Chapter 5

Endothelial and Accessory Cell Interactions in Neuroblastoma Tumor Microenvironment

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1. Introduction

Early childhood tumors that originate from the adrenal medulla and sympathetic nervous system are classified as neuroendocrine tumors [2]. Based on immunohistological criteria, neuroendocrine tumors can be broadly categorized as either neural or epithelial. As the name implies, tumors of the neural subtype display various degrees of neuronal differentiation and they stain positive for the neuroendocrine markers, synaptophysin and chromogranin A [3, 4]. Less well-differentiated or more primitive neural tumors are referred to as neuroblastoma (NB) while tumors with more differentiated features, such as ganglion and nerve bundles, are referred to as ganglioneuroblastoma and ganglioneuroma. This chapter focuses on NB, a form of cancer that occurs in infants and young children. NB is by far the most common cancer in infants, and the fourth most common type of cancer in children [5]. There are approximately 650 new cases each year in the United States, and NB accounts for 15% of all cancer deaths in children. At present, NB patients have limited options for therapy and there is a pressing need to find better treatment options. To develop better treatment options, it is critical to understand the origins of this disease, and mechanisms involved in disease progression. The first section of this chapter is dedicated to a review of neuroendocrine embryology in order to shed some light on the cell that may be responsible for NB. The exact NB progenitor cell has not been identified, however there is evidence that these cells are derived from the neural crest (NC). Understanding the differentiation of NC to cell types that constitute the peripheral nervous system, and the mechanisms utilized during this process is critical to our knowledge of NB

progression. In particular, migration of NC cells along the dorsal-ventral axis of the developing embryo, and the role of matrix in this process is likely to benefit our understanding of mechanisms of cancer metastasis in general. Subsequent sections of this chapter will address NB progression and the many factors (including genetic alterations such as *N-Myc* (*MYCN*) amplification) and cues from the surrounding microenvironment that determine tumor cell proliferation, survival, migration and angiogenesis. The tumor microenvironment is composed of endothelial cells, immune cells, and stromal cells, and, based on their phenotype, either contribute or prevent the progression or metastasis of tumor. We will focus on the contributions of Schwann cells, extracellular matrix, endothelial and immune cells to NB progression and pathogenesis to highlight the intricacies of how the microenvironment affects tumor development.

2. Neuroblastoma developmental mechanisms

Two branching networks that often develop side-by-side during embryonic development include nerves and blood vessels [6]. During embryogenesis, the neural network comprised of both the central nervous system (CNS) and peripheral nervous system (PNS) develops first, and is composed of specialized cells called neurons that relay and transmit signals across different parts of the body [7]. The CNS includes the brain, spinal cord and retina while the PNS consists of sensory neurons, ganglia and the interconnecting nerves that connect to the CNS. Neurons project long cable like cellular extensions called axons that, via electrochemical waves, transmit signals by the release of neurotransmitters at axonal junctions or synapses.

2.1. NB: A peripheral nervous system tumor

NB is a PNS tumor derived from embryonic neural precursor cells. To understand the ontogeny of NB, the development and differentiation of neural precursor cells that are involved in PNS development will be discussed to obtain a better appreciation of the cells, signaling pathways and mechanisms involved in NB. Within the PNS there are somatic and visceral neurons. The somatic neurons innervate skin, bone joints and muscles, and their cell bodies often lie in the dorsal root ganglia of the spinal cord. The visceral neurons innervate internal organs, blood vessels and glands. The visceral component of the PNS is called the autonomic nervous system (ANS), and consists of two parts: the sympathetic nervous system (SNS) and the parasympathetic nervous system (PSNS). Both the SNS and PSNS often work in complementary but opposite fashions to maintain homeostasis in most organs. Two types of neurons, namely the pre-and post-ganglion, represent the majority of ANS, and are responsible for regulating the function of target organs. The pre-ganglionic neurons of the SNS are short while those of the PSNS are long. As a general principle, neurotransmitters are secreted at a synapse that usually occurs at the junction of two axons emerging from two neurons. One exception to this rule is observed in the chromaffin cells of the adrenal medulla. These neuroendocrine cells do not possess axons and directly release neurotransmitters (catecholamines, noradrenalin, adrenaline) into systemic circulation thereby affecting multiple organs. The chromaffin cells play an important role in the fight-or-flight response and are found in small numbers in structures

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such as the carotid aorta, vagus nerve, bladder and prostate in addition to the adrenal medulla. The origin of these chromaffin cells has been attributed to a common precursor population called sympathoadrenal (SA) cells that give rise to both sympathetic neurons and chromaffin cells. Because NB is often associated with the SA cell or its progenitors [8], the development of these cells in embryogenesis provides clues to the disease inception and progression. During early PNS development there are three overlapping stages in which NBs could arise [9]. These are (1) the formation and fate specification of NC into sympathoadrenal (SA) progenitors, (2) bilateral migration and differentiation of SA cells and their coalescence near the aorta, and (3) differentiation of PNS neurons into fully developed ganglia and the establishment of synaptic connections.

2.2. Signaling mechanisms guiding neural crest development

Since chromaffin cells and neurons of the SA system arise from neural crest (NC) cells, it has been proposed that NC cells may be the origin for NB. The NC is a transient embryonic population of cells that arise from the dorsal region of the newly formed neural tube [10]. NC cells undergo epithelial-to-mesenchymal (EMT) transition, and begin to migrate through the developing mesenchyme to differentiate into the craniofacial skeleton, melanocytes as well as the SA system.

a. Formation and fate specification of NC into SA progenitors: The NC cells form at the border between neural and non-neural tissue in the vertebrate embryo. As the neural fold elevates, cells induced to become NC are located in the dorsal neural tube. The specification of NC cells is intricately linked to neural induction since these two processes also dictate the neural-non-neural boundary. It is well accepted from evidence in multiple model systems that loss of bone morphogenetic protein (BMP) signaling coincides with neural induction and thereby NC induction. BMP signaling alone is not sufficient for NC induction, as members of the Wnt and fibroblast growth factor (FGF) families have also been associated with NC induction [11]. Studies in mice, chicken, frog and zebrafish have implicated a cascade of transcription factors that confer NC cell identity. These include NC specifier genes such as Slug, Zic5, Sox9, Sox10, FoxD3, c-Myc and AP2 [12]. These factors are expressed in premigratory, and, or early migratory NC cells and are likely involved in the induction and survival of these cells. The differentiation of NC cells into the SA progenitor pathway is poorly understood. Single cell labeling studies in zebrafish [13] support the premise that Neurogenin-2 in pre- and early migrating NC cells promotes the sensory neuron differentiation at the expense of sympathetic neurons [13]. These data imply that the SA lineage is specified at an early migratory stage; however, it is unclear which molecular mechanisms trigger expression of Neurogenin-2 in a subset of NC cells. Cells during this early less differentiated stage could contribute to NB since alteration in the transcriptional signaling cascade may lead to precocious precursor cell proliferation or lack of further differentiation of these cells into the next phase of NC development.

b. Bilateral migration and differentiation of SA cells: Once specified, NC cells must delaminate from the neural tube in order to migrate to their final destination (Figure 1). Delamination is a process of tissue splitting into separate populations regardless of cellular

mechanisms [14, 15]. With reference to NC, delamination is often used interchangeably with epithelial-to-mesenchymal transition. EMT is a series of molecular events that orchestrate changes from an epithelial cell phenotype into a mesenchymal (migratory) phenotype [16]. Several EMT-inducing transcription factors such as Snail, SoxE and Foxd3 function during multiple steps of NC development. In addition, EMT induces changes in junctional proteins such as N-cadherin and cadherin6B. These processes are reminiscent of events during general tumorigenesis whereby cancer cells undergo EMT and lose the ability to adhere to substratum leading to loss of contact-mediated inhibition. Therefore, NC cells provide a relevant model to investigate different aspects of tumorigenesis and metastasis especially with respect to NB.

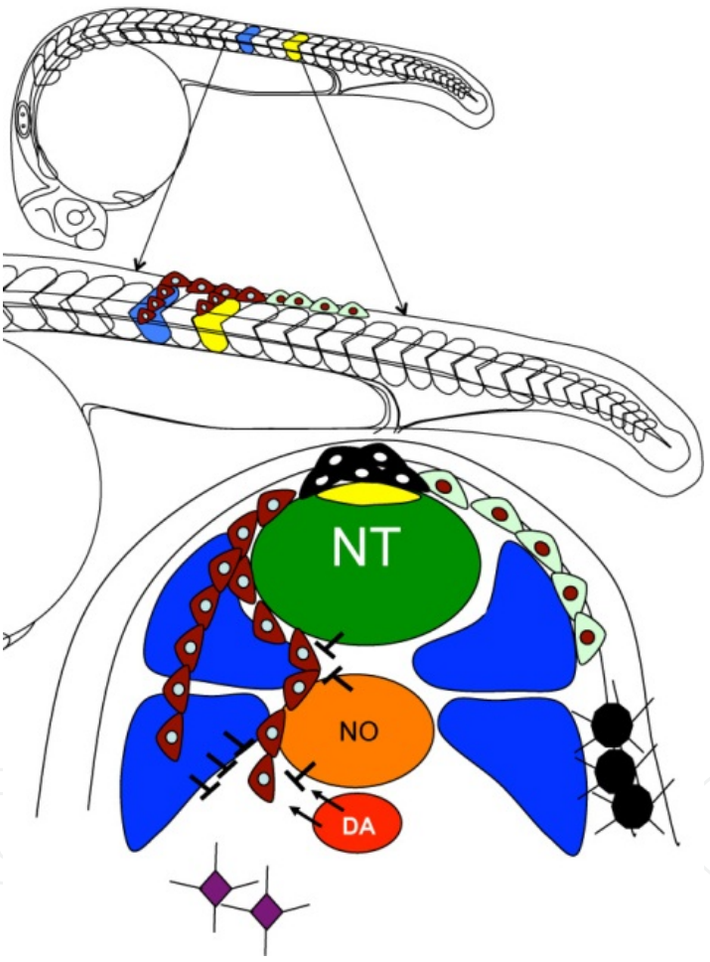

Figure 1. Neural crest cell development in zebrafish. A high power image of the trunk neural crest cell migration is depicted. A cartoon of the cross-section of the trunk region is indicated. Black cells overlying the ectoderm are neural crest cells that specify from the dorsal roof plate (yellow region) of the neural tube (NT). NC cells that migrate dorsoventrally (teal color) will differentiate into melanocytes (black shaped cells). NC cells that migrate ventromedially differentiate into sympathetic ganglia (purple cells). NC cells (red) that migrate through the somite form Schwann cells and sensory neurons and glia of DRG. NC cells that migrate between the somite and the neural tube as indicated differentiate into sympathetic ganglia.and chromaffin cells. Inhibitory signals (inverted T) and activation signals (arrows) guide the migration of the NC cells from the dorsal to ventral region. DA: dorsal aorta, NO: notochord, and blue structures are somites.

