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Neuroblastoma Aggressiveness in Relation to Sympathetic Neuronal Differentiation Stage

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Abstract

Neuroblastoma is a childhood malignancy of the sympathetic neuronal lineage. It is a rare disease, but since it is frequently diagnosed during infancy, neuroblastoma causes life-long medical follow up of those children that survive the disease. It was early recognized that a high tumor cell differentiation stage correlates to favorable clinical stage and positive clinical outcome. Today, highly differentiated tumors are surgically removed and not further treated. Cells of many established human neuroblastoma cell lines have the capacity to differentiate when stimulated properly, and these cell lines have been used as models for studying and understanding central concepts of tumor cell differentiation. One recent aspect of this issue is the observation that tumor cells can dedifferentiate and gain a stem cell-like phenotype during hypoxic conditions, which was first shown in neuroblastoma. Aberrant or blocked differentiation is a central aspect of neuroblastoma genesis. In this review we summarize known genetic and non-genetic events in neuroblastoma that might be coupled to an aberrant sympathetic neuronal differentiation and thereby indirectly influencing tumorigenesis and/or aggressive neuroblastoma behavior.

Keywords

Neuroblastoma; Differentiation; Hypoxia; Sympathetic nervous system; Tumor stem cells

Neuroblastoma - a model of tumor cell differentiation

In 1927, Cushing and Wohlbach described a case of sympaticoblastoma that spontaneously converted into a benign, differentiated tumor (1). Since this first annotation, the concept of spontaneous differentiation of neuroblast-like tumor cells into differentiated, less aggressive cells of ganglioneuroblastoma and ganglioneuroma, is textbook knowledge. The link between neuroblastoma and the sympathetic nervous system (SNS) was early recognized based on morphological similarities between neuroblastoma cells and cells of the developing SNS. The consensus of today that neuroblastoma is derived from the SNS has been gradually established, founded on observations that primary neuroblastomas localize to sites where SNS ganglia are positioned, that they express SNS marker proteins (2, 3) and produce catecholamines. The latter observation led to the early utilization of urinary catecholamine metabolites in the diagnosis of neuroblastoma (4). The finding that some human neuroblastoma cell lines have the capacity to differentiate into a more mature sympathetic differentiation stage by phorbolester (5) and retinoic acid (6, 7) treatments, together with similar observations in hematopoietic tumor cell lines, established the concept of tumor cell differentiation and *in vitro* model systems to study this phenomenon (reviewed in (8)).

Neural crest and development of the sympathetic nervous system

The sympathetic nervous system (SNS) originates from the neural crest, an ephemeral structure during vertebrate development. Located along the neural fold, cells on the neural tube in the ectoderm give rise to the neural crest, in turn generating different derivatives, such as the cranial, hindbrain, vagal and trunk neural crest (reviewed in (9)). Trunk neural crest cells migrate from the neural tube along two defined routes, with one group of cells migrating early along a ventral pathway, giving rise to glial cells and neurons, whereas a later migrating group of cells follows a lateral pathway and gives rise to melanocytes in the skin (10, 11).

When trunk neural crest cells migrate from the neural tube, fate restriction occurs as more than 85% of trunk neural crest progenitor cells become committed within 30-36 hours post-migration (11). However, it has been suggested that subpopulations of progenitor cells with maintained multi-lineage differentiation potential are retained even after migration to sites of homing, as such multipotent cells have been found in dorsal root ganglia and in gut of rodents and avians (12-18), and in peripheral nerves in rat (19).

Depending on environmental cues, such as neurotrophins, bone morphogenetic proteins (BMPs), endothelin 3, members of the wingless (wnt) family and neuregulins, trunk neural crest cells differentiate into neurons, peripheral glial cells and melanocytes. It is believed that the main cell types of the SNS (the sympathetic neurons, the small intensely fluorescent (SIF) cells with unknown function, and the chromaffin cells of the adrenal medulla and paraganglia) derive from a common ancestor cell, the committed sympathoadrenal (SA) progenitor, in turn derived from aggregated neural crest cells at the dorsal aorta (20, 21). The different growth factors and cognate receptors influencing neural crest cell fate are temporally expressed at appropriate places and growth factor gradients serve as cues leading to receptor-mediated migration of neural crest-derived cells. BMPs are expressed in the dorsal aorta and gut, structures where autonomic neurons develop (22, 23), whereas endothelin 3 is expressed along the dorsal migration path of melanocytic precursors, where it regulates self-renewal and melanocytic differentiation of bipotent glial-melanocytic neural crest-derived stem cells (24, 25).

