The Role of Stromal-Derived Factors in Neuroblastoma Differentiation

by

Angela Lynn Gaviglio

Department of Pharmacology and Cancer Biology
Duke University

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Daniel S. Wechsler

Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Pharmacology and Cancer Biology in the Graduate School of Duke University

2016
ABSTRACT

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Abstract

Neuroblastoma is a pediatric cancer arising from undifferentiated neural crest-derived precursor cells. Treatment strategies for neuroblastoma aim to promote neuroblast differentiation, however current therapies available are only modestly effective. The tumor stroma contributes to the suppression of tumor growth by releasing soluble factors that act to promote neuroblast differentiation, though the precise factors released and their mechanism of action in neuroblastoma remains unclear. Here, we identify a novel component of the differentiating stroma secretome and harness stroma biology to inform the use of a combination therapy for neuroblastoma treatment.

*HBEGF* expression is decreased in neuroblastoma compared to benign disease, correlating to an increase in mortality. HBEGF protein is expressed only in stromal compartments of tumor specimens, with tissue from late-stage disease containing very little stroma or HBEGF. Addition of soluble HBEGF to neuroblastoma cell lines leads to increased neuroblast differentiation and decreased proliferation. Heparan sulfate proteoglycans (HSPGs) and heparin derivatives further enhance HBEGF-induced differentiation by forming a complex with the epidermal growth factor receptor (EGFR), leading to activation of the ERK1/2 and STAT3 pathways and upregulation of the inhibitor of DNA binding 1 transcription factor.
Expression of the type III TGF-β receptor (TβRIII), an HSPG, is epigenetically regulated in neuroblastoma cells via direct binding of the N-Myc transcription factor to Sp-1 sites on the TβRIII promoter. Analysis of patient microarray data demonstrate that other members of the differentiating stroma secretome, including HBEGF and EGFR, are positively correlated with TβRIII expression, suggesting that these proteins may be co-regulated. Treatment with inhibitors aimed at blocking N-Myc function, including inhibitors of histone deacetylases, DNA methyltransferases (DNMTs), and aurora kinase A (AurkA) can promote neuroblast differentiation and decrease proliferation. The combination of the DNMT inhibitor decitabine with the AurkA inhibitor MLN8237 enhances differentiation and reduces proliferation compared to either agent alone. Importantly, the combination of clinically achievable doses of these targeted agents dramatically reduces tumor growth in orthotopic xenograft models of neuroblastoma, identifying a novel combination therapy that may benefit children with this disease.

In conclusion, these studies dissect the tumor microenvironment to identify an important member of the differentiating stroma secretome, while also revealing a combination therapy for clinical development that has the potential to decrease adverse side effects and increase effectiveness of neuroblastoma treatment.
Dedication

I dedicate this dissertation to my fiancé, Ryan and to my late grandmother, Geraldine.

Ryan, meeting you seven years ago changed my life. You have supported, encouraged, inspired, and loved me throughout my scientific journey. I truly could not have done this without you. I love you.

To my grandma, you paved the way for women in science by becoming one of the first women admitted to a university biology program. Your life is a testament that anything is possible and I carried that with me as I completed my thesis research. Thank you for sharing your curiosity and perseverance with me. I love you and I miss you.
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1. Introduction

1.1 Neuroblastoma

1.1.1 Neuroblastoma pathogenesis

Neuroblastoma is a pediatric cancer responsible for the majority of infantile cancer cases and is also the most common and lethal extracranial solid tumor in children (CDC 2013). Neuroblastomas arise from neural crest-derived sympathoadrenal precursor cells called neuroblasts (Brodeur 2003, Maris et al. 2007). Tumors develop mainly in the adrenal glands, but can also be found in the ganglia along the sympathetic trunk. Most neuroblastomas occur sporadically, although a family history is observed in about 1% of patients (Janoueix-Lerosey et al. 2010). Mutation in the PHOX2B gene was the first germline alteration implicated in familial neuroblastoma (Mosse et al. 2004, Trochet et al. 2004). Since this finding, ALK mutations have also been associated with hereditary neuroblastomas (Janoueix-Lerosey et al. 2008, Mosse et al. 2008). In sporadic cases, somatic mutations are uncommon with alterations occurring most frequently in the ALK (9.2%), PTPN11 (2.9%), ATRX (2.5%), MYCN (1.7%), and NRAS (0.83%) genes (Pugh et al. 2013). The absence of common somatic mutations suggests that epigenetic mechanisms may be an important factor in neuroblastoma pathogenesis.

Currently, neuroblastomas are staged based on the International Neuroblastoma Staging System (INSS) that incorporates both lymph node invasion and extension across the midline into staging prior to surgery (Smith et al. 1989, Brodeur et al. 1993).
However, due to the striking heterogeneity observed in neuroblastomas, the propensity for some tumors to spontaneously regress, and the high treatment morbidity associated with tumor resection (De Bernardi et al. 2008, Retrosi et al. 2011, Yeung et al. 2015), there has been a need to distinguish those patients who may be spared from surgery or intensive chemotherapy (Brodeur 2003, Janoueix-Lerosey et al. 2010, Maris 2010). These distinguishing features include increased cellular differentiation and stroma content, as well as lack of MYCN amplification, all of which are associated with improved prognosis (Brodeur et al. 1984, Shimada et al. 1984, Seeger et al. 1985). The International Neuroblastoma Risk Group classification system has been adapted to aid in treatment decisions based on these distinguishing features, in addition to stage, age, DNA ploidy, and chromosome 11q status (Cohn et al. 2009). While low-risk tumors are treated effectively with surgery or may spontaneously regress via differentiation or apoptosis, high-risk tumors are not as responsive to current treatments. The standard regimen for high-risk patients consists of surgery followed by myeloablative chemotherapy and retinoic-acid based differentiation therapy to target residual tumor cells (Matthay et al. 2009). Though this treatment provides some benefit, the overall survival rate is <40% and has only modestly improved over the last 40 years, likely due to a majority of tumors being metastatic at diagnosis (Maris et al. 2007, Maris 2010) and the high propensity for high-risk patients to relapse (Matthay et al. 1993, Matthay et al. 1999, Maris et al. 2007, Mullassery et al. 2009). The lack of effective treatment options for high-
risk patients urges a greater understanding of the growth factors, receptors, and pathways involved in neuroblastoma pathogenesis, which may lead to the development of novel therapies for this disease.

1.1.2 Neuroblast differentiation in neuroblastoma

Neuroblastoma is thought to arise from a cell differentiation block (Tee et al. 2012). The process of differentiation in neuroblastoma was first demonstrated in vitro in neuroblastoma cell lines more than 30 years ago (Pahlman et al. 1981). Neuroblast differentiation is an important distinguishing feature of this disease and is used to help determine patient prognosis and treatment strategies. Patients considered high-risk tend to have poorly differentiated tumors, while low-risk tumors tend to be more differentiated. Evidence that neuroblast differentiation is an important mediator of neuroblastoma pathogenesis also comes from the observation that a proportion of neuroblastomas will spontaneously regress, partially due to neuronal differentiation (D’Angio et al. 1971, Evans et al. 1971). Further support for the significance of differentiation in neuroblastoma pathogenesis is the use of 13-cis-retinoic acid (13-cis RA) as treatment for high-risk, undifferentiated disease (Matthay et al. 2009). Though this treatment is modestly effective, over half of patients will be refractory to or relapse from this treatment (Cotterill et al. 2001, Maris 2010, London et al. 2011). Indeed, single-agent 13-cis RA has recently been challenged as the most effective differentiating therapy (Yu et al. 2010), indicating the importance of identifying novel therapeutic
strategies. Since the initial discovery of differentiation-promoting agents in neuroblastoma 30 years ago, several other growth factors, receptors, and pharmacologic agents have been shown to regulate neuroblast differentiation both in vitro and in vivo (Figure 1); though no additional differentiating therapies have been successful clinically. Further understanding of these pro-differentiating pathways may provide insight into the regulation of neuroblastoma pathogenesis and could lead to the discovery of novel differentiating therapies.

