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Neuroblastoma pathogenesis: deregulation of embryonic neural crest development

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Abstract

Neuroblastoma (NB) is an aggressive pediatric cancer that originates from neural crest tissues of the sympathetic nervous system. NB is highly heterogeneous both from a clinical and molecular perspective. Clinically, this cancer represents a wide range of phenotypes ranging from spontaneous regression of 4S disease to unremitting treatment-refractory progression and death of high risk metastatic disease. At a cellular level, the heterogeneous behavior of NB likely arises from an arrest and deregulation of normal neural crest development. In the present review, we summarize our current knowledge of neural crest development as it relates to pathways promoting 'stemness' and how deregulation may contribute to the development of tumor initiating CSCs. There is an emerging consensus that such tumor subpopulations contribute to the evolution of drug resistance, metastasis, and relapse in other equally aggressive malignancies. As relapsed, refractory disease remains the primary cause of death for neuroblastoma, the identification and targeting of CSCs or other primary drivers of tumor progression remains a critical, clinically significant goal for neuroblastoma. We will critically review recent and past evidence in the literature supporting the concept of CSCs as drivers of neuroblastoma pathogenesis.

Keywords

Neuroblastoma; Neural Crest; Cancer Stem Cells; CD114

1. Introduction

Neuroblastoma (NB) is a pediatric solid tumor of neural crest origin that encompasses between 8–10% of all pediatric cancers, and up to 15% of all pediatric cancer deaths (Goodman M.T., 1999). Treating NB remains a significant challenge due to the

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heterogeneous course the disease follows, ranging anywhere from spontaneous regression to treatment resistant progression and death (Castleberry, 1997). High-risk NB, usually metastatic disease with unfavorable histology and high-risk genetic modifications, poses the most significant challenge to treatment (Cohn, et al., 2009). Despite intensive induction chemotherapy, radiation therapy, and surgery, the long term survival for patients with high-risk NB remains less than 50% and less than 10% in those patients that suffer disease relapse (Maris, et al., 2007). The biological heterogeneity seen in NB largely correlates with tumor histology and differentiation status.

Histologically, NB tumors consist of two cell types, neuroblastic ganglionic cells and reactive Schwannian stromal cells, and can be classified into four basic morphologies (Shimada, et al., 1999). These morphologies consist of ganglioneuroma (Schwannian stroma dominant), intermixed ganglioneuroblastoma (Schwannian stroma rich), nodular ganglioneuroblastoma (composite tumor composed of aggressive and non-aggressive clones) and neuroblastoma (Schwannian stroma poor). These morphologic characterizations are thought to represent levels of tumor differentiation mediated by chemotaxis of Schwann cells that secrete anti-proliferative and differentiating factors onto the neuroblasts (Liu, et al., 2005). During differentiation and maturation, a majority of neuroblasts will die before reaching maturity leaving a persistent and dominant Schwannian stroma. Thus, ganglioneuromas represent a NB that has fully matured and differentiated (Shimada and Roald, 2000). While the factors that leads a NB tumor to recruit Schwann cells and differentiate remains unknown, retinoic acid therapies have been used as maintenance therapies to differentiate residual disease with significant increase in patient event free survival (Matthay, et al., 1999). Recent findings from single cell analyses and global transcriptional profiling are beginning to shed light on the molecular control of tumor heterogeneity (Louis and Shohet, 2015, van Groningen, et al., 2017). Taken together, NB represents a tumor of developmental arrest and delayed differentiation. Understanding the normal development of the neural crest-derived sympathoadrenal system can help us understand and identify the pathways involved in the maintenance of undifferentiated clones of tumor initiating neuroblasts.

2. Neural Crest Development and NB

2.1 Neural Crest is a multipotent transient embryonic tissue

The neural crest (NC) is a transient embryonic cell population that arises during gastrulation and neurulation and migrates extensively throughout the developing embryo, differentiating into diverse tissues including much of the craniofacial skeleton, sympathetic and peripheral nervous system, adrenal chromaffin cells, and melanocytes. The complexity of tissues derived from the NC suggests the presence of multipotent progenitors. Indeed, early investigation into the potency of NC precursors found that injection of lineage tracers into NC precursors within avian embryos found that NC was multipotent; however, this technique was limited by technical challenges of intracellular injections (Bronner-Fraser and Fraser, 1989, Bronner-Fraser and Fraser, 1988). Using advances in lineage tracing models, the R26R-*Confetti* mouse model, investigators were able to identify that both individual pre-migratory and migratory NC cells were multipotent rather than pools of fate-restricted

progenitors (Baggiolini, et al., 2015). Development, maintenance, and differentiation of this multipotent cell population is a highly complex process and correspondingly, NC induction, speciation, delamination, and differentiation are tightly controlled pathways orchestrated by a multifaceted gene regulatory network (Sauka-Spengler and Bronner-Fraser, 2008). Initiating mutations or derangements during any number of these processes may generate tumor initiating neuroblasts. As detailed below, signaling pathways and transcription factors regulating various aspects of NC development are also implicated in NB pathogenesis clinical phenotypes.

2.2 NC Induction

NC development begins during gastrulation with formation of tissues involved in neural tube development. The primitive neural tube consists of non-neural ectoderm and neural plate (NP) tissues with the junction giving rise to the neural plate border (NPB). Induction of genes within this NPB leads to the expression of NC specifier genes. Induction is mediated by interconnected signaling pathways of bone morphogenic protein (BMP), Wntless/Int (WNT), Fibroblast growth factor (FGF), and to a lesser extent Notch/Delta signaling. This induction activates key transcription factors that specify the NPB and prime the NPB tissue for induction of genes that allow for NC speciation.

2.2.1 BMP in NC induction—BMP is a protein from the transforming growth factor beta (TGF β) family that is secreted by neighboring non-neural ectoderm. Signaling through BMP receptors activates the Smad family of transcription factors and leads to transcription of genes involved in growth and differentiation (Miyazono, et al., 2005). Studies using BMP gradients have shown that NPB speciation occurs in regions with intermediate BMP levels (Marchant, et al., 1998). Using a NC specific conditional knockout of BMP signaling (Pax3-Cre-BMPRIa) in mice, researchers found that mice without NC BMP had no detectable cranial or truncal NC, as determined by Cad6 and Sox10 expression, respectively (Stottmann and Klingensmith, 2011). Using a human ESC model, early inhibition of BMP (day 0–2) with antagonist noggin led to a significant decrease in NC induction whereas later inhibition with noggin (day 3–4) only had a partial reduction in induction (Leung, et al., 2016). These studies indicate that early and consistent BMP expression is essential for NC induction.

In NB, BMP has been associated with NB differentiation. In the IMR-32 NB cell line, combination therapy using BMP-6 and retinoic acid derivatives led to synergistic differentiation of NB cell lines into dopaminergic neurons as evidenced by increased expression of tyrosine hydroxylase, morphological neuronal maturation, and inability to resume cell division (Sumantran, et al., 2003). Furthermore, incubation of mouse NB cell line Neuro2a with BMP2 led to a decrease in Id expression (inhibitor of differentiation, discussed below) and upregulation of neural specific transcription factors (Dlx2, Brn3a, NeuroD6) promoting the differentiation of NB cell lines to neural lineages (Du and Yip, 2010). Thus, suppression of BMP signaling may represent a pathway derangement to maintain multipotency in NB.

