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EXPLORING NOVEL ROLES OF TUMOR PERICYTES

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EXPLORING NOVEL ROLES OF TUMOR PERICYTES
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To my family

ABSTRACT

Tumor biology has been extensively studied over the last few decades, with a principal focus on how neoplastic cells obtain cellular immortality. A number of oncogenes and tumor suppressor genes have been uncovered that regulate cancerous transformation. However, tumors are now believed to be complex tissues consisting of various kinds of tumor stromal cells as well as transformed cancer cells. The tumor stroma is mainly comprised of tumor-infiltrating leukocytes, cancer-associated fibroblasts, vascular endothelial cells, lymphatic endothelial cells, tumor pericytes, and extracellular matrix. These cells have an extensive interplay with one another or with cancer cells, from the initiation stage of tumor development to its metastatic dissemination.

The aim of this thesis was to investigate the tumor pericytes—one of the tumor stroma constituents that has not been widely explored—in order to determine their novel roles in tumor malignancy. The use of a pericyte-deficient mouse model (*pdgfb*$^{ret/ret}$), in paper I, confirmed that the myeloid-derived suppressor cells (MDSCs), one of the most aggressive types of tumor-infiltrating leukocytes, are significantly increased both at the tumor site and in the peripheral blood in B16 melanoma and Lewis lung carcinoma (LLC) subcutaneous mouse models of *pdgfb*$^{ret/ret}$, compared to their littermate controls. The increase in the MDSC number was dependent on expression of tumor-derived IL-6, induced by the hypoxic tumor microenvironment in pericyte-deficient B16 and LLC tumors. Analysis of gene expression in human samples (253 breast cancer patients of an Uppsala dataset) showed an inverse correlation between human pericyte-related genes and human MDSC markers and a subsequent relevance to the survival rate of breast cancer patients.

The relevance of the tumor pericytes to other tumor stroma cells was studied in paper II, which revealed a comparable abundance of PDGFRα- expressing perivascular cells in pericyte-deficient B16 melanomas. A further investigation identified the PDGFRα-expressing perivascular cells as “specialized myofibroblasts” with gene signature features of both fibroblast-related (Fap, Pdgfra, Hgf) and pericyte-related (Cspg4, Pdgfrb, Asma) gene sets. Moreover, pericyte-deficient, B16-melanoma-bearing mice showed an elevated level of the serum S100B protein, which is widely considered to be a distinctive prognostic marker for malignant melanoma patients. The B16 melanoma tumor cell-derived S100B was then confirmed to pass into the peripheral blood. Presumably “activated endothelial cells” in *pdgfb*$^{ret/ret}$ mice would facilitate more rapid transport of S100B protein via endothelial caveola-mediated transcytosis.

In conclusion, tumor pericytes directly interact with adjacent endothelial cells, thereby controlling the tumor vasculature and further changing the tumor microenvironment. Tumor pericytes favor tumor immunogenicity by blocking systemic MDSC bursts in experimental mouse models (B16, LLC) and are also negatively involved in recruitment of the perivascular myofibroblasts. However, the biological relevance of the perivascular myofibroblasts should be further investigated.

LIST OF SCIENTIFIC PAPERS

I. **Hong, J**., N.P. Tobin, H. Rundqvist, T. Li, M. Lavergne, Y. Garcia-Ibanez, H. Qin, J. Paulsson, M. Zeitelhofer, M.Z. Adzemovic, I. Nilsson, P. Roswall, J. Hartman, R.S. Johnson, A. Ostman, J. Bergh, M. Poljakovic, and G. Genove. 2015. Role of tumor pericytes in the recruitment of myeloid-derived suppressor cells. *Journal of the National Cancer Institute*. 107(10):pii:djv209.

II. **Hong, J**., Y. Garcia-Ibanez, H. Qin, M. Lavergne, T. Li, J. Andrae, and G. Genove. 2015. Analysis of the melanoma perivascular cells reveals recruitment of myofibroblasts in the absence of pericytes. *Manuscript in preparation*

Other publications not included in the thesis

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Zang, G., K. Gustafsson, M. Jamalpour, **J. Hong**, G. Genove, and M. Welsh. 2015. Vascular dysfunction and increased metastasis of B16F10 melanomas in Shb deficient mice as compared with their wild type counterparts. *BMC cancer*. 15:234.

CONTENTS

LIST OF ABBREVIATIONS

ANG1, ANG2	Angiopoietin 1, Angiopoietin 2
Bregs	Regulatory B cells
CAFs	Cancer associated fibroblasts
CAV1, CAV2, CAV3	Caveolin1, Caveolin2, Caveolin3
CTLs	Cytotoxic T lymphocytes
ECM	Extracellular matrix
EMT	Epithelial–mesenchymal transition
EndMT	Endothelial-mesenchymal transition
LLC	Lewis lung carcinoma
MDSCs	Myeloid-derived suppressor cells
Mo-MDSCs	Monocytic-myeloid-derived suppressor cells
G-MDSCs	Granulocytic- myeloid-derived suppressor cells
NK cells	Natural killer cells
NKT cells	Natural killer T cells
pDCs	Plasmacytoid dendritic cells
pdgfb$^{ret/ret}$	PDGFB retention motif knock out
TERT	Telomerase reverse transcriptase
TILs	Tumor-infiltrating leukocytes
TAMs	Tumor-associated macrophages
Th17 cells	T helper 17 cells
TME	Tumor microenvironment
Tregs	Regulatory T cells

1 INTRODUCTION

1.1 TUMOR MICROENVIRONMENT (TME)

In the last few decades, extensive documentation has revealed how incipient cancer cells acquire the traits of endless proliferation and aggressiveness, termed "tumor malignancy." In accordance with the advent of new molecular biology techniques, a series of discrete steps of tumorigenesis has now been well established, so that the "multistep process of tumorigenesis" is known to comprise the following: cellular transformation into neoplastic cells; angiogenesis; motility and invasion via blood and lymphatic vessels; formation of multi cell aggregates with platelets or leukocytes; embolism and circulation; arrest in capillary beds; extravasation into organ parenchyma; colonization (adaptation to new microenvironment, establishment of micrometastases and macrotumors); and the metastasis of metastatic tumors to other organs (figure 1) [1].

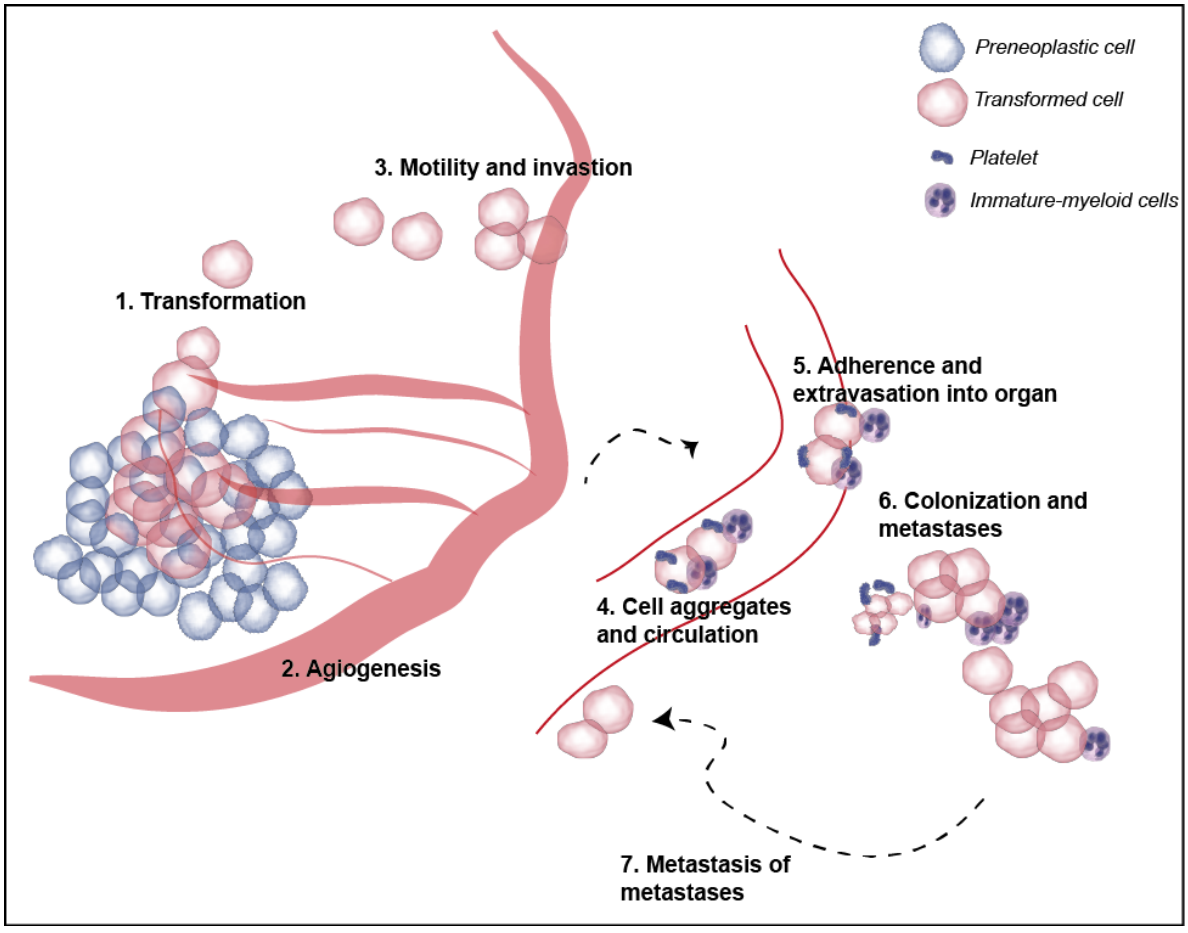


Figure. 1. Multistep process of tumorigenesis

These complex serial processes always begin with genetic changes in normal cells, whereby various kinds of extracellular or intracellular stimuli elicit aberrant gene expressions. The combination of irregular genetic changes, in turn, converts neoplastic cells into transformed cancerous cells. Many tumors show genetic instability that persists from the beginning (tumor

initiation) stage and this instability is even amplified at the late developmental stage of tumorigenesis—metastasis—as the intrinsic intracellular maintenance and repair system breaks down.