As determined by expression patterns and inhibition studies, BMP signaling is essential for initiation of differentiation of neural crest cells into sympathetic neurons (22, 26-30). The first gene expressed in neural crest-derived SNS precursor cells aggregated around the dorsal aorta

is *ASCL1*, which encodes the protein Hash-1, (Cash-1 and Mash-1 in chicken and mouse, respectively). This basic helix-loop-helix (bHLH) pro-neural gene is expressed even prior to the time when BMPs can be detected in the dorsal aorta, suggesting that BMPs rather maintain than induce *ASCL1* expression in SNS precursors (30, 31), however it has been shown that BMPs are capable of directly inducing *ASCL1* expression as well. Further differentiation and specification of these precursor cells, although not fully understood, rely on coordinated expression of transcription factor genes *ASCL1*, *PHOX2A*, *PHOX2B*, and *HAND2* at the same location as neural SNS progenitor cells reside (32-41).

BMPs can also directly initiate transcription of *PHOX2B*, encoding a homeodomain DNA binding protein, and interestingly, *PHOX2B* is mutated in a few cases of neuroblastoma. The interest for *PHOX2B* in neuroblastoma was caught when it was identified as the major disease-causing gene of congenital central hypoventilation syndrome (CCHS) (42). Patients with CCHS have a predisposition for developing tumors of the SNS, such as neuroblastoma, ganglioneuroma and ganglioneuroblastoma (43). Soon after the discovery of the role of *PHOX2B* in CCHS, four independent studies revealed heterozygous missense and frame-shift mutations in the *PHOX2B* gene, both in hereditary and sporadic neuroblastoma (44-47). These mutations were rare in all four studies indicating locus heterogeneity for predisposition of inherited disease, but still demonstrated the importance of the *PHOX2B* gene in neuroblastoma oncogenesis and initiation (Fig. 1). The Phox2b protein plays an important role in the development of the sympathoadrenal lineage, and Phox2b and Hash-1 can induce transcription of one another, whereas Phox2b alone regulates expression of related protein Phox2a and DNA-binding protein Hand2. In addition, Hand2 can, in an autocrine fashion, induce *PHOX2B* and *ASCL1* transcription, and it is suggested that two parallel but interacting pathways work to cross-activate the expression of these transcription factors. Several

heterodimeric bHLH transcription factors expressed in the nervous system are involved in specification and differentiation of neuronal progenitor cells and early neuroblasts (Reviewed in (48, 49)). Although both Hash-1 and Hand2 are frequently expressed in neuroblastoma, their expression levels are not associated with clinical stage (50).

Shortly after instructions on neural crest cell fate by *ASCL1*, *PHOX2A/B* and *HAND2* and commitment to the neuronal SNS lineage, proteins such as the bHLH transcription factor N-myc function as cues for continued sympathetic neuronal differentiation. In neuroblastoma, the *MYCN* proto-oncogene is frequently amplified, suggesting that cells at the *MYCN*-expressing stage are targeted to cause neuroblastoma (Fig. 1). *MYC*, however, is rarely amplified in neuroblastomas, and as high N-myc expression represses myc in neuroblastoma cells (51), *MYC* expression is probably not permissive at the stage where sympathetic neuroblasts express N-myc and hence are susceptible to tumorigenic transformation. During rodent SNS development, *MYCN* expression is involved in differentiation, whereas *MYC* is preferably expressed in proliferating cells (52). Whether these observations reflect that deregulated *MYCN* expression in neuroblastoma cells causes an early stage differentiation arrest in *MYCN* amplified neuroblastomas remains to be elucidated.