1.1.2.1 Epigenetic regulation by N-Myc

Somatic mutations are rare in neuroblastoma, suggesting that epigenetic regulation is critical for pathogenesis (Cole and Maris 2012). The major epigenetic mediator of neuroblast differentiation is the MYCN oncogene. This oncogene was first discovered in human neuroblastomas and neuroblastoma cell lines (Kohl et al. 1983, Schwab et al. 1983) and is amplified in 20-30% of neuroblastoma tumors (Maris 2010). MYCN amplification is associated with a poor prognosis and low cellular differentiation (Schwab et al. 1983, Seeger et al. 1985, Fredlund et al. 2008). It remains controversial whether N-Myc expression in the absence of amplification correlates with disease stage or survival (Chan et al. 1997). Evidence in support of high N-Myc protein as a pro-tumorigenic mediator of neuroblastoma is the development of the tyrosine hydroxylase (TH)-MYCN mouse model, where the TH promoter drives MYCN expression (Weiss et al. 1997). In this model, mice formed tumors that recapitulated the biological and
Pharmacologic agents, growth factors, and growth factor receptors promote neuroblast differentiation while decreasing proliferation via the JAK, MAPK/ERK, and PI3K/AKT signaling pathways. These pathways converge on the STAT or ID1 transcription factors.

Abbreviations: TPA—12-O-tetradecanoylphorbol-13-acetate, DNMTi—DNMT inhibitor, HDACi—HDAC inhibitor, TGFα—transforming growth factor alpha, IGF—inulin growth factor, BMP=bone morphogenetic protein, NGF—nerve growth factor, FGF—fibroblast growth factor, ERα—estrogen receptor alpha, IGF1R—insulin-like growth factor 1 receptor, BMPRI/II—bone morphogenetic protein receptor, RARβ—retinoic acid receptor beta, TRKA—tropomyosin receptor kinase A, FGFR—fibroblast growth factor receptor, HSPGs—heparan sulfate proteoglycans, JAK—janus kinase, MAPK—mitogen-activated protein kinase, ERK—extracellular signal-regulated kinase, PI3K—phosphatidylinositol-3-kinases, AKT—protein kinase B, TF—transcription factor, STAT—signal transducer and activator of transcription, ID—inhibitor of DNA binding
genetic characteristics of human neuroblastomas, suggesting that overexpression of N-Myc regardless of amplification can also promote neuroblastoma development (Weiss et al. 1997).

N-Myc is known to suppress neuroblast differentiation, though the critical mechanism has not been defined (Thiele et al. 1985, Harris et al. 2002, Alaminos et al. 2003, Kang et al. 2006, Fredlund et al. 2008, Loven et al. 2010, Akter et al. 2011, Iraci et al. 2011, Knelson et al. 2013). N-Myc can epigenetically repress gene transcription by creating a more compact chromatin environment that hinders transcription. A common mechanism by which N-Myc exerts these effects is by binding to Miz-1 and Sp-1 transcription factors on the promoters of target genes, resulting in the recruitment of histone deacetylases (HDAC) and/or DNA methyltransferases (DNMT) to chromatin (Westermark et al. 2011, He et al. 2013). Our work demonstrated inhibition of neuroblast differentiation via repression of the type III TGF-β receptor (TβRIII) through N-Myc recruitment to Sp-1 sites on the TβRIII promoter (Figure 2) (Knelson et al. 2013). Similarly, N-Myc has been shown to repress differentiation by transcriptionally inhibiting the pro-differentiating NGF/TRKA pathway through N-Myc binding to Sp-1 sites on the TRKA promoter and subsequent HDAC1 recruitment (Iraci et al. 2011).

Another suggested mechanism of N-Myc-mediated inhibition of neuroblast differentiation is via up-regulation of miR-17-92 (Beveridge et al. 2009, Loven et al. 2010). A better understanding of these regulatory mechanisms is critical in order to
inform the use of targeted differentiating agents that will benefit neuroblastoma patients.

Figure 2: N-Myc binds the TβRIII promoter

Chromatin immunoprecipitation in SHEP-21N cells where N-Myc expression is doxycycline (Dox)-repressible. Primers for Sp-1 binding sites in TβRIII were designed toward the sites shown. Cells were treated for 72 hours with Dox to repress N-Myc prior to ChIP assay. Image is representative of two independent experimental replicates. Adapted from Knelson, Gaviglio et al. 2013. Copyrighted material is used with permission from the journal.
1.2 Heparan sulfate signaling and the tumor stroma

1.2.1 Schwannian stroma in neuroblastoma

The tumor stroma often promotes malignant properties of solid tumors by creating and maintaining a pro-tumorigenic microenvironment (Li et al. 2007, Pietras and Ostman 2010). Conversely, the stroma is a positive prognostic factor in neuroblastoma and is favorable when designating stage of disease (Shimada et al. 1984).

The neuroblastoma tumor stroma consists mainly of Schwann cells, but can also contain fibroblasts and immune cells (Ambros et al. 1996, Zeine et al. 2009, Johann et al. 2010). The origin of Schwann cells is unclear, with evidence for both neoplastic cell-derived Schwann cells (Mora et al. 2001) and Schwann cells resulting from nearby normal cells that have undergone differentiation (Ambros et al. 1996, Du et al. 2008).

The neuroblastoma stroma functions to reduce tumorigenesis by impeding activation of cancer-associated fibroblasts and subsequent angiogenesis (Zeine et al. 2009) as well as promoting neuroblast differentiation (Kwiatkowski et al. 1998, Liu et al. 2005). Cross-talk between tumor cells and Schwann cells promotes neurogenesis and differentiation of neuroblasts in co-culture (Ambros et al. 1996) and in xenograft assays (Liu et al. 2005). Treatment of neuroblastoma cell lines with Schwann cell conditioned media has also been shown to promote neuroblast differentiation (Kwiatkowski et al. 1998), suggesting that soluble factors released from the tumor stroma are capable of inducing differentiation of neuroblastoma cells.
Figure 3: HSPGs and FGF2 co-localize with the Schwannian stroma in early-stage neuroblastoma tissue specimens

Immunofluorescence in neuroblastoma specimens using TβRIII, GPC1, GPC3, and FGF2 antibodies (green) and an S100 Schwannian stroma antibody (red). DAPI nuclear stain in blue. 40x images, scale bar=50µM. Images are representative of seven independent early-stage and seven independent late-stage tissue samples. Adapted from Knelson, Gaviglio et al. 2014a. Copyrighted material is used with permission from the journal.
The neurotrophins are secreted by Schwann cells and play an essential role in neuronal survival and differentiation (Reynolds and Woolf 1993, Ambros and Ambros 1995, Barbacid 1995). Recently, the neurotrophin nerve growth factor has been shown to promote neuroblast differentiation when secreted from Schwann cells via activation of the NTRK1 receptor on tumor cells (Pajtler et al. 2014). The stroma can also secrete soluble factors that have anti-angiogenic properties, including PEDF, TIMP-2, and SPARC (Huang et al. 2000, Crawford et al. 2001, Chlenski et al. 2002). Secreted PEDF was also capable of promoting morphologic differentiation of neuroblastoma cells (Crawford et al. 2001). Cross-talk between malignant tumor cells and Schwann cells can result in anti-metastatic effects; secretion of CXCL13, which is highly expressed in stroma-rich tumors compared to stroma-poor tumors, limited metastatic capability of neuroblastoma cells (Del Grosso et al. 2011). Our lab has also identified heparan sulfate proteoglycans (HSPGs), including TβRIII and glypicans (GPCs) 1 and 3, as well as basic fibroblast growth factor (FGF2) as stromal-derived factors (Figure 3) that have differentiation-promoting capabilities (Knelson et al. 2013, Knelson et al. 2014a). Further characterization of the differentiating-stroma secretome is essential to understand the complex relationship between the Schwannian stroma and neuroblastoma cells.
Table 1: HSPGs and their growth factor binding interactions


