Molecular and Developmental Biology of Neuroblastoma

Akira Nakagawara

Contents

5.1 Neural Crest Development and Neuroblastoma

Cancer has its own face reflecting the characteristics of the tissue from which it is derived. This can be demonstrated by histopathologic examination, by immunohistochemistry, and/or by in situ hybridization. Recent advances in molecular biology and genetics have also revealed that these morphological distinctions among cancers are associated with differences in gene expression profiles within tumor cell and stromal cell components. Furthermore, the patterns of gene expression unique for each cancer are dictated by genetic abnormalities which have occurred in progenitors of the specific developmental lineage. Neuroblastoma originates from the sympathoadrenal lineage, and its biology is closely related to that of normal sympathetic neurons. In this chapter, the molecular and cellular bases for the genesis and biology of neuroblastoma are summarized.

5.1.1 Genes of Neural Development and Molecular Targets of Neuroblastoma

During neural development, neural crest cells migrate and differentiate into several cell lineages, e.g., melanocytes, sensory neurons, enteric ganglion cells, and sympathetic neurons (Fig. 5.1). The first signaling molecules which trigger crest cells to differentiate or migrate are bone morphogenetic proteins (BMPs) and their receptors (Huber et al. 2002). The commitment to differentiate into sympathetic neurons is associated with the transient expression of (a) basic helix-loop-helix transcription factors, e.g., *MASH1* (a proneural gene homologous to *drosophila achaete-*

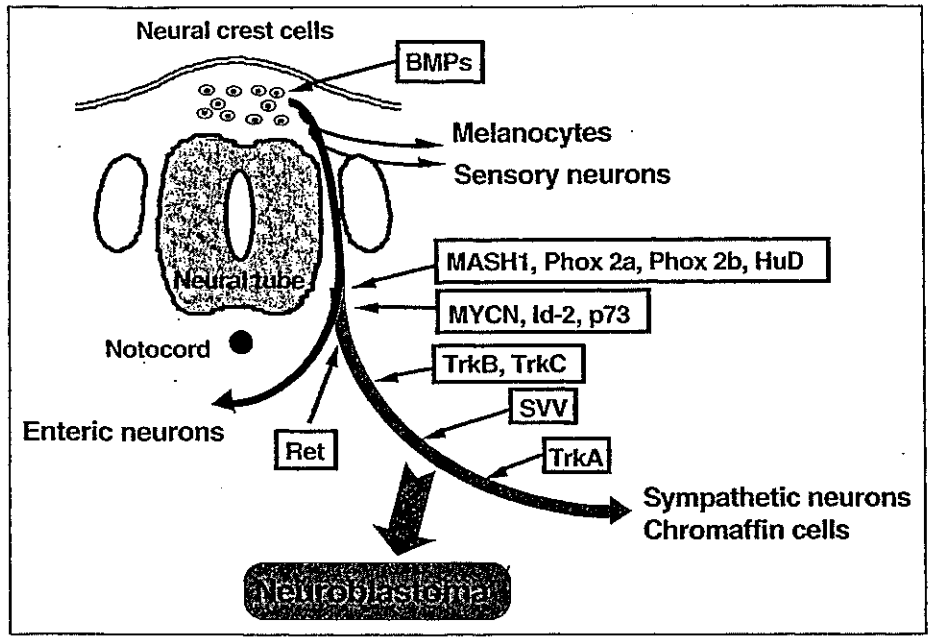


Figure 5.1

Neuroblastoma originates from the sympathoadrenal lineage of neural crest. The bone morphogenetic protein (BMP) signals may be important at the early stage of differentiation of neural crest cells. *MASH1* (*hASH1*) may function as one of the key transcription factors which define the direction of differentiation to sympathetic neurons. The other important nuclear factors, e.g., Phox2a, Phox2b, HuD, *MYCN*, Id2, and p73, may also be involved in the cell-fate determination. Some of those genes are often upregulated or amplified in aggressive neuroblastomas (Nakagawara 2004). At the stage of terminal differentiation of sympathetic neurons followed by programmed cell death, the signals through neuronal tyrosine kinase receptors, e.g., Ret, TrkB, TrkC, and TrkA, are necessary sequentially and/or in a form of crosstalk. The many genes involved in regulation of neuronal terminal differentiation or programmed cell death are often expressed at high levels in favorable neuroblastomas.

scute), *HES1*, *MYCN*, HIF1α and HuD, (b) homeobox genes, e.g., Phox2a and Phox2b, and (c) p73 (a family member of the tumor suppressor gene p53; Nakagawara 2004). Several lines of investigation support the importance of these genes. *MASH1* null mice lack sympathetic ganglion cells (Guillemot et al. 1993). Notch signaling, through its intracellular domain translocation into the nucleus, stimulates the transcriptional activation of the *HES1* and *HES5* genes whose products in turn inhibit transcription of the *MASH1* gene (Radtke and Raj 2003). *MYCN* is indispensable for the normal neural development. It induces Id2 which is a negative regulator of *HES1* and pRb, a retinoblastoma suppressor (Lasorella et al. 2000). p73 knockout mice also show abnormalities in cell survival in both the nervous and immune systems (Yang et al. 2000). Gene targeting of HIF2α disturbs the catecholamine metabolism in sympathetic neurons (Tian et al. 1998). All these genes regulate each other in an orchestrated manner to drive the correct differentiation of neural crest cells into sympathetic neurons.

Further downstream, terminal differentiation to mature sympathetic cells is strongly regulated by the signaling of neurotrophin family members and their receptors (Nakagawara 2001, 2004). In addition, other genetic aberrations associated with neuroblastoma have been mapped to specific genomic regions or genes well known to be important in regulating the normal development of neurons (Nakagawara 2001, 2004). It seems obvious that a relationship should exist between the genetic or biological targets of neuroblastoma and the key molecules involved in the normal development of neural crest cells.

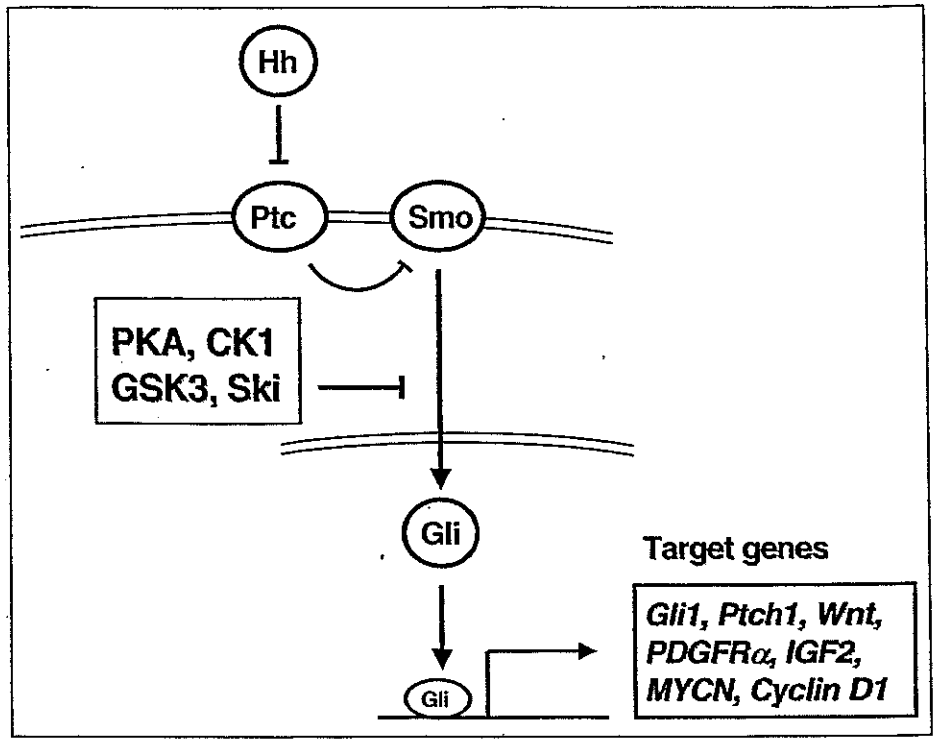


Figure 5.2
Hedgehog-Gli signaling in neural development and tumorigenesis. Sonic hedgehog (Hh) signaling activates Gli transcription factors which then induce the target genes important for regulating neural differentiation as well as neuronal tumorigenesis. They include *MYCN*, *cyclin D1*, *IGF2*, and *PDGFRα*, all of which are known to be players characterizing neuroblastoma biology. *T bars* show inhibitory interactions. *Arrows* show positive interactions

5.1.1.1 Bone Morphogenetic Proteins

Bone morphogenetic proteins (BMPs), members of the transforming growth factor-β (TGF-β) superfamily, may be the first signal that defines the early phase of differentiation and migration of neural crest cells during development (Oppenheim 1991). The ligand-dependent activation of BMP receptors transduces its signal into the nucleus through the sequential activation of Smad signaling molecules by phosphorylation. Although the role of BMPs in neuroblastoma has long been elusive, Nakamura et al. (2003) have recently reported that SH-SY5Y and RTBM1 neuroblastoma cell lines are responsive to BMP2 leading to growth arrest and differentiation. Of interest, BMP treatment also induces the downregulation of p53 family members including p53 and p73, as well as their target gene, $p21^{WAF1}$. In contrast, a similar cyclin-dependent kinase inhibitor, $p27^{KIP1}$, is markedly induced at the protein level by downregulation of Skp2, a component of its E3 ubiquitin ligase complex. BMP is also a direct transcriptional target of retinoic acid which induces neuroblastoma differentiation (see Chap. 15; Rodriguez-Leon et al. 1999). The DAN family members are inhibitors of BMP, and are also expressed in neuroblastomas (Enomoto et al. 1994). The DAN gene itself, which is mapped to chromosome 1p36, is a transcriptional target of BMP (Nakamura et al. 2003; Shinbo et al. 2002), suggesting that the BMP signaling network may be important in the differentiation and survival of neuroblastoma (Nakamura et al. 2003). The role of other important signals which function during neuronal development, including Sonic Hedgehog (Shh) and Wnt, is less well known in neuroblastoma. Interestingly, the Shh downstream signaling molecule, Gli, can transactivate *MYCN* and cyclin D1 (Altaba et al. 2004) (Fig. 5.2).