2.2.2 Wnt pathway in NC induction—Wnt is a secreted ligand that controls β -catenin signaling. Wnt binds to Frizzled and related receptors, leading to activation of Dishevelled

and inhibition of the GSK3 β /axin complex that normally targets β -catenin for degradation. Stabilization of β -catenin allows it to translocate to the nucleus and act as a co-activator with WNT effector TCF/LEF (MacDonald, et al., 2009). Secretion of Wnt by neighboring non-neural ectoderm allows it to bind to Frizzled receptors expressed on cells of the NPB, leading to induction of β -catenin genes associated with NC speciation. In *Xenopus* and avian models, expression of Wnt pathway components within ectoderm explants was sufficient to drive the expression of NC markers (Abu-Elmagd, et al., 2006, Chang and Hemmati-Brivanlou, 1998, Garcia-Castro, et al., 2002). Furthermore, blocking Wnt signaling axis (activation GSK3 β or knockdown Frizzled/Wnt) led to the loss of cells with NC marker expression (FoxD3, Snail2) (Tan, et al., 2001). Activation of Wnt signaling, through repression of GSK3 β , in human embryonic stem cells (hESCs) led to a rapid differentiation into neural-crest like cells as evidenced by expression of NC markers SOX10, PAX7 and TFAP2A (Leung, et. Al., 2016). This data suggests that canonical Wnt signaling is both sufficient and necessary for NC induction.

Deregulation of Wnt signaling has been shown to stimulate proliferation and maintenance of CSC populations in many cancers including colorectal cancer, hepatocellular carcinoma, medulloblastoma, and some leukemias (Cordeiro, et al., 2014, Dong, et al., 2016, Dow, et al., 2015, Petropoulos, et al., 2008, Wang, et al., 2016). However, studies in NB have shown conflicting evidence for the role of the Wnt pathway in tumorigenesis. Using an IGR-N-91 xenograft model, cell lines derived from the primary tumor and two metastatic foci showed a decrease in Wnt-5a expression that was reversed by retinoic acid differentiation therapy; however, the effects were due to signaling through the non-canonical calcium-dependent signaling pathway involving protein kinase C theta (PKC- θ) (Blanc, et al., 2005). Studies in NB cell line SK-N-SH have shown differential gene expression of Wnt pathway components within a CD133+ NB CSC-like population (described below) and furthermore, activation of canonical Wnt signaling through β -catenin in this subpopulation led to *in vitro* doxorubicin resistance (Vangipuram, et al., 2012).

2.2.3 FGF pathway in NC induction—Fibroblast growth factor (FGF) is a secreted protein that binds and signals through receptor tyrosine kinases, also known as fibroblast growth factor receptors (FGFRs). Signaling through FGFR activates many downstream pathways involved in proliferation and survival, including Ras/ERK and Akt/MTOR (Ornitz and Itoh, 2015). In NC induction, FGF is released by the paraxial mesoderm and acts on FGFRs in the NPB. In *Xenopus*, zebrafish, and chick models, overexpression of FGF8 ligands or their receptors led to NC marker expression via inhibition of BMP signaling and induction of Wnt signaling (Furthauer, et al., 1997, Hong, et al., 2008, Monsoro-Burq, et al., 2003, Wilson, et al., 2000). Conversely, inhibition FGF signaling through the expression of a dominant negative FGFR1 led to a loss in cells with NC marker expression (Mayor, et al., 1997). In chick embryos, ectopic expression of FGF was sufficient to drive the formation of NC-like derivatives (Yardley and Garcia-Castro, 2012). Further studies in *Xenopus* have shown that FGF signaling through FGFR4 activates STAT3 (signal transducer and activator of transcription 3) and leads to expression of NC border genes and NC specifiers, while loss of FGFR4 prevents this induction (Nichane, et al., 2010). Thus, FGF may act directly

through STAT3 activation, or indirectly through BMP pathway inhibition and Wnt pathway activation.

Signal transducer and activator of transcription 3 (STAT3) is an important transcription factor involved in the activation and regulation of genes related to proliferation, apoptosis, and tissue differentiation (Villarino, et al., 2015). In multiple CSC models, including neural tumors such as glioblastoma, STAT3 has been shown to activate transcription factors associated with pluripotency and contribute to the maintenance of CSCs (Sherry, et al., 2009). In NB cell lines, transgenic mouse models, and primary patient samples, expression of STAT3 downstream of the granulocyte stimulating factor receptor (G-CSFR) is associated with a NB CSC-like subpopulation with increased tumorigenicity and chemoresistance (Hsu, et al., 2013) [Discussed below]. Furthermore, treatment of NB cell lines and xenograft tumor models with STAT3 inhibitor Stattic led to decreased tumorigenicity, decreased metastasis, and reduction in chemoresistance (Agarwal, et al., 2015). Treatment of NB cell lines NGP and IMR-32 *in vitro* with STAT3 targeted antisense oligonucleotide AZD9150, led to decreased cell proliferation. *In vivo*, treatment of xenograft tumors with AZD9150 greatly reduced re-implantation potential of NB tumors indicating the importance of STAT3 in NB tumor initiation (Odate, et al., 2017).

2.2.4 Notch pathway in NC induction—Notch proteins are transmembrane signaling molecules that act as intracellular receptors for Delta/Jagged protein ligands. Upon binding Delta, the intracellular domain of Notch is cleaved, translocates to the nucleus and binds associated transcription factors to activate transcription of target genes (Hori, et al., 2013). In *Xenopus* and chick models, Notch cleavage and activation leads to NC induction via attenuation of BMP4 signaling. However, in mouse and zebrafish models, Notch was found to be more important in NC differentiation than induction, where knockout of Notch led to a decrease in truncal NC cell derivatives, but not cranial derivatives (Cornell and Eisen, 2000, Cornell and Eisen, 2002, Hernandez-Lagunas, et al., 2011). Therefore, the role of Notch signaling in NC induction appears to be species specific, but in general maintains the NC progenitor population in a more proliferative state through modulation of other induction signaling pathways.

Notch plays a critical role in embryological development including many pathways that maintain cells in a proliferative state with blocked differentiation. In neural systems, Notch1 is responsible for the maintenance of neural stem cells via regulation of cell cycle exit and prevention of neural differentiation. Here, knock down of Notch signaling components (RBPj) led to depletion of stem cell pool and premature cessation of neurogenesis (Ables, et al., 2010, Ehm, et al., 2010, Hitoshi, et al., 2002). In NB, inhibition of Notch1 signaling within the human NB cell line SH-SY5Y, led to neuronal differentiation via a JNK-CRT mediated pathway. Correspondingly, treatment of NB xenograft mice with Notch inhibitors (γ -secretase inhibitors, GSIs) led to suppression of tumor progression (Chang, et al., 2010). Treatment of SH-SY5Y and IMR-32 human NB cells lines with combination of GSIs and retinoic acid derivatives led to synergistic complete cell growth arrest, neuronal differentiation, and reduced cell motility (Ferrari-Toninelli, et al., 2010). Furthermore, expression of constitutively active Notch1 protein in the mouse Neuro2a cell line led to inhibition of both spontaneous and RA-induced cell differentiation (Grynfeld, et al., 2000).