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As NC cells delaminate from the neural tube, tissues surrounding the neural tube produce both positive and negative cues that guide NC along defined pathways [17]. Trunk NC migration is guided by signals emerging from adjacent somites [18], which falls under three distinct phases, (1) Directed migration resulting from contact with the ectoderm and cues from the microenvironment, (2) Contact-mediated guidance facilitating homing to the target site, and (3) Contact-inhibition of movement upon entry and colonization of the target site (i.e. the trunk for SA) [15]. These migratory behaviors occur as streams of cells, and once in the trunk, NC cells migrate either ventromedially or dorsolaterally (Figure 1) [8]. NC cells migrating via the ventromedial route (without invading the somite) become neurons and glia of the sympathetic ganglia and adrenal chromaffin cells. NC cells that take the ventromedial route and invade and remain in the sclerotome coalesce to form Schwann cells, and the sensory neurons and glia of the dorsal root ganglia (DRG). We will discuss the role of Schwann cells in NB Pathogenesis later in this chapter. NC cells that take a dorsolateral route, in between the dorsal Ectoderm and the dermamyotome, differentiate into melanocytes (Figure 1) [17]. Because NBs often emerge in the sympathetic ganglia, it is conceivable that during the migratory process, NC cells that carry mutations in critical genes implicated in NBs, such as MYCN, may lose contact inhibition and prematurely proliferate in response to molecular signals that emanate from surrounding tissue. In terms of molecular cues, trunk NC cells that migrate via the ventromedial route enter the somite via attraction cues from CXCR4/CXCL12 signaling, which has also been implicated in breast cancer metastasis [19, 20]. These NC cells are confined to the rostral sclerotome by Neuropilin2/Semaphorin3F repulsion molecules working in concert with Eph/ephrin signalling, F-spondin and proteoglycans, which, reinforce this migration route. Similarly, signaling through CXCR4/CXCl12, ErbB2 and 3/Neuregulin, and GFRá3/artemin mediate NC cell attraction past the somite and work in concert with Neuropilin1/Semaphorin4A repulsion cues from surrounding tissues that restricts NC cell migration to the dorsal aorta [17].

c. Differentiation of PNS neurons into fully developed ganglia: Once the migrated partially differentiated NC cells (SA progenitors) reach the vicinity of dorsal aorta, bone morphogenetic proteins secreted by SA cells trigger a molecular signaling cascade that is essential and sufficient to initiate the differentiation into both the noradrenergic sympathetic neurons and the cholinergic parasympathetic neurons of the ANS. Interestingly, BMP signaling is used at the first (NC induction) and third (NC differentiation into PNS neurons) stage of NC development implying its critical role in this pathway, and perhaps in NB. BMP-2 treatment of human NB cell lines (RTBM1 and SH-SY5Y) leads to growth arrest and differentiation [21]. BMP receptor IA expressed on SA progenitors responds to BMP inducing the expression of the proneural gene mammalian achaete-scute homolog (*Mash-1*) and the paired-like homebox2B (*PHOX2B*) transcription factors. PHOX2B is essential for maintaining Mash-1 expression and proliferation of SA progenitors. Human NB cell lines show high Hash-1 expression, and retinoic acid treatment decreases Hash-1 expression and promotes neurite extension [22, 23]. Germline mutations of *PHOX2B* in both a familial case of NB and in a patient with a genetically determined congenital malformation of NC-derived cells-namely, Hirschsprung disease (HSCR) exemplifies the underlying contribution of late stage genes in NC development to NB pathogenesis [24].

2.3. Model system contribution to neuroblastoma pathogenesis

Much information contributing to the pathogenesis of NB has been generated from in vitro studies performed on cell lines derived from patients [8]. Similar to other tumors, oncogene amplification or allelic loss has been linked to NB progression. Proto-oncogenes v-myc myelocytomatosis viral related oncogene (*MYCN)*, anaplastic lymphoma kinase (*ALK*) and, more recently, transforming tyrosine kinase receptor type A *(TrkA)* [25] have been widely suspected as likely contributors in NB pathogenesis. In fact, *MYCN* amplification is one of the few predictors of a poor clinical outcome for patients with NB. Tumors without *MYCN* amplification that correlate with poor survival frequently show an aberrant up regulation of genes in the *MYC* pathway, and down regulation of genes in the SA lineage differentiation pathway emphasizing the importance of MYC signaling in NB pathogenesis. BMP signal transducers in SA cells, namely transcription factors *PHOX2A* and *PHOX2B*, bind and activate noradrenergic marker genes, tyrosine hydroxylase (TH) and dopamine-β-hydroxylase that are essential enzymes for noradrenaline production. Evidence shows that *MYCN* overexpression under the tyrosine hydroxylase promoter in mice drives tumor formation that resembles human NB [26]. Recently, a dopamine-β-hydroxylase promoter driving *MYCN* and *ALK* genes in zebrafish [27] also resulted in NB formation. In this fish model, the authors demonstrated that upregulated *MYCN* mediated sympathetic neuroblast proliferation, which is eventually mitigated by a developmentally timed apoptosis of neuroblast cells. The concomitant activation of ALK blocks the neuroblast apoptosis at a critical window in development thereby establishing a novel synergistic mechanism for these two oncogenes in NB pathogenesis. These tumor models clearly imply that turning on oncogenes in cells that are undergoing transitions during NC development is at the heart of NB ontogeny and progression.

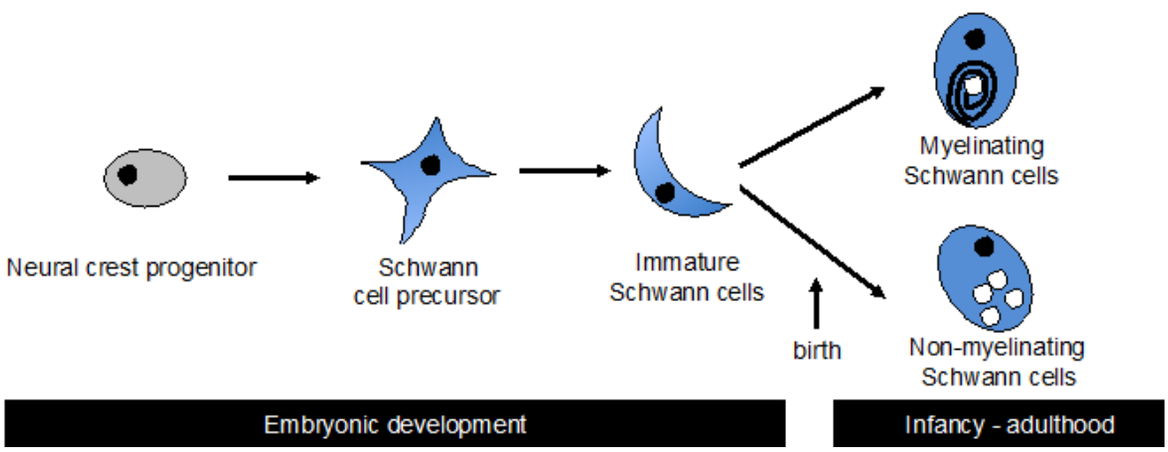

Figure 2. Schwann cell development. Schematic representation of different developmental stages of Schwann cells showing the transitory cell types being involved during embryogenesis and after birth.

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3. Role of Schwann cells in NB tumor microenvironment

NB is characterized by the co-existence of both stromal Schwann cells and neuroblastic tumor cells. The NC origin of Schwann cells suggests that they may co-evolve with tumor cells from common neural progenitors. However, the origin and functional relevance of tumor-associated Schwann cells remain controversial. Although widely assumed to be infiltrating normal Schwann cells, the finding of common genetic alterations shared with neuroblastic tumor cells argues for the same origin as tumor cells. It is well established that stroma-rich NB is associated with differentiated tumors of favorable prognosis. On the contrary, stroma-poor NB is associated with metastatic diseases and poor outcomes. Among the various organs, a high fraction of NB disseminates to the bone and bone marrow. How Schwannian stroma affect tumor dissemination has not been extensively studied. To this end, several soluble factors have been isolated from Schwann cells that have proliferative, survival and angiogenic activities in the tumor microenvironment. These include Chemokine C-X-C motif ligand 13 (CXCL13), Secreted Protein Acidic and Rich in Cysteine (SPARC), and Pigment Epithelium-Derived Factor (PEDF). Determining their roles in NB progression will aid in future development of novel treatments for this childhood malignancy, and will be discussed in detail here.

3.1. Schwann cells in normal development and NB

In the PNS, Schwann cells serve as the major glial cell type for individual neurons. During development, NC progenitors differentiate into multiple lineages including neurons, glial cells, pigment cells, endocrine cells and mesenchymal cells [28]. Based on the hierarchical organization of lineage segregation, NC-derived stem cells (NCSC) first undergo gliogenesis to generate a pool of Schwann cell precursors (SCP)(Figure 2). The helix-loop-helix transcription factor, Sox10, is required for this event by promoting the survival of NCSC and glial cell specification [29-31]. Sox10 also plays an instructive role in determining how NCSC response to neuregulin-1 (NRG-1)[32, 33]. In the PNS, NRG-1 stimulates Schwann cell proliferation, migration, and myelination [34]. NRG-1 also regulates the migratory behavior of NC cells [35, 36].

The maturation of SCP gives rise to immature Schwann cells, which in E15 mouse embryos, encapsulate bundles of axons through a process of radial sorting [37-39]. At this stage the ratio of Schwann cells to axons is 1:1 and this fine balance is partly achieved by axon-driven proliferation of immature Schwann cells. A host of factors are implicated that includes NRG-1 [40], transforming growth factor-β (TGF-β) [41-44], and laminins. Further differentiation of immature Schwann cells generates myelinating Schwann cells which surround large diameter axons, while smaller diameter axons are covered with nonmyelinating Schwann cells [45]. The functional importance of Schwann cells in neuronal survival is well established. For example, mice lacking the NRG-1 receptor, ErbB3, are devoid of SCP and have extensive neuronal cell death in the dorsal root ganglia [46]. Apart from NRG-1, additional trophic factors implicated in neuronal survival include brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), leukemia inhibitory factor (LIF), and hepatocyte growth factor (HGF) [47].

In early childhood, tumors originating from the adrenal medulla and sympathetic nervous system are classified as neuroendocrine tumors. Based on immunohistological criteria, neuroendocrine tumors can be broadly categorized into two types – neural and epithelial. As its name implies, tumors of the neural subtype display various degrees of neuronal differentiation and they stained positive for neuroendocrine markers, synaptophysin and chromogranin A [3, 4]. Less well-differentiated or more primitive neural tumors are referred to as NB while tumors with more differentiated features, such as ganglion and nerve bundles, are referred to as ganglioneuroblastoma (GNB) and ganglioneuroma (GN) (Figure 3A). Overall, GNB and GN show greater immunoreactivities towards the three neurofilament (NF) phosphoisoforms, NF-L (light), NF-M (medium), and NF-H (heavy)[48]. Also, well-differentiated tumors have higher expression of neuronal markers such as microtubule associated proteins (MAPS) and tau. Furthermore, in GNB and GN, both glial cell markers, glial fibrillary acidic protein (GFAP) and myelin basic protein (MBP) are detected, providing evidence of differentiation into non-myelinating and myelinating Schwann cells, respectively [48].