5.1.1.2 *MASH1/hASH1*

Achaete-Scute homolog-1 (*MASH1* in rodents and *hASH1* in humans) is a basic helix-loop-helix transcription factor which plays an important role in the early development of neural and neuroendocrine progenitor cells (Ball 2004). Helix-loop-helix proteins include achaete-scute homologs, E proteins, *MYCN*, *Math*, *NeuroD*, *neurogenin*, *Id*, and *HES*. Targeted disruption of *MASH1* in mice has led to the absence of

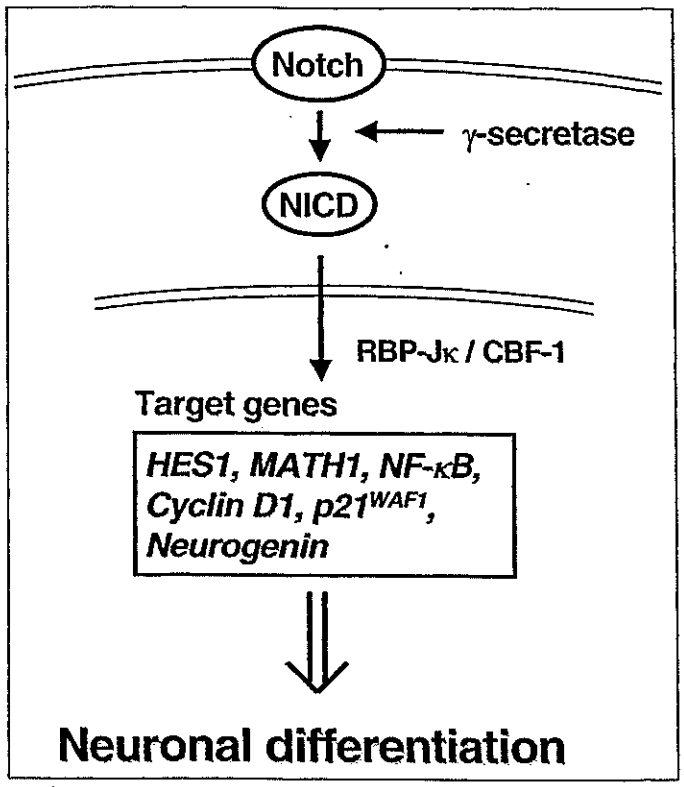


Figure 5.3

Notch signaling transactivates gene expression to induce neuronal differentiation. Binding of the ligand delta to its receptor notch triggers intramembrane proteolytic cleavage by γ-secretase. This results in the release of the notch intracellular domain (NICD), which then translocates to the nucleus where it associates with the CSL family of DNA binding proteins and transactivates gene expression. The target genes include *HES1, MATH1, NF-kB, cyclin D1, p21,* and *neurogenin. HES1* then inhibits transactivation of *MASH1 (hASH1)*

sympathetic neurons, suggesting the important role of *MASH1* in sympathetic differentiation (Guillemot et al. 1993). *MASH1* is transiently induced during neural development to promote neuronal cell differentiation; however, high *hASH1* expression persists in neuroblastoma tumors and cell lines (Soderholm et al. 1999; Ichimiya et al. 2001). Retinoic acid treatment decreases the expression of *hASH1* and induces neurite extension (Ichimiya et al. 2001). *hASH1* also directly represses the expression of PACE4, a mammalian subtilin-like proprotein convertase that activates TGF-β-related proteins (e.g., BMPs) in neuroblastoma cell lines (Yoshida et al. 2001). The Notch signaling pathway also plays a key role during neuronal development (Axelson 2004). One of the important regulators of *hASH1* is a basic HLH protein, *HES1* (Fig. 5.3). *HES1* is regulated, at least in part, by Notch signaling and is induced at the transcription level. *HES1* directly binds to the promoter of *hASH1* and inhibits its transcriptional activation. A constitutively active form of Notch could block neurite extension during the induced differentiation of human neuroblastoma cells, possibly by inhibiting *hASH1* through the induction of *HES1* (Radtke and Raj 2003).

5.1.1.3 Phox2a and Phox2b

Phox2a and Phox2b are paired-like homeodomain transcription factors with complete conservation in their homeodomain. They are specifically expressed in noradrenergic neurons and activate the tyrosine hydroxylase and dopamine-β-hydroxylase genes (Schneider et al. 1999; Stanke et al. 1999; Ernberger 2000). While the expression of Phox2a is regulated by *MASH1*, Phox2b is not (Lo et al. 1999) (Fig. 5.4). The genetic disruption of either Phox2a or Phox2b gene de-monstrated that both genes are essential for the development of autonomic neural crest derivatives (Morin et al. 1997; Pattyn et al. 1999). Interestingly, Trochet et al. (2004) reported that the Phox2b gene was mutated in a family case of neuroblastoma and in a neuroblastoma patient with Hirschsprung's disease.

5.1.1.4 Id

Id proteins generally function as inhibitors of differentiation and as positive regulators of proliferation in neuronal development (Lavarone and Lasorella 2004). Id is a protein with the helix-loop-helix domain without a basic region and forms heterodimers with bHLH proteins, e.g., *MASH1* and *HES1* to inhibit their transactivation function (Massari and Murre 2000). In pediatric cancers, *MYC* oncoproteins and EWS-Ets fusion proteins are targeted to induce Id2 which in turn inhibits Rb and other target proteins including bHLH proteins, Ets and Pax. In neuroblastoma, *MYCN* has been shown to induce Id2 which stimulates cell proliferation by inhibiting Rb function (Lasorella et al. 2000).

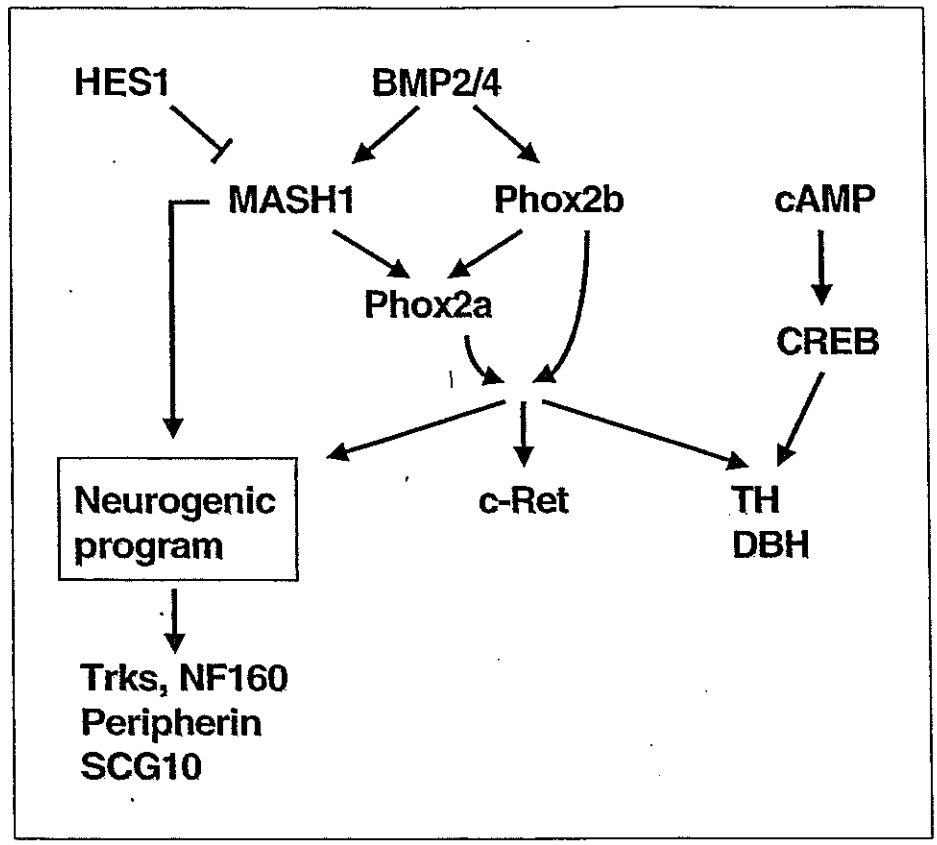


Figure 5.4

Regulatory network controlling sympathetic neuron development. BMP2 and BMP4 are required for the expression of *MASH1* and *Phox2b*. *HES1*, induced by notch signaling, inhibits expression of *MASH1*. *MASH1* and *Phox2b* are genetically upstream of *Phox2a*, and *Phox2b* is genetically upstream of *Gata3*. Expression of tyrosine hydroxylase (TH) and dopamine-β-hydroxylase (DBH) depends on *MASH1*, Phox2b, and Gata3. Cyclic AMP also controls expression of TH and DBH. Phox2a and Phox2b may affect induction or maintenance of *MASH1* expression. *MASH1*, Phox2a, and Phox2b regulate the downstream neurogenic program, leading to terminal differentiation of sympathetic neurons by inducing the genes, e.g., Trks, NF160, peripherin, and SCG10

5.1.1.5 *MYCN*

MYCN is a member of the group of *MYC*-box genes, and its product is a bHLH protein (Schwab et al. 2003). *MYCN* is transiently expressed during normal neural development and defines the direction of neuronal differentiation. *MYCN* is frequently amplified in advanced-stage neuroblastoma (Schwab et al. 1983, 1984; Brodeur et al. 1984; Seeger et al. 1985), and the biology of high-risk neuroblastoma is influenced by the subsequent overexpression of *MYCN* oncoprotein and its targets including telomerase and those functioning in ribosome biogenesis and protein synthesis (Mac et al. 2000; Boon et al. 2001).

5.2 Molecular Bases of Differentiation and Programmed Cell Death

5.2.1 Molecular Aspect of Spontaneous Regression

It is well known that some subsets of neuroblastoma can regress spontaneously. One of the most important hints to understand the mechanism of spontaneous regression is age of the patient at the onset of neuroblastoma. Regression rarely occurs when the tumor is found in patients over 1 year of age. The dramatic regression of the stage 4s tumor after its rapid growth usually occurs within 6 months after birth; therefore, it is plausible that epigenetic regulations, timed with the development of sympathetic neurons, might also control neuroblastoma regression. It is well known that massive death of sympathetic neurons is induced during the perinatal period – a process called developmentally regulated neuronal programmed cell death following deprivation of tar-

get tissue-derived neurotrophins (Oppenheim 1991). This same death mechanism appears to be conserved in primary neuroblastomas found in infants, leading to the induction of their spontaneous regression (Nakagawara 1998b).

5.2.2 Neurotrophic Factors and Their Receptors

5.2.2.1 Neurotrophins and Their Receptors in Neuroblastoma

The neurotrophin family of growth factors consists of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5; Huang and Reichardt 2003). The corresponding high-affinity neurotrophin receptors with tyrosine kinase activity have been identified as TrkA, TrkB, and TrkC (Snider 1994) (Fig. 5.5 a, b). TrkA is a preferred receptor for NGF, TrkB for BDNF and NT-4/5, and TrkC for NT-3. All of the neurotrophins also bind similarly to a lower-affinity neurotrophin receptor $p75^{NTR}$, a member of the tumor necrosis factor receptor (TNFR)/Fas family (Snider 1994). The targeted disruption of neurotrophins and their receptors has demonstrated that NGF/TrkA signaling supports the survival and differentiation of sympathetic and sensory neurons responsive to temperature and pain, while BDNF/TrkB, NT-4/TrkB, and NT-3/TrkC signaling supports those of sensory neurons responsive to tactile stimuli and motor and sensory neurons responsive to limb movement and position, respectively (Klein 1994). These results suggest that neural development and maintenance of the neural network are spatiotemporally controlled by neurotrophin signaling with or without some redundancy in both peripheral and central nervous systems.