2.3. Neural plate border specification

NPB cells have the ability to differentiate into NC, as well as spinal roof plate, dorsal interneurons, and sensory neurons. Activation of a regulatory network of homeobox transcription factors confers competence on this region to form NC. These transcription factors can be divided into two groups (early, late) based on their temporal expression. The early factors, Gbx2 and AP2, are expressed during gastrulation and are critical for NP border specification. Both transcription factors are activated by Wnt signaling to induce expression of downstream NP specifiers and loss of these two factors leads to loss of expression of downstream NC specifier Snail2 (de Croze, et al., 2011, Li, et al., 2009). Msx1 expression is tightly controlled by graded BMP signaling. It functions to activate further NC specifiers Snail2, FoxD3, and Twist1, and may play a role in suppressing NP markers such as Sox2 (Liu, et al., 2004). Pax3 and Zic1 are direct activators of critical NC specifiers including Snail1/2, FoxD3, and Sox9 (Plouhinec, et al., 2014). Ectopic expression of both Pax3 and Zic1 in ventral ectoderm led to the formation of NC indicating that these two factors are sufficient to drive NC induction (Monsoro-Burq, et al., 2005). However, expression levels of these factors are tightly controlled as elevated expression drives cells into non-NC fates (Hong and Saint-Jeannet, 2007).

In NB, Msx1 has been shown to modulate both Notch/Delta signaling and Wnt signaling pathways. Inducible expression of Msx1 in NB cell line SJNB-8 led to inhibition of proliferation and colony formation as well as strong activation of Notch pathway target genes (Revet, et al., 2008). Msx1 was also found to activate transcription of Wnt pathway antagonists; however, these gene products did not interfere with Wnt signaling (Revet, et al., 2010). Pax3 is a well characterized proto-oncogene (Linardic, 2008). Expression of Pax3 in human NB cell lines (SH-SY5Y, SH-EP1, LA-1-55N, and LA-1-5S) was correlated with more malignant morphologic phenotype. Furthermore, knockdown of Pax3 in these cell lines led to G1 cell cycle arrest and growth inhibition, induction of apoptosis, a decrease in migration and invasion, and increased sensitivity to chemotherapy (Fang, et al., 2014). The role of Gbx2 and AP-2 in NB pathogenesis is yet to be elucidated.

2.4. Neural Crest Speciation

Following activation of NPB specifiers and priming of NPB for NC development, activation of a second wave of transcription factors coordinates commitment of NPB cells to the NC lineage and primes these early NC cells for delamination and migration. These factors can be split into two groups based on their function in NC speciation.

2.4.1 Early Onset NC Specifiers—Factors expressed early in NC speciation are involved in promoting proliferation, suppressing neural differentiation, and maintaining these early NC cells in a multipotent state. The main factors involved in progenitor maintenance are c-Myc and its direct downstream target Id3 (inhibitor of differentiation 3). C-Myc and Id3 act to control the cell cycle and mediate balance between proliferation and apoptosis. Loss of both c-Myc and Id3 led to a loss of detectable NC progenitor cells and excess CNS progenitors whereas overexpression led to maintenance of the NC in a prolonged multipotent state (Kee and Bronner-Fraser, 2005, Light, et al., 2005). This indicates the role of c-Myc and Id3 in the maintenance of NC progenitor multipotency.

c-Myc and related N-Myc are transcriptional regulators that are directly involved in the regulation of thousands of genes involved in the maintenance of pluripotency (Chappell and Dalton, 2013). C-Myc was first implicated for its role in pluripotency when it was found activated downstream of LIF/STAT3 signaling (Cartwright, et al., 2005). Myc, one of the four Yamanaka transcription factors used to reprogram terminally differentiated cells into pluripotent stem cells, has the strongest effect on establishing a pluripotent-like gene signature and acts early in the reprogramming process through modulation of epigenetic modulators (Mikkelsen, et al., 2008, Sridharan, et al., 2009). While the expression of c-Myc is normally tightly regulated in embryologic development, deregulation of Myc expression is seen in many cancers (Dang, 2012, Gabay, et al., 2014).

Amplification of MYCN leading to N-myc protein overexpression is present in approximately 50% of high-risk tumors (overall incidence is approximately 25% of tumors) (Huang and Weiss, 2013). MYCN amplification, identified over 30 years ago, remains one of the strongest indicators of poor prognosis in high-risk NB. MYCN amplified tumors are typically high-grade, aggressive cancers consisting of primarily undifferentiated or poorly differentiated neuroblasts (Seeger, et al., 1985). Furthermore, high-risk tumors without MYCN amplification tend to have activated c-Myc or downstream signaling pathways contributing to undifferentiated phenotype. In an analysis of 357 diagnostic patient samples, 38/39 tumors with elevated Myc protein expression occurred without MYCN amplification and protein expression, and like MYCN amplified tumors, Myc-expressing tumors had unfavorable histology and poor 3-year event-free survival (46.5%) indicating the importance of both MYCN and c-Myc in maintaining an undifferentiated state in NB (Wang, et al., 2015). Using conditional expression of N-Myc in human NB cell line Tet21N, an unbiased ChIP-chip assay for N-Myc bound genes identified that in NB, N-Myc regulates genes associated with critical embryonic stem cell factors, including LIF, KLF2, KLF4, and LIN28B (Cotterman and Knoepfler, 2009).

2.4.2 Late onset NC Specifiers—Late onset NC specifier genes function to initiate the epithelial to mesenchymal transition allowing NC cells to adopt a migratory phenotype and delaminate. Transcription factors involved in this program include Sox9, Sox 10, FoxD3, Snail 2 and Twist1. Sox9 is activated by Ap2 signaling and is important in initiating the EMT program and in trunk NC specifically, Sox9 expression promotes NC survival through inhibition of apoptosis and cell cycle arrest via regulation of Snail2 (Cheung and Briscoe, 2003, Cheung, et al., 2005). FoxD3 and Snail2 are critical factors in initiating the EMT program. FoxD3 acts to inhibit expression of N-cadherin and activate expression of Integrin-B1, while Snail 2 acts to inhibit occludins and E-cadherin while also promoting the expression of MMPs (Cheung, Chaboissier, Mynett, Hirst, Schedl and Briscoe, 2005, Taneyhill, et al., 2007). Overall this leads to reduced cell adhesion and increased synthesis of enzymes involved in matrix resorption, freeing the cells for delamination. In addition to activation of the EMT program, Sox10 is known to promote the survival and inhibit multi-lineage differentiation of NC stem cells (John, et al., 2011).

Analysis of 42 primary NB tumor specimens by IHC revealed that higher FoxD3 expression levels correlated with tumors with good differentiation status while RT-PCR and western blotting revealed that 20 primary samples and three human cell lines had lower FoxD3

mRNA and protein levels compared to normal dorsal ganglion (Li, et al., 2013). Ectopic re-expression of FoxD3 in human NB cell lines SH-SH5Y and SK-N-SH led to downstream suppression of genes associated with angiogenesis (VEGF) and invasion (MMP-9) and attenuation of growth, migration, invasion, and angiogenesis. Furthermore, ectopic expression of FoxD3 in SH-SY5Y cells led to decreased tumor formation compared to xenografts of cells expression endogenous levels (Li, et al., 2013). Expression of Snail2 in NB is critical to migration, invasion, and metastasis. Activation of Snail2 expression within human NB cell line LA-N-5 was associated with increased migration and invasion through matrigel membrane (Tanno, et al., 2010). In human NB cell lines LAN-1, SK-N-LP, SK-N-JD, SH-SY5Y, and SK-N-AS, expression of Snail2 and cofactor LMO4 is critical in repression of E-Cadherin expression and activation of epithelial to mesenchymal transition (Ferreira, et al., 2013). In an analysis of 144 high-risk primary NB tumor samples, TWIST1 expression in correlated significantly with MYCN and Myc protein expression. Furthermore, in SHEP and Tet21N human NB cell lines, Twist1 was found to be a direct transcriptional target of both N-Myc and c-Myc and functioned to inhibit p53-mediated apoptosis, allowing aberrant cell cycle progression (Selmi, et al., 2015). Sox9 and Sox10 are used as markers of pre- and post-migratory NC in gene expression profiling of NB; however, any role they play in NB tumorigenicity has yet to be elucidated.