From committed progenitor cells, cell-type specific genes, together with mainly *HAND2* and *PHOX2A/B* induce differentiation into sympathetic neurons expressing noradrenaline-producing enzymes tyrosine hydroxylase (TH) and dopamine beta-hydroxylase (DBH), and pan-neuronal markers such as *SCG10* and neurofilaments.

Late stage and terminal differentiation of sympathetic neurons requires receptor stimulation by the neurotrophins NT-3 and NGF. When committed progenitor cells are instructed for

further differentiation, TrkC, the receptor for NT-3, is upregulated and proper TrkC-NT-3 signaling is induced. This further stimulates expression of TrkA and the neuroblasts become dependent on NGF, which is an important limiting factor for neuroblast survival and differentiation. Secretion of NT-3 and NGF will attract Trk-expressing neuroblasts, a central mechanism determining the patterning of the SNS, and the Trks continue to be expressed in SNS structures throughout human fetal development (53-55). Interestingly, high expression of TrkA and TrkC, i.e. a differentiated sympathetic neuronal tumor phenotype, correlates with favorable outcome in neuroblastoma ((56, 57), reviewed in (58), and Fig. 1).

Neuroblastoma differentiation stage and clinical outcome

There is a well-documented difference in aggressive behavior between the morphologically undifferentiated (neuroblastoma proper) and the differentiated (ganglioneuroblastoma, ganglioneuroma) forms of neuroblastoma, where the latter forms are mostly classified as clinically low-stage tumors. Employing single markers of neuronal differentiation such as neuron-specific enolase, the neuronal form of $pp60^{c\text{-}src}$ and the neurotrophin receptors $p140^{trkA}$ and $p145^{trkC}$ (2, 56, 59, 60), a positive correlation between low clinical stage and a differentiated morphology with high expression of these differentiation markers has been shown.

By using a global gene expression approach combined with pathway analyses of two large sets (251 and 101 patients, respectively) of neuroblastoma specimens with clinical follow-up data (61, 62), a more extensive analysis of tumor differentiation stage and relation to patient outcome was recently presented (63). In this study, a gene expression signature of nine genes associated with late sympathetic neuronal differentiation was constructed and the expression activity score ("pathway activity") of this gene set was calculated for individual tumors and

correlated to clinical stage, risk assessment, event-free survival and overall survival. There was a strict co-variation between neuronal pathway activity and all four of these clinical variables in that high stage of differentiation associated with less aggressive disease (63). In the same report, a gene expression signature for myc pathway activity (myc, N-myc and L-myc downstream target genes) was also constructed. As expected, high myc pathway activity correlated to aggressive disease and low overall survival, as well as to the immature phenotype (63). When combining MYC and differentiation pathway data distinct clusters of patients with different event-free and overall survival could be identified. Interestingly, some of these clusters of patients had tumors with high MYC pathway activity despite the fact that they were classified as highly differentiated. More important and clinically relevant, several of the low and intermediate risk patients that relapsed grouped according to their gene expression signature together with more aggressive, high stage tumors of low differentiation grade and high MYC pathway activity (63). Based on these findings, it can be concluded that the risk-assessment criteria used in the clinic today would benefit and be strengthened by inclusion of selected pathway activation analyses as exemplified in Fredlund et al. (63).

Cellular adaptation to hypoxia

The biological consequences of hypoxia involve induced angiogenesis, a switch in glucose metabolism and increased invasion and migration capacities, changes that stimulate tumor progression. The hypoxic response is mainly mediated by stabilization of the hypoxia inducible factor (HIF) proteins. HIFs are heterodimeric transcription factors composed of an oxygen sensitive α subunit and a constitutively expressed β subunit. At physiological oxygen levels, the α subunit is hydroxylated by the oxygen-dependent prolyl hydroxylases (PHDs), leading to interaction with the von Hippel Lindau-ubiquitin ligase complex, followed by ubiquitination and proteasomal degradation. At hypoxia, the PHDs are inactive leading to the

stabilization of the HIF-α subunits, and by dimerization with the β subunit the complex induces transcription of a number of genes regulating the plethora of cellular functions constituting the cellular adaptation to diminished oxygen concentrations. The two α isoforms HIF-1α and HIF-2α are similar to some extent but also show exclusive stabilization patterns and specific target genes (64). The third and less studied HIF-3α subunit seems to be a negative regulator of the hypoxic response due to its lack of a transcriptional activation domain (65, 66). HIF-α transcriptional activity can also be regulated without affecting protein stability through factor inhibiting HIF (FIH). At normoxia, FIH hydroxylates HIF-α at an asparagine residue, which prevents interaction with co-activators p300/CBP and thereby HIF-dependent transcription.

