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Alexander Birbrair *Editor*

Tumor Microenvironment

Signaling Pathways – Part A

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Preface

This book's initial title was *Tumor Microenvironment*. However, due to the current great interest in this topic, we were able to assemble more chapters than would fit in one book, covering tumor microenvironment biology from different perspectives. Therefore, the book was subdivided into several volumes.

This book *Tumor microenvironment: Signaling Pathways - Part A* presents contributions by expert researchers and clinicians in the multidisciplinary areas of medical and biological research. The chapters provide timely detailed overviews of recent advances in the field. This book describes the major contributions of different signaling pathways in the tumor microenvironment during cancer development. Further insights into these mechanisms will have important implications for our understanding of cancer initiation, development, and progression. The authors focus on the modern methodologies and the leading-edge concepts in the field of cancer biology. In recent years, remarkable progress has been made in the identification and characterization of different components of the tumor microenvironment in several tissues using state-of-the-art techniques. These advantages facilitated identification of key targets and definition of the molecular basis of cancer progression within different organs. Thus, this book is an attempt to describe the most recent developments in the area of tumor biology which is one of the emergent hot topics in the field of molecular and cellular biology today. Here, we present a selected collection of detailed chapters on what we know so far about the signaling pathways in the tumor microenvironment in various tissues. Eight chapters written by experts in the field summarize the present knowledge about distinct signaling pathways during tumor development.

Olivier Meurette from Lyon University discusses how Notch signaling shapes the tumor microenvironment. Yaacov Ben-David and colleagues from Guizhou Medical University describe the role of erythropoietin signaling in the microenvironment of tumors. Stephan Niland and Johannes A. Eble from the University of Münster update us with what we know about Neuropilin signaling in the tumor microenvironment. Ciuffreda Ludovica and colleagues from Regina Elena National Cancer Institute summarize current knowledge on mTOR signaling in integrating cues between cancer cells and their microenvironment. Fabian Benencia and colleagues from Ohio University address the importance of Toll-like receptors signaling in the cancer microenvironment. Marina Pajic and colleagues from the University of New South Wales focus on how Rho-ROCK signaling is contributing in the tumor

microenvironment. Gabriela Schneider from the University of Louisville introduces our current knowledge about S1P signaling in the tumor microenvironment. Finally, Xue-Feng Bai and colleagues from Ohio State University give an overview of the CD200-CD200R signaling in the regulation of the tumor immune microenvironment.

It is hoped that the articles published in this book will become a source of reference and inspiration for future research ideas. I would like to express my deep gratitude to my wife Veranika Ushakova and Mr. Murugesan Tamilsevan from Springer, who helped at every step of the execution of this project.

Belo Horizonte, Minas Gerais, Brazil Alexander Birbrair

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1 Shaping of the Tumor Microenvironment by Notch Signaling

Olivier Meurette

Abstract

The tumor microenvironment (TME) has become a major concern of cancer research both from a basic and a therapeutic point of view. Understanding the effect of a signaling pathway—and thus the effect of its targeting—in every aspect of the microenvironment is a prerequisite to predict and analyze the effect of a therapy. The Notch signaling pathway is involved in every component of the TME as well as in the interaction between the different parts of the TME. This review aims at describing how Notch signaling is impacting the TME and the consequences this may have when modulating Notch signaling in a therapeutic perspective.

Keywords

Cancer · Notch signaling · Microenvironment · Heterotypic interaction · Jag1 · CAF · Stem cell · Tumor angiogenesis · Targeted therapy

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1.1 Introduction

The Notch signaling pathway is a juxtacrine signaling pathway that mediates cell fate decision between neighbor cells. Recent reviews described precisely the molecular signaling of Notch pathway [1, 10, 11]; we will therefore only briefly introduce it. The Notch receptor is a transcription factor anchored at the membrane, which is released following interaction with a cognate ligand. Unfolding of the Notch regulatory region (NRR), due to the pulling force exerted by the ligand/extracellular part of the receptor endocytosis, unmasks the cleavage sites for ADAM10/17 and the γ -secretase, allowing the release of the Notch intracellular domain (NICD) [2] (Fig. 1.1). Stability of NICD is regulated through posttranslational modifications such as ubiquitination [3] and phosphorylation [4]. Glycosylation of the receptors is also important in the regulation of interaction between ligands and receptors [5]. The dynamic [6] and strength [7] of NICD production depend on the ligands. Cycling or continuous production of NICD can discriminate ligands by differently affecting transcription regulation [1, 6]. In the nucleus, NICD forms a complex with CBF-1/Su(H)/LAG1 (CSL) transcription factor and induces the recruitment of the transcriptional co-activator mastermind-like (MAML) [8] (Fig. 1.1). The developmental stage and the genetic and epigenetic landscape of the receiving cell will also modulate the response to

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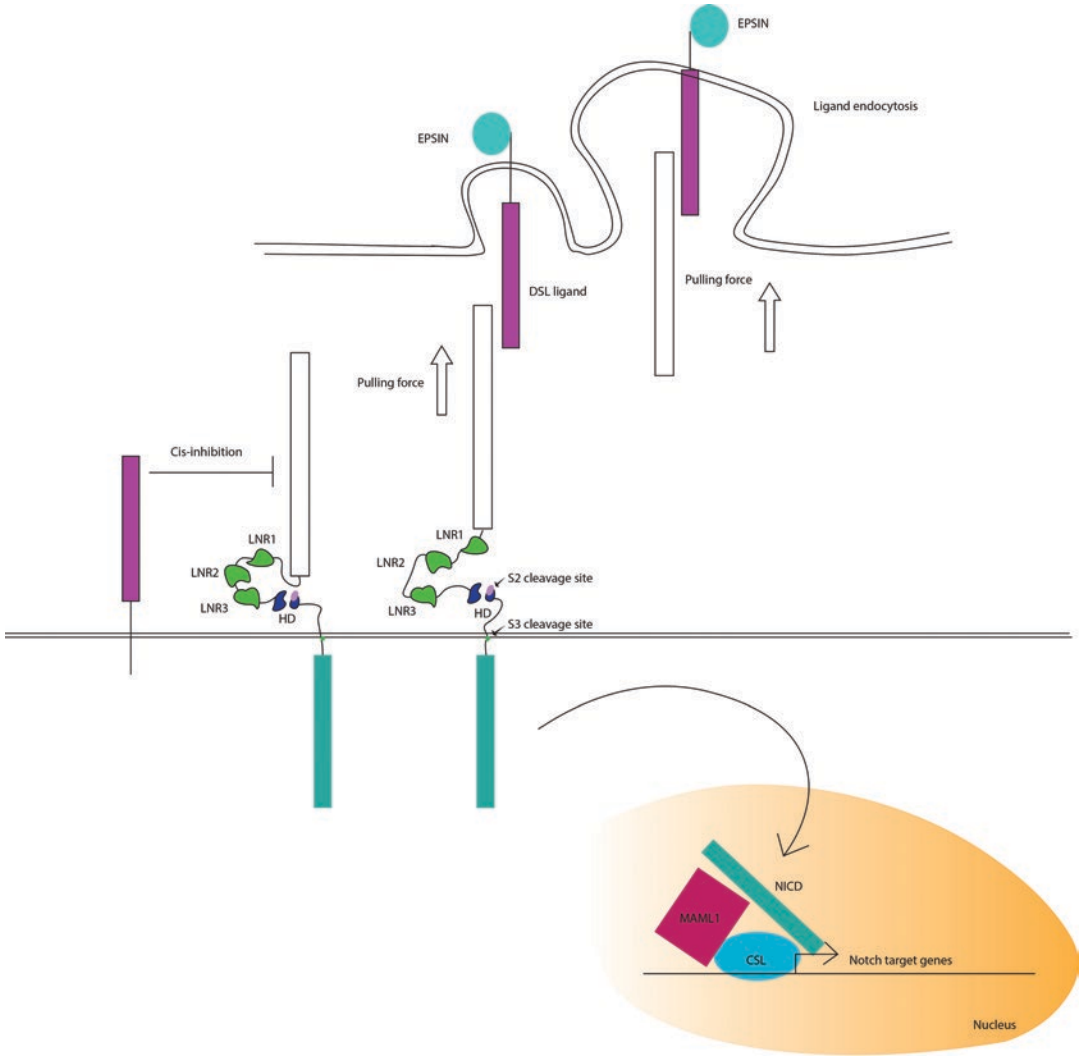

Fig. 1.1 Juxtacrine Notch signaling. Notch signaling is initiated in a juxtacrine manner between a signal-sending cell expressing the ligands (DSL ligands, Jag1–2, Dll1, Dll3 or Dll4) activating the receptor of a neighboring cell expressing the receptor (Notch 1–4). Ligand expressed on the signal-sending cell inhibits signaling through the receptor expressed on the same cell (cis-inhibition). Epsin-dependent pulling force exerted by ligand endocytosis is inducing transconformation of the NRR (Notch Regulatory Region) allowing exposure of S2 and next S3 cleavage sites. These two sequential cleavages mediated by ADAM (S2 cleavage) and γ-secretase (S3-cleavage) leads to the release of the Notch intracellular domain (NICD). Upon translocation into the nucleus, NICD recruits Mastermind-like (MAML) and CSL to activate transcription

the Notch activation. Ligands expressed by the signal-sending cells are transmembrane proteins of the Delta/Serrate/LAG-2 (DSL) family. In mammals, this family is composed of three delta-like ligands (Dll1, Dll3, and Dll4) and two jagged ligands (Jag1 and Jag2). Although ligand expressed in the signal-receiving cell might activate signaling (cis-activation) [9], it is widely accepted that they have an inhibition ability (referred to as cis-inhibition) which maintain the different fate of the signal-sending and the signal-receiving cells. Four receptors (Notch1–4) have been described in mammals and can mediate the canonical signaling [10].

Extensively studied for its important role in cell fate decision during development, the

implication of Notch signaling is now well described in cancers [10–12]. In T-cell acute lymphoblastic leukemia (T-ALL), activating mutations in the Notch1 receptor are present in more than 50% of the cases [13]. Notch signaling is aberrantly activated in different solid tumors, but mutations are sparse [10]. This raises the possibility that the Notch receptor is activated upstream through ligand interactions [14] and therefore implicates a signal-sending and a signal-receiving cell. Furthermore, Notch ligands and receptors are widely expressed in the different compartments of the tumor microenvironment (TME). Occurrence of heterotypic activation between distinct cell populations is thus likely. For example, in small cell lung cancers, Notch signaling maintains heterogeneity between different populations of tumor cells, and Notch activation presents a salt and pepper pattern [15]. Although the view that cancer is a dynamic structure evolving through interaction between the altered cells and their environment emerged a century ago, the field of TME research exploded in the 1970s with the study of the role of angiogenesis and the immune system [16]. It is now well accepted that to have a therapeutic action on cancers, one must have a comprehensive understanding of all its components. It was proposed to define the tumor environment in six different layers from the tumor cell-only environment, the niche, the confined, proximal, peripheral and organismal environment [17]. We focus here on the modulation of Notch signaling by the interaction between cells belonging to the confined environment, knowing that modulation of Notch signaling in the different tumor compartments will participate in shaping all the layers of the tumor environment. Notch signaling has been described to regulate angiogenesis, activation of fibroblasts, maintenance of the stem cell niche, the immune infiltrate and may also be regulated by physical and chemical heterogeneity in the TME (Fig. 1.2). We will first look at the expression of Notch ligands and receptors in the TME and address their regulation through cross talk with other signaling pathways. We will then artificially separate the TME according to cell types, treating the role of Notch signaling in the vasculature, the cancer stem cell niche, the metastatic niche, the immune infiltrate. We will then discuss how one should take these data in consideration when inhibiting Notch in cancers and finally suggest open questions and new avenues.

1.2 Notch Receptors and Ligands in the TME

As Notch receptors and ligands are widely expressed in the different compartments of the TME, heterotypic interactions involving different cell types have been described [18] (Table 1.1). A striking fact is that every type of cells composing the microenvironment expresses ligands and receptors, which allow Notch signaling to participate to many features of the TME (Fig. 1.2). We also observe that Notch signaling can be activated from the stroma toward the tumor, from the tumor toward the stroma and also between stromal cells. It is interesting to note that regarding endothelial cells (as discussed in more details in the next section) many studies describe a role of ligands expressed by endothelial cells in activating Notch receptors in cancer cells. Regarding stroma/stroma and cancer cell/cancer cell interactions, few studies described a role for Notch signaling in this context. Whereas in different situations, specific ligands and/or receptors may be expressed in different parts of the TME, an extensive literature search reveals that the expression of Notch ligands and receptors in every component of the TME is a general observation. It is also to be noted that looking at ligand expression at a specific time does not describe the dynamic regulation of ligands and receptors in the TME. Indeed, Notch pathway members are regulated though cross talks with the other TME-regulated pathways.

1.3 Regulation of Notch Ligands and Receptors in the TME

Notch receptors and ligands can be regulated by characteristics of the TME. This is an important point that emphasizes the potential dynamic

Table 1.1. Heterotypic induction of Notch signaling in the confined TME

Signal-sending cell	Signal-receiving cell	Ligand/ receptor	Function in the TME	Tumor type	References
Stroma toward tumor communication					
Endothelial cells	Cancer cells	Dll4/Notch3	Dormancy escape	T-ALL	[48]
Endothelial cells	Cancer cells	sJag1 (soluble Jagged-1)/ ND	Cancer stem cell phenotype	Colorectal cancer	[46]
Endothelial cells	Cancer stem cells	Jag1/Notch1	Cancer stem cells self-renewal	Glioblastoma	[47]
Endothelial cells	Cancer cells	Jag1/Notch2	Resistance and aggressiveness	B cell lymphoma	[31]
Endothelial cells	Cancer cells	Dll4/Notch1	Cancer cell survival	Ovarian cancer	[45]
Endothelial cells	Cancer cells	Dll4/Notch1	Metastasis formation	Colorectal cancer	[49]
CAF (Cancer Associated Fibroblasts)	Cancer stem cells	ND/Notch3	LDS1-dependent increase of stem cells	HCC	[58]
Fibroblasts	Cancer cells	Jag1/Notch3	Resistance to chemotherapy	Breast cancer	[34]
Astrocyte	Cancer cells	Jag1/Notch1	Cancer stem cells self-renewal	Breast cancer brain metastasis	[20]
Cancer cell toward stroma communication					
Tumor cells	Stroma	Jag1/ND	Stroma activation	Prostate cancers	[42]
Tumor cells	Bone marrow stromal cells	Jag1/ND	Differentiation of bone marrow stromal cells into CAFs	Colon cancer	[56]
Tumor cells	Mesenchymal stromal cells	Jag1, Jag2, Dll1/Notch2	Activation of wnt pathway in CLL	CLL	[30]
Tumor cells	Endothelial cell	Dll4, Jag1/ Notch1	EC senescence and increased metastasis	Lung, ovary, breast cancers	[44]
Tumor cells	Osteoblasts	Jag1/ND	Increased metastasis	Breast cancer	[68]
Cancer cell toward cancer cell					
Tumor cells	Cancer stem cell	Jag1/ND	Stem cell phenotype	TNBC	[19]
Neuroendocrine tumor cells	Non-neuroendocrine tumor cells	Jag1, Jag2, Dll3/Notch1, 2 and 3	Intra-tumoral differentiation	SCLC	[15]

ND not determined

CAF Cancer Associated Fibroblasts; *sJag1* soluble Jagged-1; *EMT* epithelial to mesenchymal transition, *TNBC* triple negative breast cancer, *SCLC* small cell lung carcinoma, *CLL* chronic lymphocytic leukemia, *HCC* hepato cellular carcinoma

regulation and activation of Notch signaling in the microenvironment. Inflammatory cytokines have been described as major regulators of Notch ligands. NF-κB activation leads to Jag1 expression in triple negative breast cancer cells [19]. Cancer cell-derived IL1-β mediates Jag1 upregulation through NF-κB in astrocytes [20]. Jag1 is also regulated by TGF-β [21] and by IL-6, secreted by cancer cells in breast cancer [22]. The IL6/STAT3/Notch axis is important in communication between cancer cells and CAFs in HCC [23], between mesenchymal stem cells and cancer cells in colon cancer [24], and between myeloid-derived suppressor cells (MDSC) and cancer cells [25]. Notch signaling also regulates IL6 secretion which has been shown to activate Notch signaling in multiple myeloma participating to remodeling the bone marrow niche [26]. Wnt signaling is also an important cross-talk partner of Notch signaling both in development and in disease [27]. Jag1 is a target of Wnt oncogenic activation in breast and colon cancers [28, 29]. In Chronic lymphocytic leukemia (CLL), Notch2 expression in mesenchymal stromal

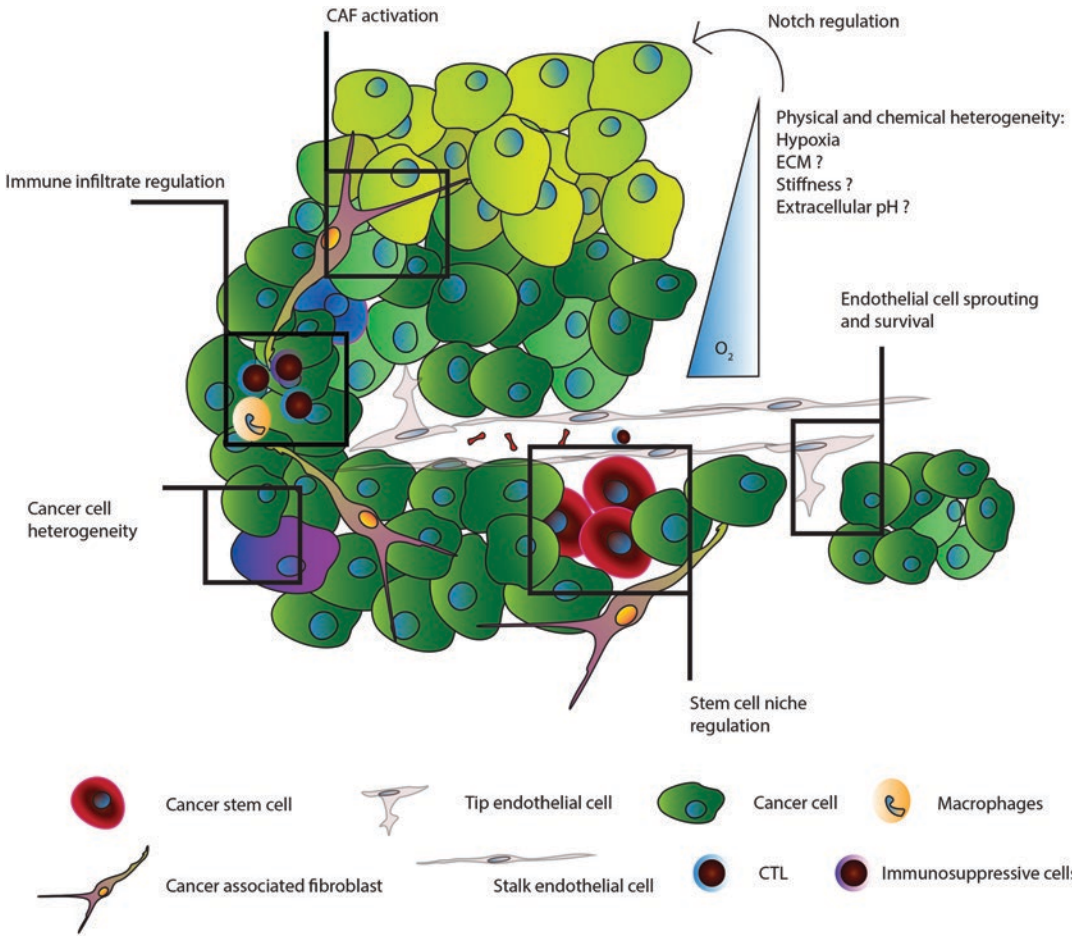

Fig. 1.2 The varied roles of Notch signaling in the tumor microenvironment

cells is responsive to ligands expressed by CLL cells to activate Wnt signaling [30]. In B-cell lymphoma, FGF4 secretion by cancer cells induces Jag1 expression in neighbor EC that in turn activates Notch2 in lymphoma cells [31]. In breast cancer, fibroblast-derived CCL2 induces Notch1 in breast cancer cells [32]. Notch ligands and receptors are also modulated in the immune infiltrate [33], and this will be discussed in a dedicated section.

Atypical activation of Notch signaling through exosomes and microvesicles may also regulate the TME. Stromal cells-derived exosomes foster notch activation though activation of STAT1 [34]. Notch activation may also be mediated through plasma-derived extracellular microvesicles containing Notch2 receptor [35].

1.4 Notch-Mediated Regulation of the Tumor Vasculature

1.4.1 Regulation of the Tip/Stalk Ratio

The tumor induces sprouting of existing vessels, recruiting vessels which supply the tumor cells with nutrients. Sprouting angiogenesis depends on the regulation of the endothelial cells (EC) phenotype. Upon stimulation with pro-angiogenic factors, EC adopt a tip cell phenotype, which enable initiation of new vessels [36]. Notch signaling is determinant in the control of this balance between tip and stalk cell. Indeed, Dll4 is expressed in tip cells and activates Notch

signaling in stalk cell inducing downregulation of VEGFR2 and therefore preventing the cell to respond to VEGF stimulation [37]. Interestingly, Jag1 has the opposite effect, probably acting as a competitive inhibitor of Dll4-Notch interaction [38]. This mechanism is regulated in the context of the tumor where the balance between endothelial expression of Dll4 and Jag1 has a major impact on the tumor vasculature architecture. Indeed, high Jag1 levels may lead to poorly perfused and chaotic angiogenesis by destabilizing the tip/stalk phenotype [39]. It was observed using endothelial specific manipulation of Jag1 levels that Jag1 overexpression in endothelial cells (EC) favors tumor vasculature. In contrast, a loss of function of Jag1 in EC leads to decreased vasculature and tumor growth [40].

1.4.2 Regulation of the Vasculature through Ligands Expressed by the Tumor

As endothelial cells are susceptible to Notch activation, ligands presented by other cells can affect vascularization. In particular, Jag1 expressed by tumor cells has an important impact on promoting tumor vascularization [41–43] (Table 1.1). It was first described that overexpressing Jag1 in cancer cell induced an increased tumor growth and microvessel density when co-injected with EC [43]. Tumor-derived Jag1 can also limit EC cell death, which could account for this effect [41]. Jag1 overexpression by prostate cancer cells has also been shown to increase tumor vascularization [42]. Tumor-induced Notch activation in the endothelium can also lead to endothelial cells senescence through Notch1 activation in EC by tumor and myeloid cells that induces inflammation and increases metastasis [44]. Many studies point to a role of Jag1 expressed by tumor cells in controlling the vasculature. Other ligands are expressed by tumor cells, but no reports of their role in activating Notch signaling in the vasculature has been described.

1.4.3 Regulation of Notch Signaling in the Tumor Compartment by EC-Derived Ligands

Notch ligands are expressed by EC, and these ligands can also affect Notch signaling in the tumor compartment (Table 1.1). For example, specifically targeting mouse Dll4 in xenograft model reduces Notch activity in cancer cells, showing that Dll4 expressed by EC activates Notch signaling in adjacent cancer cells [45]. It has also been shown in glioblastoma that Notch activity in cancer cells is higher at proximity of EC [46, 47]. This has been demonstrated in many different cancer types and may involve different Notch receptors and ligands. For example, Dll4 expressed by endothelial cells activates Notch3 in T-ALL cells allowing dormancy escape [48]. In colon cancer, Notch activation in cancer cells by adjacent blood vessel cells has also been shown to increase trans-endothelial migration and metastasis [49]. It was also demonstrated that expression of Jag1 by EC activates Notch signaling in local pericyte precursor cells to induce their differentiation [50]. Endothelial cell-expressed ligands can also regulate cancer stem cell traits, which will be discussed later in this review. Many aspects of the tumor vasculature are thus regulated by Notch signaling which controls differentiation and survival of EC, through intrinsic mechanisms or through heterotypic interactions with the tumor.

1.5 Notch-Mediated Regulation of Cancer-Associated Fibroblasts

Notch signaling is also involved in regulating fibroblast activation, another major determinant of the TME. Indeed, the loss of CSL specifically in mesenchymal cells induces dermal atrophy and inflammation leading to multifocal keratinocytes tumors in mice [51]. Alteration of Notch signaling in the stroma is thus sufficient to

induce tumors. CSL directly represses fibroblast senescence and CAF activation [52]. Notch1 is a major regulator of the senescence-associated secretory phenotype (SASP) in fibroblasts [53] and is implicated in secondary senescence [54]. Loss of Notch1 may orientate the senescence-associated secretory phenotype (SASP) toward a pro-inflammatory one, promoting tumor initiation. Fibroblasts are also involved in cross talk with the epithelial compartment since epidermal Notch1 loss is associated with non-cell autonomous change in the stroma showing an increase in immune infiltrate associated with the activation of dermal fibroblasts which express α-SMA, as well as fibroblast-derived epidermal mitogens [55]. In other models, activation of Notch rather than its loss leads to activation of fibroblasts. In colon cancer, Notch signaling mediates activation of bone marrow-derived stromal cell (BMSC) into activated fibroblasts [56]. In a prostate cancer model, Jag1 expression by cancer cells promoted an increase in activated fibroblasts expressing α-SMA and development of a reactive stroma with increased tenascin-C and collagen [42].

CAF may also activate Notch signaling in the cancer cells. For example, in breast cancers, paracrine secretion of IL6 by CAF induced Notch activation in breast cancer cell lines [57], and CAF can also promote the cancer stem cell phenotype by secreting CCL2 [32]. CAF can also induce Notch3 leading to increased cancer stem cell phenotype in hepatocellular carcinoma (HCC) [58]. The Notch-mediated interaction between cancer cells and the mesenchymal compartment is also involved in resistance to chemotherapy. In breast cancer, fibroblasts express Notch ligand Jag1 that can interact with Notch3 in breast cancer cells and regulate resistance [34].

Notch signaling-mediated communication between CAFs and cancer cells is thus involved in controlling the inflammatory environment in cancer initiation and participates in mediating resistance and regulating stem cell in cancer progression.

1.6 Notch Signaling in the Stem Cell Niche

As it is the case for stem cells in normal tissues, the cancer-associated stem cells (cancer stem cells) are localized in specific niches. The role of Notch signaling in cancer stem cell maintenance has been well described and participates to the interest of targeting Notch in cancers [59]. Among DSL ligands, Jag1 has been extensively described as an important cue in the stem cell niche. In glioblastoma, Notch signaling is activated in cancer cells by ligands presented by the vascular niche [47, 60], and coculture of cancer cells with endothelial cells increases the population of cancer stem cells in a Notch-dependent manner [61]. In B-cell lymphomas, the vascular niche is implicated in presenting Jag1 to cancer cells [31]. In human colorectal cancers, N1ICD colocalized with CD133 marker of stem cells, at proximity of CD31 expressing endothelial cells and a soluble form of Jag1, secreted by endothelial cells, favors cancer stem cell self-renewal in vitro [46]. In vivo, in APC-deficient model of intestinal cancers, Jag1 is also involved in niche formation and stem cell proliferation [62]. In head and neck cancer cell lines, K lf 4 expression is regulated by Jag1 leading to the stem cell phenotype [63]. Therefore, among Notch ligands, Jag1 was described to be involved in maintaining the stem cell phenotype. Other ligand might be involved, but this still need to be confirmed. The stem cell phenotype is maintained in breast cancer through Notch1 induced by fibroblast-derived CCL2 [32]. Extra-cellular matrix proteins have also been shown to regulate Notch signaling in the stem cell niche. In breast cancer, tenascin-C has been shown to favor establishment of lung metastasis by stimulating the stem cell phenotype [64]. Tenascin-C has also been shown to be important in inducing the tumor-initiating cell phenotype in glioma through Notch activation [65].

As described, endothelial cells, to a lesser extent fibroblasts and the extracellular matrix, are thus involved in shaping the stem cell niche

through activation of Notch signaling. In physiological conditions, immune cells have been described to regulate the stem cell compartment through Notch signaling. It is the case of hair follicle stem cells that are maintained by Jag1 expressed by Tregs [66]. In the normal mammary gland, macrophages secrete Wnt ligands in response to mammary stem cells-expressed Dll1, which maintains the stem phenotype in the niche [67]. There is therefore evidence that Treg and macrophages can regulate the stem pool in normal organs. Assessing the role of immune cells in maintaining the pool of cancer stem cells still needs to be studied.

1.7 Notch-Mediated Shaping of the Metastatic Niche

Establishment by cancer cell of a favorable environment is crucial for the spreading of the disease and colonization of metastatic sites. The role of Notch signaling is here also of major importance in different metastatic contexts. Concerning the bone metastatic niche, cancer cell-derived Jag1 induces osteoclast differentiation through Notch activation in osteoblasts [68]. As already mentioned, interaction with tenascin-C is also important in the establishment of lung metastasis by breast cancer cells through activation of Notch signaling in the metastatic niche [64]. In brain metastasis, breast cancer cell secretion of IL-1β induces Jag1 expression in astrocytes, which then activates Notch signaling in cancer cells [20]. By regulating the tip/stalk ratio, Notch is also implicated in regulating the escape of metastasizing cancer cells from dormancy, as tip cells are associated with this process [69]. Notch is therefore not only involved in shaping the TME of the primary cancer site but also participated in the establishment of a favorable environment for metastatic spreading.

1.8 Notch-Mediated Shaping of the Tumor Immunity

The revolution of immunotherapies has shed new light on the importance of the tumor infiltrate in the treatment of cancers. It is now well accepted that many tumors elicit a specific anti-tumor response that is inhibited by the tumor through establishment of an immunosuppressive environment [70]. The tumor infiltrate is composed of both anti-tumor cells (cytotoxic T cells, M1 macrophages) and immunosuppressive cells (myeloid-derived suppressor cells (MDSC), Treg, M2 macrophages). In cancer, Notch signaling is determinant both in myeloid and lymphoid lineages [33, 71] and may have anti- and pro-tumor action questioning the effect of Notch targeting on the immune infiltrate [72]. Although the role of Notch signaling in the immune system has been well documented [73], its activity in regulating the tumor immunity is more difficult to study. We will in the following section discuss current data first on the regulation of the anti-tumor infiltrate by Notch and secondly its role in the regulation of the immunosuppressive environment (Fig. 1.3).

Notch signaling is regulating differentiation and activity of the cytotoxic $CD8^+$ T cells (cytotoxic T cell (CTL)) [74, 75]. In the context of cancer, anti-tumor CTL response was shown to depend on Notch2 expression by T cells [76]. In the same study, dendritic cells overexpressing Dll1 increased the anti-tumor response. Further supporting a role for Dll1 in this context, treatment using multivalent Dll1 elicited lymphocyte T differentiation and enhanced antigen-specific cytotoxicity [77]. Triggering Dll1-Notch pathway in bone marrow precursors restores T-cell function and restores immunosurveillance [78]. These data therefore demonstrate that Notch activity is in favor of cytotoxic T-cell activation. It has to be noted that Notch may in some context upregulate PD-1 expression and therefore inhibit CTL [79].

Regarding the immunosuppressive cells, tumor-associated macrophages (TAMs) express a transcriptomic signature showing activation of the Notch pathway [80]. The deletion of CSL in monocyte lineages blocked TAM-associated immunosuppressive functions [80]. TAM may be activated through heterotypic interaction in the TME since Jag1 expression by therapy-resistant cells could increase TAM markers in macrophages [81]. However, other studies showed that CSL was necessary for the induction of M1 versus

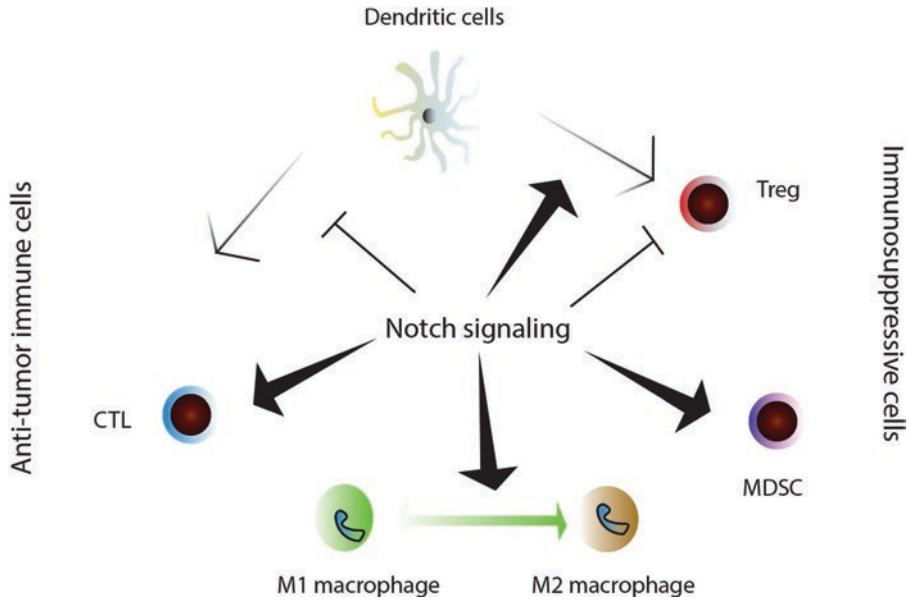

Fig. 1.3 Pleiotropic roles of Notch signaling in the tumour immunity. Notch signaling is regulating many features of tumor immune infiltrate including anti-tumor as well as immunosuppressive immune functions

M2 phenotype in response to LPS stimulation [82]. In the same line, forced activation of Notch signaling by expressing N1ICD in macrophages abrogated TAM function and reduced tumor growth [83]. Notch activity is thus controlling macrophages polarization and interactions with other cell types expressing Notch ligands in the TME could play a role. Given the ambivalent effect of CSL deletion and N1ICD overexpression, it is possible that the level of Notch activity controls TAM differentiation. Myeloid-derived suppressor cells (MDSC) are also important mediators of the tumor immunosuppressive environment in which Notch signaling plays an important role [33, 71]. Indeed, Notch may also be important for the expansion of MDSC [84].

Regarding the role of Notch in regulatory T cells (Tregs), activating Notch signaling may reduce their fitness [85] and therefore limit their immunosuppressive function. Tregs have also been shown to regulate normal stem cells in the hair follicle, through Jag1-mediated activation of Notch signaling [66]. A similar role in cancer stem cell could be investigated. Finally, CSL-deficient dendritic cells are less prone to induce Tregs differentiation [86], suggesting that Notch activity may favor DC activation of Treg.

Notch signaling thus regulates the immunosuppressive environment by acting on macrophages and MDSCs and is also implicated in anti-tumor response by directly modulating the cytotoxic ability of $CD8^+$ T cells.

1.9 Can we Predict the Effect of Notch Inhibition in the TME?

A whole panoply of Notch signaling inhibitors has been developed, targeting every step of Notch signalization: γ-secretase inhibitors (GSI), antibodies targeting ligands or receptors [10, 59] as well as compounds targeting transcription activation [87, 88], receptor glycosylation [89], or Notch trafficking [90] (Fig. 1.4a). Anticancer therapies are designed either to target the cancer cell-specific alterations through targeted therapies or to target the microenvironment with anti-angiogenic therapies or CAF-directed therapies. In an era of "precision medicine" and "targeted therapy," targeting pathways that are activated in different parts of the TME will have pleiotropic effect that one must aim at understanding. We have shown that Notch signaling is regulating

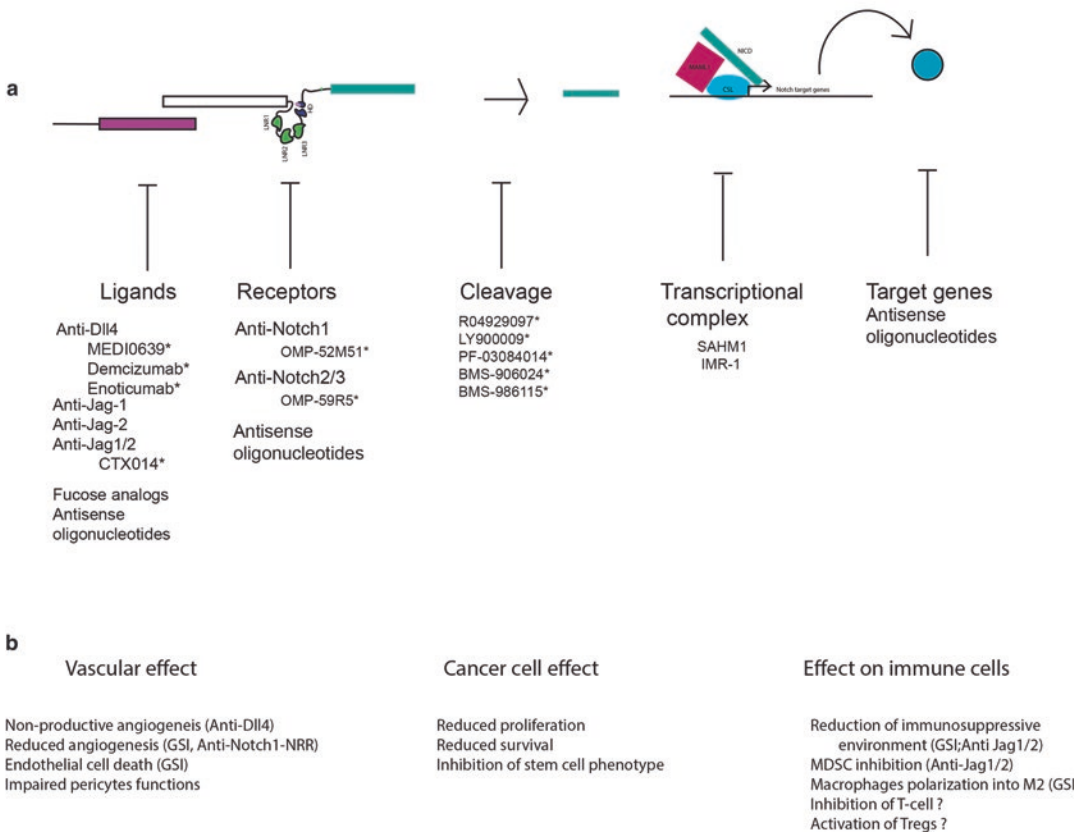

Fig. 1.4 Notch targeting strategies (**a**) and effect of Notch inhibition on the TME (**b**). *compounds under clinical trials

each component of the microenvironment. It is therefore logical that inhibiting Notch signaling will affect many different aspects of the microenvironment (Fig. 1.4b). Many in vitro and in vivo studies showed the effect of Notch inhibition on cancer cell proliferation, migration, and reducing stem cell phenotype [91] (Fig. 1.4b). Regarding the effects on the TME, the best-characterized effect of inhibiting Notch signaling on the TME is the effect on the tumor vasculature. Indeed, anti-Dll4 antibodies induce massive non-productive angiogenesis [92]. GSI also affect angiogenesis, but the effects are trickier to decipher, as GSI will target all Notch/ligand interactions. We, and others, showed that GSI treatment targeted EC and decreased tumor angiogenesis [41, 61, 93]. It has to be mentioned that the effect of GSI on vasculature will depend on the level of Jag1 and Dll4 in tumors since Jag1 and Dll4 have opposite effect on sprouting angiogenesis [38]. Regarding the effect of Notch inhibition on the immune infiltrate, the effect might be pro- or anti-tumoral (Figs. 1.3 and 1.4). First, Notch signaling is important for cytotoxic activity of T cells. Inhibiting Notch signaling might therefore inhibit the anti-tumor activity of these cells. Furthermore, infiltrating immune cells showed reduced expression of Notch receptors and ligands which may participate to immunosuppression [33]. In line with this, restoring Notch signaling in T cell bypassed tumor-induced suppression [94]. Restoring Notch expression by T cells might therefore be an interesting strategy. Triggering Notch signaling by multivalent Dll1 has also been shown to stimulate anti-tumor T cells [77]. Notch has been shown to limit Tregs function in different context [85, 95]. Inhibiting Notch signaling might therefore enforce Tregs function. On the other hand, several studies using different Notch inhibiting strategies showed a

reduction in the immunosuppressive environment [94, 96]. It has also to be noted that inhibiting Notch signaling may also limit M1 macrophages polarization and therefore limit tumor growth reduction [82].

The effects of Notch inhibition on the TME are thus complex and may be pro- or anti-tumoral. Given the existence of cross talk between the different compartments of the TME, more studies are needed to understand the overall effects and the contribution of Notch modulation in the different compartments.

1.10 Open Questions and New Avenues

1.10.1 Notch Regulation in the Physical and Chemical Tumor Environment

We would like to address in this section the potential impact of all the physical and chemical cues that influence the phenotypic diversity in the TME. Hypoxia is a common feature of solid tumors and is correlated with poor prognosis [97]. Regulation of Notch signaling by HIF pathway has been described at different steps of Notch signaling: production of NICD by potentiating γ-secretase activity through a direct interaction between HIF1-α and the γ-secretase complex [98], stabilizing NICD [99]. Notch activation pattern will therefore depend on the repartition of hypoxia in the tumor. For example, local hypoxia has been shown to regulate Notch signaling in the lung [100]. Tumors are also characterized by an abnormal acidic extracellular milieu [101], and this may affect receptor/ligands interactions. Another unexplored area is the impact of tumor mechanics on Notch signaling. Alteration of tissue stiffness is important in controlling tumor progression [102]. As Notch receptors are mechanosensors [2, 103], stiffness heterogeneity and force repartition in the TME may affect Notch signaling activation. Furthermore, remodeling of the ECM, which participates to mechanics alteration, has a major impact on tumor progression [104, 105], and Notch signaling is responsive to the ECM composition [106]. The influence of the ECM, the repartition of forces, and the stiffness of the TME on the Notch activation pattern in the tumor may be of interest. Indeed, these roles may be of major importance when considering the therapeutic targeting of Notch signaling in cancer. Notch receptors glycosylation is a major determinant of Notch activation by ligands and induces a bias in different ligands [103]. The TME-induced change in glycosylation is impacting tumor progression [107], this may therefore affect Notch signaling [5] and need further investigations.

1.10.2 Improving the Study of Notch Signaling in the TME

Interpreting the effect of manipulating Notch signaling in the TME is not straightforward. Indeed, given the cross talk and interdependence of the different component of the TME, an endpoint effect on angiogenesis or on the immune infiltrate may in part be due to an indirect consequence of affecting other component of the TME. The first step to better understand the role of Notch in the TME is to describe, in each specific context, the expression pattern of Notch signaling components. Analysis of transcriptomic data from bulk tumors has proven efficacy, but cell type deconvolution should be applied and emergence of new technologies such as single cell sequencing could be of use to better describe the Notch activation pattern in the TME. Expression of Notch ligands and receptors in each compartment of the TME is mandatory to understand the role of Notch signaling. Antibodies directed against NICDs are also interesting to characterize the activation and contribution of specific Notch receptors. N1ICD-specific [108] and more recently N3ICD-specific antibodies [109] have been developed and validated. N2ICD and N4ICD would also be of great interest. In order to better characterize Notch activation pattern in the TME, reporter mouse expressing fluorescent proteins under the control of Notch target genes promoter gives relevant information. The study of Lim and colleagues [15] very elegantly used the HES-1 GFP mouse model to sort Notch active and Notch inactive

cells from small cell lung cancers. This kind of approach could also enable to examine the topology of Notch activation in the TME. Regarding the effect of Notch modulation in the initiation and progression of cancers, using transgenic expression of NICD gave important foundation to the field of Notch in cancer but regarding study in the TME, it does not allow to study the dynamic involvement of receptor–ligand interaction. Furthermore, to study the involvement of specific Notch ligands and receptors in different compartment of the TME, specific deletion of Notch signaling component using CRE expression in a specific part of the TME is of major interest. Given the importance of Notch signaling in regulating the function of the immune infiltrate, syngeneic graft or genetically engineered mouse models should be favored instead of xenografts.

1.10.3 Specific Targeting of Notch in the TME

Notch targeting specificity should aim at targeting specific Notch pathway component as well as specific cell types in the TME. Indeed, targeting specific components of the TME may limit toxicity. Nanoparticles encapsulating GSI have already been used to target GSI in tumors through enhanced permeability and retention effect [110]. Delivering GSI or antisense oligonucleotides directed against specific cell population by antibody-conjugated nanoparticles may be an interesting approach. Antibody directed against tumor endothelium [111], CAF, TAM, or cancer stem cell-specific markers could be used to achieve this aim. Tools targeting specific members or signaling step of the Notch signaling axis are now available, and combining these approaches with approaches targeting a specific cell population may be the avenue for a safe and efficient targeting of Notch signaling in cancer. In order to develop these strategies, it is necessary to study, as mentioned before, the expression pattern of Notch ligands and receptors in the TME in each context and is a prerequisite to better understand data from preclinical and clinical models.

1.11 Conclusion

Notch is a major determinant of all aspects of the TME. Notch receptors and ligands are expressed in every component of the TME and dynamically regulated by signaling pathways involved in modeling the TME. We described the role of Notch pathway in shaping the tumor vasculature, controlling CAF activation, and modulating both the anti- and pro-tumor immune cells. The oncogenic role of Notch signaling in the cancer compartment is not to be underestimated and has been extensively reviewed. Our aim here was to insist on heterotypic Notch activation and regulation in the TME. Both approaches need to be merged to improve our understanding and develop new therapeutic strategies modulating Notch signaling in the TME.

References

1. Henrique D, Schweisguth F (2019) Mechanisms of Notch signaling: a simple logic deployed in time and space. Development 146. https://doi.org/10.1242/dev.172148
2. Langridge PD, Struhl G (2017) Epsin-dependent ligand endocytosis activates notch by force. Cell 171:1383–1396. e1312
3. Lim R, Sugino T, Nolte H, Andrade J, Zimmermann B, Shi C, Doddaballapur A, Ong YT, Wilhelm K, Fasse JWD et al (2019) Deubiquitinase USP10 regulates Notch signaling in the endothelium. Science 364:188–193
4. Fryer CJ, White JB, Jones KA (2004) Mastermind recruits CycC:CDK8 to phosphorylate the notch ICD and coordinate activation with turnover. Mol Cell 16:509–520
5. Pakkiriswami S, Couto A, Nagarajan U, Georgiou M (2016) Glycosylated notch and cancer. Front Oncol 6:37
6. Nandagopal N, Santat LA, LeBon L, Sprinzak D, Bronner ME, Elowitz MB (2018) Dynamic ligand discrimination in the notch signaling pathway. Cell 172:869–880. e819
7. Gama-Norton L, Ferrando E, Ruiz-Herguido C, Liu Z, Guiu J, Islam AB, Lee SU, Yan M, Guidos CJ, Lopez-Bigas N et al (2015) Notch signal strength controls cell fate in the haemogenic endothelium. Nat Commun 6:8510
8. Kopan R, Ilagan MX (2009) The canonical Notch signaling pathway: unfolding the activation mechanism. Cell 137:216–233
9. Nandagopal N, Santat LA, Elowitz MB (2019) Cis-activation in the Notch signaling pathway. Elife 8:e37880. https://doi.org/10.7554/eLife.37880

10. Ntziachristos P, Lim JS, Sage J, Aifantis I (2014) From fly wings to targeted cancer therapies: a centennial for Notch signaling. Cancer Cell 25:318–334
11. Aster JC, Pear WS, Blacklow SC (2017) The varied roles of Notch in cancer. Annu Rev Pathol 12:245–275
12. Ranganathan P, Weaver KL, Capobianco AJ (2011) Notch signalling in solid tumours: a little bit of everything but not all the time. Nat Rev Cancer 11:338–351
13. Weng AP, Ferrando AA, Lee W, Morris JPT, Silverman LB, Sanchez-Irizarry C, Blacklow SC, Look AT, Aster JC (2004) Activating mutations of NOTCH1 in human T cell acute lymphoblastic leukemia. Science 306:269–271
14. Indraccolo S, Minuzzo S, Masiero M, Amadori A (2010) Ligand-driven activation of the Notch pathway in T-ALL and solid tumors: why Not(ch)? Cell Cycle 9:80–85
15. Lim JS, Ibaseta A, Fischer MM, Cancilla B, O'Young G, Cristea S, Luca VC, Yang D, Jahchan NS, Hamard C et al (2017) Intratumoural heterogeneity generated by Notch signalling promotes small-cell lung cancer. Nature 545:360–364
16. Maman S, Witz IP (2018) A history of exploring cancer in context. Nat Rev Cancer 18:359–376
17. Laplane L, Duluc D, Larmonier N, Pradeu T, Bikfalvi A (2018) The multiple layers of the tumor environment. Trends Cancer 4:802–809
18. Meurette O, Mehlen P (2018) Notch signaling in the tumor microenvironment. Cancer Cell 34:536–548
19. Yamamoto M, Taguchi Y, Ito-Kureha T, Semba K, Yamaguchi N, Inoue J (2013) NF-kappaB non-cell-autonomously regulates cancer stem cell populations in the basal-like breast cancer subtype. Nat Commun 4:2299
20. Xing F, Kobayashi A, Okuda H, Watabe M, Pai SK, Pandey PR, Hirota S, Wilber A, Mo YY, Moore BE et al (2013) Reactive astrocytes promote the metastatic growth of breast cancer stem-like cells by activating Notch signalling in brain. EMBO Mol Med 5:384–396
21. Zavadil J, Cermak L, Soto-Nieves N, Bottinger EP (2004) Integration of TGF-beta/Smad and Jagged1/notch signalling in epithelial-to-mesenchymal transition. EMBO J 23:1155–1165
22. Sansone P, Storci G, Tavolari S, Guarnieri T, Giovannini C, Taffurelli M, Ceccarelli C, Santini D, Paterini P, Marcu KB et al (2007) IL-6 triggers malignant features in mammospheres from human ductal breast carcinoma and normal mammary gland. J Clin Invest 117:3988–4002
23. Xiong S, Wang R, Chen Q, Luo J, Wang J, Zhao Z, Li Y, Wang Y, Wang X, Cheng B (2018) Cancer-associated fibroblasts promote stem cell-like properties of hepatocellular carcinoma cells through IL-6/STAT3/notch signaling. Am J Cancer Res 8:302–316
24. Lin JT, Wang JY, Chen MK, Chen HC, Chang TH, Su BW, Chang PJ (2013) Colon cancer mesenchymal stem cells modulate the tumorigenicity of colon cancer through interleukin 6. Exp Cell Res 319:2216–2229
25. Peng D, Tanikawa T, Li W, Zhao L, Vatan L, Szeliga W, Wan S, Wei S, Wang Y, Liu Y et al (2016) Myeloid-derived suppressor cells endow stem-like qualities to breast cancer cells through IL6/STAT3 and NO/NOTCH cross-talk signaling. Cancer Res 76:3156–3165
26. Colombo M, Galletti S, Bulfamante G, Falleni M, Tosi D, Todoerti K, Lazzari E, Crews LA, Jamieson CH, Ravaioli S et al (2016) Multiple myeloma-derived jagged ligands increases autocrine and paracrine interleukin-6 expression in bone marrow niche. Oncotarget 7:56013–56029
27. Collu GM, Hidalgo-Sastre A, Brennan K (2014) Wnt-notch signalling crosstalk in development and disease. Cell Mol Life Sci 71:3553–3567
28. Ayyanan A, Civenni G, Ciarloni L, Morel C, Mueller N, Lefort K, Mandinova A, Raffoul W, Fiche M, Dotto GP, Brisken C (2006) Increased Wnt signaling triggers oncogenic conversion of human breast epithelial cells by a notch-dependent mechanism. Proc Natl Acad Sci U S A 103:3799–3804
29. Rodilla V, Villanueva A, Obrador-Hevia A, Robert-Moreno A, Fernandez-Majada V, Grilli A, Lopez-Bigas N, Bellora N, Alba MM, Torres F et al (2009) Jagged1 is the pathological link between Wnt and notch pathways in colorectal cancer. Proc Natl Acad Sci U S A 106:6315–6320
30. Mangolini M, Gotte F, Moore A, Ammon T, Oelsner M, Lutzny-Geier G, Klein-Hitpass L, Williamson JC, Lehner PJ, Durig J et al (2018) Notch2 controls non-autonomous Wnt-signalling in chronic lymphocytic leukaemia. Nat Commun 9:3839
31. Cao Z, Ding BS, Guo P, Lee SB, Butler JM, Casey SC, Simons M, Tam W, Felsher DW, Shido K et al (2014) Angiocrine factors deployed by tumor vascular niche induce B cell lymphoma invasiveness and chemoresistance. Cancer Cell 25:350–365
32. Tsuyada A, Chow A, Wu J, Somlo G, Chu P, Loera S, Luu T, Li AX, Wu X, Ye W et al (2012) CCL2 mediates cross-talk between cancer cells and stromal fibroblasts that regulates breast cancer stem cells. Cancer Res 72:2768–2779
33. Kelliher MA, Roderick JE (2018) NOTCH signaling in T-cell-mediated anti-tumor immunity and T-cell-based immunotherapies. Front Immunol 9:1718
34. Boelens MC, Wu TJ, Nabet BY, Xu B, Qiu Y, Yoon T, Azzam DJ, Twyman-Saint Victor C, Wiemann BZ, Ishwaran H et al (2014) Exosome transfer from stromal to breast cancer cells regulates therapy resistance pathways. Cell 159:499–513
35. Wang Q, Lu Q (2017) Plasma membrane-derived extracellular microvesicles mediate non-canonical intercellular NOTCH signaling. Nat Commun 8:709
36. Hellstrom M, Phng LK, Hofmann JJ, Wallgard E, Coultas L, Lindblom P, Alva J, Nilsson AK, Karlsson L, Gaiano N et al (2007) Dll4 signalling through Notch1 regulates formation of tip cells during angiogenesis. Nature 445:776–780

37. Benedito R, Hellstrom M (2013) Notch as a hub for signaling in angiogenesis. Exp Cell Res 319:1281–1288
38. Benedito R, Roca C, Sorensen I, Adams S, Gossler A, Fruttiger M, Adams RH (2009) The notch ligands Dll4 and Jagged1 have opposing effects on angiogenesis. Cell 137:1124–1135
39. Boareto M, Jolly MK, Ben-Jacob E, Onuchic JN (2015) Jagged mediates differences in normal and tumor angiogenesis by affecting tip-stalk fate decision. Proc Natl Acad Sci U S A 112:E3836–E3844
40. Pedrosa AR, Trindade A, Carvalho C, Graca J, Carvalho S, Peleteiro MC, Adams RH, Duarte A (2015) Endothelial Jagged1 promotes solid tumor growth through both pro-angiogenic and angiocrine functions. Oncotarget 6:24404–24423
41. Lin S, Negulescu A, Bulusu S, Gibert B, Delcros JG, Ducarouge B, Rama N, Gadot N, Treilleux I, Saintigny P et al (2017) Non-canonical NOTCH3 signalling limits tumour angiogenesis. Nat Commun 8:16074
42. Su Q, Zhang B, Zhang L, Dang T, Rowley D, Ittmann M, Xin L (2017) Jagged1 upregulation in prostate epithelial cells promotes formation of reactive stroma in the Pten null mouse model for prostate cancer. Oncogene 36:618–627
43. Zeng Q, Li S, Chepeha DB, Giordano TJ, Li J, Zhang H, Polverini PJ, Nor J, Kitajewski J, Wang CY (2005) Crosstalk between tumor and endothelial cells promotes tumor angiogenesis by MAPK activation of Notch signaling. Cancer Cell 8:13–23
44. Wieland E, Rodriguez-Vita J, Liebler SS, Mogler C, Moll I, Herberich SE, Espinet E, Herpel E, Menuchin A, Chang-Claude J et al (2017) Endothelial Notch1 activity facilitates metastasis. Cancer Cell 31:355–367
45. Kuhnert F, Chen G, Coetzee S, Thambi N, Hickey C, Shan J, Kovalenko P, Noguera-Troise I, Smith E, Fairhurst J et al (2015) Dll4 blockade in stromal cells mediates antitumor effects in preclinical models of ovarian cancer. Cancer Res 75:4086–4096
46. Lu J, Ye X, Fan F, Xia L, Bhattacharya R, Bellister S, Tozzi F, Sceusi E, Zhou Y, Tachibana I et al (2013) Endothelial cells promote the colorectal cancer stem cell phenotype through a soluble form of Jagged-1. Cancer Cell 23:171–185
47. Zhu TS, Costello MA, Talsma CE, Flack CG, Crowley JG, Hamm LL, He X, Hervey-Jumper SL, Heth JA, Muraszko KM et al (2011) Endothelial cells create a stem cell niche in glioblastoma by providing NOTCH ligands that nurture self-renewal of cancer stem-like cells. Cancer Res 71:6061–6072
48. Indraccolo S, Minuzzo S, Masiero M, Pusceddu I, Persano L, Moserle L, Reboldi A, Favaro E, Mecarozzi M, Di Mario G et al (2009) Cross-talk between tumor and endothelial cells involving the Notch3-Dll4 interaction marks escape from tumor dormancy. Cancer Res 69:1314–1323
49. Sonoshita M, Aoki M, Fuwa H, Aoki K, Hosogi H, Sakai Y, Hashida H, Takabayashi A, Sasaki M, Robine S et al (2011) Suppression of colon cancer metastasis by Aes through inhibition of Notch signaling. Cancer Cell 19:125–137
50. Patenaude A, Woerher S, Umlandt P, Wong F, Ibrahim R, Kyle A, Unger S, Fuller M, Parker J, Minchinton A et al (2015) A novel population of local pericyte precursor cells in tumor stroma that require Notch signaling for differentiation. Microvasc Res 101:38–47
51. Hu B, Castillo E, Harewood L, Ostano P, Reymond A, Dummer R, Raffoul W, Hoetzenecker W, Hofbauer GF, Dotto GP (2012) Multifocal epithelial tumors and field cancerization from loss of mesenchymal CSL signaling. Cell 149:1207–1220
52. Procopio MG, Laszlo C, Al Labban D, Kim DE, Bordignon P, Jo SH, Goruppi S, Menietti E, Ostano P, Ala U et al (2015) Combined CSL and p53 downregulation promotes cancer-associated fibroblast activation. Nat Cell Biol 17:1193–1204
53. Hoare M, Ito Y, Kang TW, Weekes MP, Matheson NJ, Patten DA, Shetty S, Parry AJ, Menon S, Salama R et al (2016) NOTCH1 mediates a switch between two distinct secretomes during senescence. Nat Cell Biol 18:979–992
54. Teo YV, Rattanavirotkul N, Olova N, Salzano A, Quintanilla A, Tarrats N, Kiourtis C, Muller M, Green AR, Adams PD et al (2019) Notch signaling mediates secondary senescence. Cell Rep 27:997–1007, e1005
55. Demehri S, Turkoz A, Kopan R (2009) Epidermal Notch1 loss promotes skin tumorigenesis by impacting the stromal microenvironment. Cancer Cell 16:55–66
56. Peng Y, Li Z, Yang P, Newton IP, Ren H, Zhang L, Wu H, Li Z (2014) Direct contacts with colon cancer cells regulate the differentiation of bone marrow mesenchymal stem cells into tumor associated fibroblasts. Biochem Biophys Res Commun 451:68–73
57. Studebaker AW, Storci G, Werbeck JL, Sansone P, Sasser AK, Tavolari S, Huang T, Chan MW, Marini FC, Rosol TJ et al (2008) Fibroblasts isolated from common sites of breast cancer metastasis enhance cancer cell growth rates and invasiveness in an interleukin-6-dependent manner. Cancer Res 68:9087–9095
58. Liu C, Liu L, Chen X, Cheng J, Zhang H, Zhang C, Shan J, Shen J, Qian C (2017) LSD1 stimulates cancer-associated fibrobasts to drive Notch3-dependent self-renewal of liver cancer stem-like cells. Cancer Res 78:938–949
59. Takebe N, Miele L, Harris PJ, Jeong W, Bando H, Kahn M, Yang SX, Ivy SP (2015) Targeting notch, hedgehog, and Wnt pathways in cancer stem cells: clinical update. Nat Rev Clin Oncol 12:445–464
60. Bayin NS, Frenster JD, Sen R, Si S, Modrek AS, Galifianakis N, Dolgalev I, Ortenzi V, Illa-Bochaca I, Khahera A et al (2017) Notch signaling regulates metabolic heterogeneity in glioblastoma stem cells. Oncotarget 8:64932–64953

61. Hovinga KE, Shimizu F, Wang R, Panagiotakos G, Van Der Heijden M, Moayedpardazi H, Correia AS, Soulet D, Major T, Menon J, Tabar V (2010) Inhibition of Notch signaling in glioblastoma targets cancer stem cells via an endothelial cell intermediate. Stem Cells 28:1019–1029
62. Nakata T, Shimizu H, Nagata S, Ito G, Fujii S, Suzuki K, Kawamoto A, Ishibashi F, Kuno R, Anzai S et al (2017) Indispensable role of notch ligand-dependent signaling in the proliferation and stem cell niche maintenance of APC-deficient intestinal tumors. Biochem Biophys Res Commun 482:1296–1303
63. Chen HF, Huang CH, Liu CJ, Hung JJ, Hsu CC, Teng SC, Wu KJ (2014) Twist1 induces endothelial differentiation of tumour cells through the Jagged1-KLF4 axis. Nat Commun 5:4697
64. Oskarsson T, Acharyya S, Zhang XH, Vanharanta S, Tavazoie SF, Morris PG, Downey RJ, Manova-Todorova K, Brogi E, Massague J (2011) Breast cancer cells produce tenascin C as a metastatic niche component to colonize the lungs. Nat Med 17:867–874
65. Sarkar S, Mirzaei R, Zemp FJ, Wu W, Senger DL, Robbins SM, Yong VW (2017) Activation of NOTCH signaling by tenascin-C promotes growth of human brain tumor-initiating cells. Cancer Res 77:3231–3243
66. Ali N, Zirak B, Rodriguez RS, Pauli ML, Truong HA, Lai K, Ahn R, Corbin K, Lowe MM, Scharschmidt TC et al (2017) Regulatory T cells in skin facilitate epithelial stem cell differentiation. Cell 169:1119–1129. e1111
67. Chakrabarti R, Celià-Terrassa T, Kumar S, Hang X, Wei Y, Choudhury A, Hwang J, Peng J, Nixon B, Grady JJ et al (2018) Notch ligand Dll1 mediates cross-talk between mammary stem cells and the macrophageal niche. Science Jun 29;360(6396).
68. Sethi N, Dai X, Winter CG, Kang Y (2011) Tumor-derived JAGGED1 promotes osteolytic bone metastasis of breast cancer by engaging Notch signaling in bone cells. Cancer Cell 19:192–205
69. Ghajar CM, Peinado H, Mori H, Matei IR, Evason KJ, Brazier H, Almeida D, Koller A, Hajjar KA, Stainier DY et al (2013) The perivascular niche regulates breast tumour dormancy. Nat Cell Biol 15:807–817
70. Fridman WH, Zitvogel L, Sautes-Fridman C, Kroemer G (2017) The immune contexture in cancer prognosis and treatment. Nat Rev Clin Oncol 14:717–734
71. Hossain F, Majumder S, Ucar DA, Rodriguez PC, Golde TE, Minter LM, Osborne BA, Miele L (2018) Notch signaling in myeloid cells as a regulator of tumor immune responses. Front Immunol 9:1288
72. Janghorban M, Xin L, Rosen JM, Zhang XH (2018) Notch signaling as a regulator of the tumor immune response: to target or not to target? Front Immunol 9:1649
73. Radtke F, MacDonald HR, Tacchini-Cottier F (2013) Regulation of innate and adaptive immunity by notch. Nat Rev Immunol 13:427–437
74. Cho OH, Shin HM, Miele L, Golde TE, Fauq A, Minter LM, Osborne BA (2009) Notch regulates cytolytic effector function in CD8+ T cells. J Immunol 182:3380–3389
75. Maekawa Y, Minato Y, Ishifune C, Kurihara T, Kitamura A, Kojima H, Yagita H, Sakata-Yanagimoto M, Saito T, Taniuchi I et al (2008) Notch2 integrates signaling by the transcription factors RBP-J and CREB1 to promote T cell cytotoxicity. Nat Immunol 9:1140–1147
76. Sugimoto K, Maekawa Y, Kitamura A, Nishida J, Koyanagi A, Yagita H, Kojima H, Chiba S, Shimada M, Yasutomo K (2010) Notch2 signaling is required for potent antitumor immunity in vivo. J Immunol 184:4673–4678
77. Biktasova AK, Dudimah DF, Uzhachenko RV, Park K, Akhter A, Arasada RR, Evans JV, Novitskiy SV, Tchekneva EE, Carbone DP et al (2015) Multivalent forms of the notch ligand DLL-1 enhance antitumor T-cell immunity in lung cancer and improve efficacy of EGFR-targeted therapy. Cancer Res 75:4728–4741
78. Huang Y, Lin L, Shanker A, Malhotra A, Yang L, Dikov MM, Carbone DP (2011) Resuscitating cancer immunosurveillance: selective stimulation of DLL1-notch signaling in T cells rescues T-cell function and inhibits tumor growth. Cancer Res 71:6122–6131
79. Yu W, Wang Y, Guo P (2017) Notch signaling pathway dampens tumor-infiltrating CD8(+) T cells activity in patients with colorectal carcinoma. Biomed Pharmacother 97:535–542
80. Franklin RA, Liao W, Sarkar A, Kim MV, Bivona MR, Liu K, Pamer EG, Li MO (2014) The cellular and molecular origin of tumor-associated macrophages. Science 344:921–925
81. Liu H, Wang J, Zhang M, Xuan Q, Wang Z, Lian X, Zhang Q (2017) Jagged1 promotes aromatase inhibitor resistance by modulating tumor-associated macrophage differentiation in breast cancer patients. Breast Cancer Res Treat 166:95–107
82. Wang YC, He F, Feng F, Liu XW, Dong GY, Qin HY, Hu XB, Zheng MH, Liang L, Feng L et al (2010) Notch signaling determines the M1 versus M2 polarization of macrophages in antitumor immune responses. Cancer Res 70:4840–4849
83. Zhao JL, Huang F, He F, Gao CC, Liang SQ, Ma PF, Dong GY, Han H, Qin HY (2016) Forced activation of notch in macrophages represses tumor growth by Upregulating miR-125a and disabling tumor-associated macrophages. Cancer Res 76:1403–1415
84. Saleem SJ, Conrad DH (2011) Hematopoietic cytokine-induced transcriptional regulation and Notch signaling as modulators of MDSC expansion. Int Immunopharmacol 11:808–815
85. Charbonnier LM, Wang S, Georgiev P, Sefik E, Chatila TA (2015) Control of peripheral tolerance by regulatory T cell-intrinsic Notch signaling. Nat Immunol 16:1162–1173
86. Zaman TS, Arimochi H, Maruyama S, Ishifune C, Tsukumo SI, Kitamura A, Yasutomo K (2017) Notch balances Th17 and induced regulatory T cell

functions in dendritic cells by regulating Aldh1a2 expression. J Immunol 199:1989–1997
87. Moellering RE, Cornejo M, Davis TN, Del Bianco C, Aster JC, Blacklow SC, Kung AL, Gilliland DG, Verdine GL, Bradner JE (2009) Direct inhibition of the NOTCH transcription factor complex. Nature 462:182–188
88. Astudillo L, Da Silva TG, Wang Z, Han X, Jin K, VanWye J, Zhu X, Weaver K, Oashi T, Lopes PE et al (2016) The small molecule IMR-1 inhibits the notch transcriptional activation complex to suppress tumorigenesis. Cancer Res 76:3593–3603
89. Schneider M, Kumar V, Nordstrom LU, Feng L, Takeuchi H, Hao H, Luca VC, Garcia KC, Stanley P, Wu P, Haltiwanger RS (2018) Inhibition of Delta-induced Notch signaling using fucose analogs. Nat Chem Biol 14:65–71
90. Nolin E, Gans S, Llamas L, Bandyopadhyay S, Brittain SM, Bernasconi-Elias P, Carter KP, Loureiro JJ, Thomas JR, Schirle M et al (2019) Discovery of a ZIP7 inhibitor from a notch pathway screen. Nat Chem Biol 15:179–188
91. Takebe N, Nguyen D, Yang SX (2014) Targeting Notch signaling pathway in cancer: clinical development advances and challenges. Pharmacol Ther 141:140–149
92. Noguera-Troise I, Daly C, Papadopoulos NJ, Coetzee S, Boland P, Gale NW, Lin HC, Yancopoulos GD, Thurston G (2006) Blockade of Dll4 inhibits tumour growth by promoting non-productive angiogenesis. Nature 444:1032–1037
93. Cook N, Frese KK, Bapiro TE, Jacobetz MA, Gopinathan A, Miller JL, Rao SS, Demuth T, Howat WJ, Jodrell DI, Tuveson DA (2012) Gamma secretase inhibition promotes hypoxic necrosis in mouse pancreatic ductal adenocarcinoma. J Exp Med 209:437–444
94. Sierra RA, Thevenot P, Raber PL, Cui Y, Parsons C, Ochoa AC, Trillo-Tinoco J, Del Valle L, Rodriguez PC (2014) Rescue of notch-1 signaling in antigen-specific CD8+ T cells overcomes tumor-induced T-cell suppression and enhances immunotherapy in cancer. Cancer Immunol Res 2:800–811
95. Bassil R, Zhu B, Lahoud Y, Riella LV, Yagita H, Elyaman W, Khoury SJ (2011) Notch ligand delta-like 4 blockade alleviates experimental autoimmune encephalomyelitis by promoting regulatory T cell development. J Immunol 187:2322–2328
96. Mao L, Zhao ZL, Yu GT, Wu L, Deng WW, Li YC, Liu JF, Bu LL, Liu B, Kulkarni AB et al (2017) Gamma-secretase inhibitor reduces immunosuppressive cells and enhances tumour immunity in head and neck squamous cell carcinoma. Int J Cancer 142:999–1009
97. Denko NC (2008) Hypoxia, HIF1 and glucose metabolism in the solid tumour. Nat Rev Cancer 8:705–713
98. Villa JC, Chiu D, Brandes AH, Escorcia FE, Villa CH, Maguire WF, Hu CJ, de Stanchina E, Simon MC, Sisodia SS et al (2014) Nontranscriptional role of Hif-1alpha in activation of gamma-secretase and Notch signaling in breast cancer. Cell Rep 8:1077–1092
99. Gustafsson MV, Zheng X, Pereira T, Gradin K, Jin S, Lundkvist J, Ruas JL, Poellinger L, Lendahl U, Bondesson M (2005) Hypoxia requires Notch signaling to maintain the undifferentiated cell state. Dev Cell 9:617–628
100. Xi Y, Kim T, Brumwell AN, Driver IH, Wei Y, Tan V, Jackson JR, Xu J, Lee DK, Gotts JE et al (2017) Local lung hypoxia determines epithelial fate decisions during alveolar regeneration. Nat Cell Biol 19:904–914
101. Corbet C, Feron O (2017) Tumour acidosis: from the passenger to the driver's seat. Nat Rev Cancer 17:577–593
102. Butcher DT, Alliston T, Weaver VM (2009) A tense situation: forcing tumour progression. Nat Rev Cancer 9:108–122
103. Kovall RA, Gebelein B, Sprinzak D, Kopan R (2017) The canonical Notch signaling pathway: structural and biochemical insights into shape, sugar, and force. Dev Cell 41:228–241
104. Harisi R, Jeney A (2015) Extracellular matrix as target for antitumor therapy. Onco Targets Ther 8:1387–1398
105. Multhaupt HA, Leitinger B, Gullberg D, Couchman JR (2016) Extracellular matrix component signaling in cancer. Adv Drug Deliv Rev 97:28–40
106. LaFoya B, Munroe JA, Mia MM, Detweiler MA, Crow JJ, Wood T, Roth S, Sharma B, Albig AR (2016) Notch: a multi-functional integrating system of microenvironmental signals. Dev Biol 418: 227–241
107. Chandler KB, Costello CE, Rahimi N (2019) Glycosylation in the tumor microenvironment: implications for tumor angiogenesis and metastasis. Cell 8:E544. https://doi.org/10.3390/cells8060544
108. Kluk MJ, Ashworth T, Wang H, Knoechel B, Mason EF, Morgan EA, Dorfman D, Pinkus G, Weigert O, Hornick JL et al (2013) Gauging NOTCH1 activation in cancer using immunohistochemistry. PLoS One 8:e67306
109. Choy L, Hagenbeek TJ, Solon M, French D, Finkle D, Shelton A, Venook R, Brauer MJ, Siebel CW (2017) Constitutive NOTCH3 signaling promotes the growth of basal breast cancers. Cancer Res 77:1439–1452
110. Mamaeva V, Rosenholm JM, Bate-Eya LT, Bergman L, Peuhu E, Duchanoy A, Fortelius LE, Landor S, Toivola DM, Linden M, Sahlgren C (2011) Mesoporous silica nanoparticles as drug delivery systems for targeted inhibition of Notch signaling in cancer. Mol Ther 19:1538–1546
111. Li JL, Harris AL (2007) The potential of new tumor endothelium-specific markers for the development of antivascular therapy. Cancer Cell 11: 478–481

2 Erythropoietin Signaling in the Microenvironment of Tumors and Healthy Tissues

Wuling Liu, Krishnapriya M. Varier, Klarke M. Sample, Eldad Zacksenhaus, Babu Gajendran, and Yaacov Ben-David

Abstract

Erythropoietin (*EPO*), the primary cytokine of erythropoiesis, stimulates both proliferation and differentiation of erythroid progenitors and their maturation to red blood cells. Basal *EPO* levels maintain the optimum levels of circulating red blood cells. However, during hypoxia, *EPO* secretion and its expression is elevated drastically in renal interstitial fibroblasts, thereby increasing the number of erythroid progenitors and accelerating their differentiation to mature erythrocytes. A tight regulation of this pathway is therefore of paramount importance. The biological response to *EPO* is commenced through the involvement of its cognate receptor, EPOR. The receptor–ligand complex results in homodimerization and conformational changes, which trigger downstream signaling events and cause activation or inactivation of critical transcription factors that promote erythroid expansion. In recent years, recombinant human *EPO* (rEPO) has been widely used as a therapeutic tool to treat a number of anemias induced by infection, and chemotherapy for various cancers. However, several studies have uncovered a tumor promoting ability of *EPO* in man, which likely occurs through EPOR or alternative receptor(s). On the other hand, some studies have demonstrated a strong anticancer activity of *EPO*, although the mechanism still remains unclear. A thorough investigation of EPOR signaling could yield enhanced understanding of the pathobiology for a variety of disorders, as well as the potential novel therapeutic strategies. In this chapter, in addition to the clinical relevance of *EPO*/EPOR signaling, we review its anticancer efficacy within various tumor microenvironments.

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2.1 Introduction

Erythropoietin is the chief controller of erythropoiesis, and its expression and functions are important for oxygenation of cells during steady-state and hypoxic conditions. Until 2001, the chief purpose of erythropoietin was considered to be in "erythropoiesis," where *EPO* binds to its receptor (EPOR), expressed on erythroid precursor cells, to stimulate the proliferation and differentiation of hemoglobin-containing erythrocytes [1]. However, Yasuda et al. [2] demonstrated the expression of mRNA as well as the presence of EPOR protein in murine embryos at the premature postimplantation stage. At this developing stage (6–7 days of pregnancy), the murine embryo grows extremely rapidly by accelerating the doubling time to around 4.8–8.1 h [3]. These findings led to the hypothesis that the EPO–EPOR interactions could be involved in carcinogenesis. As anticipated, the EPO–EPOR signaling was found to be highly prevalent in malignant reproductive organs [4], breast [5, 6], prostate [7], and cervical cancers [8]. Intriguingly, most cancers express EPO as well as EPOR, regardless of their cellular origin. Moreover, studies validated that EPO–EPOR signal inhibition impaired the cancer cell proliferation and the cancer-associated blood vessels [9]. Despite this evidence, multiple studies also demonstrated the tumor inhibitory effect of EPO in erythroleukemia and multiple myeloma [10–16]. These studies emphasize the controversial role of *EPO* in cancer, which highlights the need for further discussion and investigation.

2.1.1 *EPO* and the *EPO* Receptor

Genomic *EPO* was initially cloned after purification by Miyake et al. [17], who isolated it from an aplastic anemic patient's urine. Structurally, *EPO* is located on chromosome 7q22 and consists of four exons (Fig. 2.1). *EPO* is transcriptionally regulated under the control of a variety of transcriptional factors, including the activator hypoxia-inducible factor 2-alpha (EPAS1) and suppressor GATA-binding factor 2 (GATA2) [18, 19]. During healthy oxygen conditions (normaxia), the expression of HIF2α is dramatically reduced by a VHL-specific E3-ubiquitin complex, which leads to the suppression of the *EPO* transcription (Fig. 2.2). Conversely, in conditions of low oxygen availability (hypoxia), HIF2α, which is constrictively expressed, translocates to the nucleus and forms a heterodimer with the aryl hydrocarbon receptor nuclear translocator (ARNT2). The HIF2α-ARNT2 heterodimer complex then interacts with the critical

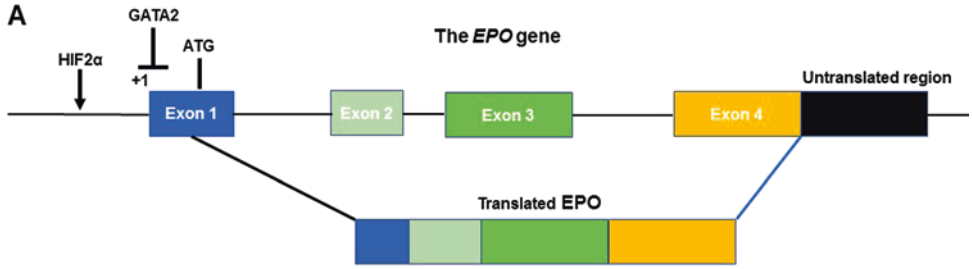

Fig. 2.1 Structure of the *EPO* gene. The exon-intron structure of the human *EPO* gene. Binding sites for transcription factor GATA2 and HIF2α are shown on the *EPO* promoter. Translation of the *EPO* gene results in the generation of a 34 kDa *EPO* protein

transcription initiation factors such as CREB-binding protein (CBP) and p300. This complex subsequently binds to the promoter of *EPO* and initiates transcription [20].

EPO is a glycoprotein protein which is predominately produced in (and secreted by) the kidney and embryological liver cells [21–23]. The corresponding gene encodes a 193-amino acid polypeptide chain, but further processing results in the production of a mature 166-amino acid protein, which is secreted into the circulating blood [24] (Fig. 2.3). Mature *EPO* gets heavily glycosylated on its three N-linked and one O-linked acidic oligosaccharide side chains [25]. The glycosylated chains are responsible for its stable biological activity and receptor interaction [26, 27]. Indeed, rEPO has great clinical utility in the treatment of anemia through the promotion of red blood cell production [28].

EPOR is *EPO*'s primary receptor, whose binding induces the activation of a defined signal transduction pathway. EPOR is a typical type-I cytokine receptor, where the ligand interacts with the extracellular domain (Fig. 2.4). Its transmembrane region spans the phospholipid bilayer and its tail portion contains eight tyrosine phosphorylation sites, which serve as locations for signaling adaptors. EPOR itself lacks an intrinsic kinase activity and thus recruits many kinases and adaptor molecules to initiate the signal [23, 30].

After maturation in the endoplasmic reticulum, EPOR is translocated to the Golgi apparatus. During this process, a minor section of the receptor is processed by heavy chain glycosylation to a mature form, which is transported to the plasma membrane (Fig. 2.4). Under stimulus-free conditions, mature EPOR expression on the plasma membrane is relatively low, having <100 receptors available at any one moment in time [23, 30]. The receptor transportation and its maturation procedure are reliant on communication

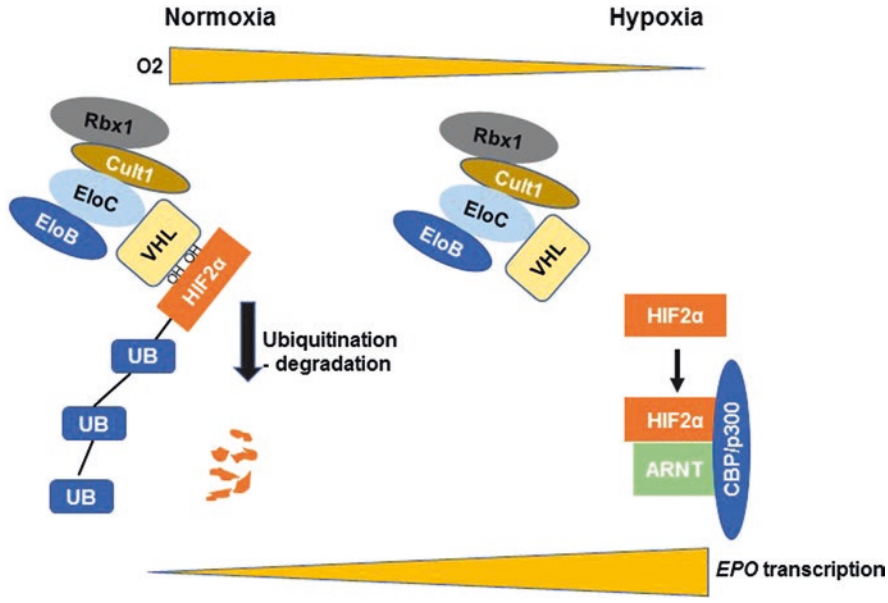

Fig. 2.2 Regulation of the *EPO* gene by HIF-2. During normoxia, the E3-ubiquitin ligase complex, consisting of VHL, RBX1, Cult1, ElonginB (EloB), and ElonginC (EloC), specifically binds to PHD-induced hydroxylated HIF2α. The C-terminal α-domain of HIF2α then links VHL to the E3-ligase via EloC to initiate ubiquitination and proteasome-mediated degradation. Under hypoxia when level of O_2 is low, the E3-ubiquitin complex remains inactive. HIF-2α, which is constitutively expressed, is no longer degraded; it translocates to the nucleus where it forms heterodimer with the aryl hydrocarbon receptor nuclear translocator (ARNT). HIF-2α/ARNT heterodimers bind to the HIF consensus binding site 5′-*RCGTG*-3′ in the *EPO* gene promoter and increase *EPO* transcription in the presence of transcriptional coactivators, such as CREB-binding protein (CBP) and p300

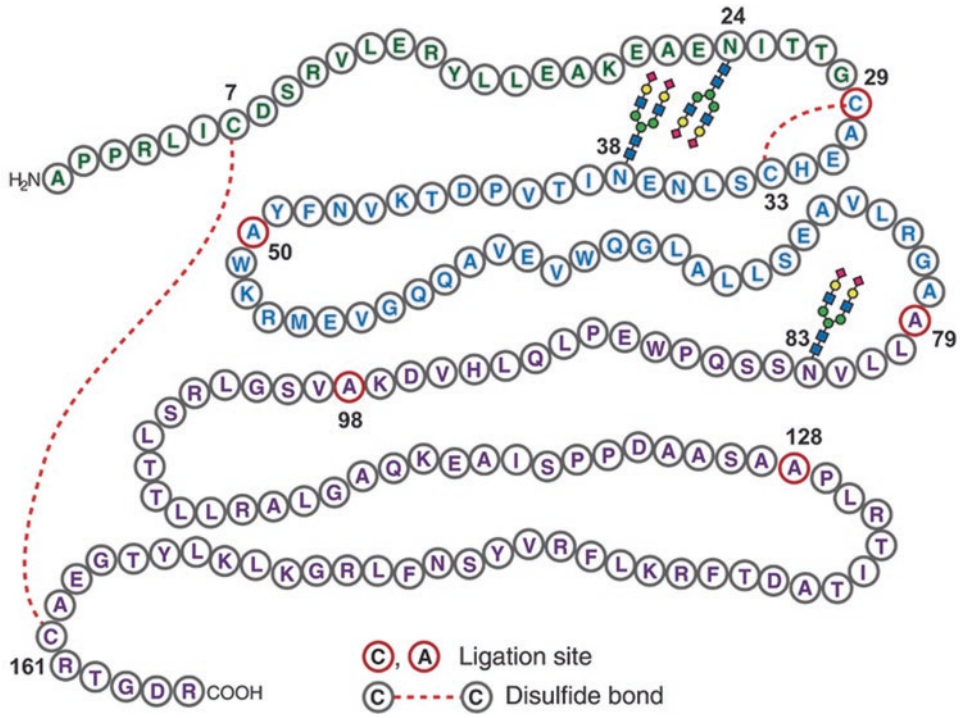

Fig. 2.3 Structure of the *EPO*. The primary structure of *EPO* and its amino acid sequence. Sites of glycosylation, ligation sites (red circle), and disulfide bonds (red dotted lines) (Permission for publication received by http://advances.sciencemag.org)

with Janus Kinase 2 (JAK2) [31]. JAK2 is the chief effector kinase of the EPO/EPOR signaling. Binding of the ligand to the receptor induces conformational changes and homodimerization of EPOR, which results in the autophosphorylation of JAK2. The autophosphorylation in turn causes phosphorylation of the cytoplasmic receptors to recruit a numeral mediator, leading to the production of erythrocytes.

EPOR signaling can be regulated by several negative-feedback inhibitors, including negative-regulating phosphatases, suppressor of cytokine signaling (SOCS) proteins, receptor internalization or ubiquitination as well as proteasome degradation in its lysine (K) residues [32]. The subtilty of the interplay between EPOR maturation and turnover is remarkable in the sense that they result in fine regulation according to external stimulus. Thus, alterations in this tight regulation pathway can result in defects that lead to fluctuations in the total red cell mass and in turn blood disorders [33].

2.2 Erythropoietin and Stem Cell Factor

Directional migration of healthy red blood cells to various vital organs is controlled in and around bone marrow niche through multifaceted mechanisms including the interaction of cytokines, chemokines, and growth factors with their cognate receptors. As a consequence of hypoxia, injury, or pathogen insult, there is an increased flow of hematopoietic and non-hematopoietic cells into the circulatory system, which triggers further transcriptional activation of *EPO*/EPOR to encourage erythropoiesis [34]. Accordingly, Rankin et al. [34] have recently shown that augmentation of HIF signaling can cause higher expression of *EPO* in osteoprogenitors of the bone marrow, resulting in higher numbers of hematopoietic stem cells/progenitors and subsequently increased erythroid differentiation. This effect of EPO on bone formation has been further confirmed by others [35].

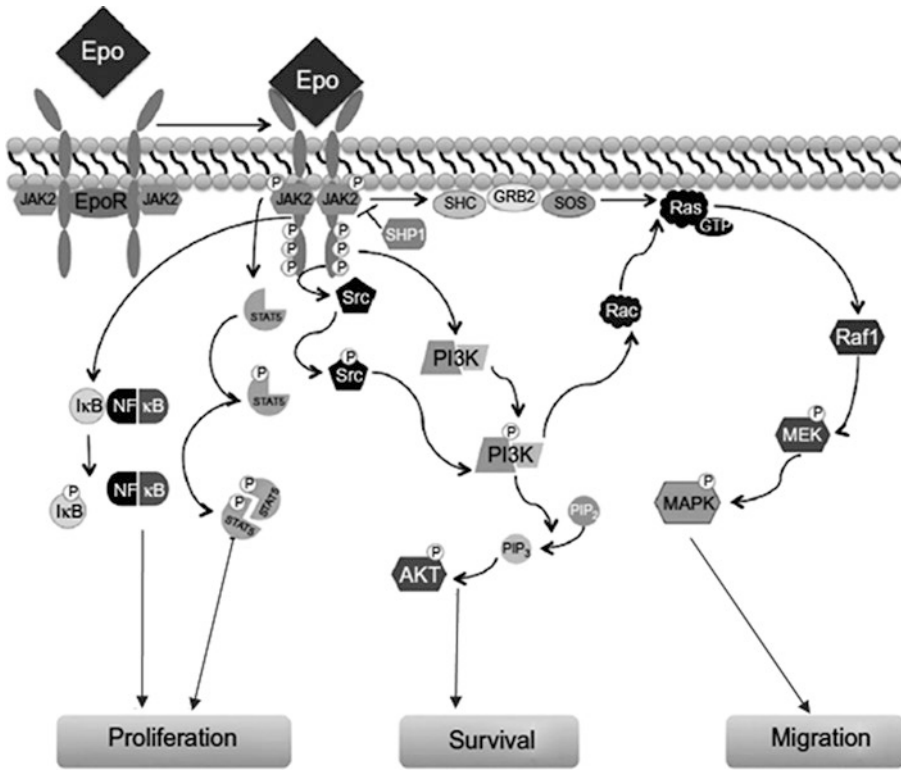

Fig. 2.4 Summary of the signal transduction pathways activated by *EPO*. Three different signal transduction pathways are predominantly activated after binding of *EPO* to its receptor: JAK2/STAT5, PI3K/AKT and RAS/RAF/MAPK, which are associated with cell proliferation, survival, and migration, respectively (Permission for publication received by Vazquez-Mellado et al. [29])

The binding of the cytokine "stem cell factor" (SCF also known KITLG) to its receptor KIT, which is caused by stress signals, also results in proliferation and differentiation of hematopoietic stem cells, resulting in the migration of new erythroid progenitors to blood stream [36].