Classically, the genetic changes that occur subsequent to genetic instability in cancer cells are categorized as follows: the activation of proto-oncogenes (e.g., RAS, EGFR, MYC, and ABL1), the inactivation of tumor-suppressor genes (e.g., BRCA1, BRCA2, PTEN, RB, and TP53), and the inactivation of genomic stability genes that encode telomerase or DNA mismatch repair machinery-related proteins [2]. A number of cancer cell-associated genes, oncogenes, and tumor suppressor genes have been reported in various cancer types and at different developmental stages of tumorigenesis, but their expression patterns show considerable heterogeneity. The recent advancements in techniques for high throughput sequencing of DNA or mRNA (whole genome sequencing and whole transcriptome sequencing) have allowed resolution and refinement of these heterogeneous patterns in certain human tumors; for example, in prostate cancer, chromosomal rearrangements of the ETS transcription factor genes with the androgen responsive promoter, TMPRSS2–ERG fusions, were discovered in up to 50% of cases, while in melanoma, mutations in the TERT promoter appear in approximately 70% [3].

The notion that gene expression profiles vary in different developmental stages of tumorigenesis or in discrete cancer types can be explained by the following two features. First, the traits of transformed cancer cells mostly resemble the traits of their normal counterparts. Second, neoplastic cells cross talk with their normal neighboring cells and, in turn, establish unique and divergent genetic aberrations. The latter concept has been aggressively studied during the last two decades and is now well appreciated to be a crucial part of the development of tumorigenesis. Various kinds of host-originated cells are interconnected with neoplastic cancer cells during the whole process of tumorigenesis; these include tumor cell-neighboring regional fibroblasts, new blood or lymphatic vessels sprouted from neighboring tissues, pericytes, many types of bone marrow-derived cells, and circulating or regional inflammatory cells. Their reciprocal crosstalk change both cancer cells and host-derived cells, thereby establishing a unique tumor tissue. Most of the tumor stroma components are reprogrammed and distorted from their normal counterparts to favor tumor malignancy (figure 2). In some human solid tumors, patho-oncologists have measured tumor–stroma ratios using conventional histopathological analysis with hematoxylin & eosin stained tumor sections and have showed that stroma-enriched tumors are associated with a poor prognosis in breast (triple negative), colon, esophagus, lung (non-small cell), liver (hepatocarcinoma), and cervical cancers [4]. As such, the tumor microenvironment is implicated in the support of tumor progression rather than in its restraint. This notion, however, is still controversial and should be addressed with more research.

The recent study of pancreatic ductal adenocarcinoma in a mouse model can be exemplified as countering the concept of a tumor-supportive role of the tumor microenvironment, as depletion of αSMA-positive myofibroblasts in the tumor microenvironment by genetic alteration induced greater malignancy in transformed pancreatic cancer cells [5]. As such,

some controversy exists regarding whether tumor-neighboring tissues are bystanders that perform some passive actions or whether they actively participate in tumorigenesis in a supportive or suppressive way. A better understanding of how the reprogramed tumor stroma, termed the tumor microenvironment (TME), interplays with cancer cells should help in establishing targeted therapies to eliminate tumor progression and metastasis.

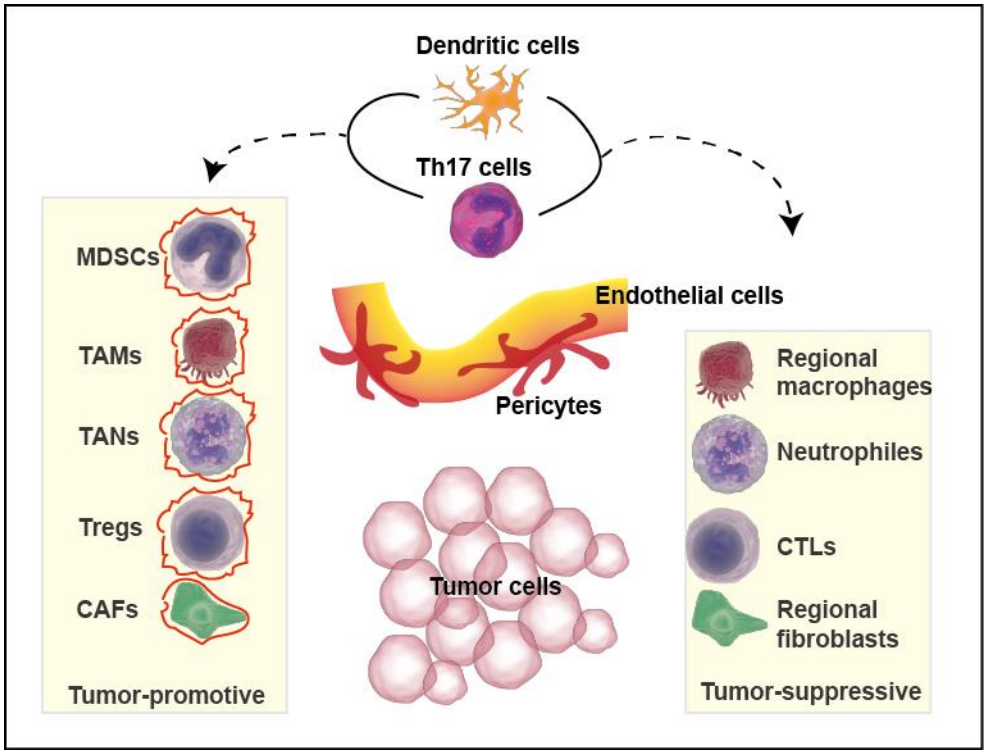


Figure. 2. The tumor microenvironment (TME)

1.1.1 Tumor-associated endothelial cells

The multistep development of tumorigenesis requires that tumor cells receive a supply of nutrients and oxygen, and that waste products be extravasated through blood vessels, in the same manner as required by normal cells. The establishment of new blood vasculature within tumor tissues therefore represents a key step in the excessive and abrupt proliferation of cancer cells, which rarely occurs in normal cells under physiological conditions in adults.

A well-known concept in tumor biology is that the formation of new blood vasculature is generated by “angiogenesis,” the sprouting of new vessels from pre-exiting ones in response to pro-angiogenic signals (e.g., VEGFA, FGFs) that react with the endothelial cells via the extracellular domains of their membrane-anchored receptors [6]. During the embryonal development stage, angiogenesis occurs vigorously and triggers blood vessel-sprouting from the existing vessel tubing, in which endothelial progenitor cells have assembled and differentiated into endothelial cells (vasculogenesis). In adults, wound healing represents a rare case that requires angiogenesis to form new vessels in lesions.

The normal vasculature is well organized, differentiated, and classified into arteries, arterioles, veins, venules and capillaries, and each type of vasculature has its own phenotype

and function [7]. In contrast, tumor vasculature is chaotic, having considerable variations in phenotype and is essentially unclassifiable. Phenotypically, tumor vessels appear irregularly branched, enlarged, hemorrhaging, and irregular in their blood flow. During the whole developmental stage of tumor progression and metastasis, the proangiogenic signals, which are mainly secreted by tumor parenchyma and partially by the tumor stroma, overwhelm their antiangiogenic counterparts, thereby causing the angiogenic switch to be invariably turned on [8].

Previous reports have confirmed that tumor angiogenesis occurs from a capillary or venule in the nearby tissue, where the tumor grows, and the newly formed vessels branch into the intratumoral region. Recent studies, however, have suggested that vasculogenesis also includes the new formation of blood vessels from endothelial progenitor cells during tumor-associated neovascularization. Endothelial progenitor cells are mostly originated from bone marrow-derived cells, including hematopoietic stem cells, myeloid progenitors, and mesenchymal stem cells. Bone marrow transplantation using EGFP or LacZ-marked reporter mice confirmed that various subsets of bone marrow-derived cells are recruited at an implanted tumor locus and become integrated into nascent vessels [9]. Whether the endothelial progenitor cells affect to any great extent the tumor-associated neovascularization remains arguable, due to the very low rate of integration and the invalidated identification of endothelial progenitor cells in tumor vessels. In addition, tumor-associated neovascularization has been shown to be accomplished by the neoplastic cancer cells themselves. In glioblastoma, a fraction of cancer cells expressing CD133, believed to be a cancer-stem cell marker, differentiates into endothelial progenitor cells as well as tumor cells, which thereafter have the features of endothelial hyperplasia in the glioblastoma tissue [10]. As such, tumor-associated endothelial cells can be obtained from three different routes: neighboring vessels and host-derived and cancer cell-derived endothelial progenitor cells.

In the context of cancer therapy, tumor-associated endothelial cells have been recognized as an “attractive target.” Tremendous efforts have been made to block tumor angiogenesis in the last few decades. A few drug candidates, including bevacizumab (a neutralizing monoclonal anti VEGFA antibody) have gone through clinical trials and have been approved for clinical use in combination with chemotherapy or cytostatic reagents for patients with some advanced cancers, including metastatic colorectal cancer, advanced non-small cell lung cancer, advanced ovarian cancer, advanced cervical cancer, metastatic renal cell carcinoma, and recurrent glioblastoma, which reflects their limited efficacy [11-13].

Recent preclinical and clinical studies have focused on a newer aspect of angiogenesis regulation: the endogenous inhibitors of angiogenesis (angiostatin and endostatin). These are counter-balancing molecules against proangiogenic factors, and play important roles in the development of tumorigenesis by suppressing tumor angiogenesis, thereby inducing dormancy, especially in distant micrometastases. Nevertheless, single targets against tumor-associated endothelial cells are recognized as still insufficient for cancer treatment [11].