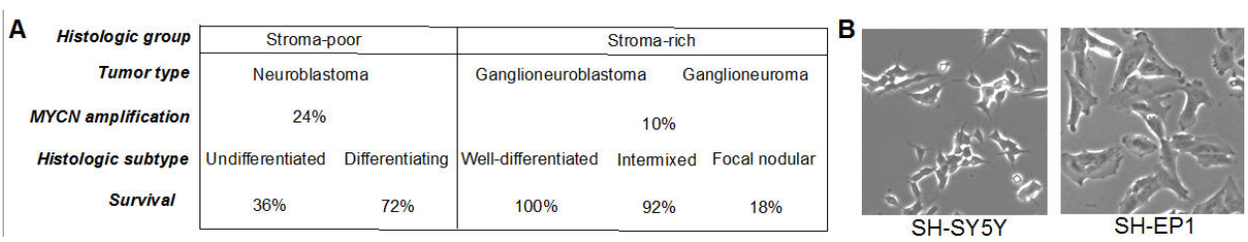

Figure 3. Neural crest tumor typesA. The different histologic groups and subtypes of neural crest tumors with their characteristics depicted [1]. **B.** Brightfield photomicrographs of neuroblastic SH-SY5Y and Schwannian SH-EP1 cell lines (gifts from Dr. Robert A. Ross).

3.2. Histology and origin of Schwannian stroma in NB

The relevance of Schwannian stroma in the diagnosis and prognosis of NB has been addressed by the seminal study by Shimada *et. al.* [1]. In general, NB can be subdivided into stroma-poor and stroma-rich groups. Stroma-poor tumors have diffuse growth patterns of neuroblastic tumor cells divided by thin septa of fibrovascular tissues. This subgroup represents the classical NB and has either an undifferentiated or differentiating histology with various degrees of mitoses and karyorrhexis or nuclear fragmentation (MKI). In general, stroma-poor tumors are considered as favorable if diagnosed <1.5 yr old, with a low MKI and a differentiating histology. This group has a survival rate of 84%. On the contrary, stroma-poor tumors that are unfavorable have a high MKI (for <1.5 yr old), undifferentiated histology (1.5-5 yr old) and occur in patients greater than 5 years of age. This group has a survival rate of only 4.5%.

Tumors of the stroma-rich group have extensive Schwannian stroma and are representative of the GNB and GN histological types. This group can be further classified into three histological subtypes – well-differentiated, intermixed and focal nodular (Figure 3A). The overall survival for stroma-rich tumors is 67% as compared to 47% for stroma-poor counterparts. Expectedly, patients with stroma-rich tumors that are well-differentiated or intermixed have

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90-100% survival survival. Interestingly, patients with the focal nodular subtype has the poorest survival of only 18%. Thus, tumors with good prognosis are the favorable stroma-poor and well-differentiated or intermixed stroma-rich. These tumors have non-advanced staging. In contrast, unfavorable stroma-poor and focal nodular stroma-rich lead topoor prognosis, and they are frequently stage III and IV diseases.

At the molecular level, stroma-poor tumors have a higher frequency (24%) of *MYCN* gene amplification as opposed to 10% for stroma-rich tumors [49, 50]. In this case, the overexpression of *MYCN* most likely leads to the expansion of the NC progenitor population. Indeed, silencing *MYCN* in NB cell lines promotes differentiation [51-53]. *MYCN* expression appears to be differentially regulated in neuroblastic and Schwannian S-type cells. For instance, LA1-55n, a neuroblastic tumor subline, has readily detectable *MYCN* expression while this oncoprotein was not present in the S-type counterpart, LA1-5s [54]. Similarly, ALK mutant protein can be detected in the neuroblastic subline of SK-N-SH while absent in several S-type sublines [55]. Thus, while *MYCN* amplification drives the expansion neuronal progenitors [56], this oncogenic event does not appear to impede differentiation into either neuronal or Schwann cell lineages [57, 58].

The common NC origin of Schwann cells and neurons would argue that Schwannian stroma in NB is derived from a common cancer initiating cell [59]. However, this assertion is not without controversy. An earlier study using cytogenetic analysis of 19 NBs demonstrated that 18 of these tumors displayed near-triploidy while no chromosomal aberrations were detected in Schwann cells [60]. This leads to the conclusion that Schwann cells in tumor stroma are reactive in nature and most likely from infiltrating normal Schwann cells. With the advent of laser-capture microdissection and allelotyping techniques, Mora *et. al.* have demonstrated in 27 of 28 NBs, S100-positive Schwann cells have identical allelic compositions as the neuroblastic tumor cells [59, 61]. Also, Schwannian stromal cells isolated from bone metastases have identical marker chromosomes as neuroblastic tumor cells [62]. Finally, the Schwannian S-type cell line, SH-EP1, harbors a F1174L mutation in the *ALK* gene that is also present in the neuroblastic N-type tumor cell line, SH-SY5Y (author's unpublished results)(Figure 3B). Both cell lines are derived from the widely used SK-N-SH NB cells [63]. All these data provide evidence that Schwann cells are tumor-derived.

3.3. The role of trophic factors in Schwannian stromal and NB pathogenesis

Since Schwannian stroma-rich tumors are associated with favorable prognosis, it is logical to assume that Schwann cells harbor tumor-suppressing properties. To this end, experiments aiming to address the biological relevance of Schwann cells in NB are limited and confined mostly to *in vitro* studies. By co-cultivation experiments, neuroblastic tumor cells have been shown to stimulate the proliferation of Schwann cells [64]. This observation may explain the rapid expansion of Schwannian stromal during NB maturation. In the same study, Schwann cells also promote neurite outgrowth in neuroblastic tumor cells. Similar survival and differentiation promoting activities were also reported when Schwann cell conditioned medium was tested on four NB cell lines [55, 65]. These results are consistent with the differentiated histology associated with stroma-rich tumors. One caveat is that Schwann cells

used in these studies were isolated from normal human peripheral nerves. It will be of interest to compare tumor-derived versus normal Schwann cells in their abilities to promote differentiation and survival of neuroblastic tumor cells. Several trophic factors have been implicated in neuronal homeostasis. These include NGF, BDNF, LIF, and CNTF [66]. The biological effects of conditioned medium mentioned above are most likely the results of a combination of these soluble factors. Clearly defining their specific biological activities, for example, differentiation versus survival may have therapeutic implications. For instance, factors that only promote differentiation but not growth can have therapeutic effects in stroma-poor tumors. Alternatively, targeting the receptors for survival promoting factors such as the TrkB receptor for BDNF may be a plausible treatment strategy [25].

The paracrine effects of trophic factors produced by Schwann cells are not restricted to neuroblastic tumor cells. For instance, three factors secreted by Schwann cells are known to inhibit angiogenesis. These include tissue inhibitor of metalloproteinase-2 (TIMP-2)[67], PEDF [68] and SPARC [69]. TIMP-2 was identified as a potential anti-angiogenic mediator in the conditioned medium of Schwann cells derived from both adult nerves and stroma-rich GN [67]. The negative effects of TIMP-2 on angiogenesis are independent of its ability to inhibit metalloproteinase (MMP) activities [70]. Instead TIMP-2 binds directly to endothelial cells through $\alpha 3\beta 1$ integrin and dampens $\beta 1$-mediated signaling and cell proliferation. PEDF, on the other hand, is a 50 kDa glycoprotein that belongs to the SERPIN family of serine protease inhibitors [71], and it binds to a PLA2/nutrin/patatin-like phospholipase domain-containing 2 (PNPLA2) receptor [72]. PEDF suppresses angiogenesis by inducing apoptosis in endothelial cells, blocking motility and tube formation [73]. In NB, PEDF enhances Schwann cell growth and inhibits basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) induced endothelial cell migration [68]. Consistent with these activities, the least differentiated NB show weak staining for PEDF while high levels are observed in well-differentiated GNB and GN. Finally, SPARC is a matricellular protein implicated in adipogenesis [74]. Surface receptors such as Stabilin-1 and $\alpha 5\beta 3$ integrin have been implicated in mediating SPARC biological activities [75, 76]. Its anti-angiogenic activities are mediated by the direct binding to a host of angiogenic mediators such as VEGF, and platelet-derived growth factor (PDGF)[77, 78]. High levels of SPARC are associated with favorable outcomes in NB [69]. *In vivo* experimental proof further supports the anti-tumorigenic role of Schwannian stroma. Using an NB xenotransplant model, NB cells implanted in sciatic nerve have greater number of infiltrating Schwann cells, more differentiated neuroblasts and reduced vascularity when compared to tumor cells injected outside of the sciatic nerves [79]. All these findings reinforce the notion that the favorable prognosis in stroma-rich NB is the consequence of a host of anti-angiogenic factors produced by the Schwannian stromal compartment.

3.4. Plasticity of Schwannian stroma

During NB progression, there is evidence of dynamic remodeling of the Schwannian tumor microenvironment that involves additional stromal cell types. One such cell type is cancer-associated fibroblasts (CAFs). CAFs are frequently detected in epithelial tumors such as breast carcinomas [80]. CAFs are "reactive" in nature and differ from normal fibroblasts by having

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a more motile or myogenic phenotype [81, 82]. In addition, CAFs also confer a pro-tumorigenic microenvironment by remodeling the extracellular matrices and producing pro-angiogenic and pro-mitogenic trophic factors. In one study, an evaluation of 60 NBs revealed an inverse correlation between the existence of CAFs and Schwannian stroma [83]. In stroma-rich GNs, alpha-smooth muscle actin (α-sma)-positive and h-Caldesmon-negative CAFs are rarely detected. On the contrary, ~90% of stromal cells in Schwannian stroma-poor NB stained positive for CAFs. Indeed, the presence of CAFs in NB is associated with microvascular proliferation. All these findings reiterate the role of Schwann cells in conferring homeostasis in NB tumor microenvironment and this may be achieved by blocking the expansion of CAFs. However, it is unclear how the relative fractions of Schwann cells versus CAFs are being regulated and whether neuroblastic tumor cells can play an instructive role in these events.

One plausible link between Schwann cells and CAFs is the well-established role of bone marrow-derived human mesenchymal stem cells (hMSCs) in the formation of tumor stroma. hMSCs are pluripotent and can differentiate into multiple cell types such as bone, cartilages, and adipose tissues [84]. hMSCs when co-mixed with weakly metastatic breast cancer cells greatly enhance their metastatic potential [85]. Interestingly, hMSCs co-mixed with NB undergo a conversion to a cell type expressing the Schwann cell markers, S100 and Egr-2 [86]. Similarly, prolonged exposure of hMSCs to tumor-derived conditioned media also results in their transition to myofibroblasts [87]. Thus, it is plausible that neuroblastic tumor cells may dictate the composition of tumor stroma by instructing hMSCs to differentiate into either Schwann cells or CAFs. Another interesting aspect of hMSCs in NB is that bone marrow is a common site of metastatic spread [88]. The ability of the chemokine, stromal-derived factor (SDF-1/CXCL12), in bone marrow homing by binding to its receptor, CXCR4, on neuroblastic tumor cells has been reported [89]. Following seeding in the bone marrow, neuroblastic tumor cells may instruct hMSCs to differentiate into Schwann cells, thereby creating a favorable metastatic niche in an otherwise non-permissive environment.