<table>
<thead>
<tr>
<th>HSPG</th>
<th>Growth Factors</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>sulfate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD44</td>
<td>FGF2, HGF, VEGF, HBEGF</td>
<td>(Jones et al. 2000, Yu et al. 2002, Volz et al. 2015)</td>
</tr>
<tr>
<td>Agrin</td>
<td>FGF2, NRG1</td>
<td>(Meier et al. 1998, Cotman et al. 1999)</td>
</tr>
</tbody>
</table>
1.2.2 Heparan sulfate signaling in neuroblastoma

HSPGs lack intrinsic signaling motifs, yet they mediate signaling by regulating ligand binding. Furthermore, HSPGs can be cleaved, producing a soluble receptor that can also form signaling complexes (Mythreye and Blobe 2009a). HSPGs interact with heparin-binding ligands (Table 1), including FGF2 and heparin-binding epidermal growth factor-like growth factor (HBEGF), as well as their receptors, including fibroblast growth factor receptor 1 (FGFR1) to mediate signaling and biology (Yayon et al. 1991, Higashiyama et al. 1993, Aviezer and Yayon 1994, Knelson et al. 2013, Knelson et al. 2014b). TβRIII, GPCs, and syndecans (SDCs) are the most abundant HSPGs in mammalian cells and have demonstrated roles in neuronal development and nervous system FGF signaling (Carey 1996, Kinnunen et al. 1998, Akita et al. 2004, Knelson et al. 2013, Knelson et al. 2014a). SDC3, also referred to as neuronal SDC, was initially identified in rat Schwann cells and is expressed in the developing rat brain, suggesting an involvement in nervous system maturation (Carey et al. 1992, Chernousov and Carey 1993). Furthermore, GPC1 and SDC3 enhance neurite outgrowth, indicating a role in neuronal differentiation (Kinnunen et al. 1998, Akita et al. 2004). Our work has demonstrated an important function for heparan sulfate signaling in neuroblastoma. Several HSPGs including TβRIII, GPCs, and SDCs are decreased in neuroblastoma with decreased expression correlating to poor prognosis (Iolascon et al. 2000, Albino et al. 2008, Iolascon et al. 2000, Albino et al. 2008, Knelson et al. 2013, Knelson et al. 2014a).
We have shown that these HSPGs can promote neuroblast differentiation while suppressing proliferation both \textit{in vitro} and \textit{in vivo} (Figure 4) (Knelson et al. 2013, Knelson et al. 2014a).

\textbf{Figure 4: HSPGs and FGF2 promote neuroblastoma differentiation to suppress proliferation}

HSPGs cleaved from the stromal cell surface and released in soluble form bind FGF2 and FGF receptors to enhance ERK1/2 signaling (inset). ERK1/2 is then translocated to the nucleus leading to activation of transcription factors, including ID1. Together, this pathway enhances neuroblastoma differentiation and decreases neuroblastoma proliferation. Adapted from Knelson et al. 2014b.
1.2.3 TβRIII in neuroblastoma

TβRIII is a transmembrane proteoglycan that is critical for embryonic development, as TβRIII-null mice are embryonic lethal and display both liver and cardiac defects (Stenvers et al. 2003). The importance of TβRIII in regulating normal cellular function is further demonstrated by its loss in many human cancers, including ovarian, prostate, lung, pancreatic, and breast carcinomas (Bristow et al. 1999, Venkatasubbarao et al. 2000, Dong et al. 2007, Hempel et al. 2007, Sharifi et al. 2007, Turley et al. 2007, Finger et al. 2008, Gordon et al. 2008). Transcriptional regulation by TGF-β1, as well as epigenetic regulation and loss of heterozygosity have all been implicated in the loss of TβRIII expression in different contexts (Gatza et al. 2010). TβRIII interacts with TGF-β1, TGF-β2, and TGF-β3, as well as activin-A, inhibin-A, BMP2, BMP4, BMP7, GDF-5, and FGF2 (Kirkbride et al. 2005, Bernabeu et al. 2009, Knelson et al. 2013) to activate a variety of signaling pathways. The canonical pathway involves SMAD-mediated signaling; however, TβRIII can also activate non-SMAD pathways, including p38, NFκB, ERK, and CDC42 (Mythreye and Blobe 2009b, Gatza et al. 2010, Gatza et al. 2011, Knelson et al. 2013). These signaling events lead to a variety of biological consequences. TβRIII has been shown to have effects on epithelial and cancer cell migration (Hempel et al. 2007, Gordon et al. 2008, Mythreye and Blobe 2009b), invasion (Dong et al. 2007, Hempel et al. 2007, Finger et al. 2008, Gordon et al. 2008), cell growth (Bandyopadhyay et al. 1999, Bandyopadhyay et al. 2002a, Bandyopadhyay et al.

TβRIII exerts some of its effects via its membrane-bound form, but can also be cleaved and released as a soluble form (sTβRIII). sTβRIII has roles in ligand sequestration leading to effects on canonical SMAD signaling (Bandyopadhyay et al. 1999, Vilchis-Landeros et al. 2001). In colon and breast cancer xenograft models, sTβRIII acts as a TGF-β neutralizing agent, leading to antitumor effects (Bandyopadhyay et al. 2002a, Bandyopadhyay et al. 2002b). Conversely, sTβRIII may also enhance TGF-β signaling in some models (Lopez-Casillas et al. 1991, Fukushima et al. 1993), demonstrating the complexity of TβRIII signaling and suggesting it exerts its biological effects in a context-specific manner. The investigation of the antitumor activity of sTβRIII in different tumor environments is an ongoing area of research and understanding its regulation and function will be crucial to the discovery of novel anti-cancer therapies.

TβRIII expression is downregulated in late-stage neuroblastomas compared to early-stage neuroblastomas (Iolascon et al. 2000, Knelson et al. 2013) and in neuroblastoma tumors compared to the benign neuroblastic ganglioneuroma or ganglioneuroblastoma tumors (Albino et al. 2008, Janoueix-Lerosey et al. 2010). In addition, in stroma-poor tumors and neuroblastoma cell lines, TβRIII expression is decreased compared to stroma-rich tumors or stromal-like cell lines (Albino et al. 2008,
Figure 5: N-Myc suppresses TβRIII expression

(A) Microarray dataset analysis for TβRIII expression in a meta-dataset consisting of 5 publicly available microarray datasets from the Gene Expression Omnibus. Data are presented as median and inter-quartile range. Mann-Whitney test. ****p<0.0001. NA=Non-amplified, Amp=Amplified

(B) Linear regression of N-Myc and TβRIII expression in the microarray meta-dataset.

(C) Western blot and 125I TGF-β binding and crosslinking with TβRIII pull-down of SK-N-AS-N-MycER-inducible cell line in the presence and absence of 4-hydroxytamoxifen (4OHT) to stabilize N-Myc. (D) SHEP-21N repressible cell line in the presence and absence of doxycycline (Dox) to repress N-Myc expression. Dox was replenished at day 3 for the 5-day treatment.

(E) 125I TGF-β binding and crosslinking with TβRIII pull-down in the presence and absence of the HDAC inhibitors trichostatin A (TSA; 1 and 4 hour treatments) and valproic acid (VPA; 3 and 6 day treatments) at the concentrations shown. Western blots for acetyl-lysine (Ac Lys), and TβRIII in the presence and absence of TSA (4 hour treatment). Background and β-actin normalized integrated density for TβRIII shown as percent control. Images in C-E are representative of at least three independent experiments. Adapted from Knelson, Gaviglio et al. 2013. Copyrighted material is used with permission from the journal.
Knelson et al. 2013). Our lab has demonstrated that this loss in TβRIII expression is due to epigenetic regulation by N-Myc and can be rescued by HDAC inhibition (Figures 2 and 5). Decreased TβRIII expression correlates with decreased survival and contributes to the undifferentiated phenotype often seen in late-stage, stroma-poor tumors (Knelson et al. 2013, Knelson et al. 2014a). Both membrane-bound and stromal-derived TβRIII can interact with FGF2 and FGFR1 to enhance ERK1/2 signaling, leading to upregulation of the inhibitor of DNA binding 1 (ID1) transcription factor. This signaling cascade promotes neuroblast differentiation to decrease proliferation, tumor growth, and metastasis (Knelson et al. 2013, Knelson et al. 2014a).

