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REVIEW

Linking neural crest development to neuroblastoma pathology

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ABSTRACT

Although rare, childhood (paediatric) cancers are a major cause of death in young children. Unlike many adult cancers, paediatric cancers, such as neuroblastoma (NB), are developmental diseases that rarely show genetic predispositions. NB is the most common extracranial solid tumour in children, accounting for ∼15% of paediatric cancer deaths. This heterogeneous cancer arises from undifferentiated neural crest-derived progenitor cells. As neural crest cells are multipotent and migratory, they are often considered the embryonic paradigm of cancer stem cells. However, very little is known about the events that trigger tumour initiation and progression. Here, we discuss recent insights into sympathoadrenal lineage specification, as well as genetic factors associated with NB. With this in mind, we consider the molecular underpinnings of NB in the context of developmental trajectories of the neural crest lineage. This allows us to compare distinct subtypes of the disease and gene-function interactions during sensitive phases of neural crest development.

KEY WORDS: Embryo, Epithelial-to-mesenchymal transition, Neural crest, Neuroblastoma

Introduction

During the mid-1800s, novel ideas emerged on evolution, disease and development. Embryology was at the core of these disciplines. In the case of cancer pathology, the classic book *Cellular Pathology* by Rudolf Virchow explained it thus:

> *'…upon a more careful examination of cancer […], we shall find that everything depends upon our searching for that stage in their development, in which they are exhibited in their perfect form…' The idea was that '…a physiological type may be found for every pathological formation…'*
>
> (Virchow and Chance, 1978)

Historically, it was well accepted that an understanding of cancer progression could be derived from studying aberrant regulation of embryonic growth (Pierce, 1985; Cofre and Abdelhay, 2017). However, with the emergence of genetics and the 'somatic mutation theory' cancer research shifted away from developmental biology towards an explanation of cancer pathology through aberrant gene expression. In classic experiments, researchers induced differentiation *in vitro* and looked for common genetic markers among the cancer cells and the tissues of origin (Cooper et al., 1992; Tsokos et al., 1987). This has led to the identification of gene mutation patterns in different types of cancer (Watson et al., 2013); but the cellular history of cancers is harder to define.

The somatic mutation theory, originated by Boveri, assumes that cancer arises from a single somatic cell that has accumulated multiple DNA mutations controlling cell cycle and proliferation (Boveri, 2008). Following this, mutations found in cancer occur in two different gene groups: proto-oncogenes and tumour suppressor genes. Proto-oncogenes drive cell differentiation, growth, proliferation and apoptosis, which are all necessary for development and homeostasis. However, when mutated or overexpressed, they become oncogenes, causing abnormal cell transformation into cancerous cells. Conversely, tumour-suppressor genes generally act to limit cell division and DNA replication, and crucially control growth during embryogenesis. This role illustrates the importance of considering developmental cancers as distinct to adult cancers, because embryonic tissues are largely proliferative in contrast to the broadly quiescent adult tissues.

Clearly, cancer research is not homogeneous or straightforward. We are now in an 'omic' era where we have the ability to acquire an enormous amount of data. However, this comes with challenges in processing and interpretation of large datasets, and we risk losing sight of the biological context. Fortunately, cancer research has turned back toward the cellular processes and behaviours that resemble phases in tissue development (i.e. cell proliferation, invasion and migration, stemness or differentiation) (Hanahan and Weinberg, 2011). Focusing on neuroblastoma (NB), we revisit the idea that both the risk of NB and the disease state are linked to specific steps in sympathoadrenal lineage development (Jansky et al., 2021). Contemporary experiments, including the transplantation of NB cells into animal models, such as mouse (Cardoso et al., 2010; Cohen et al., 2020), zebrafish (Her et al., 2021) or chicken (Delloye-Bourgeois et al., 2017; Moghadasi Boroujeni et al., 2019), have made it possible to trace NB migration and differentiation. Other developmental models are also emerging, such as 3D cultures or organoids (Kholosy et al., 2021; Poli et al., 2019; Yin et al., 2016), which allow manipulation of the microenvironment. Together, developmental biology can set the basis for understanding and predicting cellular behaviours in specific environments and, thus, understanding cancer development.

Paediatric cancers

Paediatric cancers differ from adult cancers

Paediatric cancers are a major cause of death in children under 14 years old (Siegel et al., 2021). In the UK, although the five-year survival rate has improved dramatically over the years, from ∼60% in the late 1970s to ∼84% for those under 15 diagnosed between 2012-2016 (https://phw.nhs.wales/services-and-teams/welsh-cancer-intelligence-and-surveillance-unit-wcisu/research/children-teenagers-and-young-adults-uk-cancer-statistics-report-2021/; Smith et al., 2010), many childhood malignancies do not have clear treatment regimes, either due to the rarity of patients or to our incomplete understanding of the biological underpinnings of the cancers. The

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Development (2022) 149, dev200331. doi:10.1242/dev.200331

current approaches for treatment are largely limited to a combination of traditional methods used for adult cancers (e.g. surgery, chemotherapy, radiotherapy), which have long-term complications including higher risk of pulmonary, gastrointestinal, neurological and renal conditions and poor physical health-related quality of life (Moreno et al., 2013). Recent improvements in genetic and molecular profiling now provide us with an opportunity to better understand the biology of childhood cancers, which will improve risk classification, and help identify patient-specific therapies.

The origin of paediatric cancers clearly differs from that of adult cancers; almost all adult cancers are derived from epithelial tissue (e.g. prostate, breast, lung, colon, uterus and ovary) (Filbin and Monje, 2019). However, most childhood cancers arise from immature tissues, causing leukaemias, lymphomas, sarcomas, cancers of the central nervous system or kidney (e.g. Wilms' tumour or nephroblastoma) (Amatruda, 2021; Calandrini et al., 2020). The 'latency period' in which tumours progress to metastasis is also relatively short (1-10 years) in paediatric cancers. In contrast, adult cancers can take decades to become pathological (Lam et al., 2019). This difference is probably because paediatric cancers involve relatively fewer 'hits' (i.e. the acquisition of additional genetic mutations) for cancer induction (Alexandrov et al., 2013; Gröbner et al., 2018). In fact, 10% of paediatric cancers do not show any genetic mutation at all, suggesting epigenetic contributions (Kattner et al., 2019). In addition, although many adult cancers can be prevented (i.e. smoking/lung cancer), childhood cancers do not seem to develop from lifestyle or environmental factors. A key challenge in understanding the genomic underpinnings of NB is the lack of association with somatic mutations.

