-TAg2 mouse model"). Briefly, PanNETs are highly dependent on angiogenesis for growth and invasion and have provided great insight into the development of tools for targeting several players of the tumor stroma including pericytes and ECs.

PDGF signaling and pericytes

The PDGF system

Ligands

In both mouse and human, PDGF is a conserved family of four polypeptide chains that assemble into disulphide-linked dimers via homo- or heterodimerization, and can form five full-length dimeric isoforms, i.e. PDGF-AA, -BB, -AB, -CC, and -DD.

The classical PDGFs, PDGF-AA, -BB and -AB, were discovered more than 30 years ago and only 15 years ago have the novel PDGF isoforms, PDGF-CC and PDGF-DD, been described, demonstrating that PDGF-mediated cellular signaling is more complex than previously envisioned.

All four PDGF chains contain a highly conserved core growth factor domain of approximately 100 amino acids in length, called the PDGF/VEGF homology domain, as it is also shared by members of the VEGF family. The growth factor domain is responsible and sufficient for dimerization, binding and activation of the receptors. N- and C- terminal extensions regulate in distinct ways the biological activity of the PDGFs. PDGF-AA and –BB are structurally different from the novel PDGFs. They contain a short N-terminal extension that undergoes intracellular proteolytic cleavage for activation. As such, both PDGF-AA and -BB are secreted in their active form (100). Additionally, PDGF-A has an alternatively-spliced messenger RNA that can generate two isoforms, short and long (101). Both the long PDGF-A isoform and PDGF–BB contain a short C-terminal retention motif responsible for the binding to components of the ECM (102, 103). In turn, PDGF-CC and PDGF-DD have an N-terminal CUB domain. PDGF-CC and –DD are secreted as latent proteins and in order to bind to their cognate receptors, the CUB domain needs to be proteolytically cleaved. The serine proteases, tissue plasminogen activator (tPA) (104) and urokinase plasminogen activator (uPA) (105, 106), have been identified as enzymes involved in cleavage of the CUB domain and activation of PDGF-CC and PDGF-DD respectively.

Receptors

The PDGF ligands exert their effects on target cells by binding and activating two structurally related membrane tyrosine kinase receptors, PDGFRα and PDGFRβ. PDGFRα and –β can also form heterodimers, the PDGFRα/β. The extracellular domains of the PDGF receptors contain five immunoglobulin-like domains and a split tyrosine kinase domain in the cytoplasm (107-109).

As dimeric isoforms, PDGF ligands bind to the two receptors at the same time causing them to dimerize and cross-phosphorylate, thereby creating docking sites for signaling molecules. Activation of different signaling pathways promotes cell growth, survival and migration in a ligand- and cell- dependent manner. Activation of PDGFRs leads to internalization and degradation in proteasomes or lysosomes. Alternatively, PDGFRs can be recycled and reappear in the plasma membrane initiating a new cascade of signaling events. PDGFRs are mainly expressed by cells of mesenchymal origin, i.e. fibroblasts, pericytes and SMCs. However, to a lesser extent ECs, epithelial cells and immune cells (specifically macrophages and dendritic cells) can express PDGFRβ. Besides, PDGFRα is also expressed by mesenchymal and oligodendrocyte precursors and astrocytes (110). Based on *in vitro* assays, it is known that PDGF ligands have different affinities for the αα, α/β and ββ receptors (Figure 2). Of note, PDGFs are structurally similar to members of the VEGF family (VEGF-A, VEGF-B, VEGF-C, and VEGF-D) and placental growth factor (PlGF), all sharing the conserved growth factor domain (111).

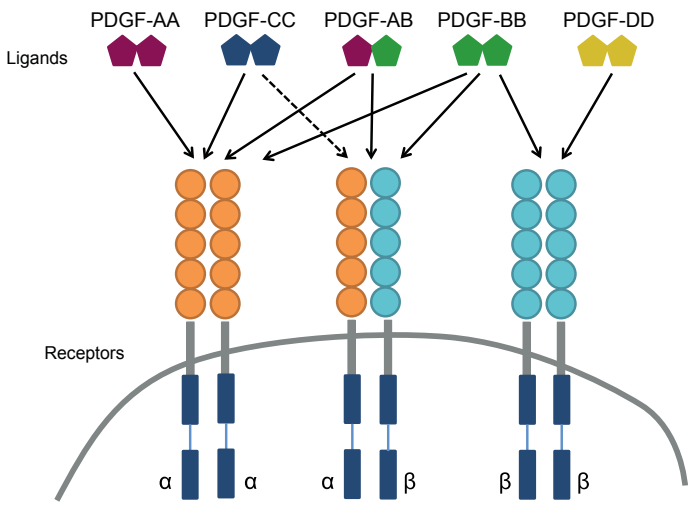

Figure 2.
Interaction between the platelet-derived growth factor (PDGF) ligands and their receptors. PDGFs are produced as homodimers or heterodimers: PDGF-AA, -BB, -AB, -CC, and –DD that bind and activate two PDGF receptors: PDGFRα and PDGFRβ. Ligand binding initiates a cascade of events involved in cell proliferation survival and migration.

PDGF-BB/PDGFRβ

In normal development

PDGF isoforms are secreted by ECs or epithelial cells and act as essential mitogens for cells expressing PDGFRs. PDGFs have fundamental roles during embryogenesis and normal development, being involved in a multitude of cellular processes including proliferation and migration.

Gene targeting studies in mice have been elucidative regarding the roles of the different PDGFs during normal development and pathological conditions. Inactivation of the *Pdgfrβ* and *Pdgfb* genes in mice leads to perinatal death, demonstrating their importance during embryonic development (112). Mice deficient for PDGFRβ or PDGF-BB show severe kidney developmental defects and a highly leaky vasculature, which has been shown to result from their inability to properly recruit pericytes and SMCs (113, 114). It has been demonstrated that, ECs secrete PDGF-BB that binds to the ECM components by the C-terminal retention motif, forming a chemotaxis-like gradient that induces pericyte recruitment (115).

Moreover, deletion of *Pdgfb*, contrary to *Pdgfrβ,* also leads to heart malformations with mice having bigger hearts with defects in the myocardium. This suggests that PDGF-BB may signal through PDGFRα expressed in the heart.

Members of the PDGF family are also involved in oligodendrocyte and neural development in the central nervous system (CNS) (116). PDGFRβ has also been shown to affect development of white adipocytes (117). Despite their role during development, evidences about the role of PDGFs in adult physiology are still limited. So far, it is known that PDGFR signaling is involved in wound healing processes (118). PDGFRβ has also been shown to regulate IFP, affecting transport from the blood vessels to the extracellular compartment (119, 120).

In tumor development

One of the described hallmarks of cancer is the self-sufficiency of growth factors (3). Deregulation of signaling pathways involving growth regulatory mechanisms is known to contribute to uncontrolled proliferation of cancer cells. Autocrine signaling of growth factors in cancer cells has been shown to stimulate cell growth in GBM (121-123). In addition, genetic abnormalities can lead to growth factor receptor amplification or constitutive activation in the absence of stimulation by growth factors (124, 125). Several receptor tyrosine kinases and their growth factor ligands have been implicated in tumor development and metastasis. PDGFs are expressed in most solid tumors and the roles of the different members of the family in cancer growth and invasion have been extensively examined (126, 127). Despite the already overwhelming literature, new functions of PDGFs in tumor development are continuously being deciphered.

Signaling by PDGFs in tumors can affect malignant cells directly in an autocrine fashion or stromal cells by paracrine stimulation. On the one hand, regarding autocrine signaling, several reports show that PDGFR signaling is often deregulated in a number of human tumors, e.g. DFSP, GBM, GIST, ovarian and endometrial tumors, as well as in CMML. Some of the genetic alterations that can give rise to deregulated signaling include amplification of the PDGFRα (in a subset of high grade GBM) (128, 129), activating point mutations in the PDGFRα (GIST) (130), translocations involving PDGFRβ, rendering the receptor constitutively active in CMML (124) and translocations involving PDGF-BB in DFSP (126, 131). In addition, upregulation of PDGFRs and its ligands has also been documented in soft tissue sarcomas and gliomas (132). Low frequency mutational activation of PDGFR has been found in common cancers such as breast and lung (133, 134). Upregulation of PDGFR has been also associated with increased metastatic dissemination of a subpopulation of malignant cells via EMT (135, 136) and regulation of IFP affecting tumor drug uptake (137). On the other hand, paracrine signaling by PDGFs, leading to recruitment and growth of mesenchymal cells in the tumor stroma facilitating tumor progression, renders stromal PDGFR expression as an attractive candidate target (see section "Pericytes in tumor development"). Together, this has encouraged the development of several PDGF antagonists currently under preclinical or clinical evaluation. Some of these drugs include antibodies, DNA aptamers, soluble extracellular domains of the receptors (138), and small molecule RTKI (132). Imatinib, sunitibib and sorafenib are part of a small but growing list of RTKI that target PDGFRβ but also other kinases such as VEGFRs and FGFRs (127). In addition to affecting tumor and/or stromal cells, targeting PDGFRβ was shown to decrease tumor IFP, which is believed to affect the delivery of therapeutic drugs aimed at the tumor compartment (139).

PDGF-DD

In normal development

PDGF-DD, as mentioned before, is the latest identified member of the PDGF-DD family (140, 141). *In vitro* assays show that activated PDGF-DD binds and phosphorylates PDGFRβ but not PDGFRα or any of the VEGFRs. These assays confirmed that proteolytic release of the CUB domain is necessary for proper binding to and activation of PDGFRβ. However, others have shown that PDGF-DD can also bind and activate the heterodimer PDGFRα/β (141). Previous studies have shown that *PDGFD* is strongly expressed by ECs from human umbilical vein and microvessels; however, human fibroblasts and SMCs also seem to express *PDGFD (142)*. Additionally, PDGF-DD has also been shown to stimulate proliferation of SMC and fibroblasts (142, 143). Overexpression of human *PDGFD* cDNA in mouse basal epidermal cells showed an increase in macrophage recruitment during wound healing and increased IFP (144). Other functions have been attributed to PDGF-DD, such as regulation of adipose-derived stem cells (145) and human neocortical development (146). To better understand the biological function of PDGF-DD

in normal development, a *Pdgfd* knockout mouse (*Pdgfd*$^{-/-}$) was generated on the C57Bl/6 background. Briefly, the exon 1 of the mouse *Pdgfd* gene was replaced by a LacZ expression cassette, which allows monitoring of *Pdgfd* expression. Contrary to *Pdgfb*$^{-/-}$ *mice*, *Pdgfd*$^{-/-}$ mice are viable, fertile and present with no gross histological abnormalities. Further characterization of adult mice showed that deficient PDGF-DD signaling was associated with a mild alteration of the systemic arterial blood pressure. LacZ expression was found in the vasculature of different organs, suggesting that PDGF-DD might be predominantly expressed by ECs. Interestingly, a significant reduction in pericyte coverage was detected in the heart of *Pdgfd*-deficient mice but not in other organs. However, vessel functionality and density appeared unaffected by lack of PDGF-DD signaling (147).