1.3 Growth factor signaling in cancer

1.3.1 EGF family signaling in neuroblastoma

The epidermal growth factor (EGF) family of ligands consists of 11 members that share similar structural and functional characteristics. This class of proteins includes epidermal growth factor (EGF), transforming growth factor alpha (TGFα), epigen (EPGN), betacellulin (BTC), epieregulin (EREG), HBEGF, amphiregulin (AREG), and neuregulins 1-4 (NRG). These proteins induce homo- or hetero-dimerization of their respective ERBB receptors leading to induction of the receptor’s intrinsic tyrosine kinase activity resulting in initiation of downstream signaling. Three EGF family members, including HBEGF (Higashiyama et al. 1992, Aviezer and Yayon 1994), AREG (Cook et al. 1991), and NRG1 (Loeb and Fischbach 1995, Meier et al. 1998) can bind to their
respective ERBB receptors as well as heparan sulfate moieties to promote different biological consequences.

EGF treatment in neuroblastoma cell lines has been shown to lead to increased cellular proliferation (da Motta et al. 1997, Ho et al. 2005). One of these studies attributed this increase to PI3K/AKT signaling, as inhibition of this pathway attenuated EGF-induced proliferation (Ho et al. 2005). In addition, another study suggested that this pro-proliferative effect relied on cooperation with other proteins; EGF could only induce proliferation in the presence of fetal bovine serum, which contains many other growth factors (da Motta et al. 1997).

The tumor-promoting effects of EGF, along with data demonstrating that neuroblastoma cell lines express the epidermal growth factor receptor (EGFR) and this expression correlates with multi-drug resistance (Meyers et al. 1988, Ho et al. 2005, Tamura et al. 2007), have led to studies seeking to inhibit EGFR signaling in hopes of reducing tumor growth. The FDA-approved EGFR inhibitor gefitinib has been shown to promote apoptosis (Tamura et al. 2007), decrease cell survival (Hatzigapiou et al. 2011), and has demonstrated modest results in a small study of children with relapsed neuroblastoma in combination with chemotherapeutics (Donfrancesco et al. 2010). In contrast, other results have not been so promising. One study found that the levels of gefitinib necessary to produce anti-proliferative effects was not achievable in pediatric patients and this study suggested that clinical trials were not justified (Rossler et al.
In support of this study, another group performed a small clinical study involving 19 neuroblastoma patients treated with gefitinib and the chemotherapy irinotecan. Though the drug combination was tolerated well, they did not observe a significant response level and thus did not recommend further evaluation of this treatment (Furman et al. 2012). Our work also cautions against the use of EGFR inhibitors, as we demonstrate that EGFR is important for neuroblast differentiation and high expression is associated with improved survival (see Chapter 3). Further studies are needed to identify if there is a subgroup of patients with hyperactive EGFR who may respond to receptor inhibition.

EGF family members can also display tumor-suppressive roles in neuroblastoma. TGFα treatment has been shown to enhance biochemical differentiation marker expression as well as morphological differentiation in an EGFR-expressing human cell line, LAN-5 (Scarpa et al. 1994). Unexpectedly, this report also demonstrated an increase in cellular proliferation with TGFα treatment, rather than the expected decrease in proliferation often seen with cellular differentiation (Goya and Timiras 1991, Lange et al. 2011, Cruz and Matushansky 2012). These results could be partially explained by a more recent study that identified the requirement for estrogen receptor-alpha to convert TGFα from a pro-proliferative molecule to a pro-differentiating factor (Ciana et al. 2003).

Another EGF family member, EREG, has also been shown to promote neuroblast differentiation. In a recent study, treatment of human SK-N-BE neuroblastoma cells with
EREG led to a decrease in cellular proliferation as well as an increase in morphological differentiation as visualized by neurite outgrowth (Rizzi et al. 2013). This research attributed these effects to enhanced ERK/MAPK activity, a pathway that has been extensively linked to neuroblast differentiation (Kim et al. 2004, Passiatore et al. 2011, Wang et al. 2011, Qiao et al. 2012).

1.3.1.1 HBEGF

HBEGF is a transmembrane glycoprotein that was initially identified in conditioned media from macrophages, demonstrating a high affinity for immobilized heparin and displaying mitogenic properties for fibroblasts and smooth muscle cells (Higashiyama et al. 1991). HBEGF is essential for lung and heart function, as demonstrated by the lethality associated with HBEGF-null mice (Iwamoto et al. 2003, Jackson et al. 2003, Yamazaki et al. 2003). As with other EGF family ligand members, HBEGF is produced as a 208kD pro-form, which undergoes ectodomain shedding to produce a soluble form (sHBEGF) and a C-terminal fragment (CTF) (Figure 6) (Vinante and Rigo 2013). Prior to cleavage, the membrane-anchored pro-form acts in a juxtacrine manner to influence signaling of nearby cells (Nakamura et al. 2001, Murayama et al. 2002), as well as functioning as the diphtheria toxin receptor (Naglich et al. 1992, Mitamura et al. 1995). The CTF also has biological activity; interaction with the transcriptional repressors PLZF or Bcl6 leads to nuclear export or degradation of these proteins, thus inhibiting their repressive actions (Nanba et al. 2003, Kinugasa et al. 2007,
HBEGF binds to both EGFR and ERBB4 to activate signaling (Raab and Klagsbrun 1997) and requires binding to HSPGs via its heparin-binding domain to achieve maximal activation (Figure 6) (Higashiyama et al. 1993, Aviezer and Yayon 1994). HBEGF can interact with the HSPGs, SDC3 (Hienola et al. 2006) and CD44 (Bennett et al. 1995, Yu et al. 2002) to affect neural migration and tissue remodeling. Downstream, HBEGF is able to initiate ERK/MAPK, PI3K/AKT, NFκB, and STAT signaling, leading to cell context specific outcomes.

Several drugs and proteins, including all-trans retinoic acid (ATRA), phorbol 12-myristate 13-acetate, TNFα, 1α,25-(OH)2D3, GM-CSF, and CXCL12 can induce HBEGF expression, likely through regulation by putative AP1, Sp-1, and SP3 binding sites located in the HBEGF promoter (Fen et al. 1993, Yoshimura et al. 2003, Edwards et al. 2009, Vinante and Rigo 2013, Miyata et al. 2014). A better understanding of HBEGF regulation in different cellular contexts will be necessary to identify potential therapies that will inhibit or activate this important cancer target.

1.3.1.2 HBEGF in neuroblastoma

HBEGF is highly expressed in the developing and adult nervous systems, and similar to FGF2, stimulates ERK signaling (Farkas and Krieglstein 2002). Along with its receptor, EGFR, HBEGF has previously been shown to promote neurogenesis (Nakagawa et al. 1998 and Jin et al. 2002). Importantly, HBEGF can also promote
neuroblast differentiation of pheochromocytoma cells, a process that depends on EGFR activation and ERK1/2 signaling (Zhou and Besner 2010). In neuroblastoma cell lines, it has been suggested that HBEGF may be a valid target for therapy. This research demonstrated that HBEGF inhibition with a diphtheria toxin mutant, which binds specifically to both membrane-bound and soluble HBEGF, leads to increased apoptosis in two neuroblastoma cell lines (Nam et al. 2015). However, this study did not address the lack of efficacy in the other neuroblastoma cell lines tested, though it is likely attributed to the higher basal HBEGF expression observed in the cell lines that responded to treatment. In contrast, our work has identified that increased HBEGF expression is associated with improved prognosis in neuroblastoma patients and may have a tumor-suppressive role in this disease, resulting in enhanced differentiation and decreased proliferation (see Chapter 3).