As shown in Fig. 2.4, the homodimerization of EPOR molecules, which as a part of its signal initiation, causes transactivation of JAK2 molecules that bind to its intracellular domain [37]. Upon its phosphorylation, JAK2 triggers the activation of various receptor tyrosine kinases (RTKs) [such as Src homology-2 (SH2) domain-containing proteins, STAT1/3/5] and subsequently stimulates downstream mediators of erythroid differentiation [38, 39]. The JAK/STAT phosphorylation triggers activation of many other signaling pathways, including the phosphatidylinositol-3 kinase (PI3K)/AKT pathway, extracellular signal-related kinase (ERK) 1/2 pathway, and the mitogen-activated protein kinase (MAPK) pathways, which ultimately leads to enhanced cell survival/proliferation [40, 41].

In addition to its role as a hematopoietic factor, *EPO* has also been documented as a potent chemotactic agent. It can readily persuade directional migration of mesenchymal stem cells to the tumor microenvironment in organs [42, 43]. This process is mainly due to elevated levels of stromal cell-derived factor-1 (SDF1), which is a known ligand of bone marrow-derived C-X-C chemokine receptor type 4 (CXCR4). Injury in major vital organs such as the kidney and heart can cause elevation in the concentrations of SDF1, which are sufficient to attract CXCR4-expressing cells to the site of injury. Due to this chemotactic property, the cytokine-induced infil-

tration is attributed in hypoxia-induced pulmonary hypertension [44] in ischemic heart disease [45]. Interestingly, *EPO* was also shown to block the endothelial differentiation capacity of cardiac progenitors to prevent heart failure during anticancer therapy [46]. Therefore, it would be interesting to show if this action is also mediated through SDF1 regulation by *EPO*.

EPO synergistically works with SCF to monitor erythropoiesis inside the bone marrow microenvironment. The SI locus on human chromosome 12 produces a precursor of the 248-amino acid membrane-bound homodimeric SCF [47]. The membrane-bound SCF undergoes posttranscriptional proteolytic cleavage at a specific site to yield a monomeric, soluble 165-amino acid SCF; although both forms of SCF can serve as KIT ligands, the dimeric form is more active [48] and offers prolonged receptor activation, while the soluble counterpart causes a transient KIT activation and promotes its rapid degradation [49].

KIT is a class III RTK and belongs to the PDGFR family. It is produced as a glycosylated protein (Fig. 2.5) with five immunoglobulin-like repeats in its extracellular domain, a transmembrane region, followed by an autoinhibitory domain as well as two intracellular tyrosine kinase domains [50]. The binding of KIT to its ligand SCF causes homodimerization and activation of its enzymatic activity thorough autophosphorylation in signal-transducing proteins containing SH2 domains. This in turn triggers downstream signaling pathways [51], including JAK/STAT and RAS/ERK (Fig. 2.6).

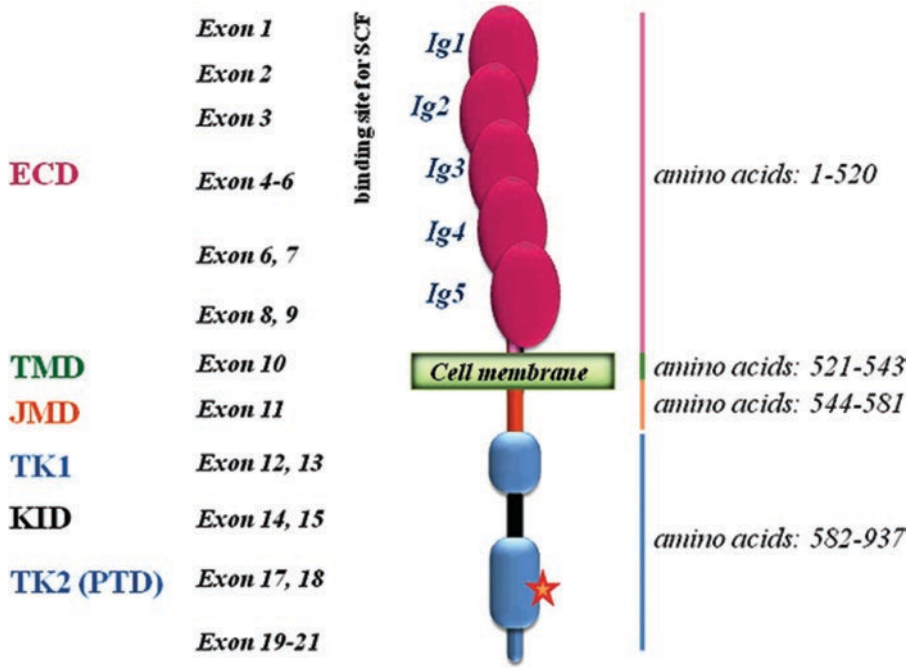

Fig. 2.5 Structure of KIT. KIT is located on chromosome 4q12 in man and contains 21 exons. After translation, KIT forms a 145-kDa (976 amino acids) transmembrane RTK. KIT is structurally characterized by five immunoglobulin-like subunits in its extracellular domain (ECD) that contain a ligand binding site (SCF) for KIT and a dimerization site, which are linked to a cytoplasmic region by a single transmembrane helix. The cytoplasmic region of KIT consists of an autoinhibitory juxtamembrane domain (JMD) and a kinase domain (KD), which are arranged in a proximal (N-) and a distal (C-) lobe, and linked by a hinge region. The C-lobe of type III RTKs include a large kinase insert domain (KID) of ~60–100 residues. The red star represents the position (amino acid 816) where an Asp to Val point mutation (KIT D816V) is found in 48% of adult systemic mastocytosis patients [29]. The EPOR receptor is presented in its monomeric form, whereas a dimer is formed from SCF ligation. TMD, transmembrane domain. (Permission for publication received by Vazquez-Mellado et al. [29])

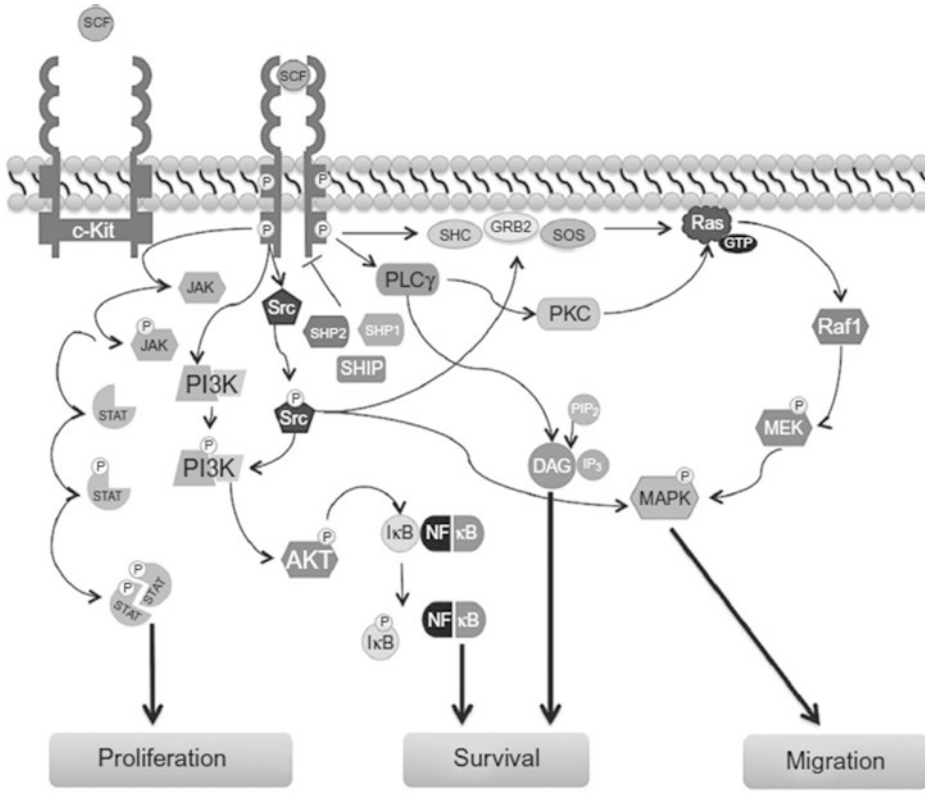

Fig. 2.6 Summary of signal transduction pathways activated by SCF/Kit regulation. (Permission for publication received by Vazquez-Mellado et al. [29])

Directional cell migration is closely monitored by the activities of SCF and KIT molecules. Both proteins are expressed during embryogenesis in migratory pathways and homing sites for melanoblasts, germ, and hematopoietic cells [52]. It is suspected that SCF/KIT interaction activates capillary tube formation in adults, though this was demonstrated using human umbilical vein endothelial cells [53]. More recently, it was reported that cardiac stem cells expressing KIT were able to migrate in response to SCF. This migration was facilitated by the ability of SCF-induced chemotaxis to induce the PI3K/AKT signal activation process, which can upregulate the expression of matrix metalloproteinases 3 and 9 [54]. Accordingly, results from Cervi et al. [12] suggest that the treatment HB60–5 leukemic cells with the anti-inflammatory drug Celebrex reduces KIT expression as well as EPOR phosphorylation, which in turn causes apoptosis. KIT overexpression does indeed enhance survival by up to twofold after treatment with Celecoxib.

2.3 Erythropoietin: Role in Proliferation and Migration in Various Cancer Types

The physiological significance of the *EPO*/EPOR expression in cancer cells was originally considered controversial due to the lack of a specific antibody to detect EPOR in the pioneering studies [55, 56]. However, very recently, specific anti-EPOR antibodies were developed, which have revealed the specific expression of this receptor in tumor cells. For instance, Cy5.5 (a near-infrared dye) conjugated rEPO was used as a probe to monitor EPOR

expression noninvasively by fluorescence-mediated tomography [57]. Likewise, by coupling Ga-DOTA to the carbohydrate side chain of recombinant *EPO*, a radiotracer was developed for monitoring EPOR status in developing tumors in vivo using PET (positron emission tomography) imaging [58]. Some studies demonstrated EPOR expression as a factor for the promotion of cancer cell proliferation, whereas in others rEPO suggested opposite results. Examples of *EPO*/EPOR signaling and their functions in both tumor promotion and suppression are given below.

2.3.1 Breast Cancer

- Quantitative reverse transcription-PCR (RT-PCR) analysis revealed the presence of EPOR as between 104 and 1608 copy numbers/10 ng of RNA in many breast cancer cell lines [59].
- Um et al. [60] projected that around 50 high-affinity *EPO* binding sites were optimum for EPO-mediated signal activation in cancer cells.
- Breast cancer-derived cell lines MDA468, SKBR3, MCF7, and MDA453 produce and discharge *EPO* into the extracellular matrix, promoting the autocrine/paracrine *EPO*/EPOR in the cells. Further study on the SKBR3 cell line coupled with siRNA-mediated downregulation of *EPO* revealed that the expression of this hormone regulates cell proliferation, migration, invasion, and stemness [61].
- In a recent study, Pradeep et al. [62] identified the Ephrin receptor B4 (EphB4) as a new receptor for EPO. In human breast and ovarian cancer, the binding or *EPO* to EPHB4 stimulated the phosphorylation and activation of STAT3, causing tumor progression (Fig. 2.7; [62]).

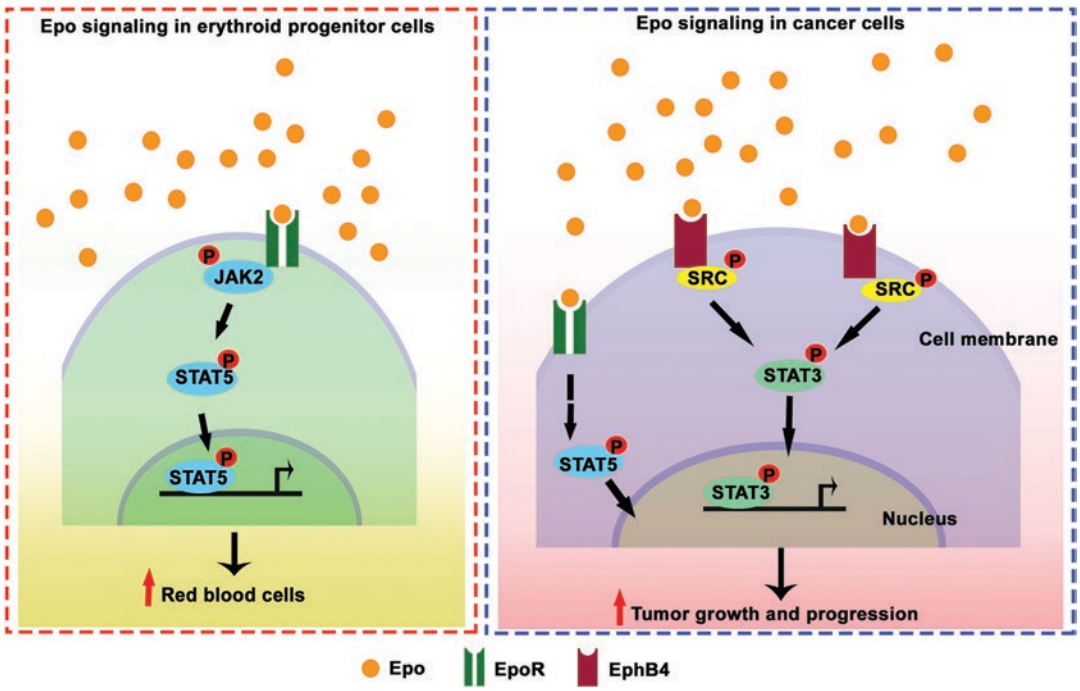

Fig. 2.7 Summary of signal transduction pathways activated by STAT3. (Permission for publication received by Pradeep et al. [62])

2.3.2 Ovarian Cancer

- A restriction digest of semiquantitative RT-PCR products and DNA sequencing confirmed the expression of EPOR in CaOV, SKOV, OVCAR-3, and A2780 human ovarian cancer cell lines [63].
- Inhibition of EPOR by a neutralizing antibody was able to block cell proliferation in A2780 cells. Moreover, fluorescence microscopy discovered the presence of EPOR in A2780 cells [64].

2.3.3 Lung Cancer

- Following bronchoscopy, EPOR transcription was detected by quantitative RT-PCR in stage III–IV non-small cell lung adenocarcinoma [65]. The study also demonstrated that 33.45% of the tumors had an elevated expression of *EPOR*, which was associated with a good prognosis. Moreover, rEPO and gemcitabine treatments increased the proliferation on endothelial as well as H1975 xenograft tumors (Fig. 2.8).

2.3.4 Cervical Cancer

- Autocrine activation of the EPO/EPOR signaling in cervical cancer cells was shown to be mediated through the JAK/STAT pathway, resulting in increased cell proliferation [8].
- *EPO* induced the growth of xenograft cervical cancer tumors in vivo and activated the phosphorylation of JAK1 (pJAK1), JAK2 (pJAK2), JAK3 (pJAK3), STAT1 (pSTAT1), STAT3 (pSTAT3), and STAT5 (pSTAT5) in the cells implanted with 1×10^5 HeLa and SiHa cells, after 15 days. Phosphorylation levels were evaluated in mice treated with EPO or vehicle by Western blot [8].

2.3.5 Leukemia

- In multiple melanoma (MM), rEPO treatment blocked the proliferation of MM cell line MOPC-315 in culture, as well as its progression in a mouse xenograft model [66]. EPO-induced tumor regression was later shown to be associated with a gain of immunity against MM cells, and also bone density loss [13, 14]. The importance of *EPO* as a therapy for multiple myeloma was later shown by other groups [15, 16].
- In erythroleukemia cell lines induced by friend murine leukemia virus (F-MuLV), *EPO* expression was detected in many cell lines. In some cell lines, this expression was associated with rearrangement of the *epo* genetic structure [67]. *epo* expression in these cells was shown to promote EPO-dependent proliferation in culture [68]. Despite this, in erythroleukemias induced by F-MuLV, rEPO, treatment of leukemic mice delayed significantly the disease progression via a compensatory erythropoietic response in combination with natural killer cell action [11]. In this and a subsequent study, both VEGF and *EPO* were shown to induce a condition in mice akin to normoxia, in contrast to hypoxia, which can block leukemia progression in vivo [11, 69, 70].
- Additionally, the rEPO administration into leukemic mice induced a polycythemia-like condition with an expansion of $SCA1^+/KIT^-$ progenitors, causing immune cell progression and reduction of leukemia. This study suggested that the combination therapy efficacy of $SCA1^+/KIT^-$ progenitors could present an alternative therapeutic strategy for leukemia [71].
- Ectopic *FLI1* expression in an erythroblastic cell line developed EPO-induced proliferation, rather than differentiation. This was made possible through the activation and inactivation of many downstream target of

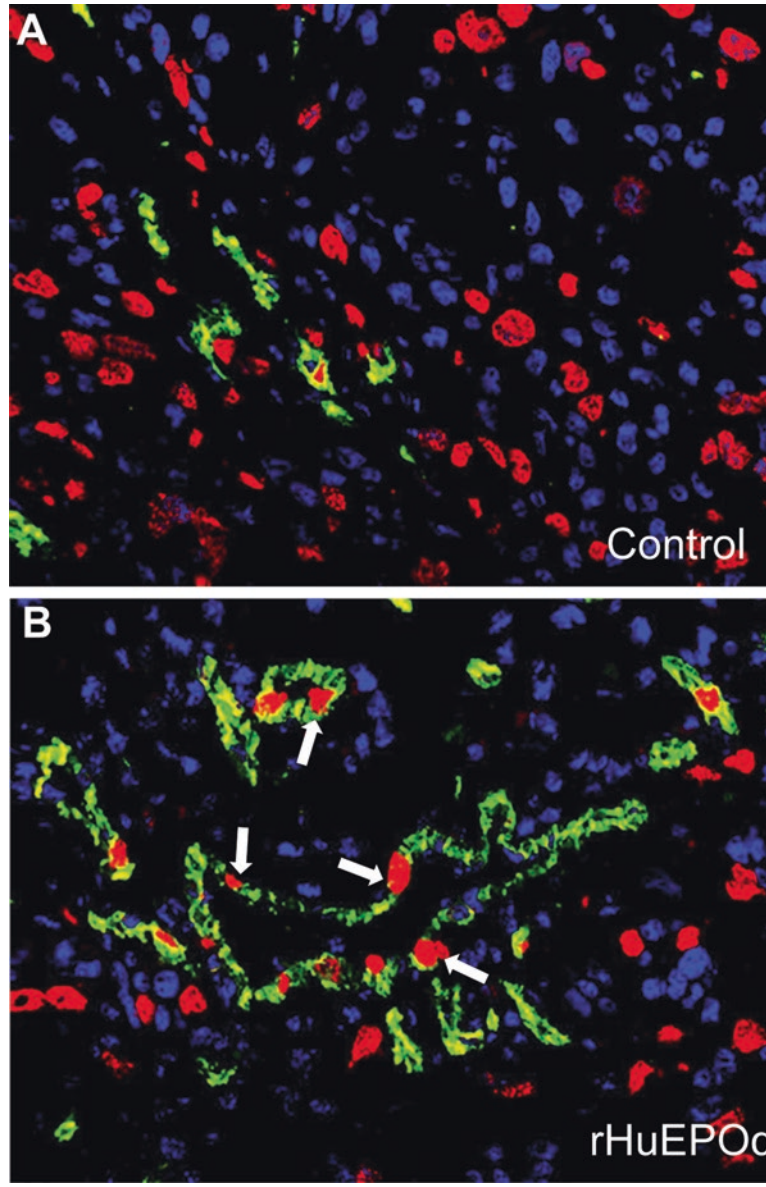

Fig. 2.8 The tumor and endothelial cells in rEPO-treated and control H1975 tumors. (**a**) Control untreated and rEPO-treated. Tumor sections are stained for the endothelial marker, CD31 (green), the proliferation-associated marker, BrdU (red) and for TOTO-3 (blue) highlighting EC as well as tumor cell nuclei. Arrows in (**b**) point at proliferating endothelial cells. (Permission for publication received by Rozsas et al. [65])

the transcription factor FLI1 [72, 73]. Despite this, leukemias carrying an activated FLI1 can still be inhibited with *EPO* therapy [11, 69]. These results suggest that *EPO* may induce the activation of immune system surrounding the tumor microenvironment to block leukemia progression, a notion that may require future investigation.

2.4 Clinical Significance of Erythropoietin Signaling

Even though cancer-associated anemia is genetically multifactorial, it may worsen after chemotherapy and radiotherapy. Statistics claim that around 40.7% of cancer patients show anemic symptoms at their treatment time [74]. Cancer-

associated anemia is therefore treated with rEPO in most cases. Administration of *EPO* is indeed reported to cause a progression-free tumor microenvironment and increase the overall survival times of cancer patients [33]. However, in some other clinical trials, the outcome of *EPO* administration was negative [75]. This may be due to an excessively high red blood cell level induced after high exogenous *EPO* administration, which could cause thrombosis in some patients [76, 77]. These results also raised the possibility that *EPO* administration may be beneficial for some types of cancer and harmful for authors. It is also possible that the inhibitory effects of *EPO* may work in early/medium stage tumors; yet not in late-stage cancers. Furthermore, the genetic situation with the tumors (i.e., activation/inactivation of additional oncogene\suppressor genes) may override *EPO's* tumor inhibitory effects and result in a greater proliferation. It is also possible that some types of cancer, such as Leukemia, are more responsive to the tumor inhibitory effect of *EPO* than solid tumors. Thus, it is critical to uncover the mechanism of tumor inhibition and progression ability of *EPO* to identify biomarkers for the response to this cytokine.

2.5 Concluding Remarks

EPO is primarily known for its role as a hormone, which stimulates the generation of red blood cells. However, the expression of *EPOR* was also identified in many studies outside the hematopoietic tissues, indicating a role for this hormone in non-hematopoietic cells. The expression of *EPO* is indeed found in various non-hematopoietic cells and more abundantly in various cancer types. Due to this expression pattern, the EPO/EPOR signaling was also connected to tumor microenvironment, causing changes in biological activities such as the immune system, migration, and tumor progression. In the clinic, *EPO* is widely used to overcome anemia, yet its administration in cancer patients has been associated with both tumor inhibition and progression. Understanding how *EPO* affects non-hematopoietic tissue as well as its underlying mechanism of action in cancer cells may result in the development of a better strategy for the treatment of cancer-related anemia and potentially even the use of this hormone (alone or in combination with other factors) for the treatment of some cancers.

References

1. Youssoufian H, Longmore G, Neumann D, Yoshimura A, Lodish HF (1993) Structure, function, and activation of the erythropoietin receptor. Blood 81:2223–2236
2. Yasuda Y, Nagao M, Okano M, Masuda S, Sasaki R et al (1993) Localization of erythropoietin and erythropoietin-receptor in post-implantation mouse embryos. Dev Growth Differ 35(6):711–722. https://doi.org/10.1111/j.1440-169X.1993.00711.x
3. Snow MHL (1977) Gastrulation in the mouse: growth and regionalization of the epiblast. J Embryol Exp Morphol 42:293–303. https://dev.biologists.org/content/42/1/293
4. Yasuda Y, Fujita Y, Musha T, Tanaka H, Shiokawa S, Nakamatsu K et al (2001) Expression of erythropoietin in human female reproductive organs. Ital J Anat Embryol 106:215–222
5. Acs G, Zhang PJ, Rebbeck TR, Acs P, Verma A (2002) Immunohistochemical expression of erythropoietin and erythropoietin receptor in breast carcinoma. Cancer 95:965–981. https://doi.org/10.1002/cncr.10787
6. Arcasoy MO, Amin K, Karayal AF, Chou SC, Raleigh JA et al (2002) Functional significance of erythropoietin receptor expression in breast cancer. Lab Investig 82:911–918. https://doi.org/10.1097/01.LAB.0000020415.72863.40
7. Feldman L, Wang Y, Rhim JS, Bhattacharya N, Loda M, Sytkowski AJ (2006) Erythropoietin stimulates growth and STAT5 phosphorylation in human prostate epithelial and prostate cancer cells. Prostate 66:135–145. https://doi.org/10.1002/pros.20310
8. Lopez TV, Lappin TRJ, Maxwell P, Shi Z, Lopez-Marure R, Aguilar C et al (2011) Autocrine/paracrine erythropoietin signaling promotes JAK/STAT-dependent proliferation of human cervical cancer cells. Int J Cancer 129:2566–2576. https://doi.org/10.1002/ijc.25935
9. Yasuda Y, Fujita Y, Matsuo T, Koinuma S, Hara S, Tazaki A et al (2003) Erythropoietin regulates tumour growth of human malignancies. Carcinogenesis 24:1021–1029. https://doi.org/10.1093/carcin/bgg060
10. Mittelman M, Neumann D, Peled A, Kanter P, Haran-Ghera N (2003) Erythropoietin induces tumor regression and antitumor immune responses in murine myeloma models. Proc Natl Acad Sci U S A 98(9):5181–5186. https://www.ncbi.nlm.nih.gov/pubmed/?term=Erythropoietin+induces+tumor+regression+and+antitumor+immune+responses+in+murine+myeloma+models

11. Cervi D, Shaked Y, Haeri M, Usenko T, Lee CR et al (2007) Enhanced natural-killer cell and erythropoietic activities in VEGF-A-overexpressing mice delay F-MuLV-induced erythroleukemia. Blood 109(5):2139–2146. https://doi.org/10.1182/blood-2005-11-026823
12. Cervi D, Truong AH, Lee JS, Sukhai N, Li YJ, Koki A, Ben-David Y (2004) Phosphorylation status of c-kit and EPO receptors, and the presence of wild-type p53 confer in vitro resistance of murine erythroleukemic cells to Celecoxib. Oncogene 23(13):2305–2314. https://doi.org/10.1038/sj.onc.1207400
13. Deshet-Unger N, Hiram-Bab S, Haim-Ohana Y, Mittelman M, Gabet Y, Neumann D (2016) Erythropoietin treatment in murine multiple myeloma: immune gain and bone loss. Sci Rep 6:30998. https://doi.org/10.1038/srep30998
14. Katz O, Gil L, Lifshitz L, Prutchi-Sagiv S, Gassmann M, Mittelman M, Neumann D (2007) Erythropoietin enhances immune responses in mice. Eur J Immunol 37(6):1584–1593. https://doi.org/10.1002/eji.200637025
15. Lamanuzzi A, Saltarella I, Ferrucci A, Ria R, Ruggieri S, Racanelli V et al (2016) Role of erythropoietin in the angiogenic activity of bone marrow endothelial cells of MGUS and multiple myeloma patients. Oncotarget 7(12):14510–14521. https://doi.org/10.18632/oncotarget.7587
16. Vatsveen TK, Sponaas AM, Tian E, Zhang Q, Misund K, Sundan A et al (2016) Erythropoietin (EPO)-receptor signaling induces cell death of primary myeloma cells in vitro. Hematol Oncol 9(1):75. https://doi.org/10.1186/s13045-016-0306-x
17. Miyake T, Kung CK, Goldwasser E (1977) Purification of human erythropoietin. J Biol Chem 252(15):5558–5564
18. Obara N, Imagawa S, Nakano Y, Suzuki N, Yamamoto M, Nagasawa T (2003) Suppression of erythropoietin gene expression by cadmium depends on inhibition of HIF-1, not stimulation of GATA-2. Arch Toxicol 77(5):267–273. https://doi.org/10.1007/s00204-003-0444-0
19. Tabata M, Tarumoto T, Ohmine K, Furukawa Y, Hatake K, Ozawa K et al (2003) Stimulation of GATA-2 as a mechanism of hydrogen peroxide suppression in hypoxia-induced erythropoietin gene expression. J Cell Physiol 186(2):260–267. https://doi.org/10.1002/1097-4652(200002)186:2<260::AID-JCP1025>3.0.CO;2-K
20. Haase VH (2013) Regulation of erythropoiesis by hypoxia-inducible factors. Blood Rev 27(1):41–53. https://doi.org/10.1016/j.blre.2012.12.003
21. Ebert BL, Bunn HF (1999) Regulation of the erythropoietin gene. Blood 94(6):1864–1877
22. Fisher JW (2003) Erythropoietin: physiology and pharmacology update. Exp Biol Med 228(1):1–14. https://doi.org/10.1177/153537020322800101
23. Lacombe C, Mayeux P (1998) Biology of erythropoietin. Haematologica 83(8):724–732
24. Sasaki R, Masuda S, Nagao M (2000) Erythropoietin: multiple physiological functions and regulation of biosynthesis. Biosci Biotechnol Biochem 64:1775–1793. https://doi.org/10.1271/bbb.64.1775
25. Jelkmann W (2003) Erythropoietin. J Endocrinol Investig 26:832–837. https://doi.org/10.1007/BF03345232
26. Maiese K, Chong ZZ, Hou J, Shang YC (2008) Erythropoietin and oxidative stress. Curr Neurovasc Res 5:125–142
27. Toyoda T, Itai T, Arakawa T, Aoki KH, Yamaguchi H (2000) Stabilization of human recombinant erythropoietin through interactions with the highly branched N-glycans. J Biochem 128:731–737. https://doi.org/10.1093/oxfordjournals.jbchem.a022809
28. Singbartl G (1994) Adverse events of erythropoietin in long-term and in acute/short-term treatment. Clin Investig 72(6):36–43
29. Vazquez-Mellado MJ, Monjaras-Embriz V, Rocha-Zavaleta L (2017) Erythropoietin, stem cell factor, and cancer cell migration. Vitam Horm 105:273–291. https://doi.org/10.1016/bs.vh.2017.02.008
30. D'Andrea AD, Zon LI (1990) Erythropoietin receptor. Subunit structure and activation. J Clin Invest 86(3):681–687. https://doi.org/10.1172/JCI114763
31. Huang LJ, Constantinescu SN, Lodish HF (2001) The N-terminal domain of Janus kinase 2 is required for Golgi processing and cell surface expression of erythropoietin receptor. Mol Cell 8(6):1327–1338. https://doi.org/10.1016/S1097-2765(01)00401-4
32. Kuhrt D, Wojchowski DM (2015) Emerging EPO and EPO receptor regulators and signal transducers. Blood 125(23):3536–3541. https://doi.org/10.1182/blood-2014-11-575357
33. Temkin SM, Hellmann M, Serur E, Lee YC, Abulafia O (2006) Erythropoietin administration during primary treatment for locally advanced cervical carcinoma is associated with poor response to radiation. Int J Gynecol Cancer 16:1855–1861. https://doi.org/10.1111/j.1525-1438.2006.00709.x
34. Rankin EB, Wu C, Khatri R, Wilson TL, Andersen R, Araldi E et al (2012) The HIF signaling pathway in osteoblasts directly modulates erythropoiesis through the production of EPO. Cell 149(1):63–74. https://doi.org/10.1016/j.cell.2012.01.051
35. McGee SJ, Havens AM, Shiozawa Y, Jung Y, Taichman RS (2012) Effects of erythropoietin on the bone microenvironment. Growth Factors 30(1):22–28. https://doi.org/10.3109/08977194.2011.637034
36. Munugalavadla V, Kapur R (2005) Role of c-kit and erythropoietin receptor in erythropoiesis. Crit Rev Oncol Hematol 54:63–75. https://doi.org/10.1016/j.critrevonc.2004.11.005
37. Witthuhn BA, Quelle FW, Silvennoinen O, Yi T, Tang B, Miura O et al (1993) JAK2 associates with the erythropoietin receptor and is tyrosine phosphorylated and activated following stimulation with erythropoietin. Cell 74:227–236. https://doi.org/10.1016/0092-8674(93)90414-l

38. Kirito K, Nakajima K, Watanabe T, Uchida M, Tanaka M et al (2002) Identification of the human erythropoietin receptor region required for Stat1 and Stat3 activation. Blood 99:102–110. https://doi.org/10.1182/blood.v99.1.102
39. Klingmuller U, Bergelson S, Hsiao JG, Lodish HF (1996) Multiple tyrosine residues in the cytosolic domain of the erythropoietin receptor promote activation of STAT5. Proc Natl Acad Sci U S A 93:8324–8328. https://doi.org/10.1073/pnas.93.16.8324
40. Bao H, Jacobs-Helber SM, Lawson AE, Penta K, Wickrema A, Sawyer ST (1999) Protein kinase B (c-Akt), phosphatidylinositol 3-kinase, and STAT5 are activated by erythropoietin (EPO) in HCD57 erythroid cells but are constitutively active in an EPO-independent, apoptosis-resistant sub clone (HCD57-SREI cells). Blood 93:3757–3773
41. Damen JE, Wakao H, Miyajima A, Krosl J, Humphries RK, Cutler RL et al (1995) Tyrosine 343 in the erythropoietin receptor positively regulates erythropoietin-induced cell proliferation and Stat5 activation. EMBO J 14:5557–5568. https://doi.org/10.1002/j.1460-2075.1995.tb00243.x
42. Lin JS, Chen YS, Chiang HS, Ma MC (2008) Hypoxic preconditioning protects rat hearts against ischaemia-reperfusion injury: role of erythropoietin on progenitor cell mobilization. J Physiol 586:5757–5769. https://doi.org/10.1113/jphysiol.2008.160887
43. Liu N, Tian J, Cheng J, Zhang J (2013) Effect of erythropoietin on the migration of bone marrow-derived mesenchymal stem cells to the acute kidney injury microenvironment. Exp Cell Res 319:2019–2027. https://doi.org/10.1016/j.yexcr.2013.04.008
44. Satoh K, Kagaya Y, Nakano M, Ito Y, Ohta J, Tada H et al (2006) Important role of endogenous erythropoietin system in recruitment of endothelial progenitor cells in hypoxia-induced pulmonary hypertension in mice. Circulation 113:1442–1450. https://doi.org/10.1161/CIRCULATIONAHA.105.583732
45. Heeschen C, Aicher A, Lehmann R, Fichtlscherer S, Vasa M, Urbich C et al (2003) Erythropoietin is a potent physiologic stimulus for endothelial progenitor cell mobilization. Blood 102:1340–1346. https://doi.org/10.1182/blood-2003-01-0223
46. Hoch M, Fischer P, Stapel B, Missol-Kolka E, Sekkali B, Scherr M et al (2011) Erythropoietin preserves the endothelial differentiation capacity of cardiac progenitor cells and reduces heart failure during anticancer therapies. Cell Stem Cell 9(2):131–143. https://doi.org/10.1016/j.stem.2011.07.001
47. Ropers HH, Craig IW (1989) REPOrt of the committee on the genetic constitution of chromosomes 12 and 13. Cytogenet Cell Genet 51:259–279. https://doi.org/10.1159/000132794
48. Hsu YR, Wu GM, Mendiaz EA, Syed R, Wypych J, Toso R et al (1997) The majority of stem cell factor exists as monomer under physiological conditions. Implications for dimerization mediating biological activity. J Biol Chem 272:6406–6415. https://doi.org/10.1074/jbc.272.10.6406
49. Langley KE, Bennett LG, Wypych J, Yancik SA, Liu XD, Westcott KR et al (1993) Soluble stem cell factor in human serum. Blood 81:656–660
50. Liu H, Chen X, Focia PJ, He X (2007) Structural basis for stem cell factor-kit signaling and activation of class III receptor tyrosine kinases. EMBO J 26:891–901. https://doi.org/10.1038/sj.emboj.7601545
51. Ronnstrand L (2004) Signal transduction via the stem cell factor receptor/c-kit. Cell Mol Life Sci 61:2535–2548. https://doi.org/10.1007/s00018-004-4189-6
52. Matsui Y, Zsebo KM, Hogan BL (1990) Embryonic expression of a haematopoietic growth factor encoded by the Sl locus and the ligand for c-kit. Nature 347:667–669. https://doi.org/10.1038/347667a0
53. Matsui J, Wakabayashi T, Asada M, Yoshimatsu K, Okada M (2004) Stem cell factor/c-kit signaling promotes the survival, migration, and capillary tube formation of human umbilical vein endothelial cells. J Biol Chem 279:18600–18607. https://doi.org/10.1074/jbc.M311643200
54. Guo J, Jie W, Shen Z, Li M, Lan Y, Kong Y et al (2014) SCF increases cardiac stem cell migration through PI3K/AKT and MMP-2/−9 signaling. Int J Mol Med 34:112–118. https://doi.org/10.3892/ijmm.2014.1773
55. Brown WM, Maxwell P, Graham AN, Yakkundi A, Dunlop EA, Shi Z et al (2007) Erythropoietin receptor expression in non-small cell lung carcinoma: a question of antibody specificity. Stem Cells 25:718–722. https://doi.org/10.1634/stemcells.2006-0687
56. Elliott S, Busse L, Bass MB, Lu H, Sarosi I, Sinclair AM et al (2006) Anti-EPO receptor antibodies do not predict EPO receptor expression. Blood 107:1892–1895. https://doi.org/10.1182/blood-2005-10-4066
57. Doleschel D, Mundigl O, Wessner A, Gremse F, Bachmann J, Rodriguez A et al (2012) Targeted near-infrared imaging of the erythropoietin receptor in human lung cancer xenografts. J Nucl Med 53:304–311. https://doi.org/10.2967/jnumed.111.091124
58. Fuge F, Doleschel D, Rix A, Gremse F, Wessner A, Winz O et al (2015) In-vivo detection of the erythropoietin receptor in tumours using positron emission tomography. Eur Radiol 25:472–479. https://doi.org/10.1007/s00330-014-3413-5
59. Shi Z, Hodges VM, Dunlop EA, Percy MJ, Maxwell AP, El-Tanani M et al (2010) Erythropoietin-induced activation of the JAK2/STAT6, PI3K/Akt, and Ras/ERK pathways promotes malignant cell behaviour in a modified breast cancer cell line. Mol Cancer Res 8:615–626. https://doi.org/10.1158/1541-7786.MCR-09-0264
60. Um M, Gross AW, Lodish HF (2007) A "classical" homodimeric erythropoietin receptor is essential for the antiapoptotic effects of erythropoietin on differentiated neuroblastomaSH-SY5Y and pheochromocytoma PC-12 cells. Cell Signal 19:634–645. https://doi.org/10.1016/j.cellsig.2006.08.014
61. Liang K, Qiu S, Lu Y, Fan Z (2014) Autocrine/paracrine erythropoietin regulates migration and invasion potential and the stemness of human breast cancer cell. Cancer Biol Ther 15:89–98. https://doi.org/10.4161/cbt.26717

62. Pradeep S, Huang J, Mora EM, Nick AM, Cho MS, Wu SY et al (2015) Erythropoietin stimulates tumor growth via EphB4. Cancer Cell 28(5):610–622. https://doi.org/10.1016/j.ccell.2015.09.008
63. Jeong JY, Feldman L, Solar P, Szenajch J, Sytkowski J (2008) Characterization of erythropoietin receptor and erythropoietin expression and function in human ovarian cancer cells. Int J Cancer 122:274–280. https://doi.org/10.1002/ijc.23068
64. Solar P, Hrckova G, Varinska L, Solarova Z, Kriska J, Uhrinova I et al (2012) Location and the functionality of erythropoietin receptor(s) in A2780 cells. Oncol Rep 28:141–146. https://doi.org/10.3892/or.2012.1795
65. Rozsas A, Berta J, Rojko L, Horvath LZ, Keszthelyi M, Kenessey I et al (2013) Erythropoietin receptor expression is a potential prognostic factor in human lung adenocarcinoma. PLoS One 8:e77459. https://doi.org/10.1371/journal.pone.0077459
66. Mittelman M, Neumann D, Peled A, Kanter P, Haran-Ghera N (2001) Erythropoietin induces tumor regression and antitumor immune responses in murine myeloma models. Proc Natl Acad Sci U S A 98(9):5181–5186. https://doi.org/10.1073/pnas.081275298
67. Howard JC, Berger L, Bani MR, Hawley RG, Ben-David Y (1996) Activation of the erythropoietin gene in the majority of F-MuLV induced erythroleukemias results in growth factor independence and enhanced tumorigenicity. Oncogene 12:1405–1415
68. Howard JC, Yosefi S, Cheong G, Bernstein A, Ben-David Y (1993) Temporal order of the mutation of Fli-1, p53 and erythropoietin genes during the multistep erythroleukemias induced by F-MuLV. Oncogene 8:2721–2729
69. Shaked Y, Cervi D, Neuman M, Klement G, Michaud CR, Haeri M et al (2005) The splenic microenvironment is a source of angiogenesis/inflammatory mediators accelerating the expansion of murine erythroleukemic cells. Blood 105:4500–4507. https://doi.org/10.1182/blood-2004-08-3210
70. Vecchiarelli-Federico LM, Cervi D, Haeri M, Li Y, Nagy A, Ben-David Y (2010) Vascular endothelial growth factor--a positive and negative regulator of tumor growth. Cancer Res 70(3):863–867. https://doi.org/10.1158/0008-5472.CAN-09-3592
71. Usenko T, Li YJ, Haeri M, Li Y, Vecchiarelli-Federico LM, Zhao X et al (2009) Enrichment of $Sca1^+$ hematopoietic progenitors in polycythemic mice inhibits leukemogenesis. Blood 114:1831–1841. https://doi.org/10.1182/blood-2008-11-187419
72. Cui JW, Li YJ, Sarkar A, Brown J, Tan YH et al (2007) Retroviral insertional activation of the Fli-3 locus in erythroleukemias encoding a cluster of microRNAs that convert EPO-induced differentiation to proliferation. Blood 110:2631–2640. https://doi.org/10.1182/blood-2006-10-053850
73. Li Y, Luo H, Liu T, Zachsenhaus E, Ben-David Y (2015) The transcription factor Fli-1 in development, cancer and disease. Oncogene 35:2022–2031. https://doi.org/10.1038/onc.2014.162
74. Knight K, Wade S, Balducci L (2004) Prevalence and outcomes of anemia in cancer: a systematic review of the literature. Am J Med. 116:11S–26S. https://www.ncbi.nlm.nih.gov/pubmed/15050883
75. Debeljak N, Solár P, Sytkowski AJ (2014) Erythropoietin and cancer: the unintended consequences of anemia correction. Front Immunol 5:563. https://doi.org/10.3389/fimmu.2014.00563
76. Moebus V, Jackisch C, Schneeweiss A, Huober J, Lueck HJ, du Bois A et al (2013) Adding EPOetin alfa to intense dose-dense adjuvant chemotherapy for breast cancer: randomized clinical trial. J Natl Cancer Inst 105(14):1018–2610. https://doi.org/10.1093/jnci/djt145
77. Tonia T, Mettler A, Robert N, Schwarzer G, Seidenfeld J, Weingart O et al (2012) Erythropoietin or darbEPOetin for patients with cancer. Cochrane Database Syst Rev 12:CD003407. https://doi.org/10.1002/14651858.CD003407.pub5

3 Neuropilin: Handyman and Power Broker in the Tumor Microenvironment

Stephan Niland and Johannes A. Eble

Abstract

Neuropilin-1 and neuropilin-2 form a small family of transmembrane receptors, which, due to the lack of a cytosolic protein kinase domain, act primarily as co-receptors for various ligands. Performing at the molecular level both the executive and organizing functions of a handyman as well as of a power broker, they are instrumental in controlling the signaling of various receptor tyrosine kinases, integrins, and other molecules involved in the regulation of physiological and pathological angiogenic processes. In this setting, the various neuropilin ligands and interaction partners on various cells of the tumor microenvironment, such as cancer cells, endothelial cells, cancer-associated fibroblasts, and immune cells, are surveyed. The suitability of various neuropilin-targeting substances and the intervention in neuropilin-mediated interactions is considered as a possible building block of tumor therapy.

Keywords

Cancer cell · Endothelial cell · Neuropilin interacting partners · Neuropilin ligands · Neuropilin signaling · Semaphorin · Tumor-penetrating peptides · Tumor angiogenesis · Tumor microenvironment · Tumor stromal cell · Vascular endothelial growth factor

Abbreviations

3′-UTR	3′-Untranslated region
ADAM	A disintegrin and metalloproteinase domain containing protein
ADAMTS	A disintegrin and metalloproteinase with thrombospondin motifs
AGO	Argonaute
AKT	Protein kinase B
ALK	Anaplastic lymphoma kinase
ALK1	Activin receptor-like kinase; serine/threonine-protein kinase receptor R3
ALK5	Activin receptor-like kinase; TGF-β receptor 1
BMP	Bone morphogenetic protein
BRAF	Rat/rapidly accelerated fibrosarcoma, isoform B
CAF	Cancer-associated fibroblasts
CD	Cluster of differentiation
CendR	Carboxy-terminal end rule
CSC	Cancer stem cell
CUB domain	Cubilin homology domain
DDR	Discoidin domain receptor

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Dlg domain	Discs large domain
EC	Endothelial cell
ECM	Extracellular matrix
EGF(R)	Epidermal growth factor (receptor)
EMT	Epithelial to mesenchymal transition
EphA2	Erythropoietin-producing human hepatocellular (EPH) receptor A2
ER	Endoplasmic reticulum
ErbB	Erythroblastosis oncogene B
ERK	Extracellular-signal-regulated kinase
FAK	Focal adhesion kinase
FGF(R)	Fibroblast growth factor (receptor)
Frzb	Frizzled-related protein
GAIP	G alpha interacting protein
GAP	GTPase activation protein
gC1qR	Globular head of complement factor C1q binding protein/ receptor
GEF	Guanine nucleotide exchange factor
GIPC	GAIP interacting protein, C-terminus
GIPC1	GIPC PDZ domain containing family member 1, synectin
GLI1	Glioma-associated oncogene homolog 1
GLUT1CBP	Glucose transporter 1 C-terminal binding protein
Her2	Human epidermal growth factor receptor 2
HGF(R)	Hepatocyte growth factor (receptor)
HH	Hedgehog
IGF1R	Insulin-like growth factor 1 (IGF-1) receptor
IIP1	Insulin-like growth factor-1 receptor-interacting protein 1
Jnk	c-Jun N-terminal kinase
KRAS	Kirsten rat sarcoma
L1CAM	L1 cell adhesion molecule
LAMC2	Laminin subunit γ2
lncRNA	Long noncoding RNA
LRP5	Low-density lipoprotein receptor related protein 5
MAM domain	Meprin/A5-protein/PTPmu
MAP(K)	Mitogen-activated protein (kinase)
MET	Mesenchymal-epithelial transition factor (MET) proto-oncogene
miR	microRNA
MMP	Matrix metalloproteinase
NIP	Neuropilin-1 interacting protein
NRP	Neuropilin
p130Cas	CRK-associated substrate
PDGF(R)	Platelet-derived growth factor (receptor)
PDZ	Postsynaptic density/discs large/zonula occludens-1
PI3K	Phosphoinositide 3-kinase
PKC	Protein kinase C
PlGF(R)	Placenta growth factor (receptor)
PSD-95 domain	Postsynaptic density protein 95 domain
PTEN	Phosphatase and tensin homolog
PTPmu	Protein tyrosine phosphatase μ
RAS	Rat sarcoma
RhoGEF	Rho guanine nucleotide exchange factor 1
RTK	Receptor-type tyrosine kinase
SAPK1	Stress-activated protein kinase 1
SEMA	Semaphorin
SEMCAP1	Semaphorin 4C (SEMA4C)-interacting protein 1
SMAD	sma(ll) and Daf-4 homolog
sNRP	Soluble neuropilin
Src	Sarcoma
Syx	Synectin-binding GEF
TAM	Tumor-associated macrophage
TEC	Tumor endothelial cell
TFPI1	Tissue factor pathway inhibitor
TGF-β(R)	Transforming growth factor-β (receptor)

TIE	Tyrosine kinase with immunoglobulin-like and EGF-like domains
TIP2	Tax-interacting protein 2
TORC2	Rapamycin-sensitive TOR complex 2
Treg	Regulatory T cell
uPA	Urokinase plasminogen activator
VCAM-1	Vascular adhesion protein-1
VEGF(R)	Vascular endothelial growth factor (receptor)
VM	Vasculogenic mimicry
WIF1	Wnt inhibitory factor 1
Wnt	Wingless-related integration site
YAP1	Yes-associated protein 1
ZO-1 domain	Zonula occludens-1 domain

3.1 Introduction

The two types of neuropilins, NRP1 and NRP2, form a small family of transmembrane receptors with a broad tissue distribution, which, due to the lack of a cytosolic protein kinase domain, act primarily as co-receptors for various ligands [1–3]. At both molecular and cellular level, they perform the executive and organizing functions of a handyman as well as of a power broker. They are instrumental in controlling the signaling of various receptor tyrosine kinases, integrins, and other molecules involved in different cells and tissues in the regulation of physiological and pathological angiogenic processes. As pleiotropic co-receptors, NRPs are thus involved in various canonical and alternative signaling processes that are important for a wide variety of processes, such as cell proliferation and survival, cell adhesion and migration, matrix remodeling, and endothelial to mesenchymal transition. This book chapter is a revised and updated version of an earlier review of neuropilins in a tumorbiological setting [4], with a special focus on the multifaceted functions of neuropilins in the tumor microenvironment. In this setting, the various neuropilin ligands and interaction partners on various cells of the tumor microenvironment, such as cancer cells, endothelial cells, cancer-associated fibroblasts, and immune cells, are surveyed. Based on this situation, the suitability of various neuropilin-targeting substances and the intervention in neuropilin-mediated interactions is considered as a possible building block of tumor therapy.

3.2 Molecular Structure of Neuropilins

Neuropilin-1 and -2 represent a small family of evolutionarily conserved transmembrane glycoproteins with a broad tissue distribution in adult vertebrates [1, 2]. The genes NRP1 and NRP2 at chromosome loci 10p12 and 2q34 encode a 120-kDa and 112-kDa protein, respectively [1, 5–9]. Alternative splicing and optional insertion of 5, 17, or 22 amino acids C-terminal to amino acid 808 in the membrane-proximal part of the NRP ectodomain yields different membrane-bound and soluble variants (Fig. 3.1) [10–14].

The extracellular part of the NRPs comprises two cubilin homology (CUB) domains (a1/a2), two FV/VIII domains (b1/b2), and a MAM (c) domain [2]. The CUB domains are homologous to the complement factor C1s/C1r, the bone morphogenetic protein 1 (BMP1), and tolloid proteins, while the FV/VIII domains show homology to coagulation factor FV/VIII, a tyrosine kinase DDR, and discoidin-1. The c- or MAM domain structurally resembles the proteins meprin, A5 (former name of NRP) and receptor protein tyrosine phosphatases μ and κ (PTPμ, κ) [15, 16]. The soluble NRP variants sNRP1 and sNRP2 consist only of the tandem domains a1/a2 and b1/b2 and lack the MAM domain as well as the transmembrane and cytoplasmic domains. The ectodomain is linked by a single-pass transmembrane domain to a short cytoplasmic region comprising 44 amino acids in NRP1 and 42 and 46 amino acids in NRP2A and NRP2B, respectively, and lacking tyrosine kinase activity [2, 10]. NRP1 and NRP2A, but not NRP2B, have a PSD-95/Dlg/

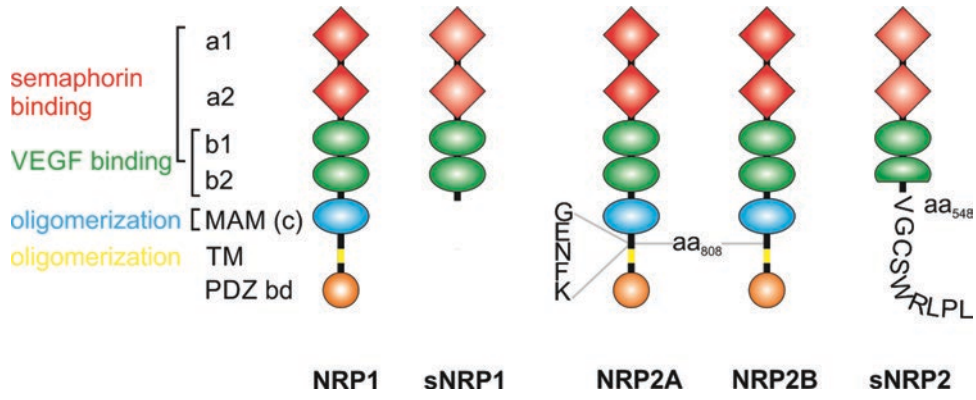

Fig. 3.1 Neuropilins have a modular domain structure to perform diverse functions. The extracellular part of NRP1 comprises two CUB domains (red), designated as a1 and a2, and two factor V/VIII homology domains termed b1 and b2 (green), that are connected via a short linker to a c- or MAM domain (blue). A single-pass transmembrane domain (TM, yellow) connects the extracellular moiety to a C-terminal PDZ binding domain motif (orange) with the characteristic amino acid sequence SEA. Semaphorin binding requires the a1/a2 tandem domain together with the b1 domain, while VEGF binds to the b1/b2 tandem domain. The MAM domain mediates NRP oligomerization. The four known soluble NRP1 isoforms (sNRP1) are truncated at the C-terminus of the b2 domain. Despite different amino acid sequence, NRP2 has the same domain structure as NRP1 except for an insertion of five amino acids between the MAM and TM domain, while soluble NRP2 (sNRP2) differs by a truncated b2 domain and nine additional C-terminal amino acids

ZO-1 (PDZ) binding motif at the intracellular carboxyl terminus [2].

Both NRP proteins can be glycosylated differently, especially in different cell types [17–19]. NRP1 is predominantly N-glycosylated and/or carries a chondroitin sulfate on Ser612 in vascular smooth muscle cells [19], while on endothelial cells (ECs) NRP1 is glycosylated with chondroitin sulfate and heparan sulfate chains [17]. NRPs, especially NRP2, is one of the few proteins that can be polysialylated to regulate, for example, trafficking of dendritic cells to secondary lymphoid organs and their interaction with T cells [20–22].

The structural diversity of NRPs in ECs is further enhanced by ADAM9- and ADAM10-mediated proteolytic processing leading to membrane-anchored NRP isoforms lacking the extracellular a1/a2 and b1/b2 tandem domains, or even the MAM domain [23].

NRP homodimerizes in particular by interaction of the α-helical transmembrane domains and presumably also the MAM domains [24–26]. In addition, NRP1 and NRP2 can also form heterodimers [27]. Whether dimers are formed immediately after translation into the ER or during vesicular transport to the cell surface is not yet known.

3.3 Neuropilin Signaling

3.3.1 Neuropilins Associate with Other Receptors into Functional Holoreceptors

Due to their modular structure, NRPs have defined, not necessarily overlapping binding sites for miscellaneous soluble ligands and, moreover, can interact with different receptors to form holoreceptor complexes of various functions (Fig. 3.2) [28]. For example, Nrp1 homodimers preferentially bind SEMA3A, whereas NRP2 homodimers interact with SEMA3F to subsequently form supramolecular holoreceptor complexes in the plasma membrane [29, 30]. As NRPs lack an intracellular kinase domain, they transmit stimulatory or inhibitory signals by recruiting various receptor kinases, which are chosen in response to the particular extracellular ligand.

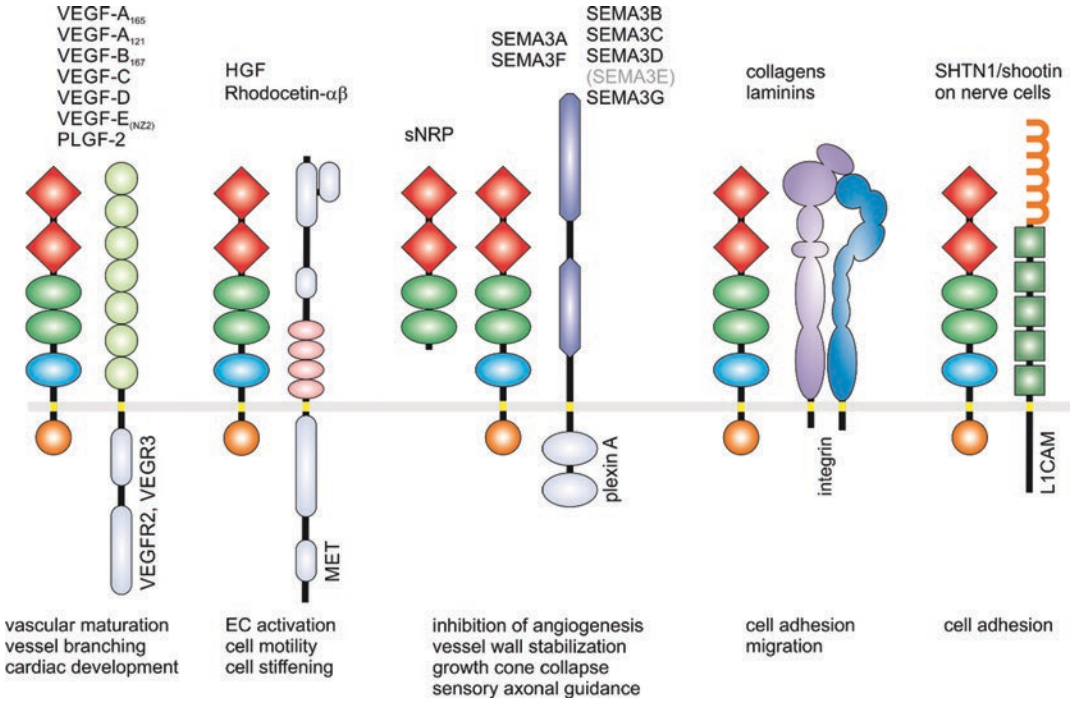

Fig. 3.2 NRP is a pivotal component of various holoreceptor complexes. For clarity, receptor and co-receptor molecules are shown as monomers only, although they actually exist as dimers. In signaling, an NRP dimer binds a likewise dimerized ligand and then interacts with a pair of receptor-type kinases or plexin receptors. Soluble sNRP isoforms may interfere with the signaling of NRP-containing holoreceptors. Physiological NRP ligands relevant to angiogenesis and tumor angiogenesis, vascular branching and maturation, and cardiovascular development include VEGF-A165, VEGF-A121, VEGF-B167, VEGF-C, VEGF-D, VEGF-E, and PIGF-2. Among the semaphorins important for the nervous system, the secreted SEMA3A and SEMA3F as well as the membrane-bound SEMA3C and SEMA3D are also of great importance in the tumor vasculature. SEMA3E is an exception in that it binds plexinD1 directly and independently of the NRP to modulate vascular patterning, although the extracellular domain of NRP1 can modulate SEMA3E-induced plexin D1 signaling [31, 32]. In addition to ipsilateral interactions, NRP is also capable of trans-cellular interactions. Tumor-penetrating peptides interact with the arginine-binding pocket within the b1 domain of NRP, to which also the snake venom-derived rhodocetin αβ binds, thereby recruiting NRP1 to the hepatocyte growth factor (HGF) receptor, MET. The color-coding of the NRP domains corresponds to the information in Fig. 3.1

3.3.2 Ligands of Neuropilin-Containing Signaling Complexes

VEGF-A is the best-understood binding partner of NRP1-containing signaling complexes forming the basis for the development of drugs that target the binding of VEGF-A to the NRP1/VEGFR2 complex [33, 34]. In this way, such drugs can inhibit tumor angiogenesis in cancer therapy as well as angiogenesis in other diseases where neovascularization also plays a key role, such as age-related macular degeneration, rheumatoid arthritis, psoriasis, diabetes-induced neovascularization of the eye, inflammatory diseases, ischemia/reperfusion injury, infant hemangioma, and atherosclerosis [35].

VEGF-A is encoded at the chromosomal locus 6p21.1, and all six splice variants are encoded by the first five exons containing the amino acids required for binding to the VEGF receptors VEGFR1 and VEGFR2 [35, 36]. The amino acid residues relevant for binding to NRP1 are encoded in exons 7 and 8a [35], which is why NRP1 can bind the VEGF-A splice variants VEGF-A165 and VEGF-A189, but not the

smaller variants with a length of 145, 121, or 120 amino acids [1, 37–41]. Furthermore, the amino acid residues relevant for the binding of VEGF to glycosaminoglycan chains of extracellular matrix proteins (ECM proteins), however, are encoded in exons 6 and 7 [35]. Accordingly, NRP1 can bind only the longer splice variants VEGF-A165 and VEGF-A189 but not the shorter forms VEGF-A145, VEGF-A121, and VEGF-A120 [1, 37–41], while NRP2 can bind VEGF-A165 and VEGF-A145 but not VEGF-A121 [42]. VEGF-A, dimerized by two disulfide bridges, binds to a holoreceptor complex of a NRP1 homodimer and a likewise homodimeric receptor, VEGFR1 or VEGFR2, forming a ternary VEGF-A/VEGFR/NRP1 complex with a putative 2:2:2-stoichiometry [35, 43, 44]. Here, two not directly adjacent arginine and glutamate residues near the C-terminus of VEGF-A are responsible for the high affinity of binding to VEGFR. Not immediately adjacent to each other are two arginine and two glutamate residues near the C-terminus of VEGF-A, which are responsible for its high-affinity binding to VEGFR [33–35, 45]. In particular, the C-terminal arginine residue of VEGF-A165 fits perfectly into a pocket within the NRP1-b1 domain formed by the side chains of residues Y297, Y353, D320, and S346 [33, 34]. The finding that various other NRP1 ligands also possess such a C-terminal arginine residue led to the concept of the carboxy-terminal end rule (CendR), according to which peptides with a C-terminal arginine residue preferentially bind to the binding pocket of the NRP b1 domain [46].

Dimerized VEGF-A165, which, in contrast to its shorter splice variant VEGF-A121, can simultaneously bind with its C termini to both the VEGFR2 ectodomain and the NRP1 b1/b2 tandem domain, was first identified as a ligand for NRP1 [1, 37–41]. Thus, a ternary signaling complex is formed in which all partners interact with one another, mainly to promote angiogenic sprouting of arterial ECs [47, 48]. In contrast, VEGF-A121, although capable of binding directly to NRP1, does not induce the formation of a NRP1/VEGFR2 holoreceptor [49].

NRP2 binds not only VEGF-A145 but also VEGF-C and can form a ternary complex together with the latter and VEGFR3 on lymphoid ECs to promote lymphangiogenesis [50, 51].

Meanwhile, other soluble ligands and other receptors for which NRP1 acts as a co-receptor have been described, demonstrating the versatility with which NRP1 is involved in the regulation of various signaling pathways. In each case, NRP1 acts as a co-receptor for a particular growth factor receptor by binding the respective growth factor and promoting the formation of a ternary signaling complex. Thus, NRP1 regulates the signaling of the following growth factors and their respective receptors: placenta growth factor (PlGF) and its receptor PlGFR [52, 53], hepatocyte growth factor (HGF) and its receptor MET [54, 55], as well as fibroblast growth factor-2 (FGF-2) [56], keratinocyte growth factor (KGF) [57], platelet-derived growth factors C and D (PDGF-C and PDGF-D) [58–60], tumor growth factor-β (TGF-β) [61–63] and their respective receptors. Although NRP1 itself does not bind to epidermal growth factor (EGF), it is involved with its extracellular domain in ligand-mediated EGFR oligomerization and endocytosis [64].

All NRP1-recruiting growth factor receptors, with the exception of the SMAD2 and SMAD3 activating receptor-type serine kinases TGF-βRI and TGF-βRII, are receptor-type tyrosine kinases. By acting as a mediator within the holoreceptor, NRP affects the interaction of the growth factors and their corresponding receptors, which autophosphorylate themselves after agonistic stimulation and recruit adapter proteins, thereby triggering an intracellular signaling cascade [63]. Thus, for example, the functional SEMA3A receptor in ECs consists of a ternary complex of NRP1 with plexin A1 and plexin D1, in which all three components are dimerized [65, 66]. In the same way, other members of the semaphorin family, as physiological mediators of antiangiogenic signals, also bind to NRPs and, similar to their effect as soluble chemorepellents in neuronal development, inhibit tumor angiogenesis and tumor growth [28, 67, 68].

Besides PlexinD1, ECs also express the other three A-plexins, albeit to a less extent [31, 65]. Holoreceptor complexes containing plexin A or plexin D in conjunction with NRP1 or NRP2

regulate tumor angiogenesis as well as various developmental processes, depending on the binding to class 3 semaphorins, SEMA3A-G [69, 70]. While SEMA3A binds to NRP1 and SEMA3F to NRP2-containing holoreceptors, promoting tumor cell normalization and inhibiting metastasis [71, 72], the affinities of SEMA3C for NRP1 and NRP2 are very similar [73].

While semaphorin binds SEMA3A to the NRP1 a1/a2 tandem domain, NRP interacts with plexin mainly via motifs within the transmembrane domains [74] and also via juxtamembrane regions of NRP, including the MAM domain [2], causing collapse and retraction of the nerve growth cone [75]. In a 2:2:2 complex of Sema3A, NRP1 and plexinA1, the contact area between the chains of an unbound SEMA3A dimer formed by the upper surface of the 7-sheet β-propeller domain (SEMA domain) is disrupted, and instead of a homophilic interaction within the dimeric SEMA3A, this dimer opens, allowing access of the NRP a1/a2 tandem domain to the top of its SEMA domains [66]. The interaction of both plexin A1 molecules, which also interact with more membrane-proximal domains of dimerized NRP1, with the SEMA domains of NRP1-bound SEMA3A leads to a conformational change and exposure of autoinhibitory contact sites, which ultimately triggers the signal for growth cone collapse in neurons [76]. Accordingly, NRP1 is not only the matchmaker between semaphorin ligand and plexin receptor but also a helper protein, supporting the switch from a homophilic SEMA domain interaction within the SEMA3A dimer to a heterophilic interaction between the SEMA3A and plexinA1 SEMA domains.

NRP2 was originally described as a receptor for SEMA3F (formerly called Sema IV), which mediates a repulsive effect on growing neurons and can heterooligomerize with NRP1 [30]. Its repertoire of semaphorin ligands partially overlaps with that of NRP1 and includes SEMA3F and SEMA3G, as well as the semaphorins SEMA3B, SEMA3C, and SEMA3D, which can interact with both NRPs [77].

In addition, NRPs can interact with members of the family of integrin cell adhesion molecules, each consisting of an α and a β subunit, both of which span the cell membrane with an α-helical transmembrane domain [48, 78–83]. Both integrin subunits together form an extracellular head domain capable of binding ECM molecules, which is linked to a small cytoplasmic domain by a stalk and transmembrane domain of each subunit. Like the NRPs, both integrin subunits lack a kinase activity [82–86]. Ligand binding to the head domain causes a drastic change in the conformation of an integrin, its arrangement in distinct adhesome complexes, and signal transduction between ECM and the cell by recruitment of cytoskeletal adapter proteins and signaling molecules [83, 87–90].

NRPs appear to influence integrin function in several ways. In addition to a lateral interaction of NRP2 with integrin α6β1 on cancer cells and an interaction of integrin α5β1 with an NRP1-VEGF complex on ECs resulting in remodeling of the fibronectin matrix [48, 78–81, 91], NRP2 on ECs can promote the spread and metastasis of adjacent cancer cells through trans-interaction with integrins α5β1 and α9β1 on the latter [92, 93]. Although NRPs are present in integrin-containing adhesomes, direct contact, as in the complex formation of NRPs with receptor kinases, has not yet been observed between NRP1 and integrins [48, 81, 91]. The presence of NRPs in adhesomes explains their regulatory effects on integrins, such as the upregulation of the collagen-binding integrin α2β1 upon stimulation with the NRP1 agonist SEMA3A [94] and the binding of integrin αvβ3 to the adhesion-modulating ECM protein tenascin C in breast cancer cells [95], the expression of αvβ3 integrin upon blockage of NRP1 [96], and, conversely, the inhibitory sequestration of NRP1 from NRP1-VEGFR2 signaling complexes by αvβ3 integrin in ECs [97].

In addition, NRP1 can interact with L1 cell adhesion molecule (L1CAM), belonging to the immunoglobulin superfamily, in both cis and trans-cellular manner and, thereby, regulate intercellular contacts between neurons [98, 99]. The association of NRP1 to a holoreceptor complex is likely mediated by the respective α-helical transmembrane domains [74], and it causes disassembly of adhesomes and growth cone collapse due

to recruitment of FAK and activation of MAPK signaling [100].

During tumor progression, proteolytic cleavage of chromogranin A, a circulating vasoregulatory neurosecretory protein, yields a proangiogenic fragment that efficiently binds to the VEGF-binding site of NRP1 [101]. Cleavage of chromogranin A in tumors and subsequent removal of the NRP1-binding fragment in the blood represent an important "on/off" switch regulating tumor angiogenesis and, hence, may be a novel therapeutic target [101].

Moreover, by forming a SEMA3A holoreceptor complex with the p75 neurotrophin receptor on neurons, NRPs can also regulate neuronal apoptosis and inhibit myelin growth [102].

In addition, heparin and heparan sulfate have been described as neuropilin ligands [1]. From a minimum chain length of eight monosaccharide units, heparin is capable of binding directly to NRP1, and with a chain length greater than 20–24 monosaccharides, it significantly enhances the binding of VEGF-A165 and PlGF-2 to the b1b2 tandem domain of NRP1 and, thus, regulates their effects in ECs [53]. With its "heparin-mimetic" site, NRP1 can also interact with the heparin-binding site of other proteins, such as FGF-2 and HGF, thereby regulating the activity of these heparin-binding proteins [56].

Since cell surfaces bear heparan sulfates instead of heparin, heparin-binding VEGF-A isoforms, for example, can regulate angiogenesis via NRP1-VEGFR signaling by being differentially retained at or released from heparan sulfate proteoglycans of the ECM to bind to heparan sulfate proteoglycans on the EC surface [103–107].

In addition, NRP1 is also a target of soluble toxins, such as the αβ subunit of rhodocetin, a venom component derived from the Malayan pit viper (*Calloselasma rhodostoma*). The C-type lectin-related protein rhodocetin αβ (RCαβ) is the first known nonenzymatic protein from a snake venom that binds to the b1/b2 tandem domain of NRP1 on ECs. On endothelial and tumor cell membranes, it induces the formation of a ternary complex with the hepatocyte growth factor, MET [108]. Like the physiological MET ligand, HGF, rhodocetin-αβ induces a restructuring of adhesomes and thereby increases cell mobility [108, 109]. Furthermore, rhodocetin-αβ causes inflammatory activation of coherent ECs via NRP1-MET signaling [110].

NRPs mostly interact with co-receptors in the plasma membrane of the same cell, but, as in the case of NRP1 and VEGFR2, are also able to interact with receptors of adjacent cells [111, 112]. A similar trans-interaction of NRP1 and membrane-bound SEMA4A plays a major role in the immune synapse between tightly interacting antigen-presenting dendritic cells and T_{reg} cells [113, 114]. Via a similar immune synapse, NRP1 can also be transferred from ECs to T lymphocytes, causing the T cells to express VEGF-A165, which in turn amplifies NRP1-VEGFR2 signaling in ECs during inflammation [115].

3.3.3 Signaling of Neuropilin-Containing Complexes

NRPs are versatile non-tyrosine kinase co-receptors for VEGF, TGF-β, and semaphorins that affect various growth-promoting signal transduction pathways regulating axonal guidance, tumor cell proliferation, angiogenesis, and cell survival (Fig. 3.3) [99, 100, 116]. As matchmakers and effectors, NRPs can affect the specificity and affinity of various holoreceptors through their extracellular domains by binding different growth factor receptors and their respective ligands. In addition, they influence the way in which ligand–receptor interaction sites are presented, such as the SEMA3A-1 and plexin A1 SEMA contact surface. Furthermore, NRP affects trafficking of VEGFR and sequestering of its ligand VEGF-A by interacting with them through its extracellular domains [117, 118]. The cytoplasmic domain of NRP1, on the other hand, does not appear to be critical for triggering NRP1-autonomous signaling, as knock-in mice with cytoplasmic domain-deficient NRP1 show only minor vascular defects, whereas a global knockout of the entire NRP1 molecule causes severe vascular malformations and lethality.

NRP1 modulates VEGF signaling as a co-receptor of VEGF receptors -1 and -2 and imparts

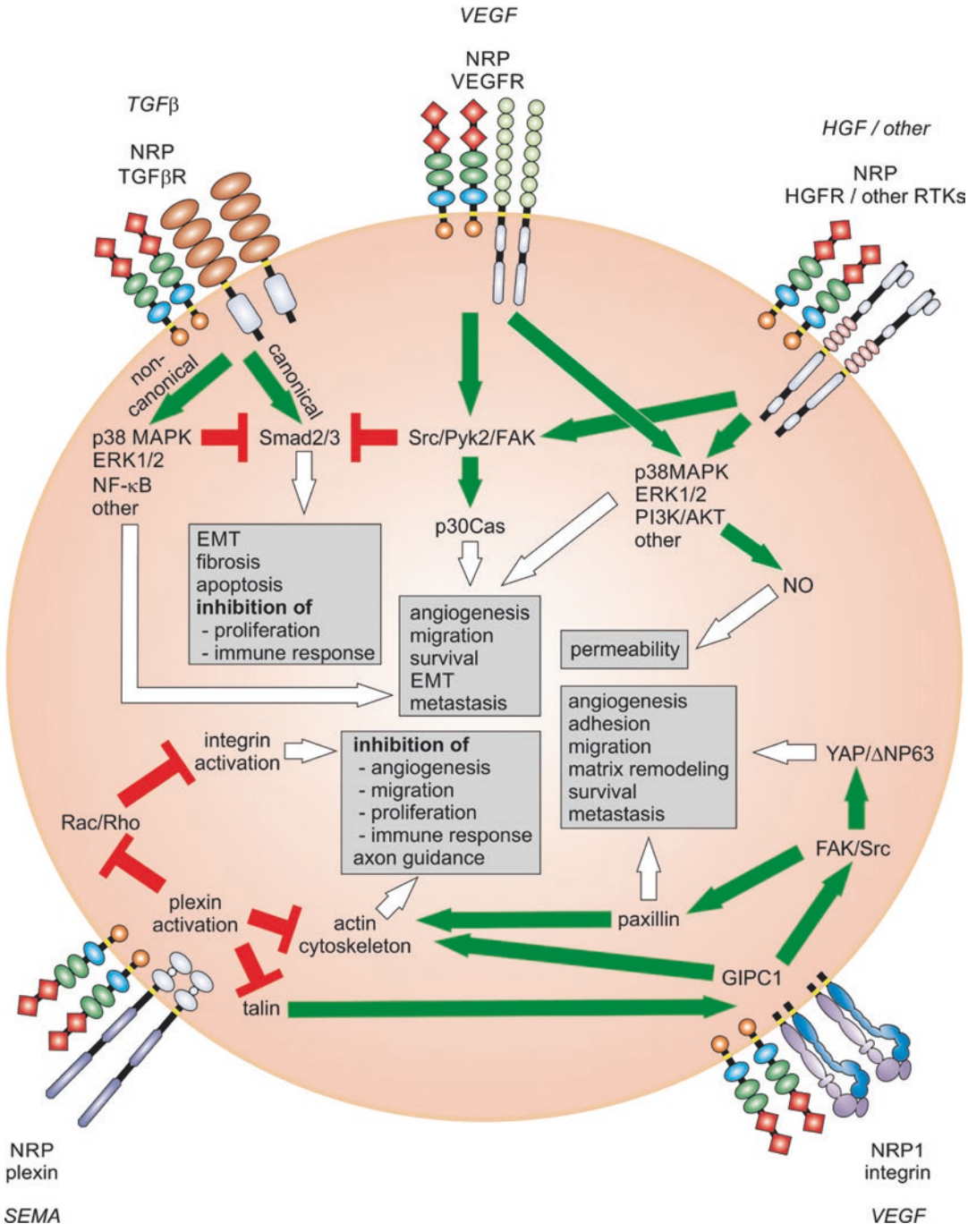

Fig. 3.3 Neuropilin, as partner in several receptor complexes, affects various signaling pathways in the tumor microenvironment. The NRPs are part of different receptor/co-receptor complexes and are therefore involved in many signaling events, thereby affecting a variety of cellular processes. In functional holoreceptor signaling, dimerized ligands bind to an NRP dimer interacting with a pair of receptor kinases or plexins. Activating signals are indicated by green arrows and inhibiting signals by red bar-headed lines, while white arrows indicate the resulting effects. (Modified from [4, 119])

the chemorepulsive activity of semaphorins in a VEGF-receptor-independent way as a co-receptor of plexin A [1, 120]. Cancer cells of solid tumors probably bind VEGF-A mainly via NRP, as they express different levels of VEGFR1, but hardly VEGFR2 and -3 [117, 118, 121, 122]. Whether ECs are capable of VEGFR-independent NRP1/VEGF-A signaling is still unknown [105, 106]. Probably, signaling is triggered by mutual phosphorylation and thus activation of the cytoplasmic kinase domains of the two VEGFR2 receptor molecules within the trimeric NRP1/VEGFR2/VEGF-A complex, thereby activating downstream signaling molecules of the two major activation axes via PI3-kinase, including protein kinase B (AKT), and via PLCγ, including RAS-RAF-ERK [48, 106]. However, skin cancer cells, prostate cancer cells, and glioblastoma cells have a high deficiency of VEGF receptors -1 and -2 so that VEGF-A-induced RhoA activation in these cells is mediated mainly by NRP1 [121]. The binding of VEGF-A to NRP1 induces the interaction of NRP1 with the scaffold protein GIPC1 (also known as NIP, SEMCAP1, Synectin, IIP1, TIP2, and GLUT1CBP), thereby promoting the formation of a molecular complex of GIPC1 with the guanine nucleotide exchange factor (GEF) for RhoA, Syx, which consequently increases the GTP-bound active form of RhoA [121, 123]. In contrast, NRP1-mediated RhoA activation stimulates EC motility via the PI3K pathway [124]. Intervention in the VEGF-A/RhoA signaling may be promising for cancer therapy [123] because RhoGEF expression of ECs is dependent on VEGF-A in the tumor microenvironment [125], and increased levels of active RhoA and ROCK due to impaired sensing of mechanical forces between tumor ECs and their surrounding ECM affect tumor vascularization [126]. VEGF-C-induced activation of AKT requires formation of a VEGFR3/VEGFR2/NRP1 complex, while activation of ERK1/2 occurs predominantly without the involvement of NRP [127].

NRP1 not only induces the expression of EGF in tumor cells but may also form a complex with EGFR, which, also known as Her2 and ErbB2 (erythroblastosis oncogene B), is overexpressed and active in many cancer cells [128–130].

EGFR, the eponymous member of the EGFR family of RTKs, is a co-receptor for EGFR, ErbB3, and ErbB4 holoreceptors [131]. Remarkably, the ectodomain of NRP1 can selectively trigger the phosphorylation of EGFR without affecting receptor activation by EGF [64]. EGF and TGF-β each trigger AKT signaling by inducing NRP1-dependent clustering and endocytosis of EGFR, in which the latter is controlled independently from tyrosine autophosphorylation by NRP1-mediated receptor oligomerization and clustering [64]. However, anti-EGFR therapy in the context of cancer treatment may show cardiotoxic side effects because EGFR, which is normally involved in EGF/neuregulin signaling, can also form a holoreceptor with NRP1, which then triggers dysfunctional, repulsive SEMA3D signaling in venous ECs [132].

Surface expression of EGFR on cancer cells and EGF-mediated signaling as well as response to EGFR-targeted therapy also depend on NRP2 [133]. NRP2 decreases the amount of EGFR on the cell surface, thereby slowing down tumor growth and suppressing an EGFR "rescue" pathway of cancer cells, which they activate as a protective response to MET-directed tumor therapy [133]. Conversely, in developing resistance to MET-directed therapy, the expression of NRP2 is lost and the NFκB signaling pathway is activated, while the EGFR-associated protein cell migration inducing hyaluronidase 1 (CEMIP, KIAA1199), which inhibits the degradation of activated EGFR kinase, is upregulated [133].

Whether and to what extent NRP1 functions as a co-receptor for FGFs in (tumor) vessels is not yet known. Although NRP1 can bind various FGFs [56], it does not affect FGF-2-induced proliferation of HUVECs, while in contrast, SEMA3A inhibits FGF-2-induced proliferation-promoting ERK1/2 activation downstream of RTKs in ECs [29].

At the epithelial-to-mesenchymal transition (EMT) of cancer cells, the interaction of NRP1 with PDGF-A and -B seems to be involved [117, 134]. Independent of GIPC1, PDGF, as

well as VEGF and HGF, stimulates cell motility by phosphorylation of p130Cas involving the cytoplasmic domain of NRP1 [19, 134–138]. Moreover, in fibrotic processes and various cancers, binding of PDGF to a holoreceptor consisting of NRP1 and PDGFRβ stimulates cell proliferation [124, 139–141]. Such a PDGF-D-induced interaction between NRP1 and PDGFRβ is also possible in trans between ECs and pericytes [60]. PDGF-D attenuates VEGFR2 signaling by transferring NRP1 into intercellular junctions, independently of PDGFRβ [60].

On ECs, NRP1 and NRP2 are involved in the binding of HGF as co-receptors of the hepatocyte growth factor receptor/scatter factor receptor MET [55, 142]. HGF stimulates motility and proliferation of these cells via NRP1 and tyrosine phosphorylation of p130Cas [138]. NRP1 is also involved in the activation of tumor growth and invasiveness-promoting signaling pathways via p38MAPK, Src, and PI3K, as well as in the internalization of NRP1/Met complexes in carcinoma cells [54].

Although showing no homology to HGF, the snake venom component rhodocetin αβ (RCαβ) binds to NRP1 on tumor cells and ECs, inducing the formation of a ternary complex with MET, which leads to its phosphorylation at Y1234/1235 and subsequent paxillin phosphorylation at Y31 [108]. As a result, cell-matrix anchoring complexes of focal adhesions are rearranged into focal contacts and the actin cytoskeleton is reorganized, thereby reducing cell adhesiveness and increasing cell motility [108, 109]. In this way, blood vessels in the tumor tissue, but not in normal tissues, are destroyed by RCαβ in in vivo tumor models by triggering responses of the tumor cells and subsequently of nearby ECs in the tumor microenvironment [109].

Frequently oncogenes become essential regulators of proliferation and survival of tumor cells by redirecting signaling pathways, which is referred to as oncogene addiction [143]. For example, an EGFR-dependent resistance to targeted therapies arises from downregulation of NRP2 in MET-addicted cancer cells and consequent compensatory enhancement of EGFR signaling [133]. NRP1 may be a useful target to inhibit oncogene addiction, tumor angiogenesis, and proliferation of cancer cells because its depletion or inactivation would inhibit various signaling pathways such as those initiated by VEGFR2, EGFR, or MET. The feasibility of this strategy is substantiated by the fact that in a xenograft mouse model of gastric cancer, NRP1 depletion causes upregulation of p27 and downregulation of both cyclin E and cyclin-dependent kinase-2 (CDK-2) resulting in cell cycle arrest in G1/S phase [144]. Similarly, NRP1 upregulates EGFR and IGF1R as alternative tumor-promoting effector kinases via a c-Jun N-terminal kinase (JNK)-dependent signaling cascade, which is why a reduction in NRP1 levels counteracts the adverse effects of acquired resistance to, for example, EGFR, MET, or BRAF (rat/rapidly accelerated fibrosarcoma, isoform B) inhibitors [145]. While expression of NRP1 is generally induced by growth factors via the RAS/MAPK pathway [129, 146, 147], a de novo expression of NRP1 in BRAF-dependent melanoma cells significantly contributes to the development of secondary drug resistance by altered gene expression, such as an upregulated expression of EGFR [145, 148, 149].

In addition, NRP1 is capable of forming holoreceptors for TGF-β with the TGF-β receptors I, II, and III, thereby controlling angiogenic sprouting independently of VEGFR2 [62, 150–152]. NRP1 has a negatively charged cleft in its b1 domain, to which various ligands such as TGF-β can bind [16, 61]. On breast cancer cells, NRP1 shows high affinity for both latent and active TGF-β1 [61]. In addition, TGF-β promotes a myofibroblast phenotype via NRP1 [61, 150], whereas downregulation of NRP1 decreases TGF-β-induced SMAD2/3 phosphorylation in stromal fibroblasts, thereby reducing their expression of smooth muscle α-actin [62, 150]. TGF-β1 and Ras signaling converge and act on NRP1 expression, with reduced NRP1 expression in KRAS-transformed cells resulting in decreased SMAD2 phosphorylation and increased tumor growth [63, 153]. In addition, microRNA (miR)-206 negatively regulates TGF-β levels and down-

stream expression of NRP1 and SMAD-2 in breast cancer cells, while overexpression of miR-206 inhibits EMT, migration, and invasion of breast cancer cells [154]. On the other hand, TGF-β1 inhibits miR-196a-3p, resulting in activation of NRP2 and thus promoting a metastatic phenotype of breast cancer cells [155].

Class 3 semaphorins appear to counteract the binding and signaling of VEGF and related growth factors. Additionally, soluble NRP isoforms modulate these signaling events [2]. Although NRP1 is considered a specific SEMA3A ligand, it cannot transduce SEMA3A signals on its own because of its short intracellular domain and has to form a complex with PlexinA receptors for this purpose [156]. Upon binding of SEMA3, the intracellular plexin domain can inactivate small GTPases such as R-Ras, thus promoting integrin-mediated cell–matrix interaction [69, 157].

SEMA3C binds to NRP1 and plexinD1 in ECs, thereby inducing internalization of VE-cadherin and shutdown of VEGF-triggered signaling via AKT, FAK, as well as p38MAPK and causing disassembly of EC junctions and focal adhesions together with respective cytoskeletal rearrangements [158]. Normally, SEMA3C signaling can thus induce EC apoptosis and inhibit pathological angiogenesis, whereas in cancer, SEMA3C and its receptors are frequently highly expressed and associated with invasion and metastasis [158, 159]. Hence, SEMA3C is discussed as a potential target of cancer therapeutics [160].

SEMA3A acts as an endogenous angiogenesis inhibitor on ECs of premalignant lesions, but its expression is lost as the cancer progresses [73]. Because NRP2 inhibits tumor development and metastasis through a strong antiangiogenic cascade, SEMA3F overexpressing melanoma cells form poorly vascularized tumors [161, 162]. NRP2 signaling increases Jagged1 levels and promotes tumor angiogenesis in pancreatic adenocarcinoma, while overexpression of Jagged1 in cancer cells similarly leads to neovascularization and growth of experimental tumors in mice [163, 164], in particular, because Jagged1 is an important regulator of tip-cell differentiation in angiogenic endothelia, by modulating delta-like 4 (Dll4)-mediated Notch signaling [165].

For the development, stabilization, and maturation of the vasculature, recruitment of pericytes to nascent vessels is essential and is mediated among others by the SEMA3A/NRP1 signaling, [166–170]. Compared with normal blood vessels, the tumor vasculature has fewer pericytes, which is one of the causes of its leakiness [171]. Invasive cancer cells recruit fewer pericytes than non-invasive cancer cells in tumor angiogenesis in vitro [137]. In addition, NRP1, as a PDGF-B co-receptor, is involved not only in the recruitment of pericytes but also in their differentiation of mesenchymal stem cells [137]. The inadequate pericyte coverage can be normalized by expression of SEMA3A, which also reduces angiogenesis and tumor growth [169, 172].

3.3.4 NRP-Triggered Signaling Pathways

Although NRPs lack a kinase domain for signal transduction, they are relevant to diverse signaling pathways because of their versatility in interacting with different signaling receptors. Moreover, the effect of their signaling depends on other signaling cascades that integrate NRPs into an intricate network of interdependent signaling pathways, such as integrin- and galectin-related, as well as Wnt, Hedgehog (HH), and Sonic hedgehog (SHH) signaling cascades.

Integrins that, similarly to NRPs, lack an intracellular signaling domain can be activated by interaction with NRPs and associate in adhesomes with various kinases, such as members of the Src family and focal adhesion kinase (FAK) [67, 97, 173, 174]. The inside-out signaling of β1, β3, and β5 integrins is mediated via the PI3K/AKT/PTEN signaling axis with NRP1 acting as VEGFR2 co-receptor [175]. Not only can β1 integrins interact directly with NRPs, but both NRP1 and NRP2 can control the activity of various integrins contributing to tumor initiation and progression, such as integrins α2β1, α5β1, and β3 [81, 91, 116, 176]. Via NRP1, an autocrine feedback loop activates the serine/threonine kinase

GSK-3, thus inhibiting the expression of integrin α2β1 and attenuating migration and invasion of SEMA3A-expressing breast cancer cells [94]. Similar inhibitory autocrine SEMA3 feedback loops tweak cell adhesion to the ECM via integrins α5β1 and αvβ3, thereby conferring the required flexibility and mobility to ECs during angiogenesis [176]. The interaction between NRP1 and a5β1 ensues in ECs by binding GIPC1 to PDZ binding motifs in the cytoplasmic tails of both receptors [78]. SEMA3A binds to an NRP1/plexinD1 holoreceptor, thereby controlling the activity of integrin α5 in integrin-containing focal complexes at dynamic cell protrusions during cell migration [78, 176]. By means of its intracellular GTPase-activating protein domain (GAP domain), plexinD1 activates the small GTPase Rap1 (Ras-related protein), which then implements the conformational activation of integrins during cell migration via RIAM1 and talin [177]. By recruitment of NRP1 by β3 integrins, angiogenesis can also be controlled by the number of NRP1/VEGFR2 holoreceptors available for VEGF-A signaling [97].