2.5 Neural crest delamination

NC delamination, also referred to as epithelial to mesenchymal transition (EMT) describes a process by which NC cells lose adhesion to neighboring neuroepithelial cells and acquire a migratory mesenchymal phenotype, allowing the cells to migrate away from the developing neural tube and colonize distant sites for further speciation and differentiation (Lamouille, et al., 2014, Theveneau and Mayor, 2012). NC delamination is initiated by NC specifier genes Sox9/10, FoxD3, Snail2 and Twist to promote cell survival, loss of cell to cell adhesion, loss of cell to matrix adhesion, and digestion of extracellular matrix.

The first step in the EMT program is transition in cell-to-cell contacts from tight junctions to gap junctions and thus eliminating cell apical to basal polarity. Snail2, activated during NC speciation, downregulates the expression of claudins and occludins, critical mediators of tight junctions that regulate signaling and calcium dependent adhesion, respectively (Ikenouchi, et al., 2003, Shin, et al., 2006). During this time, the cell also undergoes major cytoskeletal rearrangement to switch cadherins from type I (strong) to type II (weak). FoxD3 and Snail2 regulate this process by downregulating N-cadherin and E-cadherin, respectively (Cheung, Chaboissier, Mynett, Hirst, Schedl and Briscoe, 2005, Taneyhill, Coles and Bronner-Fraser, 2007). Coordinated with this, FoxD3 and Sox10 upregulate the expression of type II cadherins, including Cadherin 7 and 11 (Chalpe, et al., 2010).

Once the NC is more mobile, with weak cell-cell contacts and weak cell-ECM contacts, it begins expressing proteases to digest matrix and allow for migration away from the neural tube. Snail is thought to upregulate the expression of matrix metalloproteinases, although the mechanism in NC is unknown (Miyoshi, et al., 2005). Two key MMPs involved in NC migration are MMP2 and ADAM13. In studies of avian embryos, MMP2 was found to be a critical factor in the NC migration, where pharmacologic inhibition of MMP2 led to

significantly reduced enteric nervous system network (Anderson, 2010). In *Xenopus*, ADAM13 (A disintegrating and metalloproteinase 13) is expressed in both premigratory and migratory NC where it assists in detachment from neuroepithelium and cleavage of ECM barriers (Alfandari, et al., 2001). Expression of B1 integrin by Fox3D allows the cell to rapidly assemble and disassemble focal contacts with extracellular matrix, an essential process in directional cell migration (Delannet, et al., 1994, Lallier and Bronner-Fraser, 1993).

The process of EMT has long been implicated in cancer, with loss of cell adhesion and upregulation of MMPs as a mechanism of invasion and metastasis (Ye and Weinberg, 2015). Nevertheless, the requirement for EMT pathways and metastasis remains debated and may be tissue specific (Trimboli et al 2008, Fischer et al 2015). However, increasing evidence now suggests that EMT pathways are enriched within CSCs and may contribute to their tumorigenicity (Mitra, et al., 2015). The process of EMT is dynamic and likely consists of states with mixed gradient of mesenchymal and epithelial phenotypes. These mixed partial EMT states have been correlated to cell populations with increased aggressiveness and stemness as compared to strict epithelial and mesenchymal states (Jolly, et al., 2015). In NB, general EMT pathways have been associated with the development of drug resistance and correlate with lower overall survival. In an analysis of cisplatin resistant human NB cell lines, CHP-212Cis100, KellyCis83, and SK-N-ASCis24, all resistant lines compared to their cisplatin-sensitive counterparts were found to be enriched in gene pathways of a mesenchymal phenotype, including actin cytoskeleton signaling, integrin-linked kinase signaling, and epithelial adherens junction signaling (Piskareva, et al., 2015). In a similar analysis, SK-N-SH and SK-N-BE doxorubicin resistant cell lines had enriched expression of genes associated with epithelial to mesenchymal transition and had a more invasive phenotype as compared to their parenteral strains (Naiditch, et al., 2015).

2.6 Sympathoadrenal Speciation

NB arises exclusively within components of the sympathetic nervous system including the adrenal medulla (chromaffin cells) and paraspinal sympathetic ganglia. These cells share a common fate-restricted sympathoadrenal (SA) progenitor which develops from NC cells that have aggregated at the dorsal aorta (Anderson, et al., 1991, Shtukmaster, et al., 2013). Following EMT, migratory NC destined to become SA precursors follow a ventral migration pattern from the neural tube and aggregate at the dorsal aorta to form the primary sympathetic ganglia (Loring and Erickson, 1987). BMPs released from the wall of the dorsal aorta are essential for SA speciation and the ability of the progenitors to acquire neuronal and cholinergic properties. Studies in avian and mouse embryos have shown that expression of BMP 2/4/7 in the wall of the dorsal aorta is required for SA speciation and expression of Phox2b, a factor critical to the differentiation of SA progenitors (Reissmann, et al., 1996, Schneider, et al., 1999).

Phox2b (paired-like homebox 2b) is a transcription factor expressed in all noradrenergic neurons and is critical for the neurogenesis in the autonomic nervous system. In the absence of Phox2b expression, SA progenitors at the dorsal aorta and primitive adrenal gland lack all markers of autonomic development and lineage except for MASH-1 (Huber, et al., 2005,

Pattyn, et al., 1999). Phox2b normally functions in the activation of further SA specifiers including MASH-1, Hand2, and GATA2/3 (Huber, et. Al., 2005). MASH-1 (Mammalian achaetescute homolog 1, now ASCL1, Achaete-scute homolog 1) is a transcription factor activated by BMP signaling that is critical to functional development of the SA system. In the absence of MASH-1, migratory NC still assemble into the primary sympathetic ganglia in the vicinity of the dorsal aorta and undergo partial differentiation; however, they then undergo spontaneous apoptosis and leads to eventual loss of entire autonomic nervous system (Guillemot and Joyner, 1993, Hirsch, et al., 1998). MASH-1 functions to activate Phox2a, which activates transcription of genes associated with noradrenergic traits including expression of dopamine biosynthesis enzymes including tyrosine hydroxylase (TH) and dopamine beta-hydroxylase (DBH) (Lo, et al., 1998). Overexpression of Phox2a in is sufficient to promote autonomic neurogenesis *in vivo*; however, knockout of Phox2a expression does not affect development of sympathetic neurons or chromaffin cells and only affects development of locus cereuleus (Morin, et al., 1997, Stanke, et al., 1999). This indicates that Phox2a is sufficient to promote SA development, but not required. Hand2, GATA2, and GATA3 are transcription factors activated by Phox2b that control expression of noradrenergic markers including TH and DBH (Howard, et al., 2000, Morikawa, et al., 2005, Tsarovina, et al., 2004).

Phox2b is commonly expressed in NB and is used as a marker to detect minimal residual disease in patients following chemotherapy (Stutterheim, et al., 2008). However, gene dosage studies of Phox2b in Zebrafish revealed a dose sensitive role of Phox2b in NB tumorigenicity, where heterozygous loss or dominant negative mutations in Phox2b led to a block in SA cell differentiation and increased susceptibility to secondary transforming events in NB (Pei, et al., 2013). Correspondingly, studies in human primary tumor samples revealed that Phox2b mutations and chromosomal losses of the Phox2b loci (4p13) are early activating mutations in NB tumorigenesis (Krona, et al., 2008). Expression of known NB Phox2B variants within immature chick sympathetic neurons led to increased cell proliferation and dedifferentiation confirming the role Phox2B has in normal sympathoadrenal development as well as insights into its mechanism in NB tumorigenesis (Reiff, et al., 2010). Using the MHH-NB-11 xenograft model, knockdown of Phox2b within cell lines derived from metastatic foci led to increased primary tumor and micrometastatic burden when re-transplanted into mice indicating the importance of Phox2b as a suppressor of tumor progression and metastasis (Naftali, et al., 2016). Taken together, Phox2b, functions as a suppressor of NB tumorigenesis in a dose dependent manner and partial loss of Phox2b represents a susceptibility to NB initiation. Phox2a, Hand2, and GATA 2/3 have all been investigated in NB, but there are no clear indications of their function in relation to tumorigenicity or a block in differentiation.