Because paediatric cancers have a low incidence of somatic mutations (Padovan-Merhar et al., 2016; Savary et al., 2020; Schramm et al., 2015), familial cases are rare; thus, what causes a tumour to initiate and develop in a child is still largely unknown. The age at which each paediatric cancer is diagnosed varies; some are diagnosed during the early years after birth, or in some cases *in utero* (Jennings et al., 1993), whereas others predominate during adolescence. As paediatric cancers largely affect immature tissues, it is unsurprising that the cells of origin in paediatric cancers are embryonic or foetal cells. Indeed, histologically, many paediatric cancers are known as 'blastomas' or embryonal tumours, due to their peculiar resemblance to their corresponding foetal tissue. Paediatric cancers develop during a period of rapid changes in tissue and organ morphogenesis when developmental signalling pathways, such as WNT, Notch, transforming growth factor beta (TGFβ) and hedgehog are activated (Filbin and Monje, 2019). These signalling pathways are well known to contribute to the normal formation of tissues during embryogenesis, where these pathways are tightly regulated. Thus, slight alterations in the signalling balance can lead to congenital anomalies. Similarly, cancer susceptibility windows may arise during this time (Kelleher et al., 2006). The tumours themselves are composed of rapidly cycling cells, which share many characteristics of cancer stem cells. These characteristics, namely cells with proliferative, self-renewing, migratory and invasive capacities, are typical of cancer cell behaviour and the response of these cells to treatments often mimics developmental responses to signal modulation (Sever and Brugge, 2015). The proportion of each of these cells is unique to individual tumours (Capp et al., 2021), so careful assessment is necessary to stage and identify tumour subtypes accurately. For example, Wilms' tumour contains stromal and epithelial cells, as well as undifferentiated 'blastemal' cells that comprise the most aggressive component of the tumour (Popov et al., 2016).

Neuroblastoma is a paediatric cancer

NB is the most commonly diagnosed cancer during the first year of life, accounting for 7-10% of paediatric cancers and 15% of all paediatric cancer deaths (Trigg and Turner, 2018). Around 90% of cases are diagnosed by the age of 5, with most patients diagnosed at a late stage of the disease. The classification of NB patients considers both stage (i.e. tissue localisation and spread) and risk, defined by genetics, age and histology (Sokol and Desai, 2019). The primary tumour site of NB can be distributed at regions where the neural crest (NC) populates, such as adrenal, abdominal/retroperitoneal, neck, thorax and pelvis (Vo et al., 2014). However, most tumours arise within the adrenal medulla and paraspinal sympathetic ganglia, from which they metastasize towards other tissues, such as bone marrow, bone, liver or lung (Morgenstern et al., 2018). Thus, disease presentation is also highly heterogeneous, ranging from localised, non-metastatic tumours to widely disseminated disease. Patients diagnosed with high-risk NB are generally over 1 year of age, with large primary tumours, bone marrow infiltration and are associated with genetic factors, such as chromosome 1p deletions and *MYCN* amplification (Otte et al., 2020). Outcomes also vary widely, ranging from severe, treatment-resistant metastases to spontaneous regression (Trigg and Turner, 2018).

Unusually, a specific subtype of NB tumours, called stage 4S, shows the highest rate of spontaneous regression of all cancers (Lam et al., 2019). Stage 4S patients present with small primary tumours that are widely disseminated. Due to the spontaneous regression, these patients have a 75% chance of survival, often with minimal intervention (Miale and Kirpekar, 1994). This tumour type shares the same abnormal genomic features with aggressive stage 4 NB, but their different behaviour and prognosis has raised questions about aetiology (Trigg and Turner, 2018). Until now, one important finding has been that the epigenetic signature differs in stage 4S NB, providing new avenues to characterise, diagnose and treat the disease (Decock et al., 2016, and reviewed by Fetahu and Taschner-Mandl, 2021). Another study comparing stage 4 and stage 4S NB has found that autophagy-related genes are significantly enriched in low-risk NB groups, which may be involved in spontaneous regression (Meng et al., 2020). Interestingly, spontaneous regression is more common before 18 months of age, whereas NB diagnosed in older children appears to be higher risk (Brodeur, 2018).

Like other paediatric cancers [e.g. Wilms' tumour (Szychot et al., 2014)], the proportion of undifferentiated 'neuroblasts' within the NB tumour appears to indicate aggressiveness (Jansky et al., 2021). These neuroblasts represent a transition cell during sympathoadrenal lineage specification. Recent studies have demonstrated that nerve-associated multipotent Schwann cell precursors (SCPs), which generate sympathetic neurons and adrenal chromaffin cells (Furlan et al., 2017; Kamenev et al., 2021; Kastriti et al., 2019; Lumb et al., 2018), may underlie NB. As many tumour cells resemble the specific embryological tissue from which they derive, it is tempting to speculate that NB cells remain in an undifferentiated state because they lack the appropriate differentiation cues. The susceptibility of the sympathoadrenal lineage to perturbation underlies the fundamentals of NB research and developmental biology.

The neural crest lineage and neuroblastoma

Neural crest development

During embryogenesis, NC induction occurs in the dorsal neural plate border dependent on key signalling pathways, notably bone

Development (2022) 149, dev200331. doi:10.1242/dev.200331

morphogenetic protein (BMP), Wnt and fibroblast growth factors (FGFs) (reviewed by Seal and Monsoro-Burq, 2020). Once induced, a core characteristic of neural crest cells (NCCs) is their ability to undergo epithelial-to-mesenchymal transition (EMT), allowing them to leave the neuroectodermal epithelium and become migratory cells (Fig. 1A). During this migratory phase, the modulation of cell-cell adhesion, chemotaxis and proliferation is required for NCCs to successfully reach their destinations, while additional cues, such as FGFs and retinoic acid (RA) signalling, confer axial identity (reviewed by Rothstein et al., 2018). NCCs ultimately integrate with surrounding tissues and differentiate (reviewed by Trainor, 2013). These cells are surprisingly multipotent, with the capacity to differentiate into a variety of cell types including the cranial skeleton, pigment cells (melanocytes), neurons and glia of the peripheral nervous system and sympathetic nervous system (Bronner and Simões-Costa, 2016). Most NC progenitors only exist for a brief period because they rapidly differentiate to generate these broad-ranging derivatives (Trainor, 2013). However, longer-lived NC-like stem cells can be obtained in culture from specific sources, such as hair follicles. It is possible that such NC-like stem cells function in tissue maintenance (e.g. pigment and glial homeostasis in adults); however, the origin of NC-like stem cells in culture is unclear (reviewed by Mehrotra et al., 2020). Consequently, adult NC-derived cancers support the notion that some parts of the NC genetic programme can be reactivated during tumorigenesis (Maguire et al., 2015). These include pheochromocytomas derived from the adrenal medulla (reviewed by Maguire et al., 2015) and, most significantly, melanoma – a common and aggressive cancer arising from the transformed melanocytes (Schadendorf et al., 2015).