In neuroblastoma, high levels of TrkA are expressed in subsets of tumors with good prognosis, often showing spontaneous regression (Nakagawara et al. 1992, 1993; Suzuki et al. 1993; Kogner et al. 1993). Such tumors usually occur in patients under 1 year of age, and their DNA ploidy is aneuploid. A very limited amount of NGF may be supplied from stromal cells, e.g., Schwannian cells and fibroblasts, which at least partly regulate the differentiation and programmed cell death of neuroblastoma cells (Nakagawara 1998a). On the other hand, TrkA expression is strongly downregulated in tumors with aggressive behavior that usually possess amplification of the *MYCN* oncogene and allelic loss of chromosome 1p36 (Nakagawara et al. 1992, 1993). TrkB is preferentially expressed in aggressive neuroblastomas together with its preferred ligands BDNF and NT-4/5 which stimulate in an autocrine/paracrine manner, conferring an enhanced malignant phenotype to the tumor cells (Nakagawara et al. 1994; Matsumoto et al. 1995). TrkC is expressed in favorable neuroblastomas at variable levels (Yamashiro et al. 1996), but its preferred ligand, NT-3, is nearly undetectable by RT-PCR in primary neuroblastomas (Nakagawara 1998a); thus, in regressing neuroblastomas, tumor cells expressing the TrkA receptor may be dependent on a limited amount of NGF supplied from stromal cell. In the presence of NGF the cells mature, whereas they will die in the absence of this ligand (Nakagawara 1998a,b); however, in clinically aggressive neuroblastomas, the TrkA is downregulated and the downstream signaling cascades are disturbed, and these cells utilize the BDNF or NT-4/TrkB autocrine system for efficient growth. Neurotrophin signaling may also regulate tumor metastasis (Matsumoto et al. 1995), proliferation (Matsumoto et al. 1995), and angiogenesis (Canete et al. 2000). The role of $p75^{NTR}$ in neuroblastoma is unclear. The $p75^{NTR}$ receptor is expressed in both neuroblastoma cell lines (Azar et al. 1990) and primary neuroblastomas (Nakagawara et al. 1993). Interestingly, the expression levels of $p75^{NTR}$ mRNA are significantly higher in favorable neuroblastomas (stages 1, 2 and 4s) as compared with the advanced stage tumors, especially those with *MYCN* amplification (Nakagawara et al. 1993).

5.2.2.2 Neurotrophin Signaling in Neuroblastoma

In a rat pheochromocytoma cell line PC12, differentiation signals by NGF may be mediated through the tyrosine phosphorylation of the Trk receptor and through the subsequent activation of Shc/Grb2/SOS, Ras, Raf, MEK, and ERKs, while survival signals in the same cells may be transduced through the direct

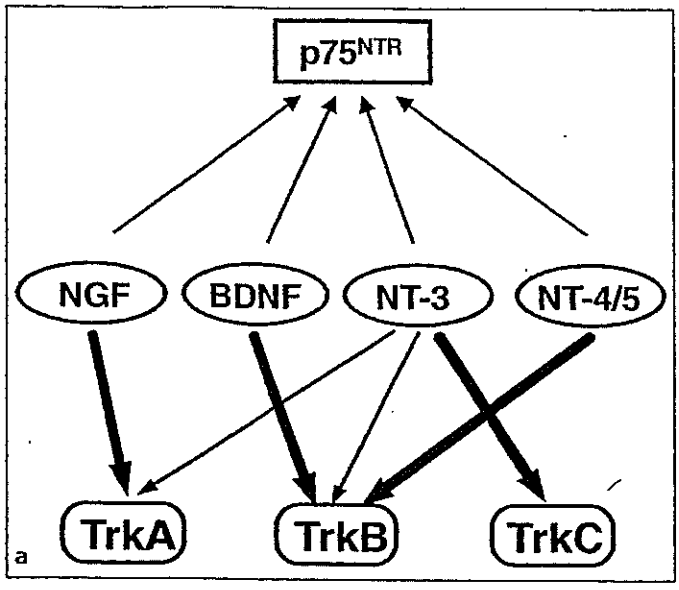
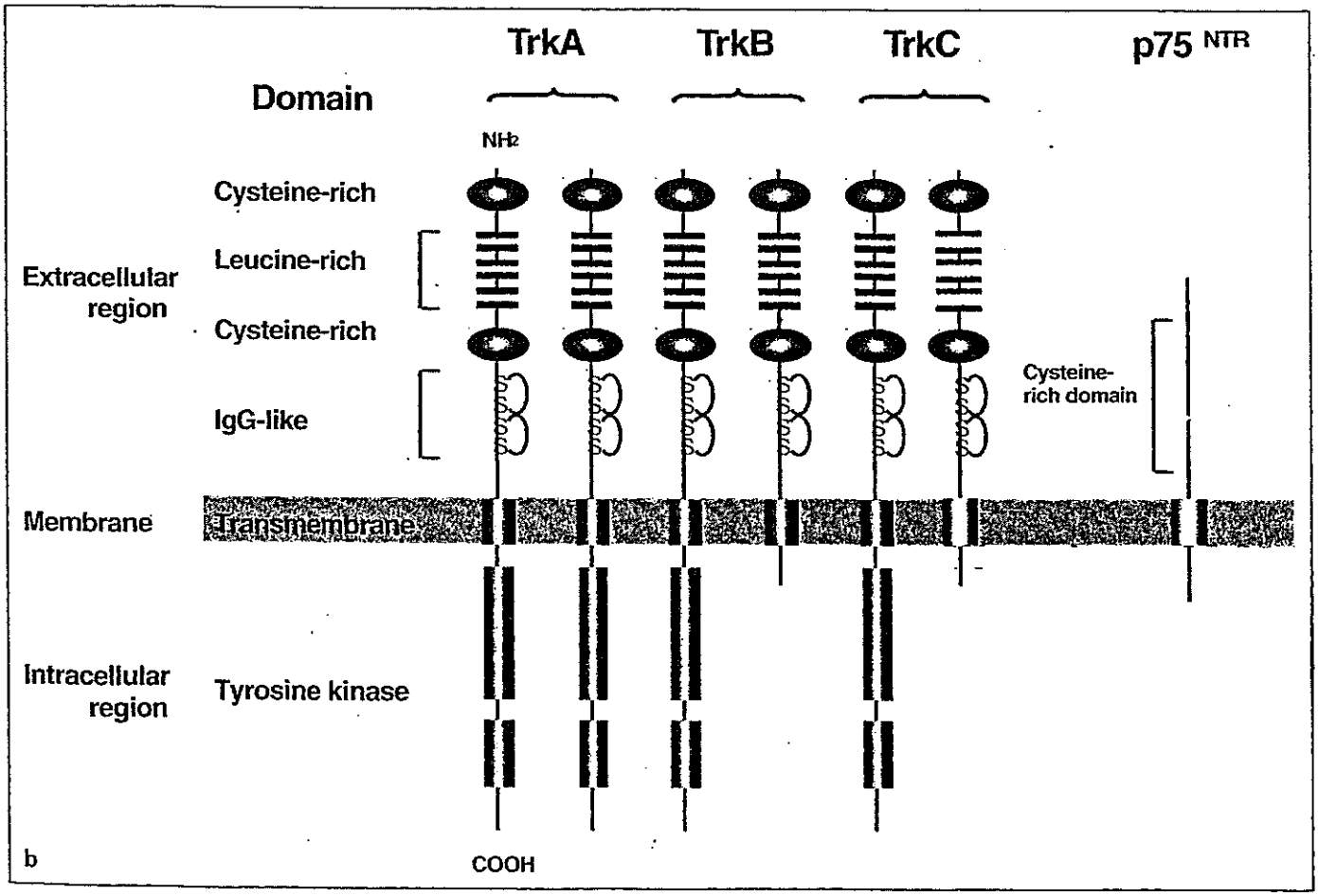


Figure 5.5 a, b

Neurotrophins and their receptors. a TrkA is a preferred high-affinity receptor for NGF, TrkB for BDNF, and NT-4/5, and TrkC for NT-3. All of the neurotrophins also bind similarly to a lower affinity neurotrophin receptor $p75^{NTR}$. b The structures of neurotrophin family receptors. The extracellular domains of TrkA, TrkB, and TrkC have high structural similarity. The intracellular domain of Trks possesses tyrosine kinase activity. TrkB and TrkC receptors have truncated forms which lack the tyrosine kinase domain. The low-affinity receptor, $p75^{NTR}$, has a short intracellular region containing the death domain, and belongs to the Fas/TNFR family of the receptors.



activation of PI3-kinase which in turn activates downstream molecules, e.g., Akt and Bad (Klesse and Parada 1999). On the other hand, in normal sympathetic neurons, the activation of PI3-kinase is mediated not by the tyrosine phosphorylation of the receptor but by the Ras activation which promotes neuronal survival, suggesting that the Trk intracellular signaling pathway might be deregulated in cancer cells. This is also the case in neuroblastoma. In the neuroblastoma cell lines with a single copy of *MYCN*, NGF can induce differentiation when exogenous TrkA is overexpressed (Eggert et al. 2000). In the cell lines with *MYCN* amplification, however, the NGF-stimulated TrkA receptors which were overexpressed cannot normally activate downstream signaling molecules, resulting in unresponsiveness to the ligand. Furthermore, it is surprising that BDNF/TrkB signaling appears to be functioning in the same cells by promoting survival (Nakagawara et al. 1994; Hishiki et al. 1998), although the signaling pathway might be different from that of sympathetic neurons (Klesse and Parada 1999).

5.2.2.3 GDNF Family Receptors

Neurotrophic factors of the glial cell line-derived neurotrophic factor (GDNF) family, which include GDNF, artemin and neurturin, are secreted by neuroblastoma cells as well as stromal cells and activate their receptor complex composed of Ret tyrosine kinase and the GFRα co-receptors expressed in neuroblastoma cells (Hishiki et al. 1998; Ichikawa et al. 2004). In contrast to NGF/TrkA and BDNF/TrkB, however, the GDNF/Ret/GFRα autocrine system is functioning in both favorable and unfavorable neuroblastomas to enhance the survival and differentiation of tumor cells (Hishiki et al. 1998).

5.2.2.4 Other Factors and Receptors

Neuroblastoma cells express other growth factors and receptors. Both pleiotrophin (PTN) and midkine (MK) are factors in the same family with neurotrophic function (Kadomatsu et al. 1990; Li et al. 1990; Kadomatsu and Muramatsu 2004). PTN is expressed significantly at high levels in favorable neuroblastomas, while MK is highly expressed in almost all neuroblastomas with a tendency to be expressed at high levels in tumors in advanced stages (Nakagawara et al. 1995). Neuroblastoma also expresses many other receptors, e.g., fibroblast growth factor receptor (FGFR; Schweigerer et al. 1991), insulin-like growth factor (IGFR; El-Badry et al. 1991), DCC (deleted in colon cancer) (Reale et al. 1996), and neuronal leucine-rich repeat receptors (NLRRs; Hamano et al. 2004), as well as a novel plasma membrane enzyme ECEL1, which is significantly highly expressed in favorable neuroblastomas (Kawamoto et al. 2003). The biological significance of these factors and receptors in neuroblastoma are not currently known.