Hypoxia and tumor cell differentiation

In a subset of neuroblastomas, tumor cells with a differentiated phenotype similar to neuroendocrine cells can be distinguished from immature neuroblast-like tumor cells based on morphology and expression of lineage specific marker genes (60, 67). In these tumors, which often have a distinct lobular growth pattern with necrotic zones in the center of the lobules (Fig. 2), the cells with neuroendocrine features are detected in regions close to necrotic zones and they are non-proliferating as determined by PCNA and Ki67 expression (67). The fact that the cells with a neuroendocrine differentiated phenotype are positioned adjacent to necrotic zones led to the speculation that the *in vivo* spontaneous lineage shift from a neuronal to a neuroendocrine phenotype observed in this subset of neuroblastomas could be driven by hypoxia (68). In an effort to test this hypothesis, Jögi et al. (68) exposed a panel of human neuroblastoma cell lines to hypoxic conditions (1% O_2) and evaluated the expression of sympathetic neuronal and chromaffin lineage marker genes, respectively, as well as markers associated with the neural crest stage. Unexpectedly at that time, the cells exposed to hypoxia

did not develop a neuroendocrine phenotype, but the result rather suggested that hypoxia drives neuroblastoma cells towards an undifferentiated, stem cell-like phenotype ((68) and Fig. 2). Specifically, the expression of chromaffin marker genes *CHGA/B*, ganglionic/neuronal marker genes *NPY, GAP-43, SCG10*, and SNS lineage specifying genes *ASCL1* and *HAND2* were all downregulated in hypoxia, while neural-crest marker genes *ID2*, *NOTCH1, HES1* and *KIT* were upregulated (68). The observation that hypoxia induces dedifferentiation of neuroblastoma cells was later confirmed in studies based on expression patterns obtained by micro-array analyses (69, 70). Although basically similar data as those we have reported, Hedborg and co-workers argue that hypoxia drives neuroblastoma cells towards a differentiated state with induction of chromaffin features and expression of NESP55 (71, 72). This raises the question on the definition of a sympathetic chromaffin cell phenotype. Few would dispute the fact that high expression of chromogranin A and B are obligate hallmarks of sympathetic chromaffin cells. Chromogranins together with a number of neuronal and neuroendocrine marker genes are consistently downregulated when cultured neuroblastoma cells are exposed to hypoxic conditions (68-70). We concluded that these cells dedifferentiated towards a neural crest-like phenotype rather than gaining chromaffin features (68). In neuroblastomas with lobular structures, we have reported the contradictory observation that hypoxic cells present with chromaffin features (e.g. increased expression of chromogranin A/B) (3), but it is likely that neuroblastoma cell lines are not derived from such tumors as they generally belong to the more highly differentiated subgroup of neuroblastomas.

Dedifferentiation as a consequence of hypoxia does not seem to be restricted to neuroblastoma, but if this is a general mechanism in cancer biology remains to be elucidated. Ductal breast carcinomas in situ (DCIS) have well-defined lesions of hypoxic regions

surrounding necrotic areas, and are thus suitable for *in vivo* investigation of the hypoxic response. In addition, breast cancer cells are clinically assessed regarding their differentiation status according to established morphological criteria. Helczynska et al., showed that hypoxic cells in DCIS were less differentiated compared to non-hypoxic cells (73). In glioma, hypoxia promotes self-renewal of both the stem and non-stem cell population, as well as pushing the non-stem cells into a stem cell-like phenotype (74). Colorectal cancer stem cells cultured at hypoxia show increased clonogenicity, inhibited differentiation and induced expression of markers associated with a stem cell-like phenotype (75). Also, prostate carcinoma LNCaP cells exposed to hypoxia downregulate differentiation-specific proteins such as prostate-specific antigen and androgen receptor, suggesting that dedifferentiation in response to hypoxia occurs also in prostate cancer (76).