In tumor development

Contrary to the other ligands of the PDGF family, the exact role of PDGF-DD in tumor development is still a conundrum. Although it has been suggested that the major source of PDGF-DD are ECs, high expression of *PDGFD* has been detected in breast, ovarian, lung, renal and brain tumor cell lines (142, 148, 149) and in breast cancer tissue (150). These observations suggest that PDGF-DD might play a role in tumor development. Exogenous expression of PDGF-DD in NIH/3T3 cells induced tumor formation and angiogenesis (143). As for PDGF-BB, PDGF-DD signaling has also been implicated in the recruitment of pericytes to the tumor microvasculature in orthotopic mouse models of human RCC (151) and breast cancer (150) or in the recruitment of fibroblasts in cholangiocarcinomas (152). Overexpression of *PDGFD* in an experimental model of breast cancer promoted metastatic dissemination (150). Involvement of PDGF-DD in EMT has been shown in a mouse model of prostate cancer when overexpressed in malignant cells (153, 154) and in breast cancer cell lines (155, 156). Excessive expression of PDGF-DD has been associated with worse prognosis in endometrial cancer (157, 158). Altogether, there is a growing literature reporting the role of PDGF-DD in tumor development, however, the mechanisms of action of PDGF-DD remain to be properly elucidated.

Importantly, all previous studies reporting the role of PDGF-DD in tumorigenesis have been performed using modulation of *PDGFD* expression in various tumor cell lines. As part of this thesis, we investigated the role of PDGF-DD during PanNET development making use of the first *Pdgfd* knockout mouse.

Pericytes

The vascular unit is composed of an inner lining of ECs surrounded by perivascular mural cells, i.e. pericytes and vascular smooth muscle cells (vSMC). Whereas big caliber vessels are normally enfolded by vSMC, pericytes are most commonly associated with the microvasculature, i.e. arterioles, venules and capillaries (159).

Pericytes in normal development

Identification

Pericytes are highly complex and heterogeneous cells identified and described for the first time more than 100 years ago (160, 161). The term pericyte was coined later on by Zimmerman indicating their close location to the vascular wall (162). Pericytes are contractile cells with a prominent nuclei and a small content of cytoplasm, embedded within the basement membrane (BM) shared with ECs. Pericytes extend long cytoplasmic processes around the abluminal endothelial wall, contacting more than one EC at the same time.

Pericyte coverage

As discussed later on for tumors, pericyte coverage in normal tissue seems to be variable and organ-specific. Analysis of different tissues led to the rough estimation that EC-to-pericyte ratios can vary between 1:1 to 10:1 and pericyte coverage spans from 10-70% (163). Generally it is assumed that the more stringent the EC barrier is in a specific tissue, the higher pericyte coverage it displays. As such, increased pericyte coverage is found in brain compared to lungs or muscles (164). Also, vessels found in the torso and in the legs, where increased blood pressure is needed, are more abundant in pericytes compared to the upper body vessels (165). These observations have suggested that pericytes play an important role in regulating blood flow, EC homeostasis and vessel stability.

The definition of pericytes is controversial given the phenotypical heterogeneity and similarities with vSMC. Usually, a combination of different features are used to define and distinguish pericytes from vSMC, i.e. location, morphology and marker expression. A handful of markers are used to identify pericytes histologically, such as Neural-glial antigen 2 (NG2) (166, 167), PDGFRβ (113, 168), α-SMA (169), regulator of G protein signaling 5 (RGS5) (170, 171), desmin (172) and others (164, 173-175). However, marker expression appears to be dynamic during development and varies in a tissue-specific manner. More importantly, these markers are not exclusively expressed by pericytes (176). Given this, identification of pericytes in tissue sections should be done by using a combination of markers and counterstaining with EC markers.

Origin

The origin of pericytes has been extensively debated and to date there is no coherent theory. It is believed that pericytes can originate from different precursor cells depending on the organ they are found in, and on their function. While some pericytes in the brain and thymus are thought to originate from the neural crest (177), coronary vessel perivascular cells are believed to stem from the mesoderm (178). Others have shown that bone marrow precursor cells can differentiate into pericytes (179). The most prevalent theories however suggest that pericytes derive from mesenchymal precursors through interaction with TGF-β (115, 180, 181). Nonetheless, the dynamics of this process remains unclear.

Recruitment and function in angiogenesis

Despite the vast literature on pericytes, their role is still an enigma, partly due to the lack of a definitive pan-marker and partly to the difficulty of isolating pure primary pericytes or to study them *in vivo*. The role of PDGF-BB secretion by tip ECs for proper recruitment and survival of pericytes to newly formed blood vessels has been comprehensively demonstrated (113, 115, 182). Similarly, EC-specific ablation of PDGF-BB leads to inefficient pericyte recruitment (183). However, pericytes in the liver (hepatic stellate cells) seem to be recruited to vessels in a PDGF-BB independent manner (115). Accordingly, other signaling pathways in different organs have been implicated in the recruitment of pericytes, i.e. heparin-binding epidermal growth factor (HB-EGF) through EGF receptors (184), the stromal derived factor 1-a (SDF-1a)/CXCR4 axis (185) and others (173).

The close contact between pericytes and ECs allows for communication by both direct physical contact and paracrine signaling (186, 187). Recently, chemomechanical signaling has been described as yet another mechanism involved in pericyte-EC communication (188). While deposition of basal lamina components by pericytes has been also observed (189, 190), it is still debatable whether interaction between pericytes and ECs contributes to BM assembly.

In normal tissues, several studies indicate that pericytes actively support vascular formation and maturation during embryonic development by stabilizing blood vessels and controlling vascular tone through vasoconstriction and vasodilation (191-193). Pericytes have been shown to secrete pro-angiogenic factors like VEGF-A at the beginning of angiogenesis, inducing EC proliferation and guiding migration through the BM (194), while stabilizing newly formed vessels by inducing EC quiescence and maturation (195).

Apart from their role in vascular formation and maturation, pericyte function seems to vary considerably depending on the organ where they are located. In the brain, pericytes are an essential component of the neurovascular unit, where they help maintaining integrity of the blood brain barrier (196-198), whereas in the kidney, where pericytes are called mesangial cells, they are essential for the development of the glomerulus (199). Moreover, a number of studies indicate that pericytes display stem and/or progenitor cell properties and behave like mesenchymal stem cells (MSC) (200, 201), white adipocyte progenitors (202) or even neural stem cells giving rise to neuronal cell lineages (176). Similarly, a growing number of studies show that tissue resident stem cells are preferentially found in perivascular niches, suggesting that pericytes might be involved in the maintenance of stem cell populations (203-206).

Pericytes in tumor development

It is becoming increasingly appreciated that genetic aberrations in tumor cells are not sufficient to drive tumor development but also the interaction with surrounding non-malignant cells, soluble factors, stromal cells and the ECM. While the role of ECs and immune cells in cancer development is better understood, the function of pericytes has confounded researchers for years and conflicting studies continuously puzzle our understanding about this cell type.

In contrast to the healthy microvasculature, vessels in tumors are irregularly shaped and leaky, with an indistinct hierarchical organization (207). Similarly, tumor pericytes seem to differ from normal pericytes, i.e. they are often found loosely attached to vessels and extend cytoplasmic processes deep into the tumor tissue, away from the vascular wall. Differences in the interaction between pericytes and ECs in tumor vessels compared to normal tissues have also been reported (208). These properties of tumor pericytes have been suggested to partly contribute to the abnormalities in tumor microvessels (208, 209). In tumors, similarly to normal tissues, the extent of pericyte coverage seems to be organ-dependent. In general, pericytes appear to be less abundant in tumor tissue compared to the microvasculature in the normal organ. Benjamin and colleagues showed that 38% of vessels in prostate tumors are covered by pericytes compared to 75% of the normal counterpart (191). In GBM, only 19% of vessels display pericytes. Conversely, Morikawa *et al.* found that pericytes are present in more than 97% of tumor vessels of experimental PanNET, breast carcinoma and Lewis Lung Carcinoma (LLC). Surprisingly, marker expression was also altered, i.e. tumor vessels in PanNETs and GBM seem to be more invested by α-SMA+ pericytes than the respective normal tissue (208). Recently, Keskin and colleagues further demonstrated that pericyte coverage and marker expression are dynamic during tumor progression (210). Indeed, clinical studies have attempted to correlate the extent of pericyte coverage with tumor prognosis. In clear cell RCC patients, increased vessel coverage with α-SMA+ pericytes was associated with more aggressive tumors and poorer outcome (211). Previously, however, absence of α-SMA+ pericytes in CRC specimens was detected in immature and leaky microvasculature of poorly differentiated tumors and associated with increased metastatic dissemination (212). In urothelial carcinoma, high pericyte coverage correlated with decrease in PFS (213). Moreover, low numbers of vessel-associated pericytes significantly correlated with a poor prognosis in invasive ductal breast carcinoma (20).

It is important to consider that most studies in tumor pericytes rely on one or two markers to detect pericytes and the choice of these markers differs between analyses. As previously mentioned, not all the classical pericyte markers are expressed in all pericytes within a tumor and there is no definitive marker that can identify all the tumor pericytes and at the same time distinguish them from normal pericytes. Consequently, this has severely hampered our interpretation of the interplay between pericytes and ECs and their effect on tumor development and drug response.

Recruitment to tumor blood vessels

As for normal tissues, recruitment and survival of pericytes in the tumor microvasculature is dependent on signaling from heparan-sulphate bound PDGF-BB, secreted by ECs (181) and to a lower extent, by tumor cells (209). When recruited to the tumor blood vessels, pericytes have been shown to secrete VEGF-A (214), Ang-1 (215) and possibly other signaling molecules promoting EC proliferation and migration, thereby affecting tumor angiogenesis.