1.1.1.1 ***Endothelial Caveolin 1***

Caveolae are cholesterol- and sphingolipid-rich lipid rafts that occur as ≈100nm flask-shaped invaginations of the plasma membrane and are involved in clathrin-independent membrane transport [13, 14]. They are ubiquitously present in most cells and are highly enriched in endothelial cells (≈ 10,000/cell). They mainly function in transport (e.g., endocytosis, exocytosis, and transcytosis) of macromolecules such as albumin, iron-transferrin, insulin, low-density lipoprotein (LDL), high-density lipoprotein, and CCL-2 [15, 16]. They are also involved in signal transduction through their constituents, including caveolins, eNOS, cavins, IL1R, gp130, INSR, RAGE, EGFR, LDLR, HDLR, and gp60 (an albumin receptor) [17]. Among these many signaling molecules, caveolins are the major structural proteins and include caveolin1 (Cav1), caveolin2 (Cav2), and caveolin3 (Cav3). Cav1 and Cav2 are expressed in most cells, while Cav3 is expressed in cardiac and skeletal muscle cells [18].

Cav1 has an essential role in caveolar biogenesis. Cav1 null mice show a loss of caveolae in most cell types, including endothelial cells, adipocytes, and cardiac and skeletal muscle cells [19, 20] and Cav1 is therefore a compulsory component. Cav1 directly interacts with eNOS in endothelial caveolae, thereby sequestering eNOS and maintaining NO production at a basal level. The Cav1/eNOS complex is closely involved with angiogenesis, vascular permeability, and vasodilation [18, 20, 21]. Cav1 also binds to the transient receptor potential channel-1 (TRPC-1) and thus enables caveolae to store calcium ions in endothelial cells [22]. Cav1 also senses shear stress in the vascular luminal side by dissociating eNOS to produce NO, which triggers the translocation of Cav1to the abluminal side [23].

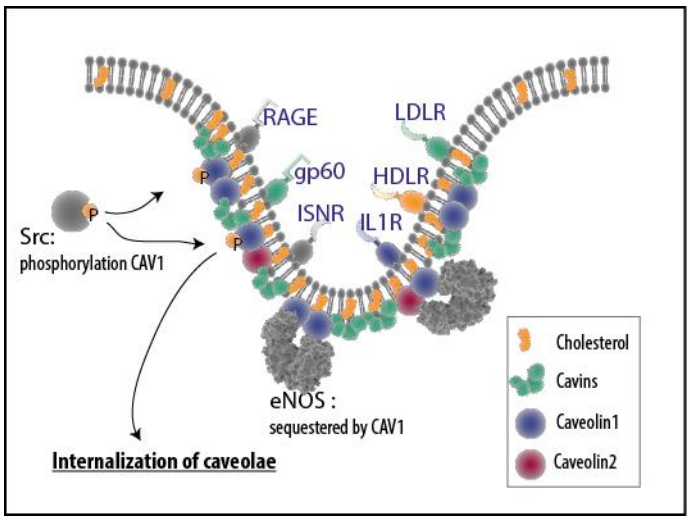


Figure.3. Endothelial caveolae

Endothelial cells require an enrichment of passive and active transport systems to pass nutrients, oxygen, and bioactive molecules bidirectionally from the blood luminal to the abluminal side, and vice versa. Caveolae exist most abundantly in the capillary endothelium (≈30% of cell surface), the interface between the blood and tissue (figure 3). Not surprisingly, therefore, caveolae, as the major component of transcytosis, are also associated with

pathological conditions including atherosclerosis, diabetes, and hyperlipemia [17, 24]. The manner in which caveolae-mediated transcytosis and endocytosis occur and which molecular interactions control each step of the serial processes remain largely unresolved questions. Recent evidence, in part, has shown that the initial step of the caveola-mediated transcellular pathway requires the phosphorylation of Cav1 (pT14). In the lung microvessel, tyrosine phosphorylation of Cav1, mediated by oxidant stress (hydrogen peroxidase), induced transcellular albumin hyperpermeability, with Src identified as the upstream kinase that performed the phosphorylation. Interestingly, a high degree of oxidant stress also induced paracellular permeability by dissociating VE-cadherin from membrane-bound β-catenin. In mouse embryonal fibroblasts (MEFs), phospho-Cav1 mediated the internalization of cholesterol-dependent clusters of GM1 and GPI-linked protein (caveolae), implying that phospho-Cav1 is not limited to the endothelial caveolae pathway [25, 26].
In tumor endothelial cells, the irregularly active state of transcellular signaling pathway is presumed to occur in the tumor endothelium, where it positively modulates TME for tumor malignancy, taking into account both notions that 1) the aberrant tumor vessels are the main passage for the considerable amount of tumor-derived factors, and 2) only small molecules (< 3nm) pass through via the paracellular pathway.

1.1.2 Tumor pericytes

Pericytes are specialized mesenchymal cells that regulate angiogenesis and vascular integrity. As inferred by their name, pericytes lie in the perivascular niche, where the basement membrane anchors pericytes and endothelial cells, enabling their close interconnection. Pericytes are present in the microvessels—capillaries, postcapillary venules, and terminal arterioles [27]. Vascular smooth muscle cells (vSMCs) encircle the large blood vessels, including precapillary arterioles, arterioles, veins, and arteries. Spindle-like and flattened vSMCs constitute a dense layer surrounding the abluminal region of blood vessels and are responsible for vasocontraction and vasodilation. By contrast, pericytes are umbrella-like round cells, with protruding cytoplasmic processes on the abluminal surface of the endothelial tubing. The possibility that pericytes also function in vasocontraction to control blood flow in the microvessels remains controversial [28]. Nevertheless, pericytes are similar to vSMCs, when gene and protein expression signatures are taken into account. Pericytes, therefore, are considered to be the closest relatives to vSMCs.

Other cell types also anchor or stand in the vascular beds in similar fashion to pericytes; these are called “perivascular mesenchymal cells” and include bone marrow mesenchymal stem cells, bone marrow-derived progenitor cells, infiltrating leukocytes, fibroblasts, myofibroblasts, and regional progenitor cells. An exact categorization of these cell types has not been established. Under physiological conditions, pericytes are defined as already described above; namely, as cells embedded in the basement membrane with endothelial cells, present in the microvessels, morphologically featuring protruding-umbrella like shapes, and expressing multiple pericyte marker proteins. So far, no single unique marker for

pericytes has been identified; instead, multiple marker proteins (e.g., NG2, PDGFRβ, CD13, αSMA, DESMIN, RGS5, IPTG7, and CD248) are used to identify pericytes [29, 30]. NG2 has been used as the most reliable marker for pericytes, but it is also expressed in the skin, bone, fat, and brain in regional progenitor cells, including oligodendrocyte precursor cells, osteoblasts, chondroblasts, epidermal and hair follicle progenitor cells, and adipose stem cells [31-33]. PDGFRβ is also expressed in other cell types, including myofibroblasts, neuronal stem cells, and mesenchymal stem cells. Interestingly, PDGFRα, which has rarely been considered as a pericyte marker, has recently been demonstrated by electron microscopy analysis to be expressed in a considerable proportion of the pericytes in the mouse spinal cord. In skeletal muscle, $NG2^{+}$/$NESTIN^{-}$perivascular cells express PDGFRα and function in fat deposition during skeletal muscle regeneration, further indicating the complexity of pericyte identification [34-36].

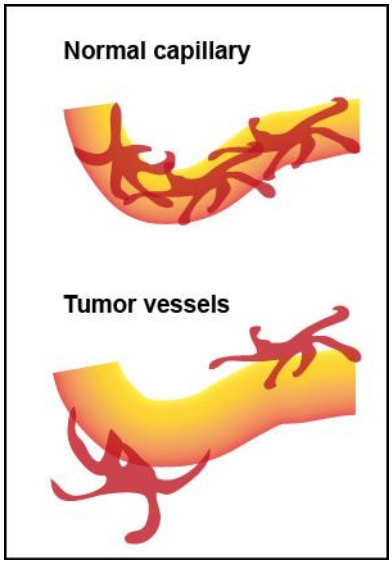
In their anatomical and biochemical respects, mature pericytes reside in close proximity to the quiescent endothelium and are embedded and interconnected with the basement membrane. At distinctive points in the basement membrane, pericytes directly contact the endothelial cells by peg-pocket type interactions, where the pericyte cytoplasmic processes (pegs) are inserted into endothelial invaginations (pockets), or where the two membranes of the pericytes and endothelial cells align together. These mechanical close contacts mediate active biochemical interactions in paracrine and juxtacrine manners, and vice versa.

Several signaling pathways are involved in pericyte/endothelium interactions: PDGFB/PDGFRβ, TGFβ1/TGFRβ2/ALK1 or ALK5, TIE2/ANG1 or ANG2, and HB-EGF/EGFR [28]. Classically, PDGFB/PDGFRβ is the best-known molecular axis, recruiting the developing pericytes to the angiogenic endothelial tip cells. PDGFB is expressed in the endothelial cells, and then retained in the extracellular matrix neighboring the endothelial cells, where it binds to a receptor tyrosine kinase, PDGFRβ, in pericytes. This interaction is the crucial and dominating factor for mural cell recruitment and subsequent vascular integrity. In a mouse model, both *Pdgfb* and *Pdgfrb* null mice exhibited perinatal lethality, mainly caused by vascular dysfunction, and particularly the loss of mural cell recruitment. Interestingly, the pattern of mural cell deficiency was heterogeneous between different organs, alluding to the fact that other pathways are also somehow actively implicated in the mural cell recruitment and that the ontogeny of mural cells varies in different organs [37, 38]. A smarter attempt has also contributed to generate a pericyte-deficient mouse model ($pdgfb^{ret/ret}$), in which the PDGFB retention motif is deleted to protect PDGFB binding to the extracellular matrix, which leads to hypoplasia and partial detachment of the pericytes [39]. In TGFβ signaling, complex patterns of regulation arise between endothelial cells and various kind of mesenchymal cells, including pericytes or vSMCs, Once TGFβ1 binds to the TGFβ type 1 receptor (ALK1 or ALK5, respectively in the endothelium and pericytes), two confronting signaling pathways are implicated. ALK1 leads to proliferation and ALK5 leads to differentiation via phosphorylation of Smad1/5 and Smad2/3, respectively. The resulting effects on vascular integrity depend on the expression ratio of ALK1/ALK5, which appears to undergo a dynamic change depending on the vascular developmental stage.