2.7 Role of MYCN in SA development and NB initiation

As mentioned previously, MYCN amplification was identified in NB over 30 years ago. It remains one of the strongest indicators of poor prognosis in high risk NB, thought to be due to maintenance of tumors in an undifferentiated or poorly differentiated state (Seegler, et. Al., 1985). In NC development, while c-Myc is involved in the maintenance of multipotent NC progenitors, transient expression of MYCN stimulates ventral migration of NC and

promotes differentiation of sympathetic neurons (Wakamatsu, et al., 1997). Overexpression of MYCN, a common model for NB initiation, has vastly different effects than transient exposure.

Transgenic mouse models of NB have been developed using MYCN driven by the promoter for tyrosine hydroxylase (TH-MYCN), an enzyme involved in dopamine synthesis that expressed in the early migrating SA precursors of the adrenal gland and sympathetic ganglia. Homozygous TH-MYCN transgenic mice develop rampant abdominal and paraspinal NB tumors with high penetrance by 2 months of age (Weiss, et al., 1997). Sympathetic ganglia from non-transgenic and TH-MYCN mice showed significant peri-natal neuroblasts hyperplasia that regressed in the absence of MYCN expression but persisted and led to tumor formation within TH-MYCN mice, indicating the importance of peri-natal MYCN expression in the initiation of NB (Hansford, et al., 2004). However, expression of MYCN within SA progenitors cultured from the adrenal glands of postnatal day 0/1 mice induced proliferation and neuronal differentiation, as seen previously, but was not sufficient to promote tumorigenesis (Mobley, et al., 2015). Taken together these data suggest that NB initiation occurs embryonically during early migration or SA speciation and requires continued peri-natal expression of MYCN to drive tumorigenesis. Expression of MYCN in JoMa1, a transgenic precursor NC cell line, was sufficient to drive tumor formation in xenograft mouse model (Schulte, et al., 2013). Further evidence of this embryologic tumorigenicity comes from overexpression of MYCN within primary NCCs generated from mouse neural tube, where transformed truncal NCC explants resulted in robust generation of tumors that histologically and molecularly represent MYCN amplified NB (Olsen, et al., 2017).

2.8 Summary of NC development & NB

Studies of NB initiation using MYCN as a driver indicate that initiating mutations likely occur during NC development leading to developmental arrest. NC development is a tightly regulated process of coordinated expression of both pro-proliferative and pro-differentiation factors (summarized in Table 1). The pathways that promote stemness (FGF/STAT3, c-Myc/ID3) and differentiation (BMP, FoxD3, Phox2b) have well documented dysregulation in NB. Activation or repression of these pathways in NB likely contribute the development and maintenance of NB CSCs.

3. Cancer stem cells - controversy and definitions

3.1 Origin of CSC Model

It has long been thought that tumors retain features and pathways of normal embryologic development, where early studies of teratomas revealed striking similarities to embryologic tissue (Conheim, 1875). The more modern view on tumor development is that a tumor retains the hierarchical structure similar to its tissue of origin, with tumor “stem cells” that give rise to more differentiated tissues within the tumor, first proposed from evidence of multipotent tumor cells in testicular carcinomas (Kleinsmith and Pierce, 1964, Pierce, 1977, Pierce and Dixon, 1959). With the advancement of fluorescence automated cell sorting technology, investigators were first able to distinctly identify a tumor developmental

hierarchy in studies of acute myelogenous leukemia, with CD34+CD38– leukemia initiating cells mirroring the function of hematopoietic stem cells in normal blood development and maturation (Cashman, et al., 1997, Lapidot, et al., 1994). Subsequent studies in solid tumors found CSC subpopulations in many solid tumors including those in brain, breast, colon, lung, pancreatic, and prostate cancers (Al-Hajj, et al., 2003, Collins, et al., 2005, Eramo, et al., 2008, Hermann, et al., 2007, O'Brien, et al., 2007, Singh, et al., 2004).

A formal definition of a CSC is a tumor subpopulation that can self-renew and generate differentiated progeny that recapitulate the heterogeneous tumor of origin (Clarke, et al., 2006). One working CSC model is that within a tumor, a small subset of cells has the capacity to both divide and to expand the reservoir of CSCs as well as differentiate into the non-tumorigenic cancer cells that make up the bulk of the tumor. These CSCs have been found to be relatively refractory to modern cancer treatments including both chemotherapy and radiation through multiple mechanisms including repression of apoptosis, induced dormancy, upregulated DNA damage repair and altered drug responses (Dean, 2005)}. The ability to escape conventional radiation and chemotherapy may allow outgrowth of highly tumorigenic, drug-resistant clones leading to refractory disease and relapse. Targeting CSCs is a major movement in cancer research to develop better combination therapies that can effectively treat heterogeneous tumor populations and fully eliminate disease. However, to effectively target CSCs, we must first be able to clearly and accurately identify them.

3.2 Identifying and assaying CSCs

CSCs are generally identified by restricted expression of surface markers, combinations of surface markers, or by properties intrinsic to the cells (i.e. ability to exclude Hoechst dye through expression of drug efflux pumps).

3.2.1 In vivo analysis of CSCs—The cardinal property for identification of a CSC subpopulation is self-renewal, which can be tested rigorously only through *in vivo* serial re-implantation experiments to measure asymmetric division, proliferation of subpopulations, and long-term clonal growth (Dick, et al., 2014). In this assay, purified populations of CSCs are injected into either syngeneic or immunocompromised animals and allowed to form tumors. Tumors are then removed, dissociated, re-purified into CSC and non-CSC fractions, and re-implanted into new animals to assay continued tumor formation (McCauley and Guasch, 2013). This assay rigorously tests the ability of a CSC subpopulation to give rise to a complete heterogeneous tumor, as well as ability of CSCs self-renew and have continued long-term clonal growth.

Other *in vivo* assays include limiting dilution experiments to assay tumorigenicity and lineage tracing to assay clonality and asymmetric growth. In limiting dilution assays, pure populations of prospective tumor initiating cells are injected into syngeneic or immunocompromised animals in increasing amounts to calculate the tumor initiating frequency based on the proportion of animals that develop tumors (Blackburn, et al., 2011, Dick, et al., 1997). Lineage tracing experiments using fluorescent or chromogenic reporters allow investigators to track the portions of tumors that arise from CSC subpopulations (asymmetric growth and clonal expansion) (Humphries, et al., 2013, Wuidart, et al., 2016).