An additional intriguing finding is that Schwann cells isolated from quail sciatic nerves can undergo transdifferentiation into myofibroblasts [90]. In vitro, TGF-β drastically enhanced the conversion of cultured Schwann cells to α-sma$^+$ and sox10$^+$ myofibroblasts. When transplanted into the first branchial arch of E2 chick embryos, these Schwann cells incorporate into the perivascular space of developing vessel walls as α-sma$^+$ cells [90]. Based on these observations, it is tempting to speculate that neuroblastic tumor cells secrete TGF-β to remodel tumor stromal by converting Schwannian-rich to a CAF-rich tumor microenvironment. In summary, this level of plasticity in stromal remodeling may allow tumor cells to adapt to local hypoxic environment or in seeding of metastatic cells.

3.5. The role of Schwannian stromal in NB therapy

From a treatment standpoint, NB in infants has a more favorable prognosis with low-grade tumors that resolved spontaneously. However, the overall survival for patients greater than 4 year old remains around 40%. Also, there are few options once tumors are refractory to conventional chemo- and radiation-therapies. How can studying the role of Schwann cells in

NB can translate into better treatment? As mentioned above, the ability of NB to differentiate into neurons and Schwann cells even in the presence of *MYCN* gene amplification can be explored in the clinical settings. Resident cancer initiating (stem) cells or "intermediate I-type" cell lines such as NUB-7 and BE(2)-C can be differentiated into neurons by retinoic acid (RA) exposure or into Schwann cells by 5-bromo-2'-deoxyuridine (BrdU) exposure [58, 91, 92]. The current standard therapy for high risk NB includes initial induction chemotherapy, followed by autologous hematopoietic stem cell transplantation, and residual disease is treated with a maintenance dose of 13-cis-RA [93, 94]. Under this aggressive treatment regimen, only one-third of patients survived [95]. It will be of interest to test if a combination of RA and BrdU is more effective in differentiating residual NB. In fact, the role of BrdU as a radiosensitizing agent is well established [96, 97].

Another treatment modality is inhibition of angiogenesis. Bevacizumab (Avastin), a humanized monoclonal antibody against VEGF has been shown to enhance the efficacy of topotecan in a NB xenograft model [98]. It has moderate toxicity with overall severe adverse events of 17% [99]. Extensive clinical trial data of Bevacizumab for NB is lacking and its therapeutic efficacy in treating this pediatric tumor is yet to be determined. Nevertheless, the fact that Schwann cells secrete a host of soluble anti-angiogenic factors can be harnessed for therapeutic use. For example, PEDF is effective in blocking growth in a wide variety of tumors [73, 100, 101]. In fact, the delivery of PEDF by adenoviral-mediated gene transfer in NB suppresses angiogenesis and blocks tumor growth [102].

One of the overarching concerns in treatment-resistant high risk NB is the involvement of developmental plasticity inSchwann cells. Indeed, Schwann cells have the capacity to dedifferentiate into less mature progenitors *in vivo* under regenerative conditions. This level of plasticity in Schwann cells has been observed in injured axons wherethis activity requires an active Raf kinase [103]. One scenario is that following intense chemotherapies, while most hyperproliferative neuroblastic tumor cells are expected to be eradicated, residual stromal cells survive and undergo dedifferentiation into neural progenitors to repopulate the primary tumor site. Alternatively, as reported by our group, treatment of the ALK-positive tumor cell line SK-N-SH with an ALK inhibitor leads to the outgrowth of S-type cell populations while N-type cells are mostly eliminated [55]. Conditioned media from these Schwann-like cells confer striking survival toward N-type cells. Thus, tumor-associated Schwann cells or CAFs may provide a chemoresistant niche to support tumor recurrence from the few neuroblastic tumor cells that survive.

In summary, while Schwannian stroma have been considered as a benign byproduct of maturing NB, their presence is intimately linked to the survival and differentiation of neuroblastic tumor cells. The development of transgenic animal models that can recapitulate features of stroma-rich and stroma-poor tumors will be necessary to better understand this interaction. These *in vivo* models will be useful for deciphering the biological effects of Schwannian stroma on tumor cells, the paracrine factors involved and their intracellular signaling. Although Schwannian stroma is an attractive target for NB therapy, the NB tumor stroma/microenvironment, which is composed of the extracellular matrix plays an equally important role in NB pathogenesis, which is discussed next.

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4. Cell-matrix and cell-cell molecular interactions in the neuroblastoma tumor microenvironment

In 1986 Harold F. Dvorak coined the phrase: "Tumors are wounds that never heal". His comment was based on similarities in the content of new blood vessels, lymphocytes, macrophages, and connective tissue components (including cellular and extracellular matrix elements) present in healing wounds and tissue surrounding tumor cells [104]. During tumor (parenchyma) development, the wound repair resolution stage fails, resulting in a microenvironment (stroma) that never "heals". Multiple factors in the "wounded" tumor microenvironment promote NB progression. In this section, we highlight the role of the extracellular matrix (ECM) in this process.

4.1. Biochemical and biophysical cues from the extracellular matrix

4.1.1. ECM stiffness conveys differentiation signals

The NB tumor microenvironment provides biochemical and mechanical signals similar to the microenvironments of other tumor types, but there is specificity in how NB tumor cells respond to these signals. It is well recognized that the interaction of tumor with stroma occurs via biochemical signaling and that the ECM provides a source of signals that instruct cellular behavior. Our understanding of how biomechanical signaling generated by shear stress, compression, and tension affect survival, proliferation, migration, and gene expression is increasing [105]. Changes in tension homeostasis occur in cancer, with breast cancer as one of the best studied examples [106]. Mechanical cues from the ECM may influence retinoic acid-mediated differentiation, which in turn may regulate clinically relevant aspects of NB biology. Recent studies show that ECM stiffness provides a physical cue that reduces NB proliferation and promotes differentiation [107]. Increasing ECM stiffness enhances neurite extension (neuritogenesis) and suppresses cell proliferation. Increased ECM stiffness also reduces expression of the oncogenic *MYCN* transcription factor. Furthermore, the addition of RA enhances ECM stiffness. Together, the data suggest that the mechanical signals from the cellular microenvironment influence NB differentiation in synergy with the RA biochemical differentiation factor [107].

4.1.2. SPARC and cell survival

One of the matrix proteins with a documented role in tumor progression is SPARC (osteonectin or BM-40). SPARC is a 34 kDa calcium-binding glycoprotein shown to associate with the cell membrane and membrane receptors [108, 109]. SPARC appears to have a cancer-type specific effect on tumor metastasis. In prostate cancer, SPARC is linked with increased migration and prostate cancer metastasis to bone. This occurs via activation of integrins $\alpha v\beta 3$ and $\alpha v\beta 5$ expressed on tumor cells [110]. In contrast, SPARC appears to act as a tumor suppressor in NB. This tumor suppressor effect has been studied in the context of radiation therapy. Irradiation of NB tumor cells was shown to inhibit SPARC expression. Interestingly, SPARC expression was significantly decreased in radiation-therapy resistant cancer cells [111]. Exogenous

overexpression of SPARC significantly suppressed the activity of AKT. This suppression was accompanied by an increase in the PTEN tumor suppressor protein both in vitro and in vivo, [112] and sensitized NB cells to radiation by inhibiting irradiation-induced cell cycle arrest. Therefore, SPARC expression restored NB radiosensitivity. In addition to this function, SPARC expressed by NB cells appears to affect endothelial cells in the immediate vicinity. Interestingly, SPARC overexpression and secretion by NB cells induced endothelial cell apoptosis, inhibited angiogenesis and suppressed expression of the pro-angiogenic molecules, VEGF, FGF, PDGF and MMP-9 in endothelial cells. This suppressed expression of growth factors was mediated by inhibition of the Notch signaling pathway [113]. Therefore, promoting SPARC expression may be a plausible anti-NB therapy.

4.2. Role of cell adhesion molecules in NB progression

4.2.1. NCAM and NB progression

Intercellular communication is a fundamental biological property that is regulated during cellular growth and differentiation. In general terms, abnormalities in gap junction intracellular communication (GJIC) and cell-cell adhesion correlate with poor prognosis for cancer treatment [114-117]. Loss of either cell-cell adhesion or GJIC occurs in cancers, and gain of communication or adhesion suppresses tumor growth [118, 119]. Cell adhesion molecules (CAMs) have been reported to regulate tumor progression and metastasis, acting as oncogenes or tumor suppressors [120-122], and one such molecule namely Neural cell adhesion molecule (NCAM) is of particular importance for both, normal brain development [123] and NB regulation. NCAM is the main protein carrier of polysialic acid (polySia), a major regulator of cell-cell interactions in the developing nervous system that is required for neuronal plasticity. Studies in NCAM knockout mice showed that the effects of polySia occur via the expression of NCAM [123]. During normal neuronal differentiation or upon RA induced differentiation of a NB cell line, NCAM appears in non-polysialated form. This allows for its hemophilic interactions, and in turn triggers enhanced ERK signaling and MAPK-dependent neuritogenesis [124]. Therefore, it can be expected that inhibition of polysialation will promote neuronal differentiation and may inhibit NB progression.

4.2.2. N-cadherin and NB progression

Clinical studies suggest that tumor invasiveness, not the ability to detach from the primary tumor are determinants of the progression to metastasis [125]. In epithelial-derived tumors, metastasis is often preceded by the loss of E-cadherin cell-cell adhesion [126, 127]. The loss of E-cadherin is often accompanied by de novo expression of N-cadherin, which promotes cell motility and migration; a phenomenon called “the cadherin switch” [128-130]. Further, N-cadherin homophilic interactions between tumor cells and surrounding tissue such as tumor vessel endothelium and stroma facilitate the transit and survival of tumor cells in distant organs [131-133]. N-cadherin thus may play a role in preventing metastasis in NB through such homotypic and heterotypic cell-cell interactions. In line with this hypothesis, N-cadherins are expressed on various NB tumors and NB cell lines, with lowest levels in patients undergoing metastasis. Therefore, its expression negatively correlates with metastasis [134].

4.2.3. Reelin signaling in NB

Reelin is an extracellular secreted protein of the Cajal-Retzius cells located in the marginal zone of the developing cerebral cortex, and is required for the organization of the cortex into layers of neurons [135]. In the absence of reelin, neurons exhibit a broader and irregular pattern of positioning [136]. Although reelin interacts with integrins and cadherins, signals from reelin are transduced by cell membrane receptors: ApoER2 and Very Low Density Lipoprotein Receptor (VLDLR) and by the intracellular regulatory protein disabled-1 (Dab1) [137, 138]. Downstream signaling involves adapter protein crk and the small GTPase Rap1 [139]. Reelin triggers the activation of Rap1 in migrating cerebral cortical neurons when they are midway through their migration path (from the ventricular zone toward the cortical plate). This activation of Rap1 by reelin is critical for neuronal multipolar polarization and migration along glia, and therefore, normal cerebral cortex organization [140-143]. However, reelin expression is not limited to the normal tissues such as brain, but is also detected in several different tumor pathologies where it has been linked with tumor aggressiveness [144]. A recent study suggests that reelin signaling regulates a migratory switch promoting metastasis in NB [145]. Reelin expression is negatively regulated by miR-128, a brain-enriched microRNA. miR-128 is downregulated in untreated NB patients, and ectopic miR-128 overexpression reduced NB cell motility and invasiveness and impaired cell growth. Furthermore, a small series of primary human NBs showed an association between high levels of miR-128 expression and favorable features, such as a favorable stage score based on the International Neuroblastoma Staging System Classification (Shimada category, [146]). In addition to the autocrine function in differentiating tumors, reelin acts as a chemoattractant for several NB cell lines. It is also expressed in blood vessels in several NB cell lines, but not in normal tissue. Therefore, it is postulated that in addition to the autocrine function, paracrine reelin presented by NB blood vessels may act as a chemoattractant and promote hematogenic and lymphogenic dissemination in NB progression [145].