The concept underlying the combinational or reciprocal TGFβ signaling networks would be to control and maintain vascular integrity [40, 41]. TIE2 is a receptor tyrosine kinase, mostly expressed in endothelial cells, that binds to angiopoietin 1 (ANG1) and angiopoietin 2 (ANG2). ANG1 is secreted by pericytes, perivascular mesenchymal cells, fibroblasts, or cancer cells and functions as a key stabilizer for vascular permeability and integrity by maintaining or increasing pericyte coverage in the endothelium. On the other hand, ANG2, in large part, is secreted by endothelial cells and counterbalances ANG1 by competing to bind the same receptor, TIE2, in the endothelium. ANG2 binding to TIE2 results in pericyte detachment and angiogenic sprouting. Hypoxia upregulates ANG2 secretion in the endothelium, as well as that of VEGFA, thereby turning on an angiogenic switch and forming aberrant and immature vasculature. As such, TIE2/ANG1, or the ANG2 signaling pathway, also participates in the homeostasis of the vascular system.

Interestingly, in the endocrine pancreas of *pdgfb*$^{ret/ret}$ mice, which are pericyte deficient mice, a similar level of pericyte coverage to that seen in littermate controls is observed, representing an exception to recruitment by the PDGF-B/PDGFRβ axis (unpublished data). A recent study in a mouse pancreatic neuroendocrine tumor model showed that endothelial- and pericyte-derived HB-EGF binds to EGFR in the pericyte, thereby increasing pericyte coverage and vascular stability and providing a clue to the identity of the alternative recruiting factor in the pancreas [42-44].

Figure. 4. Tumor pericytes



The active state of the tumor vasculature has long been postulated to lack pericyte coverage. Recent studies, however, have revealed that tumor vasculature is covered by a certain number of pericytes, and its integrity is also, in large part, controlled by the tumor pericytes. Unique traits of tumor pericytes appear to be a loose detachment from the endothelium and a more heterogeneous frequency (figure 4). The recruitment of tumor pericytes mostly occurs in an analogous manner to that of their normal counterparts. PDGF-B/PDGFRβ axis plays an important role in recruitment and maintenance of tumor pericytes in the endothelium. In the *pdgfb*$^{ret/ret}$ mouse model, implanted melanomas, lung carcinomas, and sarcomas caused a greater than 50% reduction in pericyte coverage when compared to the littermate controls [45, 46]. The TIE2/ANG1 and ANG2 signaling pathways are also implicated in tumor pericyte coverage and vascular integrity, as described above.

The gene and protein signatures of tumor pericytes might appear more variable that those of normal pericytes, as inferred by their prolonged dynamic states in tumor vasculature. The NG2 protein has been widely used as a marker for tumor pericytes in mouse models. This is

because other markers for pericytes—for example, αSMA, DESMIN and PDGFRβ—can be expressed more often in major tumor stroma constituents, cancer associated fibroblasts, and myofibroblasts. This is exemplified by the fact that NG2-tk^{+}GCV transgenic mice, which show inducible and transient pharmacological targeting of pericytes, are well appreciated as a mouse model for tumor pericyte studies, in addition to the *pdgfb*$^{ret/ret}$ pericyte-deficient mice model [47].

The role of tumor pericytes in the multistep process of tumorigenesis has been recently documented in several papers. Tumor pericytes clearly function to stabilize tumor vasculature, thereby supporting tumor vessels that deliver more oxygen and nutrients, which seems to favor tumor progression. This assumption is supported by two respective mouse experimental models. In the 4T1 murine breast tumor model, tumor growth rate is decreased when pericytes are targeted, while in T241 murine sarcoma, the ectopic expression of N-cadherin stimulates pericyte coverage and tumor growth [48, 49]. By contrast, in murine lung carcinoma in the *pdgfb*$^{ret/ret}$ mice, the partial depletion of pericytes instead results in an increased tumor growth, while in the same context of a mouse setting with a B16 murine melanoma, targeting of pericytes does not result in any difference in tumor growth [45]. These discrepancies might imply that crosstalk by the tumor pericytes with other tumor stroma constituents or tumor cells may differ among discrete tumors. However, regarding metastasis or tumor cell dissemination, consistent outcomes have been that poor pericyte coverage in tumor vasculature correlates with more advanced metastasis [47-49]. Paper I of this thesis discusses tumor pericytes and tumor malignancy in greater detail.

Investigation of the identity, ontogeny, and function of pericytes or pericyte-like cells continues in physiologic and the pathologic conditions, including tumor biology. With respect to targeting tumor pericytes as a therapeutic approach, the following factors need to be addressed in further investigations: 1) The difference between the closest relative, αSMCs, and pericytes, regarding their recruitment and maintenance, 2) A more refined definition of pericytes, focusing on how their discrete features differ from perivascular cells or pericyte-like cells, 3) The origin of pericytes or tumor pericytes—whether they arise from endothelial progenitor cells, pre-exiting pericytes, bone marrow-derived cells, or mesenchymal stem cells, and 4) The role of tumor pericytes in the tumor microenvironment—whether they are tumor supportive or suppressive.

1.1.3 Cancer-associated fibroblasts

Fibroblasts, the most abundant mesenchymal cells in the connective tissues, originate from the mesodermal layer at the embryonal stage. Morphologically, they differ in various locations and stages; thus, they are still characterized rather vaguely as non-vascular, non-epithelial, and non-inflammatory cells in the connective tissue [50]. Fibroblasts reside in the connective tissues and are embedded within the ECM, which is mostly synthesized and established by the fibroblasts. Their roles in physiological conditions are to maintain ECM homeostasis by secreting most of the ECM components, including various subtypes of

collagen fibers, fibronectin, and the collective proteases that degrade the ECM, such as the family of matrix metalloproteases (MMPs). In addition, fibroblasts support the neighboring tissue to keep its integrity in terms of paracrine and juxtacrine interactions [51].

The crucial supportive role of fibroblasts is emphasized in wound healing processes. Once an injury occurs, regional fibroblasts or distant cells respond to lesion-derived stimuli, subsequently enabling the reprograming of fibroblasts to invade the lesion, facilitate contraction, and restore the ECM, which acts as the scaffold for other recruited cells. The reprogramed fibroblasts, mostly composed of myofibroblasts defined as αSMA-expressing fibroblasts, undergo apoptosis after completing the wound healing process [52]. In the context of tumorigenesis of a solid tumor, the tumor tissue is considered to reflect a chronic wound region, in which fibroblasts are constantly being stimulated by dynamically evolving cancer cells. The fibroblasts in the tumor tissue are therefore termed "cancer-associated fibroblast (CAFs)." The CAFs can be further subtyped as discrete cells that function in tumor suppression and tumor promotion. However, CAFs, in large part, have been widely documented to orchestrate cancer cells and the tumor microenvironment, promoting tumor malignancy from the very beginning—from tumor initiation to the last metastatic colonization [53].

Fibroblasts, as mentioned earlier, are the most common cell type in the connective tissue, which means that the fibroblasts would be the first barrier that pre-transformed cells would have to overcome during the multistep process of tumorigenesis. The fibroblasts (which, in this case, are not the CAFs) have been reported in several studies to block the growth of neoplastic cells. A recent *in vitro* and *ex vivo* study showed that isolation and co-culture of normal human and mouse quiescent fibroblasts of diverse origins with the PC3 prostate carcinoma cell line revealed a prominent suppression of the proliferation of PC3 cells by secretion of different sets of ECM components and bioactive molecules responsible for cell to cell contact-dependent inhibition of cell proliferation [54, 55]. The normal fibroblasts, though not yet programmed, are somehow able to restrain the development of tumor initiation and metastatic colonization. On the other hand, a few neoplastic cells continuously evolve and acquire the ability to resist and destroy the fibroblast-derived defense armaments, thereby changing the property of the fibroblasts in a tumor-favoring way. TGFβ is the major cancer cell-derived factor affecting CAF activation and the PDGF, COX2, and IL6 signaling pathways have also been implicated in CAF activation [53, 56, 57].

The manner by which CAFs orchestrate or modulate tumorigenesis remains in question. The answer evidently lies in their unique feature as a dense reservoir of bioactive molecules, including growth factors, proinflammatory cytokines, ECM-degrading enzymes, and chemokines. Once activated, CAFs start secreting a plethora of bioactive molecules, which stimulate not only neoplastic cells or transformed cancer cells, but also neighboring tumor environment components or distant cells, in a paracrine manner. Various experiments using *in vitro* co-culture and transplantation studies with tumor cells and CAFs have shown that abundant grow factor secretions (e.g., EGF, bFGF and HGF) might support tumor growth. In some cases, tumor cells can be stimulated by CAFs to express COX2 and elicit changes in the

tumor microenvironment, including angiogenesis and tumor immunity, although the exact underlying mechanisms are not fully elucidated [53, 58]. In human ductal carcinoma in situ (DCIS), in neoplasia of breast cancer, and in xenograft tumor experiments, the COX2 level was highly upregulated by interaction with CAFs via the NF-kB mediated signaling pathway, thus enabling tumor cells to secrete MMP14 and VEGF-A. Interestingly, a neoplastic skin tumor in a transgenic mouse model showed that tumor cells stimulated neighboring CAFs to express a variety of proinflammatory molecules, including COX2, via the NF-kB signaling pathway. These two studies confirmed that CAFs are eventually implicated in tumor progression and metastasis [59], thereby providing growing evidence of a complex reciprocal crosstalk occurring between cancer cells and neighboring CAFs. The CAF-derived CXCL12 has also been well studied. CXCL12 behaves as the chemo-attractant to recruit cells expressing its receptor CXCR4, such as bone marrow-derived endothelial progenitor cells, mesenchymal stem cells, tumor cells, and tumor-infiltrating leukocytes, thereby confirming its close involvement in angiogenesis, tumor immunity, and the EMT program [60].