The sympathoadrenal lineage

During embryonic development in mice and chickens, trunk NCCs migrate in the vicinity of the dorsal aorta where BMP signalling stimulates the expression of the transcription factors *Sdf1* (also known as *Cxcl12*) and *Nrg1*, which act as chemoattractants (Saito

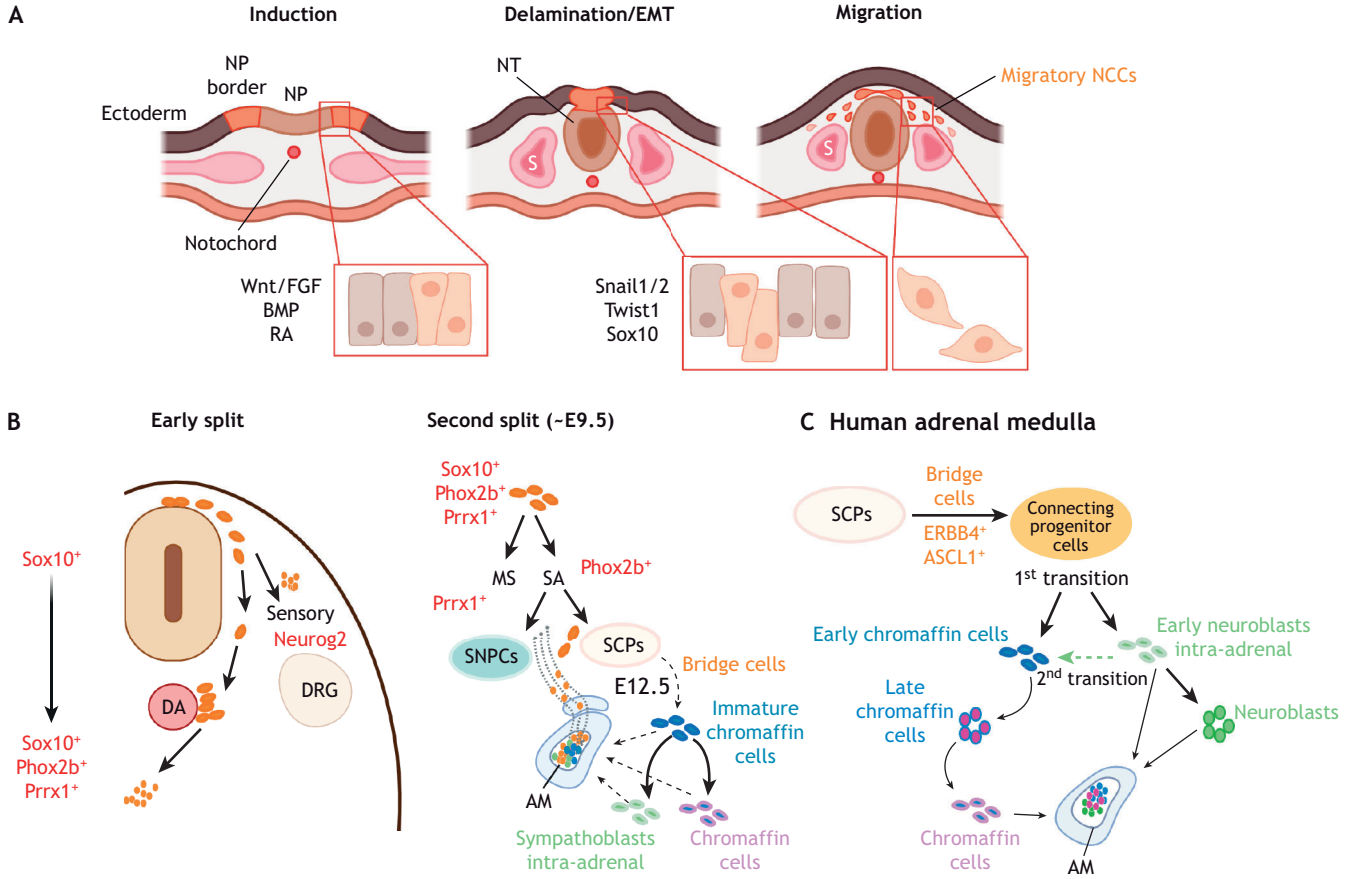

Fig. 1. Summary of sympathoadrenal development in mouse and human. (A) Neural crest cells (NCCs) are induced in the border between the developing neural plate (NP) and the adjacent non-neural epithelium. Key factors, such as Wnts and bone morphogenetic proteins (BMPs), are crucial for positioning the NP border. Additional factors, such as fibroblast growth factors (FGFs) and retinoic acid (RA) provide posterior identity, which is necessary for trunk neural crest (NC) development. Progenitor cells then delaminate while undergoing an epithelial-to-mesenchymal (EMT) transition, migrating away from the neural tube (NT). On a cellular level, the NC forms within the neurogenic epithelium. Cells begin to express EMT factors, such as Snail1/2 and Twist1, as well as the pro-migratory transcription factor Sox10. Cells dismantle their epithelial junctions and delaminate, becoming fully migratory. S, somite. (B) In mouse, the migration of trunk NCCs destined for the sympathoadrenal lineage follows two distinct paths. Early stage cells express Sox10 and migrate ventrally toward the dorsal aorta (DA). These cells are transiently Sox10^{+}Phox2B^{+} but subsequently downregulate Phox2B. An early split (left) bifurcates early fates of sensory cells including dorsal root ganglia (DRG) and Neurog2^{+} cells from bipotent progenitors. The latter follow a second split (right) to form a mesenchymal (MS) lineage and the sympathoadrenal (SA) lineage. These give rise to sympathetic neuronal progenitor cells (SNPCs) and Schwann cell precursors (SCPs). (C) In humans, analysis of the transcriptional signatures has revealed an early bridge population, followed by connecting progenitor cells, which diverge into chromaffin cells and neuroblasts. The developing adrenal medulla (AM) is populated by SCPs located inside nests of neuroblasts, surrounded by small groups of chromaffin cells, as well as a population of bridge cells (Furlan et al., 2017; Jansky et al., 2021; Kameneva et al., 2021a,b). Created with BioRender.com.