4.2.4. Gap junctions – Cellular connectivity and suppression of growth

Cell-cell interactions are mediated by specialized connections between membranes of adjacent cells called gap junctions. Gap junctions form by connecting two hemichannels (connexons) on neighboring cells, with each hemichannel comprised of a hexamer of connexin. Of the 20 known connexins, connexin 43 is the most ubiquitously expressed [147]. Gap junctional coupling in NB is negatively regulated by protein kinase C (PKC) [148]. PKC isozymes regulate various aspects of proliferation and PKC inhibitors are under study in clinical trials as potential anti-cancer therapy. Tamoxifen, an estrogen receptor antagonist, exerts some of its anti-tumor effects via PKC signaling [149, 150]. However, the exact cellular mechanisms targeted by PKC inhibitors are not known. Recently, it was shown that inhibition of PKC in NB cell lines increases GJIC via a mechanism that does not depend on the redistribution of connexin 43 or its phosphorylation [148]. Furthermore, PKC inhibition promoted cell-cell adhesion, a finding that suggests that suppression of tumor growth by PKC inhibition may be due to effects on increased GJIC and cell-cell adhesion [148].

Overall, these studies suggest that the extracellular matrix and CAMs play an important role in the biochemical and biophysical regulation of NB. The careful examination of NB environ-

ment-specific cues to fully define their effects on NB tumor progression offers an opportunity for NB targeted therapy.

5. Role of endothelial cells in NB microenvironment pathogenesis

5.1. Role of angiogenesis in NB pathogenesis

In 1962, Dr. Judah Folkman described the seminal observation that tumor angiogenesis is dependent on de novo blood vessel formation [151]. The sprouting of new blood vessels from pre-existing ones is a multi-step process consisting of endothelial cell proliferation, migration and tube formation [152]. Tumor angiogenesis is not only induced by growth factors and cytokines secreted from tumor cells [153], but also modulated by cell-cell interaction [152]. Aberrant angiogenesis is associated with excessive growth-promoting signals and a lack of sufficient "pruning," cues that spatially and temporally modulate vessel growth, remodeling and stabilization [152]. As compared to normal blood vessels, tumor vessels are more dilated and tortuous, form arteriovenous shunts, and lack the normal artery-capillary-vein hierarchy [154]. Tumor vasculature not only provides oxygen and nutrients to promote tumor proliferation and progression, but also facilitates tumor metastatic spreading. Thus, tumor angiogenesis represents an attractive new target for tumor therapy because it is well accepted that new blood vessel formation promotes tumor growth and metastatic spread [152, 155, 156].

In terms of NB, current evidence suggests that advanced and aggressive stages of NB are dependent on angiogenesis [157-159]. Meitar et al [160] demonstrated the association of the tumor angiogenesis and poor outcome in human NB. Like most solid tumors, several well-known pro-angiogenic growth factors such as VEGF-A, VEGF-B, bFGF, angiopoietin-2 (Ang-2), transforming growth factor alpha (TGF-α) and PDGF were found in advanced-stage NB tumors [161]. Human NBs produce extracellular matrix-degrading enzymes, that induce endothelial cell proliferation and are angiogenic in vivo [162]. Integrins $\alpha v\beta 3$ and $\alpha v\beta 5$ are more highly expressed in blood vessels of high-risk versus low-risk NB tumors [163]. In addition, lymphatic vessels are observed in NB [164] with higher expression of the VEGF-C lymphangiogenesis growth factor observed in advanced stage of NB [161]. These evidences suggest that tumor angiogenesis likely contributes to NB pathogenesis.

5.2. Contributions of *MYCN* amplification and trks-mediated signaling pathways to NB tumor angiogenesis

NB is an embryonic tumor that is derived from cells of the primitive NC [165]. In general, genetic abnormalities play a key role in determining tumor phenotype, [165, 166]. *MYCN* amplification is one of the first identified genetic defects in NB, and high levels of *MYCN* are associated with aggressive tumor behavior and poor survival [167]. *MYCN* is member of the MYC family of basic helix-loop-helix transcription factors that control a broad regulatory network implicated in cell cycle, DNA damage response, differentiation and apoptosis [168]. There is evidence that *MYCN* amplification is also associated with tumor angiogenesis. Several studies demonstrated that *MYCN* amplification in NB suppressed the expression of angio-

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genesis inhibitors, such as activin A, interleukin-6 and leukemia inhibitory factor [169, 170]. Activin A represses NB growth, endothelial cell proliferation and angiogenesis [171, 172]. In addition, highly expressed activin A in differentiated NB strongly correlates with a favorable NB outcome [173]. Interestingly, inhibition of PI3K/rapamycin results in the degradation of *MYCN* in NB tumor cells and results in blockage of angiogenesis indirectly [174].

A second gene family implicated in NB is the TRK family of neurotrophin receptors (NTRK) that play critical roles in the development of the CNS and PNS [25, 175]. The 3 characterized members are TrkA (NTRK1), TrkB (NTRK2) and TrkC (NTRK3) with nerve growth factor (NGF), BDNF and neurotrophin-3 (NT-3) as their primary ligands, respectively [175]. The sequential Trk expression is important for complete differentiation of normal sympathetic neurons, and the *Trk* genes expressed reflect the stage of neuronal differentiation [176]. High expression of TrkA and TrkC are associated with the ability for NB to differentiate and spontaneous regress, and are predominately found in clinically favorable NB. One mechanism that could explain this is that high expression of TrkA reduces the expression of angiogenic factors in NB cells and suppresses NB tumor xenograft growth associated with reduced angiogenic factor expression and vascularization of tumors [177]. In contrast, TrkB and its ligand, BDNF, are highly expressed in aggressive NB associated with increased cell survival, angiogenesis and drug resistance [25, 175].

5.3. Anti-angiogenesis treatments in NB - conventional anti-VEGF/VEGFR2 signaling pathways

Although targeting of the tumor vasculature represents a promising tool for cancer therapy, there are no current clinical trials of anti-angiogenesis therapy for NB [157-159]. There are several pre-clinical studies in NB animal models [157-159], and depending on the unique aspects of NB, several different approaches for anti-angiogenesis therapy is feasible. VEGF and its cognate receptor 2 (VEGFR2) are major regulators of angiogenesis. Anti-VEGF/VEGFR2 signaling pathways and inhibition of endothelial cell proliferation and migration are the most common anti-angiogenesis therapeutic approaches. The recently approved anti-angiogenesis drug, bevacizumab (Avastin), is a recombinant monoclonal antibody that binds VEGF-A and subsequently blocks the activation of its receptors. Bevacizumab reduces NB tumor growth by reducing angiogenesis [178]. In addition, treatment with bevacizumab can transiently induce tumor vasculature remodeling allowing for improved delivery and efficacy of chemotherapy in NB tumor xenografts [98]. A VEGFR-2 tyrosine kinase inhibitor Sugen 5416 (SU5416, Semoxinal) is a specific VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1) tyrosine kinase inhibitor that has shown efficacy in inhibiting angiogenesis in vivo models of NB [179]. Efficacy of inhibiting tumor growth was increased when SU5416 was given in combination with irradiation or chemotherapy [180, 181]. In addition to VEGF inhibitors, other angiogenesis inhibitors have shown efficacy on NB tumor angiogenesis and growth, which is discussed in detail elsewhere [157, 159, 182]. TNP-470 is a synthetic analog of fumagillin, an antibiotic isolated from the fungus Aspergillus fumigatus fresenius with antineoplastic activity. TNP-470 is a potent selective inhibitor of Methionine aminopeptidase-2 (MetAP-2) resulting in endothelial cell cycle arrest late in G1 phase and leading to inhibition of tumor angiogenesis [183]. TNP-470

treatment in a NB tumor xenograft model reduced the tumor growth rate and decreased capillary density [184-188], and increased the efficacy of chemotherapy [181]. Taken together, these results suggest that anti-angiogenesis is an effective approach for reducing NB growth and burden. In addition to direct approaches targeting the vasculature in NB, indirect anti-angiogenesis approaches have also shown efficacy in NB. For the most part, these approaches rely on the induction of differentiation of NB. For example, retinoids have been shown to exert their effects by inducing differentiation of NB cells. Retinoids and fenretinide, a synthetic retinoid, have demonstrated anti-angiogenic effects in NB tumor xenografts [189, 190]. The inhibitory effects were mediated by retinoic acid induced expression of thrombospondin-1 (TSP-1) in NB cells. TSP-1 is an important endogenous angiogenesis inhibitor that inhibits endothelial cell proliferation and migration. Interestingly, TSP-1 is silenced in a subset of undifferentiated advanced-stage NB tumors and NB cell lines due to promoter methylation [191]. Remarkably, ABT-510, a peptide derived from TSP-1, suppressed the growth of NB tumor xenografts [192]. In combination with valproic acid, ABT-510 showed potent inhibitory effects on the growth of NB tumor xenografts. Taken together, these results suggest that both direct and indirect approaches of targeting angiogenesis are feasible therapeutic approaches for NB.

6. Molecular and cellular mechanistic interface between endothelial and immune cells in NB

The statement that "tumors are wounds that never heal" [21] has relevance for which phenotype of immune cells are present in the tumor microenvironment, and whether these cells interact to promote or prevent tumor. During the initial stage of wound healing there is an inflammatory response that is produced by an influx of immune cells that release inflammatory mediators. The next stage of tissue remodeling is characterized by a down-regulation of the immune response, cell proliferation, and revascularization of the wound via angiogenesis [193-195]. In the resolution stage of tissue remodeling, cell proliferation is halted and vessels are stabilized. In the tumor microenvironment, there is a perpetual state of inflammation, cell proliferation and angiogenesis similar to an unhealed wound. Chronic hypoxia in the tumor microenvironment is a contributing factor as to why tumors are wounds that never heal. The cellular response to hypoxia is controlled by the expression of hypoxia inducible factors (HIF) [196]. Low oxygen tension prevents the ubiquitination and subsequent proteosomal degradation of HIF-α proteins allowing them to translocate to the nucleus and dimerize with HIF-β forming functional transcription factors (HIF-1α/HIF-β or HIF-2α/HIF-β) that promote up-regulation of angiogenic target genes. There is also evidence that HIF-1α regulates energy homeostasis and plays a role in the differentiation of immune cells that can have pro or anti-tumor effects [197]. Notably, HIF-2α expression is required to maintain an undifferentiated state of NB tumor-initiating cells, and expression of HIF-2α is associated with poor outcome in NB [197, 198]. Hypoxia and chronic inflammation are key characteristics of the tumor microenvironment that promote immune suppression and vascularization. In NB as well as other solid tumors, cytokine and chemokine mediators as well as angiogenic factors influence

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the differentiation state of immune cells which ultimately determines whether or not these cells can be activated to contribute to anti-tumor immunity. This section highlights how immune cells are affected by factors in the tumor microenvironment to become both tolerogenic and pro-angiogenic with an emphasis on the interconnection between angiogenesis and tumor immunity. In addition, future prospects for treating NB with combinations of anti-angiogenic agents and immune-based therapies as a strategy to reverse the immune suppression in the tumor microenvironment is discussed.