In breast cancer and prostate cancer cells, integrin α6β1 modulates NRP2-mediated VEGF signaling that activates TORC2/PKC and FAK signaling by activating integrin a6β1, possibly by phosphorylation of the integrin α6 subunit, and promoting its association with F-actin, which results in formation of focal adhesions and allows signaling through them [116, 178–180]. In addition, NRP1, GIPC1, and integrin α6β4 together can form a ternary receptor for VEGF-A in epidermal cancer stem cells, which triggers FAK/Src signaling to stabilize YAP1/ΔNP63α, thus promoting survival, invasiveness, and tumor angiogenesis [177].

Further, the endocytic adapter protein GIPC1 is able to bind to the cytoplasmic SEA motif (Ser-Glu-Ala) of NRP, thereby inducing internalization of integrin α5β1 in Rab5-positive early endosomes [78]. This GIPC1 integrin α5β1 complex also promotes the adhesion of ECs to fibronectin via motor protein myosin-VI and endocytosis of integrin α5β1 [78]. Binding to corresponding docking sites of NRPs, GIPC1 thus regulates recycling of clathrin-coated vesicles [2, 181–183]. In this way, NRP1 is also involved in nutrient uptake by tumor cells, with its surface expression inversely correlated with nutrient supply [184]. Like other cells, tumor cells can take up nutrients and drugs through NRP1-mediated endocytosis (micropinocytosis and a similar, but different, method) [184], in which the drugs, interestingly, need not be covalently coupled to an NRP targeting structure, but can be taken up in passing [184, 185].

Membrane trafficking is a general principle for the regulation of signaling pathways [186]. Accordingly, endocytosis of NRP1/plexin holoreceptors with bound SEMA3A in conjunction with L1-CAM occurs in neuronal as well as in other cells [99]. In addition, NRP1-directed endosomal translocation of VEGFR2 in ECs significantly regulates VEGF-A-induced ERK1/2 activation [187, 188], and stimulation of p38 MAPK also depends on endosomal NRP signaling [189]. Moreover, incorporation of transstanding NRP1 into holoreceptor complexes prevents endocytosis of VEGF-A–VEGFR2 complexes, thereby controlling angiogenesis, tumor initiation, and tumor angiogenesis [111, 112].

Galectins are mostly angiostimulatory β-galactoside binding proteins, presumably because of their involvement in receptor endocytosis [190, 191]. Unlike galectin-3 binding VEGFR, NRP1 directly binds galectin 1, which is overexpressed in tumor-associated capillary ECs in squamous cell carcinoma [192]. This enhances the phosphorylation of VEGFR2, which triggers signal transduction via the MAP kinases SAPK1 and Jnk, thereby increasing proliferation and adhesion of ECs and, together with VEGF, promoting cell migration [192].

After binding of VEGF, NRP1 triggers RAS activation followed by phosphorylation of ERK1/2 and AKT [193]. In tumors where NRP1 has tumor-promoting properties, wild-type KRAS is often present, whereas in tumors where NRP1 acts as a tumor suppressor, oncogenic KRAS mutations are often found [194]. Oncogenic KRAS and TGF-β signaling cause a transcriptional downregulation of E-cadherin by induction of the major transcription factor Snail

[63, 195]. In addition, NRP1 is downregulated by TGF-β at both transcriptional and translational levels in cells with oncogenic, but not wild-type KRAS, which reduces SMAD2 phosphorylation and stimulates tumor growth of oncogenically KRAS-transformed cells [153].

NRPs can promote intracellular signaling in cancer cells that protect them from cytostatic drugs and apoptosis-inducing drugs. For example, NRP1 and NRP2 are important positive regulators of the Hedgehog (HH) signaling pathway, which is not only important for angiogenesis and wound healing but also promotes survival of EMT and cancer stem cells (CSC) as well as tumor growth [117, 196–198]. In addition, NRPs also affect the activity of other signaling pathways, such as Wnt/β-catenin, Notch, and TGF-β via HH signaling, and, in a positive feedback loop via HH signaling, induce NRP1 transcription and, as a consequence, further activate HH target genes [100, 197]. The SEMA3-enhanced direct binding of phosphodiesterase 4B (PDE4D) to NRP increases the hydrolysis of cAMP at the plasma membrane, which in turn inhibits protein kinase A (PKA) and thus controls the HH signaling pathway [199]. While Smoothened, the canonical activator of HH signaling, is poorly expressed in the majority of lung adenocarcinomas and especially in their CSC compartment, GLI1, a downstream effector of HH signaling, is non-canonically activated in these malignancies via the MAPK/ERK pathway, which is triggered by KRAS mutation and stimulation of NRP2 with VEGF that, presumably, is auto- or paracrinely derived from CSCs or stromal cells [200]. In turn, GLI1 upregulates the important stem cell factor BMI-1 in breast cancer, thereby enhancing the expression of integrins α6β1 and NRP2 in an autocrine loop [81].

Tumor-derived Sonic hedgehog (SHH) increases PlGF production in the cerebellar stroma in medulloblastoma, thereby abetting tumor cell survival through NRP1 without involvement of VEGFR1 [201]. However, most medulloblastomas with constitutively active Wnt signal do not express NRP1 due to strong expression of miR-148a, which downregulates NRP1 by binding to its 3′-untranslated region (3′-UTR) [202].

In contrast, Wnt/β-catenin signaling increases NRP1 expression in both mammary stem cells and mouse mammary tumor virus (MMTV)-Wnt1 tumor xenografts [146]. By activating Wnt/β-catenin signaling, VEGF-A-bound NRP1 promotes in breast cancer a CSC phenotype and the formation of aggressive and highly vascularized tumors [119]. NRP1 expression of vascular progenitor cells similarly depends on Wnt and BMP4 signals, intercellular contacts, hypoxia, and hemodynamic stimuli [203]. Wnt and PI3K signaling is also associated with tumor angiogenesis in biliary tract cancer with strong expression of NRP1 and NRP2 [204]. In addition, Wnt signaling in colorectal CSCs is triggered by myofibroblast-secreted HGF, thus contributing to maintaining their stemness [205].

Although NRP2 is normally present only in small amounts in carcinomas, it is involved in their metastatic progression [206]. In a gastric cancer cell line, silencing of NRP2 that is found in gastric cancer resulted by Wnt/β-catenin signaling in a decreased expression of the metastatic mediator S100A4 and of anti-apoptotic B-cell lymphoma 2 (Bcl-2), while pro-apoptotic caspases -3 and -7 were upregulated [207]. Presumably, paracrine VEGF here causes NRP2 to trigger TGF-β1 or β-catenin/Wnt signaling [81, 208]. NRP2 expression is important for the recruitment of HUVECs by osteosarcoma cells and can be downregulated by overexpressed soluble LRP5, Frzb, or WIF1 as antagonists of Wnt signaling [209].

The binding of VEGF and related growth factors to NRPs and the corresponding signaling appears to be counteracted by class 3 semaphorins as well as soluble NRP isoforms [2]. sNRP1, lacking an MAM dimerization motif, inhibits tumor angiogenesis and tumor progression by acting as an antagonist that competes with membrane-bound receptors for binding of VEGF-A165 [10, 11]. Conversely, dimerized sNRP1 delivers VEGF-A165 to ECs, which express VEGFR2, and thereby promotes angiogenesis [12]. Furthermore, a soluble splice variant of NRP2, s9NRP2, captures VEGF-C and thus inhibits VEGF-C/NRP2 signaling in prostate cancer, which is why s9NRP2 could be therapeutically useful in the treatment of tumors whose survival strongly depends on VEGF-C/NRP2 signaling [210].

3.3.5 Regulation of NRPs at the Posttranscriptional Level

The majority of genes are subject to posttranscriptional regulation in that small noncoding RNA molecules or microRNAs (miRNAs) bind specifically to the 3′-UTR of an mRNA they regulate, either inhibiting their translation or initiating their degradation [211]. NRP1 plays a pivotal role in this type of gene regulation by efficiently binding Argonaute2 (AGO2) and AGO2/miR complexes without the involvement of its VEGF-binding site and by internalizing them to promote cellular processes such as proliferation, migration, and angiogenic tube formation, and also to regulate intercellular communication critical for the development and progression of many malignancies [212]. Long noncoding RNAs (lncRNAs) play an essential role in all stages of tumorigenesis and metastasis due to their ability to interact with miRs, among others [213]. Thus, miR-206 inhibits tumor growth and invasion in colorectal cancer by downregulating the long noncoding RNA lnc00152, thereby increasing NRP1 expression and promoting EMT [214]. As an example of mutually regulating miRNAs, miR320b in competition with miR320a abolishes in metastatic colorectal carcinoma downregulation of NRP1, β-catenin, and Rac-1 [215].

The expression of, inter alia, miR338-p, which inhibits the expression of NRP1 by phosphorylation of ERK1/2, MAPK, and AKT in gastric and oral squamous cell carcinoma, is induced by the transcription factor SOX10 [216, 217]. Due to the action of SOX10/miR-338, transcription of NRP1 is barely detectable in melanoma cells, but this downregulation of NRP1 may be reversed in response to a targeted therapy and results in drug resistance formation, whereas in carcinoma cells lacking the SOX10/miR-338 regulatory mechanism, miR-338 seems not to be associated with drug resistance [145]. In non-small cell lung cancer, miR338-3p binds to the 3′-UTR and directly controls NRP1, thus affecting EGFR tyrosine kinase inhibitor-mediated drug sensitivity [218]. miR148a in medulloblastoma cells and miR-152 in non-small cell lung cancer inhibit the translation of NRP1 in a similar fashion to miR338 in melanoma cells [202, 219], while miR320 reduces NRP1 expression in cholangiocarcinoma [220]. Likewise via downregulation of NRP1, miR320 also regulates the proliferation and migration of vascular smooth muscle cells as well as neointimal formation of blood vessels [221]. Cancer-associated fibroblasts induce a downregulation of miR-1247 in prostate cancer cells, thereby increasing the expression of NRP1, which, functioning as a co-receptor of EGFR, promotes EMT, invasiveness, and cancer stem cell characteristics [222]. Furthermore, by downregulating the expression of NRP1, miR-376a, competing with the RNA binding protein PUM2 for binding to the 3'-UTR of the NRP mRNA, inhibits the Wnt/β-catenin signaling axis in breast cancer cells and hence their proliferation, migration, and invasion, and promotes apoptosis [223, 224]. Likewise, miR-124-3p as a further suppressor of NRP1 promotes in glioblastoma multiforme proliferation and migration of tumor cells and tumor angiogenesis via PI3K/AKT/NFκB signaling pathways [225]. In contrast, loss of miR-331-3p expression results, by upregulation of NRP2, in increased proliferation and clonogenic growth of glioblastoma cells [226]. In turn, expression of NRP2 is downregulated by miR-15b, resulting in decreased MEK/ERK signaling and correspondingly reduced angiogenic tube formation in L9 rat glioma cells [227]. miR-486-5p also reduces the expression of NRP2 and thus acts as a tumor suppressor in colorectal carcinoma [228].

3.4 Neuropilins on Cells of the Tumor Microenvironment

3.4.1 Neuropilins on Tumor Cells

Cancer cells create a microenvironment that promotes their growth and drives tumor progression. NRP expression levels correlate with tumor growth, invasiveness, angiogenesis and poor prognosis [28]. In various cancers, NRPs are attributed different functions. In melanoma, NRP1 promotes metastasis, and in renal cell carcinoma and breast cancer, it stimulates the

dedifferentiation of cells and even causes CSCs to retain their stem cell properties [146, 196, 229]. When NRP1 binds VEGF-A, it promotes growth and metastasis of solid tumors, whereas SEMA3A binding generally leads to better prognosis due to less migration and invasion of tumor cells [230, 231]. This antagonism of SEMA3A and VEGF-A is also evident in leukemia cells, where NRP1 preferentially binds to SEMA3, versus the competing VEGF [232].

Additionally, NRP1 increases cell migration and invasion, both of which contribute to metastasis, by stimulating TGF-β, HH, and HGF/Met signaling, thus promoting EMT [117, 233]. NRP2 as a receptor for SEMA3F is upregulated by TGF-β1 and significantly promotes TGF-β1-induced EMT in lung cancer [234]. In addition, TGF-β-induced migration and invasion of non-small cell lung cancer cells in vitro and metastasis in vivo are stimulated by NRP2B [235]. In addition, NRP2B enhances HGF-induced AKT phosphorylation independent of its binding to GIPC1 and recruitment of PTEN, whereas inhibition of MET reduces tumor cell migration [235].

3.4.2 Neuropilins on Resident Tumor Stroma Cells

While cancer cells have been in the scientific and therapeutic focus of cancer biology and treatment for long [236], in the last two decades, the scientific view of solid tumors has expanded also other cell types, such as resident stromal fibroblasts, resident and invading immune cells, and ingrowing ECs which, together with the tumor-typical ECM, form the tumor-supportive tumor microenvironment [236–242]. Under the influence of adjacent tumor cells, such cells develop from bystanders into highly active and tumor progression-promoting cells, such as cancer-associated fibroblasts (CAFs) whose strengthened actomyosin system exerts higher forces on the stromal ECM, after their differentiation from normal fibroblasts [239, 243–247]. CAFs resemble myofibroblasts, which contribute to the regeneration of wounded tissues; therefore, the presence of CAFs in tumors has prompted tumors to be regarded as "wounds that never heal" [241, 248, 249]. The mutual interaction between tumor cells and CAFs is characterized by soluble growth factors and immobilized ECM proteins, including their stiffening by cross-linking and cell-mediated tension [239, 241, 250–252]. In CAF differentiation, soluble TGF-β, which is tethered to the ECM and tension-dependently released, plays a key role, whereupon CAFs produce additional growth factors, synthesize and deposit ECM proteins, and control the metabolic milieu of the extracellular space within a tumor mass by absorbing metabolic waste products and buffering protons, thus promoting survival and proliferation of cancer cells [247, 253, 254]. In addition to establishing the tumor microenvironment that determines the tumor differentiation state by tumor cells, CAFS and other non-tumorigenic cells, CAF-deposited ECM molecules promote tumor cell invasion and metastasis [255]. Increased deposition of fibronectin, which is integrin-dependently assembled by CAFs following NRP1 stimulation, contributes to tumor-specific desmoplasia by increasing ECM stiffness, whereas knockout of NRP1 in CAFs slows tumor progression [80].

3.4.3 Neuropilins on Infiltrating Angiogenic Tumor Vessels

ECs that grow into solid tumors are another type of cell that interacts with itself and with tumor cells in a variety of ways via NRPs. These are involved in the (patho) physiological regulation of (lymph) angiogenesis, with tumor angiogenesis and the formation of tumor-specific vasculogenic mimicry vessels (VM), which contribute significantly to the resistance to antiangiogenic therapy, of particular interest [229, 256].

In vasculogenic mimicry, ECs are partially or completely replaced by tumor cells or transdifferentiated CSCs with increased cell plasticity. Such VM conduits support blood perfusion of tumor tissue, especially in hypoxic areas. They increase the risk of metastasis [257–259]. Although VM channel-lining tumor cells phenotypically mimic ECs, they differ from normal ECs in the expression of NRP1, TIE-1, VEGF-C, endoglin, TFPI1, LAMC2, and EphA2, as well as

in the inexistent expression of TIE-2, VEGFR1, VEGFR2, P-selectin, VCAM-1, and CD31 [257]. Like the invasiveness of tumor cells, VM correlates with increased NRP1 expression due to upregulated VEGF-A, increased matrix metalloproteinase (MMP) -2 and -9 secretion, as well as integrin αvβ5 activation [58, 260]. In addition, in melanoma, VM is promoted by NRP1 and PlGF independent of VEGFR1 [229].

NRP1 is critical for VGFA-induced angiogenic signaling in ECs and tumor cells and is particularly important for the morphology of the tip-cells during sprouting angiogenesis [1, 261]. Selection of either tip- or stalk-cells is controlled via Notch-promoted expression of NRP1, with the effect of NRP1 going beyond its function as a VEGFR co-receptor [152]. The tip-cell phenotype is promoted by NRP1-mediated suppression of the stalk-cell phenotype, in which TGF-β and Bmp9/10-induced Smad2/3 phosphorylation by the activin receptor-like kinases ALK1 and ALK5 is inhibited, whereas the stalk-cell phenotype is promoted by Notch signaling and activation of ALK1 and ALK5 resulting in downregulation of NRP1 [152]. Activation of ALK receptors, together with Notch, enhances expression of HES and HEY, which promote the tip-cell phenotype through the control of tissue-specific transcription factors [44, 152, 262]. Because tumor angiogenesis requires the formation of tip-cells, which is essentially controlled by collaborating Notch and NRP1-mediated Smad2/3 signaling, inhibition of NRP1 appears attractive to curb tumor angiogenesis [152].

While PlexinD1 mediates signals of Sema3E, -A, and -F in murine developmental angiogenesis [69], NRP/SEMA3 signaling also plays a major role in tumor (lymph) angiogenesis and metastasis [263, 264]. Essential for the stabilization of the lymphatic vessel wall by pericytes is the binding of EC-produced SEMA3A to NRP1 on lymphatic pericytes [265]. Such pericyte-EC interactions can be disturbed by tumor cells, thus promoting their metastasis [171]. Expressed in ECs, SEMA3A endogenously inhibits angiogenesis by signaling via NRP1 and plexinA1/A4 [73], but SEMA3A is lost during tumor progression. Reintroduction of SEMA3A in a murine tumor model results in vessel normalization with reduced vascular density and enhanced pericyte coverage of tumor blood vessels and concomitant inhibition of tumor growth by triggering apoptosis in ECs and subsequently in tumor cells [73].

SEMA3C released from pericytes and SMCs impairs immature vessel sprouts in pathological angiogenesis, but not quiescent ECs in established vessels, by thwarting angiogenic VEGF-triggered signals [158, 266]. This selective pruning of immature vessels is due to a different composition of SEMA3C holoreceptors and strong expression of NRP1 and plexinD1 in ECs of immature vascular sprouts, suggesting that tumor angiogenesis could be inhibited by specific plexinD1 ligands [266].

In contrast to SEMA3C, which inhibits lymphangiogenesis and metastasis of tumors, its furin-cleaved form p65-SEMA3C is, at least in vitro, tumor-promoting in NRP2-expressing cancer cells, which is likely due, at least in part, to its direct binding to plexin B1 [267–271]. In lymphatic ECs, class 3 semaphorin holoreceptors consist of NRP2 and plexins A1 and D1, respectively [77]. In lymphatic ECs, VEGF-C-mediated signaling and proliferation as well as M2-macrophage-assisted angiogenesis are inhibited by furin-resistant SEMA3C, whereas caspase-3-independent apoptosis is promoted by it [267, 272]. ADAMTS-1 (a disintegrin and a metalloproteinase with thrombospondin motifs) also stimulates tumor cell migration by cleavage of SEMA3C, suggesting that proteolytic cleavage is a general principle for controlling semaphorin signaling [266, 273].

In tumor treatment, normalization of the tumor vasculature appears to be more important than inhibition of angiogenesis, to reduce hypoxia and improve perfusion, so that drugs can better reach the tumor [274, 275]. To increase the efficacy of cancer therapies by vascular normalization, the modulatory effects of SEMA3 in various cell types within the tumor vasculature and the tumor microenvironment can be utilized [69]. While secreted SEMA3, by binding to NRP1, induces the formation of plexin containing holoreceptor/ligand complexes that promote vessel normalization [69, 73, 176, 276], binding of SEMA3 to NRP1, however, also adversely increases vascular permeability and attracts

tumor-promoting macrophages [277, 278]. A parenterally administrable SEMA3A point mutant (A106K_ΔIg-b) that unlike its wild-type form does not bind to NRP1, binds strongly to plexin A4, thereby reducing vascularization, inhibiting tumor growth and metastasis, and improving the accessibility and effect of conventional chemotherapy with gemcitabine (2′,2′-difluoro-2′-deoxycytidine) in pancreatic carcinoma and RIP-Tag2 mouse models, while inhibiting retinal neovascularization in a mouse model of age-related macular degeneration [279].

NRP1 on non-ECs can block, by trans-cellular interaction, receptor internalization and thus stabilize signaling of, for example, VEGFR2, thereby inhibiting tumor angiogenesis and reducing initiation of tumor growth [112]. Moreover, vessel branching and proliferation of tumor cells are reduced in pancreatic duct adenocarcinoma as well as in murine fibrosarcoma by formation of such NRP1/VEGFR2 trans-complexes [111]. This mechanism may also explain why in apparently healthy individuals, microscopic tumors and dysplastic foci do not develop without angiogenesis for many years [280].

3.4.4 Neuropilins on Immune Cells Within the Tumor Microenvironment

As a consequence of neoplastic growth along with tissue hypoxia and necrosis, there are always numerous immune cells in the tumor microenvironment. In such cancer-associated inflammation, NRP plays an essential role as a component of semaphorin receptors [281]. Dendritic cells and other antigen-presenting cells that also express NRPs monitor the tumor tissue without, however, triggering a major immune response because immunosuppressive cytokines, TGF-β and IL-10, which support the differentiation of the anti-inflammatory phenotype M2 of macrophages, are typically found in the tumor microenvironment [21, 282]. M2 macrophages, also called tumor-associated macrophages (TAMs), express both NRPs. Their NRP2 expression correlates with their ability to efferocytose, viz. to engulf apoptotic cells, e.g., tumor cells, without eliciting inflammation or a potential immune response against tumor cell components [283]. Furthermore, NRP1, which is upregulated in cervical cancer cells in a hypoxic tumor microenvironment, correlates with TAM infiltration and causes recruited macrophages to adopt an M2 phenotype [284]. In addition to M2 macrophages, which NRP2-dependently facilitate tumor progression, other NRP-expressing myeloid cells, such as myeloid suppressor cells, also contribute to the immunosuppressive conditions of the tumor microenvironment [21]. M2 macrophages support the differentiation of CAFs and M2 macrophages themselves by secreting anti-inflammatory cytokines, such as IL10, IL4, and TGF-β, and foster regulatory T cells (Tregs, $CD4^+$ $CD25^+$ $Foxp3^+$ T cells). Bearing SEMA4D on their surface, they can activate other cells that express NRP1, such as ECs and Tregs in the tumor microenvironment [21, 285, 286]. Interaction of SEMA3A and plexinA4 with NRP1 additionally adds to immunoinhibitory signaling [129, 287, 288]. Tregs interact with different cells of the innate and adaptive immune system via NRPs and thus orchestrate immunosuppressive processes [289]. Increased expression of NRP, as found in the vast majority of tumor-infiltrating Treg cells, correlates with poor prognosis, presumably due to its immunosuppressive effect on other $CD4^+$ T helper cells, $CD8^+$ cytotoxic T cells, and natural killer cells [114, 290]. Therefore, a strategy for cancer immunotherapy could be developed that relies on alleviation of these NRP-mediated immunosuppressive conditions in the tumor microenvironment in combination with recently developed immune checkpoint inhibitors [291].

3.5 Targeting Neuropilins for Pharmacological Intervention in the Tumor Microenvironment

NRPs control a range of pivotal mechanisms in the tumor microenvironment in order to escape cytotoxic stress and therapeutic attacks and, thus,

are potential predictive biomarkers of drug response and possible targets in combination with other anticancer therapies [292]. Cancer cells often become "addicted" to certain oncogenes that activate various signal cascades, suggesting a therapeutic inhibition of precisely this signaling [293]. Unfortunately, such targeted therapies quickly become ineffective because cancer cells can evade such an attack by upregulating alternative survival- and proliferation-promoting signaling pathways [294]. Accordingly, to escape attack, tumor cells increase the expression and activity of RTKs such as EGFR, MET, and FGFR by cytokines and growth factors in the tumor microenvironment [295]. Since NRPs can form holoreceptors with the majority of these different receptors, they are promising tumor therapeutic targets, and specific and highly potent NRP1 inhibitors are of outstanding interest [296, 297].

While, for example, in colon cancer, an increased NRP1 expression is associated with a less-severe prognosis [298] and NRP1 in PANC-1 pancreas adenocarcinoma cells is tumor suppressive [299], NRP1 is usually considered a tumor-promoting co-receptor [299]. In light of this prevailing view of NRP1 being a tumor promoter, it has been tested as a therapeutic target in several studies [63]. In patients treated with the tyrosine kinase inhibitor tivozanib, low NRP1 levels are associated with better progression-free survival [300]. However, regarding the potential suitability of NRP1 as a biomarker assessing VEGF and VEGFR targeting therapies [301], there is no significant association between the expression of NRP1 and the response to treatment with VEGF antibody, bevacizumab, or the survival of patients with astrocytoma or ovarian cancer [302, 303].

Soluble sNRP as a decoy receptor damages blood vessels and causes severe bleeding as well as apoptosis of tumor cells in animal models of rat prostate carcinoma cell tumors [11]. Likewise, sNRP1 inhibits tumor angiogenesis and tumor growth in granulocytic mouse sarcoma (chloroma) [304]. Furthermore, dimerized sNRP1 decreases in a mouse model of systemic leukemia the number of circulating leukemia cells, reduces infiltration in liver and spleen, and results in reduced bone marrow neovascularization and cellularity, significantly prolonging survival time [304].

In murine tumor models, monoclonal antibodies against NRP1 or its b1/b2 domain inhibit EC migration and tumorigenesis and decrease the density of the network of tumor vessels lacking pericytes, thus delaying tumor growth if in combination with anti-VEGF therapy. When used alone, NRP1 antibody-based therapy was ineffective, suggesting that NRP1 antibodies enhance the efficacy of anti-VEGF therapy on tumor vasculature [40, 75]. However, the organism rapidly eliminates an anti-NRP1 antibody, thereby reducing its effect on tumor cells, as NRP1 is not only overexpressed by angiogenic ECs of tumor vasculature and various tumor cells [305–307] but also occurs on various cells in many tissues whose physiological functions are inevitably also affected by it [308].

Blocking NRP1/VEGFR2-mediated signaling with bevacizumab and the RTK inhibitor sunitinib to inhibit tumor angiogenesis resulted in toxic hematological and vascular side effects and hypertension in renal cancer patients [309]. Likewise, concomitant blockade of NRP1 and VEGF may be limited due to their toxicity [310]. Although a phase I study with a human monoclonal IgG1 antibody (MNRP1685A) against the VEGF-binding domain of NRP1 was promising, another phase Ib study on concomitant inhibition of NRP1 and VEGF in combination with chemotherapy showed unexpectedly high levels of proteinuria and toxicity [311, 312].

NRP1-specific tumor targeting peptides mediate tumor specificity and enhance the uptake of various therapeutic agents by tumor cells by virtue of an NRP1 binding motif [313]. Like the first-developed NRP-specific peptide inhibitor EG00229 (HY-10799), the small molecule peptide inhibitor A7R (ATWLPPR) specifically binds to NRP1 and thus inhibits VEGFR/NRP1 signaling-mediated tumor angiogenesis and tumor growth in vivo [119, 314, 315]. Similarly, the synthetic peptide EG3287 induces apoptosis of NRP1-expressing tumor cells by specifically binding to NRP1 and thereby blocking VEGF signaling [316]. Furthermore, the cyclic peptides vasotide (a retro-inverted peptidomimetic,

DCLPRC), DG1 (CRRPRMLTC), and DG2 (CRSRRIRLC) inhibit angiogenesis and tumorigenesis or invasion in preclinical mouse and primate models of human retinal diseases and non-small cell lung cancer [317, 318]. EG00086, which corresponds to the 28 C-terminal amino acids of VEGF-A and is derivatized with octanoic acid at its N-terminus, also binds to NRP1, thus efficiently inhibiting it [319]. The low molecular weight inhibitor EG01377 selectively binds to the arginine-binding pocket of NRP1 without binding to NRP2, thus inhibiting in vivo VEGF-A-induced angiogenesis, cell migration, and melanoma cell invasiveness, as well as Treg cell activation [320].

Most NRP1-specific peptides correspond to the so-called C-end rule (CendR) with the consensus sequence R/KXXR/K and have optimal binding affinity to NRP1 when two nonbasic amino acids are flanked by R and K, respectively [46, 321]. An N-terminal fragment of FGF2, LD22–4, inhibits in vitro migration of ECs, tumor cells, and fibroblasts and in vivo tumor angiogenesis and growth in animal models of breast, prostate, and lung carcinomas without cytotoxic or adverse effects by binding to NRP1 with its C-terminal CendR motif (KDPKR) [322–324]. An immunoglobulin-Fc-fused tumor tissue-penetrating peptide, Fc-TPP11 (HTPGNSKPTRTPRR), binds 1000-fold more effectively to the VEGF-binding site of NRP1 than that of NRP2, and after its NRP1-mediated internalization it downregulates VE-cadherin, thus increasing vascular and paracellular permeability in tumors and suppressing VEGF-dependent angiogenesis and tumor growth [325]. Similarly, Fc-TPP11 enhances the efficacy of coadministered doxorubicin, and when coupled to the monoclonal EGFR antibody Cetuximab (Erbitux), Fc-TPP11 significantly enhances its tumor penetration and accumulation without affecting its serum half-life [325].

Branched pentapeptides derived from the inhibitory CendR peptide KPPR by extension of its lysyl side chain with homoarginine inhibit the interaction of NRP1 with VEGF-A165 up to 30 times more effectively than the ATWLPPR peptide mentioned above [314, 315, 326]. Its plasma half-life significantly increases to 34 or 41 h by replacement of the first P with L-2,3-diaminopropionic acid or L-2,4-diaminobutyric acid, and peptidotriazoles derived from such branched peptides are remarkably resistant toward proteolysis in plasma [326, 327]. Interestingly, another peptide, pTM-NRP, interacts with the transmembrane domain of NRP1, thereby inhibiting tumor growth and metastasis in breast cancer and acting antiangiogenically in a mouse model of glioma [328, 329].

Systematically administered tumor-penetrating peptides home and penetrate the tumor tissue in consecutive steps [330]. Following integrin αvβ3- and αvβ5-mediated binding to tumor ECs and tumor cells, respectively, and subsequent proteolytic unmasking of the CendR motif by tumor-associated urokinase plasminogen activator (uPA), they bind to NRP1 and, mediated by micropinocytosis and a subsequent transcytosis cascade, penetrate deep into the tumor parenchyma [184]. In addition, NRP 1 colocalizes with the macropinocytosis marker SNX5 but not with CLCa that is involved in clathrin-mediated endocytosis [331].

Moreover, the cell-surface protein p32/gC1qR can be used as a target on tumor-associated lymphatic vessels and tumor cells. In addition to the tumor-homing peptide LyP-1 (CGNKRTRGC) [332], the disulfide-bridged TT1 (CKRGARSTC) and its linear analogue LinTT1 (AKRGARSTA) bind to p32/gC1qR on breast cancer and peritoneal carcinoma cells from stomach, ovary, and colon in murine models and in clinical explants of peritoneal carcinoma. After subsequent proteolytic exposure of its NRP1 targeting CendR motif, TT1 mediates vascular exit and tumor penetration, thus increasing the antitumor effect of coadministered therapeutics [333–335].

Irinotecan-loaded, gold-labeled silica nanoparticles coadministered with iRGD pass in transcytotic vesicles from the blood stream into perinuclear regions of cancer cells, thus reducing metastasis and enhancing survival in an orthotopic mouse model of pancreatic duct adenocarcinoma [336]. Obviously, penetration of drugs or nanoparticles into TEC and tumor cells is facilitated by triggering the CendR signaling pathway

by coupling to or coadministering with tumor-permeable peptides [185, 337]. Bispecific peptides such as RGD-ATWLPPR, which binds to NRP1 and integrin αvβ3, both overexpressed in the tumor microenvironment, accumulate more strongly than simple NRP1 or integrin ligands in the tumor, thereby reducing both the internalization of NRP1 and its turnover [338]. Since this year, a phase 1 clinical trial on the integrin-binding tumor-homing peptide CEND-1 (i.e., iRGD, CRGDKGPDC) in combination with nanoparticle albumin-bound (nab) paclitaxel and gemcitabine in metastatic pancreatic exocrine cancer is underway [339].

Docetaxel-loaded nanoparticles targeting both NRP1 and CD44 with the tLyP-1 peptide (CGNKRTR), and hyaluronic acid are taken up by metastatic tumor cells and metastasis-supporting neovasculature, thereby inhibiting tumor cell invasion and lung metastasis in three mouse models of triple negative breast cancer [340]. In addition, NRP1-coated magnetic nanoparticles may be helpful to diagnose and treat gliomas [341]. Because also nanoparticles from self-assembled amphiphilic peptides are taken up in cells via an NRP1-mediated co-endocytosis pathway, they as well may be valuable for diagnosis and therapy of NRP1-positive tumors [342].

Lipid microbubbles conjugated to peptides CRPPR and ATWLPPRD, respectively, specifically bind to NRP1-expressing cells, thus enhancing diagnostic ultrasound imaging of angiogenic tumors [307]. Polysiloxane nanoparticles, featuring the NRP1 targeting motif KDKPPR, a magnetic resonance imaging (MRI) contrast agent, and a photosensitizer, can be used theranostically, as they accumulate in the wall of glioma neovessels, where they develop cytotoxic effects when exposed to light during photodynamic therapy [343].

In addition to NRP1, CendR peptides also bind to NRP2, although with lower binding capacity, and are subsequently internalized [344]. tPlP-1, a short noncyclic tumor-homing peptide that binds to both NRP1 and NRP2, enhances the extravasation of co-injected nanoparticles into tumor tissue and may be useful for directing diagnostic and therapeutic agents to breast cancer cells [344].

Since tumor-penetrating peptides used to improve drug permeation reduce rather than enhance metastasis, the risk of facilitating the spread of metastatic cells by increasing the permeability of tumor vessels tends to be rather low [313, 345, 346]. In addition, there is no indication that the vascular system of normal tissues is compromised by systemically administered tumor-penetrating peptides [313].

In addition to normalizing the tumor vasculature to reduce intratumoral hypoxia and increased oncotic pressure, as well as to improve accessibility to chemotherapeutic agents, tumor-specific vessel disruption in combination with chemotherapeutic agents also appears to be viable in some cancers. In mouse models of fibrosarcoma and epidermoid carcinoma, intravenous administration of NRP1-specific rhodocetin αβ selectively destroys the tumor vasculature causing hemorrhage in tumors only without compromising normal vessels of healthy tissues [109].

In tumors, NRP1 occurs on the basolateral side of TECs that form intrinsically leaky tumor vessels [109]. In addition and in contrast to normal arteries, NRP1 is exposed to the bloodstream in composite vessels of tumors on the apical surface of tumor cells that have acquired an EC-like phenotype and replace ECs to varying degrees. This is even more pronounced in some cancers showing vasculogenic mimicry, where vessel-like tubes completely lined by tumor cells support the blood supply of the tumor. Because such VM vessels as well as composite vessels present NRP1 on the apical, bloodstream-facing side, they are susceptible to NRP1 targeting compounds [109]. Only in cells where NRP1 is exposed to the bloodstream, intravenously administered rhodocetin αβ can bind to the b1/b2 domain of NRP1 and trigger NRP1/MET signaling, suggesting the development of novel NRP1-targeting lead structures starting from the C-type lectin-related rhodocetin-αβ [108, 109]. Furthermore, lebein, a disintegrin from the venom of the blunt-nosed viper (*Macrovipera lebetina*), reduces the expression of both NRP1 and VEGF in an embryonic quail chorioallantoic

membrane system and in a human colon adenocarcinoma xenograft mouse model. Thus, it inhibits tumor angiogenesis [347].

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References

1. Soker S, Takashima S, Miao HQ, Neufeld G, Klagsbrun M (1998) Neuropilin-1 is expressed by endothelial and tumor cells as an isoform-specific receptor for vascular endothelial growth factor. Cell 92:735–745
2. Nakamura F, Goshima Y (2002) Structural and functional relation of neuropilins. Adv Exp Med Biol 515:55–69
3. Guo HF, Vander Kooi CW (2015) Neuropilin functions as an essential cell surface receptor. J Biol Chem 290:29120–29126. https://doi.org/10.1074/jbc.R115.687327
4. Niland S, Eble JA (2019) Neuropilins in the context of tumor vasculature. Int J Mol Sci 20. https://doi.org/10.3390/ijms20030639
5. Rossignol M, Beggs AH, Pierce EA, Klagsbrun M (1999) Human neuropilin-1 and neuropilin-2 map to 10p12 and 2q34, respectively. Genomics 57:459–460. https://doi.org/10.1006/geno.1999.5790
6. Takagi S, Hirata T, Agata K, Mochii M, Eguchi G, Fujisawa H (1991) The A5 antigen, a candidate for the neuronal recognition molecule, has homologies to complement components and coagulation factors. Neuron 7:295–307
7. Kawakami A, Kitsukawa T, Takagi S, Fujisawa H (1996) Developmentally regulated expression of a cell surface protein, neuropilin, in the mouse nervous system. J Neurobiol 29:1–17. https://doi.org/10.1002/(SICI)1097-4695(199601)29:1<1::AID-NEU1>3.0.CO;2-F
8. Fujisawa H, Kitsukawa T, Kawakami A, Takagi S, Shimizu M, Hirata T (1997) Roles of a neuronal cell-surface molecule, neuropilin, in nerve fiber fasciculation and guidance. Cell Tissue Res 290:465–470
9. Chen H, Chedotal A, He Z, Goodman CS, Tessier-Lavigne M (1997) Neuropilin-2, a novel member of the neuropilin family, is a high affinity receptor for the semaphorins Sema E and Sema IV but not Sema III. Neuron 19:547–559
10. Rossignol M, Gagnon ML, Klagsbrun M (2000) Genomic organization of human neuropilin-1 and neuropilin-2 genes: identification and distribution of splice variants and soluble isoforms. Genomics 70:211–222. https://doi.org/10.1006/geno.2000.6381
11. Gagnon ML, Bielenberg DR, Gechtman Z, Miao HQ, Takashima S, Soker S, Klagsbrun M (2000) Identification of a natural soluble neuropilin-1 that binds vascular endothelial growth factor: in vivo expression and antitumor activity. Proc Natl Acad Sci U S A 97:2573–2578. https://doi.org/10.1073/pnas.040337597
12. Yamada Y, Takakura N, Yasue H, Ogawa H, Fujisawa H, Suda T (2001) Exogenous clustered neuropilin 1 enhances vasculogenesis and angiogenesis. Blood 97:1671–1678. https://doi.org/10.1182/blood.v97.6.1671
13. Tao Q, Spring SC, Terman BI (2003) Characterization of a new alternatively spliced neuropilin-1 isoform. Angiogenesis 6:39–45
14. Cackowski FC, Xu L, Hu B, Cheng SY (2004) Identification of two novel alternatively spliced neuropilin-1 isoforms. Genomics 84:82–94. https://doi.org/10.1016/j.ygeno.2004.02.001
15. Kiedzierska A, Smietana K, Czepczynska H, Otlewski J (2007) Structural similarities and functional diversity of eukaryotic discoidin-like domains. Biochim Biophys Acta 1774:1069–1078. https://doi.org/10.1016/j.bbapap.2007.07.007
16. Lee CC, Kreusch A, McMullan D, Ng K, Spraggon G (2003) Crystal structure of the human neuropilin-1 b1 domain. Structure 11:99–108
17. Shintani Y, Takashima S, Asano Y, Kato H, Liao Y, Yamazaki S, Tsukamoto O, Seguchi O, Yamamoto H, Fukushima T et al (2006) Glycosaminoglycan modification of neuropilin-1 modulates VEGFR2 signaling. EMBO J 25:3045–3055. https://doi.org/10.1038/sj.emboj.7601188
18. Frankel P, Pellet-Many C, Lehtolainen P, D'Abaco GM, Tickner ML, Cheng L, Zachary IC (2008) Chondroitin sulphate-modified neuropilin 1 is expressed in human tumour cells and modulates 3D invasion in the U87MG human glioblastoma cell line through a p130Cas-mediated pathway. EMBO Rep 9:983–989. https://doi.org/10.1038/embor.2008.151
19. Pellet-Many C, Frankel P, Evans IM, Herzog B, Junemann-Ramirez M, Zachary IC (2011) Neuropilin-1 mediates PDGF stimulation of vascular smooth muscle cell migration and signalling via p130Cas. Biochem J 435:609–618. https://doi.org/10.1042/BJ20100580
20. Bhide GP, Fernandes NR, Colley KJ (2016) Sequence requirements for neuropilin-2 recognition by ST8SiaIV and polysialylation of its O-glycans. J Biol Chem 291:9444–9457. https://doi.org/10.1074/jbc.M116.714329
21. Roy S, Bag AK, Singh RK, Talmadge JE, Batra SK, Datta K (2017) Multifaceted role of neuropilins in the immune system: potential targets for immunotherapy. Front Immunol 8:1228. https://doi.org/10.3389/fimmu.2017.01228
22. Curreli S, Arany Z, Gerardy-Schahn R, Mann D, Stamatos NM (2007) Polysialylated neuropilin-2 is expressed on the surface of human dendritic cells and modulates dendritic cell-T lymphocyte interactions. J Biol Chem 282:30346–30356. https://doi.org/10.1074/jbc.M702965200

23. Mehta V, Fields L, Evans IM, Yamaji M, Pellet-Many C, Jones T, Mahmoud M, Zachary I (2018) VEGF (vascular endothelial growth factor) induces NRP1 (neuropilin-1) cleavage via ADAMs (a disintegrin and metalloproteinase) 9 and 10 to generate novel carboxy-terminal NRP1 fragments that regulate angiogenic signaling. Arterioscler Thromb Vasc Biol 38:1845–1858. https://doi.org/10.1161/ATVBAHA.118.311118
24. Geretti E, Shimizu A, Klagsbrun M (2008) Neuropilin structure governs VEGF and semaphorin binding and regulates angiogenesis. Angiogenesis 11:31–39. https://doi.org/10.1007/s10456-008-9097-1
25. Roth L, Nasarre C, Dirrig-Grosch S, Aunis D, Cremel G, Hubert P, Bagnard D (2008) Transmembrane domain interactions control biological functions of neuropilin-1. Mol Biol Cell 19:646–654. https://doi.org/10.1091/mbc.e07-06-0625
26. Aci-Seche S, Sawma P, Hubert P, Sturgis JN, Bagnard D, Jacob L, Genest M, Garnier N (2014) Transmembrane recognition of the semaphorin co-receptors neuropilin 1 and plexin A1: coarse-grained simulations. PLoS One 9:e97779. https://doi.org/10.1371/journal.pone.0097779
27. Herzog B, Pellet-Many C, Britton G, Hartzoulakis B, Zachary IC (2011) VEGF binding to NRP1 is essential for VEGF stimulation of endothelial cell migration, complex formation between NRP1 and VEGFR2, and signaling via FAK Tyr407 phosphorylation. Mol Biol Cell 22:2766–2776. https://doi.org/10.1091/mbc.E09-12-1061
28. Ellis LM (2006) The role of neuropilins in cancer. Mol Cancer Ther 5:1099–1107. https://doi.org/10.1158/1535-7163.MCT-05-0538
29. Guttmann-Raviv N, Shraga-Heled N, Varshavsky A, Guimaraes-Sternberg C, Kessler O, Neufeld G (2007) Semaphorin-3A and semaphorin-3F work together to repel endothelial cells and to inhibit their survival by induction of apoptosis. J Biol Chem 282:26294–26305. https://doi.org/10.1074/jbc.M609711200
30. Giger RJ, Urquhart ER, Gillespie SK, Levengood DV, Ginty DD, Kolodkin AL (1998) Neuropilin-2 is a receptor for semaphorin IV: insight into the structural basis of receptor function and specificity. Neuron 21:1079–1092
31. Gu C, Yoshida Y, Livet J, Reimert DV, Mann F, Merte J, Henderson CE, Jessell TM, Kolodkin AL, Ginty DD (2005) Semaphorin 3E and plexin-D1 control vascular pattern independently of neuropilins. Science 307:265–268. https://doi.org/10.1126/science.1105416
32. Chauvet S, Cohen S, Yoshida Y, Fekrane L, Livet J, Gayet O, Segu L, Buhot MC, Jessell TM, Henderson CE et al (2007) Gating of Sema3E/PlexinD1 signaling by neuropilin-1 switches axonal repulsion to attraction during brain development. Neuron 56:807–822. https://doi.org/10.1016/j.neuron.2007.10.019
33. Mota F, Fotinou C, Rana RR, Chan AWE, Yelland T, Arooz MT, O'Leary AP, Hutton J, Frankel P, Zachary I et al (2018) Architecture and hydration of the arginine-binding site of neuropilin-1. FEBS J 285:1290–1304. https://doi.org/10.1111/febs.14405
34. Peng K, Bai Y, Zhu Q, Hu B, Xu Y (2019) Targeting VEGF-neuropilin interactions: a promising antitumor strategy. Drug Discov Today 24:656–664. https://doi.org/10.1016/j.drudis.2018.10.004
35. Peach CJ, Mignone VW, Arruda MA, Alcobia DC, Hill SJ, Kilpatrick LE, Woolard J (2018) Molecular pharmacology of VEGF-A isoforms: binding and signalling at VEGFR2. Int J Mol Sci 19. https://doi.org/10.3390/ijms19041264
36. Robinson CJ, Stringer SE (2001) The splice variants of vascular endothelial growth factor (VEGF) and their receptors. J Cell Sci 114:853–865. PMID: WOS:000167569000004
37. Ferrara N, Gerber HP, LeCouter J (2003) The biology of VEGF and its receptors. Nat Med 9:669–676. https://doi.org/10.1038/nm0603-669
38. Klagsbrun M, Takashima S, Mamluk R (2002) The role of neuropilin in vascular and tumor biology. Adv Exp Med Biol 515:33–48
39. Neufeld G, Cohen T, Shraga N, Lange T, Kessler O, Herzog Y (2002) The neuropilins: multifunctional semaphorin and VEGF receptors that modulate axon guidance and angiogenesis. Trends Cardiovasc Med 12:13–19
40. Pan Q, Chanthery Y, Liang WC, Stawicki S, Mak J, Rathore N, Tong RK, Kowalski J, Yee SF, Pacheco G et al (2007) Blocking neuropilin-1 function has an additive effect with anti-VEGF to inhibit tumor growth. Cancer Cell 11:53–67. https://doi.org/10.1016/j.ccr.2006.10.018
41. Sarabipour S, Mac Gabhann F (2018) VEGF-A121a binding to neuropilins—a concept revisited. Cell Adhes Migr 12:204–214. https://doi.org/10.1080/19336918.2017.1372878
42. Gluzman-Poltorak Z, Cohen T, Herzog Y, Neufeld G (2000) Neuropilin-2 is a receptor for the vascular endothelial growth factor (VEGF) forms VEGF-145 and VEGF-165 [corrected]. J Biol Chem 275:18040–18045. https://doi.org/10.1074/jbc.M909259199
43. Muller YA, Li B, Christinger HW, Wells JA, Cunningham BC, de Vos AM (1997) Vascular endothelial growth factor: crystal structure and functional mapping of the kinase domain receptor binding site. Proc Natl Acad Sci U S A 94:7192–7197. https://doi.org/10.1073/pnas.94.14.7192
44. Simons M, Gordon E, Claesson-Welsh L (2016) Mechanisms and regulation of endothelial VEGF receptor signalling. Nat Rev Mol Cell Biol 17:611–625. https://doi.org/10.1038/nrm.2016.87
45. Baek DS, Kim JH, Kim YJ, Kim YS (2018) Immunoglobulin Fc-fused peptide without C-terminal Arg or Lys residue augments neuropilin-1-dependent tumor vascular permeability. Mol Pharm 15:394–402. https://doi.org/10.1021/acs.molpharmaceut.7b00761
46. Teesalu T, Sugahara KN, Kotamraju VR, Ruoslahti E (2009) C-end rule peptides mediate neuropilin-1-

dependent cell, vascular, and tissue penetration. Proc Natl Acad Sci U S A 106:16157–16162. https://doi.org/10.1073/pnas.0908201106
47. Djordjevic S, Driscoll PC (2013) Targeting VEGF signalling via the neuropilin co-receptor. Drug Discov Today 18:447–455. https://doi.org/10.1016/j.drudis.2012.11.013
48. Koch S, Tugues S, Li X, Gualandi L, Claesson-Welsh L (2011) Signal transduction by vascular endothelial growth factor receptors. Biochem J 437:169–183. https://doi.org/10.1042/BJ20110301
49. Pan Q, Chathery Y, Wu Y, Rathore N, Tong RK, Peale F, Bagri A, Tessier-Lavigne M, Koch AW, Watts RJ (2007) Neuropilin-1 binds to VEGF121 and regulates endothelial cell migration and sprouting. J Biol Chem 282:24049–24056. https://doi.org/10.1074/jbc.M703554200
50. Wang J, Huang Y, Zhang J, Xing B, Xuan W, Wang H, Huang H, Yang J, Tang J (2018) NRP-2 in tumor lymphangiogenesis and lymphatic metastasis. Cancer Lett 418:176–184. https://doi.org/10.1016/j.canlet.2018.01.040
51. Lala PK, Nandi P, Majumder M (2018) Roles of prostaglandins in tumor-associated lymphangiogenesis with special reference to breast cancer. Cancer Metastasis Rev 37:369–384. https://doi.org/10.1007/s10555-018-9734-0
52. Migdal M, Huppertz B, Tessler S, Comforti A, Shibuya M, Reich R, Baumann H, Neufeld G (1998) Neuropilin-1 is a placenta growth factor-2 receptor. J Biol Chem 273:22272–22278. https://doi.org/10.1074/jbc.273.35.22272
53. Mamluk R, Gechtman Z, Kutcher ME, Gasiunas N, Gallagher J, Klagsbrun M (2002) Neuropilin-1 binds vascular endothelial growth factor 165, placenta growth factor-2, and heparin via its b1b2 domain. J Biol Chem 277:24818–24825. https://doi.org/10.1074/jbc.M200730200
54. Matsushita A, Gotze T, Korc M (2007) Hepatocyte growth factor-mediated cell invasion in pancreatic cancer cells is dependent on neuropilin-1. Cancer Res 67:10309–10316. https://doi.org/10.1158/0008-5472.CAN-07-3256
55. Sulpice E, Plouet J, Berge M, Allanic D, Tobelem G, Merkulova-Rainon T (2008) Neuropilin-1 and neuropilin-2 act as coreceptors, potentiating proangiogenic activity. Blood 111:2036–2045. https://doi.org/10.1182/blood-2007-04-084269
56. West DC, Rees CG, Duchesne L, Patey SJ, Terry CJ, Turnbull JE, Delehedde M, Heegaard CW, Allain F, Vanpouille C et al (2005) Interactions of multiple heparin binding growth factors with neuropilin-1 and potentiation of the activity of fibroblast growth factor-2. J Biol Chem 280:13457–13464. https://doi.org/10.1074/jbc.M410924200
57. Ceccarelli S, Nodale C, Vescarelli E, Pontecorvi P, Manganelli V, Casella G, Onesti MG, Sorice M, Romano F, Angeloni A et al (2018) Neuropilin 1 mediates keratinocyte growth factor signaling in adipose-derived stem cells: potential involvement in adipogenesis. Stem Cells Int 2018:1075156. https://doi.org/10.1155/2018/1075156
58. Ruffini F, Levati L, Graziani G, Caporali S, Atzori MG, D'Atri S, Lacal PM (2017) Platelet-derived growth factor-C promotes human melanoma aggressiveness through activation of neuropilin-1. Oncotarget 8:66833–66848. https://doi.org/10.18632/oncotarget.18706
59. Ohsaka A, Hirota-Komatsu S, Araki M, Komatsu N (2015) Platelet-derived growth factor receptors form complexes with neuropilin-1 during megakaryocytic differentiation of thrombopoietin-dependent UT-7/TPO cells. Biochem Biophys Res Commun 459:443–449. https://doi.org/10.1016/j.bbrc.2015.02.124
60. Muhl L, Folestad EB, Gladh H, Wang Y, Moessinger C, Jakobsson L, Eriksson U (2017) Neuropilin 1 binds PDGF-D and is a co-receptor in PDGF-D-PDGFRbeta signaling. J Cell Sci 130:1365–1378. https://doi.org/10.1242/jcs.200493
61. Glinka Y, Prud'homme GJ (2008) Neuropilin-1 is a receptor for transforming growth factor beta-1, activates its latent form, and promotes regulatory T cell activity. J Leukoc Biol 84:302–310. https://doi.org/10.1189/jlb.0208090
62. Glinka Y, Stoilova S, Mohammed N, Prud'homme GJ (2011) Neuropilin-1 exerts co-receptor function for TGF-beta-1 on the membrane of cancer cells and enhances responses to both latent and active TGF-beta. Carcinogenesis 32:613–621. https://doi.org/10.1093/carcin/bgq281
63. Vivekanandhan S, Mukhopadhyay D (2019) Genetic status of KRAS influences transforming growth factor-beta (TGF-beta) signaling: an insight into neuropilin-1 (NRP1) mediated tumorigenesis. Semin Cancer Biol 54:72–79. https://doi.org/10.1016/j.semcancer.2018.01.014
64. Rizzolio S, Rabinowicz N, Rainero E, Lanzetti L, Serini G, Norman J, Neufeld G, Tamagnone L (2012) Neuropilin-1-dependent regulation of EGF-receptor signaling. Cancer Res 72:5801–5811. https://doi.org/10.1158/0008-5472.CAN-12-0995
65. Kigel B, Rabinowicz N, Varshavsky A, Kessler O, Neufeld G (2011) Plexin-A4 promotes tumor progression and tumor angiogenesis by enhancement of VEGF and bFGF signaling. Blood 118:4285–4296. https://doi.org/10.1182/blood-2011-03-341388
66. Antipenko A, Himanen JP, van Leyen K, Nardi-Dei V, Lesniak J, Barton WA, Rajashankar KR, Lu M, Hoemme C, Puschel AW et al (2003) Structure of the semaphorin-3A receptor binding module. Neuron 39:589–598
67. Alto LT, Terman JR (2017) Semaphorins and their signaling mechanisms. Methods Mol Biol 1493:1–25. https://doi.org/10.1007/978-1-4939-6448-2_1
68. Gaur P, Bielenberg DR, Samuel S, Bose D, Zhou Y, Gray MJ, Dallas NA, Fan F, Xia L, Lu J et al (2009) Role of class 3 semaphorins and their receptors in tumor growth and angiogenesis. Clin Cancer Res 15:6763–6770. https://doi.org/10.1158/1078-0432.CCR-09-1810

69. Serini G, Bussolino F, Maione F, Giraudo E (2013) Class 3 semaphorins: physiological vascular normalizing agents for anti-cancer therapy. J Intern Med 273:138–155. https://doi.org/10.1111/joim.12017
70. Toledano S, Nir-Zvi I, Engelman R, Kessler O, Neufeld G (2019) Class-3 semaphorins and their receptors: potent multifunctional modulators of tumor progression. Int J Mol Sci 20. https://doi.org/10.3390/ijms20030556
71. Maione F, Molla F, Meda C, Latini R, Zentilin L, Giacca M, Seano G, Serini G, Bussolino F, Giraudo E (2009) Semaphorin 3A is an endogenous angiogenesis inhibitor that blocks tumor growth and normalizes tumor vasculature in transgenic mouse models. J Clin Invest 119:3356–3372. https://doi.org/10.1172/JCI36308
72. Wong HK, Shimizu A, Kirkpatrick ND, Garkavtsev I, Chan AW, di Tomaso E, Klagsbrun M, Jain RK (2012) Merlin/NF2 regulates angiogenesis in schwannomas through a Rac1/semaphorin 3F-dependent mechanism. Neoplasia 14:84–94. https://doi.org/10.1593/neo.111600
73. Nasarre P, Gemmill RM, Drabkin HA (2014) The emerging role of class-3 semaphorins and their neuropilin receptors in oncology. Onco Targets Ther 7:1663–1687. https://doi.org/10.2147/OTT.S37744
74. Sawma P, Roth L, Blanchard C, Bagnard D, Cremel G, Bouveret E, Duneau JP, Sturgis JN, Hubert P (2014) Evidence for new homotypic and heterotypic interactions between transmembrane helices of proteins involved in receptor tyrosine kinase and neuropilin signaling. J Mol Biol 426:4099–4111. https://doi.org/10.1016/j.jmb.2014.10.007
75. Liang WC, Dennis MS, Stawicki S, Chanthery Y, Pan Q, Chen Y, Eigenbrot C, Yin J, Koch AW, Wu X et al (2007) Function blocking antibodies to neuropilin-1 generated from a designed human synthetic antibody phage library. J Mol Biol 366:815–829. https://doi.org/10.1016/j.jmb.2006.11.021
76. Barton WA, Himanen JP, Antipenko A, Nikolov DB (2004) Structures of axon guidance molecules and their neuronal receptors. Adv Protein Chem 68:65–106. https://doi.org/10.1016/S0065-3233(04)68003-X
77. Neufeld G, Kessler O (2008) The semaphorins: versatile regulators of tumour progression and tumour angiogenesis. Nat Rev Cancer 8:632–645. https://doi.org/10.1038/nrc2404
78. Valdembri D, Caswell PT, Anderson KI, Schwarz JP, Konig I, Astanina E, Caccavari F, Norman JC, Humphries MJ, Bussolino F et al (2009) Neuropilin-1/GIPC1 signaling regulates alpha-5beta1 integrin traffic and function in endothelial cells. PLoS Biol 7:e25. https://doi.org/10.1371/journal.pbio.1000025
79. Perrot-Applanat M, Di Benedetto M (2012) Autocrine functions of VEGF in breast tumor cells: adhesion, survival, migration and invasion. Cell Adhes Migr 6:547–553. https://doi.org/10.4161/cam.23332
80. Yaqoob U, Cao S, Shergill U, Jagavelu K, Geng Z, Yin M, de Assuncao TM, Cao Y, Szabolcs A, Thorgeirsson S et al (2012) Neuropilin-1 stimulates tumor growth by increasing fibronectin fibril assembly in the tumor microenvironment. Cancer Res 72:4047–4059. https://doi.org/10.1158/0008-5472.CAN-11-3907
81. Goel HL, Pursell B, Chang C, Shaw LM, Mao J, Simin K, Kumar P, Vander Kooi CW, Shultz LD, Greiner DL et al (2013) GLI1 regulates a novel neuropilin-2/alpha6beta1 integrin based autocrine pathway that contributes to breast cancer initiation. EMBO Mol Med 5:488–508. https://doi.org/10.1002/emmm.201202078
82. Campbell ID, Humphries MJ (2011) Integrin structure, activation, and interactions. Cold Spring Harb Perspect Biol 3. https://doi.org/10.1101/cshperspect.a004994
83. Luo BH, Carman CV, Springer TA (2007) Structural basis of integrin regulation and signaling. Annu Rev Immunol 25:619–647. https://doi.org/10.1146/annurev.immunol.25.022106.141618
84. Humphries JD, Byron A, Humphries MJ (2006) Integrin ligands at a glance. J Cell Sci 119:3901–3903. https://doi.org/10.1242/jcs.03098
85. Hynes RO (2009) The extracellular matrix: not just pretty fibrils. Science 326:1216–1219. https://doi.org/10.1126/science.1176009
86. Singh B, Fleury C, Jalalvand F, Riesbeck K (2012) Human pathogens utilize host extracellular matrix proteins laminin and collagen for adhesion and invasion of the host. FEMS Microbiol Rev 36:1122–1180. https://doi.org/10.1111/j.1574-6976.2012.00340.x
87. Humphries JD, Chastney MR, Askari JA, Humphries MJ (2019) Signal transduction via integrin adhesion complexes. Curr Opin Cell Biol 56:14–21. https://doi.org/10.1016/j.ceb.2018.08.004
88. Horton ER, Humphries JD, James J, Jones MC, Askari JA, Humphries MJ (2016) The integrin adhesome network at a glance. J Cell Sci 129:4159–4163. https://doi.org/10.1242/jcs.192054
89. Kanchanawong P, Waterman CM (2012) Advances in light-based imaging of three-dimensional cellular ultrastructure. Curr Opin Cell Biol 24:125–133. https://doi.org/10.1016/j.ceb.2011.11.010
90. Kanchanawong P, Shtengel G, Pasapera AM, Ramko EB, Davidson MW, Hess HF, Waterman CM (2010) Nanoscale architecture of integrin-based cell adhesions. Nature 468:580–584. https://doi.org/10.1038/nature09621
91. Goel HL, Mercurio AM (2012) Enhancing integrin function by VEGF/neuropilin signaling: implications for tumor biology. Cell Adhes Migr 6:554–560. https://doi.org/10.4161/cam.22419
92. Ou JJ, Wei X, Peng Y, Zha L, Zhou RB, Shi H, Zhou Q, Liang HJ (2015) Neuropilin-2 mediates lymphangiogenesis of colorectal carcinoma via a VEGFC/VEGFR3 independent signaling. Cancer Lett 358:200–209. https://doi.org/10.1016/j.canlet.2014.12.046

93. Cao Y, Hoeppner LH, Bach SEG, Guo Y, Wang E, Wu J, Cowley MJ, Chang DK, Waddell N et al (2013) Neuropilin-2 promotes extravasation and metastasis by interacting with endothelial alpha5 integrin. Cancer Res 73:4579–4590. https://doi.org/10.1158/0008-5472.CAN-13-0529
94. Pan H, Wanami LS, Dissanayake TR, Bachelder RE (2009) Autocrine semaphorin3A stimulates alpha2 beta1 integrin expression/function in breast tumor cells. Breast Cancer Res Treat 118:197–205. https://doi.org/10.1007/s10549-008-0179-y
95. Naik A, Al-Yahyaee A, Abdullah N, Sam JE, Al-Zeheimi N, Yaish MW, Adham SA (2018) Neuropilin-1 promotes the oncogenic tenascin-C/integrin beta3 pathway and modulates chemoresistance in breast cancer cells. BMC Cancer 18:533. https://doi.org/10.1186/s12885-018-4446-y
96. Ellison TS, Atkinson SJ, Steri V, Kirkup BM, Preedy ME, Johnson RT, Ruhrberg C, Edwards DR, Schneider JG, Weilbaecher K et al (2015) Suppression of beta3-integrin in mice triggers a neuropilin-1-dependent change in focal adhesion remodelling that can be targeted to block pathological angiogenesis. Dis Model Mech 8:1105–1119. https://doi.org/10.1242/dmm.019927
97. Robinson SD, Reynolds LE, Kostourou V, Reynolds AR, da Silva RG, Tavora B, Baker M, Marshall JF, Hodivala-Dilke KM (2009) Alphav beta3 integrin limits the contribution of neuropilin-1 to vascular endothelial growth factor-induced angiogenesis. J Biol Chem 284:33966–33981. https://doi.org/10.1074/jbc.M109.030700
98. Castellani V (2002) The function of neuropilin/L1 complex. Adv Exp Med Biol 515:91–102
99. Castellani V, Falk J, Rougon G (2004) Semaphorin3A-induced receptor endocytosis during axon guidance responses is mediated by L1 CAM. Mol Cell Neurosci 26:89–100. https://doi.org/10.1016/j.mcn.2004.01.010
100. Bechara A, Nawabi H, Moret F, Yaron A, Weaver E, Bozon M, Abouzid K, Guan JL, Tessier-Lavigne M, Lemmon V et al (2008) FAK-MAPK-dependent adhesion disassembly downstream of L1 contributes to semaphorin3A-induced collapse. EMBO J 27:1549–1562. https://doi.org/10.1038/emboj.2008.86
101. Dallatomasina A, Gasparri AM, Colombo B, Sacchi A, Bianco M, Daniele T, Esposito A, Pastorino F, Ponzoni M, Marcucci F et al (2019) Spatiotemporal regulation of tumor angiogenesis by circulating chromogranin a cleavage and neuropilin-1 engagement. Cancer Res 79:1925–1937. https://doi.org/10.1158/0008-5472.CAN-18-0289
102. Ben-Zvi A, Ben-Gigi L, Klein H, Behar O (2007) Modulation of semaphorin3A activity by p75 neurotrophin receptor influences peripheral axon patterning. J Neurosci 27:13000–13011. https://doi.org/10.1523/JNEUROSCI.3373-07.2007
103. Park JE, Keller GA, Ferrara N (1993) The vascular endothelial growth factor (VEGF) isoforms: differential deposition into the subepithelial extracellular matrix and bioactivity of extracellular matrix-bound VEGF. Mol Biol Cell 4:1317–1326. https://doi.org/10.1091/mbc.4.12.1317
104. Jonca F, Ortega N, Gleizes PE, Bertrand N, Plouet J (1997) Cell release of bioactive fibroblast growth factor 2 by exon 6-encoded sequence of vascular endothelial growth factor. J Biol Chem 272:24203–24209. https://doi.org/10.1074/jbc.272.39.24203
105. Stringer SE (2006) The role of heparan sulphate proteoglycans in angiogenesis. Biochem Soc Trans 34:451–453. https://doi.org/10.1042/BST0340451
106. Koch S, Claesson-Welsh L (2012) Signal transduction by vascular endothelial growth factor receptors. Cold Spring Harb Perspect Med 2:a006502. https://doi.org/10.1101/cshperspect.a006502
107. Vempati P, Popel AS, Mac Gabhann F (2014) Extracellular regulation of VEGF: isoforms, proteolysis, and vascular patterning. Cytokine Growth Factor Rev 25:1–19. https://doi.org/10.1016/j.cytogfr.2013.11.002
108. Niland S, Ditkowski B, Parrandier D, Roth L, Augustin H, Eble JA (2013) Rhodocetin-alphabeta-induced neuropilin-1-cMet association triggers restructuring of matrix contacts in endothelial cells. Arterioscler Thromb Vasc Biol 33:544–554. https://doi.org/10.1161/ATVBAHA.112.00006
109. Niland S, Komljenovic D, Macas J, Bracht T, Bauerle T, Liebner S, Eble JA (2018) Rhodocetin-alphabeta selectively breaks the endothelial barrier of the tumor vasculature in HT1080 fibrosarcoma and A431 epidermoid carcinoma tumor models. Oncotarget 9:22406–22422. https://doi.org/10.18632/oncotarget.25032
110. Rezaei M, Martins Cavaco AC, Seebach J, Niland S, Zimmermann J, Hanschmann EM, Hallmann R, Schillers H, Eble JA (2019) Signals of the neuropilin-1-MET Axis and cues of mechanical force exertion converge to elicit inflammatory activation in coherent endothelial cells. J Immunol 202:1559–1572. https://doi.org/10.4049/jimmunol.1801346
111. Morin E, Sjoberg E, Tjomsland V, Testini C, Lindskog C, Franklin O, Sund M, Ohlund D, Kiflemariam S, Sjoblom T et al (2018) VEGF receptor-2/neuropilin 1 trans-complex formation between endothelial and tumor cells is an independent predictor of pancreatic cancer survival. J Pathol 246:311–322. https://doi.org/10.1002/path.5141
112. Koch S, van Meeteren LA, Morin E, Testini C, Westrom S, Bjorkelund H, Le Jan S, Adler J, Berger P, Claesson-Welsh L (2014) NRP1 presented in trans to the endothelium arrests VEGFR2 endocytosis, preventing angiogenic signaling and tumor initiation. Dev Cell 28:633–646. https://doi.org/10.1016/j.devcel.2014.02.010
113. Campos-Mora M, Morales RA, Gajardo T, Catalan D, Pino-Lagos K (2013) Neuropilin-1 in transplantation tolerance. Front Immunol 4:405. https://doi.org/10.3389/fimmu.2013.00405

114. Delgoffe GM, Woo SR, Turnis ME, Gravano DM, Guy C, Overacre AE, Bettini ML, Vogel P, Finkelstein D, Bonnevier J et al (2013) Stability and function of regulatory T cells is maintained by a neuropilin-1-semaphorin-4A axis. Nature 501:252–256. https://doi.org/10.1038/nature12428
115. Bourbie-Vaudaine S, Blanchard N, Hivroz C, Romeo PH (2006) Dendritic cells can turn CD4+ T lymphocytes into vascular endothelial growth factor-carrying cells by intercellular neuropilin-1 transfer. J Immunol 177:1460–1469. https://doi.org/10.4049/jimmunol.177.3.1460
116. Goel HL, Chang C, Pursell B, Leav I, Lyle S, Xi HS, Hsieh CC, Adisetiyo H, Roy-Burman P, Coleman IM et al (2012) VEGF/neuropilin-2 regulation of Bmi-1 and consequent repression of IGF-IR define a novel mechanism of aggressive prostate cancer. Cancer Discov 2:906–921. https://doi.org/10.1158/2159-8290.CD-12-0085
117. Yoshida A, Shimizu A, Asano H, Kadonosono T, Kondoh SK, Geretti E, Mammoto A, Klagsbrun M, Seo MK (2015) VEGF-A/NRP1 stimulates GIPC1 and Syx complex formation to promote RhoA activation and proliferation in skin cancer cells. Biol Open 4:1063–1076. https://doi.org/10.1242/bio.010918
118. Smith NR, Baker D, James NH, Ratcliffe K, Jenkins M, Ashton SE, Sproat G, Swann R, Gray N, Ryan A et al (2010) Vascular endothelial growth factor receptors VEGFR-2 and VEGFR-3 are localized primarily to the vasculature in human primary solid cancers. Clin Cancer Res 16:3548–3561. https://doi.org/10.1158/1078-0432.CCR-09-2797
119. Prud'homme GJ, Glinka Y (2012) Neuropilins are multifunctional coreceptors involved in tumor initiation, growth, metastasis and immunity. Oncotarget 3:921–939. https://doi.org/10.18632/oncotarget.626
120. Takahashi T, Fournier A, Nakamura F, Wang LH, Murakami Y, Kalb RG, Fujisawa H, Strittmatter SM (1999) Plexin-neuropilin-1 complexes form functional semaphorin-3A receptors. Cell 99:59–69
121. Zhang L, Wang H, Li C, Zhao Y, Wu L, Du X, Han Z (2017) VEGF-A/neuropilin 1 pathway confers cancer stemness via activating Wnt/beta-catenin axis in breast cancer cells. Cell Physiol Biochem 44:1251–1262. https://doi.org/10.1159/000485455
122. Atzori MG, Tentori L, Ruffini F, Ceci C, Lisi L, Bonanno E, Scimeca M, Eskilsson E, Daubon T, Miletic H et al (2017) The anti-vascular endothelial growth factor receptor-1 monoclonal antibody D16F7 inhibits invasiveness of human glioblastoma and glioblastoma stem cells. J Exp Clin Cancer Res 36:106. https://doi.org/10.1186/s13046-017-0577-2
123. Shimizu A, Zankov DP, Kurokawa-Seo M, Ogita H (2018) Vascular endothelial growth factor-A exerts diverse cellular effects via small G proteins, Rho and Rap. Int J Mol Sci 19. https://doi.org/10.3390/ijms19041203
124. Wang Z, Ahmad A, Li Y, Kong D, Azmi AS, Banerjee S, Sarkar FH (2010) Emerging roles of PDGF-D signaling pathway in tumor development and progression. Biochim Biophys Acta 1806:122–130. https://doi.org/10.1016/j.bbcan.2010.04.003
125. Hernandez-Garcia R, Iruela-Arispe ML, Reyes-Cruz G, Vazquez-Prado J (2015) Endothelial RhoGEFs: a systematic analysis of their expression profiles in VEGF-stimulated and tumor endothelial cells. Vasc Pharmacol 74:60–72. https://doi.org/10.1016/j.vph.2015.10.003
126. Ghosh K, Thodeti CK, Dudley AC, Mammoto A, Klagsbrun M, Ingber DE (2008) Tumor-derived endothelial cells exhibit aberrant Rho-mediated mechanosensing and abnormal angiogenesis in vitro. Proc Natl Acad Sci U S A 105:11305–11310. https://doi.org/10.1073/pnas.0800835105
127. Deng Y, Zhang X, Simons M (2015) Molecular controls of lymphatic VEGFR3 signaling. Arterioscler Thromb Vasc Biol 35:421–429. https://doi.org/10.1161/ATVBAHA.114.304881
128. Parikh AA, Fan F, Liu WB, Ahmad SA, Stoeltzing O, Reinmuth N, Bielenberg D, Bucana CD, Klagsbrun M, Ellis LM (2004) Neuropilin-1 in human colon cancer: expression, regulation, and role in induction of angiogenesis. Am J Pathol 164:2139–2151. https://doi.org/10.1016/S0002-9440(10)63772-8
129. Ding H, Wu X, Roncari L, Lau N, Shannon P, Nagy A, Guha A (2000) Expression and regulation of neuropilin-1 in human astrocytomas. Int J Cancer 88:584–592
130. Akagi M, Kawaguchi M, Liu W, McCarty MF, Takeda A, Fan F, Stoeltzing O, Parikh AA, Jung YD, Bucana CD et al (2003) Induction of neuropilin-1 and vascular endothelial growth factor by epidermal growth factor in human gastric cancer cells. Br J Cancer 88:796–802. https://doi.org/10.1038/sj.bjc.6600811
131. Tzahar E, Waterman H, Chen X, Levkowitz G, Karunagaran D, Lavi S, Ratzkin BJ, Yarden Y (1996) A hierarchical network of interreceptor interactions determines signal transduction by Neu differentiation factor/neuregulin and epidermal growth factor. Mol Cell Biol 16:5276–5287. https://doi.org/10.1128/mcb.16.10.5276
132. Aghajanian H, Cho YK, Manderfield LJ, Herling MR, Gupta M, Ho VC, Li L, Degenhardt K, Aharonov A, Tzahor E et al (2016) Coronary vasculature patterning requires a novel endothelial ErbB2 holoreceptor. Nat Commun 7:12038. https://doi.org/10.1038/ncomms12038
133. Rizzolio S, Battistini C, Cagnoni G, Apicella M, Vella V, Giordano S, Tamagnone L (2018) Downregulating neuropilin-2 triggers a novel mechanism enabling EGFR-dependent resistance to oncogene-targeted therapies. Cancer Res 78:1058–1068. https://doi.org/10.1158/0008-5472.CAN-17-2020
134. Ball SG, Bayley C, Shuttleworth CA, Kielty CM (2010) Neuropilin-1 regulates platelet-derived growth factor receptor signalling in mesenchymal stem cells. Biochem J 427:29–40. https://doi.org/10.1042/BJ20091512
135. Banerjee S, Sengupta K, Dhar K, Mehta S, D'Amore PA, Dhar G, Banerjee SK (2006) Breast cancer cells

secreted platelet-derived growth factor-induced motility of vascular smooth muscle cells is mediated through neuropilin-1. Mol Carcinog 45:871–880. https://doi.org/10.1002/mc.20248
136. Cao S, Yaqoob U, Das A, Shergill U, Jagavelu K, Huebert RC, Routray C, Abdelmoneim S, Vasdev M, Leof E et al (2010) Neuropilin-1 promotes cirrhosis of the rodent and human liver by enhancing PDGF/TGF-beta signaling in hepatic stellate cells. J Clin Invest 120:2379–2394. https://doi.org/10.1172/JCI41203
137. Dhar K, Dhar G, Majumder M, Haque I, Mehta S, Van Veldhuizen PJ, Banerjee SK, Banerjee S (2010) Tumor cell-derived PDGF-B potentiates mouse mesenchymal stem cells-pericytes transition and recruitment through an interaction with NRP-1. Mol Cancer 9:209. https://doi.org/10.1186/1476-4598-9-209
138. Evans IM, Yamaji M, Britton G, Pellet-Many C, Lockie C, Zachary IC, Frankel P (2011) Neuropilin-1 signaling through p130Cas tyrosine phosphorylation is essential for growth factor-dependent migration of glioma and endothelial cells. Mol Cell Biol 31:1174–1185. https://doi.org/10.1128/MCB.00903-10
139. Ponten A, Folestad EB, Pietras K, Eriksson U (2005) Platelet-derived growth factor D induces cardiac fibrosis and proliferation of vascular smooth muscle cells in heart-specific transgenic mice. Circ Res 97:1036–1045. https://doi.org/10.1161/01.RES.0000190590.31545.d4
140. Andrae J, Gallini R, Betsholtz C (2008) Role of platelet-derived growth factors in physiology and medicine. Genes Dev 22:1276–1312. https://doi.org/10.1101/gad.1653708
141. Cortez E, Gladh H, Braun S, Bocci M, Cordero E, Bjorkstrom NK, Miyazaki H, Michael IP, Eriksson U, Folestad E et al (2016) Functional malignant cell heterogeneity in pancreatic neuroendocrine tumors revealed by targeting of PDGF-DD. Proc Natl Acad Sci U S A 113:E864–E873. https://doi.org/10.1073/pnas.1509384113
142. Hu B, Guo P, Bar-Joseph I, Imanishi Y, Jarzynka MJ, Bogler O, Mikkelsen T, Hirose T, Nishikawa R, Cheng SY (2007) Neuropilin-1 promotes human glioma progression through potentiating the activity of the HGF/SF autocrine pathway. Oncogene 26:5577–5586. https://doi.org/10.1038/sj.onc.1210348
143. Weinstein IB (2002) Cancer. Addiction to oncogenes—the Achilles heal of cancer. Science 297:63–64. https://doi.org/10.1126/science.1073096
144. Li L, Jiang X, Zhang Q, Dong X, Gao Y, He Y, Qiao H, Xie F, Xie X, Sun X (2016) Neuropilin-1 is associated with clinicopathology of gastric cancer and contributes to cell proliferation and migration as multifunctional co-receptors. J Exp Clin Cancer Res 35:16. https://doi.org/10.1186/s13046-016-0291-5
145. Rizzolio S, Cagnoni G, Battistini C, Bonelli S, Isella C, Van Ginderachter JA, Bernards R, Di Nicolantonio F, Giordano S, Tamagnone L (2018) Neuropilin-1 upregulation elicits adaptive resistance to oncogene-targeted therapies. J Clin Invest 128:3976–3990. https://doi.org/10.1172/JCI99257
146. Liu W, Wu T, Dong X, Zeng YA (2017) Neuropilin-1 is upregulated by Wnt/beta-catenin signaling and is important for mammary stem cells. Sci Rep 7:10941. https://doi.org/10.1038/s41598-017-11287-w
147. Lichtenberger BM, Tan PK, Niederleithner H, Ferrara N, Petzelbauer P, Sibilia M (2010) Autocrine VEGF signaling synergizes with EGFR in tumor cells to promote epithelial cancer development. Cell 140:268–279. https://doi.org/10.1016/j.cell.2009.12.046
148. Sun C, Wang L, Huang S, Heynen GJ, Prahallad A, Robert C, Haanen J, Blank C, Wesseling J, Willems SM et al (2014) Reversible and adaptive resistance to BRAF(V600E) inhibition in melanoma. Nature 508:118–122. https://doi.org/10.1038/nature13121
149. Wang J, Huang SK, Marzese DM, Hsu SC, Kawas NP, Chong KK, Long GV, Menzies AM, Scolyer RA, Izraely S et al (2015) Epigenetic changes of EGFR have an important role in BRAF inhibitor-resistant cutaneous melanomas. J Invest Dermatol 135:532–541. https://doi.org/10.1038/jid.2014.418
150. Cao Y, Szabolcs A, Dutta SK, Yaqoob U, Jagavelu K, Wang L, Leof EB, Urrutia RA, Shah VH, Mukhopadhyay D (2010) Neuropilin-1 mediates divergent R-Smad signaling and the myofibroblast phenotype. J Biol Chem 285:31840–31848. https://doi.org/10.1074/jbc.M110.151696
151. Hirota S, Clements TP, Tang LK, Morales JE, Lee HS, Oh SP, Rivera GM, Wagner DS, McCarty JH (2015) Neuropilin 1 balances beta8 integrin-activated TGFbeta signaling to control sprouting angiogenesis in the brain. Development 142:4363–4373. https://doi.org/10.1242/dev.113746
152. Aspalter IM, Gordon E, Dubrac A, Ragab A, Narloch J, Vizan P, Geudens I, Collins RT, Franco CA, Abrahams CL et al (2015) Alk1 and Alk5 inhibition by Nrp1 controls vascular sprouting downstream of Notch. Nat Commun 6:7264. https://doi.org/10.1038/ncomms8264
153. Vivekanandhan S, Yang L, Cao Y, Wang E, Dutta SK, Sharma AK, Mukhopadhyay D (2017) Genetic status of KRAS modulates the role of neuropilin-1 in tumorigenesis. Sci Rep 7:12877. https://doi.org/10.1038/s41598-017-12992-2
154. Yin K, Yin W, Wang Y, Zhou L, Liu Y, Yang G, Wang J, Lu J (2016) MiR-206 suppresses epithelial mesenchymal transition by targeting TGF-beta signaling in estrogen receptor positive breast cancer cells. Oncotarget 7:24537–24548. https://doi.org/10.18632/oncotarget.8233
155. Chen Y, Huang S, Wu B, Fang J, Zhu M, Sun L, Zhang L, Zhang Y, Sun M, Guo L et al (2017) Transforming growth factor-beta1 promotes breast cancer metastasis by downregulating miR-196a-3p expression. Oncotarget 8:49110–49122. https://doi.org/10.18632/oncotarget.16308
156. Neufeld G, Sabag AD, Rabinovicz N, Kessler O (2012) Semaphorins in angiogenesis and tumor progression. Cold Spring Harb Perspect Med 2:a006718. https://doi.org/10.1101/cshperspect.a006718

157. Sakurai A, Doci CL, Gutkind JS (2012) Semaphorin signaling in angiogenesis, lymphangiogenesis and cancer. Cell Res 22:23–32. https://doi.org/10.1038/cr.2011.198
158. Yang WJ, Hu J, Uemura A, Tetzlaff F, Augustin HG, Fischer A (2015) Semaphorin-3C signals through neuropilin-1 and PlexinD1 receptors to inhibit pathological angiogenesis. EMBO Mol Med 7:1267–1284. https://doi.org/10.15252/emmm.201404922
159. Hao J, Yu JS (2018) Semaphorin 3C and its receptors in cancer and cancer stem-like cells. Biomedicine 6. https://doi.org/10.3390/biomedicines6020042
160. Hui DHF, Tam KJ, Jiao IZF, Ong CJ (2019) Semaphorin 3C as a therapeutic target in prostate and other cancers. Int J Mol Sci 20. https://doi.org/10.3390/ijms20030774
161. Bielenberg DR, Hida Y, Shimizu A, Kaipainen A, Kreuter M, Kim CC, Klagsbrun M (2004) Semaphorin 3F, a chemorepulsant for endothelial cells, induces a poorly vascularized, encapsulated, nonmetastatic tumor phenotype. J Clin Invest 114:1260–1271. https://doi.org/10.1172/JCI21378
162. Kessler O, Shraga-Heled N, Lange T, Gutmann-Raviv N, Sabo E, Baruch L, Machluf M, Neufeld G (2004) Semaphorin-3F is an inhibitor of tumor angiogenesis. Cancer Res 64:1008–1015
163. Dallas NA, Gray MJ, Xia L, Fan F, van Buren G 2nd, Gaur P, Samuel S, Lim SJ, Arumugam T, Ramachandran V et al (2008) Neuropilin-2-mediated tumor growth and angiogenesis in pancreatic adenocarcinoma. Clin Cancer Res 14:8052–8060. https://doi.org/10.1158/1078-0432.CCR-08-1520
164. Zeng Q, Li S, Chepeha DB, Giordano TJ, Li J, Zhang H, Polverini PJ, Nor J, Kitajewski J, Wang CY (2005) Crosstalk between tumor and endothelial cells promotes tumor angiogenesis by MAPK activation of Notch signaling. Cancer Cell 8:13–23. https://doi.org/10.1016/j.ccr.2005.06.004
165. Benedito R, Roca C, Sorensen I, Adams S, Gossler A, Fruttiger M, Adams RH (2009) The Notch ligands Dll4 and Jagged1 have opposing effects on angiogenesis. Cell 137:1124–1135. https://doi.org/10.1016/j.cell.2009.03.025
166. Armulik A, Abramsson A, Betsholtz C (2005) Endothelial/pericyte interactions. Circ Res 97:512–523. https://doi.org/10.1161/01.RES.0000182903.16652.d7
167. von Tell D, Armulik A, Betsholtz C (2006) Pericytes and vascular stability. Exp Cell Res 312:623–629. https://doi.org/10.1016/j.yexcr.2005.10.019
168. Gaengel K, Genove G, Armulik A, Betsholtz C (2009) Endothelial-mural cell signaling in vascular development and angiogenesis. Arterioscler Thromb Vasc Biol 29:630–638. https://doi.org/10.1161/ATVBAHA.107.161521
169. Gu C, Giraudo E (2013) The role of semaphorins and their receptors in vascular development and cancer. Exp Cell Res 319:1306–1316. https://doi.org/10.1016/j.yexcr.2013.02.003
170. Aguilera KY, Brekken RA (2014) Recruitment and retention: factors that affect pericyte migration. Cell Mol Life Sci 71:299–309. https://doi.org/10.1007/s00018-013-1432-z
171. Xian X, Hakansson J, Stahlberg A, Lindblom P, Betsholtz C, Gerhardt H, Semb H (2006) Pericytes limit tumor cell metastasis. J Clin Invest 116:642–651. https://doi.org/10.1172/JCI25705
172. Chakraborty G, Kumar S, Mishra R, Patil TV, Kundu GC (2012) Semaphorin 3A suppresses tumor growth and metastasis in mice melanoma model. PLoS One 7:e33633. https://doi.org/10.1371/journal.pone.0033633
173. Fukasawa M, Matsushita A, Korc M (2007) Neuropilin-1 interacts with integrin beta1 and modulates pancreatic cancer cell growth, survival and invasion. Cancer Biol Ther 6:1173–1180. https://doi.org/10.4161/cbt.6.8.4363
174. Goel HL, Mercurio AM (2013) VEGF targets the tumour cell. Nat Rev Cancer 13:871–882. https://doi.org/10.1038/nrc3627
175. Byzova TV, Goldman CK, Pampori N, Thomas KA, Bett A, Shattil SJ, Plow EF (2000) A mechanism for modulation of cellular responses to VEGF: activation of the integrins. Mol Cell 6:851–860
176. Serini G, Valdembri D, Zanivan S, Morterra G, Burkhardt C, Caccavari F, Zammataro L, Primo L, Tamagnone L, Logan M et al (2003) Class 3 semaphorins control vascular morphogenesis by inhibiting integrin function. Nature 424:391–397. https://doi.org/10.1038/nature01784
177. Valdembri D, Regano D, Maione F, Giraudo E, Serini G (2016) Class 3 semaphorins in cardiovascular development. Cell Adhes Migr 10:641–651. https://doi.org/10.1080/19336918.2016.1212805
178. Goel HL, Pursell B, Standley C, Fogarty K, Mercurio AM (2012) Neuropilin-2 regulates alpha6beta1 integrin in the formation of focal adhesions and signaling. J Cell Sci 125:497–506. https://doi.org/10.1242/jcs.094433
179. Muders MH, Zhang H, Wang E, Tindall DJ, Datta K (2009) Vascular endothelial growth factor-C protects prostate cancer cells from oxidative stress by the activation of mammalian target of rapamycin complex-2 and AKT-1. Cancer Res 69:6042–6048. https://doi.org/10.1158/0008-5472.CAN-09-0552
180. Facchinetti V, Ouyang W, Wei H, Soto N, Lazorchak A, Gould C, Lowry C, Newton AC, Mao Y, Miao RQ et al (2008) The mammalian target of rapamycin complex 2 controls folding and stability of Akt and protein kinase C. EMBO J 27:1932–1943. https://doi.org/10.1038/emboj.2008.120
181. Cai H, Reed RR (1999) Cloning and characterization of neuropilin-1-interacting protein: a PSD-95/Dlg/ZO-1 domain-containing protein that interacts with the cytoplasmic domain of neuropilin-1. J Neurosci 19:6519–6527
182. Prahst C, Heroult M, Lanahan AA, Uziel N, Kessler O, Shraga-Heled N, Simons M, Neufeld G, Augustin HG (2008) Neuropilin-1-VEGFR-2 complexing requires the PDZ-binding domain of neuropilin-1. J Biol Chem 283:25110–25114. https://doi.org/10.1074/jbc.C800137200

183. Zhang G, Chen L, Sun K, Khan AA, Yan J, Liu H, Lu A, Gu N (2016) Neuropilin-1 (NRP-1)/GIPC1 pathway mediates glioma progression. Tumour Biol 37:13777–13788. https://doi.org/10.1007/s13277-016-5138-3
184. Pang HB, Braun GB, Friman T, Aza-Blanc P, Ruidiaz ME, Sugahara KN, Teesalu T, Ruoslahti E (2014) An endocytosis pathway initiated through neuropilin-1 and regulated by nutrient availability. Nat Commun 5:4904. https://doi.org/10.1038/ncomms5904
185. Sugahara KN, Teesalu T, Karmali PP, Kotamraju VR, Agemy L, Greenwald DR, Ruoslahti E (2010) Coadministration of a tumor-penetrating peptide enhances the efficacy of cancer drugs. Science 328:1031–1035. https://doi.org/10.1126/science.1183057
186. Horowitz A, Seerapu HR (2012) Regulation of VEGF signaling by membrane traffic. Cell Signal 24:1810–1820. https://doi.org/10.1016/j.cellsig.2012.05.007
187. Lanahan AA, Hermans K, Claes F, Kerley-Hamilton JS, Zhuang ZW, Giordano FJ, Carmeliet P, Simons M (2010) VEGF receptor 2 endocytic trafficking regulates arterial morphogenesis. Dev Cell 18:713–724. https://doi.org/10.1016/j.devcel.2010.02.016
188. Lanahan A, Zhang X, Fantin A, Zhuang Z, Rivera-Molina F, Speichinger K, Prahst C, Zhang J, Wang Y, Davis G et al (2013) The neuropilin 1 cytoplasmic domain is required for VEGF-A-dependent arteriogenesis. Dev Cell 25:156–168. https://doi.org/10.1016/j.devcel.2013.03.019
189. Kawamura H, Li X, Goishi K, van Meeteren LA, Jakobsson L, Cebe-Suarez S, Shimizu A, Edholm D, Ballmer-Hofer K, Kjellen L et al (2008) Neuropilin-1 in regulation of VEGF-induced activation of p38MAPK and endothelial cell organization. Blood 112:3638–3649. https://doi.org/10.1182/blood-2007-12-125856
190. D'Haene N, Sauvage S, Maris C, Adanja I, Le Mercier M, Decaestecker C, Baum L, Salmon I (2013) VEGFR1 and VEGFR2 involvement in extracellular galectin-1- and galectin-3-induced angiogenesis. PLoS One 8:e67029. https://doi.org/10.1371/journal.pone.0067029
191. Wang L, Zhao Y, Wang Y, Wu X (2018) The role of Galectins in cervical cancer biology and progression. Biomed Res Int 2018:2175927. https://doi.org/10.1155/2018/2175927
192. Hsieh SH, Ying NW, Wu MH, Chiang WF, Hsu CL, Wong TY, Jin YT, Hong TM, Chen YL (2008) Galectin-1, a novel ligand of neuropilin-1, activates VEGFR-2 signaling and modulates the migration of vascular endothelial cells. Oncogene 27:3746–3753. https://doi.org/10.1038/sj.onc.1211029
193. Cao YEG, Wang E, Pal K, Dutta SK, Bar-Sagi D, Mukhopadhyay D (2012) VEGF exerts an angiogenesis-independent function in cancer cells to promote their malignant progression. Cancer Res 72:3912–3918. https://doi.org/10.1158/0008-5472.CAN-11-4058
194. Chen H, Bagri A, Zupicich JA, Zou Y, Stoeckli E, Pleasure SJ, Lowenstein DH, Skarnes WC, Chedotal A, Tessier-Lavigne M (2000) Neuropilin-2 regulates the development of selective cranial and sensory nerves and hippocampal mossy fiber projections. Neuron 25:43–56
195. Horiguchi K, Shirakihara T, Nakano A, Imamura T, Miyazono K, Saitoh M (2009) Role of Ras signaling in the induction of Snail by transforming growth factor-beta. J Biol Chem 284:245–253. https://doi.org/10.1074/jbc.M804777200
196. Cao Y, Wang L, Nandy D, Zhang Y, Basu A, Radisky D, Mukhopadhyay D (2008) Neuropilin-1 upholds dedifferentiation and propagation phenotypes of renal cell carcinoma cells by activating Akt and Sonic hedgehog axes. Cancer Res 68:8667–8672. https://doi.org/10.1158/0008-5472.CAN-08-2614
197. Hillman RT, Feng BY, Ni J, Woo WM, Milenkovic L, Hayden Gephart MG, Teruel MN, Oro AE, Chen JK, Scott MP (2011) Neuropilins are positive regulators of Hedgehog signal transduction. Genes Dev 25:2333–2346. https://doi.org/10.1101/gad.173054.111
198. Mercurio AM (2019) VEGF/neuropilin signaling in cancer stem cells. Int J Mol Sci 20. https://doi.org/10.3390/ijms20030490
199. Ge X, Milenkovic L, Suyama K, Hartl T, Purzner T, Winans A, Meyer T, Scott MP (2015) Phosphodiesterase 4D acts downstream of neuropilin to control Hedgehog signal transduction and the growth of medulloblastoma. Elife 4. https://doi.org/10.7554/eLife.07068
200. Po A, Silvano M, Miele E, Capalbo C, Eramo A, Salvati V, Todaro M, Besharat ZM, Catanzaro G, Cucchi D et al (2017) Noncanonical GLI1 signaling promotes stemness features and in vivo growth in lung adenocarcinoma. Oncogene 36:4641–4652. https://doi.org/10.1038/onc.2017.91
201. Snuderl M, Batista A, Kirkpatrick ND, Ruiz de Almodovar C, Riedemann L, Walsh EC, Anolik R, Huang Y, Martin JD, Kamoun W et al (2013) Targeting placental growth factor/neuropilin 1 pathway inhibits growth and spread of medulloblastoma. Cell 152:1065–1076. https://doi.org/10.1016/j.cell.2013.01.036
202. Yogi K, Sridhar E, Goel N, Jalali R, Goel A, Moiyadi A, Thorat R, Panwalkar P, Khire A, Dasgupta A et al (2015) MiR-148a, a microRNA upregulated in the WNT subgroup tumors, inhibits invasion and tumorigenic potential of medulloblastoma cells by targeting neuropilin 1. Oncoscience 2:334–348. https://doi.org/10.18632/oncoscience.137
203. Kim D, Lee V, Dorsey TB, Niklason LE, Gui L, Dai G (2018) Neuropilin-1 mediated arterial differentiation of murine pluripotent stem cells. Stem Cells Dev 27:441–455. https://doi.org/10.1089/scd.2017.0240
204. Papadopoulou K, Murray S, Manousou K, Tikas I, Dervenis C, Sgouros J, Rontogianni D, Lakis S,

Bobos M, Poulios C et al (2018) Genotyping and mRNA profiling reveal actionable molecular targets in biliary tract cancers. Am J Cancer Res 8:2–15
205. Takakura N (2012) Formation and regulation of the cancer stem cell niche. Cancer Sci 103:1177–1181. https://doi.org/10.1111/j.1349-7006.2012.02270.x
206. Rizzolio S, Tamagnone L (2011) Multifaceted role of neuropilins in cancer. Curr Med Chem 18:3563–3575
207. Samuel S, Gaur P, Fan F, Xia L, Gray MJ, Dallas NA, Bose D, Rodriguez-Aguayo C, Lopez-Berestein G, Plowman G et al (2011) Neuropilin-2 mediated beta-catenin signaling and survival in human gastrointestinal cancer cell lines. PLoS One 6:e23208. https://doi.org/10.1371/journal.pone.0023208
208. Grandclement C, Pallandre JR, Valmary Degano S, Viel E, Bouard A, Balland J, Remy-Martin JP, Simon B, Rouleau A, Boireau W et al (2011) Neuropilin-2 expression promotes TGF-beta1-mediated epithelial to mesenchymal transition in colorectal cancer cells. PLoS One 6:e20444. https://doi.org/10.1371/journal.pone.0020444
209. Ji T, Guo Y, Kim K, McQueen P, Ghaffar S, Christ A, Lin C, Eskander R, Zi X, Hoang BH (2015) Neuropilin-2 expression is inhibited by secreted Wnt antagonists and its down-regulation is associated with reduced tumor growth and metastasis in osteosarcoma. Mol Cancer 14:86. https://doi.org/10.1186/s12943-015-0359-4
210. Parker MW, Linkugel AD, Goel HL, Wu T, Mercurio AM, Vander Kooi CW (2015) Structural basis for VEGF-C binding to neuropilin-2 and sequestration by a soluble splice form. Structure 23:677–687. https://doi.org/10.1016/j.str.2015.01.018
211. Bartel DP (2018) Metazoan MicroRNAs. Cell 173:20–51. https://doi.org/10.1016/j.cell.2018.03.006
212. Prud'homme GJ, Glinka Y, Lichner Z, Yousef GM (2016) Neuropilin-1 is a receptor for extracellular miRNA and AGO2/miRNA complexes and mediates the internalization of miRNAs that modulate cell function. Oncotarget 7:68057–68071. https://doi.org/10.18632/oncotarget.10929
213. Li Y, Egranov SD, Yang L, Lin C (2019) Molecular mechanisms of long noncoding RNAs-mediated cancer metastasis. Genes Chromosomes Cancer 58:200–207. https://doi.org/10.1002/gcc.22691
214. Chen ZP, Wei JC, Wang Q, Yang P, Li WL, He F, Chen HC, Hu H, Zhong JB, Cao J (2018) Long noncoding RNA 00152 functions as a competing endogenous RNA to regulate NRP1 expression by sponging with miRNA206 in colorectal cancer. Int J Oncol 53:1227–1236. https://doi.org/10.3892/ijo.2018.4451
215. Zhou J, Zhang M, Huang Y, Feng L, Chen H, Hu Y, Chen H, Zhang K, Zheng L, Zheng S (2015) MicroRNA-320b promotes colorectal cancer proliferation and invasion by competing with its homologous microRNA-320a. Cancer Lett 356:669–675. https://doi.org/10.1016/j.canlet.2014.10.014
216. Peng Y, Liu YM, Li LC, Wang LL, Wu XL (2014) MicroRNA-338 inhibits growth, invasion and metastasis of gastric cancer by targeting NRP1 expression. PLoS One 9:e94422. https://doi.org/10.1371/journal.pone.0094422
217. Liu C, Wang Z, Wang Y, Gu W (2015) MiR-338 suppresses the growth and metastasis of OSCC cells by targeting NRP1. Mol Cell Biochem 398:115–122. https://doi.org/10.1007/s11010-014-2211-3
218. Ding Z, Zhu J, Zeng Y, Du W, Zhang Y, Tang H, Zheng Y, Qin H, Liu Z, Huang JA (2019) The regulation of neuropilin 1 expression by miR-338-3p promotes non-small cell lung cancer via changes in EGFR signaling. Mol Carcinog 58:1019–1032. https://doi.org/10.1002/mc.22990
219. Zhang YJ, Liu XC, Du J, Zhang YJ (2015) MiR-152 regulates metastases of non-small cell lung cancer cells by targeting neuropilin-1. Int J Clin Exp Pathol 8:14235–14240
220. Zhu H, Jiang X, Zhou X, Dong X, Xie K, Yang C, Jiang H, Sun X, Lu J (2018) Neuropilin-1 regulated by miR-320 contributes to the growth and metastasis of cholangiocarcinoma cells. Liver Int 38:125–135. https://doi.org/10.1111/liv.13495
221. Li H, Zhao J, Liu B, Luo J, Li Z, Qin X, Wei Y (2019) MicroRNA-320 targeting neuropilin 1 inhibits proliferation and migration of vascular smooth muscle cells and neointimal formation. Int J Med Sci 16:106–114. https://doi.org/10.7150/ijms.28093
222. Taddei ML, Cavallini L, Ramazzotti M, Comito G, Pietrovito L, Morandi A, Giannoni E, Raugei G, Chiarugi P (2019) Stromal-induced downregulation of miR-1247 promotes prostate cancer malignancy. J Cell Physiol 234:8274–8285. https://doi.org/10.1002/jcp.27679
223. Zhang L, Chen Y, Wang H, Zheng X, Li C, Han Z (2018) miR-376a inhibits breast cancer cell progression by targeting neuropilin-1 NR. Onco Targets Ther 11:5293–5302. https://doi.org/10.2147/OTT.S173416
224. Zhang L, Chen Y, Li C, Liu J, Ren H, Li L, Zheng X, Wang H, Han Z (2019) RNA binding protein PUM2 promotes the stemness of breast cancer cells via competitively binding to neuropilin-1 (NRP-1) mRNA with miR-376a. Biomed Pharmacother 114:108772. https://doi.org/10.1016/j.biopha.2019.108772
225. Zhang G, Chen L, Khan AA, Li B, Gu B, Lin F, Su X, Yan J (2018) miRNA-124-3p/neuropilin-1(NRP-1) axis plays an important role in mediating glioblastoma growth and angiogenesis. Int J Cancer 143:635–644. https://doi.org/10.1002/ijc.31329
226. Epis MR, Giles KM, Candy PA, Webster RJ, Leedman PJ (2014) miR-331-3p regulates expression of neuropilin-2 in glioblastoma. J Neuro-Oncol 116:67–75. https://doi.org/10.1007/s11060-013-1271-7
227. Zheng X, Chopp M, Lu Y, Buller B, Jiang F (2013) MiR-15b and miR-152 reduce glioma cell invasion and angiogenesis via NRP-2 and MMP-3.