5.2.3 Functional Role of p53 Family Genes

Recent lines of evidence suggest that both the p53 tumor suppressor protein and its related protein p73 are involved in the induction of programmed cell death and growth arrest in neuronal cells (Pozniak et al. 2000). p73 is a recently identified candidate tumor suppressor gene mapped to chromosome 1p36.2, a frequently deleted region in many human cancers including neuroblastoma and oligodendroglioma (Ichimiya et al. 1999; Billon et al. 2004). In cultured neonatal sympathetic neurons, p53 protein levels are increased in response to NGF withdrawal as well as $p75^{NTR}$ activation, and it functions downstream of c-Jun NH_2-terminal kinase (JNK) and upstream of Bax to induce apoptosis (Aloyz et al. 1998) (Fig. 5.6). Indeed, in $p53^{-/-}$ mice, naturally occurring sympathetic neuron death is inhibited. Pozniak et al. (2000) have also reported that p73 is primarily present in developing neurons as ΔNp73, an NH_2-terminally truncated isoform, whose level is decreased when sympathetic neurons undergo apoptosis after NGF withdrawal, and that p53 becomes activated to be pro-apoptotic. In contrast to the truncated form of p73, full-length p73 has induced neuronal differentiation in a mouse neuroblastoma cell line N1E115 (Laurenzi et al. 2000). These data suggest that the neuronal apoptosis induced by NGF withdrawal is at least partly regulated by a reciprocal balance between levels of pro-apoptotic p53 and anti-apoptotic ΔNp73.

Figure 5.6

A model of signaling pathway for survival and death in sympathetic neurons regulated by NGF. NGF depletion may induce activation of JNK/p53 pathway which could be modified by p73/ΔNp73 regulatory system. $p75^{NTR}$ activation, which sends signals of both survival and death, may also regulate downstream p53/p73/ΔNp73 pathway

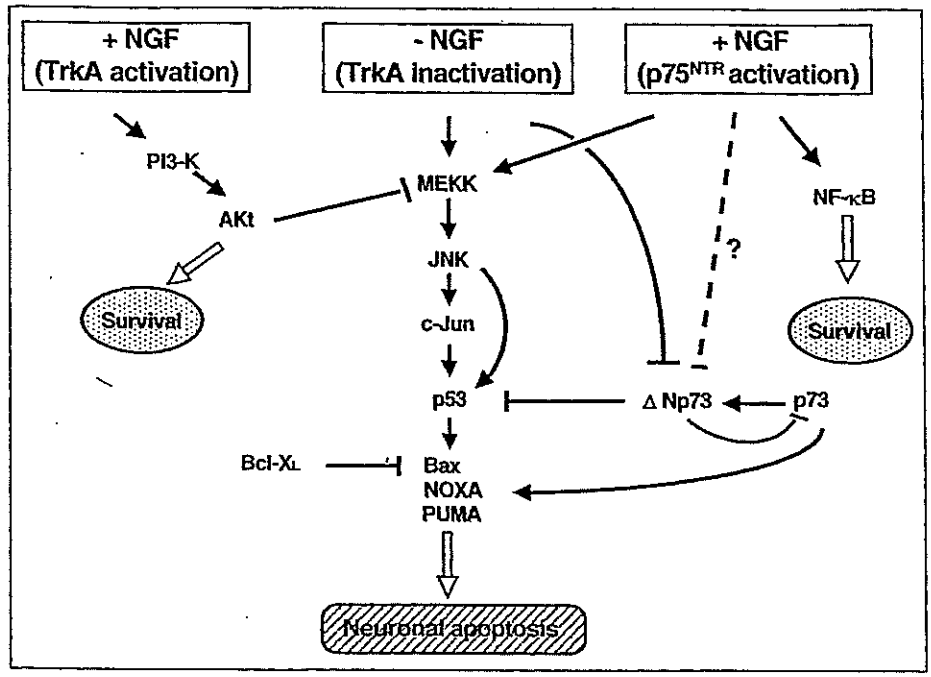
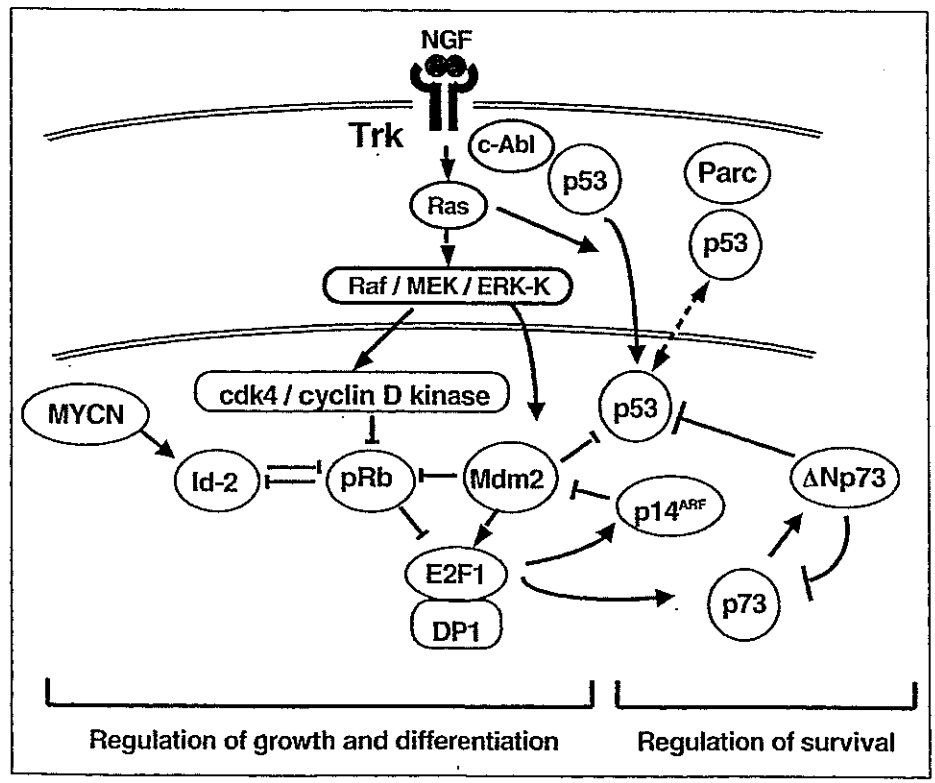


Figure 5.7

A possible signaling pathway regulating growth, differentiation and survival in neuroblastoma cells or sympathetic neurons. The NGF-triggered autophosphorylation of TrkA tyrosine kinase receptor induces activation of Ras/MAPK pathway, which in turn regulates nuclear pRB and Mdm2. In some poor-outcome neuroblastomas, p53, which is shuttling between cytosol and nucleus, is trapped in the cytosol by Parc, an anchoring protein of p53. *MYCN* induces expression of *Id-2* whose protein product in turn inhibits pRB. E2F1 negatively regulated by pRB directly induces expression of p73. p73 is regulated by ΔNp73 in a negative autoregulatory manner (Nakagawa et al. 2002), and ΔNp73 also inhibits p53



The importance of p53 and p73 has also been emphasized by the important observation that, in cultured neuroblastoma and other cancer cells, p73 directly transactivates the ΔNp73 gene by binding to its promoter after treating the cells with genotoxic reagents, e.g., cisplatin (Nakagawa et al. 2002). The induced ΔNp73 protein in turn interacts with either wild-type p53 or TAp73 and inhibits their proapoptotic function; thus, ΔNp73 can act as an oncogene and as an inhibitor of wild-type p53 and TAp73. The presence of this autoinhibitory feedback loop among p53, TAp73, and ΔNp73 may at least in part explain why there is no mutation of the p73 gene in cancers.

p53 is associated with TrkA via the proto-oncogene product c-Abl as an adaptor or bridging molecule, suggesting that it may also play a role in Trk signaling (Yano et al. 2000) (Fig. 5.7). The activation of Ras by NGF stimulation of the TrkA receptor induces p53 nuclear translocation and growth arrest in PC12 cells (Hughes et al. 2000). The c-Ha-Ras gene could be a target of p53, and protein products induce a positive feedback loop by activating $p14^{ARF}$ which counteracts the negative feedback loop mediated by mdm2 (Deguin-Chambon et al. 2000). These observations strongly suggest that p53 and p73 tumor suppressors function in neurotrophin signaling and modulate the growth, differentiation, and apoptosis of neurons.

In neuroblastoma and some other human cancers, wild type p53 is often localized in the cytoplasm (Moll et al. 1995). Although the regulatory mechanism of cellular localization of p53 and p73 is still unknown, activated Ras in NGF/TrkA signaling stimulates the nuclear translocation of p53 and leads to growth arrest by the induction of $p21^{WAF1}$ in PC12 cells (Hughes et al. 2000). Furthermore, some fractions of recurrent neuroblastomas and neuroblastoma cell lines acquire mutation of the p53 gene (Tweddle et al. 2001).

5.2.4 Apoptotic Signals in Neuroblastoma

To date, the spontaneous regression of neuroblastoma, has occurred only *in vivo*. Although this makes the analysis difficult, there are some important reports. An anti-apoptotic protein, Bcl-2, is expressed in primary neuroblastomas and neuroblastoma cell lines. The expression levels of Bcl-2 and Bcl-X_L are high in aggressive tumor cells but are low in regressing cells (Ikeda et al. 1995; Ikegaki et al. 1995). Caspase-1 and caspase-3 are expressed at significantly higher levels in favorable neuroblastomas (Nakagawara et al. 1997), and caspase-8 is silenced in aggressive neuroblastomas by the methylation of its promoter as one of mechanisms (Teitz et al. 2000). Silencing of caspase-8 is observed in 25–35% of primary neuroblastomas with a high frequency in more aggressive tumors (Teitz et al. 2000; Eggert et al. 2001; van Noesel et al. 2003). Survivin, a member of the inhibitors of apoptosis protein (IAP), is mapped to the long arm of chromosome 17. In neuroblastoma, survivin is highly expressed in high-risk tumors, and its overexpression inhibits cellular apoptosis (Islam et al. 2000). Kitanaka et al. (2002) have recently reported an interesting observation that "autophagy" may be involved in the regression of neuroblastoma cells.

5.3 Conclusions

Development of neuroblastoma may be triggered by a genetic event(s) that leads to chromosome and/or the genomic DNA abnormalities such as amplification of the *MYCN* gene and deletions or gains in chromosomal regions including 1p, 11q, and 17q. Together with other epigenetic mechanisms of gene activation or gene silencing, they affect gene and protein expression which in turn deregulate cellular signaling. In neuroblastoma the normal biology of developing neuronal cells and cancer biology appear to overlap. A further understanding of the mechanisms involved in the transformation of progenitors or the stem cells into neuroblastoma with significant cellular heterogeneity may provide clues for the development of novel therapeutic strategies for this often aggressive lethal disease.