Attempts to target CAFs for cancer therapy have not been successful. This might be because CAFs are heterogeneous and have diverse origins, arising from regional fibroblasts, bone marrow-derived progenitor cells, mesenchymal stem cells, specially differentiated stellate cells (e.g., liver and pancreas), or fibroblasts from the epithelial-mesenchymal transition (EMT) or the endothelial-mesenchymal transition (EndMT). Even though located in the same region of tumor tissue, different CAFs have various traits and stages, some of which are complementary, compensatory, or confronting in their functions regarding tumor malignancy. For instance, FAP, a type II transmembrane protein functioning as a post-prolyl protease, has been targeted for development of a CAF-related cancer therapeutic agent because the expression of FAP protein is mostly detected on the cell surfaces of CAFs, but is absent or rarely found on the normal counterparts. A study using FAP null mice showed a decrease in tumor malignancy of lung carcinoma and colon tumor models. A preclinical study of a 4T1 breast cancer mouse model showed similar results, where a decreased tumor burden was associated with the attenuated deposition of collagen I and increased recruitment of CD8 positive cells, collectively demonstrating the important role of FAP positive CAFs in modulating tumorigenesis [50, 61]. However, in a clinical trial (phase II) in human patients with metastatic colorectal cancer, humanized anti FAP-neutralizing antibody (sibrotuzumab) failed to show any significant efficacy as a treatment [62]. In addition, a recent study with αSMA-tk transgenic mice revealed the astonishing result that, in pancreatic ductal adenocarcinoma (PDAC)—one of the most stroma- rich cancers with a lethal malignancy—depletion of αSMA positive CAFs elicited a decrease in tumor vessel density, an increase in hypoxia, a subsequent recruitment of tumor-promoting inflammatory subsets, regulatory T cells (Tregs), and myeloid-derived suppressor cells (MDSCs), and eventually an increase in tumor malignancy with reduced survival. This might highlight again the complexity of CAF interplay within tumor tissues [5].

Table 1

Murine protein markers commonly used for identifying pericytes and fibroblasts

Marker (gene symbol)	*Function*	*Cell types expressing the marker*	*Other cell types expressing the marker*	*Reference*
FSP1 (*S100a4*)	S100 calcium binding protein	Fibroblasts in normal and fibrotic tissues	Epithelial ovarian carcinoma cells and macrophages, activated lymphocytes	[63-65]
Fibroblast-activation protein, FAP (*Fap*)	Serine endopeptidase	Cancer-associated fibroblasts and activated fibroblasts (myofibroblast)	Myoblast, mature cardiac fibroblasts, and activated melanocytes	[53, 66, 67]
Vimentin (*Vim*)	Intermediate filament	Fibroblasts in normal and fibrotic tissues	Endothelial cells, mesenchymal cells, and most neuronal precursor cells	[68-70]
Platelet-derived growth factor receptor-alpha, PDGFRα	Cell surface tyrosine kinase receptor for PDGFs (PDGFA and PDGFC)	Perivascular profibrotic cells in skeletal muscle, skin and small intestine, dermal fibroblasts, pericytes in spinal cord	Neural stem cells, oligodendrocytes precursor cells, renal interstitial cells	[35, 71, 72] [69, 73]
Platelet-derived growth factor receptor-beta, PDGFRβ	Cell surface tyrosine kinase receptor for PDGFs (PDGFB and PDGFD)	Bone marrow-derived or regional myofibroblasts (kidney, breast and liver), pericytes	Mesenchymal stem cells, neuronal progenitors, vSMCs	[28, 74-76]
Desmin (*Des*)	Intermediate filament	Fibroblasts in skin, pericytes	vSMCs, cardiomyocytes, skeletal muscle cells, and fetal hepatic stellate cells	[28, 53, 77]
Alpha-smooth muscle actin, αSMA (*Acta2*)	Microfilament	Myofibroblasts and pericytes	vSMCs, myoepithelial cells	[45, 78, 79]
Chondroitin sulfate proteoglycan 4, NG2 (*Cspg4*)	Integral membrane proteoglycan	Perivascular myofibroblasts and pericytes	vSMCs, oligodendrocyte precursor cells, osteoblasts, chondroblasts, epidermal and hair follicle progenitor cells, and adipose stem cells	[31-33, 80]
Endosialin (*Cd248*)	C-type lectin transmembrane receptors	Brain pericytes in embryonal stage and glioma, fibroblasts, and myofibroblasts	Vascular-associated leukocytes ($CD45^+/CD144^+$)	[81-84]
Regulator of G protein signaling 5, RGS5* (*Rgs5*)	Intracellular signal transducer (GTPase activator)	Pericytes	vSMCs, breast cancer cells, multiple myeloma cells, colon cancer cells, renal cell carcinoma cells	[85, 86]
Alanine aminopeptidase, CD13 (*Anpep*)	Membrane-integrated aminopeptidase	Brain and retinal pericytes	vSMCs, myeloid cells, and epithelial cells of normal tissues and malignant neoplasms	[87-89]

*****Obscure yet, because of lack of a validated antibody for immunostaining

1.1.4 Tumor-infiltrating leukocytes (TILs)

The use of the currently available advanced methods to detect somatic mutations in tumor biology using genome-wide analysis has indicated that some transformed cancer cells, such as melanoma, lung carcinoma, and glioma, retain hundreds to even thousands of somatic mutations on their chromosomes (somatic mutation prevalence), as well as aberrant epigenetic changes, conferring an increase in the corresponding pool of antigens to be recognized by the immune system [90]. This feature raises the question as to how pre-neoplastic or transformed cancer cells circumvent the immune surveillance system, which has evolved a sophisticated combination of innate and adaptive immune systems against external and internal hindrances that threaten the organism's lifespan. The antigens originating from host-derived transformed tumor cells are presumed to be improperly recognized and primed by the host immune system (immunologic ignorance). However, recent work with solid tumors in experimental mouse models and from human patients has shown that spontaneous tumor antigen-specific T cell responses are prevalently induced at tumor sites, even though the response varies in terms of the individual patients, the location, and the grade of tumors [91].

A complex series of anti-tumor immune response mechanisms, which are not yet fully elucidated, have been reported to suppress tumor progression and malignancy. The cells involved include granulocytes (the major immune component in the peripheral blood), regional macrophages, dendritic cells (especially the $CD8\alpha^{+}$ subset, which is implicated in type I interferon/STAT1 signaling pathway, responsible for the innate recognition of tumor cells), cytotoxic T lymphocytes (CTLs), natural killer T (NKT) cells, and natural killer (NK) cells (the complimentary innate arm to CTLs that destroy the tumor cells that downregulate expression of the MHC I molecule and thus evade attacks from CTLs). As seen in bacterial infections or skin injuries, the innate arm of the immune system first affects antitumor immunity. Regional macrophages, natural killer cells, or circulating granulocytes sense tumor cells as an aberrant stress, and some debris from dying tumor cells (DNA fragments or aberrant peptide) are exposed and are recognized by antigen presenting cells (dendritic cells), followed by the subsequent activation of the adaptive arm in host immune system.

Eventually, the activated CTLs start to destroy the tumor cells that present aberrant antigen-MHC I on their membranes. Mice that are genetically targeted for enhancement or deficiency in NK cells and CTLs confirm an inverse correlation between tumor burden and the activity of the above immune effector cells, demonstrating the importance of antitumor immunity functions against tumorigenesis [92-94]. Upon evasion of the host immune surveillance system, some, but not many, of the neoplastic or transformed cells—which have already acquired the aberrant genetic instability—proceed to evolve and thus adapt a strategy to nullify or destroy antitumor immunity, in close cooperation with the neighboring TME.

The strategy for avoiding the intrinsic surveillance immune systems can be subdivided into 4 different categories: one is that tumor cells *per se* react to the CTLs. In the case of most melanomas, the tumor cells highly express an inhibitory ligand, programmed death-ligand 1 (PD-L1), which, in turn, binds to the inhibitory receptor, PD1, in activated $CD8^+$ CTLs or NK cells, thereby inhibiting CTL or NK cell activation [95]. The tumor cells of many cancers also attempt to evade T cell-mediated immune responses through down-regulation of MHC class 1 [96].

The second category is the reprogramming of myeloid or dendritic cells in the innate immune system by tumor-derived factors—the TME even polarizes the transition-state cells into tumor-promoting state cells. Extensive investigations during the last decade have focused on these tumor-educated and tumor-favoring cell types, including tumor- associated macrophages (TAMs), tumor-associated neutrophils (TANs), and plasmacytoid dendritic cells (pDCs). TAMs, a distinct population of macrophages in the TME, promote tumor progression and metastasis. Most murine TAMs can be identified by the following markers: $CD11b^+$, $CD34^-$, $CD45^+$, $CD68^+$, $CCR2^+$, $CXCR4^+$ and $F4/80^+$. TAMs were first identified as a negative regulator of antitumor immunity by suppressing CTLs, as they express PD-L1, similarly to tumor cells and thus directly counteract CTLs or indirectly suppress CTLs by CCL22-mediated recruitment of regulatory T cells (Tregs) [97, 98]. The $TIE2^+$ TAM subset also stimulates tumor angiogenesis by expressing a proangiogenic enzyme, thymidine phosphorylase, and cathepsin B. In addition, in a mouse mammary tumor model, TAMs play a crucial role of the intravasation of invasive tumor cells within the primary tumor to form blood vessels by a paracrine interaction, termed the "paracrine loop," which includes signaling pathways such as EGF, CSF-1, and their corresponding receptors [99, 100].

Like TAMs, the tumor-educated neutrophils, the TANs, have been recently identified as an immune-suppressive cell type in murine lung carcinoma, mainly functioning to reduce CTL activity and thereby promote tumor progression. However, their actual contribution to tumorigenesis remains unclear because neutrophils are also involved in antitumor immunity. In the 1960s, patients with advanced cancer were reported to have a severe blood neutrophilia, which today is considered to result from host-tumor interactions. The nature of this blood neutrophilia, however, is still uncertain, as it may be largely tumor supportive or tumor suppressive. TANs change their properties in either way, depending on their discrete TME [101, 102].

