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MYCN, Neuroblastoma and Focal Adhesion Kinase (FAK)

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MYCN oncogene overview

The *MYCN* oncogene plays a major role in human oncogenesis. Previous data indicate that the primary function of *MYCN* is as a transcription factor, known to bind to the specific DNA E-box sequence, CACGTG (1, 2). Recent evidence supports a dual role for *MYCN*. Murphy et al recently showed that *MYCN* more commonly binds to the CATGTG E-box sequence, and *MYCN* binding correlates with DNA hypermethylation, thereby also functioning as a mediator of chromatin structure (3). Normal expression of *MYCN* is limited to embryonic brain and kidney tissues, and lymphocytes early in their differentiation (4–6). The importance of *MYCN* in embryogenesis is demonstrated by the fact that in mice, homozygous *MYCN* null is an embryonic lethal mutation, with these embryos showing a significant decrease in mature neurons of neural crest origin (7). Abnormal expression of *MYCN* is associated with neuroblastoma, the most common extracranial solid tumor of childhood. The exact function and gene targets of *MYCN* have not been completely defined (8), and a thorough investigation of which target genes are required for the proliferation and tumorigenesis of neuroblastoma have not been published (9). However, there is significant interest in this arena and some progress is being made with recent studies showing *in vitro* (10–12), and *in vivo* (9, 13) binding of *MYCN* to the genes that it regulates. For example, *MYCN* has been shown to bind to the gene promoters for various oncogenes involved in cellular proliferation, differentiation, and survival and to increase the expression of these targets. These genes include the proto-oncogenes high mobility group A1 (*HMGAI*) (14) and *Pax-3* (11), the multidrug resistance-associated protein gene (*MRPI*) (12), ATP-binding cassette (ABC) transporters that mediate efflux of chemotherapeutic agents from cancer cells (15), and the proteins nestin (10) and livin (16). There are also a number of negative transcriptional targets for N-Myc in neuroblastoma including the growth-inhibitory gene *Ndr1* (17) and leukemia inhibitory factor (*LIF*) (18). The *MYCN* protein has a number of functional regions including a basic helix-loop-helix leucine zipper motif and two highly conserved areas, Myc box I and II. These regions are important for the function of the

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MYCN protein however, the specific regions required for transcriptional regulation are segregated and appear to vary with different target genes (19).

Neuroblastoma and *MYCN* oncogene

The strongest adverse prognostic indicator in human neuroblastoma is gene amplification of greater than ten copies of the *MYCN* oncogene (20). Amplification of this gene occurs in about 20% of neuroblastomas (8, 20) and is associated with both increased recurrence of disease and decreased survival. Numerous studies have demonstrated the importance of *MYCN* in neuroblastoma tumorigenicity. The level of *MYCN* expression has been shown to correlate with the growth and proliferation of neuroblastoma cells *in vitro* (4, 21–23). Interruption of *MYCN* induces cellular differentiation in neuroblastoma cells. Nara and colleagues showed that after blocking *MYCN*, neuroblastoma cells developed multidirectional neurite extension and increased cellular and nuclear size, findings consistent with differentiation (24). Downregulation of *MYCN* with antisense oligonucleotides resulted in a decrease in both cellular proliferation and anchorage independent growth in the cells (4, 23). Other authors have utilized small interfering RNAs (siRNAs) to silence *MYCN*. Woo et al demonstrated that *MYCN* interference resulted in a decrease in the number of neuroblastoma cells in the S-phase of the cell cycle (25). In addition, other investigators have utilized this method to silence *MYCN* and have shown decreased neuroblastoma cell growth and an increase in neuroblastoma cell apoptosis (26). Finally, transgenic mice with *MYCN* over-expression develop spontaneous neuroblastomas (27) that have the same histological features seen in human tumors (28).