Development (2022) 149, dev200331. doi:10.1242/dev.200331

et al., 2012). Recent studies using lineage-tracing and single-cell RNA-sequencing (scRNA-seq) show that NCCs then undergo a series of transcriptional bifurcations that separate specific lineages (Furlan et al., 2017). An early split sets apart the sensory lineage, characterized by expression of *Neurog2*, from the precursors that will give rise to: (1) *Phox2b*$^{+}$ autonomic cell precursors to form the sympathoadrenal lineage (sympathetic neurons and chromaffin cells in the adrenal medulla) and (2) *Prrx1*$^{+}$mesenchymal precursors (Soldatov et al., 2019) (Fig. 1B). In mice, at around embryonic day (E) 9.5-E10.5, *Phox2b*$^{+}$ autonomic cell precursors split to give rise to the sympathetic neuronal progenitor cells (SNPCs) and glial cells associated with sympathetic ganglia (Soldatov et al., 2019). Later, streams of NCCs become associated with neuronal innervation and develop into SCPs, which give rise to the majority of intra-adrenal chromaffin cells and the chromaffin cells that comprise the organ of Zuckerkandl (Furlan et al., 2017; Kastriti et al., 2019) (Fig. 1B). SCPs can also give rise to a plethora of cell lineages, including melanocytes, Schwann cells, parasympathetic ganglia and the sympathoadrenal lineage. Furlan and colleagues used genetic ablations to demonstrate an early requirement for *Ascl1* in SCPs to generate chromaffin cells. Crucially, these cells activate expression of *Phox2b* but fail to become catecholaminergic (SOX10^{-}, PHOX2B^{+}, TH^{-}) (Furlan et al., 2017). Furthermore, lineage comparisons between Ascl1- and RET-dependent populations supported segregation between sympathetic neurons and chromaffin cells as early as E11.5 (Furlan et al., 2017; Furlan and Adameyko, 2018). Finally, SCPs contribute to a small number (i.e. 10%) of sympathetic neuron precursors of the posterior sympathetic chain (Kameneva et al., 2021a; Kastriti et al., 2019). SCPs can give rise to a minor population of intra-adrenal sympathetic neurons, which in mice originate from SCPs via a 'bridge' state to form immature chromaffin cells. Immature chromaffin cells can then give rise to intra-adrenal sympathoblasts and chromaffin cells (Kameneva et al., 2021a) (Fig. 1B).

Conversely, Jansky and colleagues have shown that the human sympathoadrenal lineage follows a different cell trajectory (Jansky et al., 2021) (Fig. 1C). Two transient populations of cells are derived from human SCPs: bridge cells, which express ERBB4 and ASCL1, and 'connecting progenitor cells', which express low levels of sympathoadrenal markers and progress towards the differentiation of either chromaffin cells or neuroblasts. These transitions suggest a hierarchy in stemness that places early migrating SCPs at the highest multipotency, followed by later migrating SCPs, bridge cells and connecting progenitor cells. Each step can be marked by additional gene expression profiling. For example, in human, *PHOX2B* is expressed during the transition of SCPs to bridge cells, *HAND2* is expressed at the start of bridge cell differentiation and *GATA3* is expressed towards the neuroblast transition (Jansky et al., 2021). In addition, Kameneva and colleagues have suggested through bioinformatic analysis, that a second transition, in which, a number of intra-adrenal sympathoblasts (or neuroblasts – referred to by the authors as immature sympathoblasts) form chromaffin cells (Kameneva et al., 2021b). Moreover, a recent study by Bedoya-Reina and colleagues shows the presence of a population of cells unique to the postnatal human adrenal gland, with transcriptional expression patterns that resemble chromaffin cell precursors and which express the cholinergic receptor nAChRs α7, suggesting a cholinergic nature (Bedoya-Reina et al., 2021). However, these cholinergic-like cells appear to be distinct from the SCP transcriptional signature and are absent in the mouse adrenal gland (Bedoya-Reina et al., 2021). These findings highlight the difficulties when cellular identities are defined by isolated genes, which can lead to misinterpretation of developmental trajectories. For example, in mice, sympathoblasts express the gene *Cartpt*; however, in humans, *CARTPT* is mainly expressed in chromaffin cells. Therefore, although Dong and colleagues initially attributed the *CARTPT* gene to sympathoblasts in humans (Dong et al., 2020), subsequent analysis by Kamaneva and colleagues has highlighted the importance of using an expanded expression signature of orthologous cell types when studying different animal models to understand human disease (Kameneva et al., 2021a,b). Moreover, the difficulty in designing a mouse model that recapitulates the biology of NB may be explained by the differences during sympathoadrenal development and postnatal organ development in mouse compared with that of human.

Despite the tight regulation of these developmental programmes, there are occasions in which malignancies appear during embryogenesis. As the embryonic NC lineage is uniquely multipotent, it is conceivable that cells retain a transcriptional signature akin to cancer stem cells that appear to co-opt embryonic gene expression programmes (Lobo et al., 2007). The cells of origin in NB are associated with NC-derived progenitors of the sympathoadrenal lineage, which form the sympathetic ganglia and suprarenal ganglion, as well as contributing to the population of chromaffin cells of the adrenal medulla (Fig. 1B,C) (Matthay et al., 2016). As discussed above, several recent studies have used scRNA-seq approaches to define the molecular profiles of NC-derived progenitor cells during normal mouse and human sympathoadrenal development, as well as in NB (Bedoya-Reina et al., 2021; Dong et al., 2020; Furlan et al., 2017; Hanemaaijer et al., 2021; Jansky et al., 2021; Kameneva et al., 2021a; Kildisiute et al., 2021a). These data, combined with the recent assessment of several human NB lines, demonstrate that the transcriptional states in NB resemble those of normal NCCs and that disease outcomes may correlate with distinct developmental signatures (van Groningen et al., 2017). Interestingly, further comparative analysis of the transcriptional profiles of postnatal human adrenal glands and NB tumours has revealed that an undifferentiated cluster of cells in high-risk NB highly resembles the postnatal cholinergic progenitor cell population (Bedoya-Reina et al., 2021). This sets an alternative scenario in which high-risk NB, which is more common in patients over 18 months old, develops during postnatal development.

Given the findings that the phenotype of NB is transcriptionally comparable with different stages of adrenal development, the overall prognosis may be associated with the differentiation state of NB (Hanemaaijer et al., 2021). Studies have found that low-risk NB cells transcriptionally resemble SCP cells, whereas *MYCN*-amplified and metastatic NBs resemble the most undifferentiated cell stage, which is comparable with immature cells such as bridge cells. This observation is well-supported by epigenomic and transcriptomic analysis of NB cell lines, which has shown that tumour cells can express both NCC-like (or mesenchymal) properties versus sympathoadrenal markers (Boeva et al., 2017; van Groningen et al., 2017). In addition to aggressiveness, the relative proportion of these cancer cells likely contributes to their response to chemotherapeutics (Dagogo-Jack and Shaw, 2018; Schmelz et al., 2021). Relapse is also likely to arise from the more plastic, less-differentiated NC-like subpopulations that remain (Jansky et al., 2021). Controversially, a recent paper has suggested that all NB cells have a signature akin to foetal sympathoblasts, suggesting greater homogeneity than expected (Kildisiute et al., 2021a,b); however, this may reflect the relatively early lineage expression of key drivers of NB. Nevertheless, determining the level of tumour heterogeneity regarding the differentiation state may be

Development (2022) 149, dev200331. doi:10.1242/dev.200331

crucial for therapeutic interventions. Together, these studies suggest at least two distinct phases of high-risk disease vulnerability: first, during early embryogenesis when NCC precursors maintain highly cycling and stem-like signatures; and second, at the time the committed NCC-derived precursors fail to differentiate. In the first example, control by developmental programmes may be crucial, with some cells retaining the characteristics of tumour stem cells. In the second, driver genes that prevent cell-cycle exit and differentiation appear later. Thus, developmental aberrations would be predicted to have two separable consequences: to promote growth or to regulate regression of NB.