Acknowledgements. I thank M. Ohira and T. Ozaki for reading the manuscript. I also thank K. Yagyu for preparing the figures.

References

Aloyz RS et al. (1998) p53 is essential for developmental neuron death as regulated by the TrkA and p75 neurotrophin receptors. J Cell Biol 143:1691–1703

Altaba ARi, Stecca B, Sanchez P (2004) Hedgehog-Gli signaling in brain tumors: stem cells and paradevelopmental programs in cancer. Cancer Lett 204:145–157

Axelson H (2004) The Notch signaling cascade in neuroblastoma: role of the basic helix-loop-helix proteins HASH-1 and HES-1. Cancer Lett 204:171–178

Azar CG, Scavarda NJ, Reynolds P, Brodeur GM (1990) Multiple defects of the nerve growth factor receptor in human neuroblastomas. Cell Growth Differ 1:421–428

Ball DW (2004) Achaete-scute homolog-1 and Notch in lung neuroendocrine development and cancer. Cancer Lett 159–169

Billon N et al. (2004) Roles for p53 and p73 during oligodendrocyte development. Development 131:1211–1220

Boon K et al. (2001) N-*MYC* enhances the expression of a large set of genes functioning in ribosome biogenesis and protein synthesis. EMBO J 20:1383–1393

Brodeur GM, Seeger RC, Schwab M, Varmus HE, Bishop JM (1984) Amplification of N-*MYC* in untreated human neuroblastomas correlates with advanced disease stage. Science 224:1121–1124

Canete A, Navarro S, Bermudez J, Pellin A, Castel V, Llombart-Bosch A (2000) Angiogenesis in neuroblastoma: relationship to survival and other prognostic factors in a cohort of neuroblastoma patients. J Clin Oncol 18:2789–2791

Deguin-Chambon V, Vacher M, Jullien M, May E, Bourdon JC (2000) Direct transactivation of c-Ha-Ras gene by p53: evidence for its involvement in p53 transactivation activity and p53-mediated apoptosis. Oncogene 19:5831–5841

Eggert A et al. (2000) Molecular dissection of TrkA signal transduction pathways mediating differentiation in human neuroblastoma cells. Oncogene 19:2043–2051

Eggert A et al. (2001) Resistance to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis in neuroblastoma cells correlates with a loss of caspase-8 expression. Cancer Res 61:1314–1319

El-Badry OM, Helman LJ, Chatten J, Steinberg SM, Evans AE, Israel MA (1991) Insulin-like growth factor II-mediated proliferation of human neuroblastoma. J Clin Invest 87: 648–657

Enomoto H et al. (1994) Identification of human DAN gene, mapping to the putative neuroblastoma tumor suppressor locus. Oncogene 9:2785–2791

Ernsberger U (2000) Evidence for an evolutionary conserved role of bone morphogenetic protein growth factors and phox2 transcription factors during noradrenergic differentiation of sympathetic neurons. Induction of a putative synexpression group of neurotransmitter-synthesizing enzymes. Eur J Biochem 267:6976–6981

Guillemot F, Lo LC, Johnson JE, Auerbach A, Anderson DJ, Joyner AL (1993) Mammalian achaete-scute homolog 1 is required for the early development of olfactory and autonomic neurons. Cell 75:463–476

Hamano S, Ohira M, Isogai E, Nakada K, Nakagawara A (2004) Identification of novel human neuronal leucine-rich repeat (hNLRR) family genes and inverse association of expression of Nbla10449/hNLRR-1 and Nbla10677/hNLRR-3 with the prognosis of primary neuroblastomas. Int J Oncol 24:1457–1466

Hishiki T et al. (1998) Glial cell line-derived neurotrophic factor/neurturin-induced differentiation and its enhancement by retinoic acid in primary human neuroblastomas expressing c-Ret, GFR-1 and GFR-2. Cancer Res 58:2158–2165

Huang EJ, Reichardt LF (2003) Trk receptors: roles in neuronal signal transduction. Annu Rev Biochem 72:609–642

Huber K, Combs S, Ernsberger U, Kalcheim C, Unsicker K (2002) Generation of neuroendocrine chromaffin cells from sympathoadrenal progenitors: beyond the glucocorticoid hypothesis. Ann NY Acad Sci 971:554–559

Hughes AL, Gollapudi L, Sladek TL, Neet KE (2000) Mediation of nerve growth factor-driven cell cycle arrest in PC12 cells by p53: simultaneous differentiation and proliferation subsequent to p53 functional inactivation. J Biol Chem 275: 37829–37837

Iavarone A, Lasorella A (2004) Id proteins in neural cancer. Cancer Lett 204:189–196

Ichikawa M, Murakumo Y, Takahashi M (2004) RET and neuroendocrine tumors. Cancer Lett 204:197–211

Ichimiya S et al (1999) p73 at chromosome 1p36.3 is lost in advanced stage neuroblastoma but its mutation is infrequent. Oncogene 18:1061–1066

Ichimiya S, Nimura Y, Seki N, Ozaki T, Nagase T, Nakagawara A (2001) Down-regulation of *hASH1* is associated with the retinoic acid-induced differentiation of human neuroblastoma cell lines. Med Pediatr Oncol 36:132–134

Ikeda H et al. (1995) Bcl-2 oncoprotein expression and apoptosis in neuroblastoma. J Pediatr Surg 30:805–808

Ikegaki N, Katsumata M, Tsujimoto Y, Nakagawara A, Brodeur GM (1995) Relationship between bcl-2 and *MYC* gene expression in human neuroblastoma. Cancer Lett 91:161–168

Islam A et al (2000) High expression of Survivin, mapped to 17q25, is significantly associated with poor prognostic factors and promotes cell survival in human neuroblastoma. Oncogene 19:617–623

Kadomatsu K, Muramatsu T (2004) Midkine and pleiotrophin in neural development and cancer. Cancer Lett 204:127–143

Kadomatsu K, Huang R-P, Suganuma T, Murata F, Muramatsu T (1990) A retinoic acid responsive gene MK found in the teratocarcinoma system is expressed in spatially and temporally controlled manner during mouse embryogenesis. J Cell Biol 110:607–616

Kawamoto T, Ohira M, Hamano S, Hori T, Nakagawara A (2003) High expression of the novel endothelin-converting enzyme genes, Nblao3145/ECEL1alpha and beta is associated with favorable prognosis in human neuroblastomas. Int J Oncol 22:815–822

Kitanaka C et al (2002) Increased Ras expression and caspase-independent neuroblastoma cell death: possible mechanism of spontaneous neuroblastoma regression. J Natl Cancer Inst 94:358–368

Klein R (1994) Role of neurotrophins in mouse neuronal development. FASEB J 8:738–744

Klesse LJ, Parada LF (1999) Trks: signal transduction and intracellular pathways. Microsc Res Tech 45:210–216

Kogner P, Barbany G, Dominici C, Castello MA, Raschella G, Persson H (1993) Coexpression of messenger RNA for TRK protooncogene and low affinity nerve growth factor receptor in neuroblastoma with favorable prognosis. Cancer Res 53:2044–2050

Lasorella A, Noseda M, Beyna M, Yokota Y, Iavarone A (2000) Id2 is a retinoblastoma protein target and mediates signalling by *MYC* oncoproteins. Nature 407:592–598

Laurenzi VD et al. (2000) Induction of neuronal differentiation by p73 in a neuroblastoma cell line. J Biol Chem 275: 15226–15231

Li Y-S et al. (1990) Cloning and expression of a developmentally regulated protein that induces mitogenic and neurite outgrowth activity. Science 250:1690–1694

Lo L, Morin X, Brunet JF, Anderson DJ (1999) Specification of neurotransmitter identity by Phox2 proteins in neural crest stem cells. Neuron 22:693–705

Mac SM, D'Cunha CA, Farnham PJ (2000) Direct recruitment of N-*MYC* to target gene promoters. Mol Carcinogr 29: 76–86

Massari ME, Murre C (2000) Helix-loop-helix proteins: regulators of transcription in eukaryotic organisms. Mol Cell Biol 20:429–440

Matsumoto K, Wada RK, Yamashiro JM, Kaplan DR, Thiele CJ (1995) Expression of brain-derived neurotrophic factor and $p145^{TrkB}$ affects survival, differentiation, and invasiveness of human neuroblastoma cells. Cancer Res 55:1798–1806

Moll UM, LaQuaglia M, Benard J, Riou G (1995) Wild-type p53 protein undergoes cytoplasmic sequestration in undifferentiated neuroblastomas but not in differentiated tumors. Proc Natl Acad Sci USA 92:4407–4411

Morin X, Cremer H, Hirsch MR, Kapur RP, Goridis C, Brunet JF (1997) Defects in sensory and autonomic ganglia and absence of locus coeruleus in mice deficient for the homeobox gene Phox2a. Neuron 18:411–423

Nakagawa T et al (2002) Autoinhibitory regulation of p73 by ΔNp73 to modulate cell survival and death through p73-specific target element within the ΔNp73 promoter. Mol Cell Biol 22:2575–2585

Nakagawara A (1998a) The NGF story and neuroblastoma. Med Pediatr Oncol 31:113–115

Nakagawara A (1998b) Molecular basis of spontaneous regression of neuroblastoma: role of neurotrophic signals and genetic abnormalities. Hum Cell 11:115–124

Nakagawara A (2001) Trk receptor tyrosine kinases: a bridge between cancer and neural development. Cancer Lett 169:107–114

Nakagawara A (2004) Neural crest development and neuroblastoma: the genetic and biological link. In: Aloe L, Calza L (eds) NGF and related molecules in health and disease. Elsevier, Amsterdam, pp 233–242

Nakagawara A, Arima M, Azar CG, Scavarda NJ, Brodeur GM (1992) Inverse relationship between trk expression and N-*MYC* amplification in human neuroblastomas. Cancer Res 52:1364–1368

Nakagawara A, Arima-Nakagawara M, Scavarda NJ, Azar CG, Cantor AB, Brodeur GM (1993) Association between high levels of expression of the TRK gene and favorable outcome in human neuroblastoma. N Engl J Med 328:847–854

Nakagawara A, Azar CG, Scavarda NJ, Brodeur GM (1994) Expression and function of TRK-B and BDNF in human neuroblastomas. Mol Cell Biol 14:759–767

Nakagawara A et al. (1995) Differential expression of pleiotrophin and midkine in advanced neuroblastomas. Cancer Res 55:1792–1797

Nakagawara A et al. (1997) High levels of expression and nuclear localization of ICE and CPP32 in favorable human neuroblastomas. Cancer Res 57:4578–4584

Nakamura Y, Ozaki T, Koseki H, Nakagawara A, Sakiyama S (2003) Accumulation of $p27^{KIP1}$ is associated with BMP2-mediated growth arrest and neuronal differentiation of human neuroblastoma-derived cell lines. Biochem Biophys Res Commun 307:206–213