Hypoxia and the stem cell phenotype

The correlation between hypoxia and an undifferentiated stem-cell phenotype has also been observed in normal stem cells. Early embryonic development occurs during low oxygen and by culturing human embryonic stem cells at lower oxygen concentrations (3-5% O_2), their pluripotency is maintained while the spontaneous differentiation that occurs during normoxic (21% O_2) oxygen culturing conditions is prevented (77). Furthermore, growing mesenchymal stem cells at low oxygen concentrations preserve their stem cell properties, whereas stem cells cultured during normoxic conditions become senescent (78). In addition, near-physiological oxygen tensions (5-6%) improve the efficiency of induced pluripotent stem cell generation (79). Several studies on *in vitro* differentiation of neural stem cells have shown that low oxygen levels promote proliferation, survival and multipotency as compared to normoxic oxygen levels (80-86). Hematopoietic stem cells (HSCs) in the bone marrow have been proposed to exist in a low oxygen milieu (87, 88). However, the location of HSCs has

recently been found to include, besides the endosteum close to the bone marrow, a niche adjacent to specialized blood vessels surrounded by CXCL12-secreting cells (89, 90). Similarly, neural stem cells and adipose progenitors have been shown to reside in close proximity to highly vascularized areas (91, 92). In total, this suggests that the oxygen concentration is an important factor for regulating the stem-cell phenotype.

Like normal stem cells, cancer stem cells also depend on their microenvironment for maintaining stem cell-like properties. In human neuroblastoma specimens, we have shown that small subsets of cells located adjacent to blood vessels express high levels of HIF-2α and VEGF while lacking expression of SNS marker proteins, such as TH and neuron-specific enolase (93). In the same cells, expression of neural crest and early SNS progenitor marker proteins such as Notch-1, Hes-1, Vimentin and Hand2 is detected. At least in *MYCN*-amplified tumors, we could verify that that the HIF-2α positive cells were tumor-derived by showing that they harbored the *MYCN* amplification. In neuroblastoma specimens, intense HIF-2α staining correlates with aggressive disease and poor outcome (64), and thus, we proposed that these immature and stem cell-like cells are putative neuroblastoma stem cells (64, 93, 94), however, their tumor-initiating capacity has not been demonstrated *in vivo*.

Similar to what has been proposed in neuroblastoma, glioma stem cells reside in a perivascular niche that maintains self-renewal capacity and an undifferentiated state of the cells (95). This niche is supported by tumor-secreted VEGF (96). Interestingly, the glioma stem cells express HIF-2α and targeting this protein led to reduced levels of VEGF, decreased self-renewal and lower tumorigenic capacity of glioma stem cells (97). Additional reports also support the notion that HIF-2α is an important factor for regulating the hypoxic niche in glioblastoma (74, 98). Interestingly, during normal development, neural precursors are located

close to the developing aorta suggesting that proximity to blood vessels is a stem cell feature (91, 92). As glioma and putative neuroblastoma stem cells also are found adjacent to blood vessels, this might reflect shared features with normal stem cells (93, 95).

Since hypoxia has been shown in several studies to induce an undifferentiated cell phenotype, it might seem contradictory that normal and cancer stem cells have been found close to blood vessels in presumably well-oxygenized environments. However, physiological oxygen levels at end capillaries are in the range of 5-7% and tumor vasculature is generally leaky inferring that also vascularized tumor areas might not be fully oxygenized. Nevertheless, it is only a subset of neuroblastoma and glioma cells that have high expression of HIF-2α suggesting that HIF-2α is a marker of at least neural tumor stem or stem-like cells, a hypothesis in agreement with the high and transient HIF-2α expression seen in the developing SNS (99). These observations also suggest that HIF-2α expression in these cells *in vivo* is regulated by other mechanisms additionally to oxygen level-dependent protein degradation. Several growth factor signaling pathways such as the PI3K/Akt/mTOR and MAPK pathways are known to stimulate HIF-α synthesis during normoxia (reviewed in (100)), however, this regulatory mechanism is cell-type specific and most of the studies have focused on HIF-1α whereas oxygen-independent regulation of HIF-2α remains to be elucidated.