Genetic drivers of neuroblastoma

To date, very few genes have been directly implicated in NB. Here, we discuss several known NB-associated genes and their links to NC development (Table 1). Our goal is to illustrate key crisis points occurring during normal development of the NC lineage, which may be linked to the pathology of NB (Fig. 2).

MYC proteins drive neural crest progenitors in neuroblastoma

The MYC family of oncogene proteins, including cMYC and MYCN, are basic helix-loop-helix (bHLH) transcription factors that act together with a binding partner, MAX. MYC-MAX complexes bind E-box consensus sequences (5′-CACGTG-3′) found in the enhancers and promoters of many genes, but the complex can also be found associated with promoters that lack E-box sequences (Westermann et al., 2008; reviewed by Baluapuri et al., 2020). Although binding of MYC-MAX appears to enhance gene transcription, the specificity of these interactions is unclear. Some direct targets of MYCN include the pro-apoptosis gene *TP53* (encoding p53) and the E3-ubiquitin ligase *MDM2*, which can target p53 for degradation (Westermann et al., 2008). In addition to the transcriptional activation of target genes, MYC proteins can physically interact with DNA replication origins and promote assembly of the pre-replication complex (Dominguez-Sola et al., 2007). Sustained availability of MYCN appears to interfere with the processivity of the replication machinery, causing replication stress

Table 1. Genetic drivers of neuroblastoma

Gene	Function	Expressed in NB (%)	Status in NB	Expressed in NC or NC-lineage?	Synergy	Therapeutic target
MYCN	Proto-oncogene (bHLH transcription factor of the MYC family); Involved in development of tissues and organs, e.g. nervous system (Knoepfler et al., 2002; Wakamatsu et al., 1997; Zindy et al., 2006)	20% of primary tumours; 50% of high-risk NB cases (Rickman et al., 2018)	Amplified in NB patients, alone (Lee et al., 2018) or co-expressed with ALK (Otte et al., 2020; Zhu et al., 2012)	Highly expressed during early central nervous system development and thought to be implicated in early steps of NCC migration (Wakamatsu et al., 1997). However, *Mycn* is not found expressed in migrating NC (Khudyakov and Bronner-Fraser, 2009); Active at later stages, during neurogenesis (Knoepfler et al., 2002)	*ALK* (Zhu et al., 2012)	Not direct
PHOX2B	Transcription factor (paired like homeobox 2B); Involved in the differentiation of neurons (Pattyn et al., 1997)	6-10% of familial NB cases (Raabe et al., 2008)	Mutations resulting in LOF (Di Zanni et al., 2017); Occasionally it is found co-expressed with ALK (Bachetti et al., 2010)	Expressed in NC sympathoadrenal differentiation in zebrafish (Pei et al., 2013) and mice (Pattyn et al., 1999)	*ALK* (Bachetti et al., 2010)	Not direct
ALK	Tyrosine kinase; Probably involved in neuronal development, cell cycle progression, migration, and evasion of apoptosis (reviewed by Trigg and Turner, 2018; Wulf et al., 2021)	9% of primary tumours; 14% of high-risk NB cases (reviewed by Trigg and Turner, 2018)	GOF mutations of ALK (F1174L) (Berry et al., 2012) and ALK (P1275Q) co-expressed with MYCN (Ueda et al., 2016) or PHOX2B (Bachetti et al., 2010)	Expressed in trunk migratory NC in mouse (Gonzalez Malagon et al., 2018) and *Xenopus* (Moreno et al., 2021)	*MYCN* (Berry et al., 2012; Ueda et al., 2016)	Small molecule inhibitors targeting ALK activity, e.g. crizotinib (Sahu et al., 2013), ceritinib (Ceccon, 2014); Clinical trials to target crizotinib-resistant NB (Tucker et al., 2015; Brenner and Gunnes, 2021)
ATRX	SWI/SNF-like chromatin remodelling protein; reorganisation of the nucleosome to make DNA accessible during transcription, replication and DNA repair (reviewed by Dyer et al., 2017; Tang et al., 2010)	100% of high-risk adolescent NB	In-frame deletions, point mutations and indels associated with ALT (reviewed by Dyer et al., 2017)	Not known	Synthetic lethality with MYCN amplification (Zeineldin et al., 2020)	Not direct

ALT, alternative lengthening of telomeres; GOF, gain-of-function; LOF, loss-of-function; NB, neuroblastoma; NC, neural crest.

Development (2022) 149, dev200331. doi:10.1242/dev.200331

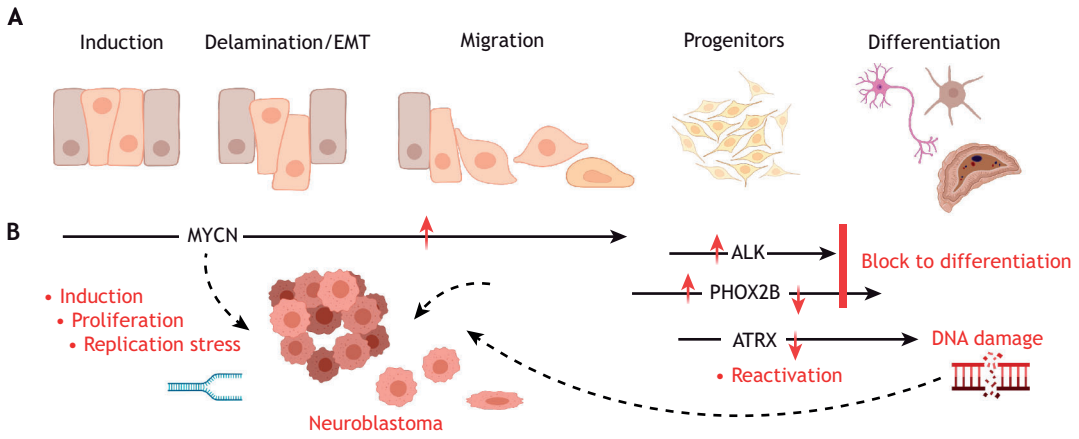

Fig. 2. Aberrant expression of oncogenes during neural crest development may lead to neuroblastoma progression. (A) The neural crest (NC) forms within the neurogenic epithelium. Cells delaminate, undergo epithelial-mesenchymal transition, become mesenchymal progenitor cells and subsequently differentiate into cells, such as sympathetic neurons, melanocytes and chromaffin cells of the adrenal medulla. (B) Several key factors have been implicated in NB progression, including MYCN, ALK, PHOX2B and ATRX. Aberrant MYCN activation (up arrow) may affect NC development at early stages, resulting in sustained expression of MYCN in NC, which may prime for neuroblastoma (NB) development. Activating mutations in the tyrosine kinase ALK (up arrow) are associated with the PHOX2B-mediated differentiation of sympathoadrenal progenitors and may lead to a failure in differentiation. PHOX2B itself is transiently expressed in sympathetic neuroblasts as they differentiate. Loss-of-function mutations in PHOX2B (down arrow) have been identified in NB, while sustained expression of PHOX2B may also block differentiation (up arrow). Finally, the DNA helicase ATRX acts as a tumour suppressor, and loss of ATRX function leads to DNA damage (down arrow). NBs in which *ATRX* is mutated have a high level of reactivation. Created with BioRender.com.

and DNA damage (Baluapuri et al., 2020), which can fuel genomic instability by disrupting the normal programme of replication initiation and causing replication fork stalling and DNA damage (Dominguez-Sola et al., 2007).