Interference with the PDGF-BB/PDGFRβ pathway in tumors severely disturbs pericyte recruitment. This was clearly demonstrated using the PDGF-BB retention mice (*Pdgfb*$^{ret/ret}$), a transgenic model with deleted C-terminal retention motif in the *Pdgfb* gene. *Pdgfb*$^{ret/ret}$ mice show accentuated defective investment of pericytes in the vascular wall (182). Fibrosarcoma cells (T241) transplanted into *Pdgfb*$^{ret/ret}$ mice display a substantial decrease in pericyte coverage around blood vessels (209). Moreover, targeting PDGF-BB with a specific DNA aptamer (AX102) led to a substantial reduction of NG2, desmin and α-SMA expressing pericytes in an experimental model of LLC (49)

Targeting pericytes

Increased pericyte coverage has been documented in experimental tumor models following anti-VEGF therapy (46). Thus, pericytes have been implicated in the development of tumor-acquired resistance (191, 216, 217).

Based on these observations, it has been proposed that depleting pericytes from tumor vessels would render ECs more responsive to anti-angiogenic therapies. Indeed, as described in the section "Anti-angiogenic therapy", drugs RTKIs targeting PDGFRβ in combination with anti-VEGF therapies more efficiently inhibited tumor vascularization in a PanNET model compared to anti-VEGF drugs alone (51, 53, 181, 218). In addition, a neutralizing antibody for PDGFRβ, enhanced the antitumor effect of anti-VEGFR2 in human xenografts of ovarian and lung cancer (219, 220). In a later study, used in combination with bevacizumab, the aptamer AX102 was shown to enhance the anti-VEGF effect in ovarian cancer xenografts (221). However, due to the unspecificity of RTKI to PDGFRβ, it remains to be clearly demonstrated that targeting pericytes enhances the effects of other anti-endothelial drugs. Attempting to address this question, Nisancioglu *et al.* treated *Pdgfb*$^{ret/ret}$ mice, upon transplantation of LLC, B16 melanoma or fibrosarcoma cell lines, with an anti-VEGF-A specific antibody, G6-31. In contrast to previous studies, reduced pericyte coverage in the *Pdgfb*$^{ret/ret}$ mice did not render tumor ECs more sensitive to the anti-VEFG-A therapy (222). Interestingly, in tumors from RIP1-TAg2 and LLC mice, Sennino and colleagues proposed that the synergistic effect of PDGF and VEGF targeting is dependent on whether pericyte or tumor cells secrete PDGF and/or VEGF (138). These results suggest that the response to targeted therapies aimed at pericytes and ECs might be tumor-dependent.

Despite the role of PDGF-BB/PDGFRβ for pericyte function in tumor vessels, others have reported contradictory observations. Overexpression of *PDGFB* in preclinical CRC and pancreatic cancers led to decrease tumor growth associated with an increase in pericyte coverage. Further analysis suggested that tumor growth was hampered by pericyte-mediated inhibition of EC proliferation *in vivo* (223). In contrast, transfection of *PDGFB* into GBM cells led to an increase in pericyte coverage, enhanced angiogenesis and subsequent tumor growth (224). Expression of PDGFRβ can also be found in GBM tumor cells, which might explain the discrepancies between these two studies. Moreover, overexpression of *PDGFB* in fibrosarcoma did not change tumor growth regardless of the increase in pericyte coverage (209). These apparently paradoxical results highlight the imperative importance of a better characterization of cell- and tumor-specific PDGF-BB/PDGFRβ signaling in tumors treated with inhibitors for this pathway.

In some experimental tumors, targeting angiogenesis has been shown to suppress metastasis (225-228) whereas in others it is associated with increased cell dissemination (46, 48). While the rationale to develop therapies against tumor pericytes is supported, some reports have shown an increase in metastatic spread upon pericyte targeting in mouse models of breast cancer (20, 210) and in a mouse model of PanNETs when crossed with *Pdgfb*$^{ret/ret}$ mice (229). Similarly, lower pericyte density in microvessels has been correlated to an increase in metastatic dissemination in CRC patients (212), in prostate cancer xenograft models (230) and other human tumors (231). Conversely, inhibition of PDGFRβ with imatinib reduced growth and metastasis in an orthotopic mouse model of human pancreatic carcinoma (232). Cooke and colleagues showed that upregulation of Met, a promoter of EMT, was associated with a reduced pericyte investment in tumor blood vessels in experimental breast cancer (20). Others suggest that pericytes affect metastatic establishment at secondary sites (233, 234). Whether pericytes actively promote metastasis or represent a physical barrier to tumor cell dissemination and/or extravasation through a compromised vascular wall has not been entirely elucidated.

The literature on pericyte biology during tumor development is clearly confounding. Evidently, there is a need to develop more powerful tools to accurately characterize pericytes, their mechanism of action *in vivo* and to fully exploit their therapeutic potential. Table 1 summarizes the diverse outcomes in experimental tumor models upon pericyte targeting or upon combination of PDGFR and VEGFR inhibitors.

Table 1

Examples of preclinical studies exploring the effect of targeting PDGF and VEGF signaling pathways on tumor growth and metastatic development.

Tumor model	Host model	Experimental conditions	Effect		Ref
			Tumor growth/ Vascularity	Metastasis	
Pericyte targeting alone					
PanNET RIP1-TAg2	*Pdgfb*$^{ret/ret}$	-	No effect on tumor growth	Increased Metastasis	(229)
MMTV-PyMT	NG2 and PDGFRβ thymidine kinase	Ganciclovir induced depletion	Reduced tumor volume	Increased metastasis	(20)
4T1 Breast carcinoma	PDGFRβ thymidine kinase	Ganciclovir induced depletion	Reduced tumor volume	Increased metastasis	(210)
PDGFR and VEGF inhibition					
B16 Melanoma LLC Lung carcinoma T241Fibrosarcoma	*Pdgfb*$^{ret/ret}$	anti-VEGF-A	No added benefit on tumor suppression	-	(222)
C6 Glioma	-	Sunitinib	Enhanced tumor suppression	-	(51)
PanNET RIP1-TAg2	-	Sunitinib/imatinib + anti-VEGFR	Enhanced tumor suppression	-	(53)
	-	Sunitinib	Reduced vascularization and tumor burden	Increased metastasis	(47)
	-	Sunitinib	Reduced vessel density and tumor burden	No difference in metastasis	(235)
	-	Nintedanib	Reduced pericyte coverage, vessel density and tumor burden	No difference in metastasis	
	-	VEGFR and PDGFR soluble receptors	Reduced pericyte coverage, vessel density and tumor burden	-	(138)
LLC Lung carcinoma	-	VEGFR and PDGFR soluble receptors	No synergistic effect	-	
PDGFR and chemotherapy					
PanNET RIP1-TAg2	-	Imatinib + Met CTX	Reduced pericyte coverage and enhanced tumor suppression	-	(55)

Functional subsets of tumor associated pericytes

In the light of intratumor heterogeneity, pericytes are a good example of a very plastic group of cells, with variable functions and phenotypes throughout tumor development. Several reports from different tumor types reveal a greater dynamic of tumor pericyte markers expression than previously envisioned. Accordingly, studies suggest the existence of functional subsets of pericytes, based on marker expression upon immunohistological analysis (Figure 3). Marker expression also appears to be temporally and spatially regulated, varying in different tumor types and throughout disease development. This phenotypic heterogeneity in the pericyte population is accompanied by distinct functional roles. The most common markers used to identify pericytes in tumors are: NG2, α-SMA, desmin, PDGFRβ, RGS5 and CD128. These markers are however not exclusive to pericytes. NG2 is a transmembrane chondroitin proteoglycan expressed mainly by pericytes in peripheral tissues but also by some tumor cells like CRC cells (236, 237). Transient expression of NG2 in tumor macrophages, as well as in adipocytes in mammary tumors, has also been documented (238). In brain tumors, besides pericytes, NG2 is also expressed by

oligodendrocyte progenitor cells. Expression of NG2 has been ascribed to a mature population of pericytes in tumors (181). Selective ablation of NG2+ pericytes caused tumor reduction in transgenic and orthotopic mouse models of breast cancer but concomitantly increased hypoxia and metastatic dissemination (20). Increased metastasis upon pericyte targeting is worrying and has been described in other studies discussed previously. NG2 expression in tumor pericytes was shown to be essential for pericyte migration and proliferation and for interaction with ECs (238). PDGFRβ expression on the other hand, appears to denote an immature subset of pericytes capable of differentiating into NG2+, α-SMA+ and desmin+ pericytes (181). PDGFRβ+ pericytes have also been shown to induce survival signals to tumor ECs in an autocrine VEGF-A-dependent way (239). Moreover, as discussed earlier, enhanced anti-VEGF effects were observed in tumors after depletion of PDGFRβ+ pericytes. Pericytes also express α-SMA, a contractile protein, and one of the most utilized pericyte markers. Prolonged treatment of tumors from RIP1-TAg2 with an anti-VEGFR2 monoclonal antibody (DC101) rendered tumors refractory to therapy and with an increased vessel coverage of α-SMA+ pericytes (240). Similarly, a clinical study in patients with recurrent malignant GBM, with subsequent resistance to bevacizumab, showed that there was no alteration in tumor vessel density but a pronounced proliferation of α-SMA+ pericytes compared to biopsies prior to treatment (241). Together, these studies suggest that increase in α-SMA+ pericytes is associated with tumors resistant to anti-VEGF-A therapy. The potential of using α-SMA expression in pericytes, for monitoring response to therapy, awaits clinical validation. RGS5 is found upregulated by pericytes in the tumor vasculature of PanNETs and GBM with still poorly understood functions (242). RGS5 expressing pericytes denoted an immature population of cells, in a model of PanNET with delayed tumor onset, the RIP1-TAg5. Loss of RSG5, associated with pericyte maturation by increased expression of α-SMA and NG2, led to vascular normalization, increased immune cell recruitment and subsequent increased anti-tumor response (243). Pericytes expressing other markers such as CD128 and desmin have been identified in tumors and have seemingly distinguished functions during tumor development and in different tumor types (244).