Plasmacytoid dendritic cells (pDCs) are the antigen-presenting cells that circulate in the blood and lie in the secondary lymphoid organs. The pDCs are the key regulators of antiviral immunity as they sense external DNA fragments and produce type I interferon (e.g., IFNα, IFNβ). They are identified in the mouse by the expression of the following markers: $B220^+$, $CD11c^+$, $PDCA1^+$, $Siglec\text{-}H^+$, $GR1^+$, and $CD11b^-$. In the context of tumor immunity, pDCs function as negative immune regulators. In human breast cancer and mouse xenograft experiments, immature pDCs showed a considerable tumor-promoting effect through a mechanism involving the endosomal Toll-like receptor (TLR7) in the pDCs, which binds to a tumor-derived or TME-derived ligand, TLR7L. This activates pDCs to produce type I

interferon, followed by the subsequent recruitment of immune-regulatory T cells (Tregs) within the tumor sites [103, 104].

A third category involves the adaptive immune arm in the immune surveillance system, which is also often distorted by tumor cells or their neighboring TME, and thereafter transformed into powerful tumor promoting constituents. The adaptive immune regulatory cells are as follows: the regulatory T cells (Tregs), the regulatory B cells (Bregs), and the IL17-producing $CD4^+$ helper T cells (Th17). The Tregs are defined as the immunosuppressive $CD4^+$ T cells and express the alpha chain of the IL2 receptor, CD25, and the immune-responsive transcription factor, forkhead box P3 (FOXP3). Tumor-derived or TME-derived CCL22 and CCL5 were first reported as Treg-recruiting factors at tumor sites. In a breast cancer model, prostaglandin E2 was also suggested to recruit Tregs, and the pleiotropic cytokine, TNF, was reported to activate Tregs by upregulating FOXP3 via a TNF/TNFR2 pathway in the presence of IL2.

The exact suppressive role of Tregs is not yet fully understood. However, it is well appreciated that Tregs would be one of the major components of immunosuppressive mechanisms for avoiding antitumor cytotoxicity. Their suppression, in large part, is directed to the activity of CTLs, NK cells, and antigen-presenting dendritic cells, by a direct cell to cell contact with FasL/Fas and PD1/PD-L1 interactions or by a TGFβ signaling pathway-mediated paracrine interaction [105-107]. Bregs are also associated with tumorigenesis. Bregs constitute a small population of B cells that have immunosuppressive functions in autoimmune diseases. Tumor-related Bregs are characterized by expression of the markers $CD25^+$, $B220^+$, and $CD19^+$. Recently, lung metastases of 4T1 breast cancer mouse model have confirmed that Bregs contribute to Treg conversion from naïve $CD4^+$ helper cells and, in the end, aid in growth of lung metastases [108]. IL17-producing Th17 cells are also involved in immune-regulatory responses relating to tumorigenesis. However, the role of Th17 cells in tumor biology remains controversial. One clear observation is that Th17 cells are more abundant at tumor sites than in the peripheral blood, alluding to a possible implication of Th17 in tumorigenesis. A recent report, using a VEGFA-blockade resistant murine lymphoma model, showed that the tumor-promoting effect of Th17 cells, which secrete IL17A, involves the modulation of the TME to induce G-CSF, thereby recruiting MDSCs. Conversely, in ovarian tumors, the increased number of Th17 cells was associated with an improved prognosis. Taken together, these data can be interpreted as indicating that Th17 cells play different roles, depending on the context of the tumors [109, 110].

The last category involves the immature myeloid cell population as a strong tumor-promoting factor for incipient cancerous cells or transformed malignant tumor cells. As mentioned previously, neutrophilia in patients with the advanced cancers results, in part, from tumor-educated, immune-regulatory activation. Tumor-bearing mice show an accumulation of immature myeloid cell populations in the bone marrow, the spleen, the blood, and at tumor sites. These populations are characterized by expression of CD11b and GR1, together with the undifferentiated myeloid-expressing proteins, S100A8 and S100A9. Today, these cells are referred to as myeloid-derived suppressor cells (MDSCs) [110, 111]. The MDSCs are a

heterogeneous population with immunosuppressive, proangiogenic, and metastasis-supportive functions (figure 5). They are subdivided into two discrete subsets: monocytic (Mo-MDSCs) and granulocytic (G-MDSCs) MDSCs. The marker proteins for murine MDSCs are $CD11b^{+}/GR1^{+}$ ($LY6C^{high}$ $LY6G^{low}$: Mo-MDSC, $LY6C^{low}$ $LY6G^{+}$: G-MDSC), and those for human MDSCs are $HLA\text{-}DR^{-}/CD33^{+}$, sub-grouped into $CD14^{+/dull}$ for Mo-MDSCs and $CD15^{+}$ for G-MDSCs. Both MDSC subsets—G-MDSCs and Mo-MDSCs—serve to suppress immune effectors, the CTLs and NK cells. G-MDSCs express a considerable amount of ROS and little NO, whereas Mo-MDSCs express little ROS but a considerable amount of NO, and both MDSC subsets express Arginase 1. When exposed to a combination of three different pathways—ROS, NO, and Arginase1—both MDSC subsets efficiently block antigen specific CTLs as well as NK cells. MDSCs can also directly sustain proliferation of tumor cells or protect against the apoptotic death of tumor cells via the S100A8/S100A9 signaling pathway in MDA231-LM2 breast cancer cells [112, 113]. Adaptively transferred MDSCs can also differentiate into $F4/80^{+}$ TAMs in HIF1α-mediated manner, thereby reflecting MDSC plasticity for the promotion of tumorigenesis in various ways [114]. Tumor angiogenesis is also positively regulated by MDSCs at tumor sites, where MDSCs interplay with the TME and thereby stimulate the production of proangiogenic factors (e.g., IL8, IL10, PGE2, VEGF, and MMP8/9). Conversely, the activated MDSC subsets can be transdifferentiated into endothelial-like cells [111, 115]. As described above, the MDSCs systemically expand, once activated by tumor- or TME-derived factors (e.g., CCL2, CXCL5, IL6, IL17, G-CSF, GM-CSF, CSF, CXCL12, TGFβ, TNF, and VEGFA) that were secreted and reached the bone marrow, a reservoir of immature myeloid cells. The systemic MDSC expansion also contributes to pre-metastatic niche formation by promoting the adherence and growth of disseminating tumor cells [112, 116].

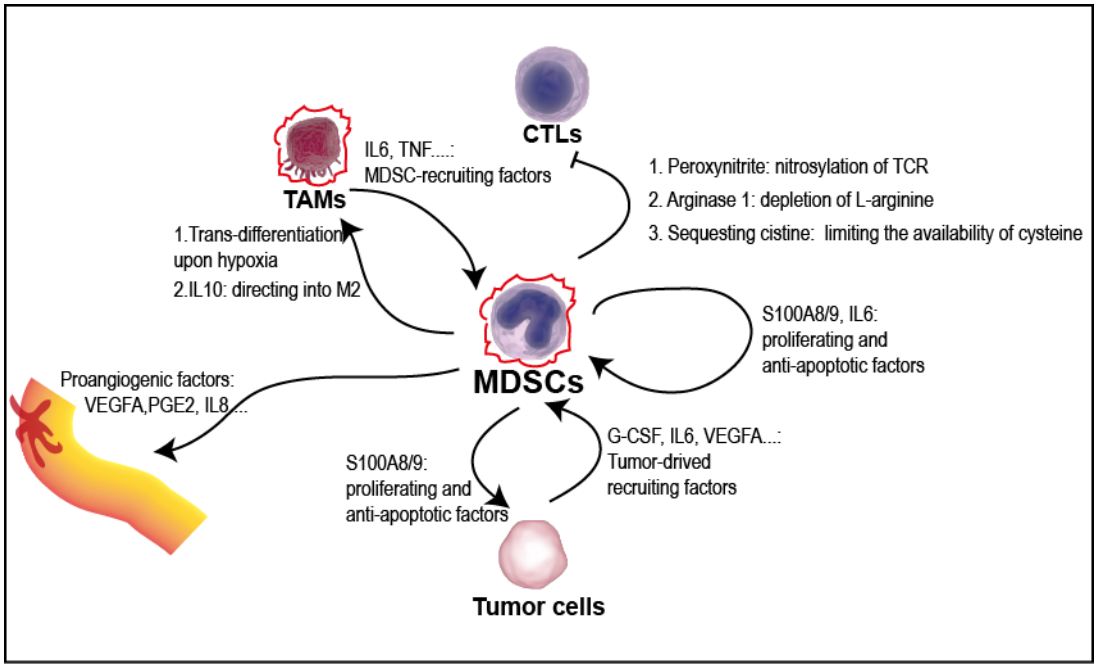


Figure. 5. MDSCs: crosstalk in the tumor

Taken together, the available evidence supports reprogramming of TILs by tumor cells through the suppression of immune effector cells and promotion of negative regulatory leukocytes, which both intrinsically function in immune homeostasis. In this way, the immune surveillance system is overcome. A better understanding of how TILs cross talk with tumor cells, the TME, or the tumor macro-environment at different stages or at different tumor loci will definitely benefit effective tumor targeting. This is especially the case when considering the accumulating, but limited, knowledge of TILs that already points to a link to clinical therapeutic approaches, such as the generation of genetically enhanced effector T cells and clinical targeting to tumor-suppressing stroma (MDSCs, Tregs and pDCs), combined with a cancer vaccine therapy [117, 118].