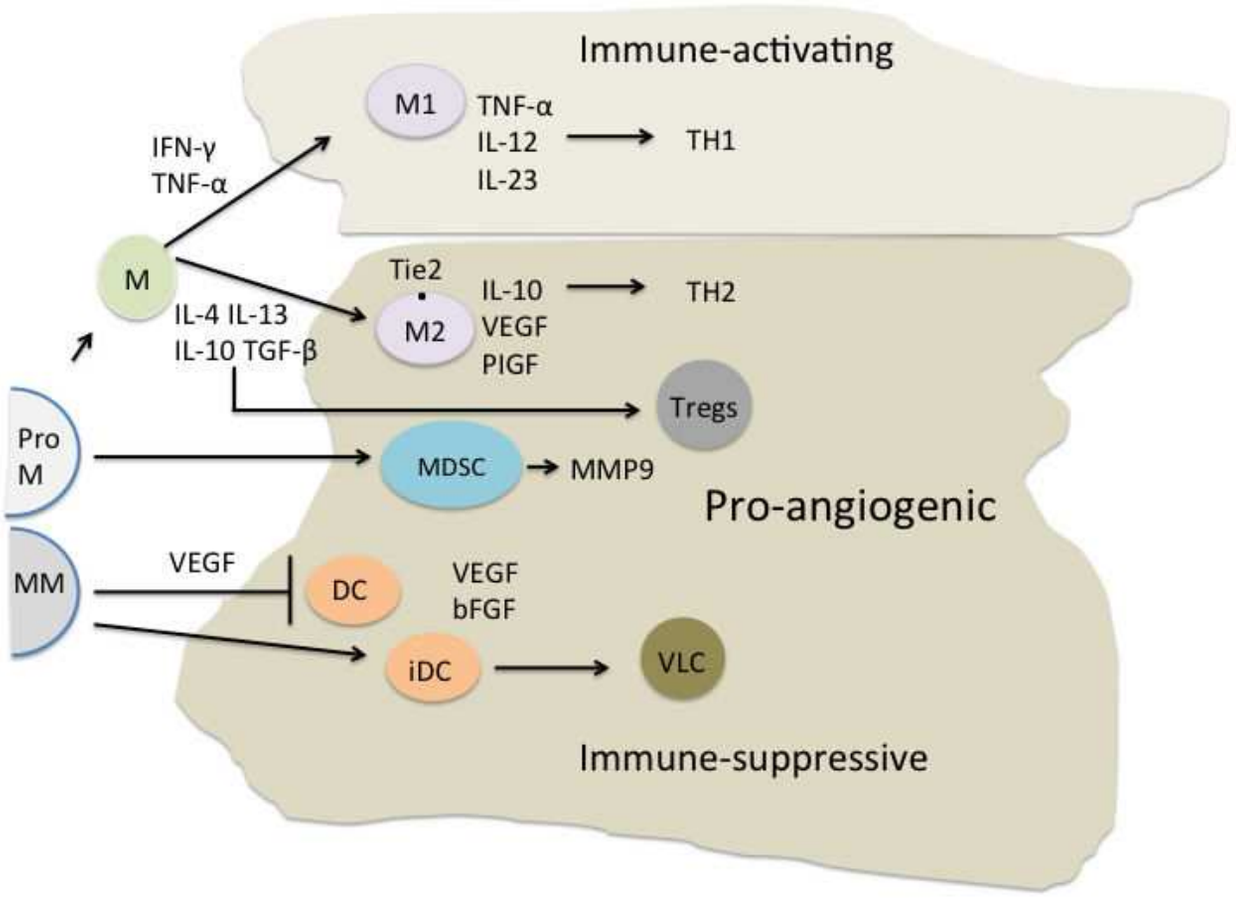

Figure 4. The contribution of immune cells in the tumor microenvironment. Pro M: pro-monocyte; MM: myelo-monocytic stem cell; M: monocyte; M1 and M2: type 1 and 2 macrophages; MDSC: myeloid-derived suppressor cell; DC: dendritic cell; iDC: immature dendritic cell; VLC: vascular leukocyte cell; Tregs: T regulatory cells; IFN-γ: interferon gamma; TNF-α: tumor necrosis factor alpha; TGF-β: transforming growth factor beta; VEGF: vascular endothelial growth factor; bFGF: basic fibroblast growth factor; PIGF: placental growth factor; and MMP9: matrix metalloproteinase 9.

6.1. Myeloid cells

Innate immune cells of the myeloid lineage, including monocytes, macrophages and dendritic cells have been implicated as drivers of angiogenesis (Figure 4). Of these cells, the contribution to angiogenesis has been best characterized for macrophages. Studies in both human tumors

and murine tumor models have shown that the presence of tumor associated macrophages (TAMs) correlates with enhanced vessel density, tumor progression and metastasis [199]. During inflammation, monocytes are attracted by chemo-attractants to damaged tissues, where they differentiate into macrophages. These macrophages are phenotypically plastic, and depending on the environmental signals within wounds or tumors, they differentiate into functional subsets with different activation states [200]. At sites of inflammation, interferon gamma (IFN-γ) and TNF-α facilitate macrophage differentiation into cytotoxic "M1" cells that secrete pro-inflammatory cytokines (TNF-α, IL-1, IL-6, IL-12 and IL-23). M1 macrophages are phagocytic, sustain tissue inflammation, and promote a T helper-1 (TH1) anti-tumor immune response [201, 202]. Alternatively, when induced in the presence of IL-4, IL-13, IL-10 and TGF-β, macrophages differentiate into "M2" cells that secrete IL-10 and participate in tissue remodeling and immune suppression. M2 macrophages also produce angiogenic factors. These factors include VEGF, placental growth factor (PIGF), arginase and the Tie2 angiopoietin cell surface receptor [203, 204]. Monocytes/macrophages that express Tie2 (referred to as TEMS) are a source of VEGF and have been found in human and murine spontaneous and orthotopic tumors [205, 206]. TEMS reside in close proximity to the tumor vasculature and are possibly recruited by angiopoietin-2-expressing endothelial cells [199].

Using a physiologic model of skin wound repair, $CCR2^{hi}$/VEGF-expressing macrophages were shown to initiate vascular sprouts during the early stages of tissue repair [195]. During the early repair period, macrophages with both M1 and M2 gene profiles were present, but cells with a M2 phenotype predominated during the later stages of repair. Results of this study imply that VEGF-expressing macrophages initiate wound-tissue vascularization. The presence of both M1 and M2 macrophages during early repair may be a reflection of the presence of M1 cells during the resolution of inflammation and the presence of M2 cells associated with initiation of an immune-suppressive tissue repair program. The data obtained from this physiologic model of wound healing parallels the process that occurs within the tumor microenvironment. In tumors, M1 cells are often found in sites of chronic inflammation, simulating the inflammatory stage of wound healing, while M2 cells are associated with vascularization, immune suppression and tissue repair [207]. However, this paradigm of tissue repair is not absolute for tumors as demonstrated by an aggressive inflammatory form of breast cancer where there is up-regulation of both VEGF and the IL-6 pro-inflammatory (M1) cytokine [208].

Dendritic cells (DCs) are professional antigen-presenting cells by nature, and they are intimately involved in the activation of tumor-specific T cells. DCs originate from $CD34^{+}$ bone marrow precursors, and they differentiate into heterogeneous subsets due to differentiation plasticity. Within this heterogeneity there are functionally 2 major distinct subtypes of dendritic cells classified as myeloid DC (mDCs) and plasmacytoid DC (pDCs). Plasmacytoid DCs produce anti-angiogenic type I interferons [209], and mDC have the capacity to function as potent antigen-presenting cells. The maturation state of DCs adds another layer of complexity as immature DCs have high endocytic activity, but they lack expression of the co-stimulatory molecules that are necessary for T cell activation. Based on these properties, immature DCs are considered as immune-tolerogenic rather than immune-activating. VEGF affects the development and maturation of DCs. Binding of VEGF to the VEGFR-1 receptor on

$CD34^+$ bone marrow progenitor cells limits differentiation along the DC lineage [210], and engagement of VEGFR-2 inhibits the maturation of DCs [210-212]. Furthermore, high levels of tumor-derived VEGF are associated with the presence of DCs with decreased co-stimulatory molecule expression [213]. There is evidence that tumor-infiltrating immature DCs also promote angiogenesis by secreting VEGF and bFGF [214, 215], and that immature DCs participate in de novo formation of blood vessels or neovascularization in the tumor microenvironment. Under the influence of VEGF or angiogenic factors, immature mDCs trans-differentiate into endothelial-like DCs (called vascular leukocytes, VLC) expressing both DC and endothelial markers such as von Willebrand factor, VEGFR-2, and VE-cadherin (CD31) [216]. Remarkably, human VLC were able to form perfusable blood vessels when transplanted into immune-deficient mice, indicating a potential to support neovascularization.

In addition to macrophages and DCs, neutrophils, eosinophils and mast cells can contribute to tumor angiogenesis. Tumor-infiltrating neutrophils and mast cells secrete VEGF and MMP-9 [217]. Secretion of MMP-9 facilitates the availability of pro-angiogenic factors through a remodeling of the extracellular matrix. Interestingly, an increase in the number of neutrophils in the tumor microenvironment correlates with increased micro-vessel density [218]. The presence of mast cells in murine models of melanoma [219], squamous cell carcinoma [220] and pancreatic islet tumors [221] has been associated with increased angiogenesis. Mast cells are present in the tumor microenvironment prior to vessel formation [222], and they congregate near tumor-derived vessels [220, 223]. Since mast cells contain pro-angiogenic factors in their secretory granules, it has been hypothesized that secretion of these factors by mast cells promotes tumor angiogenesis [199]. There is indirect evidence that eosinophils promote tumor angiogenesis, as they have been detected in human tumors [199], and they are also a source of pro-angiogenic factors [224].

Myeloid-derived suppressor cells (MDSC) are immature myeloid progenitors of monocytes, neutrophils and DCs. As tumor-resident cells, MDSC facilitate tumor progression by their immunosuppressive properties. However, these cells may also have a role in promoting angiogenesis. Studies have shown that tumor angiogenesis is decreased when the MDSC chemo-attractant, BV8 (PROK2), was neutralized [225], and tumor blood vessel density increased when MDSC were co-injected with colorectal cancer cells into mice [226]. MDSC also secrete matrix metalloproteases.