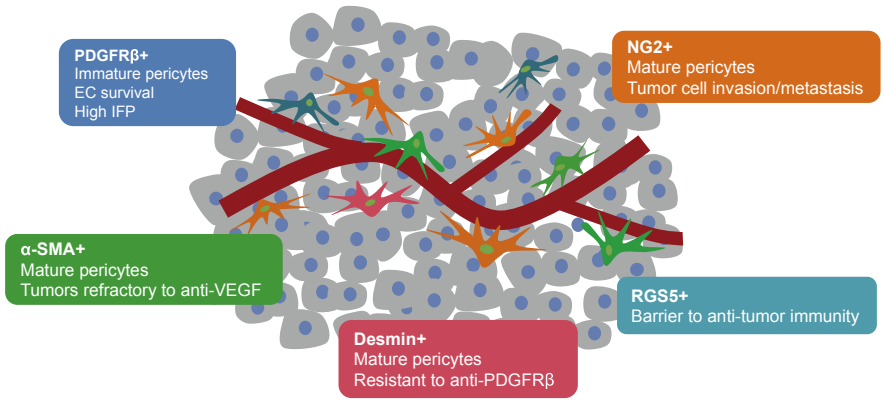

Figure 3.
Functional subsets of tumor pericytes. A summary of the current understanding on pericyte subpopulations based on expression of the most well characterized markers from studies in experimental and human tumors. Expression of pericyte markers is dynamic during tumor progression and across different tumor types. Adapted from Cortez et al. *Semin Cancer Biol*, 2014.

Pericytes as MSC and a niche for CSC

Further ambiguity arises with additional supportive roles ascribed to pericytes. It has long been suggested that pericytes exhibit multipotent properties and represent a subpopulation of MSCs both in normal and human tumor tissue (176, 245). Indeed, MSCs appear to express known pericyte markers such as α-SMA, PDGFRβ and NG2, hence suggesting a common origin (200). Pericytes are thought to be capable of developing into vSMCs (169), primary bone cells (246), adipocytes (247) and even neural cell lineages (248).

Given the aforementioned role of pericytes in the neurovascular unit, the perivascular niche (composed of pericytes, ECs, astrocytes and macrophages) has also been considered as a stem/progenitor cell supply within the brain (249). Data supporting this hypothesis originates from studies in brain tumors, particularly GBM. In brain cancer, the perivascular niche is considered to be essential for the preservation of a stem cell state in malignant cells and so far, interaction between ECs and CSC was shown to control self-renewal and differentiation of the latter, leading to increased aggressiveness (250). However, further studies are warranted to elucidate how the interaction between ECs and pericytes in the neurovascular unit contributes to the stem cell phenotype. Although in tumors most pericytes are thought to derive from precursors in the proximal normal tissue or from bone marrow derived cells, Cheng and colleagues demonstrated that TGF-β induced glioma stem cells (GSCs) have the capacity to differentiate into pericytes (251). More studies are compulsory in other tumor types, to fully elucidate the role of pericytes as potential stem cells, their origin and their stem cell promoting capacity, giving the fast emerging interest in CSCs as alternative culprits to development of resistance and recurrence of tumors in the clinic.

Pancreatic neuroendocrine tumors

Pancreatic neuroendocrine tumors represent a rare and heterogeneous group of tumors developing in the islets of Langerhans in the endocrine pancreas. PanNETs display abnormal growth of endocrine (hormone-secreting) cells. Is still debatable whether the cell of origin of PanNETs resides within the islet cells or whether exocrine and endocrine pancreatic tumors share a common precursor (252).

PanNETs account for 1-3% of all pancreatic tumors and despite the relatively low number of cases diagnosed yearly, <1 per 100,000 individuals, the incidence of PanNETs has been increasing (253). Although PanNETs have a relatively slow-growing rate, the 5-year survival of patients after resection is about 65% and the 10-year survival is 45% (254, 255).

Classification

Pancreatic neuroendocrine tumors can be divided into two major types: functional PanNETs (F-PanNET), about a third of all PanNETs, with hypersecretion of different hormone peptides including insulin, glucagon, gastrin, somatostatin etc; and non-functional PanNETs (NF-PanNET), the more prevalent and malignant tumors, with no hormonal clinical syndrome. Non-Functional PanNETs normally manifest as space-occupying tumor and metastatic lesions. The current classification for pancreatic neuroendocrine neoplasms (PanNEN) has been implemented since 2010 by the World Health Organization (WHO), based on histological differentiation and proliferative activity (ki67 labeling index). According to the new system, well-differentiated neoplasms (including benign and metastatic) with low mitotic rates are now termed neuroendocrine tumors (WD-NETs), and graded G1 (Ki67 <2%) or G2 (Ki67 2–20%), whereas poorly differentiated neoplasms are termed neuroendocrine carcinomas (PD-NECs) and graded G3 (Ki67 >20%) (256, 257).

For disambiguation, PanNET is from now on used to refer only to well-differentiated advanced tumors (functional or non-functional) and is the only focus of further discussions, excluding poorly differentiated tumors.

Diagnostics

The diagnosis of PanNET is based on histological analysis on tumor specimens, circulating biomarkers as well as different imaging tools including computed tomography (CT) and positron emission tomography (PET) scans. For years, immunostaining and serological levels of Chromogranin A has been the most widely used diagnostic and response biomarker in the clinic to monitor PanNETs as well as other NETs. However, its limited sensitivity

and specificity warrant the identification of more accurate diagnostic biomarkers. Moreover, the lack of sensitive and specific methods for early detection of PanNETs leads to that the majority of patients (about 70%) are diagnosed with advanced and often unresectable tumors associated with metastatic dissemination (253).

Treatment

The heterogeneous clinical manifestation of PanNETs, resulting in variable degrees of aggressiveness, poses a considerable challenge in the management of well-differentiated advanced PanNETs. Treatment of PanNETs is decided based on stage and tumor grade and used to be limited to surgical removal for localized tumors. For locally advanced or metastatic disease, chemotherapy including either streptozotocin monotherapy, or combination with fluorouracil or doxorubicin, were the only approved regimens for a couple of decades (258), however with limited efficacy (259). The majority of PanNETs express somatostatin receptors on the cell surface. As a result, somatostatin analogues are often given as a second line of care to reduce symptoms associated with hormonal hypersecretion (260). Although still limited, new lines of treatment are showing encouraging results.

Targeted therapy in PanNETs

An improved understanding of the different signaling mechanisms involved in PanNETs has led to the development of drugs targeting biologically relevant pathways. PanNETs are highly vascularized tumors with prominent secretion of VEGF, which has motivated clinical trials to test angiogenesis inhibitors (261). Similarly, the mammalian target of rapamycin (mTOR) pathway has been shown to be involved in the pathogenesis of PanNETs (262). Currently, targeted therapy for PanNETs is limited to sunitinib and everolimus, an mTor inhibitor, both approved by FDA in 2011 for the treatment of well-differentiated unresectable advanced PanNETs (263, 264). Although sunitinib only showed a response rate of 9.3%, there was an evident impact in median PFS from 5.5 months in the placebo group to 11.4 months in the treated arm in a randomized phase III clinical trial (263). Similarly, median PFS for the everolimus treated cohort was 11.6 months versus 4.6 months for patients treated with the placebo (264). However, none of these drugs had a significant effect on OS. As an alternative for targeted therapy, PanNET patients are also, since a few years now, given the possibility to have peptide receptor radionuclide therapy (PRRT) by labeling somatostatin analogues with different radionuclides, such as indium (111 I), yttrium (90 Y) or lutetium (177 Lu) (265, 266). Although still limited, studies of PRRT in PanNET have shown encouraging results.

Pathogenesis/molecular mechanisms

Our knowledge on the genetic basis of PanNET initiation and progression is limited. Useful markers to predict response are still unavailable. Increasing efforts are being done to identify genetic changes underlying the development of PanNETs. Jiao and colleagues performed

whole-exome sequencing of primary PanNETs and demonstrated that 45% of PanNETs had mutations in the multiple endocrine neoplasia type 1 (*MEN1*) gene (267, 268) and 28% and 15% had somatic mutations in *DAXX* and *ATRX*, respectively (269). *DAXX* and *ATRX* are two genes involved in chromatin remodeling that had previously not been associated with cancer (270). Surprisingly, mutations in *MEN1*, *DAXX* and *ATRX* were associated with a better prognosis. Moreover, 16% of the tumors had mutations in genes encoding proteins for the PI3K/AKT/mTOR pathway, already implicated in NET growth. The newly discovered mutations may offer the potential for the identification of new biomarkers and alternative therapeutic advances in PanNETs.

As highlighted in this thesis, the dynamic cellular interactions in the tumor microenvironment, and how they contribute to ITH and tumor progression, remain poorly understood. However, mounting data suggests that combining drugs affecting more than one cellular target or pathway might improve control of tumor growth and prevent development of resistance. This notion has also motivated new studies to assess the effect of combining targeted therapies for the management of human PanNETs (271-274).

The RIP1-TAg2 mouse model

One of the best-known and characterized mouse models for human PanNET, specifically insulinomas, is the RIP1-TAg2 (275). Since its development in 1985, the RIP1-TAg2 model has been used in several studies addressing questions related to tumorigenesis in general, i.e. angiogenesis, proliferation and apoptosis, and progression of clinical PanNET in particular. The RIP1-TAg2 mouse was engineered to express the large and small SV40 viral T antigens (TAg) under the control of the rat insulin promoter (RIP). TAg inhibits the function of two known tumor suppressor genes, p53 and retinoblastoma (Rb). Thus, insulin producing β-cells in pancreatic islets undergo a cascade of neoplastic transformation leading to a time-controlled and stepwise development of multifocal tumors that ultimately become invasive (Figure 4). Islets in RIP1-TAg2 mice in the C57Bl/6 background develop into fully-grown tumors at 8-10 weeks of age and tumor cells further spread and develop multifocal hepatic metastatic lesions.

The RIP1-TAg2 model has been considerably instructive and informative due to important features of the tumors, e.g. as in human PanNET, tumors in RIP1-TAg2 mice are highly vascularized and secrete several pro-angiogenic factors. The functional importance of VEGF-A for angiogenesis and β-cell tumorigenesis in the RIP1-TAg2 mouse has been convincingly demonstrated (276). This has motivated several preclinical studies targeting the tumor vasculature with agents like bevacizumab, sunitinib and everolimus. Indeed, treatment of RIP1-TAg2 mice with sunitinib or everolimus led to a significant improvement in OS (47, 262). The effectiveness of these drugs was reflected in the phase III clinical trials previously described, resulting in increased PFS in clinical PanNET (263, 264).

Similarly to other tumor models, prolonged treatment of RIP1-TAg2 mice with anti-angiogenenic drugs lead to the development of tumor resistance and increased metastatic dissemination (46, 47, 277). As such, the RIP1-TAg2 model has instructed preclinical tumor biologists about different mechanisms of acquired resistance and improved the understanding of alternative pathways a tumor can find to overcome targeted inhibition. These observations might help explain the limited improvement in PFS and OS achieved in the clinic following targeted therapies.