There are a few reports on the mechanisms by which hypoxia can regulate the stem cell phenotype and whether it is a HIF driven process. As previously described, the bHLH transcription factors Hash-1, Hand2 and N-Myc are important in the differentiation of neural crest cells into sympathetic neuroblasts. The observed down-regulation of these proteins at hypoxia suggests that they are mechanistically involved in the hypoxia-driven dedifferentiation of neuroblastoma cells. Both Hash-1 and Hand2 form functional complexes

with other ubiquitously expressed bHLH proteins, called E-proteins. The E-proteins are in turn regulated by HLH (helix-loop-helix) factors such as the ID proteins, which sequester the E-proteins from tissue specific bHLH factors, such as Hash-1 and Hand2. The observed upregulation of ID2 in hypoxic neuroblastoma cells and the fact that ID2 is a direct target gene of HIF-1α provides a mechanistic link between hypoxia and dedifferentiation in neuroblastoma (101). Another pathway suggested to be involved in dedifferentiation during hypoxia is Notch signaling. Activation of Notch leads to expression of the Hes family of transcriptional repressors, which downregulate the expression of differentiation-promoting bHLH factors, such as Hash-1. During hypoxia, HIF-1α is able to directly interact with the Notch signaling pathway by binding and stabilizing the Notch intracellular domain, leading to activation of Notch target genes and blocking of neuronal development (102).

Can the developmental stage at which neuroblastomas originate be determined?

Considering the extensive molecular characterization of the early steps of sympathetic neuronal differentiation and the global gene expression analyses of large clinical neuroblastoma materials that have been carried out, it might seem feasible to distinguish the developmental stages at which neuroblasts can be malignantly transformed. If the gene expression profile of a tumor reflects the developmental stage at which its initiating cell became transformed, collective data suggest that neuroblastoma can arise at almost any developmental stage after progenitor cell commitment to the neuronal lineage. However, as discussed here, physiological parameters such as the oxygen level and metabolic state have profound effects on the tumor cell phenotype, and the gene expression profile of a certain tumor more likely reflects the phenotype of the bulk of tumor cells, rather than reflecting the actual stage at which the tumor developed. So far, a neuroblastoma stem cell has not been defined, but if such a cell can be identified, the developmental stage at which this cell became

arrested could indicate also the stage(s) at which neuroblastoma tumors develop. However, as tumor cells can dedifferentiate under various conditions, the tumor stem cell phenotype does not need to reflect the actual stage of tumor cell transformation. We have proposed that a set of immature neural crest-like HIF-2α positive cells located adjacent to vasculature are the neuroblastoma stem cells (as discussed above), and if this notion is correct, neuroblastoma could be considered a stem cell disease of the SNS.

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Figure legends

Figure 1.

Trunk neural crest cells migrate from the neural tube instructed by environmental cues, such as the early expressed bone morphogenetic proteins (BMPs). Shortly after cells have migrated, fate restriction occurs and cells are committed to melanocytic, glial and sympathetic neuronal lineages. The main cell types of the SNS, the sympathetic neurons, the SIF and the chromaffin cells, are proposed to have a common origin, the sympathoadrenal (SA) progenitor cell. Differentiation into sympathetic neurons is orchestrated by a defined set of transcription factors, expressed at various stages of development. The *MYCN* gene is expressed after cell restriction induced by *ASCL1*, *PHOX2A/B* and *HAND2*, and it is feasible

that *MYCN*-amplified tumors arise at this developmental stage. Differentiated tumor cells, expressing high levels of the neurotrophin receptors TrkA and TrkC, associate with better patient outcome, and less aggressive neuroblastomas as well as the benign forms ganglioneuroblastomas (GNBs) and ganglioneuromas (GNs) presumably originates from cells at this stage.

Figure 2.