Oppenheim RW (1991) Cell death during development of the nervous system. Annu Rev Neurosci 14:453–501

Pattyn A, Morin X, Cremer H, Goridis C, Brunet JF (1999) The homeobox gene Phox2b is essential for the development of autonomic neural crest derivatives. Nature 399:366–370

Pozniak CD, Radinovic S, Yang A, McKeon F, Kaplan DR, Miller FD (2000) An anti-apoptotic role for the p53 family member, p73, during developmental neuron death. Science 289:304–306

Radtke F, Raj K (2003) The role of Notch in tumorigenesis: oncogene or tumour suppressor? Nat Rev Cancer 3:756–767

Reale MA et al. (1996) Loss of DCC expression in neuroblastoma is associated with disease dissemination. Clin Cancer Res 2:1097–1102

Rodriguez-Leon J, Merino R, Macias D, Ganan Y, Santesteban E, Hurle JM (1999) Retinoic acid regulates programmed cell death through BMP signalling. Nat Cell Biol 1:125–126

Schneider C, Wicht H, Enderich J, Wegner M, Rohrer H (1999) Bone morphogenetic proteins are required in vivo for the generation of sympathetic neurons. Neuron 24:861–870

Schwab M et al. (1983) Amplified DNA with limited homology to *MYC* cellular oncogene is shared by human neuroblastoma cell lines and a neuroblastoma tumour. Nature 305:245–248

Schwab M et al (1984) Chromosome localization in normal human cells and neuroblastomas of a gene related to c-*MYC*. Nature 308:288–291

Schwab M et al. (2003) Neuroblastoma: biology and molecular and chromosomal pathology. Lancet Oncol 4:472–480

Schweigerer L, Ledoux D, Fleischmann G, Barritault D (1991) Enhanced *MYCN* oncogene expression in human neuroblastoma cells is associated with altered FGF receptor expression and cellular growth response to basic FGF. Biochem Biophys Res Commun 179:1449–1454

Seeger RC et al. (1985) Association of multiple copies of the N-*MYC* oncogene with rapid progression of neuroblastomas. N Engl J Med 313:1111–1116

Shinbo J et al. (2002) p73-dependent expression of DAN during cisplatin-induced cell death and osteoblast differentiation. Biochem Biophys Res Commun 295:501–507

Snider WD (1994) Functions of the neurotrophins during nervous system development: what the knockouts are teaching us. Cell 77:627–638

Soderholm H et al. (1999) Human achaete-scute homologue 1 (HASH-1) is downregulated in differentiating neuroblastoma cells. Biochem Biophys Res Commun 256:557–563

Stanke M, Junghans D, Geissen M, Goridis C, Ernsberger U, Rohrer H (1999) The Phox2 homeodomain proteins are sufficient to promote the development of sympathetic neurons. Development 126:4087–4094

Suzuki T, Bogenmann E, Shimada H, Stram D, Seeger RC (1993) Lack of high-affinity nerve growth factor receptors in aggressive neuroblastomas. J Natl Cancer Inst 85:377–384

Teitz T et al (2000) Caspase 8 is deleted or silenced preferentially in childhood neuroblastomas with amplification of *MYCN*. Nat Med 6:529–535

Tian H, Hammer RE, Matsumoto AM, Russell DW, McKnight SL (1998) The hypoxia-responsive transcription factor EPAS1 is essential for catecholamine homeostasis and protection against heart failure during embryonic development. Genes Dev 12:3320–3324

Trochet D et al (2004) Germline mutations of the paired-like homeobox 2B (PHOX2B) gene in neuroblastoma. Am J Hum Genet 74:761–764

Tweddle DA, Malcolm AJ, Bown N, Pearson AD, Lunec J (2001) Evidence for the development of p53 mutations after cytotoxic therapy in a neuroblastoma cell line. Cancer Res 61:8–13

Van Noesel MM, Pieters R, Voute PA, Versteeg R (2003) The N-*MYC* paradox: N-*MYC* overexpression in neuroblastomas is associated with sensitivity as well as resistance to apoptosis. Cancer Lett 197:165–172

Yamashiro D, Nakagawara A, Ikegaki N, Liu X, Brodeur GM (1996) Expression of TRK-C in favorable human neuroblastomas. Oncogene 12:37–41

Yang A, Walker N, Bronson R, Kaghad M, Oosterwegel M, Bonnin J, Vagner C, Bonnet H, Dikkes P, Sharpe A, McKeon F, Caput D (2000) p73-deficient mice have neurological, pheromonal and inflammatory defects but lack spontaneous tumours. Nature 404:99–103

Yano H, Cong F, Birge RB, Goff SP, Chao MV (2000) Association of the Abl tyrosine kinase with the Trk nerve growth factor receptor. J Neurosci Res 59:356–364

Yoshida I, Koide S, Nakagawara A, Tsuji A, Matsuda Y (2001) Proprotein convertase PACE4 is a target gene of the basic helix-loop-helix transcription factor hASH-1. Biochem J 360:683–689

Progress in Brain Research, Vol. 146
ISSN 0079-6123

CHAPTER 15

Neural crest development and neuroblastoma: the genetic and biological link

Akira Nakagawara*

Division of Biochemistry, Chiba Cancer Center Research Institute, Chiba 260-8717, Japan

Abstract: Neuroblastoma is one of the most common pediatric solid tumors originating from the sympathoadrenal lineage of neural crest. The tumor shows extremely different clinical phenotypes such as spontaneous regression on one hand and aggressive growth on the other hand. The different biological behavior of neuroblastoma appears to be determined by the genetic abnormalities including amplification of *MYCN* oncogene, DNA ploidy and some allelic imbalances. However, the spontaneous regression of neuroblastoma mimics the programmed cell death normally occurring in developing sympathetic cells expressing both TrkA tyrosine kinase A and $p75^{NTR}$ neurotrophin receptor. Indeed, TrkA expression is the most important factor related to the induction of tumor cell differentiation and/or programmed cell death because without its expression spontaneous regression of neuroblastoma never occurs. Thus, the enigmatic clinical behaviors of neuroblastoma are strictly linked to the molecular mechanism of neural crest development.

Keywords: neuroblastoma; NGF; TrkA; $p75^{NTR}$; *MYCN* oncogene; MYCN oncoprotein; stem cells

Neuroblastoma, a neural crest tumor in childhood

Neuroblastoma is an embyonic tumor originating from the sympathoadrenal lineage of neural crest and one of the most common solid tumors found in children (Bolande, 1974). Its incidence is about 1/8000 births and there is no significant difference among U.S., Europe and Japan. However, after beginning the mass screening to test the urine for the levels of catecholamine metabolites (VMA and HVA) in Japan in 1985 (Sawada et al., 1984), the incidence of neuroblastoma has almost doubled without decreasing the number of the sporadic tumors (Bessho, 1996). This strongly suggested the actual presence of '*in situ* neuroblastoma', which was first proposed by Beckwith and Perrin (1963), during the development of sympathetic neurons in human fetuses. They described the detection of '*in situ* neuroblastoma' in developing human embryos at the incidence of more than 40 times that of sporadic neuroblastomas, but most of them regressed spontaneously. Therefore, it is highly possible that we detect a part of the '*in situ* neuroblastomas' by mass screening, most of which otherwise regress without giving any therapy. However, at this moment it is unclear whether the regression of *in situ* neuroblastoma is due to the developmentally regulated programmed cell death of neuronal cells.

The sporadic neuroblastomas clinically found are divided into several subsets according to the clinical behavior, biological markers and genetic abnormalities (Brodeur, 2003). One of the most important clinical factors is the patient's age. The tumors found in the patients under one year of age are usually favorable and take a good clinical course to cure. On the other hand, many of the tumors symptomatically

*Correspondence to: A. Nakagawara, Division of Biochemistry, Chiba Cancer Center Research Institute, 666-2 Nitona, Chuoh-ku, Chiba 260-8717, Japan.
Tel.: +81-43-264-5431; Fax: +81-43-265-4459;
E-mail: akiranak@chiba-ccri.chuo.chiba.jp

DOI: 10.1016/S0079-6123(03)46015-9

found in the patients over one year of age are poor prognostic and eventually kill the patients. Among the biological markers so far found, expression of the TrkA tyrosine kinase A receptor, as well as p75 neurotrophin receptor ($p75^{NTR}$) expression, is the most important indicator of prognosis (Nakagawara et al., 1992 , 1993). TrkA is a high-affinity receptor for nerve growth factor (NGF), and $p75^{NTR}$ is its low-affinity receptor. The high levels of TrkA expression are strongly associated with favorable prognosis, whereas its decreased levels are significantly correlated with poor prognosis (Nakagawara et al., 1993). The important genetic markers include DNA ploidy, amplification of the *MYCN* oncogene and an allelic loss of the distal region of chromosome 1p (1p36) (Westermann and Schwab, 2002; Brodeur, 2003). Contrary to the other cancers, neuroblastomas with hyperdiploid karyotype show a good prognosis, while those with *MYCN* amplification and/or deletion of chromosome 1p36 are strongly associated with poor prognosis. The combination of these strong prognostic indicators segregates the subsets of neuroblastoma with different clinical behavior.

Figure 1 shows three types of neuroblastoma subset. Fig. 1 (left) demonstrates a stage 1 tumor originated from the adrenal gland in a patient under one year of age. The tumor is well encapsulated without metastasis. This type of neuroblastoma usually expresses high levels of TrkA and shows triploid DNA pattern with a single copy of *MYCN*. It clinically regresses spontaneously but very slowly. The baby in Fig. 1 (middle) is the patient with stage 4s neuroblastoma. The immature tumor cells occupy