MYCN, neuroblastoma, and cell adhesion

An association between *MYCN* amplification and tumorigenicity through cell adhesion, motility, and invasiveness has been implied in some studies. *MYCN* amplified human neuroblastoma cells have both increased cellular motility and invasiveness (29) and decreased attachment (30) compared to *MYCN* non-amplified cell lines, but no mechanistic explanation for these observations has been elucidated. To this end, Ma and others recently demonstrated that *MYCN* amplification correlates with levels of microRNA-9 (miR-9) which is associated with increased cell motility and invasiveness by regulating E-cadherin in breast cancer cells (31). More specifically to neuroblastoma, Akesson and Bernards showed that rat neuroblastoma cells transfected with a *MYCN* expression vector have significant reductions in mRNA and protein expression of neural cell adhesion molecule, a specific cell-cell adhesion molecule (32). In addition, other researchers have shown that the expression of integrin subunits $\alpha 3$ and $\beta 1$ are inversely related to overexpression of the N-Myc protein in neuroblastoma (33, 34). *MYCN* overexpression in neuroblastoma cells *in vitro* also results in decreased expression of $\alpha 1$ integrin, leading to decreased attachment and increased migratory activity (35). In addition, Wu and others have demonstrated that FAK activity is required to promote integrin stimulated neuroblastoma motility through $\alpha 5\beta 1$ but not for $\alpha 4\beta 1$ integrin (36). These data provide evidence that *MYCN* is involved in neuroblastoma cell adhesion.

MYCN, neuroblastoma, and focal adhesion kinase (FAK)

The association between *MYCN* amplification and cellular motility and invasiveness in neuroblastoma implies a potential relationship between focal adhesion kinase (FAK) and *MYCN*, since FAK is a key protein involved in cellular motility. Early studies relating neuroblastoma and FAK primarily involved the use of SH-SY5Y neuroblastoma cells (non-amplified *MYCN*) to investigate the relationship between insulin-like growth factor -1 (IGF-1) and FAK activation in the role of neuronal morphology (37, 38). These investigators showed that IGF-1 stimulated cell motility was mediated through the phosphorylation of

FAK. In these studies, the researchers utilized SH-SY5Y cells not because of their cancer properties, but because these cells can be induced to differentiate, and are thereby a good experimental model for neuronal differentiation studies. In more recent studies, Kim and Feldman began investigating the effects of FAK dephosphorylation in neuroblastoma cell survival (39, 40). In the first study they showed that treatment of SH-EP neuroblastoma cells with mannitol resulted in the loss of FAK phosphorylation which was reversible by IGF-1 (39). In the subsequent study, they reported that okadaic acid treatment of SH-EP cells resulted in the loss of FAK phosphorylation and apoptosis that was not reversible with the treatment of IGF-1 (40). Until recently, however, there were no data demonstrating the expression of FAK in human neuroblastoma specimens, and none to clearly show that FAK and *MYCN* were related. An examination of 70 human neuroblastoma specimens with various INSS stage and *MYCN* amplification status revealed an increase in the expression of FAK protein, as detected by immunohistochemistry, in *MYCN* amplified human neuroblastomas compared to those tumors that were not *MYCN* amplified (Fig. 1, panels A, B) (41), although FAK expression was not correlated with overall patient survival.

Investigations into the mechanisms of FAK regulation in neuroblastoma have been ongoing. Recently, the *FAK* promoter has been cloned (42) and evaluations have demonstrated a number of binding sites on this promoter for various oncogenes, such as p53. In addition, studies have revealed E-box sequences on the *FAK* promoter that are potential binding sites for *MYCN* (Fig. 2, panel A). Further studies including electrophoretic mobility shift, chromatin immunoprecipitation (ChIP), and dual luciferase assays have shown that *MYCN* does bind to the *FAK* promoter *in vitro* and *in vivo*, resulting in an upregulation of FAK expression (43) (Fig. 2, panel B). Understanding of the effects of *MYCN* upon the *FAK* promoter in neuroblastoma impacts our understanding of the role of FAK in tumorigenesis in other tumor types. *MYCN* is reported to be amplified in human melanoma and sarcomas (44) and is associated with poor outcomes in these tumor types (45). FAK has also been shown to be overexpressed in human sarcoma and melanoma tumors (46, 47), and that downregulation of FAK results in decreased survival in human melanoma cells (48). These data provide evidence that *MYCN* is potentially a transcriptional regulator of FAK in these tumor types as well, but as of yet the role of FAK here has not been fully investigated.

Studies to determine whether the increased expression of FAK by *MYCN* amplified neuroblastoma cell lines is biologically relevant have been performed. To more fully determine the biological significance of the effects of *MYCN* upon FAK, an isogenic *MYCN* neuroblastoma cell line (SHEP Tet-21/N) (49) was utilized. Treatment of these cell lines with siRNA to FAK resulted in a significant increase in cellular apoptosis in the *MYCN* overexpressing cells compared to their non-expressing *MYCN* counterparts (43). Additionally, FAK inhibition using AdFAK-CD, an adenoviral construct that functions as a dominant-negative of FAK, in the same isogenic *MYCN* neuroblastoma cell lines resulted in decreased cellular attachment and proliferation and increased cellular apoptosis in the neuroblastoma cells that had *MYCN* overexpression compared to the non-overexpressing cell line (50).