1.3.1.3 HBEGF in other cancers

HBEGF is overexpressed in many tumors, including pancreatic (Kobrin et al. 1994), bladder (Ruck and Paulie 1998, Thogersen et al. 2001), ovarian (Miyamoto et al. 2004), liver (Inui et al. 1994, Ito et al. 2001), esophageal (Sato et al. 1996), breast, and gastric cancers (Yotsumoto et al. 2008), in addition to melanoma (Downing et al. 1997) and glioblastoma (Mishima et al. 1998). Targeting HBEGF in these tumors types is an area of active research, though the HBEGF inhibitor CRM197 (Mitamura et al. 1995) and the HBEGF neutralizing antibody Y-142 (Sato et al. 2012) are the only inhibitors to date
Figure 6: Domain structure and processing of mature HBEGF

The mature form of HBEGF consists of a heparin-binding domain and an EGF-like domain on the N-terminus end. A juxtamembrane, transmembrane, and cytoplasmic domain make up the C-terminal end. Cleavage at the juxtamembrane region by ectodomain shedding results in the release of soluble HBEGF and a cytoplasmic tail fragment. Adapted from Vinante and Rigo 2013.

that can be used to evaluate HBEGF as a potential therapeutic target.

HBEGF has been implicated as an important mediator of proliferation and cellular transformation. Transfection of HBEGF confers oncogenic properties to normal rat kidney cells and chicken embryo fibroblasts (Fu et al. 1999, Harding et al. 1999). In a pancreatic cancer genetic mouse model, HBEGF overexpression in addition to oncogenic KRAS expression was sufficient to produce rapid transformation of the exocrine
pancreas shortly before birth (Ray et al. 2014). HBEGF is also critical for epithelial proliferation in myeloma (Wang et al. 2002), glioblastoma (Mishima et al. 1998), pancreatic tissue (Means et al. 2003), and gastric (Wallasch et al. 2002) and ovarian (Miyamoto et al. 2004, Yagi et al. 2005) cancer models.

The majority of evidence for HBEGF in the malignant phenotype of tumors lies in its role in cell migration and invasion. In normal kidney cells, sHBEGF, but not membrane-bound HBEGF increases cell migration by decreasing cell-matrix and cell-cell interactions (Singh et al. 2004). HBEGF can also bind N-arginine dibasic convertase, which contributes to HBEGF-induced migration in a breast cancer cell line (Nishi et al. 2001). In addition, in estrogen receptor-negative breast cancer tumors and pancreatic tumors, HBEGF is correlated with enhanced metastasis and contributes to the invasive potential of these tumor cells (Tarbe et al. 2002, Zhou et al. 2014). Inhibition of HBEGF ectodomain shedding in fibrosarcoma, osteosarcoma, and melanoma cell lines reduces their invasive capabilities (Mammoto et al. 2002), while a neutralizing HBEGF antibody inhibits IL-8-induced migration in colon cancer cells (Itoh et al. 2005). Further investigation is necessary to identify tumor types that may respond to HBEGF-inhibiting strategies, though its importance in normal development may pose a problem when thinking about using this strategy on pediatric patients. Additional studies need to be conducted to determine if HBEGF could be used as a biomarker to better select patients who may benefit from this targeting strategy, as this will be critical to achieve maximal
results while minimizing potentially life-threatening side effects, particularly in children with these specific cancer types.

1.4 Therapeutic approaches in neuroblastoma

1.4.1 Targeted therapies

Despite improvements in survival for low-risk neuroblastoma patients over the past 40 years, high-risk patients still have a 5-year survival rate of only 40% (Maris et al. 2007, Maris 2010). Over half of neuroblastoma patients are categorized as high-risk at the time of diagnosis and 60% of these patients will suffer a relapse due to minimum residual disease (Cotterill et al. 2001, Maris 2010, Masetti et al. 2012). Post relapse, the overall survival rate is only 20% (London et al. 2011), begging for the discovery of new therapeutic options for high-risk neuroblastoma patients (Table 2).

Observations that some neuroblastomas naturally follow a biological path that includes spontaneous regression or maturation into a more differentiated, benign lesion led to the current approach for neuroblastoma therapy, which aims to promote neuroblast differentiation (D’Angio et al. 1971, Evans et al. 1971) (Adamson 1994, Masetti et al. 2012). The identification of ATRA as a differentiating drug in neuroblastoma cell lines in vitro (Sidell 1982, Sidell et al. 1983) led to the expansion of retinoids as differentiating therapies. Further studies identified 13-cis RA as a superior differentiating agent in the clinic, due to its advantageous pharmacokinetic profile (Khan et al. 1996, Matthay et al. 1999). Currently, treatment with 13-cis RA after
Table 2: Targeted therapies in clinical trials for neuroblastoma

<table>
<thead>
<tr>
<th>Agent</th>
<th>Mechanism</th>
<th>Clinical Trial Stage</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>13-cis-RA</td>
<td>Differentiation, inhibition of N-Myc</td>
<td>Standard of care</td>
<td>(Masetti et al. 2012)</td>
</tr>
<tr>
<td>ch14.18</td>
<td>Anti-GD2 monoclonal antibody</td>
<td>Superior to 13-cis-RA in Phase III trial</td>
<td>(Yu et al. 2010)</td>
</tr>
<tr>
<td>fenretinide</td>
<td>Retinoic acid derivative, promotes apoptosis</td>
<td>Phase I/II</td>
<td>(Villablanca et al. 2011, Maurer et al. 2013)</td>
</tr>
<tr>
<td>crizotinib</td>
<td>ALK inhibitor</td>
<td>Phase III</td>
<td>(Mosse et al. 2013)</td>
</tr>
<tr>
<td>alisertib/MLN8237</td>
<td>AurkA inhibitor</td>
<td>Phase I/II</td>
<td>(Masse et al. 2012)</td>
</tr>
<tr>
<td>vorinostat</td>
<td>HDAC inhibitor</td>
<td>Phase I/II</td>
<td>(Fouladi et al. 2010, Witt et al. 2012, DuBois et al. 2015b)</td>
</tr>
<tr>
<td>decitabine</td>
<td>DNMT inhibitor</td>
<td>Phase I</td>
<td>(George et al. 2010, Krishnadas et al. 2015)</td>
</tr>
<tr>
<td>^131^I-MIBG</td>
<td>systemic targeted radiopharmaceutical</td>
<td>Phase II</td>
<td>(DuBois et al. 2015a, DuBois et al. 2015b)</td>
</tr>
<tr>
<td>SF1126</td>
<td>PI3K/mTor inhibitor</td>
<td>Phase I</td>
<td>(Peirce et al. 2011)</td>
</tr>
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</table>

myeloablative therapy and autologous bone marrow transplantation is the standard of care for high-risk neuroblastoma (Masetti et al. 2012).

While some high-risk neuroblastomas respond well to ATRA or 13-cis-RA, many are resistant or will become resistant (Masetti et al. 2012). ATRA-resistant or 13-cis-RA-resistant cell lines have been shown to be sensitive, or in some cases hypersensitive, to
the retinoid derivative fenretinide, which has shown efficacy in early phase clinical trials (Reynolds et al. 2000, Villablanca et al. 2006, Villablanca et al. 2011, Maurer et al. 2013). The sensitivity of these cell lines and tumors may be due to fenretinide’s ability to promote apoptosis instead of differentiation, demonstrating a distinct mechanism of action compared to ATRA or 13-cis-RA (Delia et al. 1993, Fang et al. 2011).

The discovery of anaplastic lymphoma kinase (ALK) mutations as major contributors in familial neuroblastoma has generated interest in targeting this protein for neuroblastoma treatment (Mosse et al. 2008). The targeted therapy, crizotinib, inhibits ALK and has shown promise in preclinical testing (Bresler et al. 2011); however a Phase I/II study of crizotinib in neuroblastoma patients demonstrated limited response rates, likely due to resistance conferred by certain ALK mutations (Bresler et al. 2011, Mosse et al. 2013). Combination treatments with chemotherapy, in addition to second-generation ALK inhibitors are currently being considered and may overcome resistance to enhance clinical efficacy (Barone et al. 2013, Moreno et al. 2013).

As for many cancers, immunotherapy is currently being explored as a potential treatment option for high-risk neuroblastoma patients. The current approved approach is aimed at targeting the glycolipid GD2 with monoclonal antibodies. GD2 antigens are present in elevated amounts on neuroblastoma cells compared to healthy cells, making this antigen a good target for therapy since healthy cells will be spared from injury (Yang and Sondel 2010). Consolidation therapy with anti-GD2 immunotherapy in
combination with cytokines and 13-cis-RA has shown efficacy in increasing event-free survival and overall survival in high-risk neuroblastoma patients (Ploessl et al. 2016). It remains unclear whether anti-GD2 therapies will be effective as front-line therapy or whether tumor burden may dictate patient response to this particular treatment.