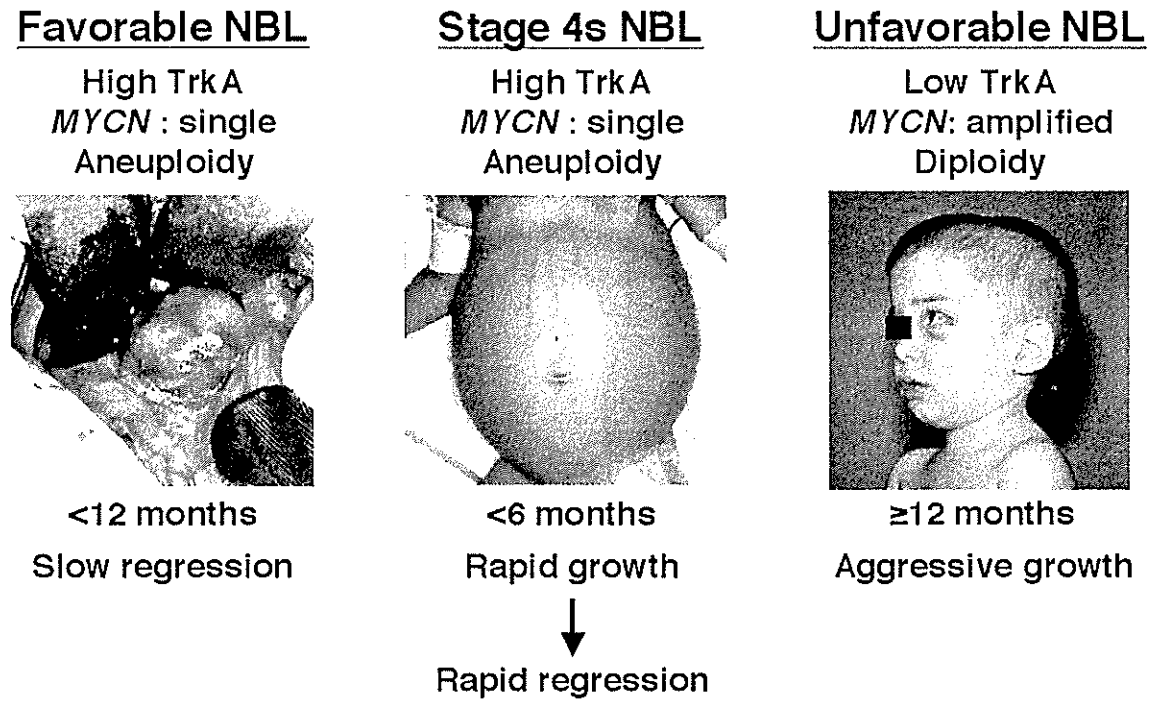


Fig. 1. Three distinct subset of human neuroblastomas with different biology and clinical behavior. Left: stage 1 neuroblastoma in a 7-month-old patient. The tumor originated from the right adrenal gland is small and well encapsulated. This kind of neuroblastoma usually regresses spontaneously. Middle: stage 4s neuroblastoma in a one-month-old baby. The primary tumor is located at the left adrenal gland. The liver is extremely enlarged and occupied by the tumor cells. The neuroblastoma cells are also positive in the bone marrow. The abdominal distension often oppresses the diaphragm to induce dyspnea. In a typical stage 4s neuroblastoma, the rapid tumor growth suddenly stops and starts to regress spontaneously. Right: stage 4 neuroblastoma in a 3-year-old boy. The tumor cells originated from the adrenal gland metastasize to long bones, skull and orbita with protrusion of the eye. The tumor cells show low TrkA expression, amplification of *MYCN*, diploid karyotype and deletion of the distal region of chromosome 1p.

the adrenal gland, liver and bone marrow (sometimes even skin), and rapidly grow at an early clinical stage. However, one day the tumor cells suddenly stop growing and start to regress spontaneously. This seems like just a miracle. The stage 4s tumor also shows high TrkA expression, triploidy and no amplification of *MYCN*. In contrast, the advanced stage of neuroblastoma shown in Fig. 1 (right) usually occurs in the patient over one year of age and metastasizes to bones and distant lymph nodes and eventually kill the patient. In this type of neuroblastoma, TrkA expression is strongly downregulated, the DNA ploidy pattern is diploid, and *MYCN* is amplified.

Genetic abnormalities of neuroblastoma

Neuroblastoma has many types of genetic abnormalities including chromosomal aneuploidy, gene amplifications, deletions, mutations, and deregulated DNA methylations. However, the pattern of the genetic aberration is different among the subsets, especially between those with favorable and unfavorable prognosis (Westermann and Schwab, 2002; Brodeur, 2003). The tumors with a tendency to regress spontaneously usually have triploidy but few abnormalities in the genome. On the other hand, the tumors with aggressive growth show a diploid or tetraploid karyotype, frequent amplification of *MYCN* oncogene, and chromosomal deletion of 1p36. The frequent gain of the chromosome 17q is reported to be associated with poor prognosis, however, it is also commonly observed in the tumors with favorable prognosis (Tomioka et al., 2003). The loss of heterozygosity at the chromosome 11q23 is reported to be frequent in the intermediate type of neuroblastoma in advanced stages with a single copy of *MYCN* and variable levels of TrkA expression (Guo et al., 1999). Thus, the subsets with different clinical behavior may be defined by the combination of the genomic aberrations.

Molecular and biological bases of neuroblastoma

Figure 2 shows a scheme of migration of the developing neural crest-derived cells, which segregate into several lineages such as melanocytes, sensory neurons, enteric neurons, and sympathetic ganglion cells. However, neuroblastoma never occurs in the other tissues than sympathetic ganglion or adrenal medulla. This suggests that the genetic events to cause neuroblastoma occur after the cell fate determination directing to sympathetic differentiation. The most likely candidate molecule to decide the direction of sympathetic differentiation at this moment is a basic helix-loop-helix transcription factor MASH1 which is transiently expressed during the neural development (Guillemot et al., 1993). In human neuroblastomas, MASH1/hASH1 is kept overexpressed (Soderholm et al., 1999; Ichimiya et al., 2001). Interestingly, induction of neuroblastoma cell differentiation in culture by treating with retinoic acid decreases the level of MASH1 mRNA. These suggest that the

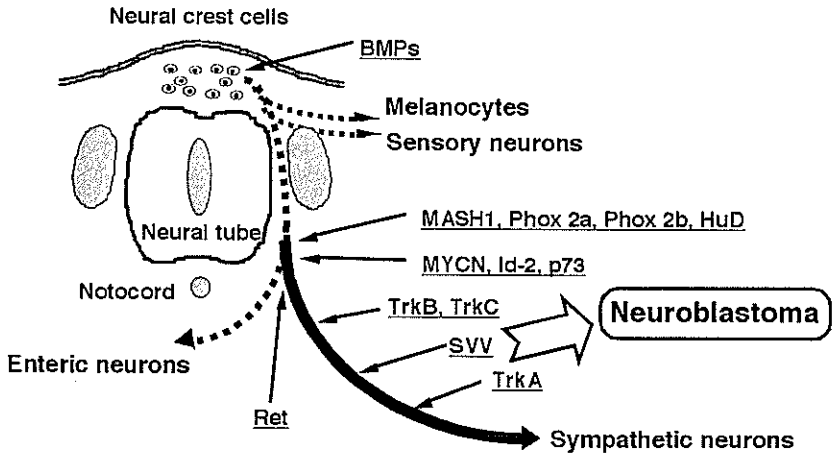


Fig. 2. Neuroblastoma occurs only from the sympathoadrenal lineage of neural crest. The important molecules regulating the sympathetic development are shown.

oncogenic events occurred in the early stage maintain the cells under arrest of differentiation by keeping the MASH1 expression at high levels, although this hypothesis must be proved. Even though the precise mechanism to regulate the oncogenic events of neuroblastoma and normal sympathetic differentiation is still elusive, it may be true that the targeting of the specific genes in such events is strictly controlled by the developmental program.

According to the accumulating evidence, it is clear that many important genes regulating normal development of sympathetic neurons are targeted to cause neuroblastoma or to modulate its biology. They include the *MYCN* gene encoding a basic helix-loop-helix transcription factor (Brodeur et al., 1984; Schwab et al., 1984), Id-2, a target of MYCN and a negative regulator of basic helix-loop-helix transcription factors (lasorella et al., 2000), MASH1 (Soderholm et al., 1999; Ichimiya et al., 2001), Phox2a and Phox2b, the homeotic proteins functioning with MASH1 (Kuno et al., unpublished data), and the downstream receptors such as Trk family members (Nakagawara et al., 1993) and Ret (Hishiki et al., 1998). These suggest that the important regulators of sympathetic differentiation are targeted to cause or maintain the cancerous status of neuroblastoma. This idea can be extended to the possible link between developmentally regulated programmed cell death of sympathetic neurons and spontaneous regression of neuroblastoma, because in both phenomena, expression of TrkA receptor is necessary (Nakagawara, 1998a, 2001). In other words, TrkA expression is almost exclusively required to induce spontaneous regression of neuroblastoma.

NGF family signaling in neuroblastoma

The NGF signals and their depletion strongly regulate survival and death of the normal sympathetic neurons, respectively. Similarly, it has recently become obvious that the NGF family signals strongly regulate the biology of neuroblastoma. Most neuroblastomas with favorable prognosis express high levels of both TrkA and $p75^{NTR}$ and functionally respond to exogenous NGF by extending neurites and promoting survival in primary culture (Nakagawara et al., 1993). The association between high levels of expression of TrkA and/or $p75^{NTR}$ and favorable outcome is statistically significant in primary human neuroblastomas. On the contrary, in aggressive neuroblastomas with *MYCN* amplification in advanced stages, expression of TrkA is extremely downregulated. The many studies about the role of Trk signaling in neuroblastoma cell lines also suggest that the intracellular TrkA signal is disturbed even though autophosphorylation of TrkA is induced by addition of NGF (Nakagawara et al., 1994). Thus, for the gain of growth advantage, the aggressive neuroblastoma cells appear to shut off the TrkA signal. Instead, they utilize a functional brain-derived neurotrophic factor (BDNF) and/or neurotrophin-4 (NT-4)/TrkB signaling system in an autocrine manner (Nakagawara et al., 1994). This BDNF/TrkB autocrine system also promotes invasion and metastasis in advanced tumors (Matsumoto et al., 1995). These suggest that spontaneous regression occurs only in neuroblastoma with high levels of TrkA expression and is induced by depletion of NGF within the tumor. The aggressive neuroblastoma cells seem to escape from the control by NGF, but to take advantage of the BDNF/TrkB autocrine loop for promotion of survival.

The family of glial cell line-derived neurotrophic factor (GDNF) mediates another important extracellular signal to regulate the survival of sympathetic neurons. Many neuroblastoma cells express the GDNF family receptors (Ret, GFRα-1, -2 and -3) and functionally respond to their ligands (GDNF, neurturin and artemin) in the primary culture (Hishiki et al., 1998). However, their expression and the responsiveness to the ligands are not associated with the disease stages or prognosis.

The other neurotrophic factors, pleiotrophin (PTN) and midkine (MK), may also be important in regulating neuroblastoma biology (Nakagawara et al., 1995). The expression of PTN is high in favorable neuroblastomas, whereas that of MK is high in all primary neuroblastomas. However, their functional roles in neuroblastoma are currently unknown.

Role of p53 and p73 in life and death of neuroblastoma

Pozniak et al. (2000) have recently reported about the crucial role of the tumor suppressor p53 and its

family member p73 in regulating survival and apoptosis during the induction of programmed cell death in mouse sympathetic neurons. Life and death of the sympathetic cervical ganglion (SCG) neurons are regulated by the balance between the levels of p53 and ΔNp73, an NH_2-terminally truncated dominant-negative form of p73. In neuroblastoma, p53 is not mutated but localized in the cellular cytoplasm especially in advanced stage tumors (Moll et al., 1995). Just recently, the anchoring molecule of p53 in the cytoplasm has been identified as Parc which is a structurally E3 ubiquitin ligase but binds to and stabilizes p53 (Nikolaev et al., 2003). It is interesting that the apoptosis-inducing stresses often trigger nuclear translocation of cytoplasmic p53 in neuroblasatoma cell lines (Ostermeyer et al., 1996).

p73 is the first family member of p53 and has occasionally been discovered as a candidate tumor suppressor of neuroblastoma (Kaghad et al., 1997). It is mapped to chromosome 1p36.2-3 which is commonly deleted in many aggressive neuroblastomas with *MYCN* amplification. The extensive mutation search has revealed that p73 is not mutated in many cancers including neuroblastoma (Ikawa et al., 1999). However, we found two mutations of the COOH-terminally located proline residues, one was somatic and the other germline. Nevertheless, most primary neuroblastomas have no mutation of p73 (Ichimiya et al., 1999).