Recent advances have been made in the development of small molecule inhibitors of FAK. For instance, TAE226, a small molecule FAK inhibitor, has been reported to decrease cell survival in human glioma cells (51) and to decrease metastasis and enhance survival in xenograft models of pancreatic and breast cancer (52, 53). FAK inhibition with TAE226 treatment of *MYCN* amplified neuroblastoma cells [SK-N-BE(2)] results in significantly decreased cell viability at low concentrations with minimal effects upon *MYCN* non-amplified cells (SK-N-AS) (Fig. 3). Golubovskaya et al have also published their results with another small molecule FAK inhibitor, 1,2,4,5-benzenetetraamine tetrahydrochloride (54). These authors reported that FAK inhibition using this small molecule resulted in

decreased breast cancer cell survival both *in vitro* and *in vivo*. Other investigators have shown the efficacy of this small molecule in treating pancreatic cancer cell xenografts (55). Use of this small molecule inhibitor in human neuroblastoma has also been reported (56). Neuroblastoma cell lines with *MYCN* amplification were more sensitive to this small molecule treatment than those that were not *MYCN* amplified. Treatment with 1,2,4,5-benzenetetraamine tetrahydrochloride resulted in decreased cellular attachment, viability and increased apoptosis *in vitro*, and decreased neuroblastoma tumor growth *in vivo*, and these effects were more pronounced in the *MYCN* amplified neuroblastoma cell lines (56). Of note, 1,2,4,5-benzenetetraamine tetrahydrochloride treatment of normal ganglion cells had no effect upon the viability of these non-cancer cells (Fig. 4).

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Figure 1. FAK protein detection in formalin-fixed, paraffin embedded human neuroblastoma specimens

A. Antibody to p125^{FAK} was utilized to detect FAK staining in 70 human neuroblastoma specimens. This photomicrograph (40×) shows a *MYCN* amplified INSS stage 4 neuroblastoma specimen with significant staining for p125^{FAK}. B. This photomicrograph (40×) shows a *MYCN* non-amplified INSS stage 4 neuroblastoma specimen with no significant p125^{FAK} staining.

Figure 2. *MYCN* regulates the FAK promoter

A. Nucleotide sequence (GenBank Accession Number AY323812) of the 5'-flanking region of the FAK gene. The major transcription initiation site is marked as +1. The putative E-box binding site for *MYCN* is underlined. B. Dual luciferase assays are utilized to detect FAK promoter activity. Graph showing fold change in FAK promoter activity in *MYCN*⁻ and *MYCN*⁺ isogenic neuroblastoma cell lines. There is a significant increase in FAK promoter activity with the P-280 construct in the *MYCN*⁺ cell line compared to the *MYCN*⁻ cells (**P*<0.01). When the E-box binding site is mutated (ΔP-280), the FAK promoter activity in the *MYCN*⁺ neuroblastoma cell lines significantly decreases compared to the wild type (P-280) promoter construct (†*P*<0.01).

Figure 3. FAK inhibition leads to decreased cell viability in *MYCN* amplified neuroblastoma cell lines

FAK inhibition is achieved with TAE226, a specific FAK kinase inhibitor. Neuroblastoma cells, SK-N-BE(2) (amplified *MYCN*) and SK-N-AS (non-amplified *MYCN*) are treated with TAE226 for 24 hours and viability is measured using Alamar Blue Assay. After 24 hours, cellular viability is significantly decreased in the *MYCN* amplified neuroblastoma cell line, SK-N-BE(2), compared to that in the non-amplified, SK-N-AS, cell line, demonstrating the biologic importance of FAK in *MYCN* amplified neuroblastoma cells.

Fig. 4. FAK inhibition has minimal effects upon normal ganglion cells

FAK is inhibited with a small molecule inhibitor, 1,2,4,5-benzenetetraamine tetrahydrochloride, for 24 and 48 hours in normal ganglion cells. Viability is measured using Alamar Blue Assay. The cellular viability in these normal cells is essentially unaffected, even after 48 hours of treatment, by FAK inhibition with 1,2,4,5-benzenetetraamine tetrahydrochloride.