The complexity and heterogeneity of PanNET was also revealed in tumors of RIP1-TAg2 mice in comparative studies using microRNA and mRNA transcriptome analyses. In a recent study, Sadanandam and colleagues elegantly demonstrated the existence of three distinct molecular subtypes in human PanNET, i.e. well-differentiated islet/insulinoma tumors (IT), metastasis-like primary (MLP, poorly differentiated and associated with metastases) and Men1-like (Men-1 mutation-enriched) with associated expression of biomarkers (278). Interestingly, tumors in RIP1-TAg2 mice closely recapitulate two of these subtypes (IT and MLP) including the expression of common biomarkers that might help guiding patient stratification and therapeutic decisions in the future.

However, the RIP1-TAg2 model also has a major limitation. Due to excessive secretion of insulin by β-cells in tumor islets, RIP1-TAg2 mice normally succumb from symptoms associated with hypoglycemia instead of compromised liver function due to the metastatic burden. Nonetheless, the multistage progress of the disease still allows the exploration of mechanisms underlying tumor cell intra- and extravasation from the blood vessel, homing and establishment into distant organs such as the liver, lung and spleen (279). Additionally, tumor initiation in the RIP1-TAg2 model is driven by inhibition of p53 and Rb. Although p53 and Rb can also be found dysregulated in certain subsets of human PanNET (280, 281), the exact molecular mechanisms underlying tumor initiation in patients are still poorly understood.

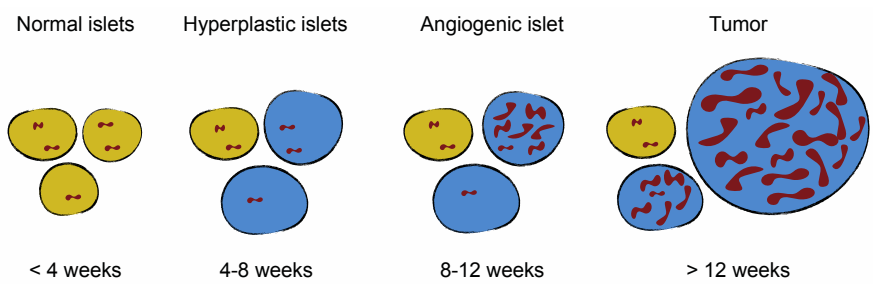

Figure 4.
Schematic representation of tumorigenesis in the RIP1-TAg2 mouse. Upon activation of T Antigen, pancreatic β-cells begin to proliferate (blue) and islets of Langerhans become hyperplastic. After 8 weeks, increased expression of pro-angiogenic factors activate the angiogenic switch leading to the development of a robust vasculature (red) that supports islets to grow continuously and become invasive tumors. Adapted from Hanahan & Folkman, *Cell* 1996.

Specific Aims

Paper I – To characterize the molecular mechanisms of pericyte-induced EC survival in tumors.

Paper II – To explore the utility of using pericyte markers as predictive of response to anti-vascular therapy.

Paper III – To generate an Affibody molecule with high specificity for human and mouse PDGFRβ, with imaging and therapeutic utility.

Paper IV – To investigate the role of PDGF-DD signaling in tumor development and progression.

Results and discussion

Paper I

Pericytes promote endothelial cell survival through induction of autocrine VEGF-A signaling and Bcl-w expression

The functional importance of pericyte coverage in tumor blood vessels remains unclear. Studies in mice have suggested that pericytes protect ECs from anti-vascular insults promoting survival and tumor revascularization. However, the molecular mechanisms involved in the crosstalk between ECs and pericytes in tumors are not well understood.

Key findings:

To disturb the pericyte-EC association, RIP1-TAg2 mice were treated during one week with two PDGFRβ signaling inhibitors, imatinib or CP-673,451. Tumors from mice treated with imatinib showed significant decrease of pericyte vessel attachment while CP-673,451 treatment led to a significant decrease of pericyte markers NG2, PDGFRβ, desmin and α-SMA. Gene expression profiling of isolated tumor ECs from treated mice revealed a significant decrease in the expression of Bcl-w, an anti-apoptotic gene, compared to untreated mice. Conversely, overexpression of PDGFB in transplanted B16 melanoma tumor cells induced expression of Bcl-w in ECs. Using a newly established pericyte-EC co-culture system, we were able to demonstrate that the presence of pericytes mediated the upregulation of Bcl-w in ECs. Moreover, we showed that pericytes protected ECs from the effect of a cytotoxic agent, seen by reduced apoptosis in ECs in close contact with pericytes. Further analysis demonstrated that Bcl-w expression was regulated by increased autocrine signaling by VEGF-A in ECs, in vitro and in vivo, modulated by the extent of pericyte coverage. Transcription of Vegfa has been shown to be regulated by NF-κb. In our study, we demonstrated that NF-κB target genes were either downregulated in pericyte-poor tumors or upregulated in pericyte-rich tumors. More mechanistic analyses revealed that blocking the activity of NF-κB significantly decreased Vegfa and subsequently Bcl-w expression in ECs in vitro. Finally, we showed that NF-κb activation in ECs was dependent on binding of integrin αv expressed by ECs to vitronectin, a component of the ECM, produced and deposited by pericytes in response to EC-secreted PDGF-BB. Altogether, we proposed that pericytes protect ECs from apoptosis by inducing Bcl-w expression in ECs in an autocrine VEGF-A dependent way.

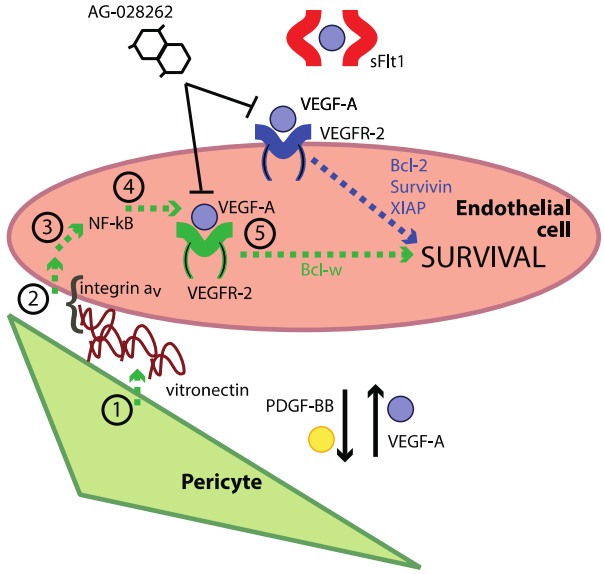

Figure 5.
A model of pericyte-induced EC survival signaling. Endothelial cells and pericytes communicate through the mutual exchange of growth factors. PDGF-BB produced by ECs recruit pericytes along angiogenic sprouts. In turn, pericytes secrete vitronectin, (1) which through an integrin α_v (2) and NF-κB (3) mediated signaling pathway induces up-regulation of intracrine VEGF-A (4) and Bcl-w expression. (5) Previously described survival factors induced by the paracrine action of pericyte- or tumor cell-derived VEGF-A, such as survivin, Bcl-2, and XIAP, are presumably sensitive to the action of VEGFR inhibitors acting both intra- and extra-cellularly (AG-028262 and sFlt1, respectively), whereas the pericyte-induced autocrine signaling by VEGF-A is only sensitive to intra-cellularly acting inhibitors. From Franco *et al. Blood*. 2011

Paper II

Use of a mouse model of pancreatic neuroendocrine tumors to find pericyte biomarkers of resistance to anti-angiogenic therapy

The development of resistance to angiogenesis therapy poses a major challenge in the clinic. Several mechanisms have been suggested to contribute to the capacity of tumors to evade therapeutic insults, e.g. upregulation of alternative pro-angiogenic factors, increased pericyte coverage, increased invasiveness and co-option of normal vessels. Increased pericyte coverage has been reported in the microvasculature of tumors refractory to therapy (46, 55). Distinct functional properties have been attributed to pericytes mainly based on marker expression, including maintenance of tumor vasculature by promoting EC proliferation and survival, regulation of immune cell infiltration and metastatic dissemination. In this study, we characterized pericyte coverage found in tumors following prolonged exposure to an anti-VEGFR2 drug.

Key findings:

RIP1-TAg2 mice were previously treated with DC101, a monoclonal antibody for VEGFR2 (46). Tumor shrinkage and substantial vessel regression were observed following one week of treatment. However, prolonged treatment for 4 weeks led to the reestablishment of the vasculature, associated with a more aggressive and metastatic tumor phenotype (46). Immunostaining of sections from tumors refractory to therapy with different pericyte markers revealed that, while perivascular PDGFRβ and NG2 expression were unchanged in abundance, there was a substantial increase in α-SMA+ pericytes in refractory tumors (4 weeks DC101) compared to responding tumors (1 week DC101) or untreated tumors.

Paper III

Engineered high-affinity affibody molecules targeting platelet-derived growth factor receptor β in vivo

High expression of stromal PDGFRβ has been associated with disease prognosis in different cancers. The potential of targeting stromal cells and angiogenesis by interfering with PDGFRβ signaling has been explored with the use of several RTKI in preclinical models and clinical trials with positive but limited outcomes. Indeed, the contribution of RTKI in substantially improving PFS and OS in the majority of patients has been bleak. Importantly, the specificity of RTKI to their targets is relatively low and off-target effects might explain the limited afforded benefits. Improved specificity can be achieved with the use of monoclonal antibodies directed against PDGFRβ. However, due to their still relative large size, complex composition and limited distribution in the stroma, there has been a demand to develop smaller and more robust molecules that can more efficiently bind and neutralize PDGFRβ with high specificity.

Key findings

Affibodies are small (≤ 6.5 Kd), non-immunoglobulin molecules that bind defined targets with great affinity. In paper III we describe the generation of a PDGFRβ-specific Affibody with the purpose to better image and potentially target PDGFRβ *in vivo*. Affibody molecules were generated from a large library using phage display selection. Sequences from binders having a desired level of selectivity and competing for binding with a natural ligand for PDGFRβ, PDGF-BB, were used to design an affinity library. From this second library, Affibody molecules with a 10-fold improvement in affinity (Kd=0.4–0.5 nM) for human PDGFRβ and a 4-fold improvement in affinity (Kd=6–7 nM) for mouse PDGFRβ were isolated and characterized. The selected molecules were highly specific for human and murine PDGFRβ, but not for human PDGFRα, and recognized the native conformation of PDGFRβ expressed in mouse and human cells. Moreover, PDGFRβ-specific Affibody

was capable of inhibiting PDGF-BB-induced receptor phosphorylation in PDGFRβ-transfected porcine aortic ECs.