6.2. T cells

Cancer patients have a decrease in immune function that can be attributed in part to the tolerogenic differentiation of innate immune cells. However, there is evidence that VEGF also interferes with T cell development. Effective T cells have the ability to specifically recognize and kill tumors. In fact, the most significant predictor of survival from solid tumors is the presence of CD8 T cells in the tumor core and invasive margins [227]. In vivo administration of a supraphysiologic concentration of recombinant VEGF blocks bone marrow-derived progenitor cells from seeding in the thymus reducing T cell production [228]. These data imply that VEGF secreted from tumors or cells in the tumor microenvironment may contribute to a systemic decrease in T cells.

As previously described, cells of the innate immune system have an important pro-angiogenic role in the tumor microenvironment. However, cells that mediate adaptive immunity also contribute to angiogenesis. The tumor microenvironment is immune suppressive due to the presence of multiple tolerogenic mechanisms. One of the most potent immune suppressive mediators arises through the differentiation of $CD4^+CD25^-$ T cells into $CD4^+CD25^+FoxP3^+$ regulatory T cells (Tregs) [229]. In addition to promoting tumorigenesis through immunosuppression, there is evidence that Tregs contribute to tumor angiogenesis. Accumulation of Tregs in the tumor microenvironment is associated with increased angiogenesis and increased microvessel density [230]. $CD4^+CD25^+$ Tregs secrete higher amounts of VEGF than $CD4^+CD25^-$ CD4 T cells, and when Tregs are depleted from the tumor microenvironment there is less VEGF and angiogenesis present [231]. Therefore, elimination of Tregs as a form of tumor immunotherapy may provide two benefits: a release from immune suppression and decreased angiogenesis.

To summarize the pro-tumorigenic role of immune cells in the tumor microenvironment, there is now convincing evidence that suppressive immune cells can contribute to tumor angiogenesis. This angiogenic activity may be a reflection of the natural wound healing process, as wounds naturally switch from an immune-activating acute inflammatory environment to one that is immune suppressive and pro-angiogenic. As an unhealed wound, the tumor microenvironment may continually cycle between one of inflammation and immune suppression. An understanding of how immune cells, tumor cells, endothelial cells and other cells in the microenvironment contribute to immune suppression and angiogenesis is key in order to devise therapies that can reprogram cells in this environment to be both immune activating and anti-angiogenic. Given the parallels between suppressed anti-tumor immunity and angiogenesis, therapies designed to relieve anti-tumor immune suppression may halt the angiogenic program, or vice versa. Studies to test synergy between immune-based and anti-angiogenic therapies have recently emerged; however, for NB, this field is in its infancy.

6.3. Anti-angiogenic and immune therapies to treat NB

The current standard of care for high-risk NB patients includes myeloablative chemotherapy followed by autologous hematopoietic stem cell transplant (AHSCT) and isotretinoin (13-*cis*-retinoic acid). While these treatments have improved the survival of patients with high-risk disease, approximately 60% of these patients will relapse and die of their disease. Recently, immune therapy has been added to standard treatment protocols as a strategy to improve survival. Post-transplant treatment with an antibody that targets the highly expressed GD2 disialoganglioside on NB tumor cells, in combination with interleukin-2 (IL-2) and granulocyte macrophage colony-stimulating factor (GM-CSF), has resulted in a 2-year 20% increase in event-free survival compared to patients treated with standard therapy alone [232]. Despite this multimodal therapy, the mortality rate remains high for patients with metastatic NB. Indicators of disease associated with a poor prognosis include a paucity of stromal Schwann cells, *MYCN* amplification, expression of the TrkB receptor tyrosine kinase and a high vascular index [233]. Infiltration of immune cell subsets has also been associated with high-risk disease.

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TAMs expressing CD68 and IL-6, as well as IL-6-expressing $CD33^{+}CD14^{+}$ myelomonocytic cells in the bone marrow, are indicators of poor survival [234]. Expression of inflammation-associated genes (IL-6, IL-6R, IL-10 and TGFβ1) also correlates with a poor 5-year event-free survival [235]. In search of new therapies aimed at targeting these high-risk factors, both preclinical and clinical studies designed to test either immune or anti-angiogenic therapies are in progress.

The goal of immune therapy is to summon immune effector cells to the tumor microenvironment and promote activation against the tumor. Much of the effort in cancer immunotherapy has focused on the activation of effector T cells, but in order to achieve an effective anti-tumor T cell response, tumor antigen, mature antigen-presenting DCs and tumor antigen-reactive T cells must be present [236]. Autologous or allogeneic whole tumor cell vaccines, tumor lysate vaccines, antigen–primed DC vaccines, and induction of endogenous tumor cell lysis are all strategies to provide a source of tumor antigens. Agents such as GM-CSF, toll-like receptor (TLR) ligands, or agonistic anti-CD40 antibody are administered to promote DC migration and maturation. Blockade of T cell inhibitory receptors with anti-CTLA-4 or anti-PD-1 antibodies, administration of T cell survival chemokines (IL-2, IL-12 or IL-15), Treg blockade, or adoptive transfer of immune cells are therapies that can promote T cell activation. Recent attention has been directed to targeting immune suppressive factors in the tumor microenvironment using molecular inhibitors or antibodies. As previously noted, the functional complexity of immune cells, and their modulation by the tumor microenvironment to become immune suppressive, is recognized as key factor in the failure of effective anti-tumor immunity.

For NB, the efficacy of several different immune therapies has been examined in both preclinical murine tumor models (Table 1) and clinical trials (Table 2). Preclinical therapies include whole-cell tumor vaccines secreting immune activating cytokines (GM-CSF, IFN-γ, IL-21) or expressing immune co-stimulatory molecules (CD54 (ICAM), CD80, CD86, CD137L). In the N2a murine tumor model, our laboratory and others have shown that depletion of Tregs using anti-CD4 or anti-CD25 mAbs increases vaccine-induced anti-tumor immunity [242, 246]. Another immune therapy designed to augment the number of anti-tumor T cells involves the adoptive cell transfer (ACT) of lymphocytes or T cells. For this therapy, autologous T cells are expanded ex vivo and returned to the patient after they have been activated against tumor antigens. Tumor-specific T cell receptors genetically attached to T cell activating domains (chimeric antigen receptors or CARS) have been transduced into T cells as a method to increase the anti-tumor cytolytic activity provided by ACT. In a preclinical model, adoptive transfer of T cells expressing an anti-GD2 CAR and the CCR2b chemokine receptor promoted trafficking of T cells to the tumor and resulted in tumor regression [245]. Of 11 patients enrolled in a clinical trial testing ACT of Epstein-Barr virus (EBV)-specific T cells expressing the anti-GD2 CAR, 2 patients had tumor regression and 2 patients experience stable disease (Table 2). One of the earliest pre-clinical strategies used a combination of anti-human GD2 antibody and IL-2 treatment in a human-mouse NB xenograft model [237]. After over a decade of study, a combination of anti-GD2, IL-2, GM-CSF and cis-retinoic acid, given in the context of autologous hematopoietic stem cell transplantation, has now been shown to improve the event-free survival of treated patients [232].

Model	Therapy	Response	References
SCID (immune deficient) mouse	Human/mouse chimeric anti-GD2 and IL-2 (ch14.8-IL-2) plus IL-2-activated human PBMCs	Suppressed dissemination of human SK-N-AS NB injected under the splenic capsule	[237]
N2a syngeneic mouse	IL-2-secreting N2a tumor vaccine	Induced protective immunity against N2a and prolonged survival of N2a-bearing mice	[238]
N2a syngeneic mouse	GM-CSF or GM-CSF and IFN-γ secreting N2a tumor vaccine	Regression of tumor in retroperitoneal inoculated N2a-bearing mice	[239]
AGN2a (N2a subclone) syngeneic mouse	AGN2a tumor vaccine transiently transfected to express CD54, CD80, CD86 and CD137L	Protection from AGN2a tumor challenge	[240]
AGN2a (N2a subclone) syngeneic mouse	Anti-CD25 mAb followed by AGN2a CD80, CD86-expressing tumor vaccine	Enhanced protection to AGN2a tumor challenge	[241]
N2a syngeneic mouse	IL-21-secreting AGN2a tumor vaccine	Protective anti-tumor immunity and detection of survivin-specific CTLs	[242]
NXS2 syngeneic mouse	Survivin DNA minigene vaccine	Increase in CD8 T cells at the tumor site and reduced tumor growth	[243]
NXS2	Tyrosine hydroxylase DNA minigene vaccine	Induced tyrosine hydroxylase-specific CTLs and eradicated primary tumor	[244]
SCID	ACT of T cells expressing anti-GD2 CAR and CCR2b	Reduction in growth of huNB xenograft and increased trafficking of CD2 CAR CCR2b T cells to the tumor	[245]
N2a	IL-21-secreting N2a tumor vaccine and anti-CD4 mAb	Reduced dissemination of intravenous inoculated N2a tumor.	[242]
AGN2a (N2a subclone)	ACT of CD25-depleted T cells following TBI and HSCT and AGN2a tumor vaccine expressing CD54, CD80, CD86, and CD137L	Increase in survival of AGN2a-bearing mice	[246]

Table 1. Immunotherapies for neuroblastoma (pre-clinical)

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Therapy	Response	Reference
IL-2	No objective tumor response.	[247]
Autologous NB transfected to produce IL-2	Of 10 patients, 1CR, 1PR and 3SD; 4 patients with anti-tumor CTLs	[248]
Allogeneic NB secreting IL-2	No cytotoxicity against the vaccinating cell line; 1PR, 7SD and 4PD	[248]
Allogeneic NB secreting IL-2 and lymphotactin	Of 21 patients with relapsed or refractory disease: 2CR 1PR; increased NK cytolytic activity	[249]
Anti-GD2 (hu14.18)/IL-2 fusion protein	Of 27 patients there were no CR or PR, 3 patients had anti-tumor activity	[250]
Anti-LI-CAM CAR	Of 6 patients, 1 with limited disease had a PR	[251]
Autologous IL-2-secreting tumor vaccine	In patients with minimal disease there was a rise in circulating CD4 and CD8 T cells specific for autologous tumor	[252]
ACT of EBV-specific T cells transduced with anti-GD2 CAR	Of 11 patients 2CR and 2SD	[253]
Anti-GD2 (hu14.18)/IL-2 fusion protein	Of 23 patients with non-bulky tumor there were 5CR	[254]
Anti-GD2 (ch14.18) GM-CSF, IL-2 and cis-retinoic acid following myeloablative conditioning and AHSCT	Improved event-free survival Incorporated into standard of care	[232]

Table 2. Immunotherapies for neuroblastoma (clinical)