Cancer Lett 329:146–154. https://doi.org/10.1016/j.canlet.2012.10.026
228. Liu C, Li M, Hu Y, Shi N, Yu H, Liu H, Lian H (2016) miR-486-5p attenuates tumor growth and lymphangiogenesis by targeting neuropilin-2 in colorectal carcinoma. Onco Targets Ther 9:2865–2871. https://doi.org/10.2147/OTT.S103460
229. Pagani E, Ruffini F, Antonini Cappellini GC, Scoppola A, Fortes C, Marchetti P, Graziani G, D'Atri S, Lacal PM (2016) Placenta growth factor and neuropilin-1 collaborate in promoting melanoma aggressiveness. Int J Oncol 48:1581–1589. https://doi.org/10.3892/ijo.2016.3362
230. Pellet-Many C, Frankel P, Jia H, Zachary I (2008) Neuropilins: structure, function and role in disease. Biochem J 411:211–226. https://doi.org/10.1042/BJ20071639
231. Janssen BJ, Malinauskas T, Weir GA, Cader MZ, Siebold C, Jones EY (2012) Neuropilins lock secreted semaphorins onto plexins in a ternary signaling complex. Nat Struct Mol Biol 19:1293–1299. https://doi.org/10.1038/nsmb.2416
232. Palodetto B, da Silva Santos Duarte A, Rodrigues Lopes M, Adolfo Corrocher F, Marconi Roversi F, Soares Niemann F, Priscila Vieira Ferro K, Leda Figueiredo Longhini A, Melo Campos P, Favaro P et al (2017) SEMA3A partially reverses VEGF effects through binding to neuropilin-1. Stem Cell Res 22:70–78. https://doi.org/10.1016/j.scr.2017.05.012
233. Chu W, Song X, Yang X, Ma L, Zhu J, He M, Wang Z, Wu Y (2014) Neuropilin-1 promotes epithelial-to-mesenchymal transition by stimulating nuclear factor-kappa B and is associated with poor prognosis in human oral squamous cell carcinoma. PLoS One 9:e101931. https://doi.org/10.1371/journal.pone.0101931
234. Nasarre P, Gemmill RM, Potiron VA, Roche J, Lu X, Baron AE, Korch C, Garrett-Mayer E, Lagana A, Howe PH et al (2013) Neuropilin-2 is upregulated in lung cancer cells during TGF-beta1-induced epithelial-mesenchymal transition. Cancer Res 73:7111–7121. https://doi.org/10.1158/0008-5472.CAN-13-1755
235. Gemmill RM, Nasarre P, Nair-Menon J, Cappuzzo F, Landi L, D'Incecco A, Uramoto H, Yoshida T, Haura EB, Armeson K et al (2017) The neuropilin 2 isoform NRP2b uniquely supports TGFbeta-mediated progression in lung cancer. Sci Signal 10. https://doi.org/10.1126/scisignal.aag0528
236. Hirata E, Sahai E (2017) Tumor microenvironment and differential responses to therapy. Cold Spring Harb Perspect Med 7. https://doi.org/10.1101/cshperspect.a026781
237. Cavaco A, Rezaei M, Niland S, Eble JA (2017) Collateral damage intended—cancer-associated fibroblasts and vasculature are potential targets in cancer therapy. Int J Mol Sci 18. https://doi.org/10.3390/ijms18112355
238. von Ahrens D, Bhagat TD, Nagrath D, Maitra A, Verma A (2017) The role of stromal cancer-associated fibroblasts in pancreatic cancer. J Hematol Oncol 10:76. https://doi.org/10.1186/s13045-017-0448-5
239. Kalluri R (2016) The biology and function of fibroblasts in cancer. Nat Rev Cancer 16:582–598. https://doi.org/10.1038/nrc.2016.73
240. Gajewski TF, Schreiber H, Fu YX (2013) Innate and adaptive immune cells in the tumor microenvironment. Nat Immunol 14:1014–1022. https://doi.org/10.1038/ni.2703
241. Otranto M, Sarrazy V, Bonte F, Hinz B, Gabbiani G, Desmouliere A (2012) The role of the myofibroblast in tumor stroma remodeling. Cell Adhes Migr 6:203–219. https://doi.org/10.4161/cam.20377
242. Mueller MM, Fusenig NE (2004) Friends or foes—bipolar effects of the tumour stroma in cancer. Nat Rev Cancer 4:839–849. https://doi.org/10.1038/nrc1477
243. Polanska UM, Orimo A (2013) Carcinoma-associated fibroblasts: non-neoplastic tumour-promoting mesenchymal cells. J Cell Physiol 228:1651–1657. https://doi.org/10.1002/jcp.24347
244. Erdogan B, Webb DJ (2017) Cancer-associated fibroblasts modulate growth factor signaling and extracellular matrix remodeling to regulate tumor metastasis. Biochem Soc Trans 45:229–236. https://doi.org/10.1042/BST20160387
245. Kuzet SE, Gaggioli C (2016) Fibroblast activation in cancer: when seed fertilizes soil. Cell Tissue Res 365:607–619. https://doi.org/10.1007/s00441-016-2467-x
246. Paszek MJ, Zahir N, Johnson KR, Lakins JN, Rozenberg GI, Gefen A, Reinhart-King CA, Margulies SS, Dembo M, Boettiger D et al (2005) Tensional homeostasis and the malignant phenotype. Cancer Cell 8:241–254. https://doi.org/10.1016/j.ccr.2005.08.010
247. Ramamonjisoa N, Ackerstaff E (2017) Characterization of the tumor microenvironment and tumor-stroma interaction by non-invasive preclinical imaging. Front Oncol 7:3. https://doi.org/10.3389/fonc.2017.00003
248. Hinz B, Phan SH, Thannickal VJ, Prunotto M, Desmouliere A, Varga J, De Wever O, Mareel M, Gabbiani G (2012) Recent developments in myofibroblast biology: paradigms for connective tissue remodeling. Am J Pathol 180:1340–1355. https://doi.org/10.1016/j.ajpath.2012.02.004
249. Dvorak HF (1986) Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. N Engl J Med 315:1650–1659. https://doi.org/10.1056/NEJM198612253152606
250. Yu Y, Xiao CH, Tan LD, Wang QS, Li XQ, Feng YM (2014) Cancer-associated fibroblasts induce epithelial-mesenchymal transition of breast cancer cells through paracrine TGF-beta signalling. Br J Cancer 110:724–732. https://doi.org/10.1038/bjc.2013.768
251. Khan Z, Marshall JF (2016) The role of integrins in TGFbeta activation in the tumour stroma. Cell

Tissue Res 365:657–673. https://doi.org/10.1007/s00441-016-2474-y
252. Mohammadi H, Sahai E (2018) Mechanisms and impact of altered tumour mechanics. Nat Cell Biol 20:766–774. https://doi.org/10.1038/s41556-018-0131-2
253. Xing Y, Zhao S, Zhou BP, Mi J (2015) Metabolic reprogramming of the tumour microenvironment. FEBS J 282:3892–3898. https://doi.org/10.1111/febs.13402
254. Marchiq I, Pouyssegur J (2016) Hypoxia, cancer metabolism and the therapeutic benefit of targeting lactate/H(+) symporters. J Mol Med (Berl) 94:155–171. https://doi.org/10.1007/s00109-015-1307-x
255. Gaggioli C, Hooper S, Hidalgo-Carcedo C, Grosse R, Marshall JF, Harrington K, Sahai E (2007) Fibroblast-led collective invasion of carcinoma cells with differing roles for RhoGTPases in leading and following cells. Nat Cell Biol 9:1392–1400. https://doi.org/10.1038/ncb1658
256. Adams RH, Alitalo K (2007) Molecular regulation of angiogenesis and lymphangiogenesis. Nat Rev Mol Cell Biol 8:464–478. https://doi.org/10.1038/nrm2183
257. Delgado-Bellido D, Serrano-Saenz S, Fernandez-Cortes M, Oliver FJ (2017) Vasculogenic mimicry signaling revisited: focus on non-vascular VE-cadherin. Mol Cancer 16:65. https://doi.org/10.1186/s12943-017-0631-x
258. Dong J, Zhao Y, Huang Q, Fei X, Diao Y, Shen Y, Xiao H, Zhang T, Lan Q, Gu X (2011) Glioma stem/progenitor cells contribute to neovascularization via transdifferentiation. Stem Cell Rev 7:141–152. https://doi.org/10.1007/s12015-010-9169-7
259. Xu Y, Li Q, Li XY, Yang QY, Xu WW, Liu GL (2012) Short-term anti-vascular endothelial growth factor treatment elicits vasculogenic mimicry formation of tumors to accelerate metastasis. J Exp Clin Cancer Res 31:16. https://doi.org/10.1186/1756-9966-31-16
260. Ruffini F, D'Atri S, Lacal PM (2013) Neuropilin-1 expression promotes invasiveness of melanoma cells through vascular endothelial growth factor receptor-2-dependent and -independent mechanisms. Int J Oncol 43:297–306. https://doi.org/10.3892/ijo.2013.1948
261. Fantin A, Vieira JM, Plein A, Denti L, Fruttiger M, Pollard JW, Ruhrberg C (2013) NRP1 acts cell autonomously in endothelium to promote tip cell function during sprouting angiogenesis. Blood 121:2352–2362. https://doi.org/10.1182/blood-2012-05-424713
262. Larrivee B, Prahst C, Gordon E, del Toro R, Mathivet T, Duarte A, Simons M, Eichmann A (2012) ALK1 signaling inhibits angiogenesis by cooperating with the Notch pathway. Dev Cell 22:489–500. https://doi.org/10.1016/j.devcel.2012.02.005
263. Guttmann-Raviv N, Kessler O, Shraga-Heled N, Lange T, Herzog Y, Neufeld G (2006) The neuropilins and their role in tumorigenesis and tumor progression. Cancer Lett 231:1–11. https://doi.org/10.1016/j.canlet.2004.12.047
264. Migliozzi MT, Mucka P, Bielenberg DR (2014) Lymphangiogenesis and metastasis--a closer look at the neuropilin/semaphorin3 axis. Microvasc Res 96:68–76. https://doi.org/10.1016/j.mvr.2014.07.006
265. Jurisic G, Maby-El Hajjami H, Karaman S, Ochsenbein AM, Alitalo A, Siddiqui SS, Ochoa Pereira C, Petrova TV, Detmar M (2012) An unexpected role of semaphorin3a-neuropilin-1 signaling in lymphatic vessel maturation and valve formation. Circ Res 111:426–436. https://doi.org/10.1161/CIRCRESAHA.112.269399
266. Serini G, Tamagnone L (2015) Bad vessels beware! Semaphorins will sort you out! EMBO Mol Med 7:1251–1253. https://doi.org/10.15252/emmm.201505551
267. Mumblat Y, Kessler O, Ilan N, Neufeld G (2015) Full-length semaphorin-3C is an inhibitor of tumor lymphangiogenesis and metastasis. Cancer Res 75:2177–2186. https://doi.org/10.1158/0008-5472.CAN-14-2464
268. Miyato H, Tsuno NH, Kitayama J (2012) Semaphorin 3C is involved in the progression of gastric cancer. Cancer Sci 103:1961–1966. https://doi.org/10.1111/cas.12003
269. Herman JG, Meadows GG (2007) Increased class 3 semaphorin expression modulates the invasive and adhesive properties of prostate cancer cells. Int J Oncol 30:1231–1238
270. Man J, Shoemake J, Zhou W, Fang X, Wu Q, Rizzo A, Prayson R, Bao S, Rich JN, Yu JS (2014) Sema3C promotes the survival and tumorigenicity of glioma stem cells through Rac1 activation. Cell Rep 9:1812–1826. https://doi.org/10.1016/j.celrep.2014.10.055
271. Bassi DE, Fu J, Lopez de Cicco R, Klein-Szanto AJ (2005) Proprotein convertases: "master switches" in the regulation of tumor growth and progression. Mol Carcinog 44:151–161. https://doi.org/10.1002/mc.20134
272. Riabov V, Gudima A, Wang N, Mickley A, Orekhov A, Kzhyshkowska J (2014) Role of tumor associated macrophages in tumor angiogenesis and lymphangiogenesis. Front Physiol 5:75. https://doi.org/10.3389/fphys.2014.00075
273. Esselens C, Malapeira J, Colome N, Casal C, Rodriguez-Manzaneque JC, Canals F, Arribas J (2010) The cleavage of semaphorin 3C induced by ADAMTS1 promotes cell migration. J Biol Chem 285:2463–2473. https://doi.org/10.1074/jbc.M109.055129
274. Jain RK (2005) Normalization of tumor vasculature: an emerging concept in antiangiogenic therapy. Science 307:58–62. https://doi.org/10.1126/science.1104819
275. Jain RK (2014) Antiangiogenesis strategies revisited: from starving tumors to alleviating hypoxia. Cancer Cell 26:605–622. https://doi.org/10.1016/j.ccell.2014.10.006

276. Maione F, Capano S, Regano D, Zentilin L, Giacca M, Casanovas O, Bussolino F, Serini G, Giraudo E (2012) Semaphorin 3A overcomes cancer hypoxia and metastatic dissemination induced by antiangiogenic treatment in mice. J Clin Invest 122:1832–1848. https://doi.org/10.1172/JCI58976
277. Acevedo LM, Barillas S, Weis SM, Gothert JR, Cheresh DA (2008) Semaphorin 3A suppresses VEGF-mediated angiogenesis yet acts as a vascular permeability factor. Blood 111:2674–2680. https://doi.org/10.1182/blood-2007-08-110205
278. Cerani A, Tetreault N, Menard C, Lapalme E, Patel C, Sitaras N, Beaudoin F, Leboeuf D, De Guire V, Binet F et al (2013) Neuron-derived semaphorin 3A is an early inducer of vascular permeability in diabetic retinopathy via neuropilin-1. Cell Metab 18:505–518. https://doi.org/10.1016/j.cmet.2013.09.003
279. Gioelli N, Maione F, Camillo C, Ghitti M, Valdembri D, Morello N, Darche M, Zentilin L, Cagnoni G, Qiu Y et al (2018) A rationally designed NRP1-independent superagonist SEMA3A mutant is an effective anticancer agent. Sci Transl Med 10. https://doi.org/10.1126/scitranslmed.aah4807
280. Albini A, Tosetti F, Li VW, Noonan DM, Li WW (2012) Cancer prevention by targeting angiogenesis. Nat Rev Clin Oncol 9:498–509. https://doi.org/10.1038/nrclinonc.2012.120
281. Franzolin G, Tamagnone L (2019) Semaphorin signaling in cancer-associated inflammation. Int J Mol Sci 20. https://doi.org/10.3390/ijms20020377
282. Schellenburg S, Schulz A, Poitz DM, Muders MH (2017) Role of neuropilin-2 in the immune system. Mol Immunol 90:239–244. https://doi.org/10.1016/j.molimm.2017.08.010
283. Roy S, Bag AK, Dutta S, Polavaram NS, Islam R, Schellenburg S, Banwait J, Guda C, Ran S, Hollingsworth MA et al (2018) Macrophage-derived neuropilin-2 exhibits novel tumor-promoting functions. Cancer Res 78:5600–5617. https://doi.org/10.1158/0008-5472.CAN-18-0562
284. Chen XJ, Wu S, Yan RM, Fan LS, Yu L, Zhang YM, Wei WF, Zhou CF, Wu XG, Zhong M et al (2019) The role of the hypoxia-Nrp-1 axis in the activation of M2-like tumor-associated macrophages in the tumor microenvironment of cervical cancer. Mol Carcinog 58:388–397. https://doi.org/10.1002/mc.22936
285. Dejda A, Mawambo G, Daudelin JF, Miloudi K, Akla N, Patel C, Andriessen EM, Labrecque N, Sennlaub F, Sapieha P (2016) Neuropilin-1-expressing microglia are associated with nascent retinal vasculature yet dispensable for developmental angiogenesis. Invest Ophthalmol Vis Sci 57:1530–1536. https://doi.org/10.1167/iovs.15-18598
286. Karkkainen MJ, Alitalo K (2002) Lymphatic endothelial regulation, lymphoedema, and lymph node metastasis. Semin Cell Dev Biol 13:9–18. https://doi.org/10.1006/scdb.2001.0286
287. Karpanen T, Heckman CA, Keskitalo S, Jeltsch M, Ollila H, Neufeld G, Tamagnone L, Alitalo K (2006) Functional interaction of VEGF-C and VEGF-D with neuropilin receptors. FASEB J 20:1462–1472. https://doi.org/10.1096/fj.05-5646com
288. Kawakami T, Tokunaga T, Hatanaka H, Kijima H, Yamazaki H, Abe Y, Osamura Y, Inoue H, Ueyama Y, Nakamura M (2002) Neuropilin 1 and neuropilin 2 co-expression is significantly correlated with increased vascularity and poor prognosis in nonsmall cell lung carcinoma. Cancer 95:2196–2201. https://doi.org/10.1002/cncr.10936
289. Shevach EM (2009) Mechanisms of foxp3+ T regulatory cell-mediated suppression. Immunity 30:636–645. https://doi.org/10.1016/j.immuni.2009.04.010
290. Zhao H, Liao X, Kang Y (2017) Tregs: where we are and what comes next? Front Immunol 8:1578. https://doi.org/10.3389/fimmu.2017.01578
291. Yu Y, Cui J (2018) Present and future of cancer immunotherapy: a tumor microenvironmental perspective. Oncol Lett 16:4105–4113. https://doi.org/10.3892/ol.2018.9219
292. Napolitano V, Tamagnone L (2019) Neuropilins controlling cancer therapy responsiveness. Int J Mol Sci 20. https://doi.org/10.3390/ijms20082049
293. Torti D, Trusolino L (2011) Oncogene addiction as a foundational rationale for targeted anticancer therapy: promises and perils. EMBO Mol Med 3:623–636. https://doi.org/10.1002/emmm.201100176
294. Fallahi-Sichani M, Becker V, Izar B, Baker GJ, Lin JR, Boswell SA, Shah P, Rotem A, Garraway LA, Sorger PK (2017) Adaptive resistance of melanoma cells to RAF inhibition via reversible induction of a slowly dividing de-differentiated state. Mol Syst Biol 13:905. https://doi.org/10.15252/msb.20166796
295. Wilson TR, Fridlyand J, Yan Y, Penuel E, Burton L, Chan E, Peng J, Lin E, Wang Y, Sosman J et al (2012) Widespread potential for growth-factor-driven resistance to anticancer kinase inhibitors. Nature 487:505–509. https://doi.org/10.1038/nature11249
296. Chaudhary B, Khaled YS, Ammori BJ, Elkord E (2014) Neuropilin 1: function and therapeutic potential in cancer. Cancer Immunol Immunother 63:81–99. https://doi.org/10.1007/s00262-013-1500-0
297. Graziani G, Lacal PM (2015) Neuropilin-1 as therapeutic target for malignant melanoma. Front Oncol 5:125. https://doi.org/10.3389/fonc.2015.00125
298. Kamiya T, Kawakami T, Abe Y, Nishi M, Onoda N, Miyazaki N, Oida Y, Yamazaki H, Ueyama Y, Nakamura M (2006) The preserved expression of neuropilin (NRP) 1 contributes to a better prognosis in colon cancer. Oncol Rep 15:369–373
299. Gray MJ, Wey JS, Belcheva A, McCarty MF, Trevino JG, Evans DB, Ellis LM, Gallick GE (2005) Neuropilin-1 suppresses tumorigenic properties in a human pancreatic adenocarcinoma cell line lacking neuropilin-1 coreceptors. Cancer Res 65:3664–3670. https://doi.org/10.1158/0008-5472.CAN-04-2229

300. Jamil MO, Hathaway A, Mehta A (2015) Tivozanib: status of development. Curr Oncol Rep 17:24. https://doi.org/10.1007/s11912-015-0451-3
301. Lambrechts D, Lenz HJ, de Haas S, Carmeliet P, Scherer SJ (2013) Markers of response for the antiangiogenic agent bevacizumab. J Clin Oncol 31:1219–1230. https://doi.org/10.1200/JCO.2012.46.2762
302. Baumgarten P, Blank AE, Franz K, Hattingen E, Dunst M, Zeiner P, Hoffmann K, Bahr O, Mader L, Goeppert B et al (2016) Differential expression of vascular endothelial growth factor A, its receptors VEGFR-1, -2, and -3 and co-receptors neuropilin-1 and -2 does not predict bevacizumab response in human astrocytomas. Neuro-Oncology 18:173–183. https://doi.org/10.1093/neuonc/nov288
303. Bais C, Mueller B, Brady MF, Mannel RS, Burger RA, Wei W, Marien KM, Kockx MM, Husain A, Birrer MJ et al (2017) Tumor microvessel density as a potential predictive marker for Bevacizumab benefit: GOG-0218 biomarker analyses. J Natl Cancer Inst 109. https://doi.org/10.1093/jnci/djx066
304. Schuch G, Machluf M, Bartsch G Jr, Nomi M, Richard H, Atala A, Soker S (2002) In vivo administration of vascular endothelial growth factor (VEGF) and its antagonist, soluble neuropilin-1, predicts a role of VEGF in the progression of acute myeloid leukemia in vivo. Blood 100:4622–4628. https://doi.org/10.1182/blood.V100.13.4622
305. Sugahara KN, Scodeller P, Braun GB, de Mendoza TH, Yamazaki CM, Kluger MD, Kitayama J, Alvarez E, Howell SB, Teesalu T et al (2015) A tumor-penetrating peptide enhances circulation-independent targeting of peritoneal carcinomatosis. J Control Release 212:59–69. https://doi.org/10.1016/j.jconrel.2015.06.009
306. Simon-Gracia L, Hunt H, Scodeller P, Gaitzsch J, Kotamraju VR, Sugahara KN, Tammik O, Ruoslahti E, Battaglia G, Teesalu T (2016) iRGD peptide conjugation potentiates intraperitoneal tumor delivery of paclitaxel with polymersomes. Biomaterials 104:247–257. https://doi.org/10.1016/j.biomaterials.2016.07.023
307. Zhang H, Tam S, Ingham ES, Mahakian LM, Lai CY, Tumbale SK, Teesalu T, Hubbard NE, Borowsky AD, Ferrara KW (2015) Ultrasound molecular imaging of tumor angiogenesis with a neuropilin-1-targeted microbubble. Biomaterials 56:104–113. https://doi.org/10.1016/j.biomaterials.2015.03.043
308. Bumbaca D, Xiang H, Boswell CA, Port RE, Stainton SL, Mundo EE, Ulufatu S, Bagri A, Theil FP, Fielder PJ et al (2012) Maximizing tumour exposure to anti-neuropilin-1 antibody requires saturation of non-tumour tissue antigenic sinks in mice. Br J Pharmacol 166:368–377. https://doi.org/10.1111/j.1476-5381.2011.01777.x
309. Feldman DR, Baum MS, Ginsberg MS, Hassoun H, Flombaum CD, Velasco S, Fischer P, Ronnen E, Ishill N, Patil S et al (2009) Phase I trial of bevacizumab plus escalated doses of sunitinib in patients with metastatic renal cell carcinoma. J Clin Oncol 27:1432–1439. https://doi.org/10.1200/JCO.2008.19.0108
310. Mahoney KM, Rennert PD, Freeman GJ (2015) Combination cancer immunotherapy and new immunomodulatory targets. Nat Rev Drug Discov 14:561–584. https://doi.org/10.1038/nrd4591
311. Weekes CD, Beeram M, Tolcher AW, Papadopoulos KP, Gore L, Hegde P, Xin Y, Yu R, Shih LM, Xiang H et al (2014) A phase I study of the human monoclonal anti-NRP1 antibody MNRP1685A in patients with advanced solid tumors. Investig New Drugs 32:653–660. https://doi.org/10.1007/s10637-014-0071-z
312. Patnaik A, LoRusso PM, Messersmith WA, Papadopoulos KP, Gore L, Beeram M, Ramakrishnan V, Kim AH, Beyer JC, Mason Shih L et al (2014) A phase Ib study evaluating MNRP1685A, a fully human anti-NRP1 monoclonal antibody, in combination with bevacizumab and paclitaxel in patients with advanced solid tumors. Cancer Chemother Pharmacol 73:951–960. https://doi.org/10.1007/s00280-014-2426-8
313. Leng Q, Woodle MC, Mixson AJ (2017) NRP1 transport of cancer therapeutics mediated by tumor-penetrating peptides. Drugs Future 42:95–104. https://doi.org/10.1358/dof.2017.042.02.2564106
314. Jarvis A, Allerston CK, Jia H, Herzog B, Garza-Garcia A, Winfield N, Ellard K, Aqil R, Lynch R, Chapman C et al (2010) Small molecule inhibitors of the neuropilin-1 vascular endothelial growth factor A (VEGF-A) interaction. J Med Chem 53:2215–2226. https://doi.org/10.1021/jm901755g
315. Binetruy-Tournaire R, Demangel C, Malavaud B, Vassy R, Rouyre S, Kraemer M, Plouet J, Derbin C, Perret G, Mazie JC (2000) Identification of a peptide blocking vascular endothelial growth factor (VEGF)-mediated angiogenesis. EMBO J 19:1525–1533. https://doi.org/10.1093/emboj/19.7.1525
316. Barr MP, Byrne AM, Duffy AM, Condron CM, Devocelle M, Harriott P, Bouchier-Hayes DJ, Harmey JH (2005) A peptide corresponding to the neuropilin-1-binding site on VEGF(165) induces apoptosis of neuropilin-1-expressing breast tumour cells. Br J Cancer 92:328–333. https://doi.org/10.1038/sj.bjc.6602308
317. Sidman RL, Li J, Lawrence M, Hu W, Musso GF, Giordano RJ, Cardo-Vila M, Pasqualini R, Arap W (2015) The peptidomimetic Vasotide targets two retinal VEGF receptors and reduces pathological angiogenesis in murine and nonhuman primate models of retinal disease. Sci Transl Med 7:309ra165. https://doi.org/10.1126/scitranslmed.aac4882
318. Hong TM, Chen YL, Wu YY, Yuan A, Chao YC, Chung YC, Wu MH, Yang SC, Pan SH, Shih JY et al (2007) Targeting neuropilin 1 as an antitumor strategy in lung cancer. Clin Cancer Res 13:4759–4768. https://doi.org/10.1158/1078-0432.CCR-07-0001
319. Jia H, Aqil R, Cheng L, Chapman C, Shaikh S, Jarvis A, Chan AW, Hartzoulakis B, Evans IM, Frolov A et al (2014) N-terminal modification of VEGF-A C

terminus-derived peptides delineates structural features involved in neuropilin-1 binding and functional activity. Chembiochem 15:1161–1170. https://doi.org/10.1002/cbic.201300658
320. Powell J, Mota F, Steadman D, Soudy C, Miyauchi JT, Crosby S, Jarvis A, Reisinger T, Winfield N, Evans G et al (2018) Small molecule neuropilin-1 antagonists combine antiangiogenic and antitumor activity with immune modulation through reduction of transforming growth factor beta (TGFbeta) production in regulatory T-cells. J Med Chem 61:4135–4154. https://doi.org/10.1021/acs.jmedchem.8b00210
321. Zanuy D, Kotla R, Nussinov R, Teesalu T, Sugahara KN, Aleman C, Haspel N (2013) Sequence dependence of C-end rule peptides in binding and activation of neuropilin-1 receptor. J Struct Biol 182:78–86. https://doi.org/10.1016/j.jsb.2013.02.006
322. Zhang L, Parry GC, Levin EG (2013) Inhibition of tumor cell migration by LD22-4, an N-terminal fragment of 24-kDa FGF2, is mediated by neuropilin 1. Cancer Res 73:3316–3325. https://doi.org/10.1158/0008-5472.CAN-12-3015
323. Ding L, Donate F, Parry GC, Guan X, Maher P, Levin EG (2002) Inhibition of cell migration and angiogenesis by the amino-terminal fragment of 24kD basic fibroblast growth factor. J Biol Chem 277:31056–31061. https://doi.org/10.1074/jbc.M203658200
324. Levin EG, Sikora L, Ding L, Rao SP, Sriramarao P (2004) Suppression of tumor growth and angiogenesis in vivo by a truncated form of 24-kd fibroblast growth factor (FGF)-2. Am J Pathol 164:1183–1190. https://doi.org/10.1016/S0002-9440(10)63206-3
325. Kim YJ, Bae J, Shin TH, Kang SH, Jeong M, Han Y, Park JH, Kim SK, Kim YS (2015) Immunoglobulin Fc-fused, neuropilin-1-specific peptide shows efficient tumor tissue penetration and inhibits tumor growth via anti-angiogenesis. J Control Release 216:56–68. https://doi.org/10.1016/j.jconrel.2015.08.016
326. Tymecka D, Puszko AK, Lipinski PFJ, Fedorczyk B, Wilenska B, Sura K, Perret GY, Misicka A (2018) Branched pentapeptides as potent inhibitors of the vascular endothelial growth factor 165 binding to neuropilin-1: design, synthesis and biological activity. Eur J Med Chem 158:453–462. https://doi.org/10.1016/j.ejmech.2018.08.083
327. Fedorczyk B, Lipinski PFJ, Puszko AK, Tymecka D, Wilenska B, Dudka W, Perret GY, Wieczorek R, Misicka A (2019) Triazolopeptides inhibiting the interaction between neuropilin-1 and vascular endothelial growth factor-165. Molecules 24. https://doi.org/10.3390/molecules24091756
328. Nasarre C, Roth M, Jacob L, Roth L, Koncina E, Thien A, Labourdette G, Poulet P, Hubert P, Cremel G et al (2010) Peptide-based interference of the transmembrane domain of neuropilin-1 inhibits glioma growth in vivo. Oncogene 29:2381–2392. https://doi.org/10.1038/onc.2010.9
329. Arpel A, Gamper C, Spenle C, Fernandez A, Jacob L, Baumlin N, Laquerriere P, Orend G, Cremel G, Bagnard D (2016) Inhibition of primary breast tumor growth and metastasis using a neuropilin-1 transmembrane domain interfering peptide. Oncotarget 7:54723–54732. https://doi.org/10.18632/oncotarget.10101
330. Simon-Gracia L, Hunt H, Teesalu T (2018) Peritoneal carcinomatosis targeting with tumor homing peptides. Molecules 23. https://doi.org/10.3390/molecules23051190
331. Wang HB, Zhang H, Zhang JP, Li Y, Zhao B, Feng GK, Du Y, Xiong D, Zhong Q, Liu WL et al (2015) Neuropilin 1 is an entry factor that promotes EBV infection of nasopharyngeal epithelial cells. Nat Commun 6:6240. https://doi.org/10.1038/ncomms7240
332. Fogal V, Zhang L, Krajewski S, Ruoslahti E (2008) Mitochondrial/cell-surface protein p32/gC1qR as a molecular target in tumor cells and tumor stroma. Cancer Res 68:7210–7218. https://doi.org/10.1158/0008-5472.CAN-07-6752
333. Paasonen L, Sharma S, Braun GB, Kotamraju VR, Chung TD, She ZG, Sugahara KN, Yliperttula M, Wu B, Pellecchia M et al (2016) New p32/gC1qR ligands for targeted tumor drug delivery. Chembiochem 17:570–575. https://doi.org/10.1002/cbic.201500564
334. Sharma S, Kotamraju VR, Molder T, Tobi A, Teesalu T, Ruoslahti E (2017) Tumor-penetrating nanosystem strongly suppresses breast tumor growth. Nano Lett 17:1356–1364. https://doi.org/10.1021/acs.nanolett.6b03815
335. Hunt H, Simon-Gracia L, Tobi A, Kotamraju VR, Sharma S, Nigul M, Sugahara KN, Ruoslahti E, Teesalu T (2017) Targeting of p32 in peritoneal carcinomatosis with intraperitoneal linTT1 peptide-guided pro-apoptotic nanoparticles. J Control Release 260:142–153. https://doi.org/10.1016/j.jconrel.2017.06.005
336. Liu X, Lin P, Perrett I, Lin J, Liao YP, Chang CH, Jiang J, Wu N, Donahue T, Wainberg Z et al (2017) Tumor-penetrating peptide enhances transcytosis of silicasome-based chemotherapy for pancreatic cancer. J Clin Invest 127:2007–2018. https://doi.org/10.1172/JCI92284
337. Ruoslahti E (2017) Tumor penetrating peptides for improved drug delivery. Adv Drug Deliv Rev 110-111:3–12. https://doi.org/10.1016/j.addr.2016.03.008
338. Thoreau F, Vanwonterghem L, Henry M, Coll JL, Boturyn D (2018) Design of RGD-ATWLPPR peptide conjugates for the dual targeting of alphaV-beta3 integrin and neuropilin-1. Org Biomol Chem 16:4101–4107. https://doi.org/10.1039/c8ob00669e
339. CEND-1 in combination with nanoparticle albumin bound-paclitaxel (Abraxane) and gemcitabine in metastatic pancreatic cancer, 27 May 2018 [cited 5 Dec 2018], 1 p. ClinicalTrials.gov [Internet]. Bethesda, MD: National Library of Medicine (US), 31 Jul 2018. Identifier: NCT03517176. Available from: https://www.clini-

caltrials.gov/ct2/show/NCT03517176?term=Cend-1&cond=cancer&rank=1

340. Liang DS, Zhang WJ, Wang AT, Su HT, Zhong HJ, Qi XR (2017) Treating metastatic triple negative breast cancer with CD44/neuropilin dual molecular targets of multifunctional nanoparticles. Biomaterials 137:23–36. https://doi.org/10.1016/j.biomaterials.2017.05.022
341. Chen L, Zhang G, Shi Y, Qiu R, Khan AA (2015) Neuropilin-1 (NRP-1) and magnetic nanoparticles, a potential combination for diagnosis and therapy of gliomas. Curr Pharm Des 21:5434–5449
342. Xiang Z, Jiang G, Yang X, Fan D, Nan X, Li D, Hu Z, Fang Q (2019) Peptosome coadministration improves nanoparticle delivery to tumors through NRP1-mediated co-endocytosis. Biomol Ther 9. https://doi.org/10.3390/biom9050172
343. Thomas E, Colombeau L, Gries M, Peterlini T, Mathieu C, Thomas N, Boura C, Frochot C, Vanderesse R, Lux F et al (2017) Ultrasmall AGuIX theranostic nanoparticles for vascular-targeted interstitial photodynamic therapy of glioblastoma. Int J Nanomedicine 12:7075–7088. https://doi.org/10.2147/IJN.S141559
344. Roth L, Agemy L, Kotamraju VR, Braun G, Teesalu T, Sugahara KN, Hamzah J, Ruoslahti E (2012) Transtumoral targeting enabled by a novel neuropilin-binding peptide. Oncogene 31:3754–3763. https://doi.org/10.1038/onc.2011.537
345. Sugahara KN, Braun GB, de Mendoza TH, Kotamraju VR, French RP, Lowy AM, Teesalu T, Ruoslahti E (2015) Tumor-penetrating iRGD peptide inhibits metastasis. Mol Cancer Ther 14:120–128. https://doi.org/10.1158/1535-7163.MCT-14-0366
346. Hamilton AM, Aidoudi-Ahmed S, Sharma S, Kotamraju VR, Foster PJ, Sugahara KN, Ruoslahti E, Rutt BK (2015) Nanoparticles coated with the tumor-penetrating peptide iRGD reduce experimental breast cancer metastasis in the brain. J Mol Med (Berl) 93:991–1001. https://doi.org/10.1007/s00109-015-1279-x
347. Zakraoui O, Marcinkiewicz C, Aloui Z, Othman H, Grepin R, Haoues M, Essafi M, Srairi-Abid N, Gasmi A, Karoui H et al (2017) Lebein, a snake venom disintegrin, suppresses human colon cancer cells proliferation and tumor-induced angiogenesis through cell cycle arrest, apoptosis induction and inhibition of VEGF expression. Mol Carcinog 56:18–35. https://doi.org/10.1002/mc.22470

4 Translational Landscape of mTOR Signaling in Integrating Cues Between Cancer and Tumor Microenvironment

Chiara Bazzichetto, Fabiana Conciatori, Italia Falcone, and Ludovica Ciuffreda

Abstract

The mammalian target of rapamycin (mTOR) represents a critical hub for the regulation of different processes in both normal and tumor cells. Furthermore, it is now well established the role of mTOR in integrating and shaping different environmental paracrine and autocrine stimuli in tumor microenvironment (TME) constituents. Recently, further efforts have been employed to understand how the mTOR signal transduction mechanisms modulate the sensitivity and resistance to targeted therapies, also for its involvement of mTOR also in modulating angiogenesis and tumor immunity. Indeed, interest in mTOR targeting was increased to improve immune response against cancer and to develop new long-term efficacy strategies, as demonstrated by clinical success of mTOR and immune checkpoint inhibitor combinations. In this chapter, we will describe the role of mTOR in modulating TME elements and the implication in its targeting as a great promise in clinical trials.

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Keywords

mTOR pathway · mTORC1 · mTORC2 · Cancer · TME · Tumor–stroma interactions · Targeted therapy · Combination therapy · Angiogenesis · Immunotherapy

4.1 Introduction

mTOR is an evolutionarily conserved serine/threonine protein kinase that regulates several anabolism processes thus promoting protein, nucleotide, and lipid synthesis. Dysregulation of mTOR signaling is associated with diverse pathological conditions including a variety of human malignancies [1, 2].

mTOR forms two multi-protein complexes, mTOR complex 1 (mTORC1) and 2 (mTORC2), each of them performs specific roles in cellular regulation (Fig. 4.1) [1].

Both complexes share the catalytic subunit mTOR, the mammalian lethal with Sec13 protein 8 (mLST8), the DEP domain-containing mTOR-interacting protein (DEPTOR), and the Tti1–Tel2 complex. mLST8 binds the catalytic domain of mTOR and is required for interaction between mTOR/Rapamycin-sensitive companion of mTOR (Rictor), but not for the mTOR/Regulatory-associated protein of mammalian target of rapamycin (Raptor) [3]. DEPTOR interacts with mTOR, thereby inhibiting its kinase activity, whereas

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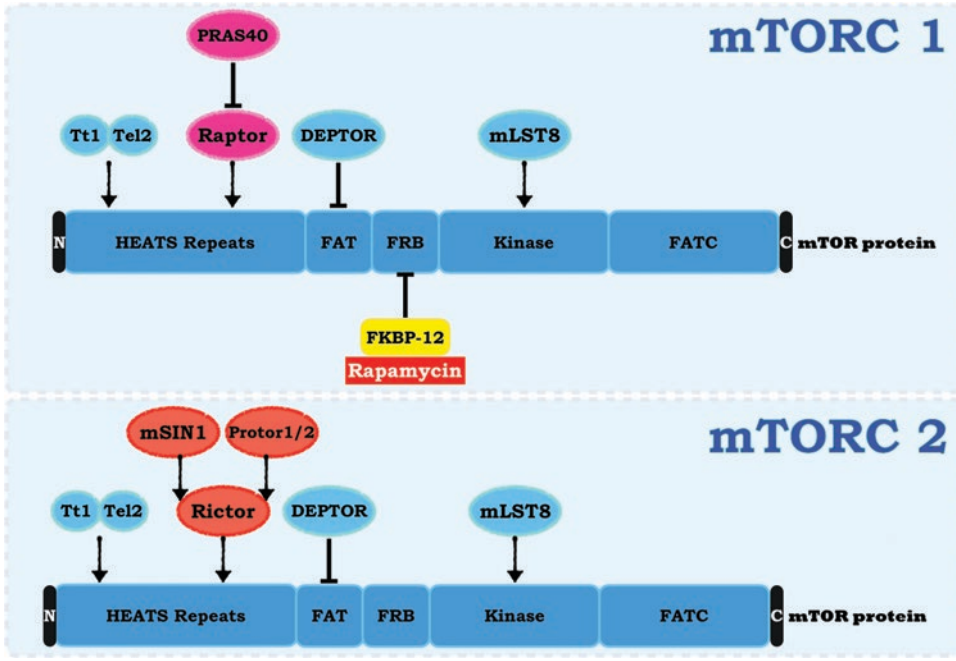

Fig. 4.1 Structural characteristics of mTOR protein. mTOR is composed of 2549 amino acids, divided into distinct protein domains: a FAT-carboxy terminal domain (FAT domain) which mediates interactions with other proteins; a FRAP-ATM-TTRAP domain (FATC domain) which senses cytosolic potential and regulates mTOR degradation; a FKBP12-rapamycin binding domain (FRB domain); a Huntingtin-Elongation factor 3-regulatory subunit A of PP2A-TOR1 repeats (HEAT repeat) which mediates protein–protein interactions

Tti1–Tel2 complex is involved in functional assembly of the mTOR complex [4, 5].

mTORC1 also includes Raptor, the proline-rich AKT substrate 40 kDa (PRAS40), whereas Rictor, the mammalian stress-activated map kinase-INteracting protein 1 (mSIN1), and the protein observed with Rictor (Protor) 1 and 2 are specific of mTORC2 complex. While Raptor is involved in amino acid sensing, assembly, substrate recognition, and phosphorylation of mTORC1 downstream proteins, DEPTOR acts as negative regulator of mTORC1 [4, 6]. Rictor and mSIN1 stabilize mTORC2 and decrease in Rictor expression levels results in reduced mSIN1 levels and vice versa [7–9].

mTORC1 and mTORC2 are triggered by nutrients and growth factors and inhibited by stress to ensure cellular homeostasis [10]. Specifically, mTORC1 regulates cell growth through protein anabolism, nucleotide biosynthesis, autophagy, glycolysis, and lipogenesis, whereas mTORC2 regulates apoptosis, glucose metabolism, lipogenesis, and the rearrangement of actin cytoskeleton (Fig. 4.2) [11–14].

Over recent years, scientific and clinical interests in mTOR-dependent regulation of TME shed light on its regulatory role in a wide range of stromal cells, including T and B cells, macrophages, and tumor fibroblasts [15, 16].

In this chapter we will summarize the current knowledge on the functional role of mTOR signaling in TME characterization and function; moreover, we will briefly discuss relevant issues and future perspectives regarding mTOR targeting as TME modulator and its possible contribution in immunotherapy.

4.2 mTOR in TME Elements

The tumor mass consists of not only cancer cells but also several resident and infiltrating host immunity cells, which constitute the cellular fraction of TME. It is now well established that the

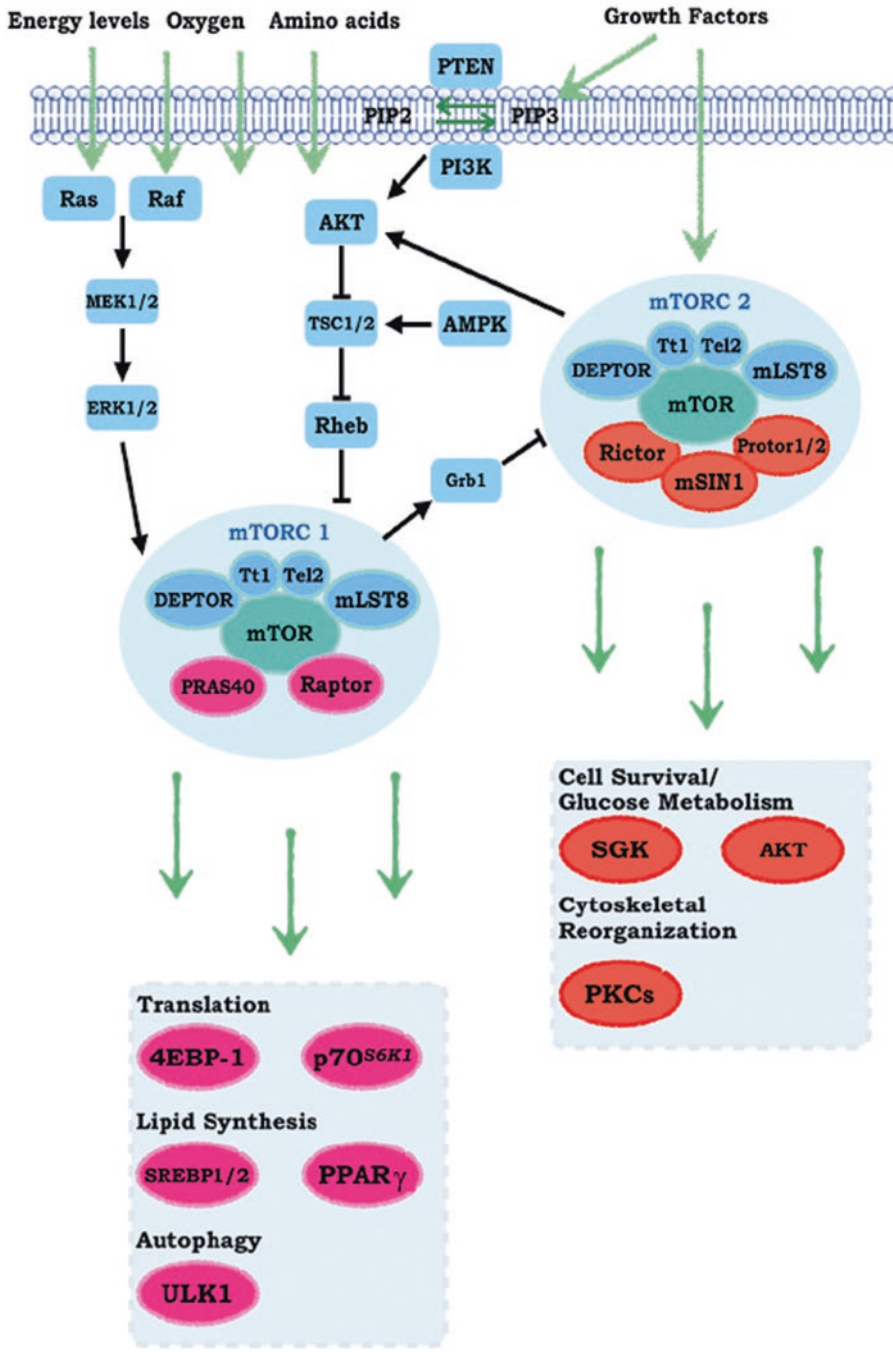

Fig. 4.2 Activation and functions of mTOR pathway. Several external cues activate intracellular mTOR signaling, through different membrane receptors. According to mTOR complex-specific activation, different biological mechanisms are activated in order to regulate tissue homeostasis

interactions and cross talk between tumor cells and elements of TME orchestrate tumor progression.

TME is composed by cytokines/chemokines, extracellular matrix (ECM) whose main constituents are collagen and hyaluronic acid, lymphatic

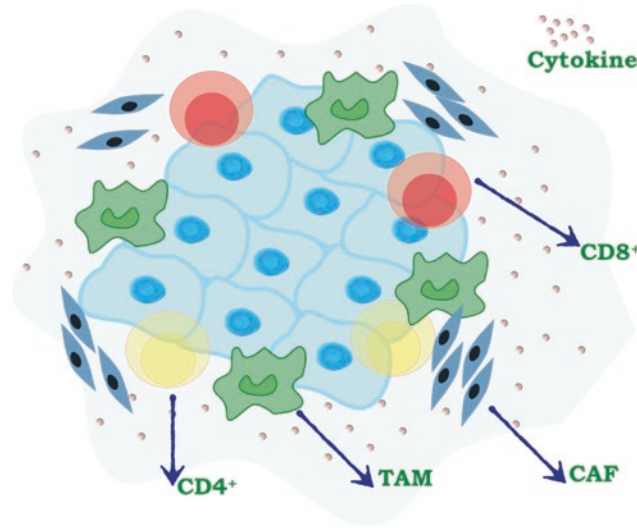

Fig. 4.3 Outline of the TME elements. TME contains different cell types including fibroblasts and infiltrating immune cells as well as their derived soluble factors (e.g., cytokines). All these elements and cancer cells influence each other's functions

vessels, and the stromal cells which include vascular endothelial cells, cancer-associated fibroblasts (CAF), adipocytes, and immune cells (Fig. 4.3) [17].

Several researches have highlighted the mTOR involvement in regulation of several cancer and stroma cells functions, including shaping immune response. Moreover, mTOR is involved in the modulation of several factors of TME including cytokines, chemokines, growth factors, and metabolites, as demonstrated by enhancement of specific cytokines/chemokines expression after mTOR hyperactivation [15, 18, 19].

CAF are important components of TME and act as promoter of tumor angiogenesis and metastasis, by both their ability to modify the ECM and the production of cytokines and growth factors that modulate activity of other stromal and cancer cells. CAF play a leading role in stroma-rich tumors like pancreatic cancer, where the stroma constitutes more than 80% of the tumor mass [20]. A recent study has suggested that mTOR is responsible for drug resistance by upregulating interleukin (IL)-6 protein synthesis in CAF, in pancreatic ductal adenocarcinoma nude mice xenografts. In the same study, Duluc and collaborators showed that somatostatin receptor inhibitor abolishes mTOR/IL-6 axis, thus reducing pancreatic tumor chemoresistance [21].

Emerging evidence suggests that upon antigen stimulation, mTOR regulates the differentiation into two distinct $CD4^+$ subsets: follicular helper (Th) and regulatory T cells (Treg) [22, 23]. Delgoffe and coworkers have shown that mTOR inhibition blocks the differentiation of $CD4^+$ T cells into Th1, Th2, and Th17, through the deletion of Rheb, a specific mTORC1 activator. This effect is principally due to the decrease in STAT phosphorylation that in turn leads to the inability of inducing the expression of lineage-specific transcription factors [24]. Conversely, Rictor deletion causes the failure of $CD4^+$ T-cell differentiation into Th2 cell type, but not into Th1 and Th17. This evidence shows that mTORC1 and mTORC2 play a specific role in lineage differentiation of $CD4^+$ T cells [24, 25]. Recent rapamycin studies have shown that mTOR activity reduction induces the development of Treg cells from naïve T cells, consistently with the ability of AKT to inhibit Treg development [26, 27]. Moreover, cell-specific PTEN deletion decreases Treg stability and function [28]. Zeng and collaborators have demonstrated that mTORC1 activity is essential for Treg function: indeed, deletion of Raptor and the consequent mTORC1 inactivity lead to the loss of Treg cells suppressive function and autoimmunity [29].

$CD8^+$ T cells, also called cytotoxic T lymphocytes, are the most important anticancer elements

of the immune system. Indeed, they are able to target and kill cancer cells and enhance memory response. Recent evidence has confirmed that mTOR is an important regulator of memory $CD8^+$ T-cell differentiation: indeed, TSC2 deletion-mediated constitutive mTORC1 activity increases of highly glycolytic $CD8^+$ T effector, thus rendering these cells unable to transition into memory $CD8^+$ T state [30, 31].

Same results were observed by enhancing mTORC1 activity through either IL-12 addition or PTEN downregulation; these mechanisms of mTOR activation lead to a reduced number of memory $CD8^+$ T cells [32, 33]. Moreover, Polizzi and colleagues have demonstrated that mTORC1 and mTORC2 regulate distinctly $CD8^+$ T-cell differentiation. Indeed, while mTORC1 influences $CD8^+$ T-cell effector responses, the genetic deletion of Rictor in $CD8^+$ T denotes that mTORC2 inactivity results in metabolic reprogramming, which increases the generation of $CD8^+$ memory cells [34].

Myeloid-derived suppressor cells (MDSC) represent another important TME immune cell populations and comprise monocytic and polymorphonuclear MDSC [35]. Granulocyte colony-stimulating factor (G-CSF) has been described to play a critical role in differentiation and recruitment of MDSCs within tumors; Welte and colleagues demonstrated that mTOR signaling promotes accumulations of these suppressor cell subsets by stimulating the production of G-CSF. Genetic and pharmacological inhibition (i.e., rapamycin treatment) of mTOR reduces G-CSF levels [36].

Tumor-associated macrophages (TAM) are present at different stages of tumor progression, recruited to tumor site by chemokines and growth factors, and are generally classified into M1 and M2. M1 macrophages promote antitumor response by inducing phagocyte-dependent inflammation, whereas M2 macrophages increase antibody response and inhibit phagocytic functions. As M2 is the predominant phenotype among TAM, they are considered as tumor promoters [37]. Recent evidence has demonstrated that mTOR also modulates TAM activation and differentiation. Indeed, the loss of mTOR activity through genetic or pharmacological inhibition results in modifications of macrophage inflammatory response toward M1-like phenotype due to the decreased cytokines production (e.g., IL-4) [38–40].

4.3 Implication of mTOR Signaling in Angiogenesis and Immunotherapy

Along with its involvement in immune system, mTOR signaling plays a key role also in tumor-related vascular formation: in keeping with all these observations, mTOR blockade has clinical implications both alone (Table 4.1) and in combination with TME-targeted agents (Table 4.2).

Angiogenesis is a physiological process that induces the formation of new vessels from the existing ones; nevertheless, cancer cells stimulate angiogenesis by releasing pro-angiogenic signals, thereby provoking not only tumor growth and progression through the distribution of nutrients but also spread metastasis process [41]. Different angiogenesis genes are principally regulated by hypoxia-inducible factors (HIF) which are induced by the low quantity of oxygen; indeed, hypoxia is one of the first step in vascular development [42]. The O_2 deprivation and hypoxic chemoattractant factors gradient (e.g., vascular endothelial growth factor (VEGF) and endothelin) are the major triggers of TAM recruitment: in this context, mTOR inhibition is responsible for TAM polarization into M1 phenotype, M2 apoptotic cell death, and angiogenesis blockade [15, 38, 43]. Consistent with these observations, it is indisputable that mTOR represents a significant hub in tumor microenvironment angiogenesis regulation with a great therapeutic potential. From several years, it is indeed well known that the inhibition of ribosomal protein S6 kinase 1 ($p70^{S6K1}$) by either PI3K or mTOR inhibitors impairs different processes in endothelial cells, such as proliferation and DNA synthesis as well as the synthesis of cyclin D1, one of the master regulators of cell cycle G1 phase [18, 44]. These results are consistent with the observation that the lacking of

Table 4.1 mTOR targeting in modulating TME features

Target	Drug	Effects on TME	Clinical implication
PI3K	Wortmannin; Ly294002	↓ DNA and cyclin D1 synthesis, endothelial cells' proliferation	↓ Angiogenesis
	IC87114	↓ Treg population	↑ Tumor regression
AKT	MK22–06	↓ PD-L1 expression	↑ Antitumor adaptive immune responses
		↓ Treg population	↑ Tumor regression
	AKT inhibitor X	↑ MDSC differentiation ↑ DC	↑ Antitumor immune response
mTOR	Rapamycin	↑ M1 TAM polarization, M2 apoptotic cell death	↓ Angiogenesis
		↓ DNA and cyclin D1 synthesis, endothelial cells' proliferation	
		↑ HIF-1α polarization	
		↓ DKK2 and lactate production	↓ Endothelial cells' growth
		↓ PD-L1 expression	↑ Antitumor adaptive immune responses
	Everolimus	↓ VEGF and CXCL12 production	↓ Angiogenesis
	Everolimus/ rapamycin	↓ T-cell proliferation ↓ PD-1-competent CD8 or CD4	↑ Immunosuppressant response

Table 4.2 Combinatorial strategies with both mTOR and TME targeting: implications in immunotherapy

mTOR drug	TME targeting	Effects on TME	Clinical implication
Everolimus	RTKi Sunitinib	↓ PDGFR and VEGFR activity in stromal cells	↓ Tumor growth and angiogenesis
INK-128	αPD-1	↓ Proliferation and clone formation in HCC cells ↓ $p70^{S6K}$ and eIF4E phosphorylation	↓ Tumor growth
Rapamycin	αCTLA-4	↑ CD8+ cells ↑ Effector cytokines productions	↓ Tumor growth
Vistusertib	αCTLA-4/PD-1/ PD-L1	↑ Th1 polarized T-cells ↓ Tumor infiltrating lymphocytes	↓ Tumor growth ↑ Survival
AZD80550	αCD40	↑ $CD8^+$, NK, mature macrophages, DC	↑ Immune response

mTORC1 negative regulator TSC1 induces the development of lymphangiosarcomas, significant increase in HIF-1α expression, and subsequent upregulation of VEGFA production [45].

Given the role of mTOR in stimulating VEGF production is not surprising that the use of everolimus reduces VEGF production [18, 46]. Nevertheless, the single mTOR inhibition is not sufficient to completely reduce angiogenesis, mainly due to the compensatory hyperactivation of other signaling pathways, such as MAPK: these cross talks put the biological rationale for new combination strategies [47–49]. For this reason, Wagle and collaborator demonstrated that, in a phase I trial, not only the presence of mTOR activating mutations (i.e., $mTOR^{E2419K}$ and $mTOR^{E2014K}$) makes tumor cells more sensitive to everolimus but also the combination of everolimus and pazopanib (a VEGF receptor inhibitor) results in an outstanding response in advanced solid tumors [46].

Different studies highlight the interaction between mTOR and HIF-1α. For example, HIF-1α is translationally increased with an activated mTORC1, thanks to the phosphorylation of 4E-BP1; moreover, HIF-1α protein is more exposed to degradation after rapamycin treatment [47, 50]. Similar to mTORC1, mTORC2 is implicated in the angiogenesis induction through the stimulation of VEGF and stromal cell-derived

factor (SDF)-1. Indeed, the absence of mTORC2 leads to deficiency in vascular development, and the use of mTOR inhibitor everolimus results in a reduction of blood vessels strengthening in cell lines with different histological origin [51]. Furthermore, it is actually known that platelet-derived growth factor (PDGF) stimulates VEGF expression in kidney cells, thus leading to angiogenesis induction through both autocrine and paracrine stimulation [52]. Kitano and coworkers recently showed that the use of a multiple tyrosine kinase inhibitor sunitinib acts in the modulation of stromal reactions by inhibiting PDGF and VEGF receptors. In this way, the combination of everolimus with sunitinib copes to significantly reduce tumor growth and angiogenesis in renal cell carcinoma (RCC) [53].

The role of mTOR in metabolism and angiogenesis modulation was also demonstrated by Bruning and collaborators. In this study they illustrate that the malonylation of mTOR at Lys1218 contributes to mTOR-reduced activity: indeed, fatty acid synthase inactivation results in increased malonil-CoA production and consequent mTOR malonylation. This regulation leads to the arrest of endothelial cell proliferation which in turn halts the process of vessel sprouting [54]. The stimulation of endothelial cells' development is also mediated through the production of lactate by aerobic glycolysis induced by DicKKopf-associated protein 2 (DKK2). Deng and collaborators showed that DKK2 induces the formation of endothelial cells by activating PI3K/mTOR pathway; the use of rapamycin, indeed, reduces the DKK2 downstream activation and the subsequent lactate production, highlighting the role of mTOR also in modulating angiogenesis independently from VEGF/VEGF receptor axis [55].

Recently, several studies highlighted the role of immunotherapy as an important component of single and combined treatment of multiple advanced cancer types. Indeed, affecting host immunity improves the clinical success, by regulating immunological components of TME and different processes, such as immune evasion [56, 57]. mTOR signaling plays a pivotal role also in the balance between immune activation/suppression. Immune suppression is another therapeutic strategy, and it is mediated by cellular subpopulation recruitment, such as Treg and MDSC, involved in immune cells regulation [58, 59]. Consistently, treatment with PI3K or AKT inhibitors causes inhibition of Treg population, which results in tumor regression [59]. Likewise, AKT inhibition limits the differentiation of the MDSC, but not of the advanced differentiated MDSC cells, in vitro [60].

Given the role of PI3K/AKT/mTOR pathway in regulating not only immune cells proliferation but also their immune surveillance activities, it is expected that targeting this signaling pathway could represent a winning therapeutic approach to counteract resistance mechanisms [61]. One of the most promising strategy of immunotherapy involves immune checkpoints and their markers, often expressed by tumor cells: in particular different studies showed the clinical benefit obtained by targeting the negative T-cell regulators programmed death (PD)-1/PD-ligand (PD-L)1 and cytotoxic T-lymphocyte protein (CTLA)-4 [62]. However, it was observed that only a small percentage of patients respond to single-agent anti-PD-1 therapy [63]. Given the importance of the immune system treatment in therapy and the role of mTOR in regulating the immune components, it could be fundamental to study the mechanisms of interaction between mTOR and the immunity, to explore in-depth the new therapeutic strategies.

The involvement of PI3K pathway in immune system is associated with PD-L1-specific expression, through the posttranscriptional regulation: indeed, the PI3K pathway hyperactivation, due to PTEN loss or knockdown, correlates with an overexpression of PD-L1 [64, 65]. Consistently, Mittendorf and her collaborators demonstrated that in PTEN-mutant triple-negative breast cancer, the use of AKT or mTOR inhibitors (MK-2206 and rapamycin, respectively) decreases PD-L1 expression [66].

It is actually known that PD-1 is physically associated with the major downstream effectors of mTORC1, $p70^{S6K1}$ and eIF4E, thus in turn inducing their phosphorylation and the subsequent mTOR pathway activation [52]. Moreover, mTOR inhibition reduces not only T-cell proliferation but also the percentage of a

specific subgroup of $CD4^+$ and $CD8^+$ lymphocytes characterized by the expression of PD-1 receptor [67, 68]. This tight connection between mTOR and PD-1 results in tumor growth decrease after PD-1 and mTOR inhibitor combination, as shown by Li and colleagues [52].

While the specific anti-PD-1 treatment leads to an increase in the CD8 T population in tumor tissue, the inhibition of CTLA-4 immune checkpoint induces the proliferation of these effectors in secondary lymphoid organ [56]. Pedicord and her collaborators have demonstrated that during the T-cell priming, the use of an mTOR inhibitor, such as rapamycin, combined with anti-CTLA-4 inhibitor causes the expansion of $CD8^+$ memory T cells and this could, in turn, have different implications in cancer immunotherapy [69].

More recently, the combinatorial benefit between mTOR and immune checkpoints inhibitors was preclinically validated using vistusertib, an mTORC1/2 inhibitor, in combination with either αPD-1 or αPD-L1 or αCTLA-4. The single use of vistusertib induces the production of Th1-promoting IL-12 cytokine and reduces the immune inhibitory IL-10 cytokine; the combination increases the frequency of intratumoral Th1 activation, thus resulting in synergistic tumor growth inhibition [70].

As opposite to negative regulators reported above, CD40 is a tumor necrosis factor (TNF) receptor family member involved in promoting T-cell-mediated immunity, and several agonistic αCD40 antibodies were developed as mimic of the natural ligand CD154. Jiang and colleagues demonstrated the synergistic interactions between AZD80550 and αCD40 in inhibiting tumor growth, as compared to head-to-head treatment of single agent. Indeed, this combination causes the activation and proliferation of $CD8^+$, natural killer, mature macrophages, and dendritic cells, thus leading to an increased immune response [71].

4.4 Commentary

As we highlighted above, mTOR signaling is involved in many aspects of TSI in each steps of carcinogenesis. In cancer, mTOR pathway hyperactivation often occurs by the loss of the negative regulator PTEN, which affects the response to not only the targeted therapy but also immunotherapy. Indeed, several studies demonstrated the tight connection between PTEN and PD-L1 expression: Song and collaborators showed that PTEN loss correlates with high levels of IFN-γ-independent PD-L1 in colorectal cancer samples, whereas Parsa et al. highlighted that PTEN-loss status induces immunoresistance through PD-L1 upregulation [65, 72]. Thus, switching off hyperactivated pathway could promote signaling through other molecular cascades, and this mechanism could be used to improve drug response. Indeed, the inhibition of PD-L1 causes tumor growth arrest and PTEN reactivation in mice [73].

These cross talks allow the biological rationale for combination approaches, and due to the limited clinical efficacy of rapamycin and rapalogs, new second- and third-generation mTOR inhibitors were developed and investigated in several clinical trials [1]. Even if combination therapy could result in synergistic effects on tumor growth, drugs are usually more toxic and expansive than monotherapy: thus, the identification of molecular contexts in which combinations are effectively synergistic is an urgent need for personalized cancer treatments. For example, SDF-1/α-CXCR4 axis causes temsirolimus resistance through VEGF signaling in pancreatic cancer. Consistently, combination of temsirolimus with the CXCR4 inhibitor AMD3465 results in synergistic effect on tumor growth arrest in xenograft models [74]. The cross talk between CXCR4 and mTOR signaling is well established also in metastatic RCC, as demonstrated by mTOR inhibition following the treatment of CXCR4 and CXCR7 antagonists. Combination between CXCR4/CXCR7/mTOR inhibitors results in additive effects in reducing migration and cell growth [75].

In summary, due to the highlighted involvement of mTOR in immunomodulation and angiogenesis, investigating the mTOR pathway reactivation feedback loops is still necessary to significantly improve personalized cancer therapy.