The amplification of MYCN is a major predictor of outcome, with those patients harboring MYCN amplification having a poor prognosis (Schwab et al. 1983, Seeger et al. 1985). Targeting the expression or oncogenic activity of N-Myc has been a major area of study for researchers. The observation that retinoids reduce N-Myc levels to promote neuroblast differentiation (Thiele et al. 1985, Thiele et al. 1988) has fueled interest in identifying ways to mediate this oncogene. Although N-Myc is difficult to target directly, many successful approaches have aimed to indirectly downregulate its expression or activity by targeting molecules upstream or downstream of N-Myc itself.

One class of inhibitors aims to target the DNA-binding functions of N-Myc. Interaction with the transcription factor Max is important for N-Myc’s ability to bind DNA and regulate transcription of its target genes. Compounds aimed at inhibiting the Myc:Max interaction have demonstrated suppression of Myc activity and reduced tumorigenicity in in vitro and in vivo models of neuroblastoma, as well as other cancers (Soucek et al. 2008, Soucek et al. 2013, Zirath et al. 2013).

Another class of inhibitors aims to inhibit the transcription of MYCN. Bromodomain and extraterminal (BET) bromodomain inhibitors have shown efficacy in
reducing tumor growth and increasing survival in preclinical models of neuroblastoma (Puissant et al. 2013, Henssen et al. 2016). Importantly, BET bromodomain inhibitors have been shown to act as differentiating agents in neuroblastoma, likely due to the disruption of BET binding to the MYCN promoter, leading to decreased transcriptional activation of the oncogene (Lee et al. 2015).


N-Myc relies on interactions with other proteins, such as Aurora kinase A (AurkA) and epigenetic modifiers including DNMTs and HDACs, to exert its oncogenic function. AurkA is critical for N-Myc stabilization, while DNMTs and HDACs act to alter N-Myc-mediated transcription (Otto et al. 2009, Westermark et al. 2011, He et al. 2013). Inhibitors of AurkA, DNMTs, and HDACs have shown efficacy in in vitro and in vivo neuroblastoma models (Yang et al. 2003, Yang et al. 2004, Yang et al. 2007, Maris et al. 2010, Gu et al. 2012, Brockmann et al. 2013, Romain et al. 2014). Currently, these agents are undergoing evaluation in clinical trials, most often in combination with 13-cis-
RA or chemotherapies (Fouladi et al. 2010, George et al. 2010, Mosse et al. 2012, Krishnadas et al. 2015).

1.4.2 Heparins as therapeutic agents

Heparins are structurally similar to HSPGs, with each consisting of repeating disaccharide units of glucosamine and glucuronic acid (Rabenstein 2002). Heparins display sulfation at the 3-O, 6-O, and N sites on glucosamine and the 2-O site on glucuronic acid, while HSPGs can be variably sulfated at these same positions. Sulfation at these sites is required for the anti-coagulant activity of heparin (Whitelock and Iozzo 2005). Heparins have been shown to be protective in terms of cancer risk and have demonstrated therapeutic effects, some of which can be attributed to its anti-coagulant action (Tagalakis et al. 2007, Zhang et al. 2013). However, the anti-neoplastic activity of heparin surpasses its role as an anti-coagulant, suggesting other contributing mechanisms exist.

Heparin has been shown to reverse cisplatin resistance in ovarian cancer cells due to extensive transcriptional reprogramming (Pfankuchen et al. 2015). Heparin can also exert anti-cancer effects by inhibiting selectin-mediated binding of platelets and leukocytes to the endothelium, leading to attenuation of metastasis in melanoma and colon cancer models (Borsig et al. 2011). Inhibition of cancer-promoting molecules, including heparanase (Borsig et al. 2011) and sulfatases (Hossain et al. 2010), is another mechanism by which heparin may demonstrate therapeutic effects. Heparanase and
sulfatases are both heparin-modifying enzymes with metastasis-promoting activity (Vlodavsky and Friedmann 2001, Lai et al. 2008a). Heparin treatment can reduce heparanase activity in a melanoma mouse model, leading to decreased lung metastasis (Vlodavsky and Friedmann 2001). Due to conflicting roles for the two known human sulfatases, hSULF-1 and hSULF-2 (Lai et al. 2008b), it is not well established if these molecules directly influence heparin’s therapeutic activity.

Recently, our lab has demonstrated a role for heparin derivatives in promoting neuroblastoma differentiation. Treatment with heparin or ODSH, a heparin derivative lacking anti-coagulant activity, can suppress xenograft growth and metastasis by enhancing differentiation in multiple orthotopic xenograft models of neuroblastoma (Knelson et al. 2014a). ODSH is currently in clinical trials for metastatic pancreatic cancer in adult patients and is being tested as a platelet recovery agent in a clinical trial for pediatric solid tumors, including neuroblastoma. It remains unknown whether antitumor effects will be observed in these settings.

**1.4.3 Future perspectives in neuroblastoma treatment**

ALK, N-Myc, and GD2 represent the three major molecular targets for which therapies have been approved for neuroblastoma treatment, though there is a crucial need to identify alternative molecular targets to improve outcome for neuroblastoma patients (Barone et al. 2013). New therapies on the horizon, including AurkA, DNMT, and HDAC inhibitors (see Chapter 3 and Table 2) as well as heparin derivatives
(Knelson et al. 2014a), may prove useful as differentiating agents that can target residual tumor cells.

Aside from developing novel therapies for neuroblastoma treatment, identification of prognostic and pharmacodynamic biomarkers is critical to avoid unnecessary treatment of these young neuroblastoma patients. Indeed, current approaches aim to utilize genetic and genomic screening to aid in treatment decisions (Hara 2012). The first prospective clinical trial in children to use this personalized therapy approach has recently started enrolling patients with relapsed or refractory neuroblastoma. The trial starts with a biopsy of the relapsed tumor or a site of relapse. Upon genomic profiling, therapy is assigned based upon the observed genetic changes, allowing for the incorporation of new therapies based on ongoing lab studies analyzing the evolution of the tumor’s genetics. It remains unclear if this tailored approach will prove efficacious for neuroblastoma patients.

In addition to using tumor biology to identify promising therapeutics, the future of neuroblastoma treatment will likely also include combination therapies consisting of targeted therapies with retinoids, chemotherapies, or other targeted agents in order to maximize efficacy (Morgenstern et al. 2013). Caution is warranted, however, as toxicities will likely increase as drug combinations increase. The ability to test the multitude of potential therapies is limiting as neuroblastoma is still relatively rare, highlighting the extreme care required when designing clinical trials for this disease. Ideal strategies will
garner insight from carefully performed studies incorporating multiple genetic and orthotopic xenograft models in addition to mechanistic cell line analyses.
2. Materials and Methods