In addition to infiltration of specific immune cellular subsets, a high NB vascular index also correlates with aggressive disease [255]. High expression of pro-angiogenic factors (HIF-2α, VEGF-A, bFGF, TGF-α, PDGF-A, angiopoietin-2, MMP-2, MMP-9, and integrins $\alpha v\beta 3$ and $\alpha v\beta 5$) as well as down-regulation the endothelial cell growth inhibitor, activin A, have been reported in advance stage or high risk NB [256-260]. Given these findings, studies have been designed to target angiogenesis with (1) Agents that directly target endothelial cells (endostatin, thrombospondin-1, thalidomide), (2) Agents that indirectly block the production or activity of pro-angiogenic molecules (antibodies to VEGF or VEGF receptors), or (3) Or agents that target both endothelial and tumor cells (receptor tyrosine kinase inhibitors (RTK) and interferon alpha (IFN-α)). For a complete review, refer to [233]. TPN-470 is an agent that inhibits the proliferation of endothelial cells by inactivating methionine aminopeptidase; however, biochemical instability may limit its application [261]. Fenretinide (N-(4-hydroxyphenyl or 4HPR) is a synthetic analog of retinoic acid that represses endothelial cell proliferation and is

associated with a reduction in VEGFR-2 and FGF-2R-2 receptor expression on endothelial cells [262]. Retinoids are promising anti-tumor agents because they also induce the differentiation of NB cells and promote the survival of tumor-reactive CD8 T cells [233, 263]. As mentioned previously, the isotretinoin retinoid has recently been added to standard care protocols for the treatment of refractory NB. Bevacizumab is an anti-VEGF monoclonal antibody that binds to VEGF receptors, blocking signaling through these receptors. A VEGF Trap decoy is another agent used to block VEGFR. This agent is composed of VEGFR-1 and VEGFR-2 segments fused to an IgG1 molecule [264]. The receptor tyrosine kinase inhibitors, SUGEN, axitinib, imatinib mesylate, sunitinib, sorafenib and ZD6474 differentially target various receptors including PDGFR, VEGFR, the stem cell factor receptor (c-KIT), the FMS-related tyrosine kinase 3, epidermal growth factor receptor (EGFR) and RET on endothelial and tumor cells [265, 266]. Preclinical studies testing the effects of these agents on human-mouse NB xenografts have been performed. For these studies, human NB cell lines were grafted (orthotopically or subcutaneously) into immune compromised mice. Tumor growth, apoptosis of tumor and endothelial cells, and tumor vascularization were examined after treatment with the anti-angiogenic agent(s). It is important to note that these mouse models cannot accurately assess impact of the immune system on tumor growth because they lack an intact human immune system. A summary of NB anti-angiogenic preclinical studies is shown in Table 3.

Model	Therapy	Response	Reference
NB xenografts into immune-compromised (Nude, SCID, NOD-SCID)	TNP-470 (AMG-1470)	Inhibited endothelial cell proliferation and migration	[267]
	Fenretinide	Prevented the induction of endothelial cell proliferation and angiogenesis	[268]
	High dose VEGF Trap decoy	Disrupted early vessel formation and vessel remodeling	[264]
	Imatinib mesylate (Gleevec)	Inhibited NB growth and suppressed PDGFR and c-Kit phosphorylation	[269]
	SUGEN (SU11657)	Reduced angiogenesis, tumor growth and increased apoptosis of NB	[265]
	Bevacizumab	Decrease in tumor micro-vessel density, tumor growth and angiogenesis	[270, 271]

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Model	Therapy	Response	Reference
	Combined treatment with a thrombospondin-1 peptide and valproic acid histone deacetylase inhibitor	Inhibited growth of NB xenografts and stabilized the growth of large tumors	[272]
	ZD6474 RTK	Inhibited tumor growth and induced endothelial cell apoptosis	[266]
	Sunitinib and sorafenib	Inhibited angiogenesis and tumor growth	[273]
	Bevacizumab and cyclophosphamide	Synergistic anti-tumor effect	[274]
	Axitinib	Tumor growth delay, but no regression	[275]

Table 3. Neuroblastoma pre-clinical anti-angiogenic therapies

Since the infiltration of immune-suppressive cells and a high vascular index both correlate with aggressive NB, interventions designed to reverse both immune suppression and angiogenesis represent promising treatment approaches. However, studies testing such combination therapies for the treatment of NB or other cancers are relatively scarce (Table 4). One ongoing phase I NB trial combines immune therapy with anti-angiogenic therapy. For this study, an iodine ^{131}I-conjugated anti-GD2 monoclonal antibody is administered in combination with bevacizumab [276]. Combination therapies for other cancers (renal cell carcinoma) have included treatment with bevacizumab and IFN-α [277] or IL-2 [278]. Surprisingly, combinations of anti-tumor immune and anti-angiogenic therapies have not been tested preclinically in NB, and there are relatively few preclinical studies in other tumor models (Table 4). However, there is evidence that these therapies can act synergistically to elicit anti-tumor responses: (1) Combinations of cytokine-secreting tumor cell-based vaccines and agents that block VEGFR signaling were tested in melanoma and breast cancer models; (2) Immune-activating cytokines, endostatin, and pigment epithelium-derived factor were tested in a hepatocellular carcinoma model; (3) Adoptive transfer of tumor-antigen specific T cells with anti-VEGF and IL-2 was tested in a melanoma tumor model; and (4) Vaccination with a viral vector encoding immune stimulatory molecules and treatment with sunitinib was tested in a colon cancer transgenic mouse model.

While it is almost certain that a combination of therapies (chemotherapy, radiation, targeted therapies, immune and/or anti-angiogenic) will be required to mount an effective anti-tumor response, the appropriate combination will likely vary among the different cancer types. For

System	Therapy	Response	Reference
Neuroblastoma clinical trial	^{131}I-labeled anti-GD2 mAb and bevacizumab	In progress	Clinicaltrials.gov
Clinical trials in other tumor models			
Renal cell carcinoma	Bevacizumab and IFN-α	Significant increase in progression-free survival compared to IFN-α alone	[277]
Renal cell carcinoma	Bevacizumab and IL-2	No clinical benefit	[278]
Preclinical Studies in other tumor models			
B16F10 melanoma	GM-CSF secreting tumor vaccine with a recombinant adeno-associated virus vector expressing a soluble VEGF receptor	A significant increase in tumor-free survival associated with a reduction in tumor-infiltrating immature DC and Tregs and an increase in effector T cells	[279]
Her2/neu breast cancer	Her2/neu expressing GM-CSF secreting tumor vaccine in combination with anti-VEGFR-1, DC101 mAb	In non-tolerant WT syngeneic mice there was accelerated tumor regression associated with expansion of CD4 and CD8 T cells. In tolerant neu transgeneic mice there was delayed tumor growth, but no regression	[280]
Woodchuck hepatocellular carcinoma	Adenovirus vectors encoding IL-12, GM-CSF, endostatin and pigment epithelium-derived factor	Regression of large tumor (>8,000 mm^2) required infusion of all vectors	[281]
B-16 melanoma	Adoptive transfer of Pmel-1 transgenic T cells with anti-VEGF, a tumor vaccine expressing melanoma tumor antigen, gp100, and IL-2 after non-myeloablative total body irradiation	There was a significant increase in survival in tumor-bearing mice when anti-VEGF was administered prior to irradiation and immune therapy	[282]
MC38-CEA colon carcinoma in CEA-transgenic mice	Sunitinib plus primary vaccination with CD80, ICAM1, LFA-3 and CEA expressing vaccinia virus and a boost with fowlpox virus	Treatment with sunitinib prior to vaccination resulted in a significant reduction in tumor growth	[283]

Table 4. Combined immune and anti-angiogenic therapy

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NB, the ideal combination is yet to be determined. Bevacizumab (Avastin®) is FDA-approved for other solid tumors and represents a promising addition to augment immune and chemotherapeutic anti-tumor efficacy for NB. Receptor tyrosine kinase inhibitors, including imatinib mesylate (Gleevec®), sorafenib (Nexavar®), and sunitinib (Sutent®) have shown some anti-tumor efficacy in NB preclinical studies, and these agents are also FDA-approved for the treatment of some solid tumors. The results from studies using combined anti-angiogenic and anti-tumor immune therapy are encouraging and offer a new avenue to explore more effective eradication of NB and other cancers. Given the multiple types of immunotherapy and anti-angiogenic agents, as well as different platforms of delivery, more studies using combinations of these therapies are warranted.

7. Conclusion

NB is an enigmatic childhood cancer that has developmental origins in NC cell lineage. MYCN, ALK and TRKA are the key target genes for NB prognosis. Extracellular matrix and cell adhesion molecules that participate in interactions and signaling across endothelial cells, immune and Schwann cells in the NB microenvironment have potential for targeting. The future for NB biology and therapy looks bright and multiple modalities affecting various cell types and signals in NB microenvironment are anticipated.

Nomenclature

ALK: anaplastic lymphoma kinase; ANS: autonomic nervous system; Ang-2: angiopoietin-2; ACT: adoptive cell transfer; BMP: bone morphogenic protein; BDNF: brain-derived neurotrophic factor; bFGF: basic fibroblast growth factor; BrdU: 5-bromo-2'-deoxyuridine; CNS: central nervous system; CNTF: ciliary neurotrophic factor; CAFs: cancer-associated fibroblasts; CAM: cell adhesion molecules; CAR: chimeric antigen receptor; CR: complete response; DRG: dorsal root ganglia; DC: dendritic cell; EMT: epithelial to mesenchymal transition; ECM: extracellular matrix; EBV: Epstein-Barr virus; EGFR: epidermal growth factor receptor; FDA: Federal Drug Administration; FGF: fibroblast growth factor; GNB: ganglioneuroblastoma; GN: ganglioneuroma; GFAP: glial fibrillary acidic protein; GJIC: gap junction intracellular communication; GM-CSF: granulocyte macrophage colony-stimulating factor; HGF: hepatocyte growth factor; hMSC: human mesenchymal stem cells; HIF: hypoxia inducible factor; IFN-γ: interferon gamma; IFN-α: interferon alpha; LIF: leukemia inhibitory factor; MYCN: v-myc myelocytomatosis viral-related protein; MAPs: microtubule associated proteins; MBP: myelin basic protein; MKI: mitosis-karyorrhexis index; MMP: metalloproteinase; mDC: myeloid dendritic cell; MDSC: myeloid-derived suppressor cell; NB: neuroblastoma; NC: neural crest; NCSC: neural crest-derived stem cell; NRG-1 neuregulin-1; NF: neurofilament; NCAM: neural cell adhesion molecule; NGF: nerve growth factor; NT-3: neurotropin-3; PBMC: peripheral blood mononuclear cell; PNS: peripheral nervous

system; PSNS: parasympathetic nervous system; PEDF: pigment epithelium-derived factor; PDGF: platelet-derived growth factor; PolySia: polysialic acid; PIGF: placental growth factor; pDC: plasmacytoid dendritic cell; PD: progressive disease; PR: partial response; RA: retinoic acid; RTK: receptor tyrosine kinase; SA: sympathoadrenal; SPARC: Secreted Protein Acidic and Rich in Cysteine; SC: Schwann cell; SCP: Schwann cell precursor; SAE: severe adverse effect; SD: stable disease; Trk: tyrosine kinase receptor; TH: tyrosine hydroxylase; TGF-β : transforming growth factor-beta; TIMP-2: tissue inhibitor of metalloproteinase-2; TGF-α: transforming growth factor-alpha; TSP-1: thrombospondin-1; TAMs: tumor-associated macrophages; TNF-α: tumor necrosis factor-alpha; TH1: T helper-1; TEMS: Tie2 monocytes/macrophages; Tregs: T regulatory cells; TLR: toll-like receptor; VLC: vascular leukocytes; VEGF: vascular endothelial growth factor

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