1.2 CANCER BIOMARKERS

1.2.1 S100A8/9 and S100B

The S100 protein family consists of 21 members: S100A1-S100A16, S100B, S100P, S100G, S100Z, and FGL, which all have calcium-binding EF hand (helix E-loop-helix F) domains in their N-terminal regions [119]. In 1965, two members of the S100 proteins, S100S1 and S100B, were first identified and termed S100 proteins because they were soluble in 100% saturated ammonium sulfate [120]. S100 proteins have bifunctional roles: In the cytoplasm, they act as calcium sensors, by binding to intracellular calcium, and as signaling modulators, by translating calcium influx into diverse target-connected signaling pathways. In the extracellular spaces, S100 proteins appear as secreted proteins that are capable of binding to several cell membrane receptors (e.g., RAGE, TLR4, FGFR1, and ALCAM), thereby affecting nearby or distant cells in complex ways, depending on the composition of their cell membrane receptors [121, 122]. The S100 proteins contribute to tumor malignancy in various types of cancers, including brain (S100B, S100A8, S100A9) [122, 123], breast (S100A4, S100A7, S100A8, S100A9) [124-127], and skin (S100A4, S100A9, S100B [121, 127]) cancers. Within the S100 protein family, S100A8/S100A9 and S100B have recently emerged as attractive therapeutic targets, with inhibitors now in clinical trials for prostate and skin cancer.

S100A8 and S100A9 are, in large part, expressed in immature $CD11b^{+}$ myeloid cells, such as the MDSCs in the tumor stroma. The prolonged expression of both S100A8 and S100A9 delays the differentiation of myeloid progenitor cells to their derivatives (e.g., macrophages and dendritic cells). In the TME, the prevalence of S100A8 and S100A9 indicates a high frequency of MDSCs and poor prognosis [128]. S100A8 and S100A9 mostly occur *in vivo* as heterodimers (S100A8/9) and their binding to the cell membrane receptor, RAGE, stimulates MAPK and NFκB signaling pathways, thereby enabling the greater expression of S100A8 and S100A9 as an autocrine feedback loop, or the maintenance of cell proliferation. However, how S100A8/9 is involved in intracellular signaling networks in different cell types remains unclear.

Tumor and serum S100B has been used as a diagnostic marker for advanced melanoma, and a glioma xenograft mouse model has shown that an aberrantly upregulated tumor S100B level promoted tumor growth and recruited tumor promoting myeloid cells to the tumor sites. In contrast to S100A8 and S100A9 (tumor stromal origin), S100B is expressed in tumor cells in melanoma and gliomas. As an intracellular regulator, the $Ca2^{+}$-bound S100B protein directly interacts with tumor suppressor protein p53, thereby sequestering p53 protein in the cytoplasmic region, which prevents tumor cells from undergoing apoptosis or cell cycle arrest [129, 130]. An association of S100B with cell migration and cell motility via a Src-mediated pathway was also reported in glial cells [131]. Once secreted into the extracellular spaces, S100B might affect the nearby cells by interacting with cell membrane receptors, as mentioned above, and then induce secretion of the proinflammatory cytokines, TNFα, IL1, and IL6 [123]. In patients with melanoma or in B16 melanoma-bearing mice, the S100B level is considerably enhanced in the blood, where circulating melanoma cells would secret S100B, but this serum S100b is viewed as a consequence of the advanced melanoma, not the cause of it [132].

1.2.2 Serum IL6

In 1983, interleukin 6 (IL6) was first identified to induce the maturation of B cells into immunoglobulin-producing cells. Accumulating evidence has now extended its role as a pleiotropic bioactive molecule that has multiple functions in the human body [133]. Its functions are associated with diverse physiologic and pathologic conditions, such as liver cell regeneration and glucose and lipid metabolism: It is a key mediator of insulin resistance, myocardial infarction, T cell priming in the secondary lymphoid organs, autoimmune disease (e.g., rheumatoid arthritis), atherosclerosis, sepsis, and tumorigenesis [133-135].

The expressions of IL6 and its receptor IL6R are detected in several cell types: hepatocytes, endothelial cells, fibroblasts, myocytes, and some leukocytes, including monocytes, macrophages, T cells, and dendritic cells [136]. The IL6/IL6R complex, however, does not turn on downstream signaling pathways; instead, this complex binds to the co-stimulatory membrane-anchored receptor, gp130, which is ubiquitously expressed in most cell types. Two IL6/IL6R /gp130 trimers form a hexamer complex, which acts as a signal transducer that mainly activates the STAT3 transcription factor, as well as PI3K and MAPK (IL6 classical signaling) [137]. Adding more complexity, IL6 in the extracellular space and in the blood can bind to the soluble form of the IL6 receptor (sIL6R), which is cleaved by the cell membrane-bound metalloproteinases, ADAM10 and ADAM17, and subsequently shed from the cellular membrane. Thereafter, this IL6/sIL6R complex binds to cell membrane-anchored gp130 and turns on the alternative IL6-mediated signaling pathway (IL6 trans-signaling). The alternative Il6 signaling mechanism enables IL6R-negative cells to respond to IL6, thereby conferring multiple availabilities to IL6 [138, 139]. Moreover, a soluble form of gp130 (sgp130) is also found in the body and acts as an antagonist to block the circulating IL6/sIL6R complex. In the steady state, sgp130 blunts the activation of IL6 signaling, but in

IL6-related pathological conditions, serum IL6 levels abruptly peak at the maximum and overwhelm the activation of IL6 trans-signaling (figure 6) [140-144].

As an inflammatory cytokine, IL6, like IL1, TNF, and TGFβ, plays an important role in the maintenance of immune homeostasis, especially in the control of the activation of T cell and humoral responses. For example, in the lymph nodes, antigen-presenting dendritic cells activate T cell priming by the IL6/IL6R/gp130 signaling pathway. By contrast, for T cell trafficking, the IL6/sIL6R/gp130 signaling pathway is activated in the high endothelial venules (HEV), increasing their adhesiveness through the expression of ICAM1. Chronic inflammation is characterized by prolonged activation of the IL6 signaling, which is often aberrantly distorted and amplified through the circulation system. In this context, IL6 signaling is presumed to be somehow closely associated with cancers, as some subtypes often develop from chronic inflammation. Interestingly, a high concentration of serum IL6 has been extensively correlated with poor prognosis in human melanoma and gastric, pancreatic, lung, breast, and colorectal cancers. IL6 in the TME can be expressed in tumor parenchyma, tumor cells, and the tumor stroma, as well as tumor-promoting cell types (e.g., CAFs, MDSCs, TAMs, and $CD4^+$ regulatory cells, Th17, and Tregs). In tumor cells, IL6 trans-signaling is, in part, aberrantly activated by genetic and epigenetic changes, which helps tumor cells maintain proliferation or block apoptotic signals. In the tumor stroma, some tumor-promoting cells (e.g., MDSCs and Tregs) are present and serve as a source of sIL6R to boost IL6 trans-signaling at tumor sites, thereby driving the establishment of a tumor-favoring niche in the TME. This includes stimulation of angiogenesis by VEGFA and bFGF, which are downstream cytokines of trans-IL6 signaling, and activation or recruitment of tumor-promoting leukocyte subsets [134].

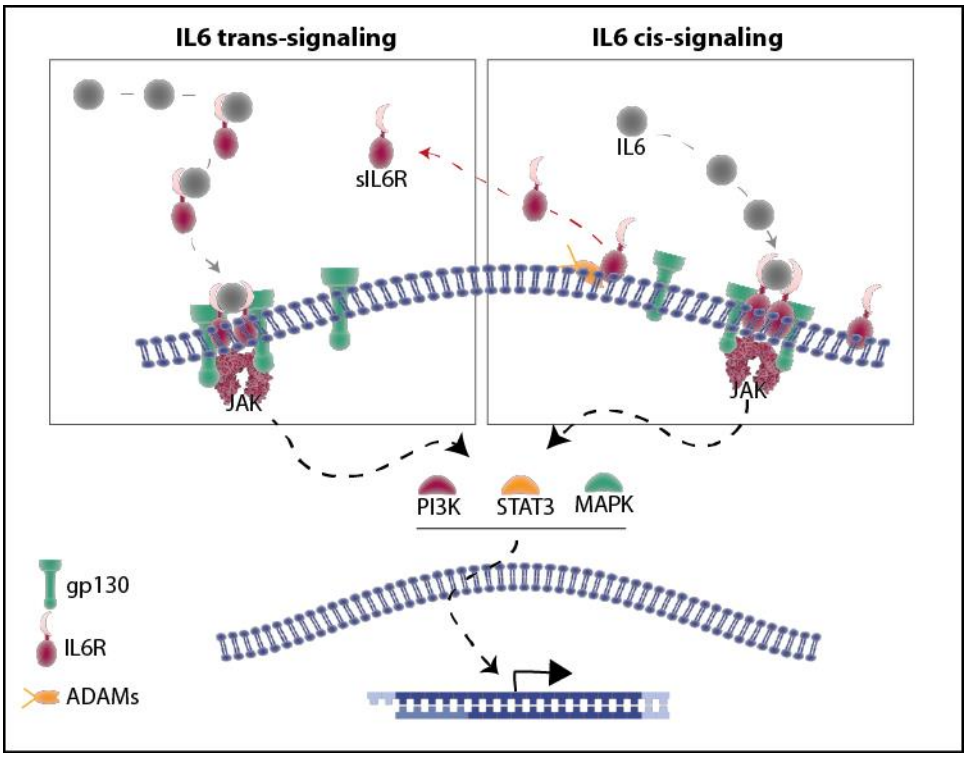


Figure. 6. Interaction of IL6 with IL6 receptor: classic (*cis*)-and *trans*-signaling

Taken together, the evidence points to an intimate involvement of cis- and trans-IL6 signaling pathways in tumorigenesis; this unexpectedly lies on the tumor-promoting side rather than in anti-tumor immunity. The origin and function of serum IL6 is still elusive in patients with cancers, but many advanced cancer types show an enhanced serum IL6 level. In the light of this, IL6 should be more extensively investigated as a therapeutic target.

2 AIM OF THE THESIS

In the last two decades, the concept of tumor biology has abruptly changed, so that tumors are indeed appreciated as complex tissues, rather than as lumps of chaotic neoplastic cells. Emerging evidence now points to an important role of the TME in regulating tumorigenesis. In this thesis, we studied the role of tumor pericytes as one of the crucial regulatory components in the TME.