2.1 Cell lines and reagents

SK-N-AS (ATCC CRL-2137), SK-N-SH-SY5Y (5Y; CRL-2266), SK-N-BE(2) (BE2; CRL-2271), and IMR-32 (IMR32, CCL-127) cells were purchased from the American Type Culture Collection (ATCC). 5Y and BE2 were grown in a 1:1 mixture of Eagle minimum essential medium and Ham’s F12 with 10% fetal bovine serum and 2mM L-Glutamine. SK-N-AS, SK-N-SH-SHEP (SHEP; gift of Michael Armstrong, Duke University, Durham, NC, USA), SK-N-AS-N-MycER, SHEP-21N (gifts of Linda Valentijn, University of Amsterdam, Amsterdam, The Netherlands), 9464D, and 975A2 (gifts of Rimas Orentas, National Cancer Institute, Bethesda, MD, USA) were grown in Dulbecco modified Eagle minimum essential medium with 10% fetal bovine serum and 1% non-essential amino acids. IMR32 were grown in modified Eagle minimum essential medium with 10% fetal bovine serum, 1mM sodium pyruvate, and 1% non-essential amino acids. 293FT and COS7 were sourced and grown as described previously (Hanks et al. 2013). All cells were grown at 37°C in 5% CO2. All human cell lines have tested negative for mycoplasma contamination. 4-hydroxytamoxifen (no. H7904) and doxycycline (no. D9891) were purchased from Sigma (St. Louis, MO, USA). The MEK1/2 inhibitor U0126 (no. 9903) was purchased from Cell Signaling (Danvers, MA, USA). The MEK1/2 inhibitor CI-1040 (no. S1020) and the STAT3 inhibitor ruxolitinib (no. S1378) were purchased from Selleck Chemical (Houston, TX, USA). The EGFR inhibitor erlotinib (no.
10483) was purchased from Cayman Chemical (Ann Arbor, MI, USA). The EGFR inhibitors lapatinib and gefitinib were acquired from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, at the National Cancer Institute. MLN8237 (no. S1133) for \textit{in vitro} experiments was purchased from Selleck Chemical. Vorinostat (no. 4652) was purchased from Tocris Bioscience (Avonmouth, Bristol, United Kingdom). Decitabine (no. 11166) for \textit{in vitro} experiments was purchased from Cayman Chemical. Recombinant soluble human HBEGF (no. 259-HE), TβRIII (no. 242-R3), GPC1 (no. 4519-GP), GPC3 (no. 2119-GP), SDC3 (no. 3539-SD), and CD44 (no. 3660-CD) were purchased from R&D Systems (Minneapolis, MN, USA). Co-culture experiments utilized restrictive 0.4μM transwells in 12-well dishes (Corning, Inc.; Corning, NY, USA).

\textbf{2.2 Microarray dataset analysis}

Our microarray meta-dataset was generated as previously described (Knelson et al. 2013). Data for the ERBB receptors are from the three U133 plus datasets only, as the Human Exon 1.0 ST Arrays lacked an EGFR probe. Expression analysis in stroma-rich and stroma-poor tumors was performed using the publicly available Albino dataset (GSE7529, (Albino et al. 2008)). Survival analysis was conducted using the Neuroblastoma Prognosis (Wei et al. 2004) dataset from the Oncogenomics website (http://home.ccr.cancer.gov/oncology/oncogenomics/). The SEQC (Consortium, Munro et al. 2014, Su et al. 2014, Wang et al. 2014) dataset (GSE49710) was used for survival,
gene expression, and linear correlation analysis. Data was visualized using the R2: Genomics Analysis and Visualization Platform (http://r2.amc.nl). The microarray probes are listed in Appendix B: Table 4. All affymetrix probes listed are from the HG-U133 Plus 2.0 platform. The GSE49710 probes listed are from the Agilent-020382 Human Custom Microarray platform.

2.3 DNA constructs, shRNA/siRNA

The TβRIII adenoviral shRNA construct used in these studies has been described previously and was used at an MOI of 50 particles/cell (Knelson et al. 2013). The EGFR-GFP overexpression plasmid was a gift from Alexander Sorkin (Addgene plasmid #32751). The EF.STAT3DN.Ubc.GFP plasmid was a gift from Linzhao Cheng (Addgene plasmid #24984). Transient DNA transfections were performed using lipofectamine 2000 (Invitrogen; Grand Island, NY, USA) or X-tremeGENE 9 (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer’s instructions.

HBEGF shRNA (62203, 62204) knockdown constructs (Sigma Mission TRC1) were purchased from the Duke University RNAi core facility. HBEGF shRNA sequences are listed in Appendix C: Table 5. Pooled ID1 siRNA (sc-29356) and control siRNA (sc-37007) were purchased from Santa Cruz Biotechnology Inc. (Dallas, TX, USA) and used according to the manufacturer’s instructions.

2.4 Western blotting

Western blotting was performed as described previously using standard
techniques (Knelson et al. 2013). Each experiment was conducted a minimum of three independent times. Antibodies for differentiation and signaling markers were purchased from Cell Signaling: NF160 (no. 2838), β3-tubulin (no. 5568), NSE (no. 9536), GAP43 (no. 5307 and 8945), phospho-ERK1/2 (p-ERK1/2; no. 9101), ERK1/2 (no. 4695), p21 (no. 2947), phospho-AKT (p-AKT; no. 4058), AKT (no. 4691), phospho-STAT3 (p-STAT3; no. 9145), STAT3 (no. 9139), and EGFR (no. 4267). The β-actin (no. A5441) antibody was purchased from Sigma-Aldrich. The ID1 (no. sc488) western antibody was purchased from Santa Cruz Biotechnology. The HBEGF (no. ab92620) western antibody was purchased from Abcam (Cambridge, MA, USA).

2.5 Iodinated TGF-β1 binding and crosslinking

Iodinated TGF-β1 binding and crosslinking was conducted with TβRIII pull down using a goat antibody to the extracellular domain (AF-242-PB, R&D Systems) in order to identify functional surface receptor expression as described previously (Dong et al. 2007, Mythreye and Blobe 2009b).

2.6 Chromatin immunoprecipitation

ChIP analysis was performed using the ChIP-IT Express Chromatin Immunoprecipitation Kit (Active Motif, Carlsbad, CA, USA) according to the manufacturer’s instructions. Briefly, chromatin was sheared (~500bp average length) by sonication with a Branson Sonifier 250 (output control 1.5; duty cycle 25%; 10 cycles of 20-second pulses at 30-second intervals). Sheared cross-linked chromatin was rotated at
4°C overnight with protein G magnetic beads and N-Myc (OP13, EMD Millipore, Billerica, MA, USA) or mouse IgG (015-000-003, Jackson ImmunoResearch Laboratories Inc., Westgrove, PA, USA). Following chromatin elution, cross-link reversal, and proteinase K digestion, samples were purified using the QIAquick PCR Purification Kit (28104, Qiagen, Germantown, MD, USA). PCR products were analyzed by quantitative RT-PCR using iQ SYBR Green Supermix (170-8882, Bio-Rad, Hercules, CA, USA) and normalized to input controls. ChIP primers are listed in Appendix D: Table 6. As a positive control, primers toward the Bmi1 promoter were used as previously described (Ochiai et al. 2010).

2.7 HBEGF immunofluorescence

Neuroblastoma tissue was obtained from the Children’s Oncology Group Biorepository with approval from the Neuroblastoma Biology Subcommittee. Tissue immunofluorescence was performed as described previously (Robertson et al. 2008, Mythreye et al. 2013) using an HBEGF antibody (no. HPA053243) from Sigma-Aldrich and the S100 Schwann cell marker antibody (no. 4066) from Abcam.

2.8 Proximity ligation assays

The interaction between EGFR and TβRIII was detected in situ using DuoLink In Situ secondary antibodies and detection reagents (no. 92002, 92006, and 92008) purchased from Sigma-Aldrich according to the manufacturer’s instructions. Secondary antibodies in the absence of primary antibodies were used as a negative control. Ten
random images per condition were taken with the CoolSNAP HQ2 camera
(Photometrics; Tucson, AZ, USA) attached to a Nikon Eclipse TE2000-U inverted
microscope (Nikon Instruments, Inc.; Melville, NY, USA) at 40X magnification (~75-100
cells total). Positive signals were quantified using an Image J Batch Processing Macro
with the following settings: rolling ball 50, threshold 255, analyze particles 5-1000, show
outlines. Data were represented as signals/cell.

2.9 Proliferation assays
Tritiated thymidine incorporation was used to measure cell proliferation as
described previously (Knelson et al. 2013). Proliferation indices (normalized to
control=1.0) were calculated and averaged for a minimum of three individual
experiments. Cells were plated in a 96-well plate at a concentration of 5x10^3 cells/well
(SK-N-AS, 5Y) or 1x10^3 cells/well (BE2). Each condition was plated and treated in
triplicate or quadruplicate prior to a 4-hour [3H] thymidine pulse (1µCi; Amersham
Biosciences/GE Healthcare; Pittsburgh, PA, USA). Following incubation with [3H]
thymidine, cells were washed with PBS and 5% trichloracetic acid prior to overnight
lysis with 0.1M NaOH. Incorporation of [3H] thymidine was determined by scintillation
counting.