References

1. Conciatori F, Ciuffreda L, Bazzichetto C, Falcone I, Pilotto S, Bria E, Cognetti F, Milella M (2018) mTOR Cross-talk in cancer and potential for combination therapy. Cancers (Basel) 10(1). https://doi.org/10.3390/cancers10010023
2. Watanabe R, Wei L, Huang J (2011) mTOR signaling, function, novel inhibitors, and therapeutic targets. J Nucl Med 52(4):497–500. https://doi.org/10.2967/jnumed.111.089623
3. Mann WA, Brewer HB Jr, Greten H (1991) Genetic variants of apo E: significance for triglyceride metabolism. Z Gastroenterol Verh 26:106
4. Peterson TR, Laplante M, Thoreen CC, Sancak Y, Kang SA, Kuehl WM, Gray NS, Sabatini DM (2009) DEPTOR is an mTOR inhibitor frequently overexpressed in multiple myeloma cells and required for their survival. Cell 137(5):873–886. https://doi.org/10.1016/j.cell.2009.03.046
5. Kim SG, Hoffman GR, Poulogiannis G, Buel GR, Jang YJ, Lee KW, Kim BY, Erikson RL, Cantley LC, Choo AY, Blenis J (2013) Metabolic stress controls mTORC1 lysosomal localization and dimerization by regulating the TTT-RUVBL1/2 complex. Mol Cell 49(1):172–185. https://doi.org/10.1016/j.molcel.2012.10.003
6. Sancak Y, Peterson TR, Shaul YD, Lindquist RA, Thoreen CC, Bar-Peled L, Sabatini DM (2008) The rag GTPases bind raptor and mediate amino acid signaling to mTORC1. Science 320(5882):1496–1501. https://doi.org/10.1126/science.1157535
7. Sarbassov DD, Ali SM, Kim DH, Guertin DA, Latek RR, Erdjument-Bromage H, Tempst P, Sabatini DM (2004) Rictor, a novel binding partner of mTOR, defines a rapamycin-insensitive and raptor-independent pathway that regulates the cytoskeleton. Curr Biol 14(14):1296–1302. https://doi.org/10.1016/j.cub.2004.06.054
8. Yang Q, Inoki K, Ikenoue T, Guan KL (2006) Identification of Sin1 as an essential TORC2 component required for complex formation and kinase activity. Genes Dev 20(20):2820–2832. https://doi.org/10.1101/gad.1461206
9. Jacinto E, Facchinetti V, Liu D, Soto N, Wei S, Jung SY, Huang Q, Qin J, Su B (2006) SIN1/MIP1 maintains rictor-mTOR complex integrity and regulates Akt phosphorylation and substrate specificity. Cell 127(1):125–137. https://doi.org/10.1016/j.cell.2006.08.033
10. Laplante M, Sabatini DM (2012) mTOR signaling in growth control and disease. Cell 149(2):274–293. https://doi.org/10.1016/j.cell.2012.03.017
11. Cybulski N, Hall MN (2009) TOR complex 2: a signaling pathway of its own. Trends Biochem Sci 34(12):620–627. https://doi.org/10.1016/j.tibs.2009.09.004
12. Oh WJ, Jacinto E (2011) mTOR complex 2 signaling and functions. Cell Cycle 10(14):2305–2316. https://doi.org/10.4161/cc.10.14.16586
13. Hagiwara A, Cornu M, Cybulski N, Polak P, Betz C, Trapani F, Terracciano L, Heim MH, Ruegg MA, Hall MN (2012) Hepatic mTORC2 activates glycolysis and lipogenesis through Akt, glucokinase, and SREBP1c. Cell Metab 15(5):725–738. https://doi.org/10.1016/j.cmet.2012.03.015
14. Saxton RA, Sabatini DM (2017) mTOR Signaling in growth, metabolism, and disease. Cell 169(2):361–371. https://doi.org/10.1016/j.cell.2017.03.035
15. Conciatori F, Bazzichetto C, Falcone I, Pilotto S, Bria E, Cognetti F, Milella M, Ciuffreda L (2018) Role of mTOR Signaling in tumor microenvironment: an overview. Int J Mol Sci 19(8). https://doi.org/10.3390/ijms19082453
16. Araki K, Ellebedy AH, Ahmed R (2011) TOR in the immune system. Curr Opin Cell Biol 23(6):707–715. https://doi.org/10.1016/j.ceb.2011.08.006
17. Bussard KM, Mutkus L, Stumpf K, Gomez-Manzano C, Marini FC (2016) Tumor-associated stromal cells as key contributors to the tumor microenvironment. Breast Cancer Res 18(1):84. https://doi.org/10.1186/s13058-016-0740-2
18. Del Bufalo D, Ciuffreda L, Trisciuoglio D, Desideri M, Cognetti F, Zupi G, Milella M (2006) Antiangiogenic potential of the mammalian target of rapamycin inhibitor temsirolimus. Cancer Res 66(11):5549–5554. https://doi.org/10.1158/0008-5472.CAN-05-2825
19. Maxwell PJ, Coulter J, Walker SM, McKechnie M, Neisen J, McCabe N, Kennedy RD, Salto-Tellez M, Albanese C, Waugh DJ (2013) Potentiation of inflammatory CXCL8 signalling sustains cell survival in PTEN-deficient prostate carcinoma. Eur Urol 64(2):177–188. https://doi.org/10.1016/j.eururo.2012.08.032
20. Kalluri R, Zeisberg M (2006) Fibroblasts in cancer. Nat Rev Cancer 6(5):392–401. https://doi.org/10.1038/nrc1877
21. Duluc C, Moatassim-Billah S, Chalabi-Dchar M, Perraud A, Samain R, Breibach F, Gayral M, Cordelier P, Delisle MB, Bousquet-Dubouch MP, Tomasini R, Schmid H, Mathonnet M, Pyronnet S, Martineau Y, Bousquet C (2015) Pharmacological targeting of the protein synthesis mTOR/4E-BP1 pathway in cancer-associated fibroblasts abrogates pancreatic tumour chemoresistance. EMBO Mol Med 7(6):735–753. https://doi.org/10.15252/emmm.201404346
22. Pepper M, Jenkins MK (2011) Origins of CD4(+) effector and central memory T cells. Nat Immunol 12(6):467–471
23. Powell JD, Delgoffe GM (2010) The mammalian target of rapamycin: linking T cell differentiation, function, and metabolism. Immunity 33(3):301–311. https://doi.org/10.1016/j.immuni.2010.09.002
24. Delgoffe GM, Kole TP, Zheng Y, Zarek PE, Matthews KL, Xiao B, Worley PF, Kozma SC, Powell JD (2009) The mTOR kinase differentially regulates effector and regulatory T cell lineage commitment. Immunity 30(6):832–844. https://doi.org/10.1016/j.immuni.2009.04.014
25. Delgoffe GM, Pollizzi KN, Waickman AT, Heikamp E, Meyers DJ, Horton MR, Xiao B, Worley PF,

Powell JD (2011) The kinase mTOR regulates the differentiation of helper T cells through the selective activation of signaling by mTORC1 and mTORC2. Nat Immunol 12(4):295–303. https://doi.org/10.1038/ni.2005
26. Valmori D, Tosello V, Souleimanian NE, Godefroy E, Scotto L, Wang Y, Ayyoub M (2006) Rapamycin-mediated enrichment of T cells with regulatory activity in stimulated CD4+ T cell cultures is not due to the selective expansion of naturally occurring regulatory T cells but to the induction of regulatory functions in conventional CD4+ T cells. J Immunol 177(2):944–949. https://doi.org/10.4049/jimmunol.177.2.944
27. Haxhinasto S, Mathis D, Benoist C (2008) The AKT-mTOR axis regulates de novo differentiation of CD4+Foxp3+ cells. J Exp Med 205(3):565–574. https://doi.org/10.1084/jem.20071477
28. Shrestha S, Yang K, Guy C, Vogel P, Neale G, Chi H (2015) Treg cells require the phosphatase PTEN to restrain TH1 and TFH cell responses. Nat Immunol 16(2):178–187. https://doi.org/10.1038/ni.3076
29. Zeng H, Yang K, Cloer C, Neale G, Vogel P, Chi H (2013) mTORC1 couples immune signals and metabolic programming to establish T(reg)-cell function. Nature 499(7459):485–490. https://doi.org/10.1038/nature12297
30. Pollizzi KN, Powell JD (2015) Regulation of T cells by mTOR: the known knowns and the known unknowns. Trends Immunol 36(1):13–20. https://doi.org/10.1016/j.it.2014.11.005
31. Salmond RJ (2018) mTOR regulation of glycolytic metabolism in T cells. Front Cell Dev Biol 6:122. https://doi.org/10.3389/fcell.2018.00122
32. Rao RR, Li Q, Odunsi K, Shrikant PA (2010) The mTOR kinase determines effector versus memory CD8+ T cell fate by regulating the expression of transcription factors T-bet and Eomesodermin. Immunity 32(1):67–78. https://doi.org/10.1016/j.immuni.2009.10.010
33. Wu T, Wieland A, Araki K, Davis CW, Ye L, Hale JS, Ahmed R (2012) Temporal expression of microRNA cluster miR-17-92 regulates effector and memory CD8+ T-cell differentiation. Proc Natl Acad Sci U S A 109(25):9965–9970. https://doi.org/10.1073/pnas.1207327109
34. Pollizzi KN, Patel CH, Sun IH, Oh MH, Waickman AT, Wen J, Delgoffe GM, Powell JD (2015) mTORC1 and mTORC2 selectively regulate CD8(+) T cell differentiation. J Clin Invest 125(5):2090–2108. https://doi.org/10.1172/JCI77746
35. Bronte V, Brandau S, Chen SH, Colombo MP, Frey AB, Greten TF, Mandruzzato S, Murray PJ, Ochoa A, Ostrand-Rosenberg S, Rodriguez PC, Sica A, Umansky V, Vonderheide RH, Gabrilovich DI (2016) Recommendations for myeloid-derived suppressor cell nomenclature and characterization standards. Nat Commun 7:12150. https://doi.org/10.1038/ncomms12150
36. Welte T, Kim IS, Tian L, Gao X, Wang H, Li J, Holdman XB, Herschkowitz JI, Pond A, Xie G, Kurley S, Nguyen T, Liao L, Dobrolecki LE, Pang L, Mo Q, Edwards DP, Huang S, Xin L, Xu J, Li Y, Lewis MT, Wang T, Westbrook TF, Rosen JM, Zhang XH (2016) Oncogenic mTOR signalling recruits myeloid-derived suppressor cells to promote tumour initiation. Nat Cell Biol 18(6):632–644. https://doi.org/10.1038/ncb3355
37. Hanahan D, Coussens LM (2012) Accessories to the crime: functions of cells recruited to the tumor microenvironment. Cancer Cell 21(3):309–322. https://doi.org/10.1016/j.ccr.2012.02.022
38. Mercalli A, Calavita I, Dugnani E, Citro A, Cantarelli E, Nano R, Melzi R, Maffi P, Secchi A, Sordi V, Piemonti L (2013) Rapamycin unbalances the polarization of human macrophages to M1. Immunology 140(2):179–190. https://doi.org/10.1111/imm.12126
39. Jiang H, Westerterp M, Wang C, Zhu Y, Ai D (2014) Macrophage mTORC1 disruption reduces inflammation and insulin resistance in obese mice. Diabetologia 57(11):2393–2404. https://doi.org/10.1007/s00125-014-3350-5
40. Hallowell RW, Collins SL, Craig JM, Zhang Y, Oh M, Illei PB, Chan-Li Y, Vigeland CL, Mitzner W, Scott AL, Powell JD, Horton MR (2017) mTORC2 signalling regulates M2 macrophage differentiation in response to helminth infection and adaptive thermogenesis. Nat Commun 8:14208. https://doi.org/10.1038/ncomms14208
41. Rajabi M, Mousa SA (2017) The role of angiogenesis in cancer treatment. Biomedicine 5(2). https://doi.org/10.3390/biomedicines5020034
42. Krock BL, Skuli N, Simon MC (2011) Hypoxia-induced angiogenesis: good and evil. Genes Cancer 2(12):1117–1133. https://doi.org/10.1177/1947601911423654
43. Chen W, Ma T, Shen XN, Xia XF, Xu GD, Bai XL, Liang TB (2012) Macrophage-induced tumor angiogenesis is regulated by the TSC2-mTOR pathway. Cancer Res 72(6):1363–1372. https://doi.org/10.1158/0008-5472.CAN-11-2684
44. Vinals F, Chambard JC, Pouyssegur J (1999) p70 S6 kinase-mediated protein synthesis is a critical step for vascular endothelial cell proliferation. J Biol Chem 274(38):26776–26782. https://doi.org/10.1074/jbc.274.38.26776
45. Sun S, Chen S, Liu F, Wu H, McHugh J, Bergin IL, Gupta A, Adams D, Guan JL (2015) Constitutive activation of mTORC1 in endothelial cells leads to the development and progression of lymphangiosarcoma through VEGF autocrine signaling. Cancer Cell 28(6):758–772. https://doi.org/10.1016/j.ccell.2015.10.004
46. Wagle N, Grabiner BC, Van Allen EM, Hodis E, Jacobus S, Supko JG, Stewart M, Choueiri TK, Gandhi L, Cleary JM, Elfiky AA, Taplin ME, Stack EC, Signoretti S, Loda M, Shapiro GI, Sabatini DM, Lander ES, Gabriel SB, Kantoff PW, Garraway LA, Rosenberg JE (2014) Activating mTOR mutations in a patient with an extraordinary response on a phase I trial of everolimus and pazopanib. Cancer Discov

4(5):546–553. https://doi.org/10.1158/2159-8290.CD-13-0353
47. Faes S, Santoro T, Demartines N, Dormond O (2017) Evolving significance and future relevance of anti-angiogenic activity of mTOR inhibitors in cancer therapy. Cancers (Basel) 9(11). https://doi.org/10.3390/cancers9110152
48. Milella M, Falcone I, Conciatori F, Matteoni S, Sacconi A, De Luca T, Bazzichetto C, Corbo V, Simbolo M, Sperduti I, Benfante A, Del Curatolo A, Cesta Incani U, Malusa F, Eramo A, Sette G, Scarpa A, Konopleva M, Andreeff M, McCubrey JA, Blandino G, Todaro M, Stassi G, De Maria R, Cognetti F, Del Bufalo D, Ciuffreda L (2017) PTEN status is a crucial determinant of the functional outcome of combined MEK and mTOR inhibition in cancer. Sci Rep 7:43013. https://doi.org/10.1038/srep43013
49. Ciuffreda L, Del Curatolo A, Falcone I, Conciatori F, Bazzichetto C, Cognetti F, Corbo V, Scarpa A, Milella M (2017) Lack of growth inhibitory synergism with combined MAPK/PI3K inhibition in preclinical models of pancreatic cancer. Ann Oncol 28(11):2896–2898. https://doi.org/10.1093/annonc/mdx335
50. Duvel K, Yecies JL, Menon S, Raman P, Lipovsky AI, Souza AL, Triantafellow E, Ma Q, Gorski R, Cleaver S, Vander Heiden MG, MacKeigan JP, Finan PM, Clish CB, Murphy LO, Manning BD (2010) Activation of a metabolic gene regulatory network downstream of mTOR complex 1. Mol Cell 39(2):171–183. https://doi.org/10.1016/j.molcel.2010.06.022
51. Kim LC, Cook RS, Chen J (2017) mTORC1 and mTORC2 in cancer and the tumor microenvironment. Oncogene 36(16):2191–2201. https://doi.org/10.1038/onc.2016.363
52. Li H, Li X, Liu S, Guo L, Zhang B, Zhang J, Ye Q (2017) Programmed cell death-1 (PD-1) checkpoint blockade in combination with a mammalian target of rapamycin inhibitor restrains hepatocellular carcinoma growth induced by hepatoma cell-intrinsic PD-1. Hepatology 66(6):1920–1933. https://doi.org/10.1002/hep.29360
53. Kitano H, Kitadai Y, Teishima J, Yuge R, Shinmei S, Goto K, Inoue S, Hayashi T, Sentani K, Yasui W, Matsubara A (2017) Combination therapy using molecular-targeted drugs modulates tumor microenvironment and impairs tumor growth in renal cell carcinoma. Cancer Med 6(10):2308–2320. https://doi.org/10.1002/cam4.1124
54. Bruning U, Morales-Rodriguez F, Kalucka J, Goveia J, Taverna F, Queiroz KCS, Dubois C, Cantelmo AR, Chen R, Loroch S, Timmerman E, Caixeta V, Bloch K, Conradi LC, Treps L, Staes A, Gevaert K, Tee A, Dewerchin M, Semenkovich CF, Impens F, Schilling B, Verdin E, Swinnen JV, Meier JL, Kulkarni RA, Sickmann A, Ghesquiere B, Schoonjans L, Li X, Mazzone M, Carmeliet P (2018) Impairment of angiogenesis by fatty acid synthase inhibition involves mTOR malonylation. Cell Metab 28(6):866–880, e815. https://doi.org/10.1016/j.cmet.2018.07.019
55. Deng F, Zhou R, Lin C, Yang S, Wang H, Li W, Zheng K, Lin W, Li X, Yao X, Pan M, Zhao L (2019) Tumor-secreted dickkopf2 accelerates aerobic glycolysis and promotes angiogenesis in colorectal cancer. Theranostics 9(4):1001–1014. https://doi.org/10.7150/thno.30056
56. Smyth MJ, Ngiow SF, Ribas A, Teng MW (2016) Combination cancer immunotherapies tailored to the tumour microenvironment. Nat Rev Clin Oncol 13(3):143–158. https://doi.org/10.1038/nrclinonc.2015.209
57. Tormoen GW, Crittenden MR, Gough MJ (2018) Role of the immunosuppressive microenvironment in immunotherapy. Adv Radiat Oncol 3(4):520–526. https://doi.org/10.1016/j.adro.2018.08.018
58. Umansky V, Sevko A (2013) Tumor microenvironment and myeloid-derived suppressor cells. Cancer Microenviron 6(2):169–177. https://doi.org/10.1007/s12307-012-0126-7
59. Abu-Eid R, Samara RN, Ozbun L, Abdalla MY, Berzofsky JA, Friedman KM, Mkrtichyan M, Khleif SN (2014) Selective inhibition of regulatory T cells by targeting the PI3K-Akt pathway. Cancer Immunol Res 2(11):1080–1089. https://doi.org/10.1158/2326-6066.CIR-14-0095
60. Gato-Canas M, Martinez de Morentin X, Blanco-Luquin I, Fernandez-Irigoyen J, Zudaire I, Liechtenstein T, Arasanz H, Lozano T, Casares N, Chaikuad A, Knapp S, Guerrero-Setas D, Escors D, Kochan G, Santamaria E (2015) A core of kinase-regulated interactomes defines the neoplastic MDSC lineage. Oncotarget 6(29):27160–27175. https://doi.org/10.18632/oncotarget.4746
61. O'Donnell JS, Massi D, Teng MWL, Mandala M (2018) PI3K-AKT-mTOR inhibition in cancer immunotherapy, redux. Semin Cancer Biol 48:91–103. https://doi.org/10.1016/j.semcancer.2017.04.015
62. Shen X, Zhao B (2018) Efficacy of PD-1 or PD-L1 inhibitors and PD-L1 expression status in cancer: meta-analysis. BMJ 362:k3529. https://doi.org/10.1136/bmj.k3529
63. Pardoll DM (2012) The blockade of immune checkpoints in cancer immunotherapy. Nat Rev Cancer 12(4):252–264. https://doi.org/10.1038/nrc3239
64. Lastwika KJ, Wilson W 3rd, Li QK, Norris J, Xu H, Ghazarian SR, Kitagawa H, Kawabata S, Taube JM, Yao S, Liu LN, Gills JJ, Dennis PA (2016) Control of PD-L1 expression by oncogenic activation of the AKT-mTOR pathway in non-small cell lung cancer. Cancer Res 76(2):227–238. https://doi.org/10.1158/0008-5472.CAN-14-3362
65. Song M, Chen D, Lu B, Wang C, Zhang J, Huang L, Wang X, Timmons CL, Hu J, Liu B, Wu X, Wang L, Wang J, Liu H (2013) PTEN loss increases PD-L1 protein expression and affects the correlation between PD-L1 expression and clinical parameters in colorectal cancer. PLoS One 8(6):e65821. https://doi.org/10.1371/journal.pone.0065821
66. Mittendorf EA, Philips AV, Meric-Bernstam F, Qiao N, Wu Y, Harrington S, Su X, Wang Y, Gonzalez-

Angulo AM, Akcakanat A, Chawla A, Curran M, Hwu P, Sharma P, Litton JK, Molldrem JJ, Alatrash G (2014) PD-L1 expression in triple-negative breast cancer. Cancer Immunol Res 2(4):361–370. https://doi.org/10.1158/2326-6066.CIR-13-0127

67. Saunders RN, Metcalfe MS, Nicholson ML (2001) Rapamycin in transplantation: a review of the evidence. Kidney Int 59(1):3–16. https://doi.org/10.1046/j.1523-1755.2001.00460.x
68. Mannick JB, Del Giudice G, Lattanzi M, Valiante NM, Praestgaard J, Huang B, Lonetto MA, Maecker HT, Kovarik J, Carson S, Glass DJ, Klickstein LB (2014) mTOR inhibition improves immune function in the elderly. Sci Transl Med 6(268):268ra179. https://doi.org/10.1126/scitranslmed.3009892
69. Pedicord VA, Cross JR, Montalvo-Ortiz W, Miller ML, Allison JP (2015) Friends not foes: CTLA-4 blockade and mTOR inhibition cooperate during CD8+ T cell priming to promote memory formation and metabolic readiness. J Immunol 194(5):2089–2098. https://doi.org/10.4049/jimmunol.1402390
70. Langdon S, Hughes A, Taylor MA, Kuczynski EA, Mele DA, Delpuech O, Jarvis L, Staniszewska A, Cosulich S, Carnevalli LS, Sinclair C (2018) Combination of dual mTORC1/2 inhibition and immune-checkpoint blockade potentiates anti-tumour immunity. Oncoimmunology 7(8):e1458810. https://doi.org/10.1080/2162402X.2018.1458810
71. Jiang Q, Weiss JM, Back T, Chan T, Ortaldo JR, Guichard S, Wiltrout RH (2011) mTOR kinase inhibitor AZD8055 enhances the immunotherapeutic activity of an agonist CD40 antibody in cancer treatment. Cancer Res 71(12):4074–4084. https://doi.org/10.1158/0008-5472.CAN-10-3968
72. Parsa AT, Waldron JS, Panner A, Crane CA, Parney IF, Barry JJ, Cachola KE, Murray JC, Tihan T, Jensen MC, Mischel PS, Stokoe D, Pieper RO (2007) Loss of tumor suppressor PTEN function increases B7-H1 expression and immunoresistance in glioma. Nat Med 13(1):84–88. https://doi.org/10.1038/nm1517
73. Zhao L, Li C, Liu F, Zhao Y, Liu J, Hua Y, Liu J, Huang J, Ge C (2017) A blockade of PD-L1 produced antitumor and antimetastatic effects in an orthotopic mouse pancreatic cancer model via the PI3K/Akt/mTOR signaling pathway. Onco Targets Ther 10:2115–2126. https://doi.org/10.2147/OTT.S130481
74. Weekes CD, Song D, Arcaroli J, Wilson LA, Rubio-Viqueira B, Cusatis G, Garrett-Mayer E, Messersmith WA, Winn RA, Hidalgo M (2012) Stromal cell-derived factor 1alpha mediates resistance to mTOR-directed therapy in pancreatic cancer. Neoplasia 14(8):690–701. https://doi.org/10.1593/neo.111810
75. Ierano C, Santagata S, Napolitano M, Guardia F, Grimaldi A, Antignani E, Botti G, Consales C, Riccio A, Nanayakkara M, Barone MV, Caraglia M, Scala S (2014) CXCR4 and CXCR7 transduce through mTOR in human renal cancer cells. Cell Death Dis 5:e1310. https://doi.org/10.1038/cddis.2014.269

Toll-Like Receptors Signaling in the Tumor Microenvironment

5

Kelly D. McCall, Maria Muccioli, and Fabian Benencia

Abstract

The involvement of inflammation in cancer progression is well-established. The immune system can play both tumor-promoting and -suppressive roles, and efforts to harness the immune system to help fight tumor growth are at the forefront of research. Of particular importance is the inflammatory profile at the site of the tumor, with respect to both the leukocyte population numbers, the phenotype of these cells, as well as the contribution of the tumor cells themselves. In this regard, the pro-inflammatory effects of pattern recognition receptor expression and activation in the tumor microenvironment have emerged as a relevant issue both for therapy and to understand tumor development.

Pattern recognition receptors (PRRs) were originally recognized as components of immune cells, particularly innate immune cells, as detectors of pathogens. PRR signaling in immune cells activates them, inducing robust antimicrobial responses. In particular, toll-like receptors (TLRs) constitute a family of membrane-bound PRRs which can recognize pathogen-associated molecular patterns (PAMPs) carried by bacteria, virus, and fungi. In addition, PRRs can recognize products generated by stressed cells or damaged tissues, namely damage-associated molecular patterns or DAMPS. Taking into account the role of the immune system in fighting tumors together with the presence of immune cells in the microenvironment of different types of tumors, strategies to activate immune cells via PRR ligands have been envisioned as an anticancer therapeutic approach.

In the last decades, it has been determined that PRRs are present and functional on nonimmune cells and that their activation in these cells contributes to the inflammation in the tumor microenvironment. Both tumor-promoting and antitumor effects have been observed when tumor cell PRRs are activated. This argues against nonspecific activation of PRR ligands in the tumor microenvironment as

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a therapeutic approach. Therefore, the use of PRR ligands for anticancer therapy might benefit from strategies that specifically deliver these ligands to immune cells, thus avoiding tumor cells in some settings. This review focuses on these aspects of TLR signaling in the tumor microenvironment.

Keywords
Toll-like receptors (TLRs) · Pathogen-associated molecular patterns (PAMPs) · Damage-associated molecular patterns (DAMPs) · Pathogen recognition receptors (PRRs) · Inflammation · Growth factors · MDA5 · RIG-I · Cancer · Tumor microenvironment · Cancer therapy · Inflammation · Macrophages · Dendritic cells · Ismmunosuppression

5.1 Introduction

Toll-like receptors (TLRs) are a particular type of pathogen recognition receptor (PRR) characterized by their association to either the plasma or the endosomal membranes of both immune and nonimmune cells. Originally described in immune cells, their expression has also been identified in nonimmune cells, and, in particular in the context of the present review, in cancerous cells. TLRs act as sensors of infection when activated by pathogen-associated molecular patterns (PAMPs) carried by microbes and are also activated by damage-associated molecular patterns (DAMPs) produced by stressed eukaryotic cells. TLR activation can lead to the production of cytokines, chemokines, and growth factors that can have an autocrine effect and can also help induce immune responses or shape the cellular microenvironment. Their expression in cancer cells has been associated with both beneficial and deleterious outcomes. As such, strategies to target these molecules for anticancer therapeutic approaches have been devised or are under study.

In this chapter, we will discuss the relationship between inflammation and cancer; how antitumor immune responses are elicited; the general characteristics of PRRs with special emphasis in TLRs; the contribution of TLR signaling in the tumor microenvironment to antitumor immune responses; and the negative consequences of TLR signaling in tumor cells.

5.2 Inflammation and Cancer

Inflammation is a broad and complex process that can have a multitude of physiological effects depending on the specific signaling pathways involved. The main function of the immune system is to recognize and clear invasion by pathogens via specific inflammatory responses. However, the immune system also plays an important role in numerous aspects of cancer initiation and progression [1–5]. Broadly, the immune response can be categorized into two parts: innate and adaptive immunity. Initially, invading pathogens (specifically, conserved pathogenic signatures, which can be protein, DNA, or RNA) are recognized by the innate immune cells, and signaling cascades are triggered to facilitate the eventual activation of the adaptive immune response [4]. Thus, innate immunity is primarily responsible for pathogen recognition and helping to mount a more specific antimicrobial response by activating the adaptive immune system [6–8]. Upon recognition of the pathogen, innate immune cells release proinflammatory cytokines, which help trigger the maturation of professional antigen-presenting cells (APCs), such as dendritic cells (DCs). APCs serve to engulf pathogens and then display them on the major histocompatibility (MHC) molecules, special surface proteins that allow for the presentation of processed foreign antigens to T cells. T cells are able to recognize and bind to specific epitopes displayed on the APCs in the context of their MHC–antigen complex, which elicits further signaling that propagates the maturation and expansion of microbe-specific T cells and later B cells. Activated cytotoxic T lymphocytes (CTLs) can recognize (via T-cell receptor)

and kill infected cells expressing the specific antigen using several mechanisms, including the perforin/granzyme pathway, as well as by activating death receptors to trigger apoptosis. Activation of B cells can also promote resolution of infection in several ways, including opsonization, neutralization, and antibody-dependent cellular cytotoxicity. Overall, the inflammatory process allows for the mounting of a more specific adaptive immune response, characterized by antibody-producing and memory B cells along with pathogen-specific cytotoxic T cells, and typically results in pathogen elimination.

It has long been known that an intricate relationship exists between the immune system, inflammation, and cancer development, growth, and regression, and it has been shown that inflammation can lead to both cancer cell growth and cancer cell death [4, 9–15]. The tumor microenvironment is defined by numerous factors, such as the tumor cells themselves, as well as by other cells at the site, including epithelial cells, fibroblasts, immune cells, and extracellular matrix (ECM) components [5, 16–21]. Altogether, the factors released by these cells dictate the nature of the tumor milieu. In fact, the tumor microenvironment is extremely important to consider when assessing cancer prognosis or individualized treatment options for immunotherapy.

In the tumor microenvironment, different cytokines and chemokines can lead to opposing outcomes in cancer progression, depending on the type of inflammation at the site. Some cytokines are immunostimulatory, while others are immunosuppressive, and oftentimes inflammatory responses that lead to immunosuppression can favor tumor growth. In this regard, an "immunosuppressive" state is characterized by immune cell populations that attenuate inflammation (e.g., T-regulatory or myeloid-derived suppressor cells), whereas an "immuno-stimulatory" state would tend to have more active immune cells, such as APCs and CTLs. Furthermore, the activation of these immune cell populations is crucial to proper function, and the tumor environment often exhibits aberrantly activated or dysfunctional immune cells [4, 5, 16, 19, 20, 22–24]. Thus, certain combinations of cytokines and chemokines may recruit and activate immune cell populations that are favorable for tumor eradication, whereas others may result in leukocyte infiltration characteristic of a tumor-favoring environment.

5.3 Antitumor Immune Responses

The immune system is known to have protective effects against cancer [14]. In fact, observations of cancer regression in patients following infection have been recorded for hundreds of years [11]. During the last few decades of the 1800s, American surgeon William Coley purposefully infected cancer patients with combinations of bacterial cocktails, following similar observations, which produced mixed and inconsistent results [11, 25]. At the time it was unclear how these infectious agents were able to sometimes trigger tumor regression, but today, it is known that the immune system can protect against cancer by recognizing specific "danger signals" on tumor cells, and swiftly eliminating them. However, cancer cells are sometimes able to escape this kind of immunosurveillance, a phenomenon that has been termed "immuno-evasion" [9, 14].

Oftentimes, tumor cells express markers on their surface that can be recognized by immune cells (danger signals) [9]. Sometimes, these "danger signals" are specific surface molecules that are upregulated in tumor cells, whereas in other cases the cancer cells can be recognized due to the loss of MHC molecules at their surface [by Natural Killer (NK) cells in particular] [26–28]. Once tumor cells are recognized, an inflammatory response is triggered, and destruction of the cancer cells occurs, thereby inhibiting further growth. Specifically, the pro-apoptotic immune response is typically characterized by immunostimulatory cytokines, such as IL-12 and IL-23, and leads to the activation of NK cells and cytotoxic T lymphocytes (CTLs). These activated leukocytes (i.e., CTLs) can directly attack and kill the tumor cells [9, 14]. Such findings have triggered a keen interest in natural immune responses against cancer in interests of exploiting such pathways to develop novel anticancer treatments. As inflammatory

pathways have a profound effect on the tumor microenvironment that can affect cancer progression, ways to modulate immune components of this environment to favor tumor eradication are rapidly being explored as we and others have shown [9, 10, 15, 29–43].

5.4 Tumor-Promoting Immune Responses

In addition to our understanding of how the immune system can inhibit cancer growth, it has also become clear that long-term (chronic) inflammation is associated with a higher risk of numerous cancers [1, 5, 13, 16, 23]. This has been particularly well-documented in the increased incidence of lung cancer among long-term cigarette smokers and liver cancer (following inflammation from "fatty liver disease"), among other types of cancers in heavy alcohol consumers [44–47]. It is noteworthy to comment that hepatocellular carcinoma can also occur in nonalcoholic fatty liver disease from chronic inflammation due to obesity.

The chronic inflammation often seen in cancers typically renders a tumor microenvironment characterized by immunosuppressive immune cells. Pleiotropic cytokines, such as TNF-α, CCL5, and IL6, can stimulate the recruitment of DCs, macrophages, and other leukocytes to the tumor site [13, 16]. In fact, the type of chronic inflammatory conditions often observed in cancers has leukocytes that tend to produce factors that can facilitate angiogenesis, the development of novel vasculature at the tumor site, thereby promoting cancer growth and potentiating metastasis. For instance, we have previously reported that tumor-infiltrating DCs contribute to this process in the presence of vascular endothelial growth factor (VEGF) [48, 49]. Thus in a scenario where chronic inflammation yields a particular cytokine profile, inflammation can contribute to tumor growth in several ways. Indeed, certain immune cell populations infiltrating the tumor environment are associated with more aggressive disease progression. Immunosuppressive cells that can repress leukocytes that may otherwise fight the tumor include T-regulatory (Treg) cells and myeloid-derived suppressor cells (MDSCs). Furthermore, anergic or dysfunctional white blood cells, including DCs, T cells, and macrophages, have been reported [19, 24, 33, 42, 48, 50–57]. Interestingly, it has been demonstrated that mouse ovarian cancer cells cocultured with macrophages actually shift the leukocytes to a phenotype resembling tumor-associated macrophages (TAM) [54]. These studies highlight the importance of the effects of the cancer cells themselves on the infiltrating immune cell population, as well as on the activation of these leukocytes.

Specific mechanisms by which leukocytes become attracted to the tumor in different cancers remain to be fully investigated. One important inflammatory switch that can trigger the recruitment of proangiogenic leukocytes to the tumor site is nuclear factor kappa B (NF-κB), a family of transcription factors that regulate the production of cytokines, chemokines, and anti-apoptotic and stress response factors [3, 4, 58–60]. Constitutive NF-κB activation has been regarded as a "master switch" associated with cancer progression and has therefore been explored as a therapeutic target [59]. Specifically in ovarian cancer, it has been shown that NF-κB activity increases the aggressiveness of ovarian cancer growth [58, 61]. However, since NF-κB downstream proteins participate in many critical cell cycle functions, the broad-scale disruption of NF-κB pathways for therapeutic purposes can lead to a multitude of undesired physiological effects. It is therefore necessary to investigate specific regulators of NF-κB in hopes of developing more targeted cancer treatments.

Potential regulators of NF-κB include the activation of PRRs, including the TLR protein family, innate immune system proteins that recognize pathogens and trigger inflammation by activating signaling pathways leading to the production of inflammatory molecules and growth factors among other molecules (Fig. 5.1), among others. When expressed in certain cancers, these pathways may thus contribute to

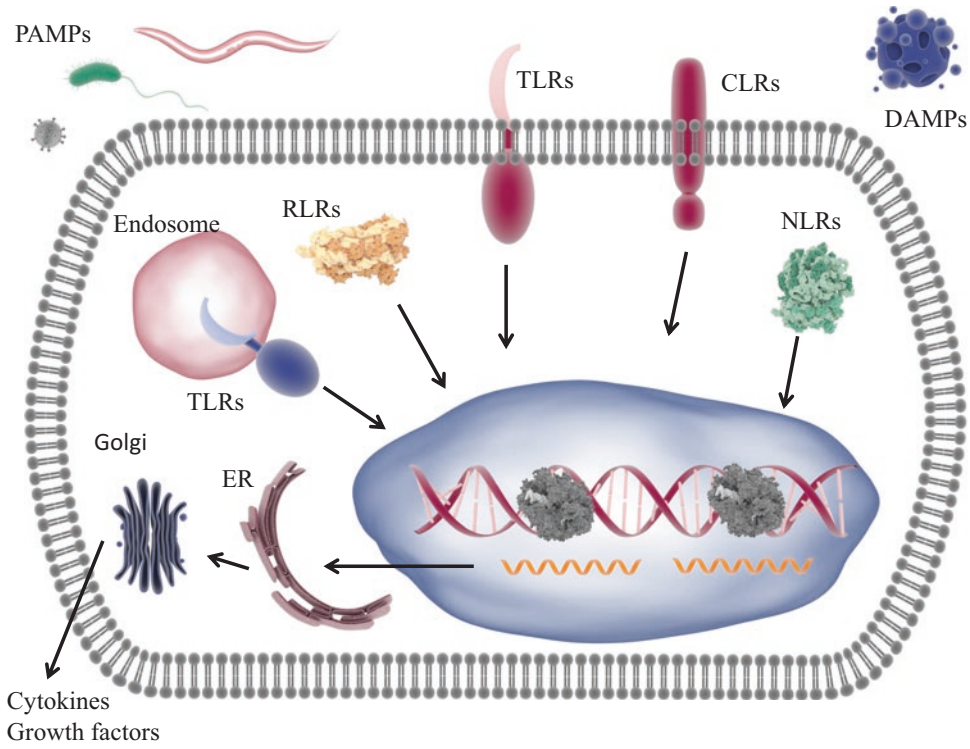

Fig. 5.1 Pattern recognition receptors activate signaling pathways resulting in activation of transduction factors. The intermembrane TLRs and C-lectin-type receptors (CLRs) along with the cytoplasmic NOD-like and RIG1-like receptors (NLRs, RLRs) recognize PAMPs or DAMPS and elicit signaling pathways converging on the activation of transduction factors, resulting in the upregulation of numerous inflammatory factors

tumor progression as we and others have shown [15, 43, 62–66]. Interestingly, TLRs have been reported to have differential effects on tumor growth, depending on the specific TLR and the type of cancer under consideration, as well as the type of cell it is expressed in (tumor cells or leukocyte) [15, 29, 32, 43, 62, 64, 65, 67–74]. Furthermore, other PRRs appear to exhibit notable cross talk with established TLR signaling pathways, underscoring the need of additional characterization of these processes, as they hold potential for novel cancer immunotherapies [41, 75–77]. Specifically, it is critical to investigate signaling pathways and downstream events in different cancer cell types as well as in immune cells to determine the overall effect of PRR-mediated signaling in the tumor environment of cancer development, as well as employ animal models to evaluate their physiological significance.

5.5 Pattern Recognition Receptors

As discussed above, the innate immune response begins with the recognition of the foreign organism or virus, where conserved PAMPs are ligated by PRRs, proteins typically expressed in various immune cells (and also found in other cell types, including tumor cells, as well as normal cells) [35, 65, 77–82]. PAMPs are nonspecific molecules, including common bacterial or viral components, such as lipopolysaccharide (LPS), DNA, or RNA. After PAMP binding to the PRR, signaling pathways ensue that further the immune response and facilitate the clearance of the pathogen. As depicted in Fig. 5.1, PRRs include the TLRs, RIG1-like receptor (RLR), NOD-like receptor (NLR), and C-type lectin receptor (CLR) families of proteins [75–77, 83]. TLRs reside in the plasma (TLR2, TLR4, TLR5) or endosomal (TLR3, TLR7, TLR9)

membranes, recognizing microbial components, including bacterial LPS (TLR4), viral dsRNA (TLR3), viral and bacterial ssDNA (TLR7/8), CpG-DNA (TLR9), and flagellin (TLR5), among others. Conversely, NLRs and RLRs are cytoplasmic factors that sense bacteria and RNA viruses, respectively, while CLRs are present in the plasma membrane and sense fungal components.

As recently reviewed by Roh and Sohn (2018), DAMPS are mainly cellular components released by apoptotic, necrotic, or dead cells. They provide information regarding tissue damage and therefore activate the immune system to fight possible infections or to participate eventually in tissue repairing processes. They are regarded as endogenous danger signals. DAMPS include products of extracellular matrix, proteins present in the cytosol, the nucleus, mitochondria, granules, or plasma membrane of eukaryotic cells. Several PRRS are involved in DAMP recognition. For example, TLR2 is able to recognize versican (extracellular matrix component), Heat shock proteins, or histones among other molecules. TLR4 is able to recognize fibronectin, fibrinogen, heat shock proteins, histones and HMGB1 (high mobility box 1, a nuclear protein), and syndecan from the plasma membrane among other DAMPs. TLR 9 is able to interact with nuclear D and mitochondrial DNA, while TLR3, 7, 8, RIG-I, and MDA5 can interact with nuclear RNA [84].

In the last decades, the interest in PRRs with respect to cancer research has heightened, as it has been consistently demonstrated that PRRs are expressed and functional not only in immune cells but also in numerous cancer types. Furthermore, it appears that PRR signaling (by both immune and tumor cells) can significantly affect disease outcome as it has been extensively reviewed by us and others [1, 2, 4, 35, 41, 62, 63, 79, 85–89]. Interestingly, it is the combinational effect of PRR activation in immune cells (DCs, macrophages, T and B cells, etc.) and PRR-activated signaling in the tumor cells themselves (i.e., ovarian epithelial cells, thyroid cancer cells, pancreatic cancer, melanoma, breast, colon, prostate, and lung cancers, among others) that help determine the overall effect of the inflammatory profile at the tumor site with regard to cancer progression or regression.

5.6 Toll-Like Receptors

TLRs are type I intermembrane proteins, containing a leucine-rich domain (that recognizes the PAMPs) and an intracellular domain that activates signal transduction (Fig. 5.2) [63, 90–92]. First discovered in *Drosophila*, ten TLRs have been identified to date in humans. As discussed, they help

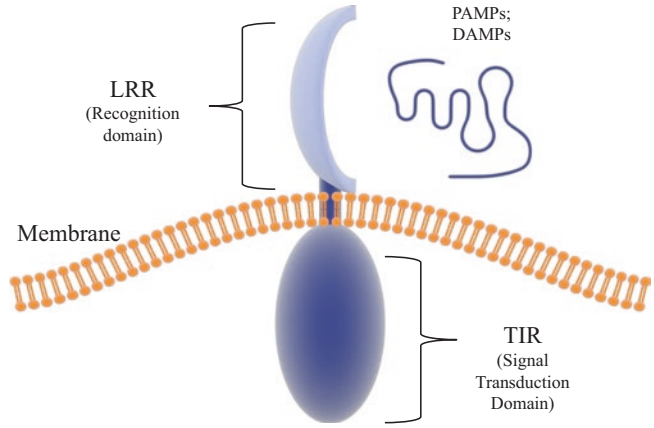

Fig. 5.2 Toll-like receptor (TLR) structure. TLRs are transmembrane receptors that reside at the cell surface or in the endosomal membrane. TIR = Toll-interleukin 1 receptor. The signaling cascades via the TIR domains are mediated by adaptor molecules including MyD88, TIRAP, TRIF, and TRAM, among others. The extracellular domain contains typically between 16 and 30 leucine-rich repeat (LRR) modules. LRRs are responsible for the interaction with PAMPs or DAMPs

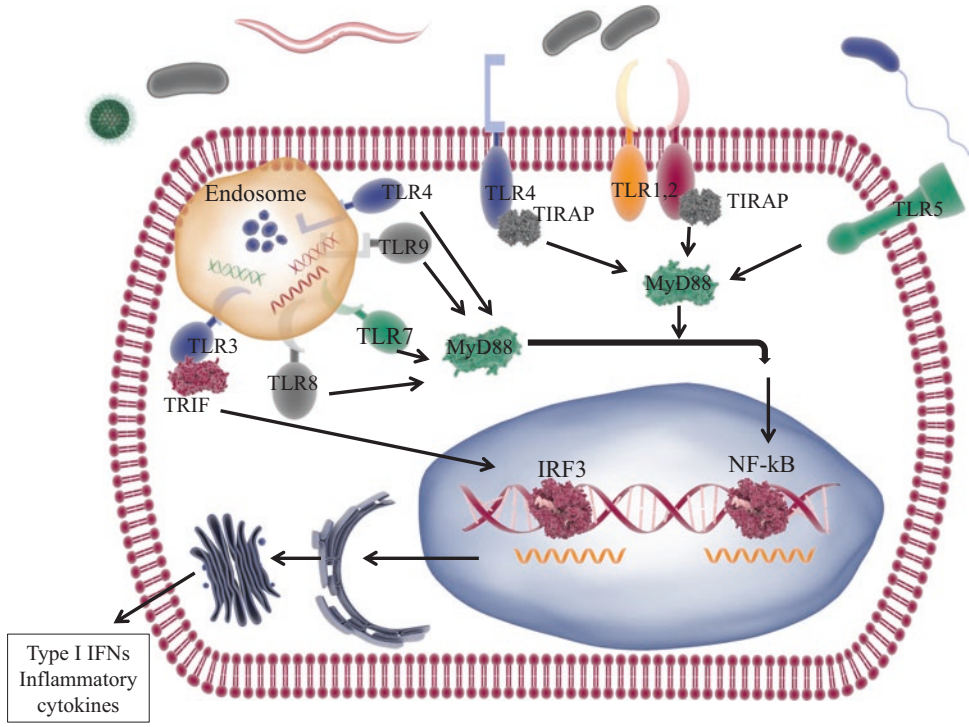

Fig. 5.3 TLR signaling pathways. TLRs reside in the cellular or endosomal membrane, recognize specific pathogen-associated molecular patterns (PAMPs), and activate pro-inflammatory transcription factors (NF-KB and IRF) [85]

serve as the first line of defense against foreign organisms, with different TLRs recognizing specific PAMPs, such as bacterial LPS or viral dsRNA, or DAMPs, and initiating an innate immune response and subsequent adaptive immune responses against infection or tissue damage. As depicted in Fig. 5.3, this response results in increases in many inflammatory cytokines and chemokines, antigen presentation molecules [e.g., major histocompatibility (MHC) genes], and co-stimulatory molecules which are critical for antigen-specific adaptive immunity [92].

In short, the signaling pathways of TLRs intersect and result in the activation of pro-inflammatory transcription factors such as NF-κB and interferon regulatory factors (IRFs) that activate the production and subsequent secretion of multiple inflammatory cytokines and chemokines. Distinct TLRs use different adaptor molecules at their intracellular signaling domains (MyD88, TRIF, TRAM, TIRAP) upon PAMP recognition as shown in Fig. 5.3. Interestingly, TLR signaling pathways have been implicated in multiple types of cancers as well as numerous autoimmune and chronic inflammatory diseases, including Crohn's disease, Hashimoto's thyroiditis, and Type 1 Diabetes, among others [15, 43, 65, 66, 81, 93–96].

5.7 dsRNA-Activated Receptors

The receptors activated and pathways triggered by dsRNA have been characterized in immune cells. Four receptors are known to recognize dsRNA: TLR3, MDA5, RIG1, and PKR. TLR3 is an endosomal receptor, MDA5 and RIG1 are cytosolic helicases, while PKR is a protein kinase activated by dsRNA and also resides in the cytosol [75–77, 83, 97]. TLR3 signaling, in particular, has been well-characterized in immune and nonimmune cells. TLR3 binds to dsRNA of dif-

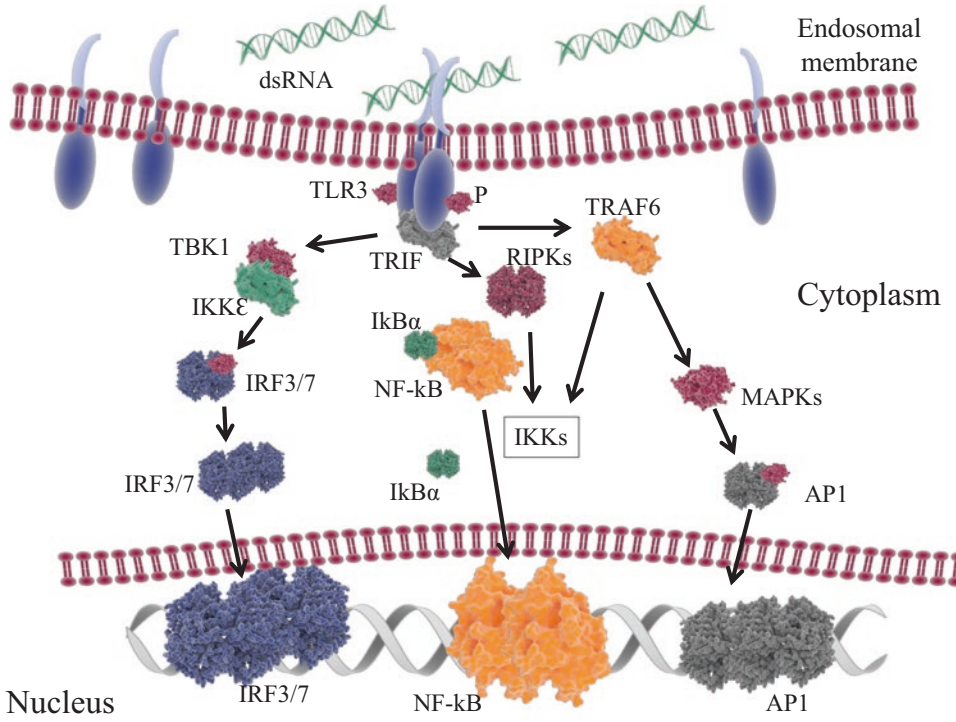

Fig. 5.4 TLR3 signaling pathway. TLR3 resides in the endosomal membrane and senses dsRNA. Upon ligating dsRNA (e.g., from viral infection or cellular debris), TLR3 utilizes the adaptor TRIF and initiates a signaling cascade that results in the activation of NF-KB, IRF3/7, and also AP1 via the MAPK signaling pathway [85]

ferent sizes, with some redundancy, and initiates inflammatory signaling cascades (Fig. 5.4). The signaling pathways downstream of dsRNA recognition converge and can activate pro-apoptotic (IRF3), as well as pro-tumorigenic NF-κB and growth-promoting AP1 transcription factors. Therefore, treatment of cells expressing functional dsRNA receptors with synthetic dsRNA would be expected to induce pro-inflammatory cytokine secretion (as a direct result of NF-κB/AP1 activation and indirect result of type I interferon production/signaling), and type 1 interferon secretion and apoptosis [91, 98]. Thus, these signaling pathways can have a multitude of downstream effects, ranging from cytotoxic T lymphocyte and natural killer cell activation, to differential leukocyte recruitment to areas of infection/damage, to autophagy and apoptosis, to pro-inflammatory cytokine upregulation capable of potentiating angiogenesis and thereby favoring tumor growth. As such, it is of utmost importance to further explore the effects of these pathways in specific tumor types.

5.8 TLR Signaling in the Tumor Microenvironment Contributes to Antitumor Immune Responses

TLR activation in tumor cells has been targeted as an anticancer therapy both in preclinical models and in clinical trials. Indeed, as described by the American Cancer Society, Bacillus Calmette-Guerin (BCG) is the most common intravesical immunotherapy for treating early-stage bladder cancer. The bacterial components are able to activate TLRs in the tumor microenvironment, prompting a localized immune response. Furthermore, the discovery that poly (I:C) treatment of tumor cells leads to apoptosis prompted the use of this TLR3 agonist in clinical trials in

past decades, but resulted in high toxicities that prevented its translation to a clinical setting [99, 100]. Since then, several other TLRs have been investigated for their ability to hinder tumor progress or as targets for anticancer therapies. In fact, the last decade has proven to be groundbreaking in TLR-targeting immunotherapeutic trials for numerous types of neoplasms, as we have previously reviewed [85, 87].Indeed many TLRs have already been targeted for cancer therapy in clinical trials, including for advanced-stage and treatment-resistant patients, mainly as adjuvants, in combination with standard-practice treatment [32, 89, 101]. For example, it has been demonstrated in a preclinical model that poly[I:C] can potentiate BCG immunotherapy for bladder cancer [102].

Regarding the use of TLR ligands on immune cells for anticancer purposes, different strategies have been investigated. APCs such as DCs can be activated in vitro with TLR ligands to induce maturation for use as live vaccines. For example, recent interest has been focused on the use of TLR7 and 8 ligands to prepare human DC vaccines [103]. Interestingly, by using a humanized mouse model, activation of CD141 and CD1c DCs was obtained in vivo via TLR3/8 combined ligation [104]. This opens the door for in vivo targeting of DCs in a cancer patient, eliminating the need for preparing expensive and GMP-approved DC vaccines. In this context, recent preclinical studies have investigated the efficacy of generating tumor antigens fused with TLR2 ligands to promote antitumor immune responses [105].

The focus on activating APCs in the TME is a matter of current interest. For example, it has been shown that intratumoral administration of TLR3 ligands can activate tumor-associated DCs [106]. In addition, intratumoral administration of the TLR9 agonist, CpG, has been shown to induce antitumor immune responses in mouse models of lymphoma and colon carcinoma [107]. Similarly, it has been recently reported that intratumoral injection of IMO-2125, a TLR9 agonist, expands CD8 T cells in a model of colon cancer and induces antitumor immune responses [108]. CpG has also been shown, in a preclinical model, to affect tumor-associated macrophage metabolism, increasing their ability to phagocytize tumor cells and to induce antitumor immune responses [109]. Furthermore, delivery of TLR ligands to DCs, monocytes, or TAMs in the TME microenvironment using nanoparticles has been investigated in order to specifically activate these immune populations [110, 111].

Recently, the ability of TLR ligands to activate NK cells and induce an enhanced killing capacity of tumor cells has been investigated. For example, it has been shown that the TLR5 agonist entomolid, the TLR8 ligand motolimod, or a combination of poly[I;C] plus CpG were able to activate NK cells to promote antitumor immune responses in preclinical models of melanoma, colon, lung, mammary, or head and neck cancer [102, 112–114].

Recent studies aim to promote in situ tumor vaccination by a combination of radiotherapy and TLR7/8 ligands. The rationale being that radiotherapy will induce an immunogenic cell death and the TLR ligands will help differentiate the tumor-associated DCs that can capture death tumor cells into robust activators of the antitumor immune response [115–117].

Finally, expression of TLRs in cancer cells has been, in some cases, associated with a better prognosis. For example, a recent study showed that high RNA levels of TLR1–3, 5–8 were associated with increased overall survival of non-small lung carcinoma patients [118]. Similarly, strong TLR2 and 4 expression were associated with a favorable prognosis in local pancreatic cancer [119], while low expression of TLR4 was associated with a poor prognosis in bladder cancer [120].

5.9 TLR Signaling in Tumor Cells: Negative Consequences

As described above for several cancer types, TLR activation can be of benefit to the patient when activated in immune cells or is associated with a better prognosis when expressed at high levels in some tumors, but in many cases it may be detrimental to clinical outcome when these receptors are triggered in the tumor cells themselves [15, 29, 32, 36, 43, 65–71, 73, 93, 101, 121–123].

With respect to cancer progression, TLRs have warranted particular interest, as the signaling pathways downstream of their activation can result in either pro-apoptotic stimuli or growth-promoting inflammatory conditions [68, 71–74, 86, 121]. While present in immune cells, TLRs are also expressed in different tumor cells, including pancreatic, breast, and ovarian cancers, and can have significant effects on the inflammatory profile of the tumor milieu [62–65, 68–71, 79, 80, 88]. In fact, it appears that tumors can exploit TLRs to their advantage via pathways that may be in part responsible for the constitutive activation of NF-κB frequently seen in cancers [4, 58, 61].

It has been demonstrated that TLR signaling pathways in tumor cells can affect cancer progression [64]. Stimulation of TLRs in tumor cells fosters chronic inflammation that drives cancer cell proliferation, migration, and angiogenesis, and establishes a tumor microenvironment that impairs the immune system, thereby allowing tumors to establish themselves and to thrive. Indeed, some of the foundational studies that laid the groundwork for implicating and understanding the role of TLR signaling in nonimmune cancer cells (and in nonimmune cells in general) were conducted in thyroid cancer. Specifically, McCall et al. reported for the first time in 2007 that functional TLR3 was present in human papillary thyroid cancer (PTC) cell lines at high basal levels consistent with its overexpression in PTC cells in vivo and that a novel inhibitor of TLR3 and TLR4 signaling, phenylmethimazole (C10), inhibited TLR3 expression and signaling in these cells along with tumor cell proliferation and migration [65]. This study was one of the first to implicate aberrant TLR expression and signaling in nonimmune cells to play a role in tumor progression and was followed by similar studies implicating TLR3 and TLR4 expression and signaling in the promotion and progression of other types of tumors including pancreatic cancer, malignant melanoma, and breast cancer, to name a few [15]. Moreover, TLR4, perhaps the best-studied TLR, has been shown to promote cancer growth in human head and neck cancers, lung cancers, and ovarian cancers, among others [63, 71]. Likely, this is at least in part due to the activation of NF-κB-controlled cytokines that can promote tumor cell proliferation, induce angiogenic factor production by tumor cells, or affects immune cells in the TME inducing a pro-tumor behavior of these cells. For example, it has been recently reported that TLR4 signaling was able to induce proliferation of esophageal cancer cells in vitro and its expression was with advanced stage and poor prognosis in esophageal adenocarcinoma [124, 125].

Furthermore, it has been recently shown via analysis of human tissues and in vitro studies that TLR4 activation can promote angiogenesis in pancreatic cancer by inducing VEGF production by cancer cells [126]. In addition, it has been recently reported that TLR4 activation in tumor cells can induce chemotherapy resistance, in particular resistance to fludarabine in acute myeloid leukemia cells and to paclitaxel in human breast and melanoma cancer cells [127, 128]. Also, a high level of polymorphism in TLR4 and other TLRs has been observed in tumor samples recovered from cancer patients [129].

Interestingly, signaling via TLR4 in cancer cells and tumor progression can vary within a particular type of cancer. For example, it has been shown that in the context of ovarian cancer, there is an association among MyD88 expression and poor survival in high-grade serous ovarian carcinoma; in contrast, MyD88 and TLR4 expression are associated with improved survival in low-grade serous carcinoma [130]. Moreover, an association between MyD88 and TLR4 levels and metastasis was also observed in some types of breast cancer [131]. In addition, recent studies show a link between HPV subtypes associated with cervical cancer and their ability to activate TLR4 to induce proliferation or resistance to apoptosis, thereby highlighting another link between microbial infection and cancer [132].

The effects of other TLRs on tumor development are being investigated, and it appears that outcomes vary greatly depending on the specific TLR and tumor type under consideration [2, 63]. In particular, TLR2 overexpression in many types of cancers seems to be associated with a worse prognosis or tumor cell proliferation. For example, it has been recently shown that signal-

ing through TLR2 induces the proliferation of different cancer cell types such as human squamous carcinoma cells, Mantle cell lymphoma cells, squamous cell carcinoma cells, colorectal cancer cells, and glioma cells [133–137]. In addition, TLR2 expression has recently been demonstrated to be upregulated and associated with a poor prognosis in gastric cancer [138]. Furthermore, TLR2 has been proposed as a marker of angiogenesis in human medulloblastoma [139]. Again, highlighting a link between cancer and infection, it has been recently demonstrated in a preclinical model that NSCL cancer metastasis is increased by gram-positive pneumonia via TLR2 activation [140].

With respect to TLR3, recent data has shown that signaling via this PRR in tumor cells can be detrimental to the patients. For example, altered expression of TLR3 was found on metastatic intestinal epithelial cells [141]. In this case, TLR3 was observed on the surface of the cells, opposed to the membrane of endosomes, which is its typical location. Furthermore, it has been recently shown in vitro that TLR3 signaling can promote tumor growth and cisplatin resistance in head and neck cancer cells, inducing the Warburg effect on these cells and therefore adaptation to hypoxia, and that it can contribute to tumor immunoescape by inducing PDL1 expression as demonstrated in human neuroblastoma cells [64, 142, 143].

Our own studies demonstrate the relevance of dsRNA-induced inflammation in both breast and ovarian tumor cells, as we found significant increases in both pro-tumor and antitumor cytokines such as IL6 and CCL5, respectively, in response to the dsRNA analogs poly (I:C) and poly (A:U) in several cell lines tested. Furthermore, we observed that in addition to TLR3, other dsRNA receptors (namely MDA5, RIG1, and PKR) may also play a role in this response [38, 40]. We determined that all of these dsRNA-sensing receptors are involved in the observed dsRNA-induced cytokine production [38]. Importantly, we found distinct differences in the contribution of each receptor to this process in the different cell lines, further underscoring the need to continue to characterize these pathways in different tumor types and subtypes [38, 40].

Finally, it has also been considered that TLR3 expression might be detrimental to tumor virotherapy by providing resistance to the oncolytic virus. Indeed, in a preclinical model it has been demonstrated that downregulation of TLR3 in colorectal cancer cells allows for a better response against oncolytic reovirus [144]. Therefore, expression of TLR3 in cancer cells might also provide information on the success of oncolytic virotherapy in a particular patient subset.

Another TLR investigated for its relevance in cancer development is TLR9. For example, it has been shown that TLR9 expression is upregulated in osteosarcoma as determined by an analysis of patient samples [145]. In a preclinical model, it has been reported that expression of this molecule in prostate cancer cells could lead to the accumulation of granulocytic MDSCs, which are known suppressors of antitumor immune responses [146]. Finally, it has been recently demonstrated that breast cancer cells with low TLR9 expression were particularly sensitive to bisphosphonates used for bone metastases [147]. In summary, the major possible outcomes of TLR activation in tumor cells and the subsequent effect on cancer growth are summarized in Fig. 5.5. Specifically, activation of the transcription factor IRF3 (downstream of TLR engagement) results in the production of type I interferons, induction of co-stimulatory molecules, and may also activate the caspase cascade leading to apoptosis [148]. In this way, IRF3 activation in tumor cells may contribute to tumor eradication [90]. However, as discussed in the section above, the intra-tumoral NF-κB activity resulting from TLR stimulation may contribute to cancer progression, likely via the increased secretion of cytokines and chemokines that can recruit immunosuppressive and proangiogenic leukocytes to the tumor site. This is due to the production of growth factors that induce cancer cell proliferation, and by increasing resistance to hypoxia or chemotherapy. In this context, the use of TLR inhibitors as a therapeutic approach to fight certain types of cancer may benefit patient survival. As discussed above, inhibition of some of these molecules in can-

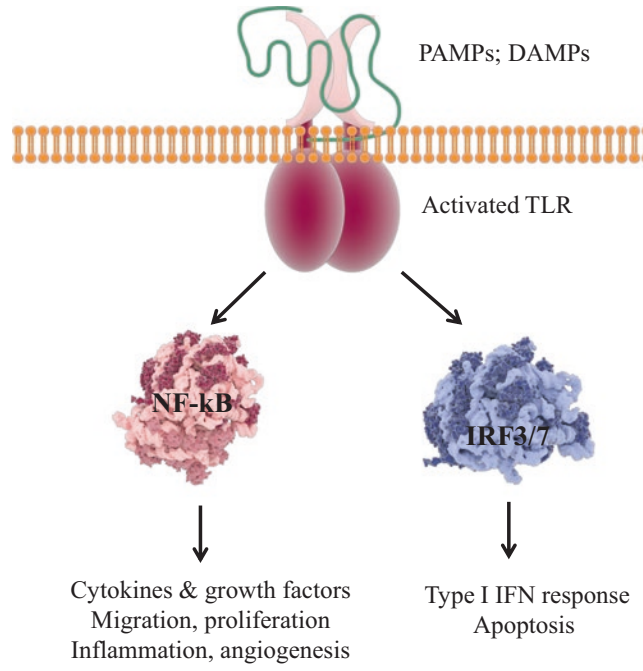

Fig. 5.5 Major potential outcomes of TLR signaling in tumor cells. TLR signaling pathways can activate several transcription factors that can have different effects on tumor growth

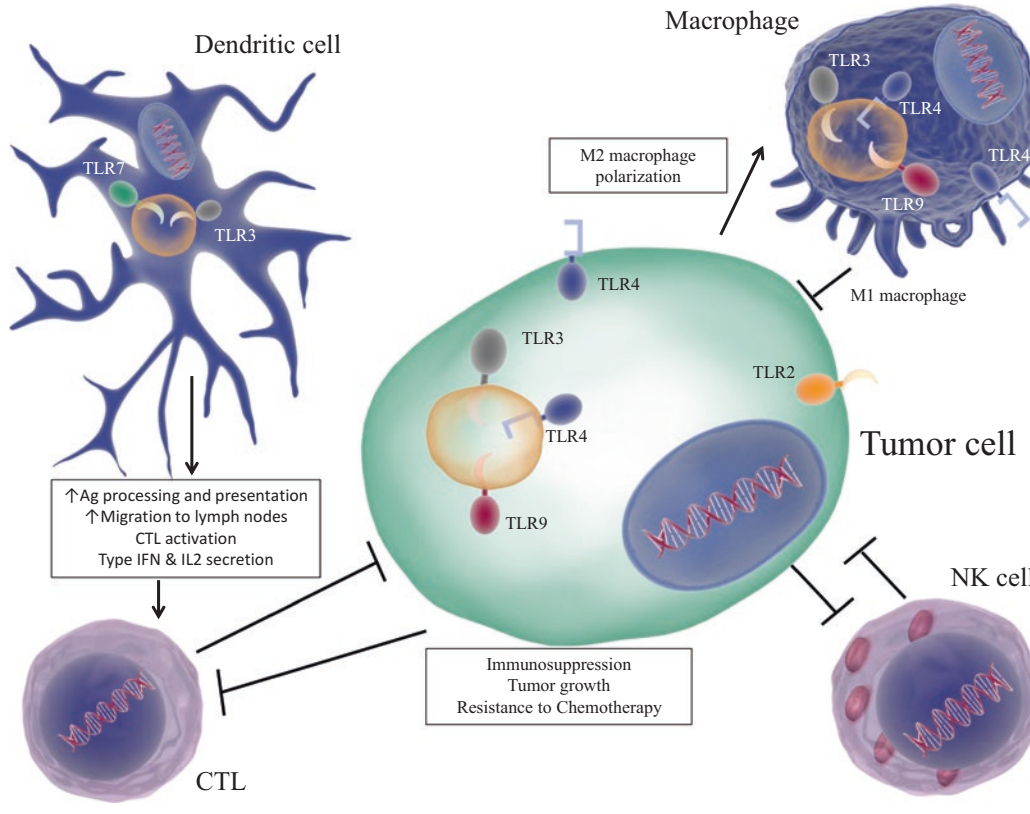

Fig. 5.6 Differential effects of TLR signaling in tumor cells and immune cells. TLR signaling in immune cells may help activate the antitumor immune response, whereas their activation in ovarian cancer cells appears to promote immunosuppression, resistance to therapy, and increased metastasis, furthering tumor growth

cers cells can lead to a decrease in tumor growth and can be used in combinatorial therapies with other anticancer agents.

5.10 Future Trends and Directions

As we have discussed, TLR engagement in tumor cells tends to have effects favoring tumor progression, including increased potential for migration, immunosuppressive effects, and resistance to chemotherapy. However, TLR stimulation in certain leukocytes, including macrophages and DCs, can help orchestrate antitumor immune response. This is illustrated in Fig. 5.6.

Since targeting TLR activity in cancer cells and possibly concurrently stimulating them in immune cells may be therapeutically beneficial in cancer treatment, TLRs have become major players in the search for novel antitumor drug targets as we have presented throughout this review. Thus, characterization of specific PRR signaling pathways, downstream cytokine secretion, along with in vivo studies examining the effects of these pathways on cancer progression, is needed to determine the best targets for immunotherapy to specific cancer types.

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References

1. de Visser KE, Eichten A, Coussens LM (2006) Paradoxical roles of the immune system during cancer development. Nat Rev Cancer 6(1):24–37
2. Fridman WH et al (2012) The immune contexture in human tumours: impact on clinical outcome. Nat Rev Cancer 12(4):298–306
3. Balkwill FR, Mantovani A (2012) Cancer-related inflammation: common themes and therapeutic opportunities. Semin Cancer Biol 22(1):33–40
4. Grivennikov SI, Greten FR, Karin M (2010) Immunity, inflammation, and cancer. Cell 140(6):883–899
5. Nelson D, Ganss R (2006) Tumor growth or regression: powered by inflammation. J Leukoc Biol 80(4):685–690
6. Medzhitov R, Janeway CA Jr (1997) Innate immunity: impact on the adaptive immune response. Curr Opin Immunol 9(1):4–9
7. Janeway CA Jr, Medzhitov R (2002) Innate immune recognition. Annu Rev Immunol 20:197–216
8. Fearon DT, Locksley RM (1996) The instructive role of innate immunity in the acquired immune response. Science 272(5258):50–53
9. Dranoff G (2004) Cytokines in cancer pathogenesis and cancer therapy. Nat Rev Cancer 4(1):11–22
10. Finn OJ (2008) Cancer immunology. N Engl J Med 358(25):2704–2715
11. Hobohm U (2001) Fever and cancer in perspective. Cancer Immunol Immunother 50(8):391–396
12. Lavoue V et al (2013) Immunity of human epithelial ovarian carcinoma: the paradigm of immune suppression in cancer. J Transl Med 11:147
13. Lin WW, Karin M (2007) A cytokine-mediated link between innate immunity, inflammation, and cancer. J Clin Invest 117(5):1175–1183
14. Schreiber RD, Old LJ, Smyth MJ (2011) Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. Science 331(6024):1565–1570
15. Schwartz AL et al (2009) Phenylmethimazole decreases toll-like receptor 3 and noncanonical Wnt5a expression in pancreatic cancer and melanoma together with tumor cell growth and migration. Clin Cancer Res 15(12):4114–4122
16. Ruegg C (2006) Leukocytes, inflammation, and angiogenesis in cancer: fatal attractions. J Leukoc Biol 80(4):682–684
17. Ronnov-Jessen L et al (1995) The origin of the myofibroblasts in breast cancer. Recapitulation of tumor environment in culture unravels diversity and implicates converted fibroblasts and recruited smooth muscle cells. J Clin Invest 95(2):859–873
18. Kerkar SP, Restifo NP (2012) Cellular constituents of immune escape within the tumor microenvironment. Cancer Res 72(13):3125–3130
19. Ostrand-Rosenberg S et al (2012) Cross-talk between myeloid-derived suppressor cells (MDSC), macrophages, and dendritic cells enhances tumor-induced immune suppression. Semin Cancer Biol 22(4):275–281
20. Egeblad M, Nakasone ES, Werb Z (2010) Tumors as organs: complex tissues that interface with the entire organism. Dev Cell 18(6):884–901
21. Maltby S, Khazaie K, McNagny KM (2009) Mast cells in tumor growth: angiogenesis, tissue remodelling and immune-modulation. Biochim Biophys Acta 1796(1):19–26
22. Conejo-Garcia JR et al (2005) Vascular leukocytes contribute to tumor vascularization. Blood 105(2):679–681
23. Coussens LM, Pollard JW (2011) Leukocytes in mammary development and cancer. Cold Spring

Harb Perspect Biol 3(3). https://doi.org/10.1101/cshperspect.a003285
24. Scarlett UK et al (2012) Ovarian cancer progression is controlled by phenotypic changes in dendritic cells. J Exp Med 209(3):495–506
25. Hoption Cann SA et al (2002) Spontaneous regression: a hidden treasure buried in time. Med Hypotheses 58(2):115–119
26. Zamai L et al (2007) NK cells and cancer. J Immunol 178(7):4011–4016
27. Marcus A et al (2014) Recognition of tumors by the innate immune system and natural killer cells. Adv Immunol 122:91–128
28. Raulet DH, Guerra N (2009) Oncogenic stress sensed by the immune system: role of natural killer cell receptors. Nat Rev Immunol 9(8):568–580
29. Scarlett UK et al (2009) In situ stimulation of CD40 and Toll-like receptor 3 transforms ovarian cancer-infiltrating dendritic cells from immunosuppressive to immunostimulatory cells. Cancer Res 69(18):7329–7337
30. Cubillos-Ruiz JR, Rutkowski M, Conejo-Garcia JR (2010) Blocking ovarian cancer progression by targeting tumor microenvironmental leukocytes. Cell Cycle 9(2):260–268
31. Benencia F, Muccioli M, Alnaeeli M (2014) Perspectives on reprograming cancer-associated dendritic cells for anti-tumor therapies. Front Oncol 4:72
32. Adams M et al (2005) The rationale for combined chemo/immunotherapy using a toll-like receptor 3 (TLR3) agonist and tumour-derived exosomes in advanced ovarian cancer. Vaccine 23(17–18):2374–2378
33. Sica A et al (2006) Tumour-associated macrophages are a distinct M2 polarised population promoting tumour progression: potential targets of anti-cancer therapy. Eur J Cancer 42(6):717–727
34. Dang Y et al (2018) TLR8 ligation induces apoptosis of monocytic myeloid-derived suppressor cells. J Leukoc Biol 103(1):157–164
35. Yu L, Chen S (2008) Toll-like receptors expressed in tumor cells: targets for therapy. Cancer Immunol Immunother 57(9):1271–1278
36. Smits EL et al (2008) The use of TLR7 and TLR8 ligands for the enhancement of cancer immunotherapy. Oncologist 13(8):859–875
37. Smith AL, Robin TP, Ford HL (2012) Molecular pathways: targeting the TGF-beta pathway for cancer therapy. Clin Cancer Res 18(17):4514–4521
38. Muccioli M et al (2018) Modulation of double-stranded RNA pattern recognition receptor signaling in ovarian cancer cells promotes inflammatory queues. Oncotarget 9(94):36666–36683
39. Guo Y et al (2012) Interleukin-6 signaling pathway in targeted therapy for cancer. Cancer Treat Rev 38(7):904–910
40. Venkatesh A et al (2018) Regulation of inflammatory factors by double-stranded RNA receptors in breast cancer cells. Immunobiology 223(6–7):466–476
41. Goutagny N et al (2012) Targeting pattern recognition receptors in cancer immunotherapy. Target Oncol 7(1):29–54
42. Guiducci C et al (2005) Redirecting in vivo elicited tumor infiltrating macrophages and dendritic cells towards tumor rejection. Cancer Res 65(8):3437–3446
43. Schwartz AL et al (2017) TLR signaling inhibitor, phenylmethimazole, in combination with tamoxifen inhibits human breast cancer cell viability and migration. Oncotarget 8(69):113295–113302
44. Hecht SS (1999) Tobacco smoke carcinogens and lung cancer. J Natl Cancer Inst 91(14):1194–1210
45. Auerbach O et al (1961) Changes in bronchial epithelium in relation to cigarette smoking and in relation to lung cancer. N Engl J Med 265:253–267
46. Seitz HK, Simanowski UA (1988) Alcohol and carcinogenesis. Annu Rev Nutr 8:99–119
47. Seitz HK, Stickel F (2007) Molecular mechanisms of alcohol-mediated carcinogenesis. Nat Rev Cancer 7(8):599–612
48. Conejo-Garcia JR et al (2004) Tumor-infiltrating dendritic cell precursors recruited by a beta-defensin contribute to vasculogenesis under the influence of Vegf-a. Nat Med 10(9):950–958
49. Huarte E et al (2008) Depletion of dendritic cells delays ovarian cancer progression by boosting antitumor immunity. Cancer Res 68(18):7684–7691
50. Curiel TJ et al (2004) Dendritic cell subsets differentially regulate angiogenesis in human ovarian cancer. Cancer Res 64(16):5535–5538
51. Riboldi E et al (2005) Cutting edge: proangiogenic properties of alternatively activated dendritic cells. J Immunol 175(5):2788–2792
52. Conrad C et al (2012) Plasmacytoid dendritic cells promote immunosuppression in ovarian cancer via ICOS costimulation of Foxp3(+) T-regulatory cells. Cancer Res 72(20):5240–5249
53. Mantovani A et al (2002) Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. Trends Immunol 23(11):549–555
54. Hagemann T et al (2006) Ovarian cancer cells polarize macrophages toward a tumor-associated phenotype. J Immunol 176(8):5023–5032
55. Heusinkveld M, van der Burg SH (2011) Identification and manipulation of tumor associated macrophages in human cancers. J Transl Med 9:216
56. Wang X et al (2013) Interaction of monocytes/macrophages with ovarian cancer cells promotes angiogenesis in vitro. Cancer Sci 104(4):516–523
57. Goyne HE et al (2014) Ovarian tumor ascites CD14+ cells suppress dendritic cell-activated CD4+ T-cell responses through IL-10 secretion and indoleamine 2,3-dioxygenase. J Immunother 37(3):163–169
58. Hernandez L et al (2010) Activation of NF-kappaB signaling by inhibitor of NF-kappaB kinase beta increases aggressiveness of ovarian cancer. Cancer Res 70(10):4005–4014

59. Karin M et al (2002) NF-kappa B in cancer: from innocent bystander to major culprit. Nat Rev Cancer 2(4):301–310
60. Wu Y, Zhou BP (2010) TNF-alpha/NF-kappa B/Snail pathway in cancer cell migration and invasion. Br J Cancer 102(4):639–644
61. Annunziata CM et al (2010) Nuclear factor kappa B transcription factors are coexpressed and convey a poor outcome in ovarian cancer. Cancer 116(13):3276–3284
62. Killeen SD et al (2006) Exploitation of the toll-like receptor system in cancer: a doubled-edged sword? Br J Cancer 95(3):247–252
63. Chen KQ et al (2007) Toll-like receptors in inflammation, infection and cancer. Int Immunopharmacol 7(10):1271–1285
64. Chuang HC et al (2012) Toll-like receptor 3-mediated tumor invasion in head and neck cancer. Oral Oncol 48(3):226–232
65. McCall KD et al (2007) High basal levels of functional toll-like receptor 3 (TLR3) and noncanonical Wnt5a are expressed in papillary thyroid cancer and are coordinately decreased by phenylmethimazole together with cell proliferation and migration. Endocrinology 148(9):4226–4237
66. Noori MS et al (2017) Phenylmethimazole and a thiazole derivative of phenylmethimazole inhibit IL-6 expression by triple negative breast cancer cells. Eur J Pharmacol 803:130–137
67. Bellora F et al (2014) TLR activation of tumor-associated macrophages from ovarian cancer patients triggers cytolytic activity of NK cells. Eur J Immunol 44(6):1814–1822
68. Chefetz I et al (2013) TLR2 enhances ovarian cancer stem cell self-renewal and promotes tumor repair and recurrence. Cell Cycle 12(3):511–521
69. Conforti R et al (2010) Opposing effects of toll-like receptor (TLR3) signaling in tumors can be therapeutically uncoupled to optimize the anticancer efficacy of TLR3 ligands. Cancer Res 70(2):490–500
70. He WG et al (2007) TLR4 signaling promotes immune escape of human lung cancer cells by inducing immunosuppressive cytokines and apoptosis resistance. Mol Immunol 44(11):2850–2859
71. Kelly MG et al (2006) TLR-4 signaling promotes tumor growth and paclitaxel chemoresistance in ovarian cancer. Cancer Res 66(7):3859–3868
72. Matijevic T, Pavelic J (2011) The dual role of TLR3 in metastatic cell line. Clin Exp Metastasis 28(7):701–712
73. Salaun B et al (2006) TLR3 can directly trigger apoptosis in human cancer cells. J Immunol 176(8):4894–4901
74. Woods DC et al (2011) TLR4 activates NF-kappa B in human ovarian granulosa tumor cells. Biochem Biophys Res Commun 409(4):675–680
75. Gordon S (2002) Pattern recognition receptors: doubling up for the innate immune response. Cell 111(7):927–930
76. Meylan E, Tschopp J, Karin M (2006) Intracellular pattern recognition receptors in the host response. Nature 442(7098):39–44
77. Takeuchi O, Akira S (2010) Pattern recognition receptors and inflammation. Cell 140(6):805–820
78. Matsukura S et al (2007) Role of RIG-I, MDA-5, and PKR on the expression of inflammatory chemokines induced by synthetic dsRNA in airway epithelial cells. Int Arch Allergy Immunol 143:80–83
79. Chen R et al (2007) Inflammation, cancer and chemoresistance: taking advantage of the toll-like receptor signaling pathway. Am J Reprod Immunol 57(2):93–107
80. Zhou MF et al (2009) Toll-like receptor expression in normal ovary and ovarian tumors. Cancer Immunol Immunother 58(9):1375–1385
81. O'Neill LA (2003) Therapeutic targeting of toll-like receptors for inflammatory and infectious diseases. Curr Opin Pharmacol 3(4):396–403
82. Matijevic T, Marjanovic M, Pavelic J (2009) Functionally active Toll-like receptor 3 on human primary and metastatic cancer cells. Scand J Immunol 70(1):18–24
83. Kawai T, Akira S (2010) The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nat Immunol 11(5):373–384
84. Roh JS, Sohn DH (2018) Damage-associated molecular patterns in inflammatory diseases. Immune Netw 18(4):e27
85. Muccioli M et al (2012) Toll-like receptors as novel therapeutic targets for ovarian cancer. ISRN Oncol 2012:642141
86. Huang B et al (2008) TLR signaling by tumor and immune cells: a double-edged sword. Oncogene 27(2):218–224
87. Muccioli M, Benencia F (2014) Toll-like receptors in ovarian cancer as targets for immunotherapies. Front Immunol 5:341
88. Chen R et al (2008) Cancers take their Toll–the function and regulation of Toll-like receptors in cancer cells. Oncogene 27(2):225–233
89. Seya T, Matsumoto M (2009) The extrinsic RNA-sensing pathway for adjuvant immunotherapy of cancer. Cancer Immunol Immunother 58(8):1175–1184
90. Akira S, Takeda K, Kaisho T (2001) Toll-like receptors: critical proteins linking innate and acquired immunity. Nat Immunol 2(8):675–680
91. Takeda K, Akira S (2004) TLR signaling pathways. Semin Immunol 16(1):3–9
92. Takeda K, Kaisho T, Akira S (2003) Toll-like receptors. Annu Rev Immunol 21:335–376
93. Harii N et al (2005) Thyrocytes express a functional toll-like receptor 3: overexpression can be induced by viral infection and reversed by phenylmethimazole and is associated with Hashimoto's autoimmune thyroiditis. Mol Endocrinol 19(5):1231–1250
94. Wong FS et al (2008) The role of Toll-like receptors 3 and 9 in the development of autoimmune diabetes in NOD mice. Ann N Y Acad Sci 1150:146–148

95. McCall KD et al (2013) Phenylmethimazole suppresses dsRNA-induced cytotoxicity and inflammatory cytokines in murine pancreatic Beta cells and blocks viral acceleration of type 1 diabetes in NOD mice. Molecules 18(4):3841–3858
96. Bsibsi M et al (2002) Broad expression of toll-like receptors in the human central nervous system. J Neuropathol Exp Neurol 61(11):1013–1021
97. Van DN et al (2012) Innate immune agonist, dsRNA, induces apoptosis in ovarian cancer cells and enhances the potency of cytotoxic chemotherapeutics. FASEB J 26(8):3188–3198
98. Kawai T, Akira S (2006) TLR signaling. Cell Death Differ 13(5):816–825
99. Krown SE et al (1985) Phase-I trials of Poly(I,C) complexes in advanced cancer. J Biol Response Mod 4(6):640–649
100. Robinson RA et al (1976) Phase 1-2 trial of multiple-dose polyriboinosinic-polyribocytidylic acid in patients with Leukemia or solid tumors. J Natl Cancer Inst 57(3):599–602
101. Geller MA et al (2010) Toll-like receptor-7 agonist administered subcutaneously in a prolonged dosing schedule in heavily pretreated recurrent breast, ovarian, and cervix cancers. Cancer Immunol Immunother 59(12):1877–1884
102. Ayari C et al (2016) Poly(I:C) potentiates bacillus Calmette-Guerin immunotherapy for bladder cancer. Cancer Immunol Immunother 65(2):223–234
103. Yi DH et al (2018) 3-day monocyte-derived dendritic cells stimulated with a combination of OK432, TLR7/8 ligand, and prostaglandin E2 are a promising alternative for cancer immunotherapy. Cancer Immunol Immunother 67(10):1611–1620
104. Pearson FE et al (2018) Activation of human CD141(+) and CD1c(+) dendritic cells in vivo with combined TLR3 and TLR7/8 ligation. Immunol Cell Biol 96(4):390–400
105. Wu CC et al (2016) A toll-like receptor 2 agonist-fused antigen enhanced antitumor immunity by increasing antigen presentation and the CD8 memory T cells population. Oncotarget 7(21):30804–30819
106. Nocera DA et al (2016) In vivo visualizing the IFN-beta response required for tumor growth control in a therapeutic model of Polyadenylic-Polyuridylic acid administration. J Immunol 196(6):2860–2869
107. Wang S et al (2016) Intratumoral injection of a CpG oligonucleotide reverts resistance to PD-1 blockade by expanding multifunctional CD8+ T cells. Proc Natl Acad Sci U S A 113(46):E7240–E7249
108. Wang D et al (2018) Modulation of the tumor microenvironment by intratumoral administration of IMO-2125, a novel TLR9 agonist, for cancer immunotherapy. Int J Oncol 53(3):1193–1203
109. Liu MG et al (2019) Metabolic rewiring of macrophages by CpG potentiates clearance of cancer cells and overcomes tumor-expressed CD47-mediated 'don't-eat-me' signal. Nat Immunol 20(3):265–275
110. Rodell CB et al (2018) TLR7/8-agonist-loaded nanoparticles promote the polarization of tumour-associated macrophages to enhance cancer immunotherapy. Nat Biomed Eng 2(8):578–588
111. Klauber TCB et al (2017) Delivery of TLR7 agonist to monocytes and dendritic cells by DCIR targeted liposomes induces robust production of anti-cancer cytokines. Acta Biomater 53:367–377
112. Dietsch GN et al (2016) Coordinated activation of Toll-like Receptor8 (TLR8) and NLRP3 by the TLR8 agonist, VTX-2337, ignites Tumoricidal natural killer cell activity. PLoS One 11(2):e0148764
113. Yang H et al (2016) The toll-like receptor 5 agonist entolimod suppresses hepatic metastases in a murine model of ocular melanoma via an NK cell-dependent mechanism. Oncotarget 7(3):2936–2950
114. Brackett CM et al (2016) Toll-like receptor-5 agonist, entolimod, suppresses metastasis and induces immunity by stimulating an NK-dendritic-CD8(+) T-cell axis. Proc Natl Acad Sci U S A 113(7):E874–E883
115. Cho JH et al (2017) The TLR7 agonist imiquimod induces anti-cancer effects via autophagic cell death and enhances anti-tumoral and systemic immunity during radiotherapy for melanoma. Oncotarget 8(15):24932–24948
116. Dovedi SJ et al (2016) Intravenous administration of the selective toll-like receptor 7 agonist DSR-29133 leads to anti-tumor efficacy in murine solid tumor models which can be potentiated by combination with fractionated radiotherapy. Oncotarget 7(13):17035–17046
117. Yoshida S et al (2018) Toll-like receptor 3 signal augments radiation-induced tumor growth retardation in a murine model. Cancer Sci 109(4):956–965
118. Bauer AK et al (2017) Toll-like receptor expression in human non-small cell lung carcinoma: potential prognostic indicators of disease. Oncotarget 8(54):91860–91875
119. Lanki MA et al (2018) Toll-like receptor 2 and toll-like receptor 4 predict favorable prognosis in local pancreatic cancer. Tumour Biol 40(9):1010428318801188
120. Kusuhara Y et al (2019) Low expression of Toll-like receptor 4 is associated with poor prognosis in bladder cancer. Anticancer Res 39(2):703–711
121. Jiang Q, Wei HM, Tian ZG (2008) Poly I: C enhances cycloheximide-induced apoptosis of tumor cells through TLR3 pathway. BMC Cancer 8:12
122. Peng GY et al (2005) Toll-like, receptor 8-mediated reversal of CD4(+) regulatory T cell function. Science 309(5739):1380–1384
123. Lu H et al (2012) VTX-2337 is a novel TLR8 agonist that activates NK cells and augments ADCC. Clin Cancer Res 18(2):499–509
124. Kohtz PD et al (2019) Toll-like Receptor-4 is a mediator of proliferation in esophageal adenocarcinoma. Ann Thorac Surg 107(1):233–241
125. Huhta H et al (2016) Toll-like receptors 1, 2, 4 and 6 in esophageal epithelium, Barrett's esopha-

gus, dysplasia and adenocarcinoma. Oncotarget 7(17):23658–23667
126. Sun YL et al (2016) Toll-like receptor 4 promotes angiogenesis in pancreatic cancer via PI3K/AKT signaling. Exp Cell Res 347(2):274–282
127. Huy H et al (2018) TLR4/NF-kappa B axis induces fludarabine resistance by suppressing TXNIP expression in acute myeloid leukemia cells. Biochem Biophys Res Commun 506(1):33–40
128. Sootichote R et al (2018) Compound a attenuates toll-like receptor 4-mediated paclitaxel resistance in breast cancer and melanoma through suppression of IL-8. BMC Cancer 18(1):231
129. Messaritakis I et al (2018) Evaluation of the detection of Toll-like receptors (TLRs) in cancer development and progression in patients with colorectal cancer. PLoS One 13(6):e0197327
130. Block MS et al (2018) MyD88 and TLR4 expression in epithelial ovarian cancer. Mayo Clin Proc 93(3):307–320
131. Wu K et al (2018) TLR4/MyD88 signaling determines the metastatic potential of breast cancer cells. Mol Med Rep 18(3):3411–3420
132. Jiang N et al (2017) Toll-like receptor 4 promotes proliferation and apoptosis resistance in human papillomavirus-related cervical cancer cells through the Toll-like receptor 4/nuclear factor-kappaB pathway. Tumour Biol 39(6):1010428317710586
133. Palani CD et al (2018) Toll-like receptor 2 induces adenosine receptor A2a and promotes human squamous carcinoma cell growth via extracellular signal regulated kinases (1/2). Oncotarget 9(6):6814–6829
134. Mastorci K et al (2016) Toll-like receptor 1/2 and 5 ligands enhance the expression of Cyclin D1 and D3 and induce proliferation in mantle cell lymphoma. PLoS One 11(4):e0153823
135. Ikehata N et al (2018) Toll-like receptor 2 activation implicated in oral squamous cell carcinoma development. Biochem Biophys Res Commun 495(3):2227–2234
136. Liu B et al (2016) TLR2 promotes human intrahepatic cholangiocarcinoma cell migration and invasion by modulating NF-kappaB pathway-mediated inflammatory responses. FEBS J 283(20):3839–3850
137. Li CL et al (2019) TLR2 promotes development and progression of human glioma via enhancing autophagy. Gene 700:52–59
138. West AC et al (2017) Identification of a TLR2-regulated gene signature associated with tumor cell growth in gastric cancer. Oncogene 36(36):5134–5144
139. Maslinska D et al (2015) Toll-like receptor 2 (TLR2) is a marker of angiogenesis in the necrotic area of human medulloblastoma. Folia Neuropathol 53(4):347–354
140. Gowing SD et al (2017) Gram-positive pneumonia augments non-small cell lung cancer metastasis via host toll-like receptor 2 activation. Int J Cancer 141(3):561–571
141. Bugge M et al (2017) Surface Toll-like receptor 3 expression in metastatic intestinal epithelial cells induces inflammatory cytokine production and promotes invasiveness. J Biol Chem 292(37):15408–15425
142. Veyrat M et al (2016) Stimulation of the toll-like receptor 3 promotes metabolic reprogramming in head and neck carcinoma cells. Oncotarget 7(50):82580–82593
143. Boes M, Meyer-Wentrup F (2015) TLR3 triggering regulates PD-L1 (CD274) expression in human neuroblastoma cells. Cancer Lett 361(1):49–56
144. Maitra R et al (2017) Toll like receptor 3 as an immunotherapeutic target for KRAS mutated colorectal cancer. Oncotarget 8(21):35138–35153
145. Jing YB et al (2015) Up-regulation of Toll-like receptor 9 in osteosarcoma. Anticancer Res 35(11):5839–5843
146. Won H et al (2017) TLR9 expression and secretion of LIF by prostate cancer cells stimulates accumulation and activity of polymorphonuclear MDSCs. J Leukoc Biol 102(2):423–436
147. Sandholm J et al (2016) Toll-like receptor 9 expression is associated with breast cancer sensitivity to the growth inhibitory effects of bisphosphonates in vitro and in vivo. Oncotarget 7(52):87373–87389
148. Honda K, Taniguchi T (2006) IRFs: master regulators of signalling by Toll-like receptors and cytosolic pattern-recognition receptors. Nat Rev Immunol 6(9):644–658

6 Rho-ROCK Signaling in Normal Physiology and as a Key Player in Shaping the Tumor Microenvironment

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Abstract

The Rho-ROCK signaling network has a range of specialized functions of key biological importance, including control of essential developmental processes such as morphogenesis and physiological processes including homeostasis, immunity, and wound healing. Deregulation of Rho-ROCK signaling actively contributes to multiple pathological conditions, and plays a major role in cancer development and progression. This dynamic network is critical in modulating the intricate communication between tumor cells, surrounding diverse stromal cells and the matrix, shaping the ever-changing microenvironment of aggressive tumors. In this chapter, we overview the complex regulation of the Rho-ROCK signaling axis, its role in health and disease, and analyze progress made with key approaches targeting the Rho-ROCK pathway for therapeutic benefit. Finally, we conclude by outlining likely future trends and key questions in the field of Rho-ROCK research, in particular surrounding Rho-ROCK signaling within the tumor microenvironment.

Keywords

Rho-ROCK signaling · Tumor microenvironment · Stroma · Pancreatic cancer · Disease · Development · Actomyosin cytoskeleton · Metastasis · Extracellular matrix · Targeted therapy · ROCK inhibitors

6.1 Introduction

The Rho-Rho-associated coiled-coil containing protein kinase (ROCK) signaling pathway is involved in a variety of key biological processes from the earliest stages of development right through to retaining important roles in adult homeostasis. Important functions of the Rho-ROCK pathway include the regulation of the cellular cytoskeleton and various transcription factors [1], allowing Rho-ROCK signaling to control cellular behaviors such as morphology, proliferation, motility, and adhesion [2]. Given its biological significance, Rho-ROCK signaling has been implicated in a plethora of disease states including neurodegenerative disorders [3], cardiovascular disorders [4], scarring/fibrosis [5–7], and various cancers, e.g., [8–11]. Consequently, Rho-ROCK signaling has been a hot topic of research for several decades, with significant

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interest in targeting the Rho-ROCK pathway with various inhibitors for therapeutic gain [2, 12].

6.1.1 Core Components of the Rho-ROCK Pathway

The intricate Rho-ROCK pathway consists of core members including the Rho family of small GTPases, Rho (A, B, and C), Rac (1, 2, and 3) and Cdc42, as well as the serine threonine kinases ROCK1/2 [13] (Fig. 6.1). ROCK1/2 are expressed in multiple tissues including the lung, liver, spleen, kidneys, testes, brain, and heart [14] and can act in several subcellular locations [15, 16]. Similarly, the Rho and Rac subfamilies of small GTPases require tight spatiotemporal control in order to elicit their specific intracellular functions [17, 18]. RhoA and RhoC are expressed ubiquitously, whereas RhoB is unstable with a short half-life [19], suggesting the importance of transcriptional control in regulating this protein. Rac1 is expressed ubiquitously, with Rac2 exclusively expressed in hematopoietic cells, and Rac3 most highly expressed in the brain [20]. There are two human isoforms of Cdc42 arising from alternative splicing with one isoform expressed ubiquitously and the other limited to the brain [20]. The Rho and Rac small GTPases cycle between active GTP-bound states and inactive GDP-bound states and thus act as molecular switches (Fig. 6.1). This process is tightly regulated by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs) [21]. GEFs and GAPs are also implicated in regulating the localization of Rho GTPases [22]. RhoA, RhoB, and RhoC are well characterized as upstream regulators of ROCK1/2 activity (Fig. 6.1) as when they are in their active

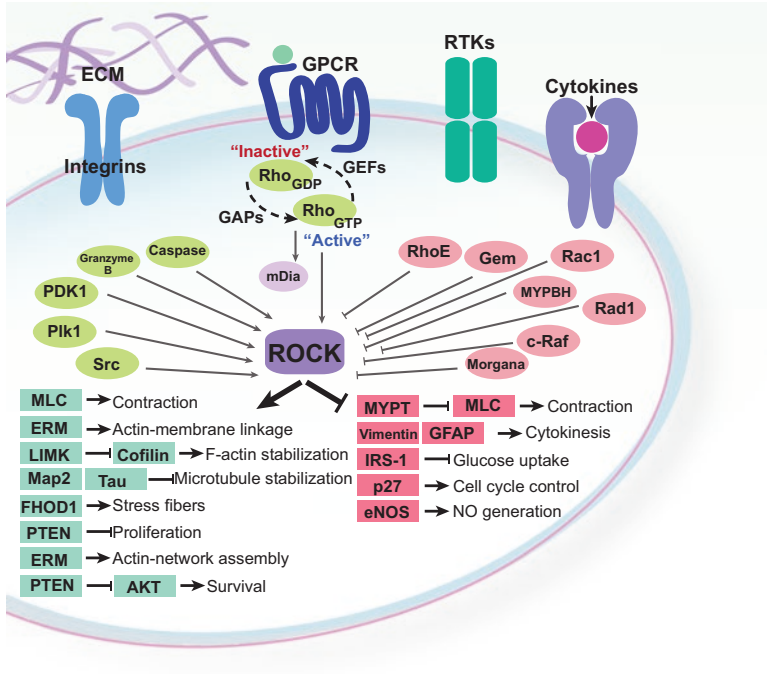

Fig. 6.1 Overview of the Rho-ROCK pathway. Key members include upstream activators/regulators of the Rho-ROCK pathway such as matricellular proteins, ligands/cytokines, and their associated receptors. These upstream regulators affect a variety of intracellular proteins (ovals) which either activate (green) or inhibit (pink) ROCK. The major downstream effectors that elicit the myriad of cellular functions controlled by the Rho-ROCK pathway are also detailed (rectangles). Positively regulated ROCK effectors/interactors are shown in dark green with negatively regulated targets shown in dark pink

states they interact with the Rho-binding domain of ROCK1/2 to enhance activity [23, 24]. The other major non-ROCK effector of the Rho family is the mammalian homolog of Drosophila diaphanous (mDia) [25]. Conversely, the Rho GTPases RhoE, Gem, and Rad reduce ROCK1/2 activity [26, 27], but several Rho-independent pathways that regulate ROCK have also been described [28, 29]. Furthermore, there is evidence that Rac1, Rac2, Rac3, and Cdc42 can compensate for RhoA functions to directly or indirectly affect ROCK1/2 functions [30].

6.1.2 Key Functions Regulated by Rho-ROCK Signaling

Once activated and translocated to specific subcellular locations, the Rho/Rac proteins cooperate with downstream effectors to activate specific signaling networks [31, 32], mostly through ROCK1/2. ROCK1/2 act on many shared downstream targets, but the two isoforms also have some distinct functions (e.g., their distinct and opposing effects on vascular smooth muscle cells [33]). Key ROCK1/2 substrates include myosin light chain (MLC) and the myosin-binding subunit of the MLC phosphatase (MYPT1), the LIM kinases (e.g., glial fibrillary acidic protein), the ERM (ezrin/radixin/moesin) complex, and intermediate filament proteins (Fig. 6.1) [34]. ROCK1/2 also interact with a variety of non-substrate proteins, including BRCA2, Dynamin I, p120-catenin, and Raf1, which affect the activity of the ROCK isoforms themselves or the protein they are interacting with [34]. The Rho-ROCK pathway has a key role in actomyosin cytoskeletal remodeling, controlling stress fiber formation [35], F-actin stabilization and network assembly [36] and microtubule stabilization [37]. As such, Rho-ROCK signaling regulates a plethora of important cellular functions including contraction [35], migration [34], cytokinesis [38], centrosome duplication [39, 40], the cell cycle [41] and proliferation [42], apoptosis [43] and differentiation [44] as well as metabolic processes such as glucose uptake [45] and nitric oxide production [46].