3.2.2 In vitro analysis of CSCs—*In vitro* assays represent a convenient way to screen for phenotypes of tumor initiating subpopulations; however, they lack the power and utility of *in vivo* assays, which are the gold standard for defining CSC subpopulations. Proliferation assays can be used to investigate the growth advantage and chemoresistance. The MTT assay is a simple method to quantify the ability of cells to reduce tetrazolium salts to purple formazan product. This method measures the metabolic viability of cells and is used as a surrogate way to measure cell proliferation (Mosmann, 1983). True measurement of proliferation can be done by staining cells or tissues with Ki67 or bromodeoxyuridine (BrdU), markers of proliferating cells that correlate with ribosomal RNA synthesis and DNA replication, respectively (Kee, et al., 2002). Soft agar colony formation assays are well-characterized methods for assaying anchorage independent cell growth, measuring the ability of cells to grow and divide independent of surrounding environment (Borowicz, et al., 2014, Puck, et al., 1956). Soft agar assays are often used as a surrogate for *in vivo* studies to quantify colony formation of CSC vs non-CSC subpopulations, and to test the efficacy of CSC-targeted therapeutics. However, only *in vivo* limiting dilution assays, ideally with primary patient-derived cells, can directly confirm CSC subpopulations. Evidence confirming targeting strategies should also derive from *in vivo* approaches, as tumor heterogeneity is likely impacted by the tumor microenvironment. Tumorsphere assays represent an additional colony formation assay using 3D spherical culture of cells within a specialized serum-free media and growth factor cocktail and may enrich for CSC subpopulations (Weiswald, et al., 2015). Tumorsphere formation and serial tumorsphere replating are techniques used as *surrogates* for *in vivo* re-implantation experiments. However, we emphasize that without prior purification of specific subpopulations based on valid phenotypic markers; these assays do not directly compare tumor-initiating CSCs to ‘non-CSC’ subpopulations. Growth of cells in special media with *ex vivo*-derived cocktails of growth factors can alter the gene expression profiles of the entire cell populations independent of CSC enrichment and thus does not represent a formal identification of tumorigenicity or self-renewal (Calvet, et al., 2014, Kreso and Dick, 2014). Again, CSCs and other tumor subpopulations are strongly influenced by local tumor microenvironment, further detracting from *in vitro* definitions of CSC phenotypes (Bissell and Labarge, 2005, Lathia, et al., 2011). While much work has focused on defining ‘tumor initiating’ cell lines (TIC lines) and other CSC-like tumor derived cell populations using surrogate *in vitro* assays, rigorous *in vivo* assays are required to validate the ‘stemness’ phenotype of tumor initiation subpopulations and define their role driving tumorigenesis, metastasis, and overall response to therapies.

4 The search for neuroblastoma CSCs

4.1. Previous markers claimed to identify neuroblastoma CSCs

Efforts to define CSCs in NB have focused on morphologies, cell efflux properties, tumorsphere culture methods, and cell surface markers to label subpopulations. The majority of these studies lack the defining *in vivo* experiments needed to quantify differences in tumor initiating capacity between subpopulations, prove self-renewal, and confirm recapitulation of a complex tumor from a limiting number of cells (summarized in Table 2). The earliest studies into the aggressive nature of high-risk NB identified cells with distinct

morphologies. NB cells can be classified as N-type (neuroblastic), S-type (Schwann-like) or I-type (Intermediate) (Biedler, et al., 1973). The I-type morphology was previously characterized as a malignant NC-like, being multipotent and capable of self-renewal based on the ability to regenerate I-type cells as well as differentiate into N and S-type morphologies (Ross, et al., 1995). Cell lines classified as I-type had 5-fold greater soft-agar plating efficiency and 6-fold greater tumor-forming capacity in SCID mice than cell lines of N and S-type morphologies (Walton, et al., 2004). Further characterization I-type NB cells identified a panel of genes consistently overexpressed in I-type compared to N and S-type, including CD133 [discussed below] as well as other 'stemness' markers, KIT, NOTCH1, GPRC5C, PIGF2, and TRKB (Ross, et al., 2015). However, I-type cells do not fit the definition of a CSC as they describe an entire population and likely represent aggressive, highly plastic cell population rather than a true CSC subpopulation.

Further studies of NB CSCs involved the identification of a side population (SP), cells that exclude Hoechst dye due to the presence of drug efflux pumps that confer multi-drug resistance. In an analysis of 23 primary NB tumor specimens, 65% had an identifiable side population that represented 0.8 – 51% of total tumor cells. Expression of SP correlated highly with expression of drug efflux pumps ABCG2 and ABCA3 (Hirschmann-Jax, et al., 2004). These cells were consistently phenotyped as CD45⁻, CD71⁻, GD2⁺, SP cells, which the authors indicate represents an early NC stem cell-like phenotype. In an analysis of paired pre-treatment and post-treatment relapse samples of NB cell lines, SP was increased in the relapsed samples as compared to their pre-treatment control (Newton, et al., 2010). While SP represents a chemoresistant subpopulation, analysis of SP *in vivo* tumorigenicity and differential gene expression of NC and SA markers has yet to be elucidated. Furthermore, analysis of ABCG2 as a marker of CSCs showed ABCG2 is associated with stemness in neural stem cells and has implicated as a CSC marker; however robust evaluation of ABCG2 mediated tumorigenicity has also yet to be elucidated (Islam, et al., 2005, Xing, et al., 2015). Overall, *in vivo* validation of these markers as identifiers of CSC subpopulations has not been demonstrated to date.

A surface protein studied as a marker of NB CSCs is CD133, a glycoprotein with an undefined role and a commonly used CSC marker in many other solid tumor (Grosse-Gehling, et al., 2013). Preliminary studies into CD133 expression in NB revealed that CD133 expression correlated with lower overall survival (Tong, et al., 2008). CD133 has also been associated with poor outcome in NB via activation of AKT pathway-mediated chemoresistance (Sartelet, et al., 2012). Loss of CD133 expression by shRNA knockdown led to decreased colony formation, induced cell differentiation, and suppressed proliferation via modulation of RET expression (Takenobu, et al., 2011). *In vivo* knockdown of CD133, via stable expression of shRNA within xenografts led to decreased tumor size, but no change in tumor initiation (Takenobu et al., 2011). Orthotopic injection of 500 CD133^{Hi} NB cells led to increased tumor formation and metastases compared to 500 CD133^{Low} cells, indicating an increased metastatic potential of CD133 positive cells; however, calculation of differential tumor formation capacity amongst the subpopulations was never completed (Cournoyer, et al., 2012). Furthermore, CD133 is only expressed in approximately 40% of NB cell lines and primary tumors, and this therefore precludes its use as a universal marker of NB CSCs (Takenobu, et al., 2011). A robust evaluation of the tumor initiating capacity

(limiting dilution tumor formation assay) and self-renewal (serial re-implantation assay) is lacking and CD133 cannot be considered a robust marker of NB CSCs.

As mentioned previously, the Wnt/B-catenin pathway is critical in NC induction and is found to regulate CSC-like populations in other tumor models. Analysis of Frizzled receptor 6 (FZD6), a natural receptor for Wnt, revealed that high-risk NB patients with high Fzd6 expression had poor overall survival (Cantilena, et al., 2011). Furthermore, the Fzd6 subpopulation was found within hypoxic tumor regions, formed tumorspheres more readily, and developed more aggressive tumors than its Fzd6-negative counterpart. Similarly, LGR5 is a Wnt-responsive g-protein coupled receptor found to label various CSCs (Kemper, et al., 2012; Liu, et al., 2014; Nakata, et al., 2013). Analysis of LGR5 expression in NB revealed that high LGR5 expression correlated with poor event free survival in high-risk NB patients and LGR5 was expressed in a higher percentage of high-grade undifferentiated tumors as compared to low grade tumors (Vieira, et al., 2015). Stimulation of LGR5 by Wnt3a ligands led to strong Wnt pathway induction and increased proliferation. Knockdown of LGR5 led to rapid induction of apoptosis independent of Wnt signaling, mediated by MEK/ERK signaling and cell cycle regulation. However, no *in vivo* assays were performed to analyze the effect of FZD6 or LGR5 expressing subpopulations on self-renewal, asymmetric growth, and tumor repopulation.