Using the RIP1-TAg2 model, we evaluated the functional biological properties of PDGFRβ-specific Affibody molecules. PDGFRβ-Affibody was injected intravenously into RIP1-TAg2 mice and allowed to circulate for 4 or 60 minutes after which tissues were collected and analyzed. We showed that PDGFRβ-Affibody accumulated around vessels in islet tumors seen by a strong signal and co-localization with a PDGFRβ antibody. A weaker signal was observed around normal vessels after 4 minutes compared to tumor tissue. However, after 1 hour, most of the PDGFRβ-Affibody molecules were cleared from the normal tissues but retained in tumor vessels. No signal was observed using the control Affibody, suggesting high specificity of the PDGFRβ-Affibody.

Discussion papers I-III

Pericytes are multifaceted and complex cells. Our understanding of their role in tumor development is still very immature, and conflicting results arise from analyses of different tumor tissues and stages of disease development. The RIP1-TAg2 model has been used extensively to study mechanisms of tumor angiogenesis ranging from tumor initiation and growth to metastatic dissemination. Experimental evidence has shown that targeting pericytes in the tumor vasculature of RIP1-TAg2 mice increases the effect of therapies aimed at ECs (47, 53, 55). In papers I, II and III we attempted to further characterize tumor pericytes using the RIP1-TAg2 model by exploring the signaling pathways involved in EC-pericyte communication (paper I), the utility of using pericyte markers to predict response to therapy (paper II) and the possibility to develop a tool to improve pericyte visualization and targeting in the tumor parenchyma (paper III).

How exactly pericytes modulate EC survival and protect the tumor vasculature upon anti-angiogenic therapy has been the focus of numerous studies. Trying to understand the molecular mechanism involved in this intricate crosstalk, we used two instructive tumor mouse models where pericyte coverage and function were affected by modulation of the PDGF-BB/PDGFRβ signaling pathway. Pericytes, upon being engaged by EC-secreted PDGF-BB, respond by secreting growth factors such as VEGF-A, further promoting EC proliferation (214). However, the belief is that this reciprocal signaling is more complex. Indeed, we found that autocrine signaling by VEGF-A in ECs was amplified in the presence of pericytes. Expression of *Vegfa* by ECs was previously shown to be regulated by α_3b_1 integrin, a component of the ECM (282). The hypothesis that the ECM composition plays a role in EC function has been previously discussed (77). In line with this idea, recruitment of pericytes by newly formed vessels has been associated with an increase in deposition of BM factors (190). Inhibition of integrin α_v reduced pericyte-induced *Vegfa* expression by ECs and their subsequent survival. One of the BM factors found to be highly secreted by

pericytes was vitronectin. Previously it was shown that binding of integrin $\alpha_v b_3$ expressed by ECs to vitronectin reduced apotosis in a NF-κB dependent manner (283, 284). Accordingly, decreased pericyte coverage following imatinib was found to decrease the expression of downstream effectors of NF-κB. We proposed a model by which, upon recruitment to the tumor vessels, pericytes deposit vitronectin that binds to integrin $\alpha_v b_3$ expressed by ECs inducing NF-κB signaling and subsequent *Vegfa* and *Bcl-w* upregulation in ECs (Figure 5).

Studies have shown synergistic effects of drugs targeting the EC and pericyte compartments simultaneously (51, 53, 55). Despite the lack of a vascular phenotype in *Bcl-w* null mice, it remains to be demonstrated *in vivo* that decreased Bcl-w signaling upon pericyte perturbation in the vascular wall renders ECs more sensitive to anti-angiogenic therapy.

It is important to consider that the cellular source of PDGF-BB might have an impact on pericyte recruitment. While PDGF-BB seems to be mostly secreted by ECs, tumor cells also produce the ligand. When overexpressing PDGF-BB in tumor cells lines, one should consider that the effect on pericyte recruitment and function might differ from when the ligand originates from ECs. In our study, increased expression of markers associated with pericytes was found in B16 melanomas upon forced expression of PDGF-BB, which was further correlated to an increase in *Vegfa* and *Bcl-w* expression by ECs. Nevertheless, tumor profiling for PDGF and VEGF expression should precede the implementation of different targeted therapies. Supporting these thoughts, Hosaka and colleagues showed that levels of PDGF-BB vary among tumors and therefore suggested that, pericyte recruitment, coverage and tumor response to anti-PDGFRβ drugs, might be dependent on the levels of available PDGF-BB (285).

It should be noted that genetic and pharmacological disruption of pericyte recruitment might also have distinct effects. Most RTKI affecting PDGFRβ signaling are promiscuous and bind other RTKs possibly enabling off-target effects that might preclude our interpretation of the results.

The identification of autocrine VEGF-A signaling in ECs fuels the speculation that small molecule RTKI like sunitinib and sorafenib, acting also intracellularly, might have a superior effect compared to monoclonal anti-VEGF-A antibodies affecting only extracellular VEGF-A. Supporting this idea, CRC xenografs with intrinsic resistance to bevacizumab showed significant upregulation of autocrine VEGF-A upon treatment. However, resistant tumors were more sensitive to nintedanib, a small molecule RTKI. This study also proposes a role for autocrine VEGF-A signaling in cell survival under low oxygen conditions (286). Nonetheless, the increased effectiveness of RTKI compared to antibodies is not consensual as e.g. both types of compounds are used to treat RCC patients (287).

In summary, in paper I we provided molecular detail on the protective role of pericytes to tumor ECs. The possibility of supplementing anti-angiogenic therapies by targeting Bcl-w signaling or other anti-apoptotic mediators in ECs should be explored.

While the PDGF-BB/PDGFRβ axis is vital for recruitment of tumor pericytes to the vasculature, the function of different markers expressed by pericytes have not been fully explored. From our briefly discussed analysis on subpopulations of pericytes, we suggested that pericytes found in the tumor parenchyma are enrolled in distinct but sometimes overlapping functions (244). Anti-vascular therapies in the clinic appear to benefit only a few patients. Thus, understanding escape and alternative mechanisms used by tumors resistant to therapy is of great need. The implication of pericytes in development of resistance has been made upon observation of increased pericyte coverage following anti-VEGF therapies (60). Augmented α-SMA+ pericyte coverage has been documented in human CRC following bevacizumab treatment (288). The utility of using pericyte markers as predictive of response to therapy has been explored in previously discussed studies. In paper II, we analyzed pericyte coverage following the re-growth of tumors upon prolonged anti-VEGFR2 therapy using the RIP1-TAg2 mouse model. Refractory tumors appeared more locally invasive and also showed increased metastatic dissemination. Our aim was to attempt to identify pericyte biomarkers of resistance to therapy by comparing pericyte marker expression between responding and refractory tumors. Vessels from refractory tumors had a significant increase in coverage by α-SMA expressing pericytes. In tumors from untreated RIP1-TAg2 mice, α-SMA was mainly found in bigger vessels in the exocrine pancreas and surrounding islet tumors, and to a lesser extent in microvessels in the tumor parenchyma. In resistant tumors from treated RIP1-TAg2 mice, α-SMA+ pericytes concomitantly expressed NG2 and PDGFRβ and were found surrounding larger vessels similar to those found primarily in the exocrine tissue. As such, we speculated that increased vascularization of tumors was likely achieved by recruitment of pericytes from vessels in the proximity of tumor islets. Vessel co-option, characterized by migration of tumor cells along preexisting vessels of host tissue, has been associated with development of tumor resistance to anti-VEGF drugs (289). Interestingly, others have also documented increased α-SMA expression in perivascular cells upon anti-vascular therapy in melanoma (290) and recurrent GBM (241). Whether α-SMA+ pericytes have an additional functional role that renders ECs more resistant to therapy should be the scope of further investigations. However, given the above-mentioned role of pericytes in providing survival cues to ECs, it is tempting to speculate that α-SMA+ pericytes further protect ECs from ongoing anti-VEGFR insults allowing tumors to become resistant and more invasive. An alternative to vascular co-option would be that increased α-SMA expression could result from differentiation of immature PDGFRβ+ pericytes (181).

Studies are needed to further confirm the predictive value of pericyte marker expression in tumor specimens from patients undergoing anti-VEGF therapy from which response has been documented in terms of tumor growth, metastatic dissemination, PFS and OS.

The dynamics of pericyte marker expression during the course of therapy strongly motivates the need to monitor and sample tumors not only before but also during and after therapy. Moreover, the low response rate observed in PanNET patients (9.3%) following sunitinib

treatment, emphasizes the heterogeneous manifestation of the disease and the need to identify patients that are most likely to benefit from anti-angiogenic therapy.

Advancing knowledge on tumor pericytes requires investigation into how and when pericyte behavior changes during the transition from physiological to pathological angiogenic states. One prospective method would involve a comprehensive characterization of pericytes isolated from normal tissue and the matching tissue during different stages of tumorigenesis. In recent years, a few reporter mice have been generated, expressing fluorescent proteins under the control of promoters for known pericyte genes, e.g. NG2DsRedBAC (291) and Tg(Pdgfrb-EGFP) (292). These mice are valuable tools, allowing for more efficient purification of pericytes from different experimental tumor models and they offer the possibility of identifying novel and more specific markers for pericytes during tumor progression.

The lack of tumor pericyte cell lines has also represented a major limitation in our analyses. Based on the prevalent idea that pericytes have organ-specific functions, results obtained from *in vitro* studies using the few available pericyte lines might not often be representative of the *in vivo* situations. The high plasticity exhibited by pericytes when in culture has been associated with the technical difficulty in generating stable cell lines.