Both cMYC and MYCN are stabilised by interactions with CIP2A (Junttila et al., 2007; Kerosuo et al., 2018). In the chick, CIP2A is first co-expressed with MycN in the neural plate and neural tube, but around Hamburger–Hamilton stage (HH) 8, CIP2A expression shifts to the NC where it is co-expressed with *cMyc* instead. Therefore, MycN is not expressed during early NC induction but only in adjacent neural precursors forming the central nervous system (Kerosuo et al., 2018). However, ectopic expression of MycN in the NC domain promotes the differentiation of NC stem cells towards a neural stem cell-like fate, thereby affecting NCC differentiation (Kerosuo et al., 2018). Furthermore, EMT factor Twist1 is a direct transcriptional target of MYCN in NB (Selmi et al., 2015). MYCN has been directly linked to poor prognosis in NB patients, notably with aggressive tumours and relapse (Look et al., 1991; Wang et al., 2020). High levels of MYC proteins, which arise from MYC-gene genomic rearrangements or upregulation of upstream regulators of MYC, including WNT (Scholz et al., 2019), are associated with many cancers (Dang, 2012). In NB, *MYCN* gene amplification occurs in 20% of NB cases, rising to 50% in high-risk tumours. In addition, somatic alterations in NB frequently leads to CIP2A-mediated stabilisation of MYCN, which is found in ∼25% of all cases (Barone et al., 2013; Campbell et al., 2019; Otte et al., 2020). Finally, MYCN-expressing tumours have decreased immune cell infiltration, due to a reduction in interferon activity and chemokine expression (Layer et al., 2017, and reviewed by Blavier et al., 2020). Consequently, MYCN-amplified, and non-MYCN-amplified NBs interact differently with adjacent immune cells, thus modulating the tumour microenvironment (Mao et al., 2016; Santilli et al., 2013).

Loss of PHOX2B prevents sympathetic neuron differentiation

PHOX2B, which encodes the homeobox transcription factor paired-like homeobox 2B, is highly expressed in the developing peripheral nervous system (Dauger et al., 2003). PHOX2B is essential for the development of the autonomic nervous system (Pattyn et al., 1999), where it functions as an activator of sympathoadrenal specification (Tomolonis et al., 2018). *PHOX2B* variants cause neurocristopathic disorders including NC migration disorders such as Hirschsprung's disease, which is characterised by failure of innervation of the gut, and congenital central hypoventilation syndrome, associated with impaired regulation of breathing by the autonomic nervous system (Aygun, 2018). In addition, *PHOX2B* was the first gene associated with NB, with 6-10% of familial cases co-segregating with loss-of-function (LOF) mutations in *PHOX2B* (Raabe et al., 2008; reviewed by Matthay et al., 2016; Trochet et al., 2004). Phox2b is a crucial regulator of NC maturation in mice, where it supports differentiation of NC-derived autonomic neurons by expressing RET, a subunit of the glial cell-derived neurotrophic factor (GDNF) receptor that is required for sympathetic neuron development (Pattyn et al., 1999). This evidence suggests that PHOX2B-associated NBs arise from NC progenitor cells that fail to differentiate.

Gain-of-function mutations in *ALK* en route to the sympathoadrenal lineage

The tyrosine kinase anaplastic lymphoma kinase (ALK) is frequently found as a fusion protein in cancers, such as anaplastic large cell lymphoma; thus, much of our understanding of its phosphorylation targets arises from analysis of pathological ALK fusions (reviewed by Hallberg and Palmer, 2016). Targets of wild-type ALK are less well understood, but include phosphoinositide 3-kinase/protein kinase B (PI3K/AKT) pathways, with phosphorylation of ERK1/2 routinely used to determine ALK activation *in vitro* (reviewed by Wulf et al., 2021).

ALK is associated with almost all familial NB cases and in many sporadic NBs, and is the most frequently mutated gene associated with primary NB (Trigg and Turner, 2018). Activating mutations in *ALK* account for most cases of congenital disease, and 5-15% of NBs acquire somatic mutations resulting in ALK activation. The most common protein variants in NB are ALK p.F1174L and ALK p.R1275Q, both of which contain amino acid changes within the ALK kinase domain that lead to impairment of cell trafficking,

Development (2022) 149, dev200331. doi:10.1242/dev.200331

ligand-independent auto-phosphorylation of ALK and increased [i.e. gain-of-function (GOF)] kinase activity (Chand et al., 2013; Mossé et al., 2008). In addition, these variants correlate with treatment resistance and poor prognosis. Although ALK is clearly oncogenic and prone to genetic insult, the importance of ALK activation during tumour initiation remains largely unknown.

In animal models, ALK plays a role in neuronal development and is expressed in NC tissues and peripheral nervous system (reviewed by Wulf et al., 2021). In embryonic and postnatal mice, *ALK* is expressed in the neural tube and brain (Iwahara et al., 1997; Vernersson et al., 2006), as well as migratory NC at E9.5 (Gonzalez Malagon et al., 2018; Moreno et al., 2021). *ALK* GOF variants overexpressed in the mouse NC mimic the onset of NB; however, the development of severe disease requires synergy with *MYCN* overexpression (Berry et al., 2012; Heukamp et al., 2012). NCC-specific overexpression of the F1174L ALK variant in mice, which causes sustained activation of ALK, appears to prevent Phox2b-mediated differentiation of sympathetic ganglia (Cazes et al., 2014; Vivancos Stalin et al., 2019). However, *in vitro* analyses suggest that Phox2b acts upstream of ALK because siRNA-mediated knockdown of *PHOX2B* in human NB cell lines reduces *ALK* expression and PHOX2B can directly bind to the *ALK* promoter (Bachetti et al., 2010). Thus, the mouse and *in vitro* models have shown the oncogenic capability of ALK and suggest reciprocal regulation between ALK and PHOX2B. However, it is still unclear how these interact in *in vivo* development.

Finally, the RET receptor tyrosine kinase, which is crucial for NC migration (Delalande et al., 2008), is both directly phosphorylated and transcriptionally regulated by ALK: both GOF experiments in ALK or RET lead to enlargement of sympathetic ganglia (Cazes et al., 2014; Ono et al., 2019; Smith-Hicks et al., 2000). Conversely, knockout of RET in ALK-activated NB cell lines leads to EMT (Siaw et al., 2021).