A. Schematic illustration of a neuroblastoma section depicting dedifferentiated hypoxic cells close to a necrotic area, tumor bulk cells, and putative neuroblastoma tumor-initiating cells adjacent to tumor blood vessel. Tumor bulk and hypoxic cells express markers of the SNS lineage to various extent, while a subset of cells close to tumor vasculature are negative for differentiation markers but stain intensely for HIF-2α. We have previously proposed that these immature, HIF-2α positive cells are the stem or tumor initiating cells in neuroblastoma.

B. Schematic illustration of cell differentiation patterns in lobular neuroblastomas, a small subset of more differentiated tumors growing in characteristic lobular structures. In these tumors, a neuronal-to-neuroendocrine shift can be detected with the neuroendocrine cells (e.g. high chromogranin A and B expression) positioned adjacent to the necrotic zones of the lobular structures (for further details, see text and (67)).

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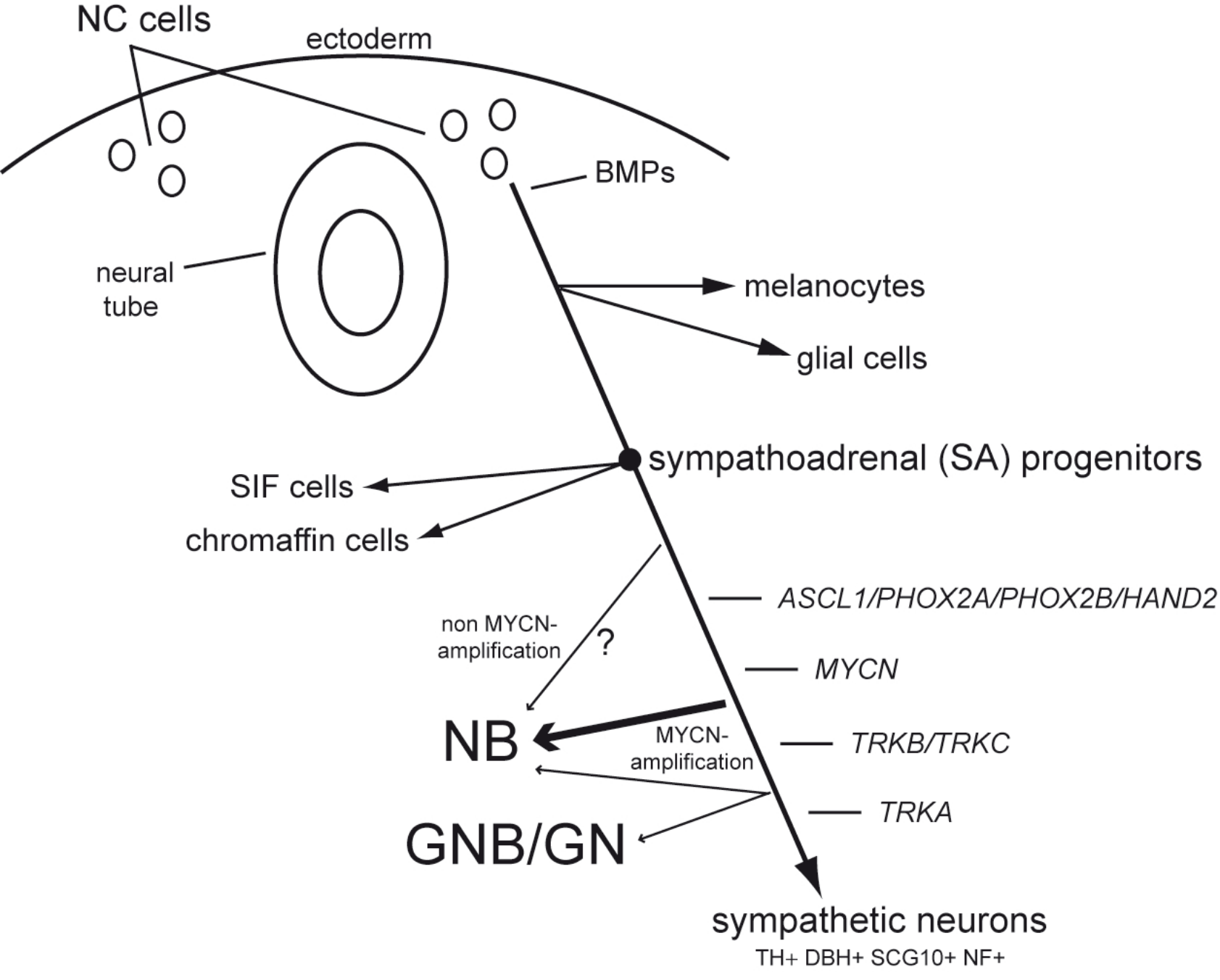
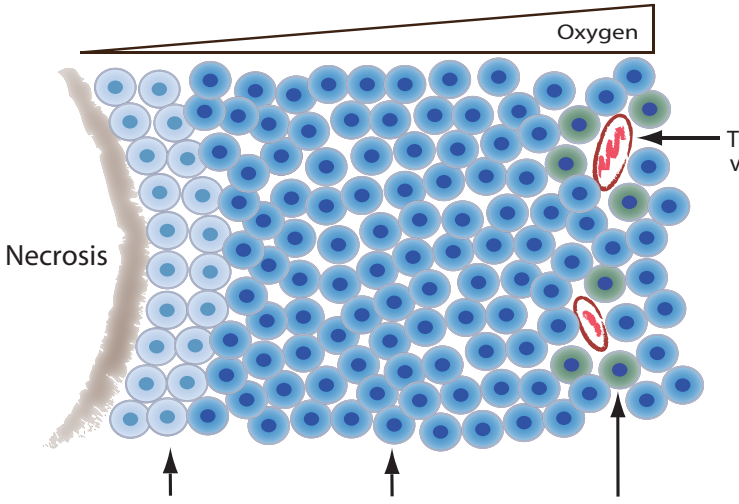
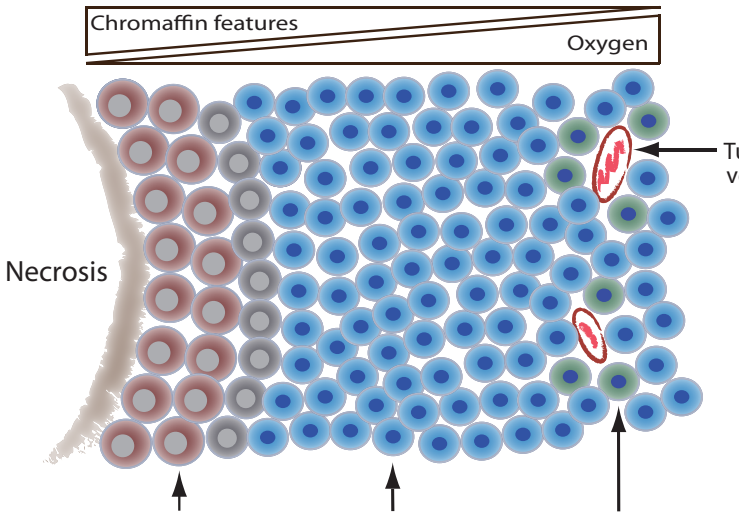

NC cells
ectoderm
BMPs
neural tube
melanocytes
glial cells
sympathoadrenal (SA) progenitors
SIF cells
chromaffin cells
ASCL1/PHOX2A/PHOX2B/HAND2
non MYCN-amplification
?
MYCN
NB
MYCN-amplification
TRKB/TRKC
TRKA
GNB/GN
sympathetic neurons
TH+ DBH+ SCG10+ NF+

A. Tumor oxygenation and differentiation status in NB



	Hypoxic cell	Tumor bulk cell	Putative NB stem cell
HIF-1α	++	–	–
HIF-2α	++	–	++
SNS lineage markers	+	++	–
Neural crest markers	–	–	+

B. Lobular NB with chromaffin differentiation features



	Hypoxic cell	Tumor bulk cell	Putative NB stem cell
HIF-1α	++	–	–
HIF-2α	++	–	++
Chromaffin markers	++	+	–