The utility of targeting pericytes or use them as a biomarker for therapy is gaining more relevance. As mentioned in this thesis, strategies to visualize and target pericytes have mostly focused on targeting PDGFRβ, one of the most studied pericyte markers. It is thus important to develop tools to properly image pericytes during tumor progression. Antibodies for PDGFRβ have been widely used for both identification and blocking of receptor activity. However, despite the capacity of antibodies to be highly specific to the desired target, there are limitations regarding their molecular properties. Antibodies have a relative large size, slow distribution, poor heat stability and additional binding sites in their multidomains, producing unspecific signals. Given this, in the last 15 years, alternative affinity proteins have been explored in order to increase thermal stability, folding properties, reduce size, improve the biodistribution and additionally simplify production methods. Affibody molecules are only 6.5 kDa in size compared to the approximately 150 kDa of antibodies (234). Affibodies have no Fc-binding motifs thus preventing unspecific binding to Fc receptors expressed in many cells of the immune compartment and some epithelial cells. Accordingly, Affibodies bind their targets with high affinity and specificity. Encouraging results have been achieved using Affibody as a diagnostic tool in patients with HER2-expressing metastasis (293) and as a radio-therapeutic tool in HER2 microxenografts (294). The aim of paper III was to develop an Affibody molecule capable of binding to human PDGFRβ with high affinity. The PDGFRβ-Affibody showed high specificity for PDGFRβ as no binding to human PDGFRα was detected. Similarly, it did not bind 16 abundant proteins found in the blood plasma, emphasizing its potential as an imaging tool. The capacity of PDGFRβ-Affibody to bind mouse PDGFRβ was also tested, as this allows experimental validation of the molecule in preclinical settings. Our major contribution to

the study was to evaluate the biological functionality of PDGFRβ-Affibody for molecular imaging and as an inhibitor of PDGFRβ activation. The specific retention of PDGFRβ-Affibody around tumor vessels in the RIP1-TA2g mouse model, compared to vessels in normal tissues such as the exocrine pancreas and the liver, is possibly due to an increased fenestration and permeability of tumor vessels, which facilitates access to PDGFRβ expressing cells. Moreover, it is possible that PDGFRβ expression is augmented in tumors where active angiogenesis and pericyte recruitment are ongoing. The absence of signal in normal tissues suggests that there is a proper clearance of unbound protein, a required property of imaging agents. Nevertheless, substantial accumulation was observed in the kidney tubuli already after 4 minutes. Whether there was an unspecific uptake of PDGFRβ-Affibody should still be investigated, as the molecules did not seem to bind PDGFRβ expressed in the glomeruli (unpublished observations). To improve molecular imaging using the PDGFRβ-Affibody, high hepatic and renal uptake should be avoided to allow proper uptake in tumors and avoid extremely rapid clearance.

Besides its potential as an imaging agent, the ultimate goal for the PDGFRβ-Affibody would be use it as a targeted agent to deliver drugs to specific tumor areas. It is still unanswered whether eradication of pericytes rather than inhibition of their function would afford an increased benefit to angiogenic therapies. Coupling Affibody molecules with cytotoxic compounds could circumvent the off-target effects of chemotherapy on normal cells, and deliver drugs to pericytes directly or to a restricted perivascular area. Such compounds could be radionuclides, small molecule toxins or fusion of toxic small protein domains. Coupling the Affibody with a small albumin binding domain (ADB) allows binding to serum albumin and has been shown to extend its circulatory half-life and promote a faster distribution (295). Importantly, we were able to demonstrate that the PDGFRβ-Affibody molecule is able to compete with a natural ligand for PDGFRβ, the PDGF-BB, *in vitro*. PDGFRβ activation, as measured by phosphorylation of the receptor upon stimulation with PDGF-BB, was abrogated after addition of the PDGFRβ-Affibody. However, the possibility of using Affibody as a neutralizing agent for PDGFRβ signaling in tumors *in vivo* should be properly investigated. Altogether, results presented in paper III show successful isolation of an Affibody molecule with high affinity and selectivity for PDGFRβ in human and mouse. Given the small size and potential better access to pericytes compared to normal antibodies, further validation should be performed to test the possibility of using PDGFRβ-Affibody molecules as both imaging and therapeutic tools for pericytes in the tumor stroma.

The three aspects we focused on in the last studies, bring new insights into pericyte biology but also stress the need to a) better understand the communication between pericytes and the other components of the tumor stroma, b) explore the therapeutic relevance of pericyte marker expression in the clinic and c) develop more powerful tools to identify and target pericytes in tumors to improve anti-angiogenic therapies.

Paper IV

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

Key findings

In this study, making use of a previously uncharacterized *Pdgfd* knockout mouse (147), we analyzed the biological effects of impairing PDGF-DD signaling in PanNET development using the RIP1-TAg2 mouse model. We found that in PanNETs, *Pdgfd* was mainly expressed in the EC compartment with a significant upregulation in the angiogenic phase of the multi-step tumor progression process. Early tumorigenesis is unaffected in the *Pdgfd*$^{-/-}$ background as seen by a comparable number of angiogenic and tumor islets with RIP1-TAg2;*Pdgfd*$^{+/+}$ mice. In later stages, tumors from RIP1-TAg2;*Pdgfd*$^{-/-}$ mice grew considerable slower resulting in a prolonged survival of the mice. The effect of *Pdgfd* depletion had no impact on the rate of apoptosis in tumors; however, the proliferative capacity of tumor cells was significantly reduced. Surprisingly, pericyte recruitment and angiogenesis were unaffected by impaired PDGF-DD signaling. PDGF-BB, the other natural ligand for PDGFRβ, which is involved in pericyte recruitment, was significantly upregulated in tumors with impaired PDGF-DD signaling. Attempting to find other cellular targets for PDGF-DD, we identified a rare subclone of malignant cells expressing PDGFRβ in primary tumors and in liver metastases from RIP1-TAg2 mice. *In vitro* analysis demonstrated that PDGFRβ+ tumor cells upregulate the expression of mitogenic factors for β-tumor cells in response to PDGF-DD, thereby potentiating the growth of the bulk population of tumor cells. Further analysis suggested that expression of PDGFRβ results from interconversion between PDGFRβ+ and PDGFRβ- populations seen by the *de novo* generation of PDGFRβ+ malignant cells in tumors originated from pure PDGFRβ- sorted tumor cells. Finally we were able to identify rare PDGFRβ+ tumor clones in human PanNETs specimens with matched metastatic lesions. Our data establishes a previously unrecognized role for PDGF-DD in tumor growth and further evidences the heterogeneous composition of the tumor cell compartment in PanNETs.

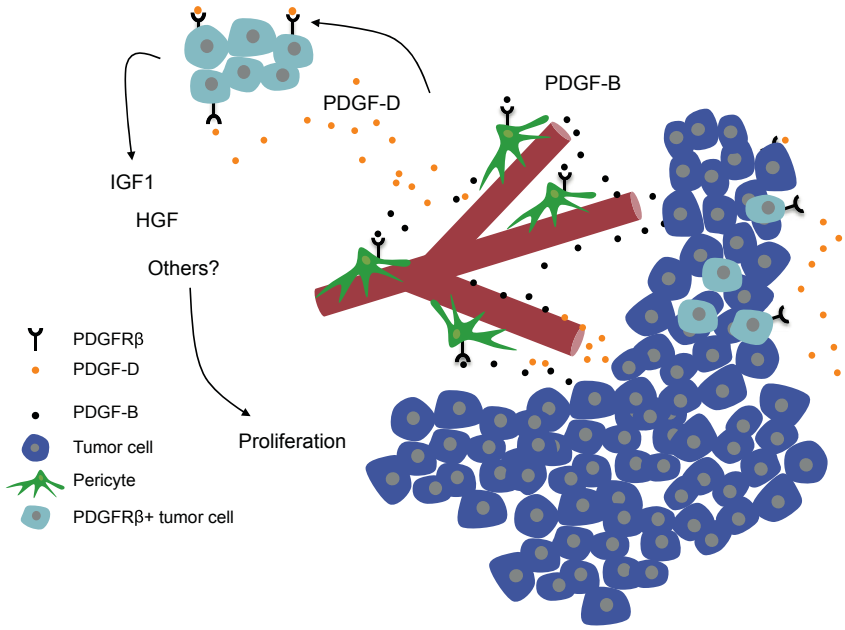

Figure 6.
Proposed model for PDGF-DD function during experimental PanNET tumorigenesis. PDGF-BB secreted by ECs binds to components of the ECM creating a gradient that attracts pericytes to tumor blood vessels. PDGF-DD secreted by ECs might have a distinct distribution in the tumor parenchyma and thus, be capable of binding and activating PDGFRβ expressed in rare malignant clones, inducing expression of mitogenic factors for pancreatic β-tumor cells.

Discussion paper IV

PDGF overactivity has been recurrently documented in human tumors and it is believed to drive tumor progression. Increased expression or mutation of *PDGF* genes have also been reported in several tumor types (126, 127). Signaling by PDGF-BB through PDGFRβ is a key event in the recruitment of pericytes to the tumor microvasculature. In turn, poor pericyte investment renders vessels more susceptible to different angiogenic therapies. Our analysis of PDGF-DD expression in tumors from RIP1-TAg2 suggests that the ligand is predominantly secreted by ECs, as seen by the substantial increase during the angiogenic phase. In accordance with our results, in a gene expression dataset composed of samples isolated from different stages of tumor development in RIP1-TAg2 mice, *Pdgfd* expression appeared to be increased during the angiogenic phase (Jonas Sjölund, personal communication). Despite a few studies showing that inhibition of PDGF-DD impacts pericyte recruitment (150, 151), we did not register any significant disturbance of the microvascular unit of the tumors, as both vessel density and pericyte coverage resembled the

normal vasculature from islet tumors in the RIP1-TAg2 mice. In rare occasions, we observed a weaker association of pericytes with ECs. However, pericyte detachment did not affect vascular functionality. Upregulation of *Pdgfb* in *Pdgfd*-deficient tumors suggests that PDGF-BB can partially compensate for PDGF-DD in the tumor stroma. As mentioned before, null mice for *Pdgfb* show embryonic lethality, demonstrating that PDGF-DD, in contrast, is not able to compensate for the lack of PDGF-BB. These observations suggest that PDGF-DD has a specific and non-overlapping function with PDGF-BB. In tumors, PDGFRβ is mainly expressed by mesenchymal cells; however, studies in human PanNETs and CRC, reported the presence of malignant cells expressing PDGFRβ as well (136, 296). Similarly, in young murine pancreatic islets, expression of PDGFRα and PDGFRβ has been documented and was associated with an increased proliferative capacity of pancreatic β-cells (297). Using FACS analysis and immunohistochemistry, we identified minor populations of tumor cells expressing PDGFRβ in RIP1-TAg2 mice. Surprisingly, PDGFRβ+ clones were enriched in hepatic metastatic lesions. Even though the metastatic burden in RIP1-TAg2 appeared unchanged upon depletion of *Pdgfd*, the increased frequency of PDGFRβ+ tumor clones suggests that PDGFRβ signaling might be important in the metastatic niche. As such, it is necessary to understand if PDGFRβ+ clones display a mesenchymal phenotype, whether they are the ones escaping the primary tumor and invading distant organs or, alternatively, if PDGFRβ is upregulated *de novo* in the metastatic niche to support e.g. cell growth. Indeed, treatment of a pancreatic β-tumor cell line (βTC) isolated from RIP1-TAg2 mice, with PDGF-DD promoted α-SMA expression, a known mesenchymal marker (unpublished observations). In accordance with these speculations, overexpression of PDGFRβ in experimental prostate cancer was shown to promote EMT (154). Moreover, PDGFRβ expression in CRC cells was recently shown to be associated with EMT and an increased invasive capacity of these cells (136). In many human tumors, however, detection of EMT can be difficult due to its transient nature. Interestingly, using a mouse model of small cell lung cancer, Calbo and colleagues demonstrated the predominance of a rare subclone of malignant cells in liver metastatic lesions compared to the primary tumors where it was outnumbered by a dominant subclone (298). This study also emphasizes the proposition that the molecular composition of metastases can be distinct compared to the primary tumors. The relevance of PDGFRβ expression in tumor cells in our experimental model, in human PanNET and other cancers should be further addressed in order to understand its functional properties.