ATRX-associated adolescent neuroblastoma have a distinct developmental aetiology

Many genes associated with epigenetic regulation and chromatin remodelling are altered in childhood cancers, including paediatric glioblastoma multiforme (Liu et al., 2012) and paediatric adrenocortical carcinoma (Assié et al., 2014). For example, the expression of DNA methyltransferases, particularly *DNMT3A/B*, is enriched in high-risk NB cells (Qiu et al., 2005). Other epigenetic markers involved in histone modifications have also been identified in several NB models, giving potential new avenues for treatment and prognosis of the disease (reviewed by Jubierre et al., 2018).

Mutations in *ATRX* (alpha thalassemia/mental retardation, X-linked), a gene that encodes an SWI-SNF-like chromatin remodelling protein, are associated with adolescent NB. Sequencing studies from 40 patients with metastatic NB have found that 100% of adolescent and young adult patients (>12 years old) present with mutations in *ATRX*. A significantly lower percentage (17%) of children aged 18 months to 12 years also have *ATRX* mutations. In contrast, *ATRX* has not been associated with any tumours from infants (0-<18 months) (Cheung et al., 2012; Watson et al., 2015). Distinct LOF mutations appear to be associated with differing severity in patients. Interestingly, patients presenting this syndrome show craniofacial defects related to NC development but do not tend to develop tumours. *ATRX* mutations found in NB patients are spread throughout the coding region (Watson et al., 2015) and are associated with an absence of ATRX protein in the nucleus, long telomeres and with tumour aggressiveness (Cheung et al., 2012). *ATRX* mutations in NB often produce in-frame deletions in the region of the heterochromatin-binding domain. These mutations probably affect the ATRX H3.3 chaperone function, resulting in genome instability and defects in deposition of H3.3 at regulatory elements of neuronal differentiation genes and leading to the cells maintaining an undifferentiated state (Qadeer et al., 2019; Zeineldin et al., 2020).

In contrast to the synergy between MYCN and ALK, it is intriguing that *ATRX* mutations found in NB are incompatible with NB carrying MYCN amplification (Zeineldin et al., 2020). Both MYCN and ATRX are indicative of high-risk NB commonly associated with a poor prognosis (Kimura et al., 2021). However, they are mutually exclusive across all ages and stages in NB.

Elevated MYCN levels promote cell reprogramming, in which mitochondria metabolism depends on exogenous glutamine (Wise et al., 2008). Amplified-MYCN NB, thus, depends on glutaminolysis to survive (Qing et al., 2012). This process may elevate reactive oxygen species production and DNA-replicative stress. *ATRX* deletions cause defects in the function of the ATRX histone chaperone complex, which may also lead to replicative stress. When the resulting replicative stress of MYCN amplification is combined with that of *ATRX* LOF, synthetic lethality results. This can eventually be exploited to design targeted treatments and improve the outcome for patients with high-risk NB (Zeineldin et al., 2020).

Developmental models of neuroblastoma

Using animal models to understand human disease has been one of the main approaches in biomedical research. Genetic and embryological manipulations allow us to probe the relevance of specific gene mutations and to investigate the function of novel genes. As discussed above, in NB only a few genes have been directly associated with disease prognosis, and some chromosomal intervals have also been found altered. However, it is difficult to engineer new models that recapitulate relevant expression patterns or complex regulatory changes, especially when we are still unable to pinpoint the precipitating events. Thus, a mouse model that entirely recapitulates a human tumour may not be possible. Here, we discuss some of the extant NB models and potential new approaches to characterise the variety of NB origin.

Cell-specific genetic tools in animal models

The majority of mouse models aimed at understanding NB employ genetic GOF strategies driving MYCN or ALK^{F1174L}. Although ubiquitous expression can be informative, as in chicken overexpression experiments (Kerosuo et al., 2018), these may lead to pleiotropic effects, obscuring the developmental progression of NB. Therefore, approaches have relied on targeting specific cells of the sympathoadrenal lineage.

Models dependent on overexpression in sympathoadrenal lineages

A widely used model is a transgenic mouse in which human *MYCN* is expressed under the control of the rat tyrosine hydroxylase (*Th*) promoter (*Th-MYCN*) (Alam et al., 2009). Tyrosine hydroxylase is the rate-limiting enzyme in catecholamine synthesis (reviewed by Daubner et al., 2011). *Th* expression in the peripheral neural system in mice is limited to sympathetic ganglia and the adrenal chromaffin cells (Anderson and Axel, 1986). However, Alam and colleagues have found that cells that overexpress *MYCN* in the *Th-MYCN* mouse are mostly proliferative $Phox2B^{+}$ neuronal progenitors, suggesting that MYCN promotes proliferation and prevents differentiation in these cells (Alam et al., 2009). Although several reports have suggested that *MYCN* overexpression is sufficient to

Development (2022) 149, dev200331. doi:10.1242/dev.200331

drive NB, the onset depends on gene dosage. For example, ∼70% of *Th-MYCN* homozygous mice develop tumours within the first 7 weeks of age, whereas only ∼45% of hemizygous mice show tumours later between 5.6-19 weeks of age (Rasmuson et al., 2012). In addition, using the *Th* promoter to express ALK^{F1174F} requires concurrent high expression of *MYCN* to drive disease in mice and zebrafish (Berry et al., 2012; Zhu et al., 2012).

Recently, the *Dbh-iCre* line allowed specific induction of *MYCN* in dopamine b-hydroxylase-expressing cells (termed *Dbh-iCre; LSL-MYCN*) (Althoff et al., 2015). Here, the *MYCN* gene is preceded by a stop codon flanked by LoxP sites (lox-stop-lox, LSL) allowing Cre-recombinase-dependent activation. Dbh catalyses the final step of norepinephrine biosynthesis, and is therefore expressed in chromaffin cells and peripheral sympathetic neurons (Fuxe et al., 1970). Over 75% of these mice develop neuroblastic tumours. As with *MYCN*, using the *Dbh* promoter to drive GOF mutations of ALK^{F1174L} is sufficient to induce NB (Heukamp et al., 2012), but without the additional requirement of high *MYCN* expression observed using the *Th* promoter discussed above. The variation between the *Th-MYCN* model and *Dbh-iCre; LSL-MYCN* model is not clear. Initially, the onset of gene expression needs to be directly compared to explain these discrepancies.

Overexpression in neural crest progenitor cells leads to perinatal lethality, precluding analysis

A *Sox10-Cre* line driving *(LSL)-MYCN* causes respiratory failure and stillborn animals but without evidence of NB (Yang et al., 2020). Similarly, targeting ALK^{F1174L} to NC using *Sox10-Cre* is also embryonic lethal (Vivancos Stalin et al., 2019). As these mice die before the age where they might develop NB, we cannot exclude the possibility that there are early priming events for NB obscured by lethality. However, in human NB cells, *SOX10* expression is mostly restricted to Schwannian stroma and is absent in neuroblasts, which is associated with good prognosis. Interestingly, the expression of *SOX9* is increased in human *MYCN*-amplified NB cell lines and is associated with unfavourable prognosis (Yang et al., 2020). The overexpression of *SOX9* is associated with migration and invasion behaviours and, in contrast to the requirements for *Sox9* during development, it appears to impair differentiation in NB cells (Yang et al., 2020). Thus, the role of SOX9 in NB may be maintaining the mesenchymal state, rather than promoting differentiation (Yang et al., 2020). However, SOX9 does not initiate stemness properties in NB cells (Yang et al., 2020).