6.1.3 Regulation of the Rho-ROCK Pathway

Given the key role of Rho-ROCK signaling in many fundamental biological processes, Rho and ROCK are subjected to a variety of regulatory mechanisms that control their activation status, ultimately influencing the kinase activity of ROCK. A key regulatory factor involves the correct subcellular localization of Rho and ROCK. RhoA, RhoB, and RhoC are highly related biochemically but differ in their subcellular targeting due to variations in their C-terminus [20, 47]. RhoA is predominantly cytosolic with a small fraction also bound to the plasma membrane [47]. RhoB is localized to the plasma membrane and within endomembrane vesicles, with RhoC associating with the cytosol and perinuclear locations [47]. The localizations of Rac1, Rac2, and Rac3, which are also highly structurally similar [20], are also controlled by posttranslational modifications to their C-termini [48]. ROCK1/2 associate with vimentin [49] and stress fibers [50] in the cytoplasm as well as locating at the plasma membrane [51].

Upstream regulation of the Rho GTPases is achieved via specific cell-surface receptors including G-protein-coupled receptors (GPCRs) and tyrosine kinase receptors as well as cell–matrix adhesion and cell–cell adhesion molecules [52–55], which can act as inputs to activate Rho proteins (Fig. 6.1). These GTP-bound, activated Rho proteins can then interact with multiple effectors for signal transduction and inputs into a variety of different pathways. Alongside multiple effectors binding with these various Rho isoforms, further regulation of Rho-ROCK signaling is accomplished by the use of multiple contact sites between Rho isoforms and their effectors. For example, RhoA is commonly thought to act by disrupting intramolecular autoinhibitory interactions to release functional domains within the effector protein [1]. This is illustrated by the fact that the kinase domain of ROCK is autoinhibited by a region of the C-terminus of ROCK that includes a Rho-binding domain (RBD) [56]. Therefore, it is thought that Rho-mediated activation of ROCK may occur via an allosteric binding mechanism

whereby Rho binds to the RBD inducing conformational changes that displace the autoinhibitory segment of the ROCK C-terminus [1].

While there are several upstream activating regulators of Rho-ROCK signaling that are well characterized, fewer examples of negative regulation of the Rho-ROCK pathway are known. The Rho GTPase-activating proteins (Rho-GAPs) and other small GTPases such as Rac1 and Rap1 are well-known negative regulators of the Rho-ROCK pathway [57, 58]. Inhibition of Rho-ROCK interaction can also block Rho-ROCK signaling. This is achieved by proteins interacting with either RhoA, for example [59, 60], or with the kinase domain of ROCK [26, 27]. Finally, there is evidence that ROCK1/2 can be regulated spatiotemporally by microRNAs (reviewed in [36]).

6.2 Rho-ROCK Signaling in Development

Rho-ROCK signaling is involved in key developmental progressions, acting to govern the earliest stages such as the first cell fate segregations, through to later processes such as organ development. Here we outline some of these examples in order to highlight how errors in the Rho-ROCK pathway function can contribute to various disease states later in development.

6.2.1 Importance of the Rho-ROCK Pathway for the Earliest Stages of Development

Inhibiting ROCK by pharmacological means using Y27632 treatment at the zygote stage of mammalian development results in failure of the first cleavage, with most embryos arresting and failing to develop to the 8-cell stage [61]. Treatment with Y27632 at the 8-cell stage leads to embryos failing to undergo compaction and not developing into blastocysts [61]. During preimplantation development of the mammalian embryo, the first cell fate segregation produces two lineages known as the trophectoderm (TE), an extra-embryonic epithelium, and the inner cell mass (ICM), which occurs by the early blastocyst stage. The ICM is pluripotent and gives rise to the fetal body and the extra-embryonic membranes that comprise the amniotic sac, whereas the TE is crucial for implantation and placentation [62]. Rho-ROCK signaling plays an important role in the establishment and maintenance of the TE. Apico-basal cell polarity is necessary for establishment of the TE [63], as mislocalization of polarity proteins results in defects in TE formation and function. The Rho-ROCK pathway is a well-established regulator of cell polarity [64], and recent work has highlighted the requirement of RhoA GTPases for retaining the gene expression program specific to the TE which is necessary at later stages for blastocyst hatching and implantation [65]. It was also recently shown that inhibition of ROCK by Y27632 in human placenta-derived trophoblasts leads to their differentiation [66]. Furthermore, Rho-ROCK signaling was demonstrated to play a key role in the survival of human embryonic stem cells (hESCs) as ROCK inhibition by Y27632 promoted their survival and proliferation [67], although the sub-embryonic origin (i.e., epiblast-derived versus ICM-derived) of the stem cells dictates the importance of Rho-ROCK signaling for survival [68]. Additionally, Rac1 and Cdc42 have been implicated in the maintenance of epiblast cells at the blastocyst stage [69]. It is therefore apparent that Rho family GTPase activity determines the fate of pluripotent stem cells within the early developing embryo.

6.2.2 The Role of Rho-ROCK in Germ Layer Establishment and Maintenance

The formation of the three primary embryonic germ layers, the ectoderm, endoderm, and mesoderm, is the first step in the generation of a multicellular organism and occurs during the developmental phase known as gastrulation. The ectoderm gives rise to the epidermis, nervous system, and various neural crest-derived tissues. The endoderm gives rise to the respiratory, gastrointestinal and urinary systems, and endocrine glands, whereas the mesoderm

goes on to form the notochord, cartilage, axial skeleton, trunk muscles, connective tissue, kidneys, and blood [70]. Work from Kim et al. suggests that blocking ROCK-dependent myosin-II activity is required for neural crest specification of hESCs to give rise to neurons, osteocytes, chondrocytes, and smooth muscle cells [71]. In addition to this, Srinivasan et al. have shown that mesenchymal stem cells isolated from the ectoderm change expression of "stemness" markers in a Rho-ROCK signaling-dependent manner when cultured on substrates of differing stiffness, affecting their adipogenic and chondrogenic differentiation potential [72].

ROCK is required for the formation of segregated tissues called the primitive endoderm and epiblast in the ICM, as Y27632 treatment leads to mingling of the primitive endoderm and epiblast populations, with treated blastocysts yielding greater fetal loss [73]. Further, the cell shape changes that occur during epiblast differentiation require ROCK, as pharmacological inhibition with Y27632 or H1152 resulted in a loss of epiblast polarization [74]. The atypical Rho GTPase, *Rhou*, has been shown to be required for maintaining F-actin polarization, epithelial morphogenesis, and differentiation of the endoderm, likely via activation of JNK-mediated pathways [75].

Prolonged treatment of human-induced pluripotent stem cells (hIPSCs) with Y27632 facilitated differentiation toward a mesendodermal lineage, accompanied by an increase in the expression of epithelial–mesenchymal transition (EMT) markers, but had inhibitory effects when cells were subjected to ectodermal differentiation [76]. Endothelial cells can be obtained from ESC-derived mesodermal precursor cells using vascular endothelial growth factor-A [77]. The differentiation and expansion of these endothelial cells can be significantly improved by using Y27632, which activates PI3-kinase-Akt signaling to promote endothelial cell proliferation [77].

6.2.3 Requirement of Rho-ROCK for Migration During Development

One of the best-characterized functions of Rho-ROCK signaling is its regulation of cell migration through reorganization of the actin cytoskeleton [78]. Unsurprisingly then, ROCK1/2 appear particularly important during development where cell migration is required for the formation of tissues and organs. The first long-range cell migration event in the mammalian embryo is the movement of endoderm cells from the surface of the ICM facing the blastocoel cavity to line the inner surface of the trophectoderm. This process has been shown to be regulated by planar cell polarity acting through Rho-ROCK signaling [79]. EMT is the first major change to cell morphology following fertilization [80], and during gastrulation, the specification of the embryonic axes is accompanied by the occurrence of EMT in distinct regions of the embryo. One such region is the primitive streak, which demarcates the initial site of mesoderm formation resulting from these EMT movements. ROCK inhibition in blastocysts using Y27632 disrupts primitive streak formation due to loss of directional cell movements [80]. Neural crest cells derive from the developing neuroepithelium to give rise to craniofacial structures and the peripheral nervous system [81]. The neural crest is a population of multipotent progenitor cells that arise at the border of neural and nonneural ectoderm, which undergo EMT and migration [82]. EMT is crucial for these neural crest cells to delaminate from the neuroepithelium and undergo migration, and this process can be perturbed with ROCK inhibitors resulting in reduced bleb-based motility and EMT [81]. Y27632 can also inhibit the migration of the bilateral heart primordia, as treatment with the ROCK inhibitor blocked the migration of precardiac mesoderm causing a failure of cardiac tube fusion [83], suggesting that ROCK1/2 regulate migration of the cardiac precursors to the ventral midline [83].

6.2.4 Rho-ROCK Signaling Has Key Roles During Tissue Formation

As discussed above, the Rho GTPases have key roles in regulating cytoskeletal organization and polarity of epithelial cells, which can impact on tissue morphogenesis and cellular differentiation [75]. Neural tube closure is a crucial morphogenetic event involving marked restructuring of both neural and nonneural tissues. Apical actomyosin-driven constriction is commonly employed to shape and bend epithelia and is a key mechanism responsible for neural plate bending [84]. During neurulation, RhoA plays an essential role in regulating actomyosin dynamics in the neural epithelium of the elevating neural folds, whereas at later steps, Rac1 is required for the formation of cell protrusions in the nonneural surface ectoderm during neural fold fusion [84]. Apical actomyosin-driven constriction is also employed during lens placode invagination during eye development [85]. During this process, a region of the ectoderm first thickens to form the lens placode followed by invagination to form the lens pit [86]. The lens placode epithelium remains in close apposition to the epithelium of the presumptive retina, and it has been demonstrated that mutual antagonism by the small Rho GTPases Rac1 and RhoA determines cell shape and tissue curvature during this coordinated invagination [87].

Rho-ROCK-regulated tension is also required for coordinated 3D tissue formation and alignment of multiple tissues during development, such as the eye, with Rho GTPase-activating protein ARHGAP18 acting as an effector downstream of YAP to control tissue tension via the cortical actomyosin network [88]. This signaling was also shown to be important for fibronectin assembly and organization to activate integrin signaling for tissue alignment and coordinated morphogenesis [88]. Most tissues are subject to external mechanical forces throughout their lifetime. Rho-ROCK signaling is an important component of the mechanotransduction process, in which external forces are converted into biochemical responses in the cell [89]. Compression-induced enhancement of actomyosin tension was shown to require ROCK activity downstream of RhoA, leading to increased proliferation and upregulated expression of EMT markers such as *vimentin*, *snai2*, and *zeb1* [90]. YAP is involved in mechanotransduction in a Rho- and actomyosin tension-dependent manner based on external stimuli such as extracellular matrix (ECM) stiffness and cell spreading [89].

Self-organizing tissues (e.g., the eye cup) have been grown from 3D cultures of ESC aggregates whereby the initial stages of morphogenesis require mechanical apical constriction processes that are ROCK inhibitor (Y27632) sensitive [91], suggesting a role for the Rho-ROCK pathway in generating these shaping forces. Stem cell-derived organoids represent promising models for studying development and disease. Great progress has been made in generating organoid structures from hESC or hiPSC cultures, with one of the key extrinsic factors added to the culture media being the ROCK inhibitor, Y27632, which blocks dissociation-induced apoptosis and promotes survival [68, 92], a complex process at least in part supported through sustained activity of sterol regulatory element binding protein transcription factors SREBP1 and SREBP2, and altered cellular lipid synthesis and accumulation [93].

6.2.5 Rho-ROCK Pathway in Organ Formation

Rho-ROCK signaling is involved in brain morphogenesis governing processes such as axonogenesis, neuronal development, and neuronal migration [94]. The Rho-ROCK pathway acts to limit axonogenesis ensuring just one axon forms during neuronal polarization [95]. Neuronal precursor migration determines the cellular architecture of the brain for subsequent neural network formation [96]. Shinohara et al. found that Rho signaling via mDia and ROCK regulates nuclear translocation through F-actin dynamics in tangential neuronal migration, whereas this mechanism is dispensable in radial neuronal migration [96]. Furthermore, Rho signaling is crucial for synaptic plasticity [94]. Rac and Rho work antagonistically for shaping dendritic spines, growth, and shrinkage, with Rac promoting spine

formation and Rho preventing it, and ROCK mediating the latter Rho action [97]. Neural precursor cells have shown differential migratory and morphological interactions with laminin under differing topographical contexts using scaffolds that mimic the developing central nervous system [98]. Neural stem cell migration is blocked on topographies consisting of large fiber structures, which can be reversed by the Y27632 ROCK inhibitor [98].

The Rho-ROCK pathway has important roles in the development of the cardiovascular system where ROCK1/2 are ubiquitously expressed, including in adult heart tissue, although ROCK2 expression is higher here suggesting a more prominent role for this isoform [99]. At the earliest stages of heart development, Rho-ROCK signaling is important for formation of the definitive cardiac tube, whereby precardiac mesoderm cells migrate toward the midline and fuse [83]. Y27632 treatment of embryos at this stage results in cardia bifida and abnormal looping of the heart tube [83]. One later example is the requirement of RhoA-ROCK for establishing the right-sided sinoatrial node as the definitive pacemaker of the heart by restricting pacemaker gene expression to the right side of the sinus venosus myocardium [100]. In the developing lungs, decreasing cytoskeletal tension with Rho-ROCK inhibitors blocks basement membrane thinning at the tips of growing epithelial buds preventing both epithelial budding and branching angiogenesis [101].

hESCs and hiPSCs are potential promising sources for regenerative therapies; however, cell-replacement therapies are currently limited by the ability to differentiate and expand larger pools of progenitor cells from these pluripotent stem cell sources. It was recently demonstrated that ROCK and nonmuscle myosin-IIs (NM-II) can inhibit the differentiation of hiPSCs to pancreatic endoderm [102, 103], suggesting a method to simplify and improve current endoderm and pancreatic differentiation protocols for potential therapeutic use. Related to this, ROCK inhibition by H1152 was recently shown to promote the maturation of human pancreatic beta-like cells from multiple hPSC lines [104]. Specifically, Ghazizadeh et al. showed that ROCK2 inhibition lead to the generation and maturation of glucose-responding cells thereby providing a possible approach for producing human beta-cells for translational use [104].

6.3 Rho-ROCK Signaling in Normal Physiology and Adult Tissue Homeostasis

In this section we discuss some of the adult physiological and homeostatic processes that involve the Rho-ROCK pathway for their regulation, again with the aim of understanding how deregulation of Rho-ROCK signaling contributes to disease.

6.3.1 Homeostasis

The human epidermis is comprised of several layers of specialized epithelial cells called keratinocytes. Epidermal homoeostasis requires tight regulation of the balance between keratinocyte proliferation and terminal differentiation [44]. ROCK1 and ROCK2 were found to play distinct roles in regulating keratinocyte-fibronectin adhesion and terminal differentiation of keratinocytes for epidermal homeostasis [44]. The role that mechanical forces play in regulating tissue homeostasis is becoming increasingly recognized, and these mechanotransduction processes must be tightly regulated to maintain tissue and cell integrity [105]. This balance between cells sensing and responding to external mechanical stimuli is known as mechano-reciprocity [106], and has been shown to be of importance in regulating skin homeostasis, in particular wound healing [105]. A key mediator of mechano-reciprocity is the Rho-ROCK pathway, which acts as a transducer of outside-in and inside-out signaling. Kular et al. have demonstrated that mechano-reciprocity during wound healing is balanced via a negative feedback mechanism that limits excessive ROCK signaling to ensure correct ECM deposition and remodeling for optimal wound closure by reepithelialization [105]. This negative regulation of Rho-ROCK signaling is achieved by 14-3-3ζ,

which binds to MYPT1, promoting dephosphorylation of activated regulatory myosin light chain (pMLC) [105]. EMT is required for regenerative processes in epithelial tissues and is influenced by mechanical cues from the substrate [107]. Markowski et al. found that substrate-induced EMT was dependent on Rho-ROCK signaling in lung epithelial cells, as Y27632 blocked EMT when RLE-6TN cells were cultured on polyacrylamide substrates of varying stiffness [108].

6.3.2 Immunity

Initial studies on the role of Rho-ROCK signaling in the immune system examined its requirement for development, activation, and migration of lymphocytes [54]. Reduced RhoA in T cells significantly reduces T-cell receptor-dependent proliferative response and T-cell migration [94]. Rho-ROCK signaling functions in both the sensitization phase and effector phase of Th2-dependent allergic inflammation with T-cell-specific deletion of RhoA impairing Th2 differentiation in vitro and reducing Th2 cytokine production in vivo [109]. The use of a ROCK inhibitor mimicked this RhoA deletion confirming involvement of the Rho-ROCK pathway. Deregulated ROCK activity may be involved in the pathogenic immune response of myasthenia gravis, where treatment with ROCK1/2 inhibitor Fasudil restored the balance of Th1/Th2/Th17/Treg subsets and ameliorated the severity of this disease in an experimental rat model [110]. In clinical trials of a ROCK2-specific inhibitor (KD025), secretion of IL-21 and IL-17 from activated peripheral blood mononuclear cells (PBMCs) was found to be significantly reduced ex vivo via a STAT3-dependent mechanism [111]. Phase 2 trials of KD025 in patients with psoriasis vulgaris normalized skin pathology via decreases in plasma levels of IL-17 and IL-23 [112]. Finally, ROCK2 has been shown to contribute to regulation of IFN-γ secretion from T cells in rheumatoid arthritis patients [113].

6.3.3 Glucose Transport and Voltage Channel Trafficking

Insulin functions to promote glucose transport in muscle and adipose cells by stimulating translocation of glucose transporter 4 (Glut4). This translocation of Glut4 is mediated by actin cytoskeleton reorganization [114, 115]. Previous studies suggest that ROCK2 plays a pivotal role in the regulation of insulin signaling and insulin-dependent glucose homeostasis in cells and tissues [116]. More recent examples have shown that ROCK1 also has a role in regulating glucose transport as targeted disruption of ROCK1 causes insulin resistance in vivo [117], although the mechanism of ROCK1 glucose transport regulation was found to be distinct to that of ROCK2 [118].

Rho-ROCK signaling has been implicated in regulating both Ca^{2+} and K+ voltage channels. Stirling et al. showed that Rho/ROCK have dual roles in regulating trafficking via voltage-sensitive potassium channels (Kv), where they act by suppressing Kv ionic current through modulating channel endocytosis via both clathrin- and cholesterol-dependent mechanisms [119]. Further, it was recently demonstrated that Y27632 inhibits voltage-dependent K+ channels in coronary arterial smooth muscle cells [120]; however, this mechanism was shown to be ROCK-independent. RhoA was observed to regulate neuronal high-voltage activated Ca^{2+} channels. Data from Rousset et al. suggested that RhoA may govern synaptic transmission during development and potentially contribute to pathophysiological processes when axon regeneration and growth cone kinetics are impaired [121]. Ca^{2+} release from the sarcoplasmic reticulum has been shown to invoke depolarization-induced Rho-ROCK activation, leading to sustained contractile activation in smooth muscle cells and subsequently sustained arterial contraction [122].

6.4 Deregulation of Rho-ROCK Signaling in Disease

Deregulated Rho-ROCK signaling has been implicated in a variety of pathologies other than cancer such as neurodegenerative disorders [3, 123, 124], cardiovascular disorders [125, 126], diabetic kidney disease [127], diseases of the bone [128], and scarring/fibrosis in multiple tissues/organs [5, 125, 129]. Here we focus on the role of the Rho-ROCK pathway in cancer, in particular highlighting the complexity of dynamic Rho-ROCK intercellular communication between the tumor and its microenvironment.

6.4.1 The Role of Rho-ROCK Signaling in Promoting Cancer Initiation and Progression

The role of deregulated Rho-ROCK signaling in cancer progression has been a popular area of research, and aberrant activation of the Rho-ROCK pathway may happen in several ways. The *HRas*, *KRas*, and *NRas* GTPases are well known to have high frequencies of activating mutations in human cancers, effectively resulting in the protein being in a continuous "on" state [130]. Mutations in Rho GTPase family members are however rare [131, 132], and aberrant activation of this pathway generally occurs through overexpression of Rho GTPases or due to changes in the levels of regulators of Rho activity, including increased activation of GEFs and inactivation of GAPs. Recently, large-scale sequencing has uncovered gain-of-function and loss-of-function mutations in several of the Rho GTPases which have been associated with immunodeficiency syndromes and cancer [133]. Mutations in Rac1 and RhoA have been identified in melanomas, sarcomas, T-cell lymphomas, neuroblastomas, and gastric cancer [134–140]. P29S, N92I, and C157Y mutants of Rac1 were shown to exist preferentially in the GTP-bound state as a result of a rapid transition from the GDP-bound state, as opposed to reduced intrinsic GTPase activity [136]. In melanoma, Rac1 P29S was shown to increase binding of the protein to downstream effectors promoting melanocyte proliferation and migration [135]. In gastric cancer, RhoA gain-of-function occurs via mutational hotspots affecting the Tyr42, Arg5, and Gly17 residues, which were associated with poorly differentiated adenocarcinomas [139]. In addition, over 600 somatic coding mutations in ROCK1 and ROCK2 have been identified in human cancers, plus several thousand single nucleotide polymorphisms (SNPs) in ROCK1 and ROCK2 have been identified [141]. The role of these mutations and SNPs in cancer progression is still emerging, but ROCK1/2 upregulation has been demonstrated in malignant tissues [140, 141]. It remains to be seen whether this enhanced ROCK signaling can facilitate cell transformation to promote tumor cell survival and growth.

Given the roles of Rho GTPases in cell adhesion and migration, Rho-ROCK signaling is heavily implicated in the processes that drive tumor cell invasion and metastasis (Fig. 6.2), which was first demonstrated in an in vivo peritoneal tumor dissemination model [142]. Importantly, in this model, continuous local infusion of Y27632 significantly reduced dissemination and tumor nodule formation [142], confirming a role for ROCK signaling. Actin polymerization drives cancer cell motility, and tumor cells can migrate as single cells or collectively in a group [143]. For single tumor cell migration, distinct methods of migration have been observed, and Rho-ROCK plays differing roles in these different migration forms (Fig. 6.2). Tumor cells in 3D matrices exhibited Rac-mediated elongated mesenchymal migration that is proteolysis-guided, and ROCK-mediated actomyosin-driven rounded amoeboid migration [144]. These two migration modes are interchangeable and employed by tumor cells in a context-dependent manner [144].

There are also differences between RhoA, RhoB, and RhoC in how they regulate cancer cell migration. Using RNAi approaches in prostate and breast cancer cell lines, it was shown that

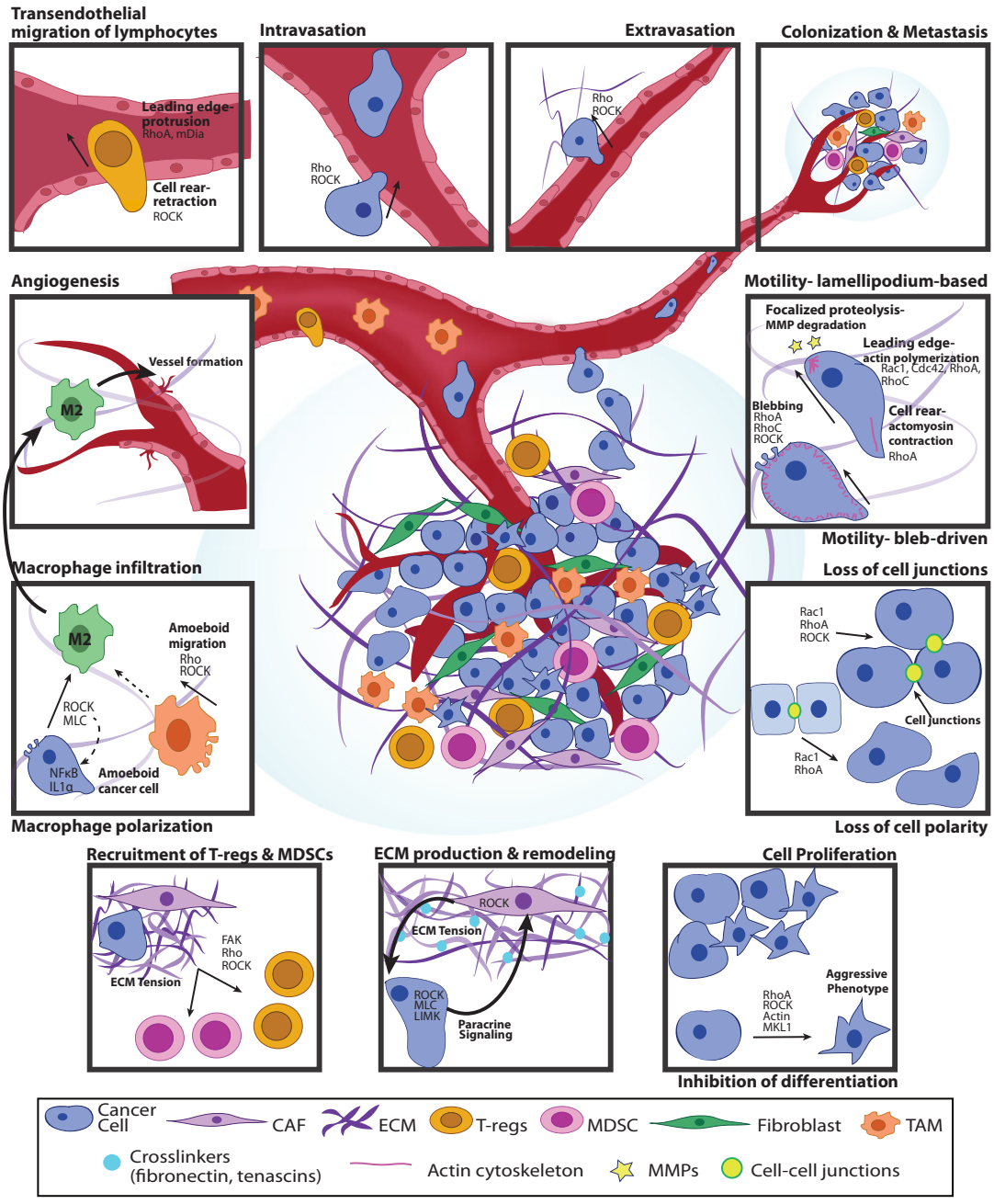

Fig. 6.2 Rho-ROCK signaling in the dynamic tumor microenvironment (TME). The Rho-ROCK pathway regulates a multitude of processes in the dynamic TME that drive disease progression, including cancer cell and stromal cell (e.g., fibroblasts and immune cells) behavior as well as their cross talk and subsequent modification of the TME. Panels detail specific examples of these diverse processes, highlighting roles for Rho-ROCK signaling at the primary site as well as its role in establishing metastatic spread

RhoA depletion induced cell elongation with narrow lamellipodia on 2D and 3D substrata [145]. This RhoA RNAi-induced elongation may facilitate invasion since RhoA-depleted cells invade more competently through matrigel-coated transwells and into a 3D matrix of matrigel versus control cells [48]. Knockdown of RhoC increased cancer cell spreading and cells presented with a thin lamellipodium around most of the cell periphery [145]. Furthermore, RhoC-depleted cells had reduced migration, chemotaxis, and invasion through matrigel-coated transwells [145]. Opposite to RhoC depletion, RhoB knockdown reduced cell spreading and increased cell migration speed on 2D substrata, potentially via reduced β1-integrin levels [146]. More recent data indicate that RhoB may directly drive membrane blebbing, enhancing blebby amoeboid 3D-migration of lymphoid, melanoma, and lung cancer cells, through a KIF13A-regulated mechanism of RhoB localization to the plasma membrane, and downstream signaling via ROCK and myosin-II [147]. Interestingly, Suwa et al. [148] examined RhoA, RhoB, and RhoC expression in 33 cases of pancreatic ductal adenocarcinoma (PDAC) and found that RhoC expression levels were higher in tumors than in nonmalignant tissues and higher in metastatic lesions than in primary tumors. Furthermore, increased RhoC levels correlated with perineural invasion, lymph node metastasis, and poorer prognosis, whereas expression of RhoA or RhoB showed no correlation with these clinicopathological findings [148].

6.4.2 What Is the Tumor Microenvironment and What Is Its Role in Promoting Cancer Progression?

The tumor microenvironment (TME) is complex and dynamic, consisting of cells such as fibroblasts, pericytes, immune cells, adipocytes, lymphocytes, and endothelial cells (collectively referred to as stromal cells), as well as a noncellular component comprised of ECM (e.g., matricellular proteins such as collagen, laminin, and fibronectin). Under healthy conditions, this microenvironment plays an important role in tissue homeostasis as discussed earlier. In cancer, the TME interacts with cells of the tumor to affect cancer initiation, progression, metastasis, and chemoresistance (Fig. 6.2). Through these interactions, tumor cells are able to hijack the functions of normal cells in the TME to drive pro-tumorigenic processes such as immune cell evasion [149] and the generation of cancer-associated fibroblasts (CAFs) [150].

6.4.3 Roles of Rho-ROCK Signaling in the TME

Most current therapies involve targeting cell-intrinsic functions of tumor cells, such as their proliferative capacity or their ability to evade apoptosis [151]. Many studies have examined the effects of inhibiting Rho-ROCK signaling in cancer, using a variety of inhibitors in in vitro and in vivo preclinical models (reviewed in ref. 2). The two best-described pharmacological ROCK inhibitors are Y27632 and Fasudil (approved for clinical use in Japan and China as vasodilators [152]). Given the tumor-promoting properties of the TME mediated by Rho-ROCK signaling, targeting key factors that are involved in the development of this environment, such as tumor-promoting CAFs, the ECM, and immune cells, are potentially novel approaches to cancer therapy. With a number of ROCK inhibitors in clinical trials for various pathologies (Table 6.1), including the early Phase I safety testing of AT13148 in advanced solid cancers (NCT01585701), there is significant potential in repurposing some of these agents as anti-cancer therapies. In support of this, inhibiting the Rho-ROCK pathway specifically in cancer cells can reduce proliferation, invasion, and angiogenesis in vitro, and in vivo has the effect of reducing

Table 6.1 ROCK inhibitors in clinical trials for various pathologies

Inhibitor	Target	Pathology	Phase	Combination	Status/findings	NIH number/trial name	References
AT13148	AGC kinase inhibitor	Advanced solid tumors	Phase 1	Monotherapy	Completed: Dose escalation reached 240mg with predictable tolerable 'on target' toxicities	NCT01585701	Papadatos-Pastos P et al., 2015, J Clin Oncol
Verosudil (AR-12286)	AGC kinase inhibitor	Glaucoma	Phase 2	Monotherapy	Completed: clinically and statistically significant reduced intraocular pressure (IOP) from baseline in patients with ocular hypertension and glaucoma	NCT00902200	Williams RD et al., 2011, Am J Ophthalmol
			Phase 2	Monotherapy	Completed: Reduced intraocular pressure (IOP) from baseline; limited difference in IOP between 0.5% or 0.7% treatments	NCT01936389	Skaat A et al., 2016, J Glaucoma
Ripasudil (K-115)	Highly selective and potent ROCK1/2 inhibitor	Fuchs' endothelial dystrophy	Phase 2	Monotherapy—hydrochloride hydrate 0.4% ophthalmic solution	Recruiting	NCT03813056, NCT03575130	
			Phase 4	Monotherapy—hydrochloride hydrate 0.4% ophthalmic solution	Recruiting	NCT03249337	
Fasudil (HA-1077)	Selective and potent ROCK1/2 inhibitor	Amyotrophic lateral sclerosis	Phase 2	Monotherapy	Recruiting	NCT03792490	
		Atherosclerosis, hypercholesterolemia	Phase 2	Monotherapy	Completed: No results posted	NCT00120718	
		Cardiovascular diseases	Phase 2	Monotherapy	Completed: No results posted	NCT03404843	
		Raynaud's phenomenon, scleroderma	Phase 3	Monotherapy	Completed: No significant benefits for preventing vasoconstriction of digital and cutaneous arteries following cold challenge	NCT00498615	
		Retinal vein occlusion	Phase 3	Combined Bevacizumab and Fasudil injection	Recruiting	NCT03391219	
		Diabetic macular edema	Phase 3	Intravitreal injection of Fasudil and Bevacizumab	Completed: Best-corrected visual acuity was significantly improved in group receiving Fasudil/Bevacizumab vs Bevacizumab alone	NCT01823081	Ahmadieh H et al., 2019, Br J Opthamol
		ST segment elevation myocardial infarction	Phase 4	Monotherapy	Not yet recruiting	NCT03753269	

KD025	Specific ROCK2 inhibitor	Autoimmune diseases, fibrosis	Phase 1	Monotherapy	Not yet recruiting	NCT03907540	
		Autoimmune diseases, fibrotic disease, drug-drug interaction	Phase 1	KD025, Itraconazole, Rabeprazole, Rifampicin, Omeprazole	Recruiting	NCT03530995	
		Idiopathic pulmonary fibrosis	Phase 2	Monotherapy	Recruiting	NCT02688647	
		Chronic graft-versus-host-disease	Phase 2	Monotherapy	Recruiting	NCT03640481	
			Phase 2	Monotherapy	Active, not recruiting	NCT02841995	Jagasia M et al., 2018, Blood
		Systemic sclerosis, diffuse cutaneous systemic sclerosis	Phase 2	Monotherapy	Recruiting	NCT03919799	
		Psoriasis vulgaris	Phase 2	Monotherapy	Completed: No results posted	NCT02106195, NCT02852967	
			Phase 2	Monotherapy	Completed: Psoriasis area and severity index scores reduced 50% from baseline in 46% of patients	NCT02317627	Zanin-Zhorov A et al., 2017, J Immunol
		Bioavailability in healthy subjects	Phase 1	Monotherapy	Completed: No results posted	NCT02557139	
Y39983 (RKI983/ SNJ-1656)	Selective and potent ROCK1/2 inhibitor	Glaucoma, ocular hypertension	Phase 1	Monotherapy	Completed: Significant IOP reductions from baseline at several hours and after repeated instillation		Tanihara H et al., 2008, Arch Ophthalmol
			Phase 1	Monotherapy	Completed: No results posted	NCT00515424, NCT00846989	

tumor growth and metastasis formation [2]. Conditional activation of ROCK has been shown to promote tumor progression in murine models of PDAC [153] and cutaneous squamous cell carcinoma [42]. Conversely, the ROCK inhibitors Fasudil and Y27632 could decrease the invasion, stress fiber organization, and migration of PDAC cells in vivo [154], and breast cancer cells (MDA-MB 231) in vitro [155]. These studies suggest an important role for Rho-ROCK in promoting tumor progression via modulation of the TME. Here we discuss some general mechanisms by which the Rho-ROCK pathway alters the TME, before highlighting distinct roles in specific cancer types.

6.4.3.1 Rho-ROCK Regulation of CAFs in the TME

CAFs are a mesenchymal cell type present in most solid tumors that share some morphological and functional properties with normal tissue fibroblasts. Activated CAFs display high contractility, enhanced proliferation, and migration, and secrete high levels of growth factors and ECM components compared with normal tissue fibroblasts [156]. CAFs can be activated by cancer cell-secreted factors such as transforming growth factor-β (TGFβ), C-X-C motif chemokine 12 (CXCL12), interleukin-6 (IL-6), platelet-derived growth factor (PDGF), and fibroblast growth factor 2 (FGF-2) [157–160]. CAFs are usually associated with disease progression, and CAF content within tumors has been shown to correlate with patient prognosis [161–163]. CAFs have a substantial role in cancer cell invasion [164], for example, in squamous cell carcinoma, where ROCK activity in the CAFs as opposed to the tumor cells leads to remodeling of collagen matrices to generate tracks for the invading squamous cancer cells [165]. RhoA inhibition reduces the expression of alpha-smooth muscle actin (αSMA), which is frequently expressed by activated CAFs [166]. Furthermore, factors secreted by CAFs (see below), such as insulin-like growth factor-1 (IGF-1), can amplify Rho-ROCK signaling in cancer cells promoting RhoA-dependent cancer cell invasion [166]. Indeed, Sanz-Moreno et al. found that cytokines in the TME can signal through the JAK1 kinase to generate actomyosin contractility through ROCK-dependent signaling leading to ECM remodeling by CAFS and amoeboid migration of tumor cells [167]. Thus, cytokine signaling can generate actomyosin contractility in both stromal and tumor cells. Interestingly, this study also found that actomyosin contractility positively modulates activity of the transcription factor STAT3 downstream of JAK1, thereby establishing a positive feedback loop within this signaling axis [167].

6.4.3.2 Rho-ROCK Regulation of the ECM in the TME

ROCK signaling has been implicated in remodeling the ECM in the TME to facilitate tumor invasion. Loss of normal tissue homeostasis during tumorigenesis can lead to stromal remodeling and subsequent activation of stromal cell populations such as CAFs, which can lead to CAFs secreting growth factors such as TGFβ, FGF, vascular endothelial growth factor (VEGF) and epidermal growth factor (EGF), chemokines (CXCL8 and CXCL12), and cytokines (IL-6) [168–172]. These secreted factors can facilitate tumor cell growth, motility, invasion, and EMT. Activation of CAFs by ECM changes can in turn lead to further ECM remodeling since CAFs secrete ECM proteins including fibronectin, laminin, collagen, periostin, tenascin C, and osteopontin as well as matrix metalloproteinases (MMPs) [173]. This CAF-secreted ECM serves to function as part of a self-sustaining feedback loop which maintains activity of the CAFs [156]. These remodeling processes change the mechanical properties of the TME and provide mechanical cues that lead to increased tumor cell growth, proliferation, and survival [174]. A further consequence of matrix stiffening is integrin clustering which leads to focal adhesion kinase (FAK) and Src activation, and subsequently, activation of Rho GTPases resulting in increased actin polymerization and actomyosin contractility [42, 175]. ECM stiffening has been widely demonstrated to increase Rho-ROCK-mediated actomyosin contractility in cancer cells via this mechanism [176].

For example, in invasive squamous cell carcinoma, mechanotransduction mediated by integrin and FAK signaling activates ROCK in both cells of the tumor and the TME where it acts via GSK3b and the transcriptional coactivator β-catenin to promote tumor progression [177]. As such, Src signaling in the TME represents an attractive therapeutic target [178].

6.4.3.3 Rho-ROCK Regulation of Immune Cells in the TME

Immune cells are an abundant and important component of the TME, which have garnered increasing interest in recent years due to the promise of immunotherapies as therapeutic strategies in cancer. Tumor progression requires the evasion of immune cells by cancer cells with the TME acting in an immunosuppressive manner here as well as the desmoplasia of the TME providing a physical barrier to T-cell infiltration [179]. Hyperactive FAK has been demonstrated to be an important regulator of the fibrotic and immunosuppressive TME [180]. Here we focus specifically on T cells and macrophages as they make up the bulk of the immune cell population in the TME, and the role of Rho-ROCK signaling in these populations is better documented.

T lymphocytes are the major adaptive immune cell population that infiltrates tumors [181]. T-cell migration in the TME is governed by attachment of the ligand ICAM to leukocyte integrin LFA-1, which allows T-cell polarization. This process is reliant on changes in the actomyosin cytoskeleton under the spatially different regulation of myosin light chain kinase (MLCK) and ROCK [182]. MLCK functions at the leading edge of the T cell—blocking its activity causes polarized T cells to retract at the front of the cell, whereas ROCK and RhoA act at the rear of the cell where they prevent detachment of the T-cell trailing edge [182]. Another important requirement for T-cell movement in the TME is transendothelial migration, the process by which T cells cross endothelial barriers during immune surveillance. T cells achieve this by extending lamellipodia and filopodia under the endothelium in order to transmigrate, a process governed by RhoA [183]. RhoA is active at the leading edge of lamellipodia and filopodia in transmigrating T cells where it acts via mDia for protrusion and via ROCK and actomyosin contractility for lamellipodial retraction [183].

T-cell function is regulated by T-cell receptor (TCR) expression with TCR function being regulated by RhoA which is required for thymocyte development [184]. Furthermore, ROCK regulates T-cell proliferation and activation via the actomyosin cytoskeleton, which controls gene expression and structural rearrangements [185]. Interestingly, ROCK1 and ROCK2 appear to have some distinct roles in regulating T-cell activity. ROCK1 has been implicated in interleukin-13 and interleukin-5 cytokine production in Type 2 helper cells [186], whereas ROCK2 regulates IL-17 and IL-21 production by T cells via phosphorylation of interferon regulatory factor 4 [187].

Macrophages are another major immune cell type present in the TME and have a broad spectrum of phenotypes or activation states, with the two opposite extremes known as M1 (pro-inflammatory) and M2 (anti-inflammatory) [188]. Zandi et al. observed that ROCK2 inhibition decreased M2-like macrophages in a mouse model of age-related macular degeneration [189]. Monocyte and macrophage migration in the TME requires dynamic changes to the actomyosin cytoskeleton, with Y27632 inhibition decreasing macrophage infiltration into breast tumor spheroids [190]. Macrophages have been documented to infiltrate tissues using either the Rho-ROCK-mediated amoeboid migration mode or the protease-dependent mesenchymal migration mode [191]. Strikingly, macrophages can alter the invasive migratory mode of tumor cells from an MMP-dependent mesenchymal migration to an amoeboid mode by remodeling the ECM and creating a path for tumor cells [190]. Seminal recent work by Georgouli et al. [192] has shown that ROCK-myosin-II activity in amoeboid invasive melanoma cancer cells directly regulates an immunomodulatory secretome, driving the recruitment and polarization of tumor-associated macrophages (TAMs). This in turn creates a further tumor-promoting TME, which is sustained via a positive feedback loop between ROCK-myosin-II-

driven secretion from amoeboid melanoma cancer cells and IL-1α/NF-κB signaling (highlighted in Fig. 6.2). Of note, blocking ROCK-myosin-II activity in melanoma cells in vitro and in vivo using pharmacological agents (Y27632, GSK269962A) or RNAi leads to reprogramming of macrophages from a "tumor-promoting" to "tumor-killing" phenotype and reduced tumor growth [192], illustrating the multifaceted role for ROCK inhibition in cancer.

6.4.3.4 Rho-ROCK Dynamics in the TME of Pancreatic Cancer: Implications for Therapeutic Targeting

PDAC is a highly lethal malignancy typified by poor 5-year survival rates of only 8% and modest responses to current standard chemotherapy [193, 194], with the early metastatic behavior of PDAC [195] being key to this cancer's lethality. PDAC is characterized by tumors with high desmoplasia consisting of stromal cells and secreted ECM components, which occupies the majority of the tumor mass [196]. The majority of this stroma consists of CAFs, with the major source for these myofibroblast-like cells being activated/differentiated pancreatic stellate cells (PSCs), characterized by the marker αSMA [196, 197]. These CAFs have been hypothesized to drive PDAC progression in an ancillary manner. Interestingly, work from Öhlund et al. recently demonstrated the existence of distinct CAF populations in PDAC with distinct spatial locations and functional roles [198]. ROCK inhibitors (Y27632 and Fasudil) have been shown to block activation of PSCs in vitro [199, 200], suggesting the importance of ROCK signaling for PDAC CAF generation and function. In support of this, Stylianou et al. have recently demonstrated that native pancreatic fibroblasts cultured on increasing collagen concentrations upregulate αSMA, have more aligned stress fibers, are mechanically softer with increased invasive ability, and have reduced expression of RhoA and ROCK [201]. These data highlight the role collagen deposition can play in creating a fibroblast-transforming permissive TME.

ROCK1/2 have elevated expression in PDAC and are associated with reduced patient survival [153, 199, 202]. Disruption of epithelial cell–cell adhesions represents an early and important stage in tumor metastasis as cells undergo EMT. In a model of PDAC epithelium (HPAF-II cells), activation of protein kinase C (PKC) was demonstrated to cause disruption and internalization of adherens junctions (AJ) and tight junctions (TJ) via ROCK2-dependent activation of NM-II [203]. Interestingly, ROCK2 inhibition by Y27632 or pharmacological inhibition of NM-II with blebbistatin resulted in attenuation of AJ/TJ disassembly, which was not seen with inhibition of RhoA [203]. Antisense morpholino oligonucleotides directed against ROCK1 reduced transwell migration of pancreatic cancer cells (MIA PaCa-2 and Panc-1) in vitro [202], with ROCK1/2 activation shown to promote invasive growth of mouse PDAC cells into 3D collagen matrices by increasing matrix-remodeling activities via upregulation of *Mmp10* and *Mmp13* [153]. In the same study, use of the ROCK inhibitor Fasudil improved survival in the KPC mouse model of PDAC, which was associated with increased tumor collagen deposition due to a reduction in MMP production/secretion. Another paper reported positive effects on KPC mouse survival following treatment with Fasudil, although these mice also received the chemotherapeutic Gemcitabine in combination [199]. Retroviral overexpression of ROCK1/2 in PDAC cells led to an increase in the expression of several genes including *Ptgs2* (encodes a key enzyme in prostaglandin biosynthesis in inflammation and mitogenesis), *Tnc* (encodes an ECM glycoprotein), *CD44* (encodes a cell-surface glycoprotein involved in cell–cell interactions, cell adhesion, and migration), and *Cyr61* (encodes an ECM protein and is a YAP-target gene) [204]. These findings give insights into how actomyosin contractility influences gene transcription to modify cell behavior in pancreatic cancer. Oncogenic KRas is one of the major drivers of PDAC [205]. Mutational activation of KRas upregulates eIF5A, and pharmacological inhibition or genetic knockdown of eIF5A reduces PDAC cell migration, invasion, and metastasis in vitro and in vivo [206].

Fujimura et al. discovered that RhoA-ROCK signaling operates downstream of eIF5A in invasive cancer cells. They demonstrated that eIF5A activation controls the protein expression of RhoA and ROCK in PDAC cells [206]. Collectively, the studies discussed in this section underline the importance of Rho-ROCK signaling for PDAC progression both within the tumor itself and for crosstalk with the stroma/TME, and highlight the potential for targeting Rho-ROCK in the TME as a therapeutic strategy in pancreatic cancer [207].

Interestingly, ROCK2 expression was increased in pancreatic cancer precursor lesions known as pancreatic intraepithelial neoplasias (PanINs) versus normal tissue [153]. Ablation of *Rac1* has been further shown to delay the formation of PanINs [208], suggesting a role for aberrant Rho-ROCK signaling at the initiating stages of disease. Finally, there is evidence to suggest that Rho-ROCK signaling also functions at even earlier stages of premalignancy, whereby Ras-transformed cells are apically extruded from otherwise normal epithelia in a process involving an EphA2-EphrinA1-Src-ROCK signaling axis. However, it remains to be seen whether this phenomenon is tumor-promoting or -suppressive, although this is likely context-dependent [209, 210].

6.4.3.5 The Rho-ROCK Pathway Functions in the TME of Breast Cancer and Represents a Potential Therapeutic Target

Breast cancer is characterized by an increase in glandular stiffness. Tumor rigidity is likely due to a combination of factors such as elevated interstitial tissue pressure and solid stress due to perturbed vasculature and tumor expansion [211], increased elastic modulus of cancer cells due to altered cytoskeletal dynamics [212], and ECM stiffening due to fibrosis [213]. Work from Paszek et al. demonstrated that matrix stiffness perturbs epithelial tensional homeostasis by causing clustering of integrins, which results in growth-factor-enhanced ERK activation and increased Rho-ROCK-generated cytoskeletal contractility and focal adhesion formation [214]. These mechano-changes in turn perturb tissue behavior, pushing it toward a malignant phenotype, highlighting the role of disrupted tensional homeostasis and Rho-ROCK signaling in breast tumor initiation.

Calvo et al. isolated CAFs from different stages of breast cancer in the PyMT mouse model to analyze their function and gene expression [215]. Through global mRNA analysis they found that gene expression of yes-associated protein (YAP), the transcriptional co-activator of the Hippo pathway, was significantly upregulated in CAFs isolated from all stages of breast cancer. Interestingly, their GSEA analysis showed significant overlap between their murine CAFs and genes of the stroma in human breast cancer [215] suggesting the relevance of their approach. YAP activation status (i.e., the proportion located in the nucleus) correlated with disease stage CAF in murine and human breast cancer, as did YAP-target gene expression. Depletion of YAP by siRNA reduced the ability of CAFs to contract collagen-rich matrices, form focal adhesions, and promote cancer cell invasion [215]. These observations were confirmed in several different human CAF lines. Interestingly, the study found that YAP regulated several well-known cytoskeletal regulators including *anillin actin binding protein* (*ANLN*), *diaphanous related formin 3* (*DIAPH3*), and *filamin A* (*FLNA*) for CAFs to remodel the ECM and promote invasion [215]. Finally, Calvo et al. observed that matrix stiffening activated YAP in CAFs via actomyosin contractility and Src function to establish a feed-forward self-reinforcing loop. Transient ROCK inhibition by Y27632 was able to disrupt the feed-forward loop by blocking the nuclear translocation of YAP, leading to a long-lasting reversion of the CAF phenotype [215], suggesting potential therapeutic approaches for targeting the stroma in breast cancer.

A novel potent small molecule inhibitor of ROCK1 and ROCK2, named RKI-1447, was shown to suppress phosphorylation of the ROCK substrates MLC-2 and MYPT-1 in human cancer cells, but had no effect on the phosphorylation of AKT and MEK, additionally blocking actin stress fiber formation [216]. As such, RKI-1447 blocked

breast cancer cell migration, invasion, and anchorage-independent tumor growth. In vivo, RKI-1447 inhibited the outgrowth of mammary tumors in the MMTV/neu transgenic mouse model [216]. The same group also reported on another novel ROCK inhibitor, RKI-18, which had similar effects to RKI-1447, additionally blocking p21-activated kinase-mediated lamellipodia and filopodia formation in breast cancer cells [217]. RKI-18 was also shown to reduce phosphorylation of MLC-2 in human lung, colon, and prostate cancer cells and inhibit the migration, invasion, and anchorage-independent growth of human breast cancer cells [217]. These two studies suggest the use of RKIs as antitumor agents in breast cancer warrant further preclinical investigation.

6.4.3.6 Rho-ROCK Signaling in the TME of Renal Cancer

Clear cell renal cell carcinomas (CC-RCCs) account for 90% of all renal cancer cases, with the tumor-suppressor gene, *von Hippel-Lindau* (*VHL*), being functionally lost in up to 90% of CC-RCC tumors [218]. RhoA expression and activity (as well as Cdc42 and Rac1) is hypoxia-inducible in renal cancer, and Rho-ROCK pathway activity can stimulate HIF activity via several mechanisms [219]. Furthermore, VHL loss leads to Hypoxia Inducible Factor (HIF) upregulation [8]. As such, Thompson et al. observed synthetic lethality when downregulating ROCK1 by siRNA or using the Y27632 inhibitor, reporting reduced colony-forming ability, increased cytotoxicity, and reduced migration of CC-RCC cells [8]. The same study also showed that Y27632 could reduce subcutaneous CC-RCC tumor growth in mice. Interestingly, downregulation of ROCK2 had no effect on CC-RCC behavior.

6.4.3.7 Rho-ROCK Signaling in the TME of Urothelial Cancers

Kamai et al. found that RhoA mRNA levels and RhoA protein levels were greater in tumor and metastatic lymph node tissues than in matched non-tumor tissues in 47 patient samples from renal pelvic/ureteric cancer [220]. Taken together, these results suggest that the Rho-ROCK pathway may be a potential therapeutic target for preventing cancer invasion and metastasis by inhibiting cancer cell migration. Further, primary tumors from patients with high RhoA mRNA/protein levels were poorly differentiated and associated with muscle invasion. High RhoA levels correlated with shorter disease-free and overall survival, suggesting RhoA may be a useful prognostic factor for this disease [220].

In a similar study, Kamai et al. noted a significant correlation between Rho-ROCK pathway activity and invasion and metastasis of bladder cancer in 107 matched tumor and non-tumor surgical samples [9]. RhoA, RhoC, and ROCK levels were higher in tumors and metastatic lymph nodes than in non-tumor bladder and uninvolved lymph nodes, and the levels of RhoA and RhoC protein correlated positively with ROCK protein expression [9]. Again, high RhoA, RhoC, and ROCK expression levels were significantly correlated with poor tumor differentiation, muscle invasion, and lymph node metastasis. Further, high RhoA, RhoC, and ROCK protein levels were associated with shortened disease-free and overall survival [9]. Immunohistochemistry demonstrated that RhoA, RhoC, and ROCK proteins were abundantly expressed in tumor cells, with the authors concluding the RhoA- and RhoC-ROCK pathway is likely to be involved in local invasion and lymph node metastasis of tumor cells, and that expression of these components may serve as a useful prognostic marker in bladder cancer. Fasudil treatment of bladder cancer cell lines suppressed cell proliferation and migration and induced apoptosis in a dose-dependent manner [221].

6.4.3.8 The Rho-ROCK Pathway in Osteosarcoma

Tumors contain populations of cancer stem cells (CSCs) which have the ability to maintain pluripotency and self-renew. In the context of osteosarcoma, these CSCs are termed osteosarcoma-initiating cells (OSi cells) and have been shown to be chemoresistant and a major contributor to tumorigenesis [222]. The ROCK inhibitor Fasudil

was shown to suppress in vitro growth and in vivo tumorigenicity of OSi cells by modulating actin cytoskeleton dynamics and causing their differentiation toward terminal adipocytes [223]. Furthermore, when Fasudil treatment was combined with doxorubicin in a sequential manner, it acted synergistically in vivo to reduce tumor growth more significantly than Fasudil or doxorubicin alone [223]. This suggests that a "transdifferentiation" approach may be an effective method for targeting CSCs therapeutically.

6.5 Targeting Rho-ROCK Signaling in the Cancer TME

Although ROCK inhibitors are not currently used as anti-cancer treatments, there is mounting evidence of their potential in this area. Here we detail a recent example demonstrating the benefits of such an approach in the PDAC TME.

6.5.1 Targeting Rho-ROCK Signaling in the Cancer TME of PDAC

There has been much effort to understand how the different components of the TME of PDAC drive disease progression and contribute to treatment failure, with a view to identifying new therapeutic targets. However, studies targeting the stroma have had somewhat unexpected outcomes. Özdemir et al. published work showing that depletion of CAFs in mouse models at the precursor PanIN or the PDAC stage resulted in more invasive, undifferentiated tumors that contained more CSCs and had elevated hypoxia, and ultimately reduced animal survival [224]. Interestingly, this study also noted that CAF depletion in combination with immunotherapy (anti-CTLA4) led to reversed disease acceleration and prolonged animal survival. Rhim et al. showed that deleting sonic hedgehog (Shh) in a mouse model of PDAC, which is a soluble ligand overexpressed by neoplastic cells in PDAC, leads to reduced stromal content as was predicted, but unexpectedly resulted in more aggressive, undifferentiated tumors that more readily metastasized and caused higher animal mortality [225]. Heterogeneity in PDAC CAFs was recently shown to exist and has been suggested to be a possible reason for the differing results obtained using stroma-targeting approaches [198]. Furthermore, findings by Laklai et al. indicate that the genotype driving the PDAC is important in stromal–epithelial interactions and affects how fibrosis in the TME occurs, and may influence how a patient would respond to anti-stromal therapy [226]. They found in PDAC patient biopsies that higher matricellular protein and activated STAT3 were associated with SMAD4 mutation and shorter survival. Using mouse models they delineated that STAT3 activation drives ROCK-dependent ECM remodeling and stiffening to enhance fibrosis and elevate mechano-signaling in PDACs [226]. Taken together, this body of work emphasizes a need for carefully considered approaches when it comes to targeting stromal elements in PDAC.

As such, recent efforts have turned toward targeting Rho-ROCK signaling in the TME to improve patient outcomes [227]. This approach to manipulating the TME can result in the targeting of several stromal elements concurrently to elicit positive changes to the TME composition that improve therapeutic response. Prior to this work, little was known about how consecutive dual targeting of tumor tissue tension and vasculature prior to chemotherapy could affect tumor response. In particular, this work highlighted the promise for a "priming" approach in targeting Rho-ROCK in the TME in a transient manner to manipulate tumor tissue [228, 229]. Intravital imaging was used to optimize this transient ROCK inhibition using the pharmacological ROCK inhibitor Fasudil and to influence cell responses to chemotherapy [227]. Combining mouse and stratified patient-derived models of pancreatic cancer with biosensor FLIM-FRET intravital imaging allowed the authors to visualize the effects of ROCK inhibition in real time in live tissues [227, 230, 231]. Using transient "priming," Fasudil was delivered for 3 days prior to che-

motherapy and resulted in synchronization of the cell cycle in pancreatic cancer cells, making them susceptible to standard-of-care chemotherapy gemcitabine/Abraxane. These effects were seen in both the primary tumors and metastatic sites [227]. Furthermore, Fasudil priming in the adjuvant setting disrupted coordinated cancer cell movement to impair metastatic colonization of the liver. Collective migration was hypothesized to be affected due to disrupted durotaxis [232]. The authors also found that Fasudil treatment increased the sensitivity of circulating tumor cells (CTCs) to shear stress that is encountered in the blood stream, impeding their ability to extravasate and colonize host tissues [227]. ROCK inhibition also reduced the ability of metastatic cells to remodel host ECM to create a permissible environment to support their growth at distant sites, as has previously been described [233]. Fasudil "priming" reduced ECM remodeling and tumor tissue stiffness, thus affecting integrin signaling and ablating mechanical cues normally provided by the matrix to cancer cells [227]. This reduction in tumor tissue stiffness was accompanied by an increase in permeability of the tumor vasculature, which may aid drug delivery.

This work strongly suggests that using ROCK inhibitors in pancreatic cancer treatment in a "priming" regime merits further attention for improving chemotherapeutic efficacy in this deadly malignancy. In particular, the study by Vennin et al. demonstrates how effective transient ROCK inhibition can be versus chronic treatment, which has a greater potential for adverse effects and toxicity. In support of the use of ROCK inhibitors as a therapeutic means in pancreatic cancer, AT13148, a novel multi-kinase inhibitor, which potently blocks ROCK1/2 (but also PKA, AKT, and p70S6K; [234]), was recently tested in the KPC mouse model and on patient-derived pancreatic cancer cell lines. The study by Rath et al. demonstrated that AT13148 induced morphological changes, reduced cellular contractile forces as well as reducing motility on pliable substrates and impairing invasion into 3D collagen matrices [11]. In vivo, AT13148 reduced subcutaneous tumor growth and metastasis in the KPC mouse model. Another benefit noted by the authors was the ability of AT13148 to maintain separation between tumor and healthy tissue boundaries, suggesting its potential as an adjuvant treatment for enabling tumor resection [11].

6.6 Conclusions, Future Trends, and Directions

Targeting the Rho-ROCK pathway in the cancer TME is an emerging and exciting avenue of research. Preclinical studies with various ROCK inhibitors suggest promise for targeting Rho-ROCK signaling therapeutically, not just in cancer but also in a range of pathologies, with the two best-characterized ROCK inhibitors being Y27632 and Fasudil. Despite this, ROCK inhibitors are not currently used as a cancer treatment, largely due to the challenge of developing anti-metastatic drugs, and uncertainties about the cancer types most likely to benefit from ROCK inhibitor therapy [228]. Furthermore, effective predictive biomarkers of treatment response to Rho-ROCK inhibition have been lacking. Although recent work from Vennin et al. has demonstrated that CD31-based or ECM-based biomarkers may be a promising avenue [227] and collectively, might further facilitate the identification of patients who could benefit from a transient "priming" stromal targeting regimen prior to chemotherapy. In future, examining the stroma for further potential biomarkers of tumor response to Rho-ROCK inhibition may provide additional important insights versus searching for responses in the tumor itself. Indeed, this will likely also prove important for assessing the effects of ROCK inhibition in primary versus metastatic lesions, as well as in precancerous lesions. An interesting challenge remains in determining which patients are most likely to benefit from therapies targeting Rho-ROCK signaling in the TME. Work toward this has already begun and is highlighted by the recent study from Georgouli et al. [192], where the authors delineated a novel role for ROCK-myosin-II dynamics in regulating melanoma cancer cell behavior aside from intrinsic control of cell motility. Inhibition of ROCK-myosin-II activity ablated the

invasive amoeboid melanoma cell phenotype they saw in human melanoma biopsies and reduced secretion of IL-1α by cancer cells, preventing a positive feedback loop involving activation of NF-κB. Likewise, targeting of NF-κB in melanoma cells diminished their amoeboid behavior and their secretory profile. However, since NF-κB is difficult to target therapeutically [235, 236], this study suggests ROCK inhibitors could be used in the context of melanoma to reprogram the innate immune microenvironment, and that melanoma patient biopsies could be used to assess patients for ROCK pathway activation to identify patients that could benefit from such an approach. Indeed, the authors propose that following resection of the primary lesion, ROCK inhibitors could be used to restrict formation/progression of an immunosuppressive microenvironment and metastatic dissemination [192].

While Fasudil is more selective against ROCK, Y27632 is less selective and furthermore, Y27632 is not optimal for in vivo use. Therefore it seems likely that these ROCK inhibitors may still compromise normal cellular functions due to both their nonspecificity and because of the pleiotropy of ROCK [181]. As an example of this, ROCK1 has been implicated as the predominant cause for the hypotensive effects of pan-ROCK inhibitors [2]. One approach to mitigate such consequences will be to examine further the downstream effector pathways of ROCK that are responsible for certain tumor-promoting activities to further enhance tumor TME-targeting strategies by identifying novel effector targets. Such strategies may also help to mitigate any resistance to specific ROCK inhibition that emerges through compensatory expression of either isoform when inhibiting these kinases, as well as helping to understand isoform-specific regulation of cancer cell behavior. Other recent efforts to mitigate unwanted effects of chronic ROCK inhibition include transient, short-term inhibition using "priming" approaches [227], which exhibited impressive efficacy in altering the TME, paving the way for future clinical studies using ROCK inhibitor "priming" approaches prior to, or perhaps even in tandem with chemotherapeutics to improve their delivery and impact on the tumor.

As research in this arena expands, newer, more potent and more selective ROCK inhibitors are likely to be identified with improved in vivo pharmacodynamics. Furthermore, identification of the tumor types that are most likely to benefit from ROCK inhibitor treatment should become clearer. As strategies to target the Rho-ROCK pathway in the cancer TME develop, these approaches are likely to yield tangible benefits, particularly in aggressive cancer types where Rho-ROCK signaling drives invasive spread of the disease.

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References

1. Amin E et al (2013) Rho-kinase: regulation, (dys)function, and inhibition. Biol Chem 394(11):1399–1410
2. Chin VT et al (2015) Rho-associated kinase signalling and the cancer microenvironment: novel biological implications and therapeutic opportunities. Expert Rev Mol Med 17:e17
3. Koch JC, Tatenhorst L, Roser A-E, Saal K-A, Tönges L, Lingor P (Sep. 2018) ROCK inhibition in models of neurodegeneration and its potential for clinical translation. Pharmacol Ther 189:1–21
4. Cai A, Li L, Zhou Y (2016) Pathophysiological effects of RhoA and Rho-associated kinase on cardiovascular system. J Hypertens 34(1):3–10
5. Knipe RS et al (2017) The rho kinase isoforms ROCK1 and ROCK2 each contribute to the development of experimental pulmonary fibrosis. Am J Respir Cell Mol Biol 58(4):471–481
6. Okimoto S et al (2019) Vitamin A-coupled liposomal rho-kinase inhibitor ameliorates liver fibrosis without systemic adverse effects. Hepatol Res 49(6):663–675
7. Lenti MV, Di Sabatino A (2019) Intestinal fibrosis. Mol Asp Med 65:100–109
8. Thompson JM et al (2017) Rho-associated kinase 1 inhibition is synthetically lethal with von Hippel-Lindau deficiency in clear cell renal cell carcinoma. Oncogene 36(8):1080–1089
9. Kamai T et al (2003) Significant association of rho/ROCK pathway with invasion and metastasis of bladder cancer. Clin Cancer Res 9:2632–2641

10. Liu S (2011) The ROCK signaling and breast cancer metastasis. Mol Biol Rep 38(2):1363–1366
11. Rath N et al (2018) Rho kinase inhibition by AT13148 blocks pancreatic ductal adenocarinoma invasion and tumor growth. Cancer Res 78(12):3321–3336
12. Defert O, Boland S (2017) Rho kinase inhibitors: a patent review (2014–2016). Expert Opin Ther Pat 27(4):507–515
13. Nakagawa O, Fujisawa K, Ishizaki T, Saito Y, Nakao K, Narumiya S (1996) ROCK-I and ROCK-II, two isoforms of rho-associated coiled-coil forming protein serine/threonine kinase in mice. FEBS Lett 392(2):189–193
14. Morgan-Fisher M, Wewer UM, Yoneda A (2013) Regulation of ROCK activity in cancer. J Histochem Cytochem 61(3):185–198
15. Kher SS, Worthylake RA (2011) Regulation of ROCKII membrane localization through its C-terminus. Exp Cell Res 317(20):2845–2852
16. Ishiuchi T, Takeichi M (2011) Willin and Par3 cooperatively regulate epithelial apical constriction through aPKC-mediated ROCK phosphorylation. Nat Cell Biol 13(7):860–866
17. McCormack J, Welsh NJ, Braga VMM (2013) Cycling around cell-cell adhesion with Rho GTPase regulators. J Cell Sci 126(2):379–391
18. Timpson P, Jones GE, Frame MC, Brunton VG (2001) Coordination of cell polarization and migration by the rho family GTPases requires Src tyrosine kinase activity. Curr Biol 11(23):1836–1846
19. Lebowitz PF, Davide JP, Prendergast GC (1995) Evidence that farnesyltransferase inhibitors suppress Ras transformation by interfering with Rho activity. Mol Cell Biol 15(12):6613–6622
20. Wennerberg K, Der CJ (2004) Rho-family GTPases: it's not only Rac and Rho (and I like it). J Cell Sci 117(8):1301–1312
21. Cherfils J, Zeghouf M (2013) Regulation of small GTPases by GEFs, GAPs, and GDIs. Physiol Rev 93(1):269–309
22. Fritz RD, Pertz O (2016) The dynamics of spatiotemporal rho GTPase signaling: formation of signaling patterns. F1000Res 5
23. Ishizaki T et al (1996) The small GTP-binding protein rho binds to and activates a 160 kDa Ser/Thr protein kinase homologous to myotonic dystrophy kinase. EMBO J 15(8):1885–1893
24. Conway A-M, James AB, O'Kane EM, Rakhit S, Morris BJ (2004) Regulation of myosin light chain phosphorylation by RhoB in neuronal cells. Exp Cell Res 300(1):35–42
25. Watanabe N et al (1997) p140mDia, a mammalian homolog of Drosophila diaphanous, is a target protein for Rho small GTPase and is a ligand for profilin. EMBO J 16(11):3044–3056
26. Ward Y et al (2002) The GTP binding proteins Gem and Rad are negative regulators of the Rho-Rho kinase pathway. J Cell Biol 157(2):291–302
27. Riento K, Guasch RM, Garg R, Jin B, Ridley AJ (2003) RhoE binds to ROCK I and inhibits downstream signaling. Mol Cell Biol 23(12):4219–4229
28. Araki S et al (2001) Arachidonic acid-induced Ca2+ sensitization of smooth muscle contraction through activation of Rho-kinase. Pflugers Arch 441(5):596–603
29. Yoneda A, Multhaupt HAB, Couchman JR (2005) The Rho kinases I and II regulate different aspects of myosin II activity. J Cell Biol 170(3):443–453
30. Strzelecka-Kiliszek A, Mebarek S, Roszkowska M, Buchet R, Magne D, Pikula S (2017) Functions of Rho family of small GTPases and Rho-associated coiled-coil kinases in bone cells during differentiation and mineralization. Biochim Biophys Acta Gen Subj 1861(5 Pt A):1009–1023
31. Jaffe AB, Hall A (2005) RHO GTPASES: biochemistry and biology. Annu Rev Cell Dev Biol 21(1):247–269
32. Pajic M et al (2015) The dynamics of rho GTPase signaling and implications for targeting cancer and the tumor microenvironment. Small GTPases 6(2):123–133
33. Wang Y et al (2009) ROCK isoform regulation of myosin phosphatase and contractility in vascular smooth muscle cells. Circ Res 104(4):531–540
34. Schofield AV, Bernard O (2013) Rho-associated coiled-coil kinase (ROCK) signaling and disease. Crit Rev Biochem Mol Biol 48(4):301–316
35. Asp P, Wihlborg M, Karlén M, Farrants A-KO (2002) Expression of BRG1, a human SWI/SNF component, affects the organisation of actin filaments through the RhoA signalling pathway. J Cell Sci 115(Pt 13):2735–2746
36. Rath N, Olson MF (2012) Rho-associated kinases in tumorigenesis: re-considering ROCK inhibition for cancer therapy. EMBO Rep 13(10):900–908
37. Kadir S, Astin JW, Tahtamouni L, Martin P, Nobes CD (2011) Microtubule remodelling is required for the front-rear polarity switch during contact inhibition of locomotion. J Cell Sci 124(Pt 15):2642–2653
38. Lowery DM et al (2007) Proteomic screen defines the Polo-box domain interactome and identifies Rock2 as a Plk1 substrate. EMBO J 26(9):2262–2273
39. Kanai M, Crowe MS, Zheng Y, Vande Woude GF, Fukasawa K (2010) RhoA and RhoC are both required for the ROCK II-dependent promotion of centrosome duplication. Oncogene 29(45):6040–6050
40. Hanashiro K, Brancaccio M, Fukasawa K (2011) Activated ROCK II by-passes the requirement of the CDK2 activity for centrosome duplication and amplification. Oncogene 30(19):2188–2197
41. Croft DR, Olson MF (2006) The rho GTPase effector ROCK regulates cyclin A, cyclin D1, and p27Kip1 levels by distinct mechanisms. Mol Cell Biol 26(12):4612–4627

42. Samuel MS et al (2011) Actomyosin-mediated cellular tension drives increased tissue stiffness and β-catenin activation to induce epidermal hyperplasia and tumor growth. Cancer Cell 19(6):776–791
43. Street CA, Bryan BA (2011) Rho kinase proteins—pleiotropic modulators of cell survival and apoptosis. Anticancer Res 31(11):3645–3657
44. Lock FE, Hotchin NA (2009) Distinct roles for ROCK1 and ROCK2 in the regulation of keratinocyte differentiation. PLoS One 4(12):e8190
45. Palanivel R, Ganguly R, Turdi S, Xu A, Sweeney G (2014) Adiponectin stimulates rho-mediated actin cytoskeleton remodeling and glucose uptake via APPL1 in primary cardiomyocytes. Metabolism 63(10):1363–1373
46. Laufs U et al (2000) Neuroprotection mediated by changes in the endothelial actin cytoskeleton. J Clin Invest 106(1):15–24
47. Wang L, Yang L, Luo Y, Zheng Y (2003) A novel strategy for specifically down-regulating individual Rho GTPase activity in tumor cells. J Biol Chem 278(45):44617–44625
48. Ridley AJ (2013) RhoA, RhoB and RhoC have different roles in cancer cell migration. J Microsc 251(3):242–249
49. Sin WC, Chen XQ, Leung T, Lim L (1998) RhoA-binding kinase alpha translocation is facilitated by the collapse of the vimentin intermediate filament network. Mol Cell Biol 18(11):6325–6339
50. Chen X, Tan I, Ng CH, Hall C, Lim L, Leung T (2002) Characterization of RhoA-binding kinase ROKalpha implication of the pleckstrin homology domain in ROKalpha function using region-specific antibodies. J Biol Chem 277(15):12680–12688
51. Kimura K et al (1998) Regulation of the association of adducin with actin filaments by Rho-associated kinase (Rho-kinase) and myosin phosphatase. J Biol Chem 273(10):5542–5548
52. Momotani K, Somlyo AV (2012) p63RhoGEF: a new switch for G(q)-mediated activation of smooth muscle. Trends Cardiovasc Med 22(5):122–127
53. Zou W, Teitelbaum SL (2010) Integrins, growth factors, and the osteoclast cytoskeleton. Ann N Y Acad Sci 1192:27–31
54. Tybulewicz VLJ, Henderson RB (2009) Rho family GTPases and their regulators in lymphocytes. Nat Rev Immunol 9(9):630–644
55. Iden S, Collard JG (2008) Crosstalk between small GTPases and polarity proteins in cell polarization. Nat Rev Mol Cell Biol 9(11):846–859
56. Dvorsky R, Ahmadian MR (2004) Always look on the bright site of Rho: structural implications for a conserved intermolecular interface. EMBO Rep 5(12):1130–1136
57. Herbrand U, Ahmadian MR (2006) p190-RhoGAP as an integral component of the Tiam1/Rac1-induced downregulation of Rho. Biol Chem 387(3):311–317
58. Zieba BJ et al (2011) The cAMP-responsive Rap1 guanine nucleotide exchange factor, Epac, induces smooth muscle relaxation by down-regulation of RhoA activity. J Biol Chem 286(19):16681–16692
59. Larrea MD et al (2009) RSK1 drives p27Kip1 phosphorylation at T198 to promote RhoA inhibition and increase cell motility. Proc Natl Acad Sci U S A 106(23):9268–9273
60. Valderrama F, Cordeiro JV, Schleich S, Frischknecht F, Way M (2006) Vaccinia virus-induced cell motility requires F11L-mediated inhibition of RhoA signaling. Science 311(5759):377–381
61. Duan X, Chen K-L, Zhang Y, Cui X-S, Kim N-H, Sun S-C (2014) ROCK inhibition prevents early mouse embryo development. Histochem Cell Biol 142(2):227–233
62. Marikawa Y, Alarcon VB (2012) Creation of trophectoderm, the first epithelium, in mouse preimplantation development. Results Probl Cell Differ 55:165–184
63. Hirate Y et al (2013) Polarity-dependent distribution of angiomotin localizes Hippo signaling in preimplantation embryos. Curr Biol 23(13):1181–1194
64. Alarcon VB, Marikawa Y (2018) ROCK and RHO playlist for Preimplantation development: streaming to HIPPO pathway and Apicobasal polarity in the first cell differentiation. Adv Anat Embryol Cell Biol 229:47–68
65. Marikawa Y, Alarcon VB (2019) RHOA activity in expanding blastocysts is essential to regulate HIPPO-YAP signaling and to maintain the trophectoderm-specific gene expression program in a ROCK/actin filament-independent manner. MHR Basic Sci Reprod Med 25(2):43–60
66. Motomura K et al (2017) A rho-associated coiled-coil containing kinases (ROCK) inhibitor, Y-27632, enhances adhesion, viability and differentiation of human term placenta-derived trophoblasts in vitro. PLoS One 12(5):e0177994
67. Watanabe K et al (2007) A ROCK inhibitor permits survival of dissociated human embryonic stem cells. Nat Biotechnol 25(6):681–686
68. Ohgushi M et al (2010) Molecular pathway and cell state responsible for dissociation-induced apoptosis in human pluripotent stem cells. Cell Stem Cell 7(2):225–239
69. Samuel MS, Olson MF (2011) Rho-GTPases in embryonic stem cells. Embryonic Stem Cells Basic Biol Bioeng
70. Kiecker C, Bates T, Bell E (2016) Molecular specification of germ layers in vertebrate embryos. Cell Mol Life Sci 73(5):923–947
71. Kim K, Ossipova O, Sokol SY (2015) Neural crest specification by inhibition of the ROCK/Myosin II pathway. Stem Cells 33(3):674–685
72. Srinivasan A, Chang S-Y, Zhang S, Toh WS, Toh Y-C (2018) Substrate stiffness modulates the mul-

tipotency of human neural crest derived ectomesenchymal stem cells via CD44 mediated PDGFR signaling. Biomaterials 167:153–167
73. Laeno AMA, Tamashiro DAA, Alarcon VB (2013) Rho-associated kinase activity is required for proper morphogenesis of the inner cell mass in the mouse blastocyst. Biol Reprod 89(5):122
74. Li L, Arman E, Ekblom P, Edgar D, Murray P, Lonai P (2004) Distinct GATA6- and laminin-dependent mechanisms regulate endodermal and ectodermal embryonic stem cell fates. Development 131(21):5277–5286
75. Loebel DAF, Tam PPL (2012) Rho GTPases in endoderm development and differentiation. Small GTPases 3(1):40–44
76. Maldonado M, Luu RJ, Ramos MEP, Nam J (2016) ROCK inhibitor primes human induced pluripotent stem cells to selectively differentiate towards mesendodermal lineage via epithelial-mesenchymal transition-like modulation. Stem Cell Res 17(2):222–227
77. Joo HJ et al (2012) ROCK suppression promotes differentiation and expansion of endothelial cells from embryonic stem cell-derived Flk1(+) mesodermal precursor cells. Blood 120(13):2733–2744
78. Lawson CD, Ridley AJ (2018) Rho GTPase signaling complexes in cell migration and invasion. J Cell Biol 217(2):447–457
79. LaMonica K, Grabel L (2012) The planar cell polarity pathway and parietal endoderm cell migration. In: Turksen K (ed) Planar cell polarity: methods and protocols. Springer, New York, pp 187–200
80. Stankova V, Tsikolia N, Viebahn C (2015) Rho kinase activity controls directional cell movements during primitive streak formation in the rabbit embryo. Development 142(1):92–98
81. Berndt JD, Clay MR, Langenberg T, Halloran MC (2008) Rho-kinase and myosin II affect dynamic neural crest cell behaviors during epithelial to mesenchymal transition in vivo. Dev Biol 324(2):236–244
82. Kuriyama S, Mayor R (2008) Molecular analysis of neural crest migration. Philos Trans R Soc Lond B Biol Sci 363(1495):1349–1362
83. Wei L et al (2001) Rho kinases play an obligatory role in vertebrate embryonic organogenesis. Development 128(15):2953–2962
84. Rolo A, Escuin S, Greene NDE, Copp AJ (2018) Rho GTPases in mammalian spinal neural tube closure. Small GTPases 9(4):283–289
85. Borges RM, Lamers ML, Forti FL, Santos MFD, Yan CYI (2011) Rho signaling pathway and apical constriction in the early lens placode. Genes N Y N 2000 49(5):368–379
86. Chauhan BK et al (2009) Cdc42- and IRSp53-dependent contractile filopodia tether presumptive lens and retina to coordinate epithelial invagination. Development 136(21):3657–3667
87. Chauhan BK, Lou M, Zheng Y, Lang RA (2011) Balanced Rac1 and RhoA activities regulate cell shape and drive invagination morphogenesis in epithelia. Proc Natl Acad Sci U S A 108(45):18289–18294
88. Porazinski S et al (2015) YAP is essential for tissue tension to ensure vertebrate 3D body shape. Nature 521(7551):217–221
89. Dupont S et al (2011) Role of YAP/TAZ in mechanotransduction. Nature 474(7350):179–183
90. Boyle ST, Kular J, Nobis M, Ruszkiewicz A, Timpson P, Samuel MS (2018) Acute compressive stress activates RHO/ROCK-mediated cellular processes. Small GTPases 1–17
91. Eiraku M et al (2011) Self-organizing optic-cup morphogenesis in three-dimensional culture. Nature 472(7341):51–56
92. Chen G, Hou Z, Gulbranson DR, Thomson JA (2010) Actin-myosin contractility is responsible for the reduced viability of dissociated human embryonic stem cells. Cell Stem Cell 7(2):240–248
93. Romani P et al (2019) Extracellular matrix mechanical cues regulate lipid metabolism through Lipin-1 and SREBP. Nat Cell Biol 21(3):338–347
94. Narumiya S, Thumkeo D (2018) Rho signaling research: history, current status and future directions. FEBS Lett 592(11):1763–1776
95. Takano T et al (2017) Discovery of long-range inhibitory signaling to ensure single axon formation. Nat Commun 8(1):33
96. Shinohara R et al (2012) A role for mDia, a rho-regulated actin nucleator, in tangential migration of interneuron precursors. Nat Neurosci 15(3):373–380
97. Tashiro A, Minden A, Yuste R (2000) Regulation of dendritic spine morphology by the rho family of small GTPases: antagonistic roles of Rac and rho. Cereb Cortex 10(10):927–938
98. Czeisler C et al (2016) Surface topography during neural stem cell differentiation regulates cell migration and cell morphology. J Comp Neurol 524(17):3485–3502
99. Julian L, Olson MF (2014) Rho-associated coiled-coil containing kinases (ROCK). Small GTPases 5(2):e29846
100. Vicente-Steijn R et al (2017) RHOA-ROCK signalling is necessary for lateralization and differentiation of the developing sinoatrial node. Cardiovasc Res 113(10):1186–1197
101. Moore KA et al (2005) Control of basement membrane remodeling and epithelial branching morphogenesis in embryonic lung by rho and cytoskeletal tension. Dev Dyn 232(2):268–281
102. Toyoda T et al (2017) Rho-associated kinases and non-muscle Myosin IIs inhibit the differentiation of human iPSCs to pancreatic endoderm. Stem Cell Rep 9(2):419–428
103. Korostylev A et al (2017) A high-content small molecule screen identifies novel inducers of definitive endoderm. Mol Metab 6(7):640–650
104. Ghazizadeh Z et al (2017) ROCKII inhibition promotes the maturation of human pancreatic beta-like cells. Nat Commun 8(1):298

105. Kular J et al (2015) A negative regulatory mechanism involving 14-3-3ζ limits Signaling downstream of ROCK to regulate tissue stiffness in epidermal homeostasis. Dev Cell 35(6):759–774
106. Butcher DT, Alliston T, Weaver VM (2009) A tense situation: forcing tumour progression. Nat Rev Cancer 9(2):108–122
107. Hay ED (1995) An overview of epithelio-mesenchymal transformation. Acta Anat (Basel) 154(1):8–20
108. Markowski MC, Brown AC, Barker TH (2012) Directing epithelial to mesenchymal transition through engineered microenvironments displaying orthogonal adhesive and mechanical cues. J Biomed Mater Res A 100(8):2119–2127
109. Yang J-Q et al (2016) RhoA orchestrates glycolysis for TH2 cell differentiation and allergic airway inflammation. J Allergy Clin Immunol 137(1):231–245.e4
110. Song J et al (2019) Inhibition of ROCK activity regulates the balance of Th1, Th17 and Treg cells in myasthenia gravis. Clin Immunol 203:142–153
111. Zanin-Zhorov A et al (2014) Selective oral ROCK2 inhibitor down-regulates IL-21 and IL-17 secretion in human T cells via STAT3-dependent mechanism. Proc Natl Acad Sci U S A 111(47):16814–16819
112. Zanin-Zhorov A et al (2017) Cutting edge: selective Oral ROCK2 inhibitor reduces clinical scores in patients with psoriasis vulgaris and normalizes skin pathology via concurrent regulation of IL-17 and IL-10. J Immunol 198(10):3809–3814
113. Zanin-Zhorov A, Waksal SD (2015) ROCKing cytokine secretion balance in human T cells. Cytokine 72(2):224–225
114. Kanzaki M, Pessin JE (2001) Insulin-stimulated GLUT4 translocation in adipocytes is dependent upon cortical actin remodeling. J Biol Chem 276(45):42436–42444
115. Török D et al (2004) Insulin but not PDGF relies on actin remodeling and on VAMP2 for GLUT4 translocation in myoblasts. J Cell Sci 117(Pt 22):5447–5455
116. Furukawa N et al (2005) Role of Rho-kinase in regulation of insulin action and glucose homeostasis. Cell Metab 2(2):119–129
117. Lee DH et al (2009) Targeted disruption of ROCK1 causes insulin resistance in vivo. J Biol Chem 284(18):11776–11780
118. Chun K-H et al (2012) Regulation of glucose transport by ROCK1 differs from that of ROCK2 and is controlled by Actin polymerization. Endocrinology 153(4):1649–1662
119. Stirling L, Williams MR, Morielli AD (2009) Dual roles for RHOA/RHO-kinase in the regulated trafficking of a voltage-sensitive potassium channel. Mol Biol Cell 20(12):2991–3002
120. Li H et al (2016) Y-27632, a Rho-associated protein kinase inhibitor, inhibits voltage-dependent K+ channels in rabbit coronary arterial smooth muscle cells. Pharmacology 98(5–6):220–227
121. Rousset M et al (2015) Regulation of neuronal high-voltage activated Ca(V)2 Ca(2+) channels by the small GTPase RhoA. Neuropharmacology 97:201–209
122. Ureña J, López-Barneo J (2012) Metabotropic regulation of RhoA/rho-associated kinase by L-type Ca2+ channels. Trends Cardiovasc Med 22(6):155–160
123. Henderson BW et al (2016) Rho-associated protein kinase 1 (ROCK1) is increased in Alzheimer's disease and ROCK1 depletion reduces amyloid-β levels in brain. J Neurochem 138(4):525–531
124. Fujimura M, Usuki F, Nakamura A (2019) Fasudil, a rho-associated coiled coil-forming protein kinase inhibitor, recovers Methylmercury-induced axonal degeneration by changing microglial phenotype in rats. Toxicol Sci 168(1):126–136
125. Shimizu T, Liao JK (2016) Rho kinases and cardiac Remodeling. Circ J 80(7):1491–1498
126. Bailey KE et al (2019) Disruption of embryonic ROCK signaling reproduces the sarcomeric phenotype of hypertrophic cardiomyopathy. JCI Insight 5
127. Komers R (2013) Rho kinase inhibition in diabetic kidney disease. Br J Clin Pharmacol 76(4):551–559
128. Stanley A, Heo S, Mauck RL, Mourkioti F, Shore EM (2019) Elevated BMP and mechanical signaling through YAP1/RhoA poises FOP Mesenchymal progenitors for Osteogenesis. J Bone Miner Res 34:1894–1909
129. Wang Y-X et al (2005) Inhibition of Rho-kinase by fasudil attenuated angiotensin II-induced cardiac hypertrophy in apolipoprotein E deficient mice. Eur J Pharmacol 512(2–3):215–222
130. Prior IA, Lewis PD, Mattos C (2012) A comprehensive survey of Ras mutations in cancer. Cancer Res 72(10):2457–2467
131. Kandoth C et al (2013) Mutational landscape and significance across 12 major cancer types. Nature 502(7471):333–339
132. Waddell N et al (2015) Whole genomes redefine the mutational landscape of pancreatic cancer. Nature 518(7540):495–501
133. Olson MF (2018) Rho GTPases, their post-translational modifications, disease-associated mutations and pharmacological inhibitors. Small GTPases 9(3):203–215
134. Hodis E et al (2012) A landscape of driver mutations in melanoma. Cell 150(2):251–263
135. Krauthammer M et al (2012) Exome sequencing identifies recurrent somatic RAC1 mutations in melanoma. Nat Genet 44(9):1006–1014
136. Kawazu M et al (2013) Transforming mutations of RAC guanosine triphosphatases in human cancers. Proc Natl Acad Sci U S A 110(8):3029–3034
137. Yoo HY et al (2014) A recurrent inactivating mutation in RHOA GTPase in angioimmunoblastic T cell lymphoma. Nat Genet 46(4):371–375
138. Sakata-Yanagimoto M et al (2014) Somatic RHOA mutation in angioimmunoblastic T cell lymphoma. Nat Genet 46(2):171–175