PDGF-DD but not PDGF-BB, promoted the formation of tumor spheres, a property commonly associated with stem cell traits. We further investigated the presumed stem cell phenotype of PDGFRβ+ tumor clones but given our results, it was inconclusive whether PDGFRβ+ malignant clones also display CSC abilities.

Equally unexpectedly, PDGF-DD, and not PDGF-BB, induced proliferation of tumor cells *in vitro*. This result further suggests that PDGF-DD has a distinct function in the tumor stroma compared to PDGF-BB. As discussed earlier (section "PDGF system – Ligands"), this might be explained by a distinct tissue distribution between the two ligands conferred

by the presence of the retention motif in PDGF-BB. An additional, but still unexplored possibility, is that the unique functions of PDGF-DD might also result from its binding to a distinct (co)-receptor in tumor cells. We further asked whether the increase in cell proliferation upon PDGF-DD stimulation was induced directly in PDGFRβ+ clones or indirectly in the PDGFRβ- bulk population. Surprisingly, we found that most proliferating tumor cells were PDGFRβ- and they were also found in the vicinity of PDGFRβ+ tumor cells. A growing literature reports on cooperation mechanisms between different subclones of cells using experimental tumor models. One suggestion of positive interaction is the secretion of growth factors by one subclone that further promotes the growth of the other subclone(s) (299). We hypothesized that PDGFRβ+ clones promoted growth of PDGFRβ- tumor cells in a paracrine fashion. Profiling of growth factors known to stimulate proliferation of β-cells in pancreas (300-303) revealed that PDGF-DD significantly induced expression of insulin growth factor 1 *(Igf1)* and hepatocyte growth factor *(Hgf)*. Whether PDGFRβ- tumor cells can also directly trigger growth factor secretion by PDGFRβ+ clones either by secreting additional PDGF-DD or by producing the proteases involved in its activation, thus regulating its bioavailability and activity, needs to be investigated. We proposed a model in which PDGF-DD secreted by ECs binds and activates PDGFRβ expressed in rare tumor cells inducing the production of growth factors that stimulate proliferation of the bulk tumor cell population (Figure 6). Clones expressing PDGFRβ appeared to be stably maintained as they were found in similar proportions in pancreatic βTCs and primary tumors from RIP1-TAg2 mice throughout time. Trying to understand the phenomena of stable co-existence of cellular subclones within the same tumor, a report in experimental breast cancer suggested that distinct isolated subclones of tumor cells, with time, tend to interconvert between states and converge to the original heterogeneous frequency (304). Moreover, Archetti and colleagues created an artificial heterogeneity model system by mixing IGF2-producer and non-producer pancreatic βTCs from RIP1-TAg2 mice. IGF2 secreted by producer clones sustained the growth of non-producer cells and over time the two subpopulations could stably co-exist (305). In agreement with this, we showed that PDGFRβ+ and PDGFRβ- isolated clones from primary tumors and cells lines could give rise to the original frequencies of both subpopulations *in vivo* and *in vitro* and thus recapitulate the heterogeneity of the original tumors. However, the fundamental question of how expression of PDGFRβ in tumor cells is regulated and maintained is still unanswered.

To confirm the clinical relevance of our findings, we analyzed a cohort of matched primary and metastatic human PanNETs. PDGFRβ expression was mainly detected in perivascular cells, as expected, but also in small isolated groups of malignant cells in the tumor parenchyma and in hepatic metastases.

The VEGFR and PDGFR RTKI sunitinib is approved for treatment of advanced PanNET (263). The effect of sunitinib on tumor angiogenesis is also thought to be achieved through the inhibition of PDGFRβ expressed by e.g. perivascular cells. In the light of our findings, it is tempting to speculate that the effect of sunitinib could be partially attained by inhibiting PDGFRβ signaling in tumor cells. Evidently this warrants further detailed investigation.

Previously, stromal PDGFRβ expression (mainly in fibroblasts and pericytes) has been associated with a worse prognosis in breast cancer patients (306, 307). Similarly, high expression of PDGFRβ was prognostic of increased risk of recurrence following surgery in clear cell RCC (308). In pediatric gliomas, high PDGFRβ expression is also associated with malignant histology (309). As mentioned earlier, a few reports document PDGFRβ expression in tumor cells (135, 136, 296). Our results suggest that the prognostic or predictive value of PDGFRβ expression in malignant cells should therefore also be explored.

PDGF-DD expression in human PanNETs has not yet been documented. Preliminary analysis of a data set of human PanNET showed that *PDGFD* is significantly upregulated in insulinomas compared to normal islets in the pancreas (Jonas Sjölund, personal communication). However, the survival data is unavailable for this data set and thus, the prognostic relevance of *PDGFD* overexpression in PanNET cannot be assessed. Liu and colleagues showed that *PDGFD* is upregulated in breast tumors compared to normal tissue (150). However, the scarcity of sensitive and specific imaging tools to detect and measure PDGF-DD in human and mouse tissue has made these characterizations rather inconclusive. With the increased interest in identifying driver mutations and understanding early molecular events during PanNET tumorigenesis, efforts to assemble larger cohorts of tumor specimens for genome sequencing are ongoing.

Conclusion and future perspectives

The complexity of the tumor microenvironment has been the focus of numerous studies in the last decades. The unceasing identification of essential signaling pathways and cell-cell interactions involving tumor cells and the supporting stromal cells, has added to our still scarce understanding of the intricate routes a tumor resorts to in order to survive, grow and spread. As a result, every year new targeted therapies are developed and implemented in the clinic in an attempt to deplete tumors of resources and circumvent disease resistance and progression.

Our studies have focused on two compartments of the tumor microenvironment: the malignant tumor cells and the pericytes. Early anti-angiogenic therapy focused mainly on ECs, such as a blockade of VEGFR/VEGF signaling. However, the field has rapidly expanded from only targeting ECs to also targeting their supporting cells. Several aspects about the biology of tumor pericytes remain unsolved. On the one hand, ECs appear to rely on pericytes for survival and function, making pericytes an attractive target to supplement angiogenic therapies. On the other hand, targeting pericytes appears to aid tumor intravasation through an unstable and hyperpermeable vasculature facilitating metastatic dissemination and disease progression. Clearly, there is a need for a better molecular characterization of signaling pathways involved in EC-pericyte-tumor cell communication to further identify novel pericyte markers in tumors and exploit pericytes as therapeutic targets, and to explore the possibility of using pericyte marker expression to estimate the likelihood of disease progression. New model systems and imaging approaches are necessary in order to advance our understanding of pericyte dynamics, functionality, and phenotypic flexibility. Moreover, we provided novel insight into the signaling between PDGF-DD and PDGFRβ in tumorigenesis, through a rare subpopulation of malignant cells expressing PDGFRβ in PanNETs. The potential use PDGF-DD/PDGFRβ signaling pathway as a therapeutic target needs to be determined. Moreover, ITH in PanNETs needs to be explored as a possible cause of the still limited effects provided by targeted therapy.

One of the proposed approaches to overcome ITH is the development of viable combinations of therapies that can attack multiple targets that have been identified as essential for tumorigenesis or resistance to therapy. Our findings stress the importance of understanding the clinical consequences of ITH both at the level of stromal cells and malignant cells. More than identifying new interactions among multiple cancer-related pathways, it is critical to understand which molecular aberrations are crucial for tumor growth and that will likely produce the most effects on tumor survival when targeted.

Popular science summary

Tumors result from abnormal and uncontrolled growth of cells and can develop potentially in any organ of our body. Tumors are composed not only of malignant cells but also of support cells that provide different elements necessary for tumor cells to grow and spread (Figure 1). These cells compose the tumor microenvironment and communicate with each other in complex ways.

For decades now, it is known that tumors, like any other organ in our body, require oxygen and nutrients to survive, grow and function. As such, tumors develop new blood vessels to sustain their growth, a process termed angiogenesis. Blood vessels in tumors are composed of two essential cells, the endothelial cells (ECs), forming the inner lining of the vessels and pericytes, cells that embrace ECs, stabilize them and regulate blood flow. It became logical after these findings that destroying the blood vessels in tumors would inhibit tumor growth and possibly kill tumor cells. Several drugs have been developed and are used in the clinic to prevent ECs in blood vessels from growing and in consequence, deprive tumors of oxygen. However, despite some beneficial results, it became apparent that ECs can become resistant to such compounds and find alternative ways to grow. Pericytes are believed to protect ECs from these anti-angiogenic drugs. However, how pericytes function in tumors is not very well understood.

To study pericytes, we used a mouse that develops pancreatic neuroendocrine tumors, similar to the human disease. In paper I we studied how pericytes communicate with ECs in tumors and demonstrated that when pericytes are close to ECs, they release substances that stimulate EC survival. We also examined the possibility of using the amount and the type of pericyte coverage of blood vessels to predict how a patient will respond to drugs against ECs (paper II). In paper III we developed a very small compound that, when injected into mice with tumors, binds to pericytes with precision. This compound will be useful to track pericytes and understand their role in tumors and it could potentially be combined with different drugs and be delivered to pericytes to kill them. Finally, in paper IV we studied how a factor/nutrient produced by ECs in tumors, called platelet derived growth factor-DD (PDGF-DD) can control tumor growth. The role of this factor has not been well understood. We showed that PDGF-DD can affect a rare group of tumor cells, different from the majority of cells and in this way regulate the growth of the whole tumor. Tumor biologists are now discovering that tumors are even more complex than assumed before. Besides the different cell types existing in the tumor microenvironment, the tumor cells themselves can be very different from each other within the same tumor. Our work from paper IV supports this idea and encourages more studies to investigate how the existence of different tumor cells in the same tumor affects the way patients respond to therapy in the clinic.

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Stockholm, where it all started…

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