These animal models have dramatically improved our understanding of potential downstream targets that govern NB cell behaviour. However, differences between mouse and human sympathoadrenal lineage development mean that animal models cannot always provide the whole perspective when considering human disease.

Human neural crest cells

Recently, human-mouse interspecies chimeras have been developed to generate tumours more closely resembling human NB. In these chimeras, pluripotent or lineage-restricted human stem cells are introduced into a host post-implantation mouse embryo (Cohen et al., 2016). Recently, Cohen and colleagues have generated mouse-human NC chimeras using human induced pluripotent stem cell (iPSC)-derived NCCs expressing doxycycline (Dox)-inducible *MYCN* and ALK^{F1174L}. Introduction of these cells into mouse embryos leads to the formation of tumours resembling primary human NBs (Cohen et al., 2020). This model opens the possibility to study immune responses towards cancer and potentially design new therapies.

An exciting new approach is to generate iPSCs directly from human NB patients that carry different mutations, such as recent generation of iPSCs carrying the ALK p.R1275Q mutation (Marin Navarro et al., 2019). These cells can now be induced towards the sympathoadrenal lineage and differentiated into chromaffin cells to follow the step-by-step mechanisms of tumours originating from specific driver mutations. Patient-derived iPSCs from NB patients could also potentially be used to understand the developmental outcomes; for example, by comparing with peculiar cases of spontaneous tumour regression.

Therapeutic implications and opportunities

The heterologous nature of NB makes it a very challenging condition to treat, especially for those high-risk NB patients (Maris, 2010). Generally, low-risk NB requires very little intervention. In this category, stage 4S NB patients undergo spontaneous regression with little or no risk of relapse. Intermediate risk groups may require surgery, chemotherapy, or both. However, high-risk NB patients are subject to aggressive treatments: these include chemotherapy and surgery, myeloablative therapy, stem cell rescue, radiotherapy, immunotherapy and a differentiation agent (reviewed by Smith and Foster, 2018). Although event-free survival has increased in recent years, a high proportion of high-risk NB patients still relapse. These patients are rarely cured, with <10% of long-term survival. Moreover, the chemicals used in treatment directly interfere with cell division by disrupting cell-cycle progression. So, although they can reduce tumour growth, there are implications for children who are in their developmental years.

We have discussed some genes that directly influence prognosis. However, these genes represent a challenge for researchers because they are difficult to target pharmacologically (Table 1). As ALK is a kinase, it is the only protein discussed that is readily targeted directly; however, *ALK*-mutated NB can show drug resistance. For example, the ALK p.F1174L mutation becomes resistant to crizotinib (Trigg and Turner, 2018).

As NB comprises undifferentiated sympathoadrenal precursors, using RA to induce differentiation in high-risk NB patients is promising (reviewed by Ferreira et al., 2020). RA signalling is highly conserved; during development, it generally functions to switch cells from proliferation to differentiation (Das et al., 2014). Some RA-targeting drugs are currently in use (e.g. Isotretinoin or 13-cis RA) or in clinical trials (e.g. fenretinide) (Matthay, 2013; Veal et al., 2013). However, the latter seems to have a cytotoxic effect rather than acting as a differentiating agent (Cuperus et al., 2008).

The family of Trk neurotrophin receptors (TrkA/B/C; NTRK1/2/3) is an interesting family to include in targeted treatment for NB. They all have essential roles in the maintenance and differentiation of the nervous system through specific neurotrophin ligands (e.g. TrkA/NGF and TrkB/BDNF) (Fagan et al., 1996; Kasemeier-Kulesa et al., 2015). Interestingly, p75NTR (NGFR), another neurotrophin receptor, is expressed throughout the trunk NC, and seems to enhance the affinity of Trk receptors to their ligands (Ho et al., 2011). One peculiar aspect of these receptors is that they show differential expression patterns in NB. Whereas TrkA is highly expressed in NB showing good prognoses, TrkB is found in high-risk NB and is a marker for poor outcomes (Brodeur, 2018). Furthermore, inhibition of Trks in NB can lead to cell death (Brodeur et al., 2009; Nakagawara, 2001).

DNA-binding proteins, especially general factors such as MYCN, are even more difficult to target. However, some recent experiments on *ATRX*-driven NB have shown some promise.

Although ATRX-associated NBs show therapy resistance, studies suggest they are sensitive to PARP inhibitors and other DNA-damaging drugs (George et al., 2020), which have the potential to move quickly into the clinic as a treatment for NB.

Conclusions

In this Review, we propose that the defined genetic associations seen in NB, combined with our knowledge of the normal NC development, can provide insights into the heterogeneity of disease prognosis and therapies. First, we have suggested that the NC is an exemplar of an embryonic stem cell; this lineage carries these stem-like characteristics during early prenatal development, showing a proliferative and migratory capacity. Consequently, some cancer cells appear to co-opt the NC-related developmental programmes. Second, it appears that the spontaneous regression seen in a subset of patients and the effectiveness of differentiation therapies lends support to the idea that NB arises early in the developmental life of an NCC.

A key remaining question is whether the cancer stem cell is a multipotent cell in the wrong place or whether it is a more differentiated cell that is reactivated or regresses towards a migratory, invasive state. In the case of NB, distinct genetic signatures may indicate different developmental phases. For example, tumours expressing high levels of *MYCN* may be able to escape the factors that limit proliferation and self-renewal. In contrast, tumours carrying GOF mutations in *ALK* appear to ignore differentiation cues, either because they have migrated to a region of the body that lacks these cues or because they cannot receive or respond to signals. Similarly, NB associated with *PHOX2B* may be unable to progress beyond the sympathoadrenal progenitor phase. Finally, *ATRX*-associated NB are unique in their manifestation in adolescents, potentially indicative of reactivation of stemness in differentiated NCCs.

Owing to the embryonal nature of NB, a detailed understanding of the normal developmental context of human sympathoadrenal specification is necessary for defining crucial periods. Recent transcriptome analysis of mouse and human sympathoadrenal development and NB have advanced our understanding of the developmental paths undertaken by transient cell populations with specific transcription expression signatures that may be vulnerable to develop NB. We need this information to understand how key genes interact with environmental stressors to drive pathology, which will also provide clues with regards to the tumour ‘cell of origin’ or the tumour ‘stem cell’ that may not only initiate cancer but also cause cancer relapse. Moreover, from the abovementioned studies, we can now identify specific species developmental trajectories that may provide a clear view of the challenges when using different disease models. Targeted therapies can then be designed, which are necessary to avoid systemic damage. Altogether, understanding the genomic landscape and developmental state will be crucial for designing effective combination therapies.

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Competing interests

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