139. Kakiuchi M et al (2014) Recurrent gain-of-function mutations of RHOA in diffuse-type gastric carcinoma. Nat Genet 46(6):583–587
140. Dyberg C et al (2017) Rho-associated kinase is a therapeutic target in neuroblastoma. Proc Natl Acad Sci U S A 114(32):E6603–E6612
141. Wei L, Surma M, Shi S, Lambert-Cheatham N, Shi J (2016) Novel insights into the roles of Rho kinase in cancer. Arch Immunol Ther Exp 64(4): 259–278
142. Itoh K, Yoshioka K, Akedo H, Uehata M, Ishizaki T, Narumiya S (1999) An essential part for Rho-associated kinase in the transcellular invasion of tumor cells. Nat Med 5(2):221–225
143. Olson MF, Sahai E (2008) The actin cytoskeleton in cancer cell motility. Clin Exp Metastasis 26(4):273
144. Sadok A, Marshall CJ (2014) Rho GTPases: masters of cell migration. Small GTPases 5:e29710
145. Vega FM, Fruhwirth G, Ng T, Ridley AJ (2011) RhoA and RhoC have distinct roles in migration and invasion by acting through different targets. J Cell Biol 193(4):655–665
146. Vega FM, Colomba A, Reymond N, Thomas M, Ridley AJ (2012) RhoB regulates cell migration through altered focal adhesion dynamics. Open Biol 2(5):120076
147. Gong X, Didan Y, Lock JG, Strömblad S (2018) KIF13A-regulated RhoB plasma membrane localization governs membrane blebbing and blebby amoeboid cell migration. EMBO J 37(17)
148. Suwa H et al (1998) Overexpression of the rhoC gene correlates with progression of ductal adenocarcinoma of the pancreas. Br J Cancer 77(1):147–152
149. Binnewies M et al (2018) Understanding the tumor immune microenvironment (TIME) for effective therapy. Nat Med 24(5):541
150. LeBleu VS, Kalluri R (2018) A peek into cancer-associated fibroblasts: origins, functions and translational impact. Dis Model Mech 11(4):dmm029447
151. Boyle ST, Samuel MS (2016) Mechano-reciprocity is maintained between physiological boundaries by tuning signal flux through the rho-associated protein kinase. Small GTPases 7(3):139–146
152. Feng Y, LoGrasso PV, Defert O, Li R (2016) Rho kinase (ROCK) inhibitors and their therapeutic potential. J Med Chem 59(6):2269–2300
153. Rath N et al (2017) ROCK signaling promotes collagen remodeling to facilitate invasive pancreatic ductal adenocarcinoma tumor cell growth. EMBO Mol Med 9(2):198–218
154. Timpson P et al (2011) Spatial regulation of RhoA activity during pancreatic cancer cell invasion driven by mutant p53. Cancer Res 71(3):747–757
155. Guerra FS, de Oliveira RG, Fraga CAM, Mermelstein CS, Fernandes PD (2017) ROCK inhibition with Fasudil induces beta-catenin nuclear translocation and inhibits cell migration of MDA-MB 231 human breast cancer cells. Sci Rep 7(1):13723
156. Tomasek JJ, Gabbiani G, Hinz B, Chaponnier C, Brown RA (2002) Myofibroblasts and mechano-regulation of connective tissue remodelling. Nat Rev Mol Cell Biol 3(5):349
157. Orimo A et al (2005) Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. Cell 121(3):335–348
158. Albrengues J et al (2014) LIF mediates proinvasive activation of stromal fibroblasts in cancer. Cell Rep 7(5):1664–1678
159. Skobe M, Fusenig NE (1998) Tumorigenic conversion of immortal human keratinocytes through stromal cell activation. Proc Natl Acad Sci 95(3):1050–1055
160. Strutz F et al (2000) Basic fibroblast growth factor expression is increased in human renal fibrogenesis and may mediate autocrine fibroblast proliferation. Kidney Int 57(4):1521–1538
161. Surowiak P et al (2006) Stromal myofibroblasts in breast cancer: relations between their occurrence, tumor grade and expression of some tumour markers. Folia Histochem Cytobiol 44(2):111–116
162. Graizel D, Zlotogorski-Hurvitz A, Tsesis I, Rosen E, Kedem R, Vered M (2019) Oral cancer-associated fibroblasts predict poor survival: systematic review and meta-analysis. Oral Dis
163. Son GM, Kwon M-S, Shin D-H, Shin N, Ryu D, Kang C-D (2019) Comparisons of cancer-associated fibroblasts in the intratumoral stroma and invasive front in colorectal cancer. Medicine (Baltimore) 98(18):e15164
164. Kalluri R, Zeisberg M (2006) Fibroblasts in cancer. Nat Rev Cancer 6(5):392–401
165. Gaggioli C et al (2007) Fibroblast-led collective invasion of carcinoma cells with differing roles for RhoGTPases in leading and following cells. Nat Cell Biol 9(12):1392–1400
166. Daubriac J et al (2017) The crosstalk between breast carcinoma-associated fibroblasts and cancer cells promotes RhoA-dependent invasion via IGF-1 and PAI-1. Oncotarget 9(12):10375–10387
167. Sanz-Moreno V et al (2011) ROCK and JAK1 signaling cooperate to control actomyosin contractility in tumor cells and stroma. Cancer Cell 20(2):229–245
168. Augsten M (2014) Cancer-associated fibroblasts as another polarized cell type of the tumor microenvironment. Front Oncol 4:62
169. Cai J et al (2012) Fibroblasts in omentum activated by tumor cells promote ovarian cancer growth, adhesion and invasiveness. Carcinogenesis 33(1):20–29
170. Giannoni E et al (2010) Reciprocal activation of prostate cancer cells and cancer-associated fibroblasts stimulates epithelial-Mesenchymal transition and cancer Stemness. Cancer Res 70(17): 6945–6956
171. Henriksson ML et al (2011) Colorectal cancer cells activate adjacent fibroblasts resulting in FGF1/

FGFR3 Signaling and increased invasion. Am J Pathol 178(3):1387–1394
172. Jia C-C et al (2013) Cancer-associated fibroblasts from hepatocellular carcinoma promote malignant cell proliferation by HGF secretion. PLoS One 8(5):e63243
173. Gascard P, Tlsty TD (2016) Carcinoma-associated fibroblasts: orchestrating the composition of malignancy. Genes Dev 30(9):1002–1019
174. Malik R, Lelkes PI, Cukierman E (2015) Biomechanical and biochemical remodeling of stromal extracellular matrix in cancer. Trends Biotechnol 33(4):230–236
175. Zhai J et al (2003) Direct interaction of focal adhesion kinase with p190RhoGEF. J Biol Chem 278(27):24865–24873
176. Provenzano PP, Keely PJ (2011) Mechanical signaling through the cytoskeleton regulates cell proliferation by coordinated focal adhesion and Rho GTPase signaling. J Cell Sci 124(8):1195–1205
177. Ibbetson SJ, Pyne NT, Pollard AN, Olson MF, Samuel MS (2013) Mechanotransduction pathways promoting tumor progression are activated in invasive human squamous cell carcinoma. Am J Pathol 183(3):930–937
178. Parkin A, Man J, Timpson P, Pajic M (2019) Targeting the complexity of Src signalling in the tumour microenvironment of pancreatic cancer: from mechanism to therapy. The FEBS Journal 286(18):3510–3539
179. Anderson KG, Stromnes IM, Greenberg PD (2017) Obstacles posed by the tumor microenvironment to T cell activity: a case for synergistic therapies. Cancer Cell 31(3):311–325
180. Jiang H et al (2016) Targeting focal adhesion kinase renders pancreatic cancers responsive to checkpoint immunotherapy. Nat Med 22(8):851–860
181. Johan MZ, Samuel MS (2019) Rho–ROCK signaling regulates tumor-microenvironment interactions. Biochem Soc Trans 47(1):101–108
182. Smith A, Bracke M, Leitinger B, Porter JC, Hogg N (2003) LFA-1-induced T cell migration on ICAM-1 involves regulation of MLCK-mediated attachment and ROCK-dependent detachment. J Cell Sci 116(15):3123–3133
183. Heasman SJ, Ridley AJ (2010) Multiple roles for RhoA during T cell transendothelial migration. Small GTPases 1(3):174–179
184. Zhang S et al (2014) Gene targeting RhoA reveals its essential role in coordinating mitochondrial function and Thymocyte development. J Immunol 193(12):5973–5982
185. Tharaux P-L et al (2003) Rho kinase promotes Alloimmune responses by regulating the proliferation and structure of T cells. J Immunol 171(1):96–105
186. Zhu M et al (2011) Role of Rho kinase isoforms in murine allergic airway responses. Eur Respir J 38(4):841–850
187. Biswas PS et al (2010) Phosphorylation of IRF4 by ROCK2 regulates IL-17 and IL-21 production and the development of autoimmunity in mice. J Clin Invest 120(9):3280–3295
188. Mantovani A, Marchesi F, Malesci A, Laghi L, Allavena P (2017) Tumour-associated macrophages as treatment targets in oncology. Nat Rev Clin Oncol 14(7):399–416
189. Zandi S et al (2015) ROCK-isoform-specific polarization of macrophages associated with age-related macular degeneration. Cell Rep 10(7):1173–1186
190. Guiet R et al (2011) The process of macrophage migration promotes matrix metalloproteinase-independent invasion by tumor cells. J Immunol 187(7):3806–3814
191. Goethem EV, Poincloux R, Gauffre F, Maridonneau-Parini I, Cabec VL (2010) Matrix architecture dictates three-dimensional migration modes of human macrophages: differential involvement of proteases and Podosome-like structures. J Immunol 184(2):1049–1061
192. Georgouli M et al (2019) Regional activation of Myosin II in cancer cells drives tumor progression via a secretory cross-talk with the immune microenvironment. Cell 176(4):757–774.e23
193. Von Hoff DD et al (2013) Increased survival in pancreatic cancer with nab-paclitaxel plus gemcitabine. N Engl J Med 369(18):1691–1703
194. Conroy T et al (2011) FOLFIRINOX versus gemcitabine for metastatic pancreatic cancer. N Engl J Med 364(19):1817–1825
195. Notta F et al (2016) A renewed model of pancreatic cancer evolution based on genomic rearrangement patterns. Nature 538(7625):378–382
196. Feig C, Gopinathan A, Neesse A, Chan DS, Cook N, Tuveson DA (2012) The pancreas cancer microenvironment. Clin Cancer Res 18(16):4266–4276
197. Vonlaufen A et al (2010) Isolation of quiescent human pancreatic stellate cells: a promising in vitro tool for studies of human pancreatic stellate cell biology. Pancreatology 10(4):434–443
198. Öhlund D et al (2017) Distinct populations of inflammatory fibroblasts and myofibroblasts in pancreatic cancer. J Exp Med
199. Whatcott CJ, Ng S, Barrett MT, Hostetter G, Von Hoff DD, Han H (2017) Inhibition of ROCK1 kinase modulates both tumor cells and stromal fibroblasts in pancreatic cancer. PLoS One 12(8):e0183871
200. Masamune A, Kikuta K, Satoh M, Satoh K, Shimosegawa T (2003) Rho kinase inhibitors block activation of pancreatic stellate cells. Br J Pharmacol 140(7):1292–1302
201. Andreas S, Vasiliki G, Maria L, Zacharia Lefteris C, Triantafyllos S (2019) Collagen content and extracellular matrix cause cytoskeletal remodelling in pancreatic fibroblasts. J R Soc Interface 16(154)
202. Kaneko K, Satoh K, Masamune A, Satoh A, Shimosegawa T (2002) Expression of ROCK-1 in

human pancreatic cancer: its Down-regulation by Morpholino Oligo antisense can reduce the migration of pancreatic cancer cells in vitro. Pancreas 24(3):251–257
203. Ivanov AI, Samarin SN, Bachar M, Parkos CA, Nusrat A (2009) Protein kinase C activation disrupts epithelial apical junctions via ROCK-II dependent stimulation of actomyosin contractility. BMC Cell Biol 10(1):36
204. Rath N, Kalna G, Clark W, Olson MF (2016) ROCK signalling induced gene expression changes in mouse pancreatic ductal adenocarcinoma cells. Sci Data 3:160101
205. Hingorani SR et al (2003) Preinvasive and invasive ductal pancreatic cancer and its early detection in the mouse. Cancer Cell 4(6):437–450
206. Fujimura K, Choi S, Wyse M, Strnadel J, Wright T, Klemke R (2015) Eukaryotic translation initiation factor 5A (EIF5A) regulates pancreatic cancer metastasis by modulating RhoA and Rho-associated kinase (ROCK) protein expression levels. J Biol Chem 290(50):29907–29919
207. Vennin C, Murphy KJ, Morton JP, Cox TR, Pajic M, Timpson P (2018) Reshaping the tumor Stroma for treatment of pancreatic cancer. Gastroenterology 154(4):820–838
208. Heid I et al (2011) Early requirement of Rac1 in a mouse model of pancreatic cancer. Gastroenterology 141(2):719–730.e7
209. Porazinski S et al (2016) EphA2 drives the segregation of Ras-transformed epithelial cells from normal neighbors. Curr Biol 26(23):3220–3229
210. Hogan C et al (2009) Characterization of the interface between normal and transformed epithelial cells. Nat Cell Biol 11(4):460–467
211. Padera TP, Stoll BR, Tooredman JB, Capen D, di Tomaso E, Jain RK (2004) Cancer cells compress intratumour vessels. Nature 427(6976):695
212. Beil M et al (2003) Sphingosylphosphorylcholine regulates keratin network architecture and viscoelastic properties of human cancer cells. Nat Cell Biol 5(9):803
213. Paszek MJ, Weaver VM (2004) The tension mounts: mechanics meets morphogenesis and malignancy. J Mammary Gland Biol Neoplasia 9(4):325–342
214. Paszek MJ et al (2005) Tensional homeostasis and the malignant phenotype. Cancer Cell 8(3): 241–254
215. Calvo F et al (2013) Mechanotransduction and YAP-dependent matrix remodelling is required for the generation and maintenance of cancer-associated fibroblasts. Nat Cell Biol 15(6):637–646
216. Patel RA et al (2012) RKI-1447 is a potent inhibitor of the rho-associated ROCK kinases with anti-invasive and antitumor activities in breast cancer. Cancer Res 72(19):5025–5034
217. Patel RA, Liu Y, Wang B, Li R, Sebti SM (2014) Identification of novel ROCK inhibitors with anti-migratory and anti-invasive activities. Oncogene 33(5):550–555
218. Cowey CL, Rathmell WK (2009) VHL gene mutations in renal cell carcinoma: role as a biomarker of disease outcome and drug efficacy. Curr Oncol Rep 11(2):94–101
219. Turcotte S, Desrosiers RR, Béliveau R (2003) HIF-1alpha mRNA and protein upregulation involves Rho GTPase expression during hypoxia in renal cell carcinoma. J Cell Sci 116(Pt 11):2247–2260
220. Kamai T et al (2003) RhoA is associated with invasion and lymph node metastasis in upper urinary tract cancer. BJU Int 91(3):234–238
221. Abe H et al (2014) The rho-kinase inhibitor HA-1077 suppresses proliferation/migration and induces apoptosis of urothelial cancer cells. BMC Cancer 14(1):412
222. Shimizu T et al (2010) C-MYC overexpression with loss of Ink4a/Arf transforms bone marrow stromal cells into osteosarcoma accompanied by loss of adipogenesis. Oncogene 29(42):5687–5699
223. Takahashi N et al (2019) ROCK inhibition induces terminal adipocyte differentiation and suppresses tumorigenesis in Chemoresistant osteosarcoma cells. Cancer Res 79(12):3088–3099
224. Özdemir BC et al (2014) Depletion of carcinoma-associated fibroblasts and fibrosis induces immunosuppression and accelerates pancreas cancer with reduced survival. Cancer Cell 25(6):719–734
225. Rhim AD et al (2014) Stromal elements act to restrain, rather than support, pancreatic ductal adenocarcinoma. *Cancer Cell* 25(6):735–747
226. Laklai H et al (2016) Genotype tunes pancreatic ductal adenocarcinoma tissue tension to induce matricellular fibrosis and tumor progression. Nat Med 22(5):497–505
227. Vennin C et al (2017) Transient tissue priming via ROCK inhibition uncouples pancreatic cancer progression, sensitivity to chemotherapy, and metastasis. Sci Transl Med 9(384):eaai8504
228. Vennin C, Rath N, Pajic M, Olson MF, Timpson P (2017) Targeting ROCK activity to disrupt and prime pancreatic cancer for chemotherapy. Small GTPases 1–8
229. Chin VT, Vennin C, Timpson P, Pajic M (2017) Effective modulation of stromal signaling through ROCK inhibition: is it all in the timing? Mol Cell Oncol 4(5):e1333973
230. Conway JRW, Warren SC, Timpson P (2017) Context-dependent intravital imaging of therapeutic response using intramolecular FRET biosensors. Methods 128:78–94
231. Conway JRW, Carragher NO, Timpson P (2014) Developments in preclinical cancer imaging: innovating the discovery of therapeutics. Nat Rev Cancer 14(5):314–328
232. Sunyer R et al (2016) Collective cell durotaxis emerges from long-range intercellular force transmission. Science 353(6304):1157–1161
233. Costa-Silva B et al (2015) Pancreatic cancer exosomes initiate pre-metastatic niche formation in the liver. Nat Cell Biol 17(6):816–826

234. Sadok A et al (2015) Rho kinase inhibitors block melanoma cell migration and inhibit metastasis. Cancer Res 75(11):2272–2284
235. Croghan GA et al (2010) A study of paclitaxel, carboplatin, and bortezomib in the treatment of metastatic malignant melanoma. Cancer 116(14):3463–3468
236. Markovic SN et al (2005) A phase II study of bortezomib in the treatment of metastatic malignant melanoma. Cancer 103(12):2584–2589

S1P Signaling in the Tumor Microenvironment

7

Gabriela Schneider

Abstract

Sphingosine-1-phosphate (S1P), together with other phosphosphingolipids, has been found to regulate complex cellular function in the tumor microenvironment (TME) where it acts as a signaling molecule that participates in cell–cell communication. S1P, through intracellular and extracellular signaling, was found to promote tumor growth, angiogenesis, chemoresistance, and metastasis; it also regulates anticancer immune response, modulates inflammation, and promotes angiogenesis. Interestingly, cancer cells are capable of releasing S1P and thus modifying the behavior of the TME components in a way that contributes to tumor growth and progression. Therefore, S1P is considered an important therapeutic target, and several anticancer therapies targeting S1P signaling are being developed and tested in clinics.

Keywords

Sphingosine-1 phosphate (S1P) · Tumor microenvironment · Sphingosine kinase (SphK) · Sphingosine-1 phosphate receptors (S1PR) · Cell motility · Chemotaxis · Immunomodulation · Macrophage polarization · TAM/M2 macrophages · Tumor angiogenesis · Cancer metastasis · Tumor growth · Hypoxia-inducible factor 1α (HIF1α) · Nuclear factor-κB (NF-κB) · Inflammation

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7.1 Introduction

The tumor microenvironment (TME) plays an important role in cancer biology contributing to tumor initiation, progression, metastasis, and responses to treatment. Cancer cells within a solid tumor influence the surrounding microenvironment through the release of extracellular signals in the form of cytokines, chemokines, and lipid mediators. These signals work to control immune responses, inflammation, as well as angiogenesis. Sphingosine-1-phosphate (S1P), a bioactive sphingolipid, has emerged over the last few decades as a new player in the TME and cancer progression. It can be produced and released into the TME from cancerous and noncancerous tissues and acts to regulate the interactions between tumor, immune, and mesenchymal cells that are present within the TME. In this chapter, we summarize the mechanisms through which S1P, present in the TME, participates in tumor progression, inhibits antitumor immune response, modulates inflammation, regulates response to hypoxic con-

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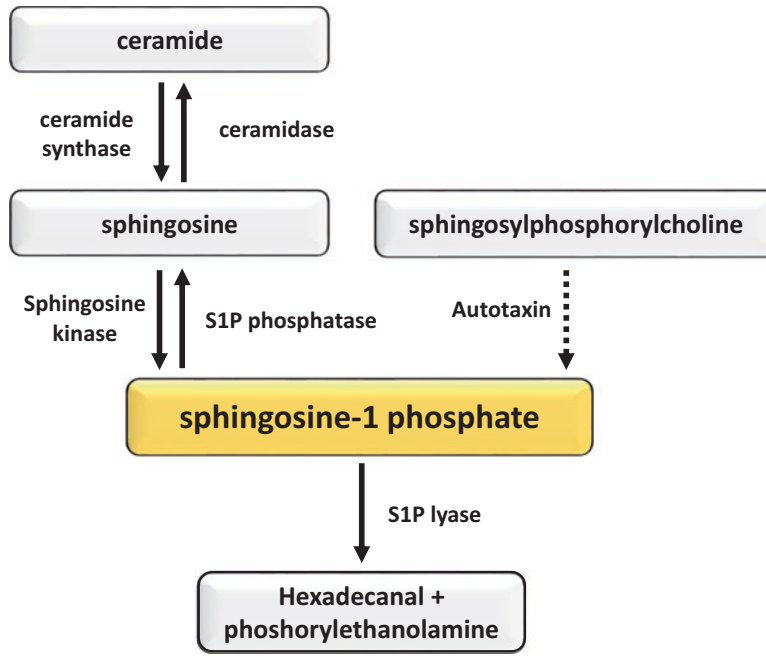

Fig. 7.1 Metabolism of sphingosine-1 phosphate (S1P). Enzymes and substrates involved in the synthesis and degradation of S1P. Solid lines represent confirmed pathways, dotted line represents the pathway identified in vitro but not yet confirmed in vivo

ditions, and facilitates the recruitment of mesenchymal cells to increase tumor angiogenesis. Additionally, we will discuss therapeutic strategies that target S1P signaling in cancer patients.

7.2 Metabolism of S1P

S1P is generated by the conversion of ceramide to sphingosine, which is catalyzed by ceramidase, and subsequent phosphorylation of sphingosine by sphingosine kinases (SphK1 and SphK2) (Fig. 7.1). SphK1 is localized mainly in the cytosol [1], whereas SphK2 can be found in the nucleus and internal membranes of the endoplasmic reticulum, Golgi, and mitochondria [2, 3] which suggest the distinct function of generated S1P. Both enzymes can be translocated to different cell compartments in response to specific signals. For example, SphK1 can be recruited to the plasma membrane in response to growth stimulating factors such as epidermal growth factor (EGF) or phorbol 12-myristate 13-acetate [4, 5] and targeted to Golgi apparatus by phosphatidic acid [6], whereas SphK2 can be translocated from the nucleus to the cytoplasm dependent on PKD-mediated phosphorylation [7].

Interestingly, S1P levels and SphK1/2 expression and/or activity were found to be increased in distinct cancer types including acute lymphoblastic leukemia [8], astrocytoma [9], breast cancer [10, 11], colon cancer [12, 13], gastric cancer [14], glioblastoma [15, 16], lung cancer [17], non-Hodgkin's lymphoma [18], prostate cancer [19], thyroid cancer [20, 21], and many others [22–24]. Several reports also indicate that increased expression of SphK1 correlated with disease progression, cancer recurrence, and reduced patient survival [9, 10, 14, 15, 23, 24] as well as invasion and lymph node metastasis [25]. In contrast, reduced expression of SphK1 and subsequently lower level of S1P in plasma were found in prostate cancer patients [26]. Moreover, S1P level correlates with patients' survival, and downregulation of SphK in erythrocytes could have implication in cancer-induced anemia [26].

It has been shown that S1P can also be generated by autotaxin (ATX) through hydrolysis of sphingosylphosphorylcholine (SPC) [27]; however, it is uncertain whether this pathway is active in vivo. First of all, the reported K_m value of ATX for SPC (~23 mM) [27] is much higher than normal SPC levels in plasma/serum (0.03–0.13 μM) [28, 29]. Moreover, in mice with down-

regulated Autotaxin, the level of S1P was not changed when compared with wild-type animals, in contrast to the main autotaxin metabolite, lysophosphatidic acid, which was decreased by ~50% [30]. This suggests that the in vivo contribution of Autotaxin to the total pool of S1P is limited.

S1P levels are the result of the balance between its synthesis and reversible conversion to sphingosine or irreversible degradation. Dephosphorylation of S1P is catalyzed by specific S1P phosphatases (SPP1 and SPP2), or lipid phosphate phosphatases (LPP1–3) and subsequent sphingosine conversion to ceramide by ceramide synthase [31] or through irreversible degradation by S1P lyase (SPL) that cleaves S1P to hexadecenal and phosphoethanolamine [32]. Similarly to SphK, enzymes responsible for S1P degradation were also found to be dysregulated in malignant tissues; lower expression of SPL was found in colon [33, 34], prostate [35], and pancreatic cancers [35], and was shown to have implications in chemo and radiotherapy resistance and cancer cells metastasis [35]. Downregulation of SPP was found in colon cancer [31], gastric cancer [31], and glioblastoma [16], and its expression was correlated with lymph node metastasis and gastric cancer patient's survival [31].

7.3 Sources of S1P in TME

S1P is present in the components of the TME such as blood, lymph, and interstitial fluid. In circulation, S1P is bound to plasma proteins, mainly high-density lipoprotein (HDL) [36] apolipoprotein M [37], and to a lesser extent to albumin [38]. The main source of plasma S1P was thought to be platelets, which are characterized by high SphK activity and lack of SPL which allows them to accumulate large amounts of S1P, up to ninefold more than erythrocytes. Although erythrocytes produce less S1P than platelets, at the same time they constitute about 95% of total blood cell number, thus their contribution to the S1P pool in the blood is considerably much higher and is estimated to be 75%. Other important contributors of plasma S1P are the vascular endothelium and endothelial cells, and in lymph, lymphatic endothelial cells, thus suggesting that stromal cells, could synthesize and release endogenous S1P also to TME. Recently, it has been shown that cancer cells themselves can also secrete high levels of S1P [39–41] hence contributing to the total S1P pool present in TME, which could explain high level of S1P in ascites fluids from ovarian cancer [42, 43] and additional observation that plasma S1P level decrease in patients after ovarian cancer surgery [44]. Moreover, S1P can also be released from dying cells (necrotic or apoptotic) and damaged tissues [45–48]. This can have important implication in anticancer therapies since it was shown that S1P levels were increased in several organs after γ-irradiation or chemotherapy, creating an unwanted prometastatic environment as a side effect of the treatment [46] (Fig. 7.2).

The structure of S1P and its relatively high solubility in water unable S1P to diffuse over the membranes to the extracellular compartments. Therefore to act as a signaling molecule, S1P has to be either generated in extracellular compartments directly or synthesized intracellularly and transported outside the cells by specific transporters. SphK1 was found to be constitutively released from endothelial cells in quantities that allow for the synthesis of extracellular S1P and obtain its physiologically relevant concentrations [49, 50]. Moreover, SphK1 was also found to be released from histiocytic lymphoma U937 cells in response to stimulation with oxidized LDL immune complexes [51] thus indicating that extracellular synthesis of S1P might be regulated by additional signaling factors. Several transporters of S1P that allows for the autocrine/paracrine signaling of S1P have been identified including Spinster 2 (SPNS2) [52, 53] and several members of the ABC-type lipid transporters family, namely ABCC1, ABCC2, and ABCA1 [54]. This diversity in the type of transporters might suggest their importance in the regulation of S1P levels in different tissues. Indeed, recently, it has been shown that SNPS2 transporter was necessary for secretion of S1P to the lymph, but it did not play an important role in the regulation of S1P levels in plasma [53, 55]. Moreover, Spns2 is not expressed in murine eryth-

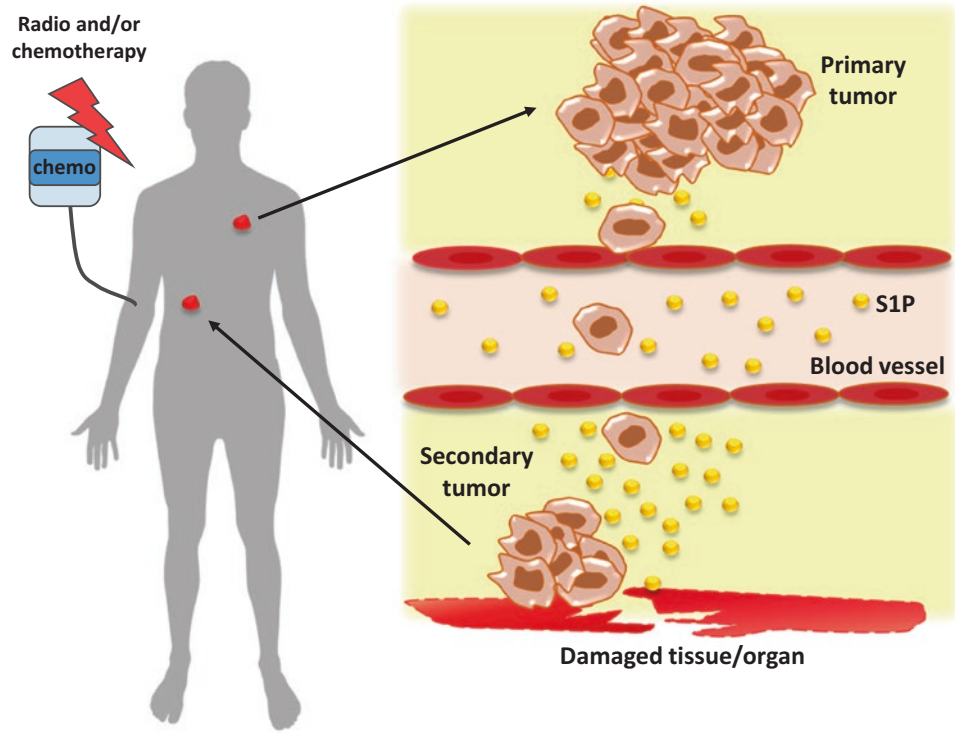

Fig. 7.2 Sphingosine-1 phosphate (S1P) plays a role in the formation of the prometastatic environment as a side effect of radio/chemotherapy. S1P released from damaged tissues (malignant and nonmalignant) induces migration of tumor cells that survive initial anticancer treatment. Such cells metastasize to distant locations where they can form secondary tumors

rocytes, and the level of S1P in the blood is not affected in Spns2 knockout mice [56]. On the other hand, several in vitro studies have revealed that ABC transporters mediate S1P release in different types of cells, including mast cells [57], erythrocytes [58], breast cancer cells [59], astrocytes [60], and also platelets [61]. Additionally, since S1P is present in the circulation, mainly in complex with HDL particles [38], it might suggest that the S1P export may be coupled with ABCA1-dependent lipoprotein formation [60].

What is worth to note, changes in SPNS2 expression were found in non–small cell lung cancer (NSCLC) patients' samples, and in vitro studies indicate that the overexpression of SPNS2 induced apoptosis, whereas its knockdown enhanced NSCLC cells migration [62]. Moreover, alterations of SPNS2 affected the expression of several enzymes involved in S1P metabolism, including SphK, SPP, and SPL1 [62], thus indicating a cross talk between the pathways involved in S1P synthesis/degradation and extracellular transport of S1P.

7.4 S1P Signaling

S1P has been shown to regulate cellular functions both via intracellular (Fig. 7.3) and extracellular (Fig. 7.4) mechanisms. Intracellular S1P was first identified as an activator of intracellular calcium channels via an inositol triphosphate-independent pathway [63], but a target ion channel has not been identified. However, several studies support this observation by demonstrating that increased level of S1P also upregulates intracellular calcium concentrations [64]. In the nucleus, S1P was found to play a role in the regulation of gene expression by binding to histone deacetylases (HDAC1 and HDAC2), and inhibiting their activ-

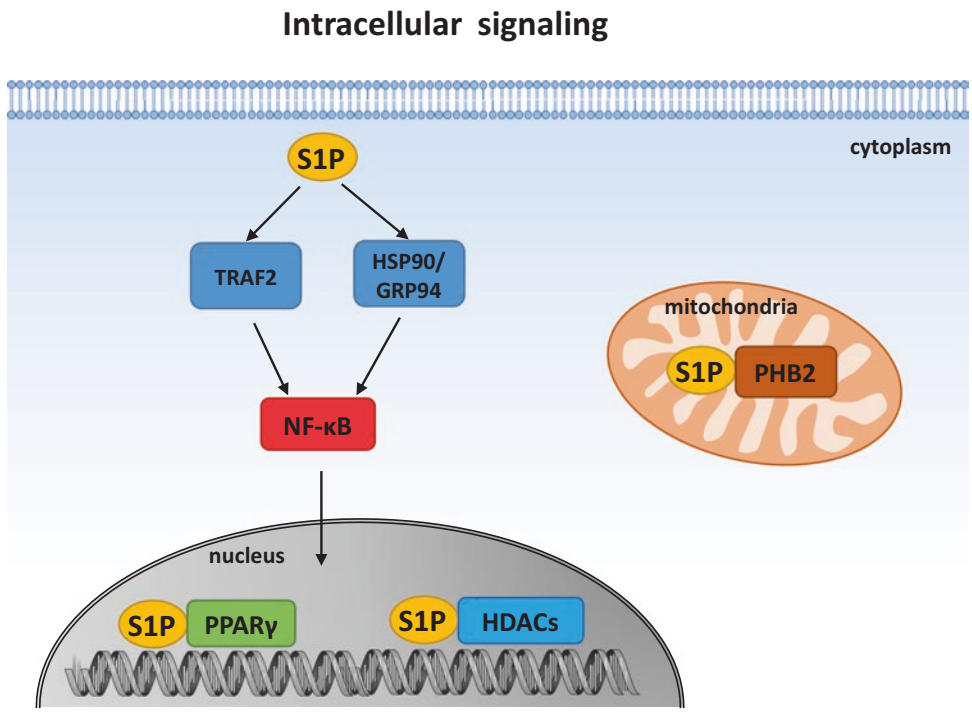

Fig. 7.3 Intracellular sphingosine-1 phosphate (S1P) signaling. Intracellular S1P regulates the nuclear factor-κB (NF-κB) signaling pathway by targeting TNF receptor-associated factor 2 (TRAF2) or heat shock protein 90 (Hsp90)/glucose-regulated protein 94 (GRP94). In mitochondria, it interacts with prohibitin 2 (PHB2) thus regulating mitochondrial respiration, whereas in the nucleus it regulates the activity of histone deacetylases (HDACs) and nuclear transcription factor PPARγ

ity thus regulating transcription of several genes including the cyclin-dependent kinase inhibitor p21 [65] (Fig. 7.3). In mitochondria, the interaction of S1P with the prohibitin 2 (PHB2) protein was found to be important for cytochrome c oxidase assembly and mitochondrial respiration [66]. Interestingly, in both cases, SphK2 was found to be the main enzyme involved in the synthesis of S1P in a particular cellular compartment [65, 66]. On the other hand, S1P generated by SphK1 was found to act as a cofactor for the TNF receptor-associated factor 2 (TRAF2) E3 ubiquitin ligase complex by which it regulates the activity of the nuclear factor-κB (NF-κB) signaling involved in inflammatory, antiapoptotic, and immune processes [67]. NF-κB activation was observed also in response to endoplasmic reticulum (ER) stress which resulted in S1P increase and subsequent interaction with HSP90 and/or GRP94 protein to form a signaling complex with an ER stress responsive protein, IRE1α, TRAF2, and RIP1 [68]. Of note, NF-κB activation may also be induced by extracellular S1P [69, 70], suggesting that S1P acts upon several stages within the same signaling cascade. S1P has also been shown to enhance the cellular inhibitor of apoptosis 2 (cIAP2)-mediated K63-linked polyubiquitination of interferon regulatory factor-1 (IRF-1), which is essential for IL-1-induced production of chemokines CXCL10 and CCL5 [71]. Moreover, S1P interacts with the transcription factor peroxisome proliferator-activated receptor γ (PPARγ) through which it can regulate angiogenesis [72].

Extracellular S1P acts through binding to G-protein-coupled receptors (S1PR1–S1PR5) by which it regulates several cell processes, including cell survival and migration [73] (Fig. 7.4). Expression patterns of S1PRs vary between tissues and can change during development and aging. S1PR1–S1PR3 are essentially ubiquitously

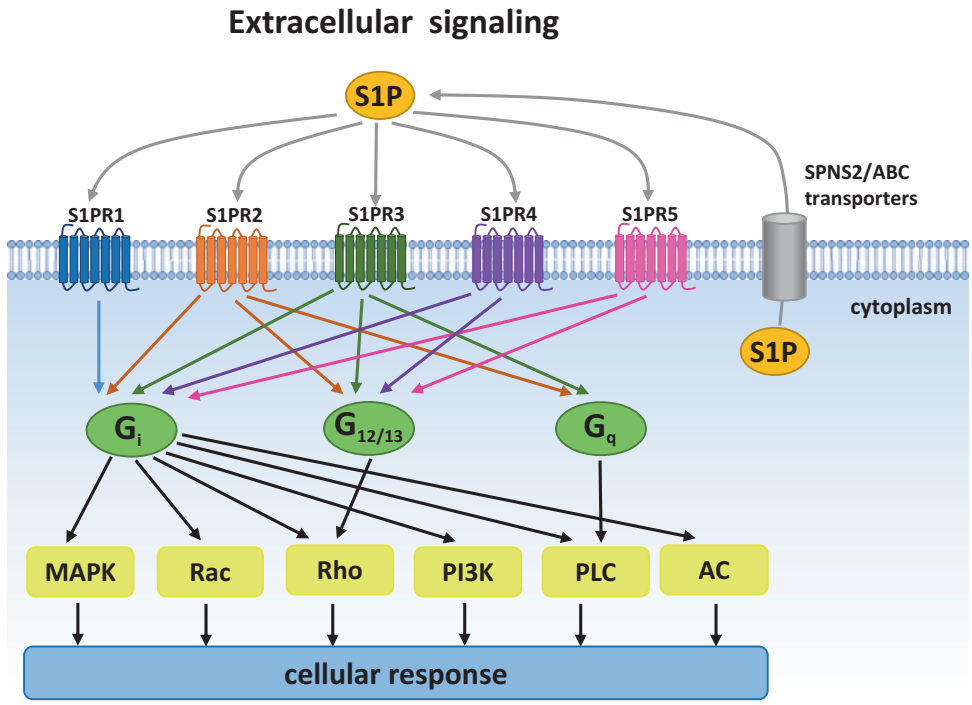

Fig. 7.4 Extracellular sphingosine-1 phosphate (S1P) signaling. Intracellularly generated S1P is exported to the extracellular compartments by Spinster 2 (SPNS2) and members of the ABC transporter family thus allowing for autocrine/paracrine signaling of S1P. Extracellular S1P binds to the specific G-coupled S1P receptors designated as S1PR1–5, regulating mitogen-activated protein kinase (MAPK), Rac, Rho, phosphatidylinositol 3-kinase (PI3K), phospholipase C (PLC), and adenyl cyclase (AC) pathways

expressed, whereas expression of S1PR4 and S1PR5 is restricted to distinct cell types [74]. S1PRs can activate several different signaling pathways. S1PR1 is coupled with G_i protein and activates Ras, mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase (PI3K), protein kinase B (AKT), and phospholipase C (PLC) pathways. Both S1PR2 and S1PR3 are coupled to G_i, G_q, and $G_{12/13}$ and can activate Ras, MAPK, PI3K, AKT, PLC, and Rho-dependent pathways [75]. Through coupling with G_i and $G_{12/13}$, S1PR4 mediates cell shape change and motility via a Rho-dependent pathway [75], whereas S1PR5 appears to activate the $G_{12/13}$ protein and the subsequent Rho/ROCK signaling pathway [76] and through coupling with G_i inhibits adenyl cyclase (AC) [77]. These studies strongly suggest that S1P can mediate diverse functions through activation of different signal transduction pathways in different cell types, as well as within the same cell, depending on the patterns of S1PR expression. What is worth noting, it has been suggested that only S1P generated by SphK1 and not SphK2 can activate S1PRs [78].

7.5 S1P as a Modulator of Cancer Biology

Extra- and intracellular S1P can activate various signaling cascades that are implicated in cancer cell proliferation, survival, migration, inhibition of apoptosis, and chemoresistance. SphK1 was found to be involved in the regulation of S1P-dependent proliferation of several cancers, including gastric [79] and colorectal cancers [80]. In contrast to SphK1, the role of SphK2 in the regulation of cell growth seems to be more context-dependent. In some cells, upregulation of SphK2 levels was found to cause cell cycle arrest, caspase-3 activation, cytochrome c release, and thus inhibited cell growth [78, 81]. Whereas in

others, such as glioblastoma, colorectal cancer, or prostate cancer, downregulation of SphK2 was associated with decreased proliferation of malignant cells in vitro and in vivo [82, 83]. Surprisingly, a study testing new SphK inhibitors on a wide variety of cancer cell lines including breast cancer, glioblastoma, melanoma, cervix, and colon cancers revealed that SphK activity was not required for tumor cell viability both in vivo and in vitro [84]. Thus, these discrepancies with opposing findings require further investigation. Several studies were able to identify receptors through which S1P affects cancer cell proliferation. In human prostate cancer PC-3 cells and glioma cells S1P attenuates cell proliferation through activation of S1PR5 [85, 86], S1PR2 is involved in growth of hepatocellular carcinoma and Willim's tumor [87, 88], downregulation of S1PR1 was associated with decreased growth of rhabdomyosarcoma xenografts [46], whereas in breast cancer higher S1PR1 expression correlates with decreased cell proliferation [89]. Interestingly, in glioblastoma S1PR1–3 were found to be involved in the stimulation of proliferation [90], while activation of S1PR5 has an opposite effect [91]. Taken together, these observations suggest that both exogenous and intracellular S1P are important for tumor growth and indicate that attenuation of S1P signaling pathway could be a promising strategy in cancer treatment.

Extracellular S1P has been found to act a potent chemoattractant or chemokinetic factor for different types of cancers, including gastric cancer [92], leukemia [93], lung cancers [94], glioblastoma [95], ovarian cancer [96], or rhabdomyosarcoma [46], thus indicating the role of S1P in tumor metastasis. In the majority of cells, S1P stimulates the motility of cancer cells through S1PR1 or S1PR3, mainly through activation of the ERK1/2 signaling pathway. However, some evidence also indicates that a S1PR1–RAC1–CDC42-dependent pathway involving the tyrosine phosphorylation of membrane-type matrix metalloproteinase 1 might play a role [97, 98]. Moreover, in some cells, such as ovarian cancer, calcium mobilization might accompany S1P-mediated invasion [96]. By contrast, S1P can inhibit cancer cell motility through a S1PR2-dependent regulation of Rho [95]. The specific effect of S1P is partially determined by the type of the receptors expressed in particular tumors, e.g., exposure to S1P of gastric cancer cell lines that dominantly expressed S1PR3 induced their migration, whereas the opposite effect was observed in gastric tumor cells that mainly expressed S1PR2 [92]. The effects of S1P on the motility of cancer cells can also be context-dependent. For example, activation of the S1P–S1PR2 axis increased glioma invasiveness by enhancing the expression of secreted, angiogenic matricellular protein CCN1 [99]. The metastatic behavior of tumor cells can also depend on S1P metabolic enzymes' pattern of expression. It has been found that SphK levels positively correlated with migration and invasion of ovarian cancer [100], breast cancer, and kidney carcinoma cells [101]. In addition to S1P-induced motility of cancer cells, this sphingolipid was also found to be involved in the regulation of invasion processes. S1P was found to induce overexpression of MMP-2 through the MAPK/ERK1/2 and NF-κB pathways and is responsible for cancer cell invasion in endothelial cells [102]. Upregulation of S1P can also lead to overexpression of MMP2, which has a major role in initiating cell invasion via H-Ras signaling in human breast cancer [103]. In the same cells, S1P was also found to induce MMP9, and this effect was S1PR3-dependent.

Angiogenesis, a process of formation of new blood vessels, which is necessary for tumor growth and invasion, can also be regulated by S1P signaling. Increased angiogenesis in tumor tissue can be at least partially explained by the S1P-induced migration of endothelial cells [104] and vascular smooth muscle cells [105, 106] which participate in the formation of new blood vessels in cancerous tissue. However, it was also shown that cross talk between S1P and VEGF signaling can additionally contribute to increase angiogenesis. S1P was found to upregulate VEGF in human prostate cancer [75] and thyroid cancer [107], whereas in bladder cancer, VEGF was found to stimulate SphK1 leading to the increase of intracellular levels of S1P [107]. S1PR1 was

found to be involved in S1P-induced regulation of vascularization in thyroid cancer [107], breast cancer cells [108], and Lewis lung carcinoma [106]. Angiogenesis of tumors was also found to correlate with SphK1 level in hepatoma cells [109], Lewis lung carcinoma [106], as well as in glioma cells [110].

Accumulating evidence indicates that factors present in the TME, including S1P, might play a role in acquiring of chemoresistance by cancer cells. Overexpression of SpK1 was found to be associated with the chemoresistance of leukemic cells to imatinib [111] and daunorubicin [112], prostate cancer to docetaxel [113], renal cancer to sunitinib [114], and gastroesophageal cancer to oxaliplatin, cisplatin, and docetaxel [115]. It has been suggested that at least part of this effect is the result of an imbalance between ceramide and S1P [116]. Also, other S1P metabolizing enzymes were shown to be involved in chemoresistance, e.g., overexpression SphK2 has been correlated to gefitinib chemoresistance in non-small cell lung cancer cells [117] and hormone-independent breast cancer [118], whereas depletion of SPL caused cisplatin resistance [119]. Interestingly, the treatment of prostate cancer cells with camptothecin upregulates both SK1 and S1P receptors, suggesting that resistance to camptothecin could involve autocrine/paracrine mechanisms [120]. Some studies also indicate the involvement of specific S1P receptors in the acquired chemoresistance of cancer cells; for example, the use of S1PR1 antagonist was found to sensitize neuroblastoma cells to etoposide [121].

Taken together, these data strongly indicate that modulation of S1P-related signaling may constitute a promising anticancer therapy; however, due to high heterogeneity between the tumors and observed opposing effects in response to S1P stimulation, more work has to be done to better understand the mechanisms of its action in cancer cells.

7.6 S1P as a Modulator of the Immune Response

The composition and characteristics of the TME vary between different types of tumors, but it is crucial in determining the antitumor response. Several different populations of immune cells are capable of playing a role in antitumor immune response, including macrophages, natural killer cells (NKs), dendritic cells (DCs), and effector T cells. However, at the same time, tumor cells protect themselves from destruction by induction of an immunosuppressive microenvironment, thus promoting the development of immunosuppressive cells such as myeloid-derived suppressor cells (MDSCs) and regulatory T cells. Accumulating evidence indicates that S1P might be one of the components of the TME that can regulate this interplay between cancer and immune cells (Fig. 7.5). The best-studied effect of S1P on immune cells includes its role in the regulation of migration and polarization of macrophages. Macrophages originate from the myeloid lineage and belong to the innate immune system. They are derived from blood monocytes that migrate into cancer tissue in response to specific chemokines present in the TME. One of their main functions is the removal of microbes and cell debris through phagocytosis, but they also play a crucial role in the initiation of inflammation [122, 123]. Based on signals from the microenvironment, macrophages can exhibit different phenotypes, and the process of acquiring different functional programs is known as polarization. Two major subsets have been proposed: M1 and M2 macrophages which correspond to the extreme phenotypes of the opposite spectrum. M1 macrophages (classically activated macrophages) are aggressive and highly phagocytic, produce large amounts of reactive oxygen and nitrogen species, secrete high levels of IL-12 and IL-23, and induce the activation, and clonal expansion of T-helpers cells thus contributes to

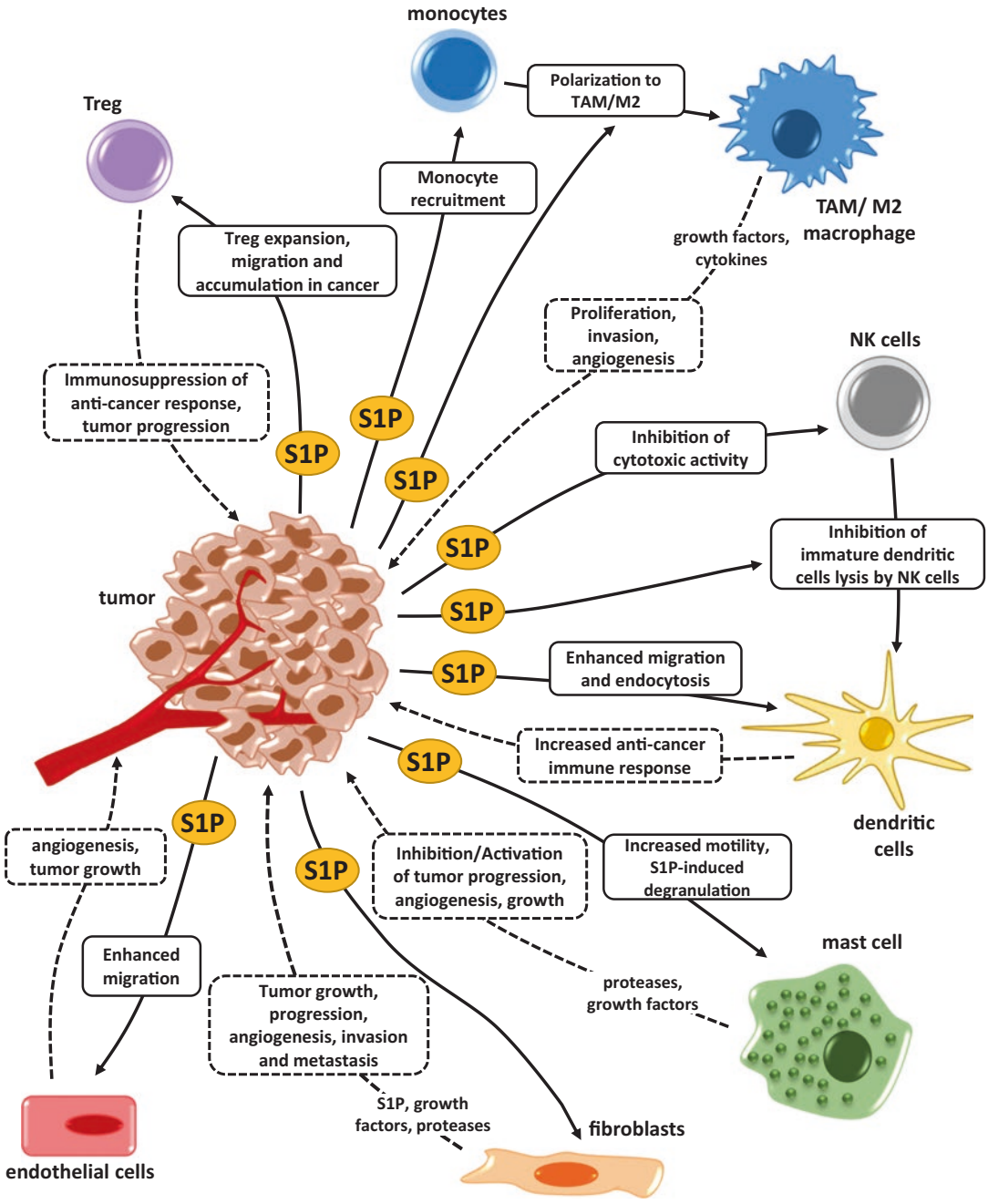

Fig. 7.5 Sphingosine-1 phosphate (S1P) is a key regulator of cell–cell interaction and modulator of the anticancer immune response in the tumor microenvironment (TME). S1P released from cancer cells chemoattract monocytes and induce their polarization into TAM/M2 macrophages, which in turn secrete growth factors and cytokines that stimulate tumor growth. S1P also inhibits cytotoxic activity of natural killer (NK) cells and promotes regulatory T-cell (Treg) expansion, migration, and accumulation in malignant tissue which results in immunosuppression of anticancer response. In the presence of S1P, NK-mediated cell lysis of immature monocyte-derived dendritic cells (DCs) is inhibited. Moreover, S1P enhances endocytosis and induces migration of mature DCs which could potentially increase immune response toward cancer cells. Mast cells respond to S1P stimulation with increased motility and degranulation which result in the release of growth factors and cytokines that depending on the context can stimulate or inhibit tumor growth and progression. Similarly, fibroblasts, in the presence of S1P, secrete growth factors, proteases, and also S1P that accelerate tumor growth, angiogenesis, and invasion. Additionally, S1P induces angiogenesis and tumor growth by enhancing migration of endothelial cells

inflammation. In contrast, M2 macrophages (alternatively activated macrophages) are anti-inflammatory and aid in the process of angiogenesis and tissue repair. In the TME, M1 macrophages are involved in the elimination of tumor cells, whereas M2 macrophages stimulate tumor growth by releasing angiogenic factors and anti-inflammatory cytokines [124]. Tumor-associated macrophages (TAMs) display an M2-like phenotype, and several studies indicate a correlation between TAM density and poor prognosis of cancer patients. Moreover, new evidence connect TAMs with chemotherapy resistance.

S1P has been identified to be a potent chemoattractant for many different types of normal and malignant cells. Interestingly, S1P released from apoptotic leukemic cells not only was found to attract monocytes, but its effect was comparable with monocyte chemoattractant protein-1 (MCP-1/CCL2) [45]. These results were confirmed in several other models including breast cancer [47, 48] and acute T-cell leukemia [125, 126], and more detailed studies indicate the involvement of S1P-S1PR1 axis in monocyte migration [125]. There are also some suggestions that SphKs are involved in the release of S1P from apoptotic cells; however, the results are contradictory; some studies showed that activation of SphK1 is necessary to facilitate this process [45, 126], whereas the other pointed out to the involvement of SphK2 [126].

Accumulating evidence point to a crucial role of S1P in macrophage polarization. In several models, including breast cancer, S1P was found to induce a M2 phenotype in macrophages which was characterized by decreased tumor necrosis factor (TNF)-alpha, IL-12, and nitric oxide synthase production, but increased formation of IL-4, IL-8, IL-10, TGF-β1, and, interestingly, SphK1 [47, 48, 127, 128]. More detailed studies indicated that this process involved suppression of NF-κB, p38 MAPK, and JNK signaling pathways [47, 128, 129], as well as activation of tyrosine kinase receptor A and ERK1/2 [48, 128]. Moreover, S1P activity was dependent on the expression of SphK2, S1PR1/3 but not S1PR2 [47, 126, 129, 130]. Additionally, IL-10, a potent, anti-inflammatory cytokine was found to stimulate macrophages to secrete prostaglandin E2 (PGE2) that induced migration of endothelial cells and increased angiogenesis, a seal of tumor progression [130]. S1P released from cancer cells also induced Bcl-X(L) and Bcl-2 upregulation, which protected macrophages from cell death [126].

The identification of S1P function in monocyte recruitment and polarization of macrophages toward the less aggressive M2 phenotype suggest that S1P present in the TME can have a positive effect on tumor growth not only by direct stimulation of cell proliferation, invasiveness, and chemoresistance but also by allowing them to evade the tumor-killing response elicited by cytotoxic macrophages. Therefore, a better understanding of S1P role in the regulation of macrophage production and polarization could lead to the development of new therapies allowing for the reprogramming of TAMs toward an M1 phenotype and activation of their antitumor response.

Even though most of the literature focus on the crucial role of S1P in monocyte/macrophage recruitment, survival, and polarization, it was also found that this phosphosphingolipid can modulate the function of other immune cells present in the TME such as regulatory T cells (Tregs) or natural killer cells (NK). Tregs are a subpopulation of T cells that act to suppress the immune response, thus maintaining homeostasis and self-tolerance. In cancers, however, their suppressive activity toward other immune cells promotes tumor progression. S1PR1 was found to be involved in the regulation of Tregs functions since the permanent deletion of this receptor from Treg cells resulted in autoimmunity [131]. Moreover, activation of S1PR1 signaling leads to Tregs accumulation in cancerous tissues and promotes tumor growth, through activation of STAT3 [132]. However, in other studies, S1PRs agonist FTY720 (fingolimod) was found to inhibit IL-2-induced STAT-5 phosphorylation, paralleled by a loss of forkhead box protein 3 (FoxP3) expression, which resulted in decreased Treg cells proliferation, both, in vitro and in vivo [133, 134]. FTY720 is an agonist of four S1PRs (S1PR1, S1PR3, S1PR4, and S1PR5) [135], therefore the discrepancy between studies might indicate that

although S1PR1 can stimulate the suppressive nature of Tregs, this effect can be counteracted by stimulation of other S1P receptors. Therefore more studies have to be done to better characterize the role of S1P and its receptors in Tregs regulation. Interestingly, S1PR1 present on cancer cells was found to regulate a cross talk between bladder cancer cells and Tregs. Overexpression of this receptor in bladder cancer cells promoted the generation of bladder cancer-induced Tregs by activation TGF-β signaling pathway, leading to the secretion of TGF-β and IL-10 from tumor cells [134].

Recently S1P was identified as a potent chemoattractant for natural killers cells (NK) that play a crucial role in antitumor immunity [136, 137]. However, at the same time it was shown that S1P stimulation activates the phosphatidylinositol 3-kinase (PI3K)-dependent signaling pathway, protein kinase A (PKA), protein kinase B (PKB/AKT), glycogen synthase kinase-3beta (GSK-3beta) and increases the level of cAMP, thus inhibiting the cytotoxic activity of NK cells [136]. Further studies confirmed that S1P can act as an anti-inflammatory molecule that significantly reduced the release of IL-17A and IFN-gamma from NK cells in an S1PR1-independent manner [138]. At the same time, it was found that S1P can modulate the interaction between NK and tumor cells since activation of S1PR1 on human myeloid leukemia K562 cells protected them from NK cells-induced lysis [138]. Interestingly, S1P was also found to modulate the interaction of NK cells with immature dendritic cells (DCs) [138]. DCs are antigen-presenting cells that play a central role in the initiation of adaptive immune responses. In the presence of S1P, NK-mediated cell lysis of immature monocyte-derived DCs was inhibited, which may favor antigen presentation to T cells [138]. Moreover, S1P enhances endocytosis and induce migration of mature DCs in an S1PR3--dependent but not S1PR1-dependent manner [139], which could potentially increase immune response toward cancer cells. This duality in S1P action cells requires more studies to better understand the effect of its signaling on migration and phenotypic modulation of NK and DCs cells.

One of the best-studied effects of S1P is its role in migration and egress of lymphocytes from lymphoid organs. However, less attention was put in resolving the role of S1P in the regulation of B and T lymphocytes in cancer progression. In diffuse large B-cell lymphoma (DLBCL) cell lines, expression of S1PR2 inversely correlated with the oncogenic transcription of FOXP1, resulting in reduced tumor growth in S1PR2 overexpressing cells [140]. Moreover, low S1PR2 expression was found to be a strong negative prognostic factor of patient survival, especially in combination with high FOXP1 expression [140]. Interestingly, different B-cell populations express different combinations of S1PRs; S1PR1 was found to promote migration, whereas S1PR4 modulates and S1PR2 inhibits S1PR1 signals [141]. Moreover, the expression of CD69 in activated B lymphocytes and B cells from patients with chronic lymphocytic leukemia (CLL) inhibited S1P-induced migration [141]. Studying B-cell lines, normal B lymphocytes, and B cells from patients with primary immunodeficiencies, Bruton's tyrosine kinase, β-arrestin 2, LPS-responsive beige-like anchor protein, dedicator of cytokinesis 8, and Wiskott-Aldrich syndrome protein were found to be critical signaling components downstream of S1PR1 [141]. S1PR1 is expressed at low levels in CLL lymph nodes as compared with normal B cells [142], increased expression of S1PR1 correlates with STAT3 activation and survival of B-cell lymphoma cells [143]. Furthermore, downregulated expression of S1PR1 in CLL B cells impairs their egress from the peripheral lymphoid organs and enhances their survival [144]. S1P was also identified as a molecule that inhibits T-cell proliferation [145].

Mast cells (MCs) are immune cells of the myeloid lineage and are present in connective tissues throughout the body. The activation and degranulation of mast cells modulate many aspects of physiological and pathological conditions in various settings [146]. Immunomodulating action of mast cells is related to the production and release of several multi-potent molecules including S1P [147, 148], Interestingly, MCs have been found to act as both tumor promotors and tumor suppressors

[147, 149, 150], and this effect can differ within the same tumor depending on the tissue compartment, e.g., in prostate tumors intratumoral MCs negatively regulate angiogenesis and tumor growth, whereas peritumoral MCs stimulate the expansion of cancer cells [150]. High numbers of mast cells have been found in several tumors including colorectal [151], pancreatic [152], melanoma, [153], NSCLC [154], squamous cell carcinomas (SCC) of the esophagus [155], mouth [156], and lip [157]. Interestingly, an elevated number of MCs was correlated with good [150, 158, 159] and poor prognosis [158, 160]. Fr example, in prostate cancer, patients with higher MCs counts had a better prognosis than the patients with lower MSc counts [159, 160]. Moreover, the MCs numbers correlated well with the clinical stages of tumors [160]. Similarly to other immune cells, S1P was found to stimulate motility of MCs [161], and this effect was S1PR1-dependent [161]. On the other hand, S1PR2 showed an inhibitory effect on MCs migration; however, it was necessary for S1P-induced degranulation [161]. Moreover, SphK1 but not SphK2 was found to be critical in MCs for antigen-induced degranulation, chemokine secretion, and migration, while both isozymes are essential for cytokine secretion [162]. Studies with MCs also led to the discovery that ABCC1 promotes the export of S1P across the plasma membrane independent of MCs degranulation [57]. Interestingly, exposure of MCs to S1P can lead to increased release of proteinases involved in tumor growth and metastasis, as well as pro-angiogenic VEGF [92, 148].

The complexity of S1P signaling that regulates immune cells in the TME (Fig. 7.5) requires further studies especially that not only S1P can directly act on different subpopulations of cells but also modulate cross talk between immune cells and tumor. Nevertheless, some of the studies indicate that the modulation of S1P-dependent signaling to increase the antitumor response of the immune system can be a promising target for anticancer therapies.

7.7 S1P as a Modulator in Inflammatory Pathways

Several studies indicate the involvement of S1P or enzymes controlling its metabolism in the regulation of inflammation. As mentioned earlier, intracellular S1P can activate NF-κB; however, recent studies indicate that this activation can also be mediated by S1PR (mainly S1PR1–3), thus involving an extracellular pool of S1P [69, 163, 164]. Interestingly, some results indicate a link between TNF signaling and NF-κB activation that involves S1P as a signaling molecule and/or as a cofactor of TRAF2 E3 ubiquitin ligase [67]. In melanoma cells, activation of NF-κB by extracellular S1P was found to be irreversibly correlated with expression of actin-binding protein FlnA [164], an interacting partner of SphK1 [165] and TRAF2 [166]. Moreover, it was also found that TRAF-interacting protein (TRIP), a binding partner of TRAF2, abrogated TNF-induced NF-κB activation by inhibiting binding of S1P to TRAF2 and thus suppressing its E3 ubiquitin ligase activity [67]. In inflammation-associated colon cancer, S1P was found to be essential for the production of the NF-κB-regulated cytokine, IL-6, crucial for persistent activation of transcription factor STAT3. This leads to upregulation of S1PR1 and reciprocally, enhanced S1PR1 expression activates STAT3 and upregulates IL-6 gene expression, thus accelerating tumor growth and metastasis in a STAT3-dependent manner [167]. The connection between S1PR1 and STAT3 activation was found to be crucial in distinct tumors, including lymphoma, adenocarcinoma, melanoma, breast, and prostate cancers [167] and decreased expression of STAT3-regulated genes by targeting S1PR1 was found to inhibit tumor progression [143]. What is interesting is S1P-induced STAT3 activation in colitis-induced colon cancer was correlated with upregulation of SphK1 or decreased level of SPL. A similar association was observed in animal models of inflammation, as S1P levels were increased in mice with dextran sulfate-

induced colitis, but not in mice lacking the SphK1 gene [168, 169]. The connection between S1P-metabolized enzymes and S1P-induced activation of STAT3 was also observed in cholangiocarcinoma cells where inhibition of SphK2 abrogated STAT3 phosphorylation and decreased cells' proliferation [170]. Also, in ER-negative breast cancer cells, SphK1 knockdown led to a significant reduction in leptin-induced STAT3 phosphorylation [171]. SphK1 and S1P were also found to be required for TNF-α-induced cyclooxygenase 2 (COX2), and prostaglandin E2 (PEG2) production [172]. Additionally, S1P in cooperation with lipopolysaccharide (LPS) was found to increase the expression of pro-inflammatory molecules such as IL-6, cyclooxygenase-2 (COX2), and prostacyclin in human endothelial cells [163]. Moreover, S1P was able to induce COX2 expression in Wilms tumor and this effect was mediated by S1PR2 [173].

Increased levels of S1P suggesting its involvement in controlling inflammation was observed in several nonmalignant conditions such as inflammatory arthritis [174, 175] multiple sclerosis [176], or asthma [177]. In contrast, increased expression of SPL and SPP2, thus indicating a decreased level of S1P, was found in the skin of patients and animals with psoriasis [178] and atopic dermatitis [179, 180], which could suggest that S1P exert anti-inflammatory actions in the skin. This hypothesis was confirmed in animal models of dermatitis or psoriasis, where the topical application of S1P reduced skin hyperproliferation and swelling [181, 182]. This mechanism of suppression involved inhibition of Langerhans cells migration to the lymph nodes or reduced antigen processing through S1PR2 activation [183]. Additionally, in a model of allergic asthma, inhalation of S1P suppressed airway by altering dendritic cell function [184]. Interestingly, only HDL-bound S1P, but not albumin-bound S1P, restrained lymphopoiesis and neuroinflammation in mice [185]. Although the described mechanisms were found in non-cancerous tissues, one cannot exclude that S1P can regulate similar processes in tumors.

7.8 S1P as a Regulator of Cells' Interaction

The role of S1P signaling in the regulation of the interaction between tumor and the immune system has been described in previous paragraphs; however, S1P can also modulate the interaction between other cell types present in the TME (Fig. 7.5). In S1PR2 knockout mice, the lack of this receptor on endothelial, vascular smooth muscle, and DC11b-positive bone marrow cells resulted in accelerated angiogenesis and growth of Lewis lung carcinoma and B16 melanoma cells [186]. The opposite effect was observed for S1PR1, whose downregulation in endothelial cells resulted in inhibited endothelial cell migration in vitro and the growth of neovessels into subcutaneous implants of Matrigel in vivo, thus leading to dramatic suppression of tumor growth [106]. In a model of melanoma, inhibition of SphK1 activity in dermal fibroblast enhanced tumor growth, whereas factors released from Sphk1 expressing melanoma cells were necessary for fibroblast differentiation into myofibroblasts [67, 187]. Moreover, myofibroblasts were found to release S1P and metalloproteinases that additionally increased melanoma growth and metastasis [67, 187]. Similarly, SphK1 expressed in the tumor stroma of serous ovarian cancer was required for the differentiation and the tumor-promoting function of cancer-associated fibroblasts through activation of TGF-β-signaling pathways via transactivation of S1PR2 and S1PR3 [188]. The importance of stromal SphK1 in tumorigenesis was confirmed in vivo in SphK1 knockout mice, where reduced tumor growth and decreased metastasis were observed [188]. S1P also mediate interactions in the pancreas between tumor and stromal cells where pancreatic cancer cell-derived S1P activates pancreatic stellate cells to release paracrine factors, including matrix metalloproteinase-9 (MMP9), which in turn promotes tumor cell migration and invasion, both in vitro and in vivo [189]. Interestingly, it has been proposed that communication between the host organism and cancer cells in a lung cancer model is transduced by S1P generated systemically rather than via tumor-

derived S1P, and that lung colonization and cancer metastasis requires S1PR2 activation [190]. Recently, some additional mechanism has also been described where stromal–cancer interaction has been facilitating through microvesicles [191]. It has been found that S1PR2 can be shed from breast cancer cells in exosomes present in conditioned medium. Moreover, when combining with fibroblast, S1PR2 was proteolyzed to produce a constitutively active form which promoted proliferation of these cells through activation of the ERK1/2 pathway [191]. S1PR2 was also found to be important in the regulation of epithelial defense against cancer (EDAC), a process in which epithelial cells eliminate neighboring cancer cells [192]. Altogether, these results strongly support the hypothesis that S1P in the TME may regulate the communication between cancerous and stromal cells to enhance tumor development (Fig. 7.5).

7.9 S1P and Hypoxia

Hypoxia is a non-physiological low level of oxygen in a tissue, and this phenomenon is observed in a majority of malignant tumors. It is the result of intensive proliferation and expansion of tumor tissue in which oxygen demand is surpassed by oxygen supply [193]. Decreased oxygenation may lead to either cancer cell death or cancer cell survival, and the type of response partially depends on the time of exposure to hypoxia [193]. Hypoxia induces several intracellular signaling pathways, including the hypoxia-inducible factor (HIF) pathway. The SphK1 promoter has two hypoxia-inducible factor-responsive elements (HREs) and both hypoxic-inducible factors HIF1α and HIF2α have been shown to regulate transcription of SphK1 [194, 195] (Fig. 7.6). Interestingly, in glioma cells, HIF1α and HIF2α had the opposite effect on SphK1 expression; while downregulation of HIF2α decreased expression of SphK1 and S1P levels, silencing of HIF1α increased SphK1 synthesis [196]. At the same time, both SphK1 and SphK2 were found to be necessary to stabilize HIF1α in normal and malignant cells [197, 198] and SphK1 was also found to control HIF1α expression through a phospholipase D-driven mechanism [196]. In lung cancer, hypoxia was found to enhance SphK2 activity and lead to sphingosine 1-phosphate-mediated chemoresistance through an autocrine/paracrine mechanism that includes activation of S1PR1 and S1PR3 in cancer cells [199]. Hypoxia-induced SphK1 also promotes endothelial cell migration in [195] and increases S1P production and release from glioma cells [200]. Additionally, conditioned medium from hypoxia-treated tumor cells resulted in neoangiogenesis in human umbilical vein endothelial cells in a S1PR-dependent manner thus providing evidence of a link between S1P production as a potent angiogenic agent and the hypoxic phenotype observed in many tumors [200]. S1P was also found to be involved in the activation of HIF1α in macrophages [201], and migration of endothelial cells [202]. Taken together, presented results indicate that regulation of S1P signaling in response to hypoxic conditions could be a potential therapeutic target leading to decreased angiogenesis in growing tumors.

7.10 S1P-Targeting Anticancer Therapies

Several strategies have been applied to the inhibition of S1P signaling in cancers targeting either metabolizing enzymes (mainly SphK1 and SphK2), specific S1P receptors, or S1P itself. The majority of SphK-targeting inhibitors either, rapidly and reversibly, inhibit catalytic activity [203] of SphK or induce ubiquitin-proteasomal degradation of SphK [204, 205], which results in a significant reduction in SphK levels in cancer cells. Unfortunately, some of them were found to be not isoform-specific, inhibit enzymes other than SphK (e.g., protein kinase C and ceramide kinase) [206, 207] or, despite showing efficacy in cancer models [208–212], were characterized with high toxicity [209]. Interestingly, some natural products like B-5354c [213], S-15183a, and S-15183b [214] have been shown to inhibit SphK in vitro and were found to reduce tumor growth in vivo [120]. Nevertheless, the efficacy and tox-

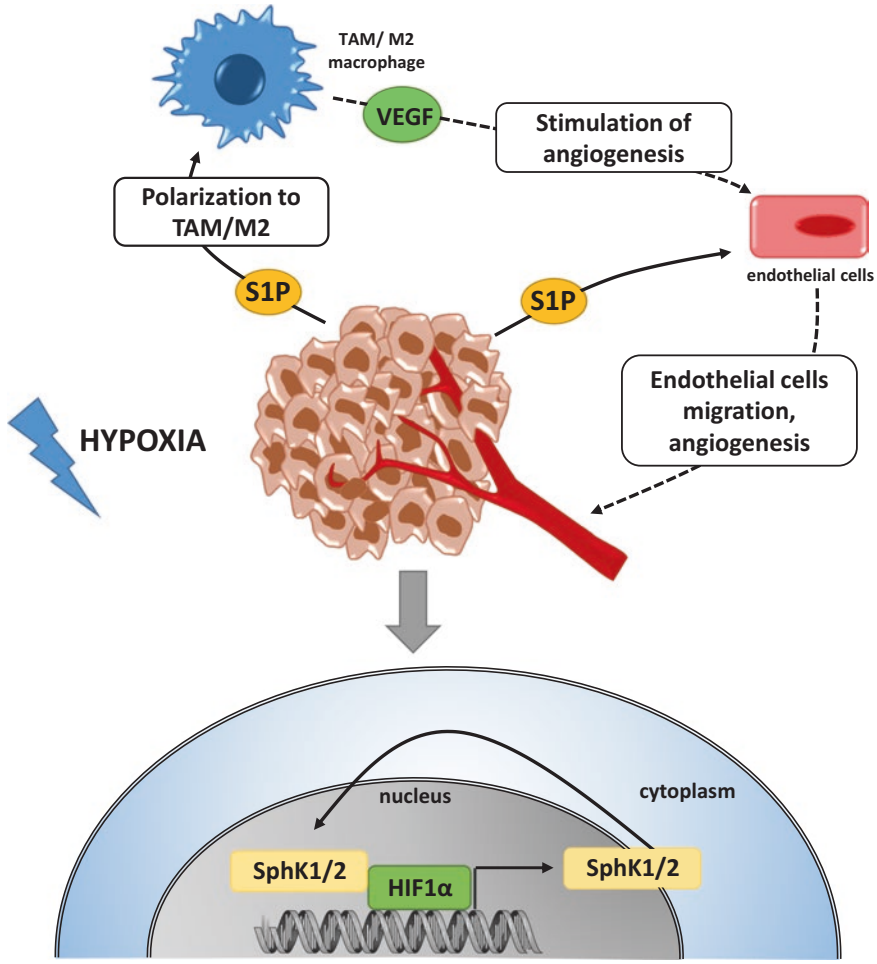

Fig. 7.6 Role of sphingosine-1-phosphate (S1P) in hypoxia. At the molecular level (lower panel), hypoxia induces expression of hypoxia-inducible factor 1α (HIF1α) that regulates expression of sphingosine kinase 1 (SphK1). At the same time, SphK1 stabilizes HIF1α and controls its expression through a phospholipase D-driven mechanism. At the cellular level (upper panel), S1P released from cancer cells promotes endothelial cell migration and subsequent angiogenesis. S1P was also found to induce TAM/M2 macrophage polarization and is involved in the activation of HIF1α in macrophages. Moreover, stabilization of HIF1α in macrophages induces vascular endothelial growth factor (VEGF) release that additionally stimulates angiogenesis

icity of three compounds that target SphK have been or are being assessed in clinical trials. Safingol (L-threo-dihydrosphingosine), which decreases the activity of SphK1, but also acts on protein kinase C [215], was shown to effectively downregulate the levels of S1P. However, although reversible, dose-dependent hepatic toxicity was observed. Currently, Safingol is being tested in patients with relapsed malignancies. Good tolerance and effectiveness in decreasing S1P levels were also shown for the selective SphK2 inhibitor ABC294640 (YELIVA) in Phase I studies in patients with advanced solid tumors [216]. Now, YELIVA is being evaluated in Phase II studies for the treatment of cholangiocarcinoma and hepatocellular carcinoma. A third compound, phenoxodiol, which was shown to reduce the activation of SphK1 [217] was assessed in clinical trials for the treatment of ovarian and prostate cancers [218–220]. However, the effect of phenoxodiol on SphK is indirect, and it also downregulates antiapoptotic proteins, induces

AKT downregulation, and inhibits topoisomerase II.

Multiple S1PR-selective agents are available on the market [135], and although some of them were tested in animals models, none of them were or are being evaluated in clinical trials as anticancer treatments. One of the explanations might be the heterogeneous pattern of S1PR expression in cancer, which can differ not only between the same types of cancer from different patients but also between cells within the same tumor, thus limiting the efficacy of S1PR treatments.

Another approach for inhibition of S1P-related signaling includes the development of monoclonal antibodies (mAbs) that bind and neutralize S1P from blood and other compartments. Sphingomab (LT1009), an S1P-specific antibody, was found to reduce tumor progression, metastasis and, in some cases, eliminated tumors in mouse xenograft and allograft models [221]. This effect was attributed to its anti-angiogenic properties since in vitro studies indicated that anti-S1P mAbs blocked endothelial cell migration and resulting capillary formation and in vivo observation indicated a reduction in tumor blood flow [221]. On the other hand, in in vivo prostate, Sphingomab blocked the activity of HIF-1α in cancer exposed to hypoxia and modified vessel architecture, thus increasing intratumoral blood perfusion [222]. This transient vascular normalization of tumor vessels sensitized it to chemotherapeutic treatment leading to decreased tumor growth and metastasis [222]. Two monoclonal S1P-specific antibodies, LT1002 and Sonepcizumab (Asonep), a humanized form of sphingomab, were shown to have high specificity for S1P but not to other structurally related lipids [223]. Sonepcizumab has recently completed Phase I clinical trials for the treatment of solid tumors, but results are not yet available. However, results from Phase II clinical study of Sonepcizumab in patients with metastatic renal cell carcinoma showed encouraging overall survival and favorable safety profile suggesting further investigation of this agent in combination with VEGF-directed agents or checkpoint inhibitors [224].

Binding and sequestering of S1P from the environment can be also obtained using Spiegelmers (Spiegel = German word for mirror) which are biostable oligonucleotides made from nonnatural mirror-image L-nucleotides that adopt complex three-dimensional structures and bind targets in a fashion comparable to antibodies [225]. NOX-S93, a high-affinity inhibitor of S1P, was shown to reduce angiogenesis in in vitro assay [225]. Moreover, in in vivo experiments, administration of NOX-S93 decreased S1P-induced spread of rhabdomyosarcoma cells [46].

7.11 Conclusions

There is no doubt that S1P plays an essential role in the regulation of the TME and modulates interactions between its components. S1P released from tumor cells allows them to inhibit anticancer immune response, increase angiogenesis, and adapt to hypoxic conditions. Cells stimulated by S1P within the TME can, in turn, secrete growth factors and cytokines that orchestrate cancer progression and chemoresistance. Thus, S1P signaling in the TME should be taken into account when designing novel therapeutic strategies for cancer patients.