The aldehyde dehydrogenase family (ALDH) is involved in the biosynthesis of retinoic acid, an agent known to differentiate NB. High expression of ALDH leading to retinoic acid tolerance is thought to be a mechanism leading to resistance of retinoic acid differentiation therapy. There are 19 known ALDH isoforms and they have found to be differentially expressed in CSC subpopulations (Marcato, et al., 2011). Researchers found that both ALDH1A2 and ALDH1A3 isoform expression correlated significantly with poor overall survival in high-risk NB patients. shRNA knockdown of ALDH1A2 reduced colony and tumorsphere formation *in vitro* (Hartomo, et al., 2015). Similarly, inhibition and knockout of isoform ALDH1A3 led to reduction in NB tumor initiating cells (TICs) as demonstrated by reduced colony formation and reduced tumorsphere passaging (Flahaut, et al., 2016). However, despite these *in vitro* results, when ALDH12A knockdown cell lines were injected subcutaneously into mice, there was no difference in tumor formation and only a slight, non-significant difference in tumor size (Hartomo, et al., 2015). These ALDH studies highlight the importance of rigorous *in vivo* testing of self-renewal rather than relying on tumorsphere re-passaging, which has been shown to be an unreliable predictor of stemness. A variety of other NB CSC markers have been used in panels, including the neurofilament Nestin, cytoplasmic protein GD2, and receptor tyrosine kinase c-KIT; however, demonstration of tumorigenicity using only *in vitro* models and/or expression within only a subset of NB cell lines and tumor specimens prevents the use of these proteins as robust markers (Walton, et al., 2004; Xing, et al., 2015).

4.2. Expression of the surface receptor CD114 as a putative marker of NB CSCs

Recently, a novel NB subpopulation was characterized based on surface expression of the granulocyte colony stimulating factor (G-CSF) receptor, also known as CD114. This G-CSF receptor positive subpopulation (CD114+) is <1% of total NB cells and demonstrates several

CSC-like characteristics (Hsu, et al., 2013). Xenograft limiting dilution assays showed that CD114+ cells are 10 times more tumorigenic than their CD114- counterparts and *in vivo* lineage tracing assays showed that CD114+ cells self-renew and can regenerate differentiated (CD114-) tumor progeny. Additionally, sensitive and specific flow cytometry methods detect surface expression of a CD114 subpopulation within *all* NB cell lines, PDXs, xenografts tested to date. Critically, this tumor subpopulation has also been detected in all the primary diagnostic NB patient samples analyzed to date (N> 30) at frequencies between 0.01% and 3%. A trend towards enrichment of the CD114+ subpopulation after chemotherapy, in tumor relapses, and in metastases, indicates a level of chemoresistance within these cells (Hsu, et al., 2013, Agarwal, et al., 2015). Furthermore, the CD114+ subpopulation has a gene expression profile similar to that of primitive NC cells including markers of early pre-migratory crest (SOX10, Twist) as well as mixed EMT markers (CHD1, CHD2, Vim, MMPs). Very recently experiments using neural tube explants have demonstrated transient expression of the CSF3R gene (coding for CD114) during ex vivo expansion and MYCN-driven transformation of these primitive crest precursors (personal communication K Freeman, St Jude's Hospital). Taken together, CD114 expression marks a NB subpopulation that is highly tumorigenic when compared directly to the CD114- subpopulation, has stem cell-like molecular phenotype, and differentiates into CD114- cells *in vivo* and *in vitro*. Additionally, this subpopulation has a gene expression profile similar to that of pre-migratory or early migratory NC, which has recently been shown to maintain multipotent capacity (Baggiolini, et al., 2015). Furthermore, researchers have shown that NB tumorigenicity and metastasis are dependent on this subpopulation through downstream activation of STAT3 target genes (Agarwal, et. Al., 2015). This tumorigenicity is abrogated through treatment with G-CSF-neutralizing antibodies or STAT3 inhibition. Further work is needed to define the role of this tumor subpopulation in neuroblastoma pathogenesis and drug responses, and to determine whether targeting them will enhance current and future treatment strategies.

5. Advancing technology for identification and characterization of CSC subpopulations

5.1. Using computation models to predict stemness and drug targets

Using a network analysis approach applied to transcriptome data, researchers at the University of Chicago identified a c-Myc regulated stem cell-like gene network common to high-risk NB regardless of MYCN amplification status (Yang, et al., 2017). As a proof of concept, they performed a drug-gene interaction analysis for this c-Myc dependent network and identified a CDK inhibitor, Ronicinib, as a potential therapeutic. Treatment of NB cell lines with Ronicinib, led to downregulation of c-Myc, cell cycle arrest, and eventually cell apoptosis. Similar analyses by the same group in breast cancer models identified markers of metastasis as well as patient prognostic indicators (Yang, et al., 2014, Yang, et al., 2013). While this approach does not identify distinct markers of NB CSCs, per say, it can help us better understand the gene networks associated with aggressive cell subpopulations and identify potential CSC targets for the treatment of high-risk disease.

5.2. Single-cell RNA sequencing as a method for identifying tumor hierarchy and CSC subpopulations

Advances in RNA sequencing technology have allowed for the analysis of single-cell transcriptomes from large populations of cells (Wang and Navin, 2015). This technology provides a huge benefit to studying heterogeneous cell populations within tumors and identify subpopulations with stem-like gene signatures and enrichments (Cloney, 2017). Single cell RNA-sequencing of 430 cells from five different glioblastoma specimens showed marked intratumoral heterogeneity in transcription of genes related to activated signaling, proliferation, immune response, and hypoxic response (Patel, et al., 2014). In a large-scale analysis of 4,347 single cell transcriptome libraries from six oligodendroglioma specimens, researchers at the Broad Institute discovered clustering of a subpopulation with increased stem cell expression signatures (high SOX2/4/11, cell cyclins). These cells had increased proliferative potential and self-renewal gene signatures, providing evidence for a developmental hierarchy and CSC model in the brain tumor oligodendroglioma (Tirosh, et al., 2016). Whole tumor single-cell RNA seq would allow us to identify the developmental hierarchy that exists in NB and better identify gene networks activated in NB CSCs.

6. Conclusion

Under genetic, epigenetic or chemical pressure, the normal developmental pathways in NC cells become dysregulated leading to NB tumor initiation (Louis and Shohet). Under the pressure of oncogenic stimuli such as aberrant expression of MYCN, dysregulation of NC pathways may generate highly malignant NB CSC subpopulations. Among different NB CSC markers studied, expression of the G-CSF receptor (CD114) labels a tumor subpopulation meeting many of the criteria for CSCs. These include a) self-renewal, b) high tumor initiation potential, c) differentiation capacity to generate primarily CD114⁺ complex tumors, as defined by *in vivo* limiting dilution and lineage tracing studies. Long-term serial transplantation comparisons of CD114⁺ and CD114⁺ subpopulations remain to be performed from primary tumor tissue. Importantly, we have found this subpopulation is consistently represented in all primary tumor samples tested today (N > 30). Human CD114⁺ neuroblastoma cells have distinct genetic and epigenetic signatures similar to early neural crest, further highlighting the role of NC development as a source of tumor heterogeneity and potential tumor initiating CSCs. Ongoing research efforts will determine the precise development switches in NC differentiation pathways that define tumor-initiating populations. These include more rigorous *in vivo* testing of putative CSC markers and improvement in technology for characterizing tumor hierarchy and gene expression patterns.