PAPER I: *To establish the role of tumor pericytes in regulating MDSC bursts*

PAPER II: *To confirm the close interplay of tumor pericytes and perivascular myofibroblasts*

3 RESULTS AND DISCUSSION

3.1 PAPER I

In paper I, tumor pericytes are studied with a focus especially on how they interplay with TILs in the TME. The use of syngeneic subcutaneous tumors (B16 melanoma and Lewis lung carcinoma) in pericyte-deficient transgenic mice (*pdgfb*$^{ret/ret}$) demonstrated that the numbers of tumor-promoting TILs and MDSCs were increased both in the blood and at the tumor sites in the pericyte-deficient, tumor-bearing group, when compared to the tumor-bearing littermate control group. This systemic MDSC boost did not occur in the pericyte-rescued B16 melanoma (B16-PDGF-B) cells, implying that the tumor pericytes did indeed regulate MDSC activation and recruitment. The observed higher frequency of MDSCs in the blood of pericyte-deficient, tumor-bearing mice suggested the occurrence of a release of MDSC-related bioactive molecules from the TME or the circulating MDSC to the blood, thereby indicating an involvement in MDSC recruitment and expansion. Surprisingly, the level of serum IL6, one of the potential MDSC-recruiting factors, was also increased, together with the MDSC population. The increased serum IL6 level was downregulated in the pericyte-rescued B16 melanoma (B16-PDGF-B).

Serum IL6 is used as a prognostic marker for human melanoma, gastric, pancreatic, lung, breast, and colorectal cancers and it has an as yet unexplained close relationship with TILs in the TME. However, how serum IL6 affects tumorigenesis, and whether it is the cause or the consequence of tumorigenesis, remains to be elucidated. In light of this, the underlying mechanism of serum IL6 as it relates to tumor pericytes is worth studying. The following mechanistic study was therefore performed using IL6 shRNA-mediated gene knockdown cell lines (B16-IL6^{KD}) and hypoxia experiments. The results confirmed that the increase in serum IL6 is tumor cell-derived in the hypoxic TME. The reduction in IL6 expression from B16 melanoma, as expected, also reversed the systemic MDSC bursts to a similar level to that seen in the tumor-bearing, littermate control group. These experiments confirm a role for serum IL6 as a hypoxia-responsive, MDSC-recruiting factor.

Pericyte-deficient tumor vasculature serves to elicit a hypoxic TME, because of the lack of integrity of this vasculature. As a tumor grows to an advanced stage, it tends to have a more irregular tumor vasculature, with less pericyte coverage, and its TME tends to be more hypoxic. This might explain why patients or animals with advanced solid tumors show a high concentration of serum IL6. This would be an interesting topic for a future study, to determine if the serum IL6 and the MDSCs in the patients are correlated with diverse types and degrees of cancers. The T241 murine sarcoma showed a similar MDSC expansion, independent of tumor pericyte coverage, which might reflect that T241 sarcoma cells, unlike B16 and LLC cells, do not upregulate IL6 under hypoxic conditions. This is one example where a different type of tumor cell might confer its own discrete TME.
Each B16 and LLC tumor in the *pdgfb*$^{ret/ret}$ mice exhibited a different type of tumor malignancy, even though both tumors acquired the same tumor-promoting armament in the

form of MDSCs. The LLC tumor in *pdgfb*$^{ret/ret}$ mice grows more rapidly, but the B16 tumor in *pdgfb*$^{ret/ret}$ mice grows similarly, while more tumor cells disseminate into the peripheral blood. This higher frequency of circulating tumor cells does not occur in the LLC tumor in these mice. This discrepancy was explained by the fact that each tumor has a different subset of MDSCs: B16 tumors have a relatively higher proportion of monocytic MDSCs, while LLC tumors have mostly granulocytic MDSCs. In this paper, the exact mechanism underlying the MDSC subset and different tumor phenotype is still elusive. Further study is needed to determine which TME constituent interplays with tumor pericytes and activates MDSC in a different way. In the blood, MDSCs belong to mostly granulocytic subset in both B16 and LLC tumor model, implying that MDSCs are first recruited by the HIF1α/IL6 axis and then activated in a different way at each tumor site.

The human study with breast cancer cohorts showed a fortunate, meaningful, and correlated outcome, where the expression of pericyte-related genes (CSPG4, RGS5,ITGA7, CD248) are inversely correlated with the expression of human MDSC markers (S100A9, CD33) and this correlation appears to have consequential relevance to the survival rate of patients with breast cancers. As discussed in the introduction, due to the lack of the specific markers for tumor pericytes, the gene expression analysis used above is limited in its accuracy. Nevertheless, it is still of importance, in that the clusters of pericyte-related genes displayed a significant correlation with MDSC markers.

3.2 PAPER II

Recently, tumor pericytes have been shown to regulate tumor-favoring TILs and MDSCs via aberrant tumor vasculature. In paper II, a new idea is proposed whereby tumor pericytes in B16 melanoma play a tumor type-specific role by regulating myofibroblast recruitment. In B16 tumors, PDGFRα-expressing cells were found in tumor vasculature and, in turn, these perivascular PDGFRα-expressing cells appeared more frequently in pericyte-deficient tumor vasculature. By contrast, in LLC tumors, PDGFRα-expressing cells were mostly localized outside of the tumor vasculature and showed no difference in frequency between pericyte-deficient, *pdgfb*$^{ret/ret}$ and littermate control mice, indicating TME heterogeneity between the tumor types.

The perivascular PDGFRα^{+} cells were further characterized using immunochemistry and quantitative PCR analysis with FACS-sorted samples: PDGFRα^{+}/PDGFRβ^{+} as the perivascular PDGFRα^{+} cells and PDGFRα^{+}/PDGFRβ^{-} as the pericytes, and identified as a subset of myofibroblasts that differentially express mRNA for FAP, HGF, CXCL12, and Wnt5a. Interestingly, the perivascular myofibroblasts (PDGFRα^{+}) express comparable amounts of NG2, PDGFRβ, and αSMA to those expressed by pericytes, but the fibroblasts do not express mRNA of RGS5, another marker for pericytes. As discussed in the introduction, no pericyte-specific marker has yet been found. Correct detection of tumor pericytes, especially by immunostaining, requires careful selection of marker proteins and must take into consideration the tumor type and stage. In the light of this notion, RGS5 should be

considered as a pericyte marker in the melanoma context. However, this study was performed with a mouse model, and requires verification by studies on human melanomas.

This study also showed that serum S100B is involved in the generation of the higher frequency of the perivascular myofibroblasts observed in the pericyte-deficient B16 tumor, as determined using the S100B-knockdown B16 (B16-S100B^{KD}) tumor model. The S100B protein is highly expressed in melanoma cells in humans and mice, and it is a bifunctional protein, as discussed in the introduction. The S100B secreted into the extracellular space binds to its receptor, RAGE, which is abundant in endothelial cells and leukocytes. This is followed by cellular responses, including secretion of pro-inflammatory cytokines and chemokines and possibly transcytosis through the endothelium. In the melanoma, serum S100B is used as a prognostic marker. Nevertheless, the way that serum S100B affects tumorigenesis in melanoma and in the blood is not yet understood. S100B expression is considered to be a consequence of the increased tumor burden. Our experimental setting revealed a distinctive function of serum S100B in recruiting the perivascular myofibroblasts.

The function of recruited myofibroblasts in terms of the increase in serum S100B remains to be established. Even though the pericyte-deficient B16 tumor showed an increased serum S100B (a marker of poor prognosis for melanoma), it did not exhibit any difference in tumor malignancy. As shown in paper I, pericyte-deficient B16 melanoma showed more MDSC bursts. Therefore, the MDSCs are presumed to behave as the major controller, driving melanoma to greater malignancy, while the perivascular myofibroblasts, as a minor modulators, react in accordance with MDSCs or against a tumor-promoting TME. Conceivably, serum S100B may enhance the recruitment of perivascular myofibroblasts as "defenders" of the host-defense system. As shown in the introduction, recent evidence obtained in murine pancreatic adenocarcinoma has suggested a protective role of the TME against tumor malignancy.

Lastly, this study has revealed a possible reason for the increased serum S100B levels observed in pericyte-deficient B16 tumors. In pericyte-detached endothelial cells, tyrosine-14 of Cav1 had a higher phosphorylation level when compared to Cav1 of pericyte-attached endothelial cells. This was confirmed both in the *in vitro* cell culture experiment and in the B16 tumor vascular fragment in *pdgfb*$^{ret/ret}$ and littermate control mice. Moreover, lung vascular fragments in tumor-free, pericyte-deficient mice also showed an enhanced phosphorylation of Cav1, indicating that pericyte coverage regulates the endothelial Cav1 state, but not in a tumor-limiting manner. The S100B receptor, RAGE, has been reported to have a caveolar localization, so that, upon S100B binding, the RAGE/S100B complex is internalized with the help of phospho-Cav1 (an essential prerequisite for S100B internalization). The manner by which pericytes control endothelial Cav1 deserves further investigation.

4 ACKNOWLEDGMENTS

I am finally writing the "Acknowledgment" section—which I never imagined I would get to before. It reminds me of a time, 5 years ago, in March, 2010. I came here for an interview with Guillem and some people in Christer's group about entering the Ph.D. program. It was actually the first time in my life that I had visited Europe. Everything was so different from the place where I lived: people's appearances, streets, downtowns, food, transportation, trees, sky—the social system and the way people thought. The most impressive thing was the coldness, around -15°C on the slushy street. As I remember, it was one of the coldest Marches recorded. However, when I arrived in the lab, I realized that all the things inside were warm and bright, even though outside was still cold and dark. Now, I am sitting in the same lab where those bright people still work next to me. I would like to express my gratitude to the bright people whom I have met during my last 5 years' special journey.

Christer Betsholtz, my co-supervisor, was the reason that I came to Karolinska for the Ph.D. program. Six years ago, in South Korea, I read his paper about pericytes, published in 2003. It was so awesome and inspiring that I began to consider whether to quit my job and do a Ph.D. So, I came here and met Christer, who was a great and humorous scientist. Even though I haven't talked much with him, his comments have always been concise and correct. I sincerely appreciate his help.

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