References

1. Pyne NJ, El Buri A, Adams DR, Pyne S (2018) Sphingosine 1-phosphate and cancer. Adv Biol Regul 68:97–106
2. Maceyka M, Sankala H, Hait NC, Le Stunff H, Liu H, Toman R et al (2005) SphK1 and SphK2, sphingosine kinase isoenzymes with opposing functions in sphingolipid metabolism. J Biol Chem 280:37118–37129
3. Igarashi N, Okada T, Hayashi S, Fujita T, Jahangeer S, Nakamura S (2003) Sphingosine kinase 2 is a nuclear protein and inhibits DNA synthesis. J Biol Chem 278:46832–46839
4. Johnson KR, Becker KP, Facchinetti MM, Hannun YA, Obeid LM (2002) PKC-dependent activation of sphingosine kinase 1 and translocation to the plasma membrane. Extracellular release of sphingosine-1-phosphate induced by phorbol 12-myristate 13-acetate (PMA). J Biol Chem 277:35257–35262
5. Paugh BS, Paugh SW, Bryan L, Kapitonov D, Wilczynska KM, Gopalan SM et al (2008) EGF reg-

ulates plasminogen activator inhibitor-1 (PAI-1) by a pathway involving c-Src, PKCdelta, and sphingosine kinase 1 in glioblastoma cells. FASEB J 22:455–465
6. Pyne S, Lee SC, Long J, Pyne NJ (2009) Role of sphingosine kinases and lipid phosphate phosphatases in regulating spatial sphingosine 1-phosphate signalling in health and disease. Cell Signal 21:14–21
7. Ding G, Sonoda H, Yu H, Kajimoto T, Goparaju SK, Jahangeer S et al (2007) Protein kinase D-mediated phosphorylation and nuclear export of sphingosine kinase 2. J Biol Chem 282:27493–27502
8. Wallington-Beddoe CT, Powell JA, Tong D, Pitson SM, Bradstock KF, Bendall LJ (2014) Sphingosine kinase 2 promotes acute lymphoblastic leukemia by enhancing MYC expression. Cancer Res 74:2803–2815
9. Li J, Guan HY, Gong LY, Song LB, Zhang N, Wu J et al (2008) Clinical significance of sphingosine kinase-1 expression in human astrocytomas progression and overall patient survival. Clin Cancer Res 14:6996–7003
10. Ruckhaberle E, Rody A, Engels K, Gaetje R, von Minckwitz G, Schiffmann S et al (2008) Microarray analysis of altered sphingolipid metabolism reveals prognostic significance of sphingosine kinase 1 in breast cancer. Breast Cancer Res Treat 112:41–52
11. Watson C, Long JS, Orange C, Tannahill CL, Mallon E, McGlynn LM et al (2010) High expression of sphingosine 1-phosphate receptors, S1P1 and S1P3, sphingosine kinase 1, and extracellular signal-regulated kinase-1/2 is associated with development of tamoxifen resistance in estrogen receptor-positive breast cancer patients. Am J Pathol 177:2205–2215
12. Kawamori T, Kaneshiro T, Okumura M, Maalouf S, Uflacker A, Bielawski J et al (2009) Role for sphingosine kinase 1 in colon carcinogenesis. FASEB J 23:405–414
13. Long J, Xie Y, Yin J, Lu W, Fang S (2016) SphK1 promotes tumor cell migration and invasion in colorectal cancer. Tumour Biol 37:6831–6836
14. Li W, Yu CP, Xia JT, Zhang L, Weng GX, Zheng HQ et al (2009) Sphingosine kinase 1 is associated with gastric cancer progression and poor survival of patients. Clin Cancer Res 15:1393–1399
15. Van Brocklyn JR, Jackson CA, Pearl DK, Kotur MS, Snyder PJ, Prior TW (2005) Sphingosine kinase-1 expression correlates with poor survival of patients with glioblastoma multiforme: roles of sphingosine kinase isoforms in growth of glioblastoma cell lines. J Neuropathol Exp Neurol 64:695–705
16. Abuhusain HJ, Matin A, Qiao Q, Shen H, Kain N, Day BW et al (2013) A metabolic shift favoring sphingosine 1-phosphate at the expense of ceramide controls glioblastoma angiogenesis. J Biol Chem 288:37355–37364
17. Johnson KR, Johnson KY, Crellin HG, Ogretmen B, Boylan AM, Harley RA et al (2005) Immunohistochemical distribution of sphingosine kinase 1 in normal and tumor lung tissue. J Histochem Cytochem 53:1159–1166
18. Bayerl MG, Bruggeman RD, Conroy EJ, Hengst JA, King TS, Jimenez M et al (2008) Sphingosine kinase 1 protein and mRNA are overexpressed in non-Hodgkin lymphomas and are attractive targets for novel pharmacological interventions. Leuk Lymphoma 49:948–954
19. Malavaud B, Pchejetski D, Mazerolles C, de Paiva GR, Calvet C, Doumerc N et al (2010) Sphingosine kinase-1 activity and expression in human prostate cancer resection specimens. Eur J Cancer 46:3417–3424
20. Guan H, Liu L, Cai J, Liu J, Ye C, Li M et al (2011) Sphingosine kinase 1 is overexpressed and promotes proliferation in human thyroid cancer. Mol Endocrinol 25:1858–1866
21. Qiu W, Yang Z, Fan Y, Zheng Q (2016) MicroRNA-613 inhibits cell growth, migration and invasion of papillary thyroid carcinoma by regulating SphK2. Oncotarget 7:39907–39915
22. French KJ, Schrecengost RS, Lee BD, Zhuang Y, Smith SN, Eberly JL et al (2003) Discovery and evaluation of inhibitors of human sphingosine kinase. Cancer Res 63:5962–5969
23. Li J, Wu H, Li W, Yin L, Guo S, Xu X et al (2016) Downregulated miR-506 expression facilitates pancreatic cancer progression and chemoresistance via SPHK1/Akt/NF-kappaB signaling. Oncogene 35:5501–5514
24. Uranbileg B, Ikeda H, Kurano M, Enooku K, Sato M, Saigusa D et al (2016) Increased mRNA levels of Sphingosine kinases and S1P Lyase and reduced levels of S1P were observed in hepatocellular carcinoma in association with poorer differentiation and earlier recurrence. PLoS One 11:e0149462
25. Kim HS, Yoon G, Ryu JY, Cho YJ, Choi JJ, Lee YY et al (2015) Sphingosine kinase 1 is a reliable prognostic factor and a novel therapeutic target for uterine cervical cancer. Oncotarget 6:26746–26756
26. Nunes J, Naymark M, Sauer L, Muhammad A, Keun H, Sturge J et al (2012) Circulating sphingosine-1-phosphate and erythrocyte sphingosine kinase-1 activity as novel biomarkers for early prostate cancer detection. Br J Cancer 106:909–915
27. Clair T, Aoki J, Koh E, Bandle RW, Nam SW, Ptaszynska MM et al (2003) Autotaxin hydrolyzes sphingosylphosphorylcholine to produce the regulator of migration, sphingosine-1-phosphate. Cancer Res 63:5446–5453
28. Liliom K, Sun G, Bunemann M, Virag T, Nusser N, Baker DL et al (2001) Sphingosylphosphocholine is a naturally occurring lipid mediator in blood plasma: a possible role in regulating cardiac function via sphingolipid receptors. Biochem J 355:189–197
29. El-Najjar N, Orso E, Wallner S, Liebisch G, Schmitz G (2015) Increased levels of Sphingosylphosphorylcholine (SPC) in plasma of metabolic syndrome patients. PLoS One 10:e0140683

30. Tanaka M, Okudaira S, Kishi Y, Ohkawa R, Iseki S, Ota M et al (2006) Autotaxin stabilizes blood vessels and is required for embryonic vasculature by producing lysophosphatidic acid. J Biol Chem 281:25822–25830
31. Gao XY, Li L, Wang XH, Wen XZ, Ji K, Ye L et al (2015) Inhibition of sphingosine-1-phosphate phosphatase 1 promotes cancer cells migration in gastric cancer: clinical implications. Oncol Rep 34:1977–1987
32. Serra M, Saba JD (2010) Sphingosine 1-phosphate lyase, a key regulator of sphingosine 1-phosphate signaling and function. Adv Enzym Regul 50:349–362
33. Oskouian B, Sooriyakumaran P, Borowsky AD, Crans A, Dillard-Telm L, Tam YY et al (2006) Sphingosine-1-phosphate lyase potentiates apoptosis via p53- and p38-dependent pathways and is down-regulated in colon cancer. Proc Natl Acad Sci U S A 103:17384–17389
34. Degagne E, Pandurangan A, Bandhuvula P, Kumar A, Eltanawy A, Zhang M et al (2014) Sphingosine-1-phosphate lyase downregulation promotes colon carcinogenesis through STAT3-activated microRNAs. J Clin Invest 124:5368–5384
35. Brizuela L, Ader I, Mazerolles C, Bocquet M, Malavaud B, Cuvillier O (2012) First evidence of sphingosine 1-phosphate lyase protein expression and activity downregulation in human neoplasm: implication for resistance to therapeutics in prostate cancer. Mol Cancer Ther 11:1841–1851
36. Hammad SM, Al Gadban MM, Semler AJ, Klein RL (2012) Sphingosine 1-phosphate distribution in human plasma: associations with lipid profiles. J Lipids 2012:180705
37. Christoffersen C, Obinata H, Kumaraswamy SB, Galvani S, Ahnstrom J, Sevvana M et al (2011) Endothelium-protective sphingosine-1-phosphate provided by HDL-associated apolipoprotein M. Proc Natl Acad Sci U S A 108:9613–9618
38. Murata N, Sato K, Kon J, Tomura H, Yanagita M, Kuwabara A et al (2000) Interaction of sphingosine 1-phosphate with plasma components, including lipoproteins, regulates the lipid receptor-mediated actions. Biochem J 352(Pt 3):809–815
39. Nagahashi M, Ramachandran S, Kim EY, Allegood JC, Rashid OM, Yamada A et al (2012) Sphingosine-1-phosphate produced by sphingosine kinase 1 promotes breast cancer progression by stimulating angiogenesis and lymphangiogenesis. Cancer Res 72:726–735
40. Nagahashi M, Yamada A, Miyazaki H, Allegood JC, Tsuchida J, Aoyagi T et al (2016) Interstitial fluid sphingosine-1-phosphate in murine mammary gland and cancer and human breast tissue and cancer determined by novel methods. J Mammary Gland Biol Neoplasia 21:9–17
41. Nagahashi M, Tsuchida J, Moro K, Hasegawa M, Tatsuda K, Woelfel IA et al (2016) High levels of sphingolipids in human breast cancer. J Surg Res 204:435–444
42. Xu Y, Xiao YJ, Baudhuin LM, Schwartz BM (2001) The role and clinical applications of bioactive lysolipids in ovarian cancer. J Soc Gynecol Investig 8:1–13
43. Wang D, Zhao Z, Caperell-Grant A, Yang G, Mok SC, Liu J et al (2008) S1P differentially regulates migration of human ovarian cancer and human ovarian surface epithelial cells. Mol Cancer Ther 7:1993–2002
44. Sutphen R, Xu Y, Wilbanks GD, Fiorica J, Grendys EC Jr, LaPolla JP et al (2004) Lysophospholipids are potential biomarkers of ovarian cancer. Cancer Epidemiol Biomark Prev 13:1185–1191
45. Gude DR, Alvarez SE, Paugh SW, Mitra P, Yu J, Griffiths R et al (2008) Apoptosis induces expression of sphingosine kinase 1 to release sphingosine-1-phosphate as a "come-and-get-me" signal. FASEB J 22:2629–2638
46. Schneider G, Bryndza E, Abdel-Latif A, Ratajczak J, Maj M, Tarnowski M et al (2013) Bioactive lipids S1P and C1P are prometastatic factors in human rhabdomyosarcoma, and their tissue levels increase in response to radio/chemotherapy. Mol Cancer Res 11:793–807
47. Weigert A, Tzieply N, von Knethen A, Johann AM, Schmidt H, Geisslinger G et al (2007) Tumor cell apoptosis polarizes macrophages role of sphingosine-1-phosphate. Mol Biol Cell 18:3810–3819
48. Ley S, Weigert A, Weichand B, Henke N, Mille-Baker B, Janssen RA et al (2013) The role of TRKA signaling in IL-10 production by apoptotic tumor cell-activated macrophages. Oncogene 32:631–640
49. Venkataraman K, Thangada S, Michaud J, Oo ML, Ai Y, Lee YM et al (2006) Extracellular export of sphingosine kinase-1a contributes to the vascular S1P gradient. Biochem J 397:461–471
50. Ancellin N, Colmont C, Su J, Li Q, Mittereder N, Chae SS et al (2002) Extracellular export of sphingosine kinase-1 enzyme. Sphingosine 1-phosphate generation and the induction of angiogenic vascular maturation. J Biol Chem 277:6667–6675
51. Hammad SM, Taha TA, Nareika A, Johnson KR, Lopes-Virella MF, Obeid LM (2006) Oxidized LDL immune complexes induce release of sphingosine kinase in human U937 monocytic cells. Prostaglandins Other Lipid Mediat 79:126–140
52. Osborne N, Brand-Arzamendi K, Ober EA, Jin SW, Verkade H, Holtzman NG et al (2008) The spinster homolog, two of hearts, is required for sphingosine 1-phosphate signaling in zebrafish. Curr Biol 18:1882–1888
53. Mendoza A, Breart B, Ramos-Perez WD, Pitt LA, Gobert M, Sunkara M et al (2012) The transporter Spns2 is required for secretion of lymph but not plasma sphingosine-1-phosphate. Cell Rep 2:1104–1110
54. Takabe K, Spiegel S (2014) Export of sphingosine-1-phosphate and cancer progression. J Lipid Res 55:1839–1846

55. Nagahashi M, Kim EY, Yamada A, Ramachandran S, Allegood JC, Hait NC et al (2013) Spns2, a transporter of phosphorylated sphingoid bases, regulates their blood and lymph levels, and the lymphatic network. FASEB J 27:1001–1011
56. Fukuhara S, Simmons S, Kawamura S, Inoue A, Orba Y, Tokudome T et al (2012) The sphingosine-1-phosphate transporter Spns2 expressed on endothelial cells regulates lymphocyte trafficking in mice. J Clin Invest 122:1416–1426
57. Mitra P, Oskeritzian CA, Payne SG, Beaven MA, Milstien S, Spiegel S (2006) Role of ABCC1 in export of sphingosine-1-phosphate from mast cells. Proc Natl Acad Sci U S A 103:16394–16399
58. Kobayashi N, Kobayashi N, Yamaguchi A, Nishi T (2009) Characterization of the ATP-dependent sphingosine 1-phosphate transporter in rat erythrocytes. J Biol Chem 284:21192–21200
59. Takabe K, Kim RH, Allegood JC, Mitra P, Ramachandran S, Nagahashi M et al (2010) Estradiol induces export of sphingosine 1-phosphate from breast cancer cells via ABCC1 and ABCG2. J Biol Chem 285:10477–10486
60. Sato K, Malchinkhuu E, Horiuchi Y, Mogi C, Tomura H, Tosaka M et al (2007) Critical role of ABCA1 transporter in sphingosine 1-phosphate release from astrocytes. J Neurochem 103:2610–2619
61. Kobayashi N, Nishi T, Hirata T, Kihara A, Sano T, Igarashi Y et al (2006) Sphingosine 1-phosphate is released from the cytosol of rat platelets in a carrier-mediated manner. J Lipid Res 47:614–621
62. Bradley E, Dasgupta S, Jiang X, Zhao X, Zhu G, He Q et al (2014) Critical role of Spns2, a sphingosine-1-phosphate transporter, in lung cancer cell survival and migration. PLoS One 9:e110119
63. Ghosh TK, Bian J, Gill DL (1990) Intracellular calcium release mediated by sphingosine derivatives generated in cells. Science 248:1653–1656
64. Himmel HM, Meyer zu Heringdorf D, Windorfer B, van Koppen CJ, Ravens U, Jakobs KH (1998) Guanine nucleotide-sensitive inhibition of L-type Ca2+ current by lysosphingolipids in RINm5F insulinoma cells. Mol Pharmacol 53:862–869
65. Hait NC, Allegood J, Maceyka M, Strub GM, Harikumar KB, Singh SK et al (2009) Regulation of histone acetylation in the nucleus by sphingosine-1-phosphate. Science 325:1254–1257
66. Strub GM, Paillard M, Liang J, Gomez L, Allegood JC, Hait NC et al (2011) Sphingosine-1-phosphate produced by sphingosine kinase 2 in mitochondria interacts with prohibitin 2 to regulate complex IV assembly and respiration. FASEB J 25:600–612
67. Alvarez SE, Harikumar KB, Hait NC, Allegood J, Strub GM, Kim EY et al (2010) Sphingosine-1-phosphate is a missing cofactor for the E3 ubiquitin ligase TRAF2. Nature 465:1084–1088
68. Park K, Ikushiro H, Seo HS, Shin KO, Kim YI, Kim JY et al (2016) ER stress stimulates production of the key antimicrobial peptide, cathelicidin, by forming a previously unidentified intracellular S1P signaling complex. Proc Natl Acad Sci U S A 113:E1334–E1342
69. Blom T, Bergelin N, Meinander A, Lof C, Slotte JP, Eriksson JE et al (2010) An autocrine sphingosine-1-phosphate signaling loop enhances NF-kappaB-activation and survival. BMC Cell Biol 11:45
70. Siehler S, Wang Y, Fan X, Windh RT, Manning DR (2001) Sphingosine 1-phosphate activates nuclear factor-kappa B through Edg receptors. Activation through Edg-3 and Edg-5, but not Edg-1, in human embryonic kidney 293 cells. J Biol Chem 276:48733–48739
71. Harikumar KB, Yester JW, Surace MJ, Oyeniran C, Price MM, Huang WC et al (2014) K63-linked polyubiquitination of transcription factor IRF1 is essential for IL-1-induced production of chemokines CXCL10 and CCL5. Nat Immunol 15:231–238
72. Parham KA, Zebol JR, Tooley KL, Sun WY, Moldenhauer LM, Cockshell MP et al (2015) Sphingosine 1-phosphate is a ligand for peroxisome proliferator-activated receptor-gamma that regulates neoangiogenesis. FASEB J 29:3638–3653
73. Blaho VA, Hla T (2014) An update on the biology of sphingosine 1-phosphate receptors. J Lipid Res 55:1596–1608
74. Chun J, Hla T, Lynch KR, Spiegel S, Moolenaar WH (2010) International Union of Basic and Clinical Pharmacology. LXXVIII. Lysophospholipid receptor nomenclature. Pharmacol Rev 62:579–587
75. Huang YL, Huang WP, Lee H (2011) Roles of sphingosine 1-phosphate on tumorigenesis. World J Biol Chem 2:25–34
76. Novgorodov AS, El-Alwani M, Bielawski J, Obeid LM, Gudz TI (2007) Activation of sphingosine-1-phosphate receptor S1P5 inhibits oligodendrocyte progenitor migration. FASEB J 21:1503–1514
77. Im DS, Heise CE, Ancellin N, O'Dowd BF, Shei GJ, Heavens RP et al (2000) Characterization of a novel sphingosine 1-phosphate receptor, Edg-8. J Biol Chem 275:14281–14286
78. Liu H, Toman RE, Goparaju SK, Maceyka M, Nava VE, Sankala H et al (2003) Sphingosine kinase type 2 is a putative BH3-only protein that induces apoptosis. J Biol Chem 278:40330–40336
79. Xia J, Wu Z, Yu C, He W, Zheng H, He Y et al (2012) miR-124 inhibits cell proliferation in gastric cancer through down-regulation of SPHK1. J Pathol 227:470–480
80. Ju T, Gao D, Fang ZY (2016) Targeting colorectal cancer cells by a novel sphingosine kinase 1 inhibitor PF-543. Biochem Biophys Res Commun 470:728–734
81. Don AS, Rosen H (2009) A lipid binding domain in sphingosine kinase 2. Biochem Biophys Res Commun 380:87–92
82. Xun C, Chen MB, Qi L, Tie-Ning Z, Peng X, Ning L et al (2015) Targeting sphingosine kinase 2 (SphK2) by ABC294640 inhibits colorectal cancer cell growth in vitro and in vivo. J Exp Clin Cancer Res 34:94

83. Schrecengost RS, Keller SN, Schiewer MJ, Knudsen KE, Smith CD (2015) Downregulation of critical oncogenes by the selective SK2 inhibitor ABC294640 hinders prostate cancer progression. Mol Cancer Res 13:1591–1601
84. Rex K, Jeffries S, Brown ML, Carlson T, Coxon A, Fajardo F et al (2013) Sphingosine kinase activity is not required for tumor cell viability. PLoS One 8:e68328
85. Chang CL, Ho MC, Lee PH, Hsu CY, Huang WP, Lee H (2009) S1P(5) is required for sphingosine 1-phosphate-induced autophagy in human prostate cancer PC-3 cells. Am J Physiol Cell Physiol 297:C451–C458
86. Rodgers A, Mormeneo D, Long JS, Delgado A, Pyne NJ, Pyne S (2009) Sphingosine 1-phosphate regulation of extracellular signal-regulated kinase-1/2 in embryonic stem cells. Stem Cells Dev 18:1319–1330
87. Cheng JC, Wang EY, Yi Y, Thakur A, Tsai SH, Hoodless PA (2018) S1P stimulates proliferation by upregulating CTGF expression through S1PR2-mediated YAP activation. Mol Cancer Res 16:1543–1555
88. Li MH, Sanchez T, Pappalardo A, Lynch KR, Hla T, Ferrer F (2008) Induction of antiproliferative connective tissue growth factor expression in Wilms' tumor cells by sphingosine-1-phosphate receptor 2. Mol Cancer Res 6:1649–1656
89. Lei FJ, Cheng BH, Liao PY, Wang HC, Chang WC, Lai HC et al (2018) Survival benefit of sphingosin-1-phosphate and receptors expressions in breast cancer patients. Cancer Med 7:3743–3754
90. Van Brocklyn J, Letterle C, Snyder P, Prior T (2002) Sphingosine-1-phosphate stimulates human glioma cell proliferation through Gi-coupled receptors: role of ERK MAP kinase and phosphatidylinositol 3-kinase beta. Cancer Lett 181:195–204
91. Hu WM, Li L, Jing BQ, Zhao YS, Wang CL, Feng L et al (2010) Effect of S1P5 on proliferation and migration of human esophageal cancer cells. World J Gastroenterol 16:1859–1866
92. Chumanevich A, Wedman P, Oskeritzian CA (2016) Sphingosine-1-phosphate/sphingosine-1-phosphate receptor 2 axis can promote mouse and human primary mast cell Angiogenic potential through upregulation of vascular endothelial growth factor-a and matrix metalloproteinase-2. Mediat Inflamm 2016:1503206
93. Abdelbaset-Ismail A, Cymer M, Borkowska-Rzeszotek S, Brzezniakiewicz-Janus K, Rameshwar P, Kakar SS et al (2019) Bioactive phospholipids enhance migration and adhesion of human leukemic cells by inhibiting heme oxygenase 1 (HO-1) and inducible nitric oxygenase synthase (iNOS) in a p38 MAPK-dependent manner. Stem Cell Rev 15:139–154
94. Schneider G, Sellers ZP, Bujko K, Kakar SS, Kucia M, Ratajczak MZ (2017) Novel pleiotropic effects of bioactive phospholipids in human lung cancer metastasis. Oncotarget 8:58247–58263
95. Malchinkhuu E, Sato K, Maehama T, Mogi C, Tomura H, Ishiuchi S et al (2008) S1P(2) receptors mediate inhibition of glioma cell migration through rho signaling pathways independent of PTEN. Biochem Biophys Res Commun 366:963–968
96. Park KS, Kim MK, Lee HY, Kim SD, Lee SY, Kim JM et al (2007) S1P stimulates chemotactic migration and invasion in OVCAR3 ovarian cancer cells. Biochem Biophys Res Commun 356:239–244
97. Fisher KE, Pop A, Koh W, Anthis NJ, Saunders WB, Davis GE (2006) Tumor cell invasion of collagen matrices requires coordinate lipid agonist-induced G-protein and membrane-type matrix metalloproteinase-1-dependent signaling. Mol Cancer 5:69
98. Nyalendo C, Michaud M, Beaulieu E, Roghi C, Murphy G, Gingras D et al (2007) Src-dependent phosphorylation of membrane type I matrix metalloproteinase on cytoplasmic tyrosine 573: role in endothelial and tumor cell migration. J Biol Chem 282:15690–15699
99. Young N, Van Brocklyn JR (2007) Roles of sphingosine-1-phosphate (S1P) receptors in malignant behavior of glioma cells. Differential effects of S1P2 on cell migration and invasiveness. Exp Cell Res 313:1615–1627
100. Zhang H, Wang Q, Zhao Q, Di W (2013) MiR-124 inhibits the migration and invasion of ovarian cancer cells by targeting SphK1. J Ovarian Res 6:84
101. Gao P, Smith CD (2011) Ablation of sphingosine kinase-2 inhibits tumor cell proliferation and migration. Mol Cancer Res 9:1509–1519
102. Wu WT, Chen CN, Lin CI, Chen JH, Lee H (2005) Lysophospholipids enhance matrix metalloproteinase-2 expression in human endothelial cells. Endocrinology 146:3387–3400
103. Moon A, Kim MS, Kim TG, Kim SH, Kim HE, Chen YQ et al (2000) H-ras, but not N-ras, induces an invasive phenotype in human breast epithelial cells: a role for MMP-2 in the H-ras-induced invasive phenotype. Int J Cancer 85:176–181
104. Wang F, Van Brocklyn JR, Hobson JP, Movafagh S, Zukowska-Grojec Z, Milstien S et al (1999) Sphingosine 1-phosphate stimulates cell migration through a G(i)-coupled cell surface receptor. Potential involvement in angiogenesis. J Biol Chem 274:35343–35350
105. Boguslawski G, Grogg JR, Welch Z, Ciechanowicz S, Sliva D, Kovala AT et al (2002) Migration of vascular smooth muscle cells induced by sphingosine 1-phosphate and related lipids: potential role in the angiogenic response. Exp Cell Res 274:264–274
106. Chae SS, Paik JH, Furneaux H, Hla T (2004) Requirement for sphingosine 1-phosphate receptor-1 in tumor angiogenesis demonstrated by in vivo RNA interference. J Clin Invest 114:1082–1089
107. Shu X, Wu W, Mosteller RD, Broek D (2002) Sphingosine kinase mediates vascular endothelial growth factor-induced activation of ras and

mitogen-activated protein kinases. Mol Cell Biol 22:7758–7768
108. Boucharaba A, Guillet B, Menaa F, Hneino M, van Wijnen AJ, Clezardin P et al (2009) Bioactive lipids lysophosphatidic acid and sphingosine 1-phosphate mediate breast cancer cell biological functions through distinct mechanisms. Oncol Res 18:173–184
109. Lu Z, Zhang W, Gao S, Jiang Q, Xiao Z, Ye L et al (2015) MiR-506 suppresses liver cancer angiogenesis through targeting sphingosine kinase 1 (SPHK1) mRNA. Biochem Biophys Res Commun 468:8–13
110. Guillermet-Guibert J, Davenne L, Pchejetski D, Saint-Laurent N, Brizuela L, Guilbeau-Frugier C et al (2009) Targeting the sphingolipid metabolism to defeat pancreatic cancer cell resistance to the chemotherapeutic gemcitabine drug. Mol Cancer Ther 8:809–820
111. Baran Y, Salas A, Senkal CE, Gunduz U, Bielawski J, Obeid LM et al (2007) Alterations of ceramide/sphingosine 1-phosphate rheostat involved in the regulation of resistance to imatinib-induced apoptosis in K562 human chronic myeloid leukemia cells. J Biol Chem 282:10922–10934
112. Sobue S, Nemoto S, Murakami M, Ito H, Kimura A, Gao S et al (2008) Implications of sphingosine kinase 1 expression level for the cellular sphingolipid rheostat: relevance as a marker for daunorubicin sensitivity of leukemia cells. Int J Hematol 87:266–275
113. Pchejetski D, Golzio M, Bonhoure E, Calvet C, Doumerc N, Garcia V et al (2005) Sphingosine kinase-1 as a chemotherapy sensor in prostate adenocarcinoma cell and mouse models. Cancer Res 65:11667–11675
114. Gao H, Deng L (2014) Sphingosine kinase-1 activation causes acquired resistance against Sunitinib in renal cell carcinoma cells. Cell Biochem Biophys 68:419–425
115. Matula K, Collie-Duguid E, Murray G, Parikh K, Grabsch H, Tan P et al (2015) Regulation of cellular sphingosine-1-phosphate by sphingosine kinase 1 and sphingosine-1-phosphate lyase determines chemotherapy resistance in gastroesophageal cancer. BMC Cancer 15:762
116. Okamoto H, Takuwa N, Yokomizo T, Sugimoto N, Sakurada S, Shigematsu H et al (2000) Inhibitory regulation of Rac activation, membrane ruffling, and cell migration by the G protein-coupled sphingosine-1-phosphate receptor EDG5 but not EDG1 or EDG3. Mol Cell Biol 20:9247–9261
117. Liu W, Ning J, Li C, Hu J, Meng Q, Lu H et al (2016) Overexpression of Sphk2 is associated with gefitinib resistance in non-small cell lung cancer. Tumour Biol 37:6331–6336
118. Antoon JW, White MD, Slaughter EM, Driver JL, Khalili HS, Elliott S et al (2011) Targeting NFkB mediated breast cancer chemoresistance through selective inhibition of sphingosine kinase-2. Cancer Biol Ther 11:678–689
119. Min J, Stegner AL, Alexander H, Alexander S (2004) Overexpression of sphingosine-1-phosphate lyase or inhibition of sphingosine kinase in Dictyostelium discoideum results in a selective increase in sensitivity to platinum-based chemotherapy drugs. Eukaryot Cell 3:795–805
120. Pchejetski D, Doumerc N, Golzio M, Naymark M, Teissie J, Kohama T et al (2008) Chemosensitizing effects of sphingosine kinase-1 inhibition in prostate cancer cell and animal models. Mol Cancer Ther 7:1836–1845
121. Lifshitz V, Priceman SJ, Li W, Cherryholmes G, Lee H, Makovski-Silverstein A et al (2017) Sphingosine-1-phosphate receptor-1 promotes environment-mediated and acquired chemoresistance. Mol Cancer Ther 16:2516–2527
122. Mantovani A, Biswas SK, Galdiero MR, Sica A, Locati M (2013) Macrophage plasticity and polarization in tissue repair and remodelling. J Pathol 229:176–185
123. Porta C, Rimoldi M, Raes G, Brys L, Ghezzi P, Di Liberto D et al (2009) Tolerance and M2 (alternative) macrophage polarization are related processes orchestrated by p50 nuclear factor kappaB. Proc Natl Acad Sci U S A 106:14978–14983
124. Hanahan D, Coussens LM (2012) Accessories to the crime: functions of cells recruited to the tumor microenvironment. Cancer Cell 21:309–322
125. Weichand B, Weis N, Weigert A, Grossmann N, Levkau B, Brune B (2013) Apoptotic cells enhance sphingosine-1-phosphate receptor 1 dependent macrophage migration. Eur J Immunol 43:3306–3313
126. Weigert A, Johann AM, von Knethen A, Schmidt H, Geisslinger G, Brune B (2006) Apoptotic cells promote macrophage survival by releasing the antiapoptotic mediator sphingosine-1-phosphate. Blood 108:1635–1642
127. He H, Zhang S, Tighe S, Son J, Tseng SC (2013) Immobilized heavy chain-hyaluronic acid polarizes lipopolysaccharide-activated macrophages toward M2 phenotype. J Biol Chem 288:25792–25803
128. Park SJ, Lee KP, Kang S, Lee J, Sato K, Chung HY et al (2014) Sphingosine 1-phosphate induced anti-atherogenic and atheroprotective M2 macrophage polarization through IL-4. Cell Signal 26:2249–2258
129. Hughes JE, Srinivasan S, Lynch KR, Proia RL, Ferdek P, Hedrick CC (2008) Sphingosine-1-phosphate induces an antiinflammatory phenotype in macrophages. Circ Res 102:950–958
130. Brecht K, Weigert A, Hu J, Popp R, Fisslthaler B, Korff T et al (2011) Macrophages programmed by apoptotic cells promote angiogenesis via prostaglandin E2. FASEB J 25:2408–2417
131. Eken A, Duhen R, Singh AK, Fry M, Buckner JH, Kita M et al (2017) S1P1 deletion differentially affects TH17 and regulatory T cells. Sci Rep 7:12905
132. Priceman SJ, Shen S, Wang L, Deng J, Yue C, Kujawski M et al (2014) S1PR1 is crucial for accumulation of regulatory T cells in tumors via STAT3. Cell Rep 6:992–999

133. Wolf AM, Eller K, Zeiser R, Durr C, Gerlach UV, Sixt M et al (2009) The sphingosine 1-phosphate receptor agonist FTY720 potently inhibits regulatory T cell proliferation in vitro and in vivo. J Immunol 183:3751–3760
134. Liu YN, Zhang H, Zhang L, Cai TT, Huang DJ, He J et al (2019) Sphingosine 1 phosphate receptor-1 (S1P1) promotes tumor-associated regulatory T cell expansion: leading to poor survival in bladder cancer. Cell Death Dis 10:50
135. Park SJ, Im DS (2017) Sphingosine 1-phosphate receptor modulators and drug discovery. Biomol Ther (Seoul) 25:80–90
136. Lagadari M, Lehmann K, Ziemer M, Truta-Feles K, Berod L, Idzko M et al (2009) Sphingosine-1-phosphate inhibits the cytotoxic activity of NK cells via Gs protein-mediated signalling. Int J Oncol 34:287–294
137. Walzer T, Chiossone L, Chaix J, Calver A, Carozzo C, Garrigue-Antar L et al (2007) Natural killer cell trafficking in vivo requires a dedicated sphingosine 1-phosphate receptor. Nat Immunol 8:1337–1344
138. Rolin J, Sand KL, Knudsen E, Maghazachi AA (2010) FTY720 and SEW2871 reverse the inhibitory effect of S1P on natural killer cell mediated lysis of K562 tumor cells and dendritic cells but not on cytokine release. Cancer Immunol Immunother 59:575–586
139. Maeda Y, Matsuyuki H, Shimano K, Kataoka H, Sugahara K, Chiba K (2007) Migration of CD4 T cells and dendritic cells toward sphingosine 1-phosphate (S1P) is mediated by different receptor subtypes: S1P regulates the functions of murine mature dendritic cells via S1P receptor type 3. J Immunol 178:3437–3446
140. Flori M, Schmid CA, Sumrall ET, Tzankov A, Law CW, Robinson MD et al (2016) The hematopoietic oncoprotein FOXP1 promotes tumor cell survival in diffuse large B-cell lymphoma by repressing S1PR2 signaling. Blood 127:1438–1448
141. Sic H, Kraus H, Madl J, Flittner KA, von Munchow AL, Pieper K et al (2014) Sphingosine-1-phosphate receptors control B-cell migration through signaling components associated with primary immunodeficiencies, chronic lymphocytic leukemia, and multiple sclerosis. J Allergy Clin Immunol 134:420–428
142. Till KJ, Pettitt AR, Slupsky JR (2015) Expression of functional sphingosine-1 phosphate receptor-1 is reduced by B cell receptor signaling and increased by inhibition of PI3 kinase delta but not SYK or BTK in chronic lymphocytic leukemia cells. J Immunol 194:2439–2446
143. Liu Y, Deng J, Wang L, Lee H, Armstrong B, Scuto A et al (2012) S1PR1 is an effective target to block STAT3 signaling in activated B cell-like diffuse large B-cell lymphoma. Blood 120:1458–1465
144. Capitani N, Patrussi L, Trentin L, Lucherini OM, Cannizzaro E, Migliaccio E et al (2012) S1P1 expression is controlled by the pro-oxidant activity of p66Shc and is impaired in B-CLL patients with unfavorable prognosis. Blood 120:4391–4399
145. Jin Y, Knudsen E, Wang L, Bryceson Y, Damaj B, Gessani S et al (2003) Sphingosine 1-phosphate is a novel inhibitor of T-cell proliferation. Blood 101:4909–4915
146. Krystel-Whittemore M, Dileepan KN, Wood JG (2015) Mast cell: a multi-functional master cell. Front Immunol 6:620
147. Oskeritzian CA (2015) Mast cell plasticity and sphingosine-1-phosphate in immunity, inflammation and cancer. Mol Immunol 63:104–112
148. Price MM, Oskeritzian CA, Milstien S, Spiegel S (2008) Sphingosine-1-phosphate synthesis and functions in mast cells. Future Lipidol 3:665–674
149. Marichal T, Tsai M, Galli SJ (2013) Mast cells: potential positive and negative roles in tumor biology. Cancer Immunol Res 1:269–279
150. Johansson A, Rudolfsson S, Hammarsten P, Halin S, Pietras K, Jones J et al (2010) Mast cells are novel independent prognostic markers in prostate cancer and represent a target for therapy. Am J Pathol 177:1031–1041
151. Gulubova M, Vlaykova T (2009) Prognostic significance of mast cell number and microvascular density for the survival of patients with primary colorectal cancer. J Gastroenterol Hepatol 24:1265–1275
152. Strouch MJ, Cheon EC, Salabat MR, Krantz SB, Gounaris E, Melstrom LG et al (2010) Crosstalk between mast cells and pancreatic cancer cells contributes to pancreatic tumor progression. Clin Cancer Res 16:2257–2265
153. Toth-Jakatics R, Jimi S, Takebayashi S, Kawamoto N (2000) Cutaneous malignant melanoma: correlation between neovascularization and peritumor accumulation of mast cells overexpressing vascular endothelial growth factor. Hum Pathol 31:955–960
154. Carlini MJ, Dalurzo MC, Lastiri JM, Smith DE, Vasallo BC, Puricelli LI et al (2010) Mast cell phenotypes and microvessels in non-small cell lung cancer and its prognostic significance. Hum Pathol 41:697–705
155. Elpek GO, Gelen T, Aksoy NH, Erdogan A, Dertsiz L, Demircan A et al (2001) The prognostic relevance of angiogenesis and mast cells in squamous cell carcinoma of the oesophagus. J Clin Pathol 54:940–944
156. Iamaroon A, Pongsiriwet S, Jittidecharaks S, Pattanaporn K, Prapayasatok S, Wanachantararak S (2003) Increase of mast cells and tumor angiogenesis in oral squamous cell carcinoma. J Oral Pathol Med 32:195–199
157. Rojas IG, Spencer ML, Martinez A, Maurelia MA, Rudolph MI (2005) Characterization of mast cell subpopulations in lip cancer. J Oral Pathol Med 34:268–273
158. Pittoni P, Tripodo C, Piconese S, Mauri G, Parenza M, Rigoni A et al (2011) Mast cell targeting hampers prostate adenocarcinoma development but promotes the occurrence of highly malignant neuroendocrine cancers. Cancer Res 71:5987–5997

159. Fleischmann A, Schlomm T, Kollermann J, Sekulic N, Huland H, Mirlacher M et al (2009) Immunological microenvironment in prostate cancer: high mast cell densities are associated with favorable tumor characteristics and good prognosis. Prostate 69:976–981
160. Nonomura N, Takayama H, Nishimura K, Oka D, Nakai Y, Shiba M et al (2007) Decreased number of mast cells infiltrating into needle biopsy specimens leads to a better prognosis of prostate cancer. Br J Cancer 97:952–956
161. Jolly PS, Bektas M, Olivera A, Gonzalez-Espinosa C, Proia RL, Rivera J et al (2004) Transactivation of sphingosine-1-phosphate receptors by FcepsilonRI triggering is required for normal mast cell degranulation and chemotaxis. J Exp Med 199:959–970
162. Oskeritzian CA, Alvarez SE, Hait NC, Price MM, Milstien S, Spiegel S (2008) Distinct roles of sphingosine kinases 1 and 2 in human mast-cell functions. Blood 111:4193–4200
163. Fernandez-Pisonero I, Duenas AI, Barreiro O, Montero O, Sanchez-Madrid F, Garcia-Rodriguez C (2012) Lipopolysaccharide and sphingosine-1-phosphate cooperate to induce inflammatory molecules and leukocyte adhesion in endothelial cells. J Immunol 189:5402–5410
164. Campos LS, Rodriguez YI, Leopoldino AM, Hait NC, Lopez Bergami P, Castro MG et al (2016) Filamin a expression negatively regulates sphingosine-1-phosphate-induced NF-kappaB activation in melanoma cells by inhibition of Akt signaling. Mol Cell Biol 36:320–329
165. Maceyka M, Alvarez SE, Milstien S, Spiegel S (2008) Filamin a links sphingosine kinase 1 and sphingosine-1-phosphate receptor 1 at lamellipodia to orchestrate cell migration. Mol Cell Biol 28:5687–5697
166. Leonardi A, Ellinger-Ziegelbauer H, Franzoso G, Brown K, Siebenlist U (2000) Physical and functional interaction of filamin (actin-binding protein-280) and tumor necrosis factor receptor-associated factor 2. J Biol Chem 275:271–278
167. Lee H, Deng J, Kujawski M, Yang C, Liu Y, Herrmann A et al (2010) STAT3-induced S1PR1 expression is crucial for persistent STAT3 activation in tumors. Nat Med 16:1421–1428
168. Snider AJ, Kawamori T, Bradshaw SG, Orr KA, Gilkeson GS, Hannun YA et al (2009) A role for sphingosine kinase 1 in dextran sulfate sodium-induced colitis. FASEB J 23:143–152
169. Pyne NJ, Ohotski J, Bittman R, Pyne S (2014) The role of sphingosine 1-phosphate in inflammation and cancer. Adv Biol Regul 54:121–129
170. Ding X, Chaiteerakij R, Moser CD, Shaleh H, Boakye J, Chen G et al (2016) Antitumor effect of the novel sphingosine kinase 2 inhibitor ABC294640 is enhanced by inhibition of autophagy and by sorafenib in human cholangiocarcinoma cells. Oncotarget 7:20080–20092
171. Alshaker H, Wang Q, Frampton AE, Krell J, Waxman J, Winkler M et al (2015) Sphingosine kinase 1 contributes to leptin-induced STAT3 phosphorylation through IL-6/gp130 transactivation in oestrogen receptor-negative breast cancer. Breast Cancer Res Treat 149:59–67
172. Pettus BJ, Bielawska A, Spiegel S, Roddy P, Hannun YA, Chalfant CE (2003) Ceramide kinase mediates cytokine- and calcium ionophore-induced arachidonic acid release. J Biol Chem 278:38206–38213
173. Li MH, Sanchez T, Milne GL, Morrow JD, Hla T, Ferrer F (2009) S1P/S1P2 signaling induces cyclooxygenase-2 expression in Wilms tumor. J Urol 181:1347–1352
174. Lai WQ, Melendez AJ, Leung BP (2010) Role of sphingosine kinase and sphingosine-1-phosphate in inflammatory arthritis. World J Biol Chem 1:321–326
175. Alvarez SE, Milstien S, Spiegel S (2007) Autocrine and paracrine roles of sphingosine-1-phosphate. Trends Endocrinol Metab 18:300–307
176. Kulakowska A, Zendzian-Piotrowska M, Baranowski M, Kononczuk T, Drozdowski W, Gorski J et al (2010) Intrathecal increase of sphingosine 1-phosphate at early stage multiple sclerosis. Neurosci Lett 477:149–152
177. Ammit AJ, Hastie AT, Edsall LC, Hoffman RK, Amrani Y, Krymskaya VP et al (2001) Sphingosine 1-phosphate modulates human airway smooth muscle cell functions that promote inflammation and airway remodeling in asthma. FASEB J 15:1212–1214
178. Mechtcheriakova D, Wlachos A, Sobanov J, Kopp T, Reuschel R, Bornancin F et al (2007) Sphingosine 1-phosphate phosphatase 2 is induced during inflammatory responses. Cell Signal 19:748–760
179. Seo EY, Park GT, Lee KM, Kim JA, Lee JH, Yang JM (2006) Identification of the target genes of atopic dermatitis by real-time PCR. J Invest Dermatol 126:1187–1189
180. Baumer W, Rossbach K, Mischke R, Reines I, Langbein-Detsch I, Luth A et al (2011) Decreased concentration and enhanced metabolism of sphingosine-1-phosphate in lesional skin of dogs with atopic dermatitis: disturbed sphingosine-1-phosphate homeostasis in atopic dermatitis. J Invest Dermatol 131:266–268
181. Reines I, Kietzmann M, Mischke R, Tschernig T, Luth A, Kleuser B et al (2009) Topical application of sphingosine-1-phosphate and FTY720 attenuate allergic contact dermatitis reaction through inhibition of dendritic cell migration. J Invest Dermatol 129:1954–1962
182. Schaper K, Dickhaut J, Japtok L, Kietzmann M, Mischke R, Kleuser B et al (2013) Sphingosine-1-phosphate exhibits anti-proliferative and anti-inflammatory effects in mouse models of psoriasis. J Dermatol Sci 71:29–36
183. Japtok L, Schaper K, Baumer W, Radeke HH, Jeong SK, Kleuser B (2012) Sphingosine 1-phosphate modulates antigen capture by murine Langerhans

cells via the S1P2 receptor subtype. PLoS One 7:e49427
184. Idzko M, Hammad H, van Nimwegen M, Kool M, Muller T, Soullie T et al (2006) Local application of FTY720 to the lung abrogates experimental asthma by altering dendritic cell function. J Clin Invest 116:2935–2944
185. Blaho VA, Galvani S, Engelbrecht E, Liu C, Swendeman SL, Kono M et al (2015) HDL-bound sphingosine-1-phosphate restrains lymphopoiesis and neuroinflammation. Nature 523:342–346
186. Du W, Takuwa N, Yoshioka K, Okamoto Y, Gonda K, Sugihara K et al (2010) S1P(2), the G protein-coupled receptor for sphingosine-1-phosphate, negatively regulates tumor angiogenesis and tumor growth in vivo in mice. Cancer Res 70:772–781
187. Albinet V, Bats ML, Huwiler A, Rochaix P, Chevreau C, Segui B et al (2014) Dual role of sphingosine kinase-1 in promoting the differentiation of dermal fibroblasts and the dissemination of melanoma cells. Oncogene 33:3364–3373
188. Beach JA, Aspuria PJ, Cheon DJ, Lawrenson K, Agadjanian H, Walsh CS et al (2016) Sphingosine kinase 1 is required for TGF-beta mediated fibroblastto- myofibroblast differentiation in ovarian cancer. Oncotarget 7:4167–4182
189. Bi Y, Li J, Ji B, Kang N, Yang L, Simonetto DA et al (2014) Sphingosine-1-phosphate mediates a reciprocal signaling pathway between stellate cells and cancer cells that promotes pancreatic cancer growth. Am J Pathol 184:2791–2802
190. Ponnusamy S, Selvam SP, Mehrotra S, Kawamori T, Snider AJ, Obeid LM et al (2012) Communication between host organism and cancer cells is transduced by systemic sphingosine kinase 1/sphingosine 1-phosphate signalling to regulate tumour metastasis. EMBO Mol Med 4:761–775
191. El Buri A, Adams DR, Smith D, Tate RJ, Mullin M, Pyne S et al (2018) The sphingosine 1-phosphate receptor 2 is shed in exosomes from breast cancer cells and is N-terminally processed to a short constitutively active form that promotes extracellular signal regulated kinase activation and DNA synthesis in fibroblasts. Oncotarget 9:29453–29467
192. Yamamoto S, Yako Y, Fujioka Y, Kajita M, Kameyama T, Kon S et al (2016) A role of the sphingosine-1-phosphate (S1P)-S1P receptor 2 pathway in epithelial defense against cancer (EDAC). Mol Biol Cell 27:491–499
193. Muz B, de la Puente P, Azab F, Azab AK (2015) The role of hypoxia in cancer progression, angiogenesis, metastasis, and resistance to therapy. Hypoxia (Auckl) 3:83–92
194. Ahmad M, Long JS, Pyne NJ, Pyne S (2006) The effect of hypoxia on lipid phosphate receptor and sphingosine kinase expression and mitogen-activated protein kinase signaling in human pulmonary smooth muscle cells. Prostaglandins Other Lipid Mediat 79:278–286
195. Schwalm S, Doll F, Romer I, Bubnova S, Pfeilschifter J, Huwiler A (2008) Sphingosine kinase-1 is a hypoxia-regulated gene that stimulates migration of human endothelial cells. Biochem Biophys Res Commun 368:1020–1025
196. Bouquerel P, Gstalder C, Muller D, Laurent J, Brizuela L, Sabbadini RA et al (2016) Essential role for SphK1/S1P signaling to regulate hypoxia-inducible factor 2alpha expression and activity in cancer. Oncogene 5:e209
197. Ader I, Brizuela L, Bouquerel P, Malavaud B, Cuvillier O (2008) Sphingosine kinase 1: a new modulator of hypoxia inducible factor 1alpha during hypoxia in human cancer cells. Cancer Res 68:8635–8642
198. Michaud MD, Robitaille GA, Gratton JP, Richard DE (2009) Sphingosine-1-phosphate: a novel non-hypoxic activator of hypoxia-inducible factor-1 in vascular cells. Arterioscler Thromb Vasc Biol 29:902–908
199. Schnitzer SE, Weigert A, Zhou J, Brune B (2009) Hypoxia enhances sphingosine kinase 2 activity and provokes sphingosine-1-phosphate-mediated chemoresistance in A549 lung cancer cells. Mol Cancer Res 7:393–401
200. Anelli V, Gault CR, Cheng AB, Obeid LM (2008) Sphingosine kinase 1 is up-regulated during hypoxia in U87MG glioma cells. Role of hypoxia-inducible factors 1 and 2. J Biol Chem 283:3365–3375
201. Herr B, Zhou J, Werno C, Menrad H, Namgaladze D, Weigert A et al (2009) The supernatant of apoptotic cells causes transcriptional activation of hypoxia-inducible factor-1alpha in macrophages via sphingosine-1-phosphate and transforming growth factor-beta. Blood 114:2140–2148
202. Williams PA, Stilhano RS, To VP, Tran L, Wong K, Silva EA (2015) Hypoxia augments outgrowth endothelial cell (OEC) sprouting and directed migration in response to sphingosine-1-phosphate (S1P). PLoS One 10:e0123437
203. Baek DJ, MacRitchie N, Anthony NG, Mackay SP, Pyne S, Pyne NJ et al (2013) Structure-activity relationships and molecular modeling of sphingosine kinase inhibitors. J Med Chem 56:9310–9327
204. Loveridge C, Tonelli F, Leclercq T, Lim KG, Long JS, Berdyshev E et al (2010) The sphingosine kinase 1 inhibitor 2-(p-hydroxyanilino)-4-(p-chlorophenyl) thiazole induces proteasomal degradation of sphingosine kinase 1 in mammalian cells. J Biol Chem 285:38841–38852
205. Byun HS, Pyne S, Macritchie N, Pyne NJ, Bittman R (2013) Novel sphingosine-containing analogues selectively inhibit sphingosine kinase (SK) isozymes, induce SK1 proteasomal degradation and reduce DNA synthesis in human pulmonary arterial smooth muscle cells. Medchemcomm 4. https://doi.org/10.1039/C3MD00201B
206. Igarashi Y, Hakomori S, Toyokuni T, Dean B, Fujita S, Sugimoto M et al (1989) Effect of chemically well-defined sphingosine and its N-methyl deriva-

tives on protein kinase C and src kinase activities. Biochemistry 28:6796–6800
207. Sugiura M, Kono K, Liu H, Shimizugawa T, Minekura H, Spiegel S et al (2002) Ceramide kinase, a novel lipid kinase. Molecular cloning and functional characterization. J Biol Chem 277:23294–23300
208. Endo K, Igarashi Y, Nisar M, Zhou QH, Hakomori S (1991) Cell membrane signaling as target in cancer therapy: inhibitory effect of N,N-dimethyl and N,N,N-trimethyl sphingosine derivatives on in vitro and in vivo growth of human tumor cells in nude mice. Cancer Res 51:1613–1618
209. Okoshi H, Hakomori S, Nisar M, Zhou QH, Kimura S, Tashiro K et al (1991) Cell membrane signaling as target in cancer therapy. II: inhibitory effect of N,N,N-trimethylsphingosine on metastatic potential of murine B16 melanoma cell line through blocking of tumor cell-dependent platelet aggregation. Cancer Res 51:6019–6024
210. Kapitonov D, Allegood JC, Mitchell C, Hait NC, Almenara JA, Adams JK et al (2009) Targeting sphingosine kinase 1 inhibits Akt signaling, induces apoptosis, and suppresses growth of human glioblastoma cells and xenografts. Cancer Res 69:6915–6923
211. Shirahama T, Sweeney EA, Sakakura C, Singhal AK, Nishiyama K, Akiyama S et al (1997) In vitro and in vivo induction of apoptosis by sphingosine and N, N-dimethylsphingosine in human epidermoid carcinoma KB-3-1 and its multidrug-resistant cells. Clin Cancer Res 3:257–264
212. French KJ, Upson JJ, Keller SN, Zhuang Y, Yun JK, Smith CD (2006) Antitumor activity of sphingosine kinase inhibitors. J Pharmacol Exp Ther 318:596–603
213. Kono K, Tanaka M, Ogita T, Kohama T (2000) Characterization of B-5354c, a new sphingosine kinase inhibitor, produced by a marine bacterium. J Antibiot (Tokyo) 53:759–764
214. Kono K, Tanaka M, Ono Y, Hosoya T, Ogita T, Kohama T (2001) S-15183a and b, new sphingosine kinase inhibitors, produced by a fungus. J Antibiot (Tokyo) 54:415–420
215. Schwartz GK, Ward D, Saltz L, Casper ES, Spiess T, Mullen E et al (1997) A pilot clinical/pharmacological study of the protein kinase C-specific inhibitor safingol alone and in combination with doxorubicin. Clin Cancer Res 3:537–543
216. Britten CD, Garrett-Mayer E, Chin SH, Shirai K, Ogretmen B, Bentz TA et al (2017) A phase I study of ABC294640, a first-in-class sphingosine kinase-2 inhibitor, in patients with advanced solid tumors. Clin Cancer Res 23:4642–4650
217. Gamble JR, Xia P, Hahn CN, Drew JJ, Drogemuller CJ, Brown D et al (2006) Phenoxodiol, an experimental anticancer drug, shows potent antiangiogenic properties in addition to its antitumour effects. Int J Cancer 118:2412–2420
218. Miyamoto M, Takano M, Aoyama T, Soyama H, Ishibashi H, Kato K et al (2018) Phenoxodiol increases cisplatin sensitivity in ovarian clear cancer cells through XIAP down-regulation and autophagy inhibition. Anticancer Res 38:301–306
219. Kelly MG, Mor G, Husband A, O'Malley DM, Baker L, Azodi M et al (2011) Phase II evaluation of phenoxodiol in combination with cisplatin or paclitaxel in women with platinum/taxane-refractory/resistant epithelial ovarian, fallopian tube, or primary peritoneal cancers. Int J Gynecol Cancer 21:633–639
220. McPherson RA, Galettis PT, de Souza PL (2009) Enhancement of the activity of phenoxodiol by cisplatin in prostate cancer cells. Br J Cancer 100:649–655
221. Zhang L, Wang X, Bullock AJ, Callea M, Shah H, Song J et al (2015) Anti-S1P antibody as a novel therapeutic strategy for VEGFR TKI-resistant renal cancer. Clin Cancer Res 21:1925–1934
222. Ader I, Gstalder C, Bouquerel P, Golzio M, Andrieu G, Zalvidea S et al (2015) Neutralizing S1P inhibits intratumoral hypoxia, induces vascular remodelling and sensitizes to chemotherapy in prostate cancer. Oncotarget 6:13803–13821
223. O'Brien N, Jones ST, Williams DG, Cunningham HB, Moreno K, Visentin B et al (2009) Production and characterization of monoclonal anti-sphingosine-1-phosphate antibodies. J Lipid Res 50:2245–2257
224. Pal SK, Drabkin HA, Reeves JA, Hainsworth JD, Hazel SE, Paggiarino DA et al (2017) A phase 2 study of the sphingosine-1-phosphate antibody sonepcizumab in patients with metastatic renal cell carcinoma. Cancer 123:576–582
225. Purschke WG, Hoehlig K, Buchner K, Zboralski D, Schwoebel F, Vater A et al (2014) Identification and characterization of a mirror-image oligonucleotide that binds and neutralizes sphingosine 1-phosphate, a central mediator of angiogenesis. Biochem J 462:153–162

8 CD200-CD200R Pathway in the Regulation of Tumor Immune Microenvironment and Immunotherapy

Jin-Qing Liu, Aiyan Hu, Jianmin Zhu, Jianyu Yu, Fatemeh Talebian, and Xue-Feng Bai

Abstract

Tumor-associated inflammation and immune responses are key components in the tumor microenvironment (TME) which regulate tumor growth, progression, and metastasis. Tumor-associated myeloid cells (TAMCs) are a group of cells that play multiple key roles including induction of tumor-associated inflammation/angiogenesis and regulation of tumor-specific T-cell responses. Thus, identification and characterization of key pathways that can regulate TAMCs are of critical importance for developing cancer immunotherapy. Recent studies suggest that CD200-CD200 receptor (CD200R) interaction may be important in regulating the TME via affecting TAMCs. In this chapter, we will give a brief overview of the CD200-CD200R axis, including the biology behind CD200-CD200R interaction and the role(s) it plays in tumor microenvironment and tumor growth, and activation/effector functions of T cells. We will also discuss CD200-CD200R's role as potential checkpoint molecules for cancer immunotherapy. Further investigation of the CD200-CD200R pathway will not only advance our understanding of tumor pathogenesis and immunity but also provide the rationale for CD200-CD200R-targeted immunotherapy of human cancer.

Keywords

CD200 · CD200 receptor · Tumor microenvironment · Tumor immunity · Tumor-associated myeloid cells (TAMCs) · Tumor-associated macrophages (TAMs) · Myeloid derived suppressor cells (MDSCs) · Regulatory T cells (Tregs) · Dendritic cells (DCs) · Cytotoxic T lymphocytes (CTLs) · Immunotherapy

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8.1 Introduction

Tumor-associated inflammation and immune responses are major contributors in regulating tumor growth and progression and establishing a tumor microenvironment (TME) [1]. Tumor-associated myeloid cells (TAMCs) are a group of cells that play key roles in inducing tumor-associated inflammation/angiogenesis [2, 3], activating tumor invasion/metastasis [4, 5], and regulating tumor-specific T-cell responses [6]. Therefore, to better understand cancer pathogenesis and pave the way for developing effective cancer immune therapy, identification and characterization of key pathways that regulate TAMCs in the TME are of critical importance. In this regard, accumulating evidence [7–9] suggests that CD200-CD200 receptor (CD200R) interaction may be important in regulating the TME. In the past decade, reports suggesting an association between CD200-CD200R pathway and prognosis in human cancer patients [10, 11] have caused an explosion of interest in these molecules and their interactions. Today, clinical trials of patients with advanced cancer are underway based on blockade of this pathway using antibodies [12]. In this chapter, we will give a brief overview of the biological aspects of the CD200-CD200R axis and its role in tumor microenvironment, tumor growth, T-cell activation, and effector functions and its potential role as "checkpoint molecules" for cancer immunotherapy.

8.2 The Biology of CD200-CD200R Axis

CD200 (also known as OX-2) is a member of the Ig super family (IgSF) of proteins and shares structural similarities with the B7 family of proteins (Fig. 8.1). It contains two extracellular immunoglobulin domains and a small 19aa intracellular domain with no known signaling motif [13]. CD200 is expressed in a variety of normal tissues including B and activated T lymphocytes [14–18]. Recent studies have revealed that CD200 is also overexpressed in a variety of human cancer cells including human melanoma [19], ovarian cancer [20], myeloid leukemia [11], some B-cell malignancies [10], and a majority of endocrine malignancies such as small cell lung carcinoma [21]. CD200R, the cognate ligand for CD200, is also an IgSF protein [22]. The expression pattern of mouse and human CD200R is similar, with strong expression in macrophages, neutrophils, and mast cells [23]. Unlike most of the IgSF receptors, CD200R lacks ITIM domains [24]. However, its 67 AA cytoplasmic tail contains three tyrosine residues, and the third tyrosine residue is located within an *NPXY* motif, which is phosphorylated upon ligation of the CD200R [25]. This leads to the recruitment and phosphorylation of Dok-2 and 1, which then bind to RasGAP and SHIP [25–27]. In macrophages and mast cells, this cascade has been shown to inhibit the phosphorylation of ERK, P38, and JNK [26], and the activation of myeloid cells [28]. CD200R signaling in macrophage appears to limit autoimmune inflammation in animal models of multiple sclerosis, arthritis [29], and lung injury caused by viral infection [30], as CD200-deficient mice exhibit hyper active macrophages with significant increases in disease severity. Notably, CD200R-deficient mice were more susceptible to arthritis, presumably due to enhanced macrophage functions [31]. These findings suggest that CD200-CD200R pathway is mainly involved in regulating the functions of myeloid lineages of cells. Although CD200R expression is mainly found in macrophages and neutrophils, further research revealed lower levels of CD200R expression in dendritic cells (DC) and some subsets of T cells [23, 32, 33], suggesting additional functions for CD200R signaling in regulating these cell types. Some laboratories reported elevated cytotoxic T-cell (CTL) responses in $CD200^{-/-}$ mice infected with influenza virus [30, 34], while other research suggested that autoantigen-specific T-cell responses were normal [29, 31]. To make matters more complicated, certain studies propose that CD200 signaling is required for the induction of T-cell tolerance [35, 36]. Although these studies yielded conflicting results, they all confer CD200-CD200R signaling contributes to T-cell response regulation.

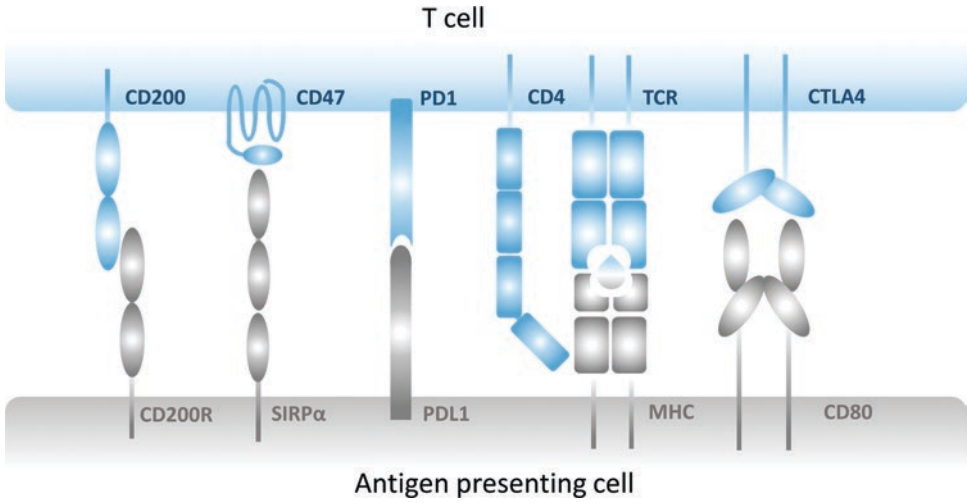

Fig. 8.1 CD200-CD200R axis is considered to be a pair of checkpoint molecules that regulate tumor-specific immune responses. CD200 and CD200R shares similar structures with other important immunoglobulin family members such as CD47-SIRPa, PD1-PD-L1, and CTLA4-B7

8.3 CD200-CD200R Interaction in Tumor Microenvironment and its Impact on Tumor Growth and Progression

In the tumor microenvironment, a number of cell types express CD200 and/or CD200R (Fig. 8.2). CD200 is overexpressed in cancer cells of a variety of human tumors including melanoma [19], ovarian cancer [20], some B-cell malignancies [10], and many endocrine malignancies such as small cell lung carcinoma [21]. Additionally, endothelial cells from tumor blood vessels and activated T, B, and myeloid cells in the TME express significant levels of CD200. TAMCs, including tumor-associated macrophages (TAM), myeloid-derived suppressor cells (MDSC), and tumor-associated dendritic cells (TADCs), are the major lineages of cells expressing CD200R in the TME [7]. Other immune cells such as Tregs also express significant levels of CD200R. The complicated interactions of CD200-CD200R among these cell types can significantly shape TME and affect tumor growth and progression (Fig. 8.2). This can explain the confounding dilemma: Why do studies on the role of CD200 expression in tumors often lead to controversial results?

A human study in 2006 suggested that CD200 mRNA expression in myeloma cells is associated with decreased survival of patients [10]. However, this result was later challenged by another report, which showed that loss of CD200 protein expression on myeloma cells is correlated with a clinically more aggressive disease, characterized by expression of a 70-gene signature [37]. CD200 expression in acute myeloid leukemia (AML) is associated with poor prognosis [11]. However, a more recent study demonstrated that CD200 expression in chronic lymphocytic leukemia (CLL) is actually associated with better prognosis [38]. Similarly, CD200 is associated with tumor grading and metastasis in bladder cancer [39], while in breast cancer, CD200 is mainly present in patients with early-stage breast cancer, which does not favor nodal metastasis [40]. Thus, it appears that tumor CD200 plays differential roles in human cancer depending on the tumor type.

In animal studies, CD200 expression was found in cancer stem cells of basal cell carcinoma and associated with tumor initiation capacity [41] or positively correlated with the metastatic capacity in squamous cell carcinoma [42]. However, these tumor types do not overexpress CD200, and it remains unclear if expression of CD200 on cancer stem cells is responsible for their capacity in tumor

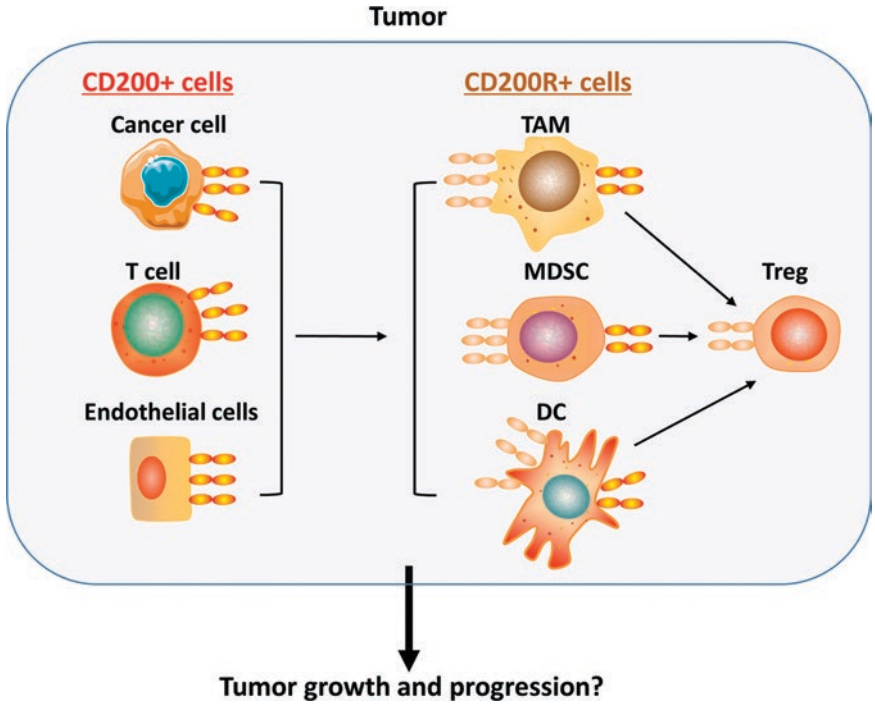

Fig. 8.2 CD200-CD200R interaction in tumor microenvironment. In the TME, CD200 is mainly expressed in some types of cancer cells, activated immune cells such as T cells, and endothelial cells, while CD200R is predominantly expressed in myeloid cells such as TAMs, MDSCs, and DCs. Tregs also express significant levels of CD200R. Interactions among these cell types in TME are likely to determine the outcome of the tumor-associated inflammation and immune response, and subsequently affect tumor growth and metastasis

initiation and metastasis. In $CD200^{-/-}$ mice, scientists observed reduced carcinogen-induced tumor development [43]. In CD200R-deficient mice, decreased growth and metastasis of CD200-positive EMT6 tumors was observed [44]. However, another work showed that 4THM breast tumors exhibit accelerated growth and metastasis in $CD200R^{-/-}$ mice compared to WT mice [45]. In a recent study [9], we found that CD200R-deficient mice exhibit accelerated growth only in CD200-positive B16 tumors (no difference was observed in CD200-negative B16 tumor growth). Strikingly, CD200R-deficient mice receiving CD200-positive B16 cells intravenously exhibited massive tumor growth in multiple organs including liver, lung, kidney, and peritoneal cavity, while the growth of the same tumors in wild-type mice was limited. CD200-positive tumors grown in CD200R-deficient mice contained higher numbers of $CD11b^{+}Ly6C^{+}$ myeloid cells and exhibited increased expression of VEGF and HIF-1α genes with increased angiogenesis. Based on these results, we hypothesize that CD200 expressed on tumor cells mainly interacts with CD200R-positive myeloid cells which inhibits myeloid cell expansion within TME (Fig. 8.3). This model may explain why tumors exhibit accelerated or reduced growth in the absence of CD200-CD200R interaction. Expansion of M2 macrophages and MDSCs will enhance tumor-associated inflammation/angiogenesis [2, 3], leading to tumor invasion/metastasis [4, 5] while also regulating tumor-specific T-cell responses [6]. All these events culminate in enhanced tumor growth. In contrast, expansion of M1 macrophages will lead to tumor growth inhibition due to their direct antitumor effects which include induction of tumor-specific T-cell responses [46]. Although this hypothetical model remains to be tested in more tumor models, current data available in literature does suggest that CD200-CD200R pathway differentially regulates tumor growth and progression in different tumor models, based on the imbalance of inflammation and immunity of various TMEs [33].

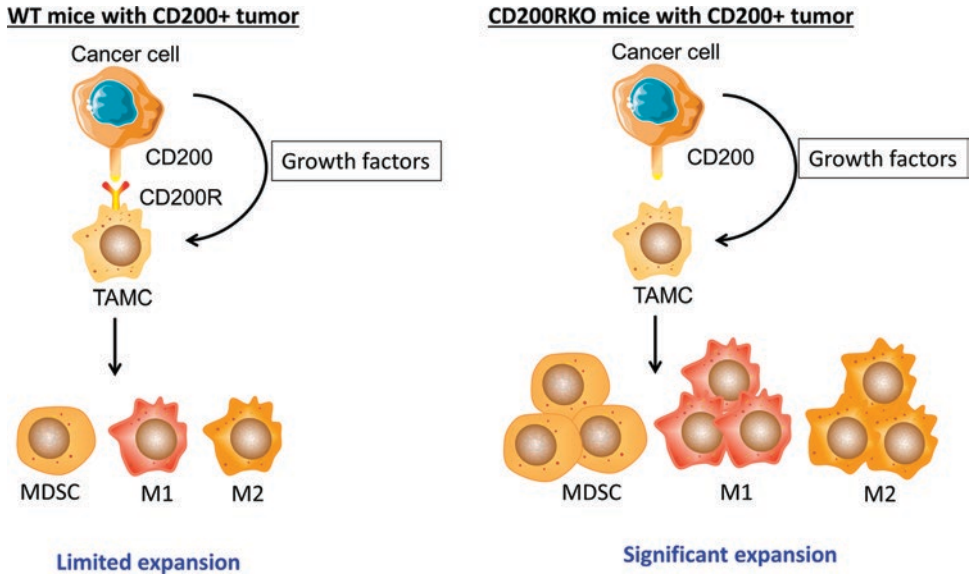

Fig. 8.3 Tumor-expressed CD200 inhibits the expansion of myeloid cells in TME. Cancer cells are known to recruit myeloid cells to the tumor microenvironment through the secretion of myeloid cell growth factors such as M-CSF-1. When the CD200-CD200R axis is intact (left panel), tumor-expressed CD200 can inhibit myeloid cell expansion via interaction with CD200R. In the absence of CD200-CD200R interaction (right panel), significant expansion of myeloid cells occurs. However, the types of myeloid cells that expand may depend on available factors driving myeloid cell differentiation in the tumor microenvironment

In addition to cancer cell-expressed CD200, endothelial cells from tumor vessels express high levels of CD200 [47]. Presently, the significance of endothelial CD200 in tumor growth and progression remains unclear. It is suggested that endothelial cell CD200 is important for immune cell–endothelial cell interactions [48] and suppresses immune cell functions [47]. It is possible that endothelial CD200 may also affect the recruitment of CD200R-positive myeloid cells into tumors, thereby affecting tumor growth and progression. Today we know that some CD200R-positive myeloid cells also express CD200 upon activation [49]. The significance of the CD200-CD200R interactions among these cell types remains to be determined.

8.4 CD200-CD200R Interaction in Regulating Activation and Effector Functions of Tumor-Specific T Cells

Dendritic cells play key roles in the induction of T-cell responses including antitumor T-cell responses. Since CD200R is expressed in DC, some types of cancer cells constitutively express CD200, and activated T cells upregulate CD200; it is expected that CD200-CD200R interaction plays a role in the induction of antitumor T-cell responses. Figure 8.4 outlines the possible checkpoints where CD200-CD200R interaction may regulate DC induction of an antitumor T-cell response. First, in TME, CD200-positive tumor cells or their debris are captured by DCs. This affects uptake of tumor antigen by DCs and subsequently DC differentiation. This process may also affect DC expansion in TME. At this time, no data concerning how CD200-CD200R interaction affects DC uptake of antigen is available. Second, after tumor antigen capture, DCs migrate to lymph nodes and present tumor antigens to T cells. After activation, T cells upregulate CD200, which in turn may affect DC function through CD200R and thereby influence T-cell activation. In this regard, Xiong et al. recently showed that tumor-derived vaccines containing CD200 indeed inhibit T-cell activation [50]. Third, activated T cells (CD200-positive) infiltrate TME, where they are reactivated by DCs. CD200 on T cells may interfere with T-cell reactivation by DC

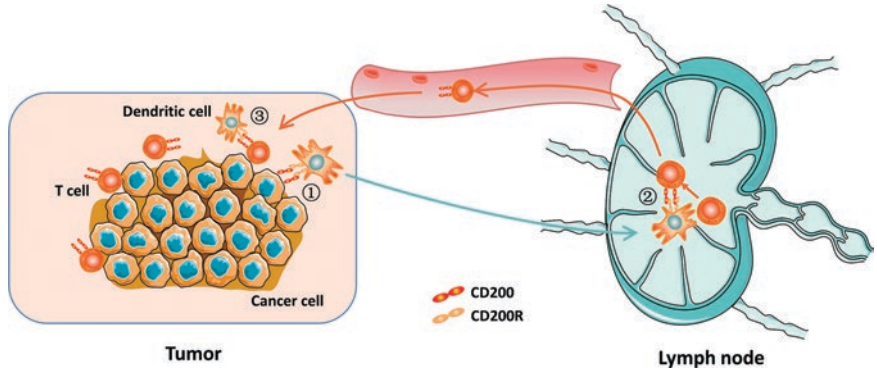

Fig. 8.4 CD200-CD200R interaction and DC induction of antitumor T-cell responses. The following are the checkpoints where CD200-CD200R may play a role during the process: (1) CD200R-positive DCs migrate to TME, where they pick up dead CD200-positive tumor cells or their debris; (2) DCs loaded with tumor antigen meet T cells in lymph nodes and activate them (upregulate CD200); and (3) CD200-positive T cells infiltrate tumors where DCs reactivate them

through interaction with CD200R and thereby affect T-cell effector functions. Whether CD200R in tumor-associated DC regulates their function remains to be investigated. However, in a subset of DC (plasmacytoid DC), CD200R signaling did induce indoleamine 2,3-dioxygenase (IDO), which initiated the immunosuppressive pathway of tryptophan [51]. In the tumor microenvironment, pDC are normally rare, therefore the general significance of this observation remains to be determined.

Based on the model proposed in Fig. 8.4, CD200-CD200R should play a regulatory role in DC-mediated activation of T cells. However, findings regarding the role of CD200-CD200R in T-cell activation and effector function are often controversial. In vitro coculture experiments using allogeneic lymphocytes and CD200-positive cancer cells such as melanoma cells suggest that blockade of CD200-CD200R interaction increases IFN-γ production by T cells [19, 52–54]. In vivo mouse studies demonstrated that in some tumor models, CD200 signal, derived from either tumor cells or host cells, inhibits antitumor immune responses [35, 55–57]. However, we found CD200-positive tumors grown in wild-type mice contained more IFN-γ/TNF-α-secreting tumor-infiltrating T cells [7–9]. On the other hand, we found that CD200-positive B16 tumors grown in CD200R-deficient mice contained much less infiltrated T cells [7]. The discrepancy in results suggests that the role of CD200 in tumor immunity may differ based on tumor types [33]. The current understanding of the role of CD200R signaling in tumor immunity is very limited. Based on the model provided in Fig. 8.2, we suggest that CD200R is predominantly expressed on DC and a group of "immune suppressors" in the TME. We anticipate that signaling from these CD200R expressing cells will affect T-cell response and T-cell effector functions. Eventually, these opposing pro- or anti-signals will determine the specific T-cell response that develops in a particular TME.

We previously tested whether CD200-positive tumors are susceptible to T-cell adoptive transfer therapy. P1CTL cells that recognize tumor antigen P1A were adoptively transferred into mice bearing CD200-positive or CD200-negative J558 tumors. Strikingly, we found that established CD200-positive tumors were often completely rejected by adoptively transferred CTLs, without tumor recurrence. In contrast, CD200-negative tumors were initially rejected by adoptively transferred CTLs, but the majority of tumors recurred due to tumor antigen mutation. Tumor expression of CD200 significantly inhibited suppressive activity and IL-10 production by tumor-associated myeloid cells. As a result, more CTLs accumulated in tumor beds and exhibited a greater capacity to produce IFN-γ in CD200-positive tumors compared to CD200-negative

tumors [7, 8]. Based on these results, we propose a cellular model to explain the mechanism by which CD200-positive tumors respond better to CTL therapy (Fig. 8.5). In this model, tumor-associated myeloid cells serve as tumor growth enhancers. In the absence of CD200-CD200R interaction, tumor cells do not inhibit myeloid cell expansion and function. The freely expanding myeloid cells (when CD200-CD200R expression is absent or inhibited) help establish tumors. In contrast, in the presence of CD200-CD200R interaction, the expansion and functions of myeloid cells are inhibited, thereby failing to help mutated tumor cells in establishing tumors.

8.5 Is Targeting CD200-CD200R Feasible for Cancer Immunotherapy?

Since the CD200-CD200R pathway regulates immune cell functions and shares similarities with other checkpoint molecules, there is a broad interest in manipulating this pathway for cancer therapy. Currently, CD200 blockade is a proposed immunotherapeutic option for CD200-positive human cancers. This therapeutic strategy is based on studies performed in a hu-SCID model, where established tumors are rejected by adoptively transferred peripheral blood mononuclear cells upon CD200 blockade [58–60]. In a phase I study, Samalizumab (an anti-human CD200 Ab) was injected into 23 patients with advanced chronic lymphocytic leukemia (CLL) and 3 patients with multiple myeloma (MM). While the treatment was ineffective in the three MM patients, reduced CD200 expression was observed in CLL cells of treated patients. Notably, antibody-mediated depletion of CD200-expressing $CD4^+$ effector T cells was observed [12]. Since CD200 is broadly expressed in normal tissues, targeting CD200 is difficult and may have potential side effects. Thus, developing non-depleting CD200 antibodies is necessary for CD200 blockade therapy. As an alternative approach, targeting CD200R should be more feasible for treating human cancer due to its limited expression pattern in normal tissues and abundant presence in TME of essentially all types of solid tumors. To determine if enhancing CD200R

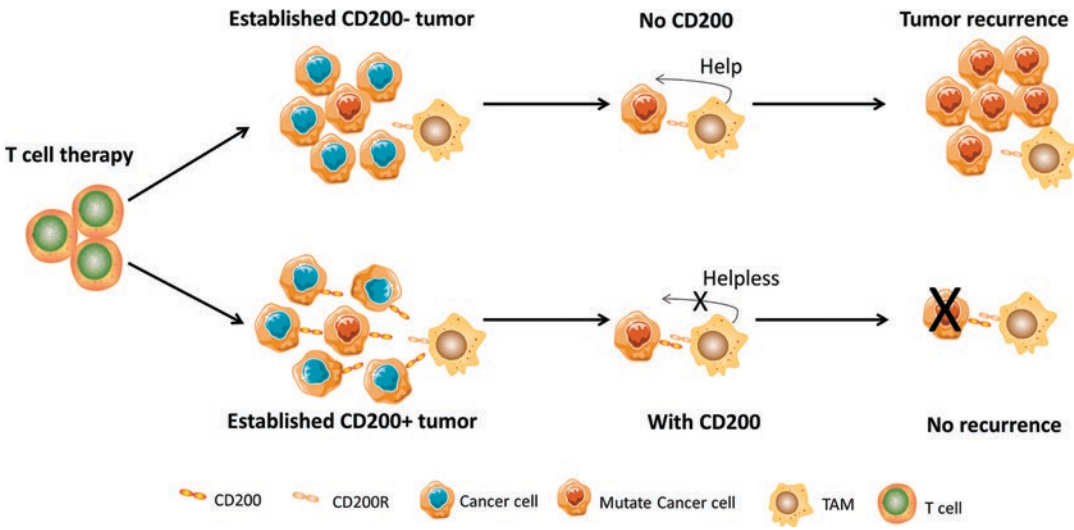

Fig. 8.5 A proposed mechanism of how tumor-expressed CD200 controls tumor evasion of T-cell therapy. Adoptively transferred tumor-specific T cells can destroy both CD200+ and CD200− cancer cells while they fail to eliminate cancer cells that mutate tumor antigen. However, in the presence of tumor CD200 (lower panel), mutated cancer cells cannot grow back to tumor due to lack of help from myeloid cells, leading to tumor rejection. In the absence of tumor CD200 (upper panel), expanded myeloid cells can help mutated tumor cells regrow into a tumor, leading to tumor recurrence

signaling could affect tumor growth, we tested the efficacy of an agonistic anti-CD200R mAb (OX110) [23, 30] in treating lung metastasis of CD200-negative melanoma [8]. We found that OX110 treatment significantly inhibited tumor foci formation in the lungs. Consistent with this study, Pilch et al. recently showed that co-injection of TLR7 agonist and anti-CD200R antibody reshaped TME and induced antitumor myeloid cells in the mouse CT26 colon tumor model [61]. Thus, targeting CD200R rather than CD200 should be a feasible approach for human cancer therapy. Furthermore, the broad expression of CD200 in some types of blood cancer has inspired novel T-cell therapies (an indirect use of the CD200-CD200R axis). For instance, the Greenberg group has designed CD19 CAR-T cells that express CD200R whose intracellular domain is replaced with a CD28 signaling motif [62]. Adoptive transfer of these CD200R manipulated, CD19-targeted CAR-T cells resulted in significant clearance of leukemic cells in treated animals. In the future, this strategy may also be utilized to treat patients with solid tumors that overexpress CD200.

8.6 Concluding Remarks and Future Perspective

Although CD200 and CD200R are considered to be a pair of checkpoint molecules that potentially regulate immune responses and immunotherapy, there are considerable differences between the CD200-CD200R axis and other important checkpoint molecules such as PD-1-PD-L1. For instance, tumor-infiltrating T cells, especially $CD8^+$ T cells, do not normally express CD200R, while tumor-associated myeloid cells are the main cell types that express CD200R. Thus, CD200-CD200R pathway does not directly regulate T cells. It actively affects and regulates the functions of myeloid cells in the TME, thereby indirectly gauging the activation level and effector functions of T cells in the respective environment. At this stage, the signaling events mediated by CD200R in these cell types and their biological effects are not very clear. Further studies on the basic biology of the CD200-CD200R axis in the TME are necessary.

Because of the complicated cellular interactions that may be regulated by CD200-CD200R in the tumor microenvironment (Fig. 8.2), in-depth studies using genetic mouse models are needed to figure out the roles and functions of these cellular interactions in different tumor types. Among such interactions, the role of CD200R signaling in Tregs is a complete mystery. We speculate that CD200-CD200R signaling in TME regulates the homeostasis and functions of Tregs. Similarly, studying the roles of CD200R signaling in tumor-associated DC (Fig. 8.4) is of paramount importance for developing CD200R-based cancer immunotherapy.

For future CD200-CD200R-targeted cancer therapy, careful studies are needed to evaluate what types of cancer patients may benefit. Since CD200-CD200R differs from other checkpoint molecules, blockade of this "checkpoint" may not unleash antitumor immune responses, depending on the tumor microenvironment. We predict that in tumors where M1 type macrophages are dominant, CD200 blockade will be beneficial. Additionally, since CD200 is more broadly expressed in normal tissues, targeting CD200R rather than CD200 is a more feasible approach to therapy of human cancer. Finally, for tumor types that overexpress CD200, CD200R signaling can be manipulated to transduce a positive signal and utilized in T-cell therapy, as exemplified by Greenberg et al. [62].

References

1. Mantovani A, Allavena P, Sica A, Balkwill F (2008) Cancer-related inflammation. Nature 454:436–444
2. Yang L, DeBusk LM, Fukuda K, Fingleton B, Green-Jarvis B, Shyr Y, Matrisian LM, Carbone DP, Lin PC (2004) Expansion of myeloid immune suppressor Gr+CD11b+ cells in tumor-bearing host directly promotes tumor angiogenesis. Cancer Cell 6:409–421
3. Murdoch C, Muthana M, Coffelt SB, Lewis CE (2008) The role of myeloid cells in the promotion of tumour angiogenesis. Nat Rev Cancer 8:618–631
4. Qian BZ, Pollard JW (2010) Macrophage diversity enhances tumor progression and metastasis. Cell 141:39–51

5. Mantovani A, Schioppa T, Porta C, Allavena P, Sica A (2006) Role of tumor-associated macrophages in tumor progression and invasion. Cancer Metastasis Rev 25:315–322
6. Gabrilovich DI, Nagaraj S (2009) Myeloid-derived suppressor cells as regulators of the immune system. Nat Rev Immunol 9:162–174
7. Wang L, Liu JQ, Talebian F, El-Omrani HY, Khattabi M, Yu L, Bai XF (2010) Tumor expression of CD200 inhibits IL-10 production by tumor-associated myeloid cells and prevents tumor immune evasion of CTL therapy. Eur J Immunol 40:2569–2579
8. Talebian F, Liu JQ, Liu Z, Khattabi M, He Y, Ganju R, Bai XF (2012) Melanoma cell expression of CD200 inhibits tumor formation and lung metastasis via inhibition of myeloid cell functions. PLoS One 7:e31442
9. Liu JQ, Talebian F, Wu L, Liu Z, Li MS, Wu L, Zhu J, Markowitz J, Carson WE 3rd, Basu S, Bai XF (2016) A critical role for CD200R Signaling in limiting the growth and metastasis of CD200+ melanoma. J Immunol 197:1489–1497
10. Moreaux J, Hose D, Reme T, Jourdan E, Hundemer M, Legouffe E, Moine P, Bourin P, Moos M, Corre J, Mohler T, De Vos J, Rossi JF, Goldschmidt H, Klein B (2006) CD200 is a new prognostic factor in multiple myeloma. Blood 108:4194–4197
11. Tonks A, Hills R, White P, Rosie B, Mills KI, Burnett AK, Darley RL (2007) CD200 as a prognostic factor in acute myeloid leukaemia. Leukemia 21:566–568
12. Mahadevan D, Lanasa MC, Farber C, Pandey M, Whelden M, Faas SJ, Ulery T, Kukreja A, Li L, Bedrosian CL, Zhang X, Heffner LT (2019) Phase I study of samalizumab in chronic lymphocytic leukemia and multiple myeloma: blockade of the immune checkpoint CD200. J Immunother Cancer 7:227
13. Barclay AN, Clark MJ, McCaughan GW (1986) Neuronal/lymphoid membrane glycoprotein MRC OX-2 is a member of the immunoglobulin superfamily with a light-chain-like structure. Biochem Soc Symp 51:149–157
14. Koning N, Swaab DF, Hoek RM, Huitinga I (2009) Distribution of the immune inhibitory molecules CD200 and CD200R in the normal central nervous system and multiple sclerosis lesions suggests neuron-glia and glia-glia interactions. J Neuropathol Exp Neurol 68:159–167
15. Ragheb R, Abrahams S, Beecroft R, Hu J, Ni J, Ramakrishna V, Yu G, Gorczynski RM (1999) Preparation and functional properties of monoclonal antibodies to human, mouse and rat OX-2. Immunol Lett 68:311–315
16. Dick AD, Broderick C, Forrester JV, Wright GJ (2001) Distribution of OX2 antigen and OX2 receptor within retina. Invest Ophthalmol Vis Sci 42:170–176
17. Rosenblum MD, Olasz EB, Yancey KB, Woodliff JE, Lazarova Z, Gerber KA, Truitt RL (2004) Expression of CD200 on epithelial cells of the murine hair follicle: a role in tissue-specific immune tolerance? J Invest Dermatol 123:880–887
18. Wright GJ, Jones M, Puklavec MJ, Brown MH, Barclay AN (2001) The unusual distribution of the neuronal/lymphoid cell surface CD200 (OX2) glycoprotein is conserved in humans. Immunology 102:173–179
19. Petermann KB, Rozenberg GI, Zedek D, Groben P, McKinnon K, Buehler C, Kim WY, Shields JM, Penland S, Bear JE, Thomas NE, Serody JS, Sharpless NE (2007) CD200 is induced by ERK and is a potential therapeutic target in melanoma. J Clin Invest 117:3922–3929
20. Moreaux J, Veyrune JL, Reme T, De Vos J, Klein B (2008) CD200: a putative therapeutic target in cancer. Biochem Biophys Res Commun 366:117–122
21. Love JE, Thompson K, Kilgore MR, Westerhoff M, Murphy CE, Papanicolau-Sengos A, McCormick KA, Shankaran V, Vandeven N, Miller F, Blom A, Nghiem PT, Kussick SJ (2017) CD200 expression in neuroendocrine neoplasms. Am J Clin Pathol 148:236–242
22. Barclay AN, Wright GJ, Brooke G, Brown MH (2002) CD200 and membrane protein interactions in the control of myeloid cells. Trends Immunol 23:285–290
23. Wright GJ, Cherwinski H, Foster-Cuevas M, Brooke G, Puklavec MJ, Bigler M, Song Y, Jenmalm M, Gorman D, McClanahan T, Liu MR, Brown MH, Sedgwick JD, Phillips JH, Barclay AN (2003) Characterization of the CD200 receptor family in mice and humans and their interactions with CD200. J Immunol 171:3034–3046
24. Zhang S, Cherwinski H, Sedgwick JD, Phillips JH (2004) Molecular mechanisms of CD200 inhibition of mast cell activation. J Immunol 173:6786–6793
25. Mihrshahi R, Barclay AN, Brown MH (2009) Essential roles for Dok2 and RasGAP in CD200 receptor-mediated regulation of human myeloid cells. J Immunol 183:4879–4886
26. Minas K, Liversidge J (2006) Is the CD200/CD200 receptor interaction more than just a myeloid cell inhibitory signal? Crit Rev Immunol 26:213–230
27. Mihrshahi R, Brown MH (2010) Downstream of tyrosine kinase 1 and 2 play opposing roles in CD200 receptor signaling. J Immunol 185:7216–7222
28. Jenmalm MC, Cherwinski H, Bowman EP, Phillips JH, Sedgwick JD (2006) Regulation of myeloid cell function through the CD200 receptor. J Immunol 176:191–199
29. Hoek RM, Ruuls SR, Murphy CA, Wright GJ, Goddard R, Zurawski SM, Blom B, Homola ME, Streit WJ, Brown MH, Barclay AN, Sedgwick JD (2000) Down-regulation of the macrophage lineage through interaction with OX2 (CD200). Science 290:1768–1771
30. Snelgrove RJ, Goulding J, Didierlaurent AM, Lyonga D, Vekaria S, Edwards L, Gwyer E, Sedgwick JD, Barclay AN, Hussell T (2008) A critical function for CD200 in lung immune homeostasis and the severity of influenza infection. Nat Immunol 9:1074–1083
31. Simelyte E, Alzabin S, Boudakov I, Williams R (2010) CD200R1 regulates the severity of arthritis but

has minimal impact on the adaptive immune response. Clin Exp Immunol 162:163–168
32. Rijkers ES, de Ruiter T, Baridi A, Veninga H, Hoek RM, Meyaard L (2008) The inhibitory CD200R is differentially expressed on human and mouse T and B lymphocytes. Mol Immunol 45:1126–1135
33. Rygiel TP, Meyaard L (2012) CD200R signaling in tumor tolerance and inflammation: a tricky balance. Curr Opin Immunol 24:233–238
34. Rygiel TP, Rijkers ES, de Ruiter T, Stolte EH, van der Valk M, Rimmelzwaan GF, Boon L, van Loon AM, Coenjaerts FE, Hoek RM, Tesselaar K, Meyaard L (2009) Lack of CD200 enhances pathological T cell responses during influenza infection. J Immunol 183:1990–1996
35. Rygiel TP, Karnam G, Goverse G, van der Marel AP, Greuter MJ, van Schaarenburg RA, Visser WF, Brenkman AB, Molenaar R, Hoek RM, Mebius RE, Meyaard L (2012) CD200-CD200R signaling suppresses anti-tumor responses independently of CD200 expression on the tumor. Oncogene 31:2979–2988
36. Gorczynski RM, Chen Z, He W, Khatri I, Sun Y, Yu K, Boudakov I (2009) Expression of a CD200 transgene is necessary for induction but not maintenance of tolerance to cardiac and skin allografts. J Immunol 183:1560–1568
37. Alapat D, Coviello-Malle J, Owens R, Qu P, Barlogie B, Shaughnessy JD, Lorsbach RB (2012) Diagnostic usefulness and prognostic impact of CD200 expression in lymphoid malignancies and plasma cell myeloma. Am J Clin Pathol 137:93–100
38. D'Arena G, Valvano L, Vitale C, Coscia M, Statuto T, Bellesi S, Lamorte D, Musto P, Laurenti L, D'Auria F (2019) CD200 and prognosis in chronic lymphocytic leukemia: conflicting results. Leuk Res 83:106169
39. Rexin P, Tauchert A, Hanze J, Heers H, Schmidt A, Hofmann R, Hegele A (2018) The immune checkpoint molecule CD200 is associated with tumor grading and metastasis in bladder cancer. Anticancer Res 38:2749–2754
40. Clark DA, Dhesy-Thind S, Ellis P, Ramsay J (2014) The CD200-tolerance signaling molecule associated with pregnancy success is present in patients with early-stage breast cancer but does not favor nodal metastasis. Am J Reprod Immunol 72:435–439
41. Colmont CS, Benketah A, Reed SH, Hawk NV, Telford WG, Ohyama M, Udey MC, Yee CL, Vogel JC, Patel GK (2013) CD200-expressing human basal cell carcinoma cells initiate tumor growth. Proc Natl Acad Sci U S A 110:1434–1439
42. Stumpfova M, Ratner D, Desciak EB, Eliezri YD, Owens DM (2010) The immunosuppressive surface ligand CD200 augments the metastatic capacity of squamous cell carcinoma. Cancer Res 70:2962–2972
43. Rygiel TP, Karnam G, Goverse G, van der Marel AP, Greuter MJ, van Schaarenburg RA, Visser WF, Brenkman AB, Molenaar R, Hoek RM, Mebius RE, Meyaard L (2012) CD200-CD200R signaling suppresses anti-tumor responses independently of CD200 expression on the tumor. Oncogene 31:2979–2988
44. Podnos A, Clark DA, Erin N, Yu K, Gorczynski RM (2012) Further evidence for a role of tumor CD200 expression in breast cancer metastasis: decreased metastasis in CD200R1KO mice or using CD200-silenced EMT6. Breast Cancer Res Treat 136:117–127
45. Erin N, Podnos A, Tanriover G, Duymus O, Cote E, Khatri I, Gorczynski RM (2015) Bidirectional effect of CD200 on breast cancer development and metastasis, with ultimate outcome determined by tumor aggressiveness and a cancer-induced inflammatory response. Oncogene 34:3860–3870
46. Mantovani A, Marchesi F, Malesci A, Laghi L, Allavena P (2017) Tumour-associated macrophages as treatment targets in oncology. Nat Rev Clin Oncol 14:399–416
47. Belkin DA, Mitsui H, Wang CQ, Gonzalez J, Zhang S, Shah KR, Coats I, Suarez-Farinas M, Krueger JG, Felsen D, Carucci JA (2013) CD200 upregulation in vascular endothelium surrounding cutaneous squamous cell carcinoma. JAMA Dermatol 149:178–186
48. Ko YC, Chien HF, Jiang-Shieh YF, Chang CY, Pai MH, Huang JP, Chen HM, Wu CH (2009) Endothelial CD200 is heterogeneously distributed, regulated and involved in immune cell-endothelium interactions. J Anat 214:183–195
49. Singh A, Falk MK, Hviid TV, Sorensen TL (2013) Increased expression of CD200 on circulating CD11b+ monocytes in patients with neovascular age-related macular degeneration. Ophthalmology 120:1029–1037
50. Xiong Z, Ampudia-Mesias E, Shaver R, Horbinski CM, Moertel CL, Olin MR (2016) Tumor-derived vaccines containing CD200 inhibit immune activation: implications for immunotherapy. Immunotherapy 8:1059–1071
51. Fallarino F, Asselin-Paturel C, Vacca C, Bianchi R, Gizzi S, Fioretti MC, Trinchieri G, Grohmann U, Puccetti P (2004) Murine plasmacytoid dendritic cells initiate the immunosuppressive pathway of tryptophan catabolism in response to CD200 receptor engagement. J Immunol 173:3748–3754
52. Siva A, Xin H, Qin F, Oltean D, Bowdish KS, Kretz-Rommel A (2008) Immune modulation by melanoma and ovarian tumor cells through expression of the immunosuppressive molecule CD200. Cancer Immunol Immunother 57:987–996
53. McWhirter JR, Kretz-Rommel A, Saven A, Maruyama T, Potter KN, Mockridge CI, Ravey EP, Qin F, Bowdish KS (2006) Antibodies selected from combinatorial libraries block a tumor antigen that plays a key role in immunomodulation. Proc Natl Acad Sci U S A 103:1041–1046
54. Wong KK, Khatri I, Shaha S, Spaner DE, Gorczynski RM (2010) The role of CD200 in immunity to B cell lymphoma. J Leukoc Biol 88:361–372
55. Gorczynski RM, Chen Z, Hu J, Kai Y, Lei J (2001) Evidence of a role for CD200 in regulation of immune rejection of leukaemic tumour cells in C57BL/6 mice. Clin Exp Immunol 126:220–229

56. Gorczynski RM, Clark DA, Erin N, Khatri I (2011) Role of CD200 expression in regulation of metastasis of EMT6 tumor cells in mice. Breast Cancer Res Treat 130:49–60
57. Gorczynski RM, Chen Z, Diao J, Khatri I, Wong K, Yu K, Behnke J (2010) Breast cancer cell CD200 expression regulates immune response to EMT6 tumor cells in mice. Breast Cancer Res Treat 123:405–415
58. Kretz-Rommel A, Qin F, Dakappagari N, Ravey EP, McWhirter J, Oltean D, Frederickson S, Maruyama T, Wild MA, Nolan MJ, Wu D, Springhorn J, Bowdish KS (2007) CD200 expression on tumor cells suppresses antitumor immunity: new approaches to cancer immunotherapy. J Immunol 178:5595–5605
59. Kretz-Rommel A, Qin F, Dakappagari N, Cofiell R, Faas SJ, Bowdish KS (2008) Blockade of CD200 in the presence or absence of antibody effector function: implications for anti-CD200 therapy. J Immunol 180:699–705
60. Kretz-Rommel A, Bowdish KS (2008) Rationale for anti-CD200 immunotherapy in B-CLL and other hematologic malignancies: new concepts in blocking immune suppression. Expert Opin Biol Ther 8:5–15
61. Pilch Z, Tonecka K, Braniewska A, Sas Z, Skorzynski M, Boon L, Golab J, Meyaard L, Rygiel TP (2018) Antitumor activity of TLR7 is potentiated by CD200R antibody leading to changes in the tumor microenvironment. Cancer Immunol Res 6:930–940
62. Oda SK, Daman AW, Garcia NM, Wagener F, Schmitt TM, Tan X, Chapuis AG, Greenberg PD (2017) A CD200R-CD28 fusion protein appropriates an inhibitory signal to enhance T-cell function and therapy of murine leukemia. Blood 130:2410–2419

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