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Figure 1. Neural crest development and neuroblastoma pathogenesis

NC development is a tightly regulated process beginning with neural tube formation and ending with the formation of terminally differentiated NC derivatives, including the sympathoadrenal lineages. Each step of the process is controlled by cascades of signaling pathways and activation of key transcription factors. The exact stage along this pathway where NB originates still remains unknown; however, key pathways highlighted below have been shown to contribute to ‘stemness’ and may be involved in the formation of tumor initiating NB CSCs.

Table 1

Summary of factors involved in NC development and their relation to NB tumorigenesis.

Gene	Function NC Development	Association in NB
NC Induction		
BMP	Induction of NP border specifiers ¹	Tumor differentiation (downregulated) ²
FGF	Induction of NP border specifiers ³	--
STAT3	Activation of NC border and NC specifiers ⁴	Stemness, tumorigenicity, chemoresistance ⁵
Wnt	Induction of NP border specifiers ⁶	Chemoresistance ⁷
Notch/Delta	Induction of NP border specifiers ⁸	Inhibition of differentiation ⁹
NP Border Specification		
Gbx2	Activation of NC Specifiers ¹⁰	--
AP2	Activation of NC Specifiers ¹¹	--
Msx1	Activation of NC specifiers: Snail2, FoxD3, Twist1 ¹²	Notch signaling; proliferation, inhibition of differentiation ¹³
Pax3	Activation of NC specifiers: Snail2, FoxD3, and Sox9 ¹⁴	Proliferation, migration, invasion, chemoresistance ¹⁵
Zic1	Activation of NC specifiers: Snail2, FoxD3, and Sox9 ¹⁴	--
NC Specification		
c-Myc	Maintenance of multipotency ¹⁶	Inhibition of differentiation; proliferation; poor overall prognosis ¹⁷
Id3	Maintenance of multipotency ¹⁶	--
Sox9	Initiation of delamination (EMT); inhibition of apoptosis ¹⁸	--
Sox10	Initiation of delamination (EMT); inhibition of differentiation ¹⁹	--
FoxD3	Initiation of delamination (EMT) ²⁰	Suppression of growth, invasion (downregulated) ²¹
Snail2	Initiation of delamination (EMT) ²²	Migration, invasion, metastasis ²³
Twist1	Initiation of delamination (EMT) ²⁴	Inhibition of apoptosis ²⁵
Sympathoadrenal Specification		
Phox2a	Activation of sympathoadrenal specifiers ²⁶	--
Phox2b	Activation of sympathoadrenal specifiers ²⁷	Suppression of tumorigenesis (dose dependent) ²⁸
MASH-1	Activation of sympathoadrenal specifiers ²⁹	--
Hand2	Activation of noradrenergic enzymes ³⁰	--
GATA2/3	Activation of noradrenergic enzymes ³¹	--

NC development is a tightly regulated process of coordinated expression of both pro-proliferative and pro-differentiation factors. The pathways that promote stemness (FGF/STAT3, c-Myc/ID3) and differentiation (BMP, FoxD3, Phox2b) have well documented dysregulation in neuroblastoma.

Note: Individual factors in EMT and delamination have not been well-documented in neuroblastoma, and have thus been omitted from this table.

¹Marchant, et. al., 1998, Stottmann and Klingensmith, 2011, Leung, et. al., 2016;

²Sumantran, et. al., 2003, Du and Yip, 2010;

³Furthauer, et. al., 1997, Hong, et. al., 2008, Mayor, et. al., 1997, Monsoro-Burq, et. al., 2003, Wilson, et. al., 2000;

⁴Nichane, et. al., 2010;

- ⁵Agarwal, et. al., 2015, Odate, et. al., 2017, Yco, et. al., 2014;
- ⁶Abu-Elmagd, et. al., 2006, Chang and Hemmati-Brivanlou, 1998, Garcia-Castro, et. al., 2002, Leung, et. al., 2016, Tan, et. al., 2001;
- ⁷Vangipuram, et. al., 2012;
- ⁸Cornell and Eisen, 2000, Cornell and Eisen 2002, Hernandex-Lagunas, et. al., 2011;
- ⁹Chang, et al., 2010; Grynfield, et. al., 2000, Ferrari-Toninelli, et. al., 2010, Liu, et. al., 2017;
- ¹⁰de Croze, et. al., 2011;
- ¹¹Li, et. al., 2009;
- ¹²Liu, et. al., 2004;
- ¹³Revet, et. al., 2008, Revet, et. al., 2010;
- ¹⁴Monsoro-Burq, et. al., 2005, Plouhinec, et. al., 2014;
- ¹⁵Fang, et. al., 2014;
- ¹⁶Kee and Bronner-Fraser, 2005; Light, et. al., 2005;
- ¹⁷Seeger, et. al., 1985, Wang, et. al., 2015, Cotterman and Knoepfler, 2009, Hasan, et. al., 2013, Selmi, et. al., 2015;
- ¹⁸Cheung and Briscoe, 2003, Cheung, et. al., 2005;
- ¹⁹John, et. al., 2011;
- ²⁰Cheung, et. al., 2005;
- ²¹Li, et. al., 2013;
- ²²Taneyhill, et. al., 2007;
- ²³Ferronha, et. al., 2007, Tanno, et. al., 2010;
- ²⁴Cheung, et. al., 2005;
- ²⁵Selmi, et. al., 2015;
- ²⁶Lo, et. al., 1998, Morin, et. al., 1997, Stanke, et. al., 1999;
- ²⁷Huber, et. al., 2005, Pattyn, et. al., 1999;
- ²⁸Krona, et. al., 2008, Pei, et. al., 2013, Naftali et. al., 2016, Reiff, et. al., 2010;
- ²⁹Guillemot and Joyner, 1993, Hirsch, et. al., 1998;
- ³⁰Howard, et., al, 2000, Morikawa, et. al., 2005;
- ³¹Tsarovina, et. al., 2004

Table 2

Summary of properties associated with putative NB CSCs

Marker	Tumorigenic Properties			Stem Cell Properties	
	Tumor Formation	Metastasis	Chemoresistance	<i>In vivo</i> self-renewal	Asymmetric growth
Properties					
I-type ¹	X	--	--	--	X
Side population (SP) ²	--	--	X	--	--
Proteins					
ABCG2 ³	X	--	--	--	--
ALDH ⁴	X	--	X	--	--
CD114 ⁵	X	X	X	X	X
CD133 ⁶	X	X	X	--	--
FZD6 ⁷	X	--	--	--	--
LGR5 ⁸	X	--	--	--	--

As evidenced by Table 2, most of the markers of NB CSCs characterized thus far describe subpopulations of cells with higher tumorigenicity as evidenced by increased tumor formation, increased metastasis, or increased chemoresistance. CD114 is the only marker characterized meets the majority criteria for defining a NB CSC subpopulation. These cells have the capacity for self-renewal, proven via lineage tracing, and asymmetric growth, proven by flow cytometry analysis of tumors formed from pure CD114+ cells.

¹Ross, et al., 1995, Walton, et al., 2004, Ross, et al., 2015;

²Hirschmann-Jax, et al., 2004, Newton, et al., 2010;

³Xing, et al., 2015;

⁴Hartono, et al., 2015, Flauhaut, et al., 2016;

⁵Hsu, et al., 2013, Agarwal, et al., 2015;

⁶Tong, et al., 2008, Takenobu, et al., 2011, Courmoyer, et al., 2012, Sartelet, et al., 2012;

⁷Cantilena, et al., 2011;

⁸Vieira, et al., 2015