Interestingly, in many malignant solid tumors, p73 has satisfactorily shown to be upregulated, though it has functionally the apoptosis-inducing ability like p53. We and the other investigators have recently found that p73 can bind to the *ΔNp73* proper promoter and induce transcription of which possesses the oncogenic function (Nakagawa et al., 2002). In addition, *ΔNp73* binds to both wild type p53 and p73 to suppress their apoptosis-inducing function (Nakagawa et al., 2002). These observations are very important because they might at least in part explain how the cancers without p53 mutation do develop the tumors with poor prognosis. In neuroblastoma, Casciano et al. (2002) have reported that both *p73* and *ΔNp73* are highly expressed in aggressive rather than favorable tumors.

Figure 3 shows the current summary of the signals for induction of neuronal apoptosis. Both p53 and p73 as well as ΔNp73 might be cooperatively functioning to regulate the programmed cell death of sympathetic as well as neuroblastoma cells.

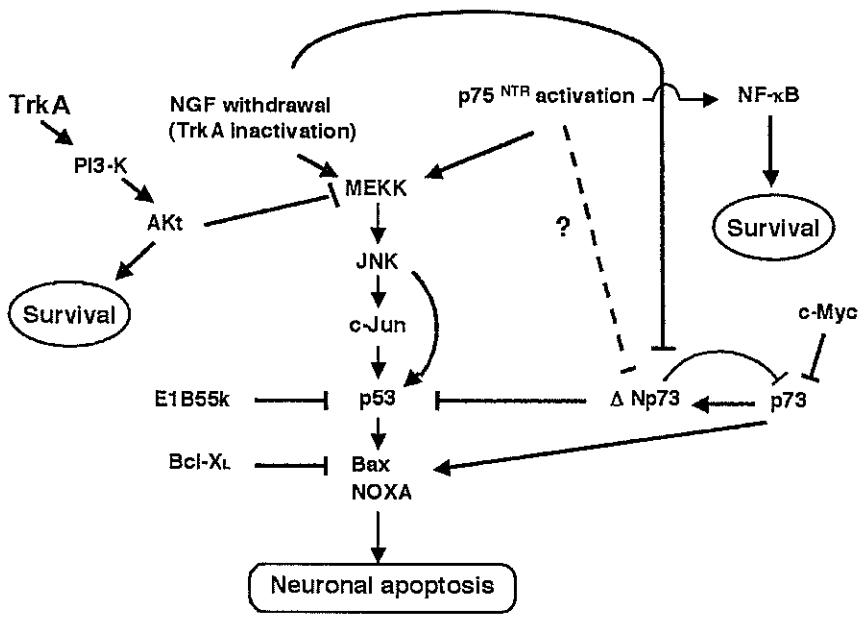


Fig. 3. Intracellular signaling of neuronal survival and apoptosis: the role of p53, p73 and ΔNp73.

Comprehensive genomics to identify the novel genes

To date, several genes functioning as landmark regulators in different subsets of neuroblastoma, such as *MYCN*, *MASH1*, *Trk*, *p53* and *p73*, have been identified. However, in the present postgenome era, we can try comprehensive approach to identify the important genes in a mass scale. For that purpose, we generated oligo-capping cDNA libraries from the primary neuroblastoma tissues of three different subsets as shown in Fig. 1. In total, we obtained 6252 gene clusters from 9729 clones randomly picked up from the cDNA libraries, among which 34% were novel genes with unknown function. The expression profiles of each subset of neuroblastoma were extremely different. By using the semi-quantitative reverse transcriptase (RT)-PCR, we have identified 757 genes differentially expressed between favorable (stage 1, high expression of *TrkA* and a single copy of *MYCN*) and unfavorable (stage 3 or 4, decreased levels of *TrkA* expression and amplification of *MYCN*) neuroblastomas. Among them, 502 are novel genes. [The results of our neuroblastoma cDNA project excluding those obtained from the stage 4s cDNA libraries were published elsewhere (Ohira et al., 2000; Ohira et al., in press).]

The expression profile of known genes was very different among the three subsets of neuroblastoma. The favorable subset frequently expressed neuronal specific genes including those related to neural differentiation, synapse, catecholamine metabolism and protein degradation. On the other hand, the unfavorable subset expressed many genes related to cell cycle control, protein synthesis and transcriptional regulation. The 4s tumor contained apoptosis-related genes, oncogenes and HLA family members which might be derived from the infiltrated lymphocytes into the tumor.

The 757 differentially expressed genes were strongly implicated in understanding of neuroblastoma biology. Of interest, vast majority of those genes was expressed at higher levels in the favorable subset as compared to the unfavorable one. The genes highly expressed in the favorable subset contained those related to neuronal differentiation, migration, cell–cell interaction, protein degradation, synaptic vesicles, catecholamine metabolism and intracellular signaling (Ohira et al., 2000; Ohira et al., in press). Most of them define the neuronal-specific phenotype and maintain the neuronal function. They also included heat shock proteins and ubiquitin/proteasome-related molecules that might sense the stress. On the other hand, only about 10% of the differential genes were expressed at high levels in the unfavorable subset. The protein products of such known genes contained many transcriptional and translational regulators including oncoproteins.

We also applied the primary culture of newborn mouse SCG neurons for screening those genes which change during the NGF-induced differentiation and/or the NGF depletion-induced apoptosis. This approach has identified 33 genes related to the former and 56 genes changeable during the latter (Isogai et al., unpublished data).

Our unique approach has identified more than several interesting genes as well as their products which include Nbla0219/BMCC1, a novel proapoptotic molecule with BCH domain, P-loop and coiled-coil domain, and Nbla0078/NEDL1, a novel E3 ubiquitin ligase with the HECT domain. The other interesting genes whose analyses have been published during our studies also include human *RIM/Nbla0761*, a Rab3-interacting molecule in the synaptic vesicles, *XCE/Nbla3145*, a new endothelin-converting enzyme and *FOG2/Nbla3139*, a coactivator of GATA transcription factor. Currently, a total of 7000 genes we cloned from the primary neuroblastomas are being fixed on the slide glass for cDNA microarray analysis.

Thus, our neuroblastoma cDNA project has provided enormous information and the gene materials for understanding of neuroblastoma biology as well as the molecular mechanism of neural crest development.

Developmental time axis and oncogenic events

Our neuroblastoma cDNA project has provided us with tremendous information about the genes expressed in different subsets with characteristic biology (Ohira et al., 2000; Ohira et al., in press). It suggested the presence of a kind of rule in the expression patterns of the subset-specific genes. Figure 4 shows the groups of genes expressed along the time axis of sympathetic neuron development. During the early

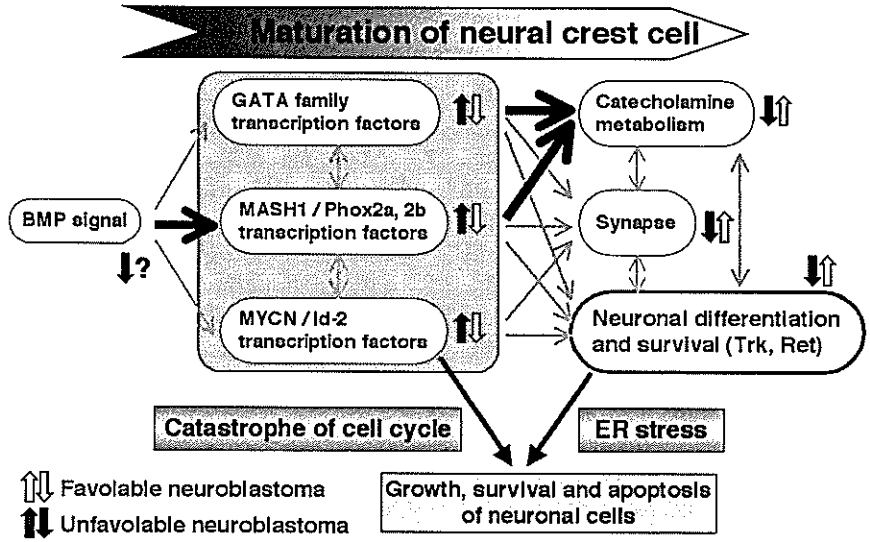


Fig. 4. The gene expression cascade along the time axis during the neural crest development, which is expected from the results obtained from the neuroblastoma cDNA project. Many transcription factors are upregulated in the unfavorable neuroblastoma, whereas the genes related to the terminal differentiation of neuron are upregulated in the favorable neuroblastoma. ER, endoplasmic reticulum.

stages of development, many transcription factors seem to function in deciding the direction of differentiation as well as regulating cell growth and survival of neural crest-derived cells. Interestingly, many genes highly expressed in unfavorable NBLs contain transcription factors and the components of their complexes. They include MYCN and Id family transcription factors that link to the regulation of Rb and p53 and regulate cell growth and apoptosis (Lasorella et al., 2000). The another basic helix-loop-helix transcription factor, MASH1, is constitutively activated in neuroblastoma, and by collaborating with Phox2a and Phox2b, it may regulate the arrest of differentiation in an unfavorable neuroblastomas (Kuno et al., unpublished data). Our neuroblastoma cDNA project has also revealed that there may be a neuronal cassette of GATA transcription factor complex that controls growth and differentiation of sympathetic progenitor cells (Ohira et al., 2003). Some molecules in this complex are upregulated in unfavorable neuroblastomas (Aoyama et al., manuscript in preparation). Thus, many important components in the transcriptional regulators appear to be highly expressed in unfavorable neuroblastomas and function to regulate the tumor cell growth or the status of de-differentiation.

On the other hand, most of a remarkable number of the genes expressed at high levels in favorable neuroblastomas encode the molecules that are necessary to maintain the neuronal function. They may be necessary for keeping catecholamine metabolism, synapse formation, neuronal cell survival, etc. We have also found many genes related to the ubiquitin-proteasome pathway and heat shock proteins in favorable neuroblastomas. They might be involved in induction of apoptosis triggered by endoplasmic reticulum stress.

Thus, the pattern of the differentially expressed genes in neuroblastoma subsets suggests the changes in the developmentally regulated gene expression along the time axis.

The hypothesis of neuroblastoma stem cells

According to the result of neuroblastoma mass screening, it may be true that most of the early stage neuroblastomas do not progress to the advanced tumors. In addition, the study of molecular mechanism linking neural development and neuroblastoma has revealed that the aggressive neuroblastoma occuring in an older patient seems to be arrested at