2.10 Orthotopic xenograft
The pcDH CMV luciferase EFI Puro vector was a gift from Christine Eyler
(Brigham and Women’s Hospital, Boston, MA) (Eyler et al. 2011). After lentiviral
transduction and puromycin selection, luciferase expression was confirmed using an IVIS 100 imaging system (Caliper Life Sciences/Perkin Elmer). SK-N-AS luciferase cell lines were implanted orthotopically (1.5 million cells per mouse in 20µL of DMEM) in the left adrenal capsule of 6-8 week old female NOD scid gamma mice (Duke Cancer Center Isolation Facility) as described previously (Knelson et al. 2013). Briefly, mice anesthetized with isoflurane underwent left subcostal laparotomy. Gentle retraction of the spleen exposed the adrenal gland for injection using a 23-gauge needle (7804-07; 2 inch PT2) on a 25µL syringe (#702; Hamilton Company). Peritoneal and cutaneous incisions were closed in two layers with 4.0 coated polyglactin 910 sutures (Vicryl 18mm J392H; Ethicon, Summerville, NJ, USA) for peritoneal closure and 4.0 silk suture (Perma-silk 18mm 683G; Ethicon, Summerville, NJ, USA) for cutaneous closure. Mice were housed under pathogen-free conditions on a 12-hour light/dark cycle. Animals were monitored closely for tumor growth and signs of illness and sacrificed at humane endpoints. MLN8237/Alisertib (5mg/kg or 10mg/kg, A1004, AdooQ Bioscience, Irvine, CA, USA) was delivered in 200µL 10% (2-Hydroxypropyl)-β-cyclodextrin and 1% sodium bicarbonate (Sigma, #H107 and #S8875) in water via oral gavage using flexible animal feeding needles (#9923B; Cadence Science, Inc., Cranston, RI, USA) as described previously (Maris et al. 2010). Decitabine (0.2mg/kg or 0.4mg/kg, #11166, Cayman Chemical) was delivered in 100µL 10% (2-Hydroxypropyl)-β-cyclodextrin in water via intraperitoneal (IP) injection as described previously (Maes et al. 2014). Firefly
D-luciferin potassium salt was purchased from Gold Biotechnology (#LUCK-1G; St. Louis, MO, USA) and injected IP at a concentration of 150mg luciferin/kg body weight 5 minutes prior to imaging as per manufacturer’s instructions. Mice were imaged for 5 seconds and bioluminescence was calculated using LivingImage software (Caliper Life Sciences, Hopkinton, MA, USA) and defined as photon flux (photons/s/cm²/steradian) over a standard-sized oval region of interest encompassing the body of the mouse.

2.11 Statistical analysis

Clinical data were analyzed using non-parametric statistics (Kruskal-Wallis global test with Mann-Whitney post-hoc tests) and presented as median with upper and lower quartiles. Kaplan-meier survival curves were analyzed with Log-Rank statistics. All in vitro experiments were analyzed using parametric statistics (ANOVA global test followed by Bonferroni-corrected two-tailed Student’s t-tests) and presented as mean +/- SEM. When data were normalized to control, one-sample Student’s t-tests were used with an expected value of 1 or 100%. Significance was set at p<0.05 for all experiments. Slope and p-value for the line of best fit, as well as R² value for the association was reported for all linear regression analyses. All statistical analyses were conducted with GraphPad Prism (GraphPad Software, Inc.; La Jolla, CA, USA).

2.12 Study approval

All patient samples were de-identified and the project was exempted by the Duke University Health System Institutional Review Board (Protocol ID 00034541).
3. Heparin-binding epidermal growth factor-like growth factor promotes neuroblastoma differentiation

A modified version of this chapter is under consideration at *The FASEB Journal*.

High-risk neuroblastoma is characterized by undifferentiated neuroblasts and low Schwannian stroma content. The tumor stroma contributes to the suppression of tumor growth by releasing soluble factors that act to promote neuroblast differentiation. Here we identify heparin-binding epidermal growth factor-like growth factor (HBEGF) as a potent pro-differentiating factor in neuroblastoma. *HBEGF* mRNA expression is decreased in human neuroblastoma tumors compared to benign tumors, with loss correlating with decreased survival. HBEGF protein is expressed only in stromal compartments of human neuroblastoma specimens, with tissue from late-stage disease containing very little stroma or HBEGF expression. In three human neuroblastoma cell lines, SK-N-AS, SK-N-BE2, and SH-SY5Y, soluble HBEGF is sufficient to promote neuroblast differentiation and decrease proliferation. Heparan sulfate proteoglycans and heparin derivatives further enhance HBEGF-induced differentiation by forming a complex with the epidermal growth factor receptor, leading to activation of the ERK1/2 and STAT3 pathways and upregulation of the inhibitor of DNA binding transcription factor. These data support a role for loss of HBEGF in the neuroblastoma tumor microenvironment in neuroblastoma pathogenesis.
3.1 Introduction

Growth factors and their receptors regulate neuronal differentiation programs during early development, still their contribution to neuroblastoma pathogenesis is unclear. Late-stage neuroblastoma tumors are often poorly differentiated likely due to their propensity to also lack stromal cells in the tumor microenvironment (Maris et al. 2007). The mechanism by which the Schwannian stroma can promote differentiation is complex and not fully understood. Many neuroblastoma patients cannot be cured with current differentiating therapies, highlighting the importance of understanding mechanisms that regulate neuroblast differentiation to inform novel differentiating therapies.

We have previously defined a critical member of the pro-differentiating secretome, demonstrating that heparan sulfate proteoglycans (HSPGs) and their soluble ectodomains released from the stroma can promote basic fibroblast growth factor (FGF2)-induced neuroblast differentiation to suppress tumor growth via enhanced ERK1/2 signaling and downstream upregulation of the transcription factor, ID1 (Knelson et al. 2013, Knelson et al. 2014a). The interrogation of stroma biology in this study inspired the identification of heparin derivatives as differentiating agents for use in neuroblastoma treatment (Knelson et al. 2014a).

As HSPGs can bind to many ligands (Table 1), we aimed to build on our knowledge of stroma biology to further characterize the differentiating stroma.
secretome. Using microarray datasets, we have previously identified that HBEGF expression is decreased in neuroblastoma patients with aggressive, stroma-poor tumors while many other heparin-binding ligands remain unchanged (Albino et al. 2008, Knelson et al. 2014a). Furthermore, HBEGF has been shown to promote neurite outgrowth of pheochromocytoma cells, a process that depends on EGFR activation and ERK1/2 signaling (Zhou and Besner 2010). These data led us to investigate the role of HBEGF in neuroblastoma.

3.2 Results

3.2.1 HBEGF expression is decreased in neuroblastoma with loss correlating with decreased survival

We determined mRNA expression of EGF family ligands using our microarray meta-dataset (Knelson et al. 2013). HBEGF expression was significantly decreased in neuroblastoma tumors compared to benign neuroblastic tumors, while EGF, AREG, BTC, EREG, TGFα, NRG1, NRG2, and NRG4 remained unchanged (Figures 7A and B). We then compared HBEGF protein expression by performing immunofluorescence microscopy on clinical specimens. In patients with Stage I/II disease, HBEGF was readily detectable, while patients with Stage IV disease displayed minimal HBEGF staining (Figure 7C). Using a publicly available dataset (2014, Munro et al. 2014, Su et al. 2014, Wang et al. 2014), we determined that HBEGF expression decreases with advanced-stage disease (Figure 8A). Interestingly, patients with stage 4S disease, which spontaneously regresses over time, have HBEGF levels comparable to early-stage patients (Figure 8A).
Figure 7: HBEGF is decreased in neuroblastoma patients

A) Microarray analysis for EGF family ligand expression in benign neuroblastic tumors (ganglioneuroma/ganglioneuroblastoma) or neuroblastoma (NB) tumors in the microarray meta-dataset. Kruskal-Wallis: \( p < 0.0001 \), Mann-Whitney for intergroup comparisons: ****\( p < 0.0001 \). Bold text indicates heparan sulfate-binding ligands. B) Microarray analysis for HBEGF expression in the microarray meta-dataset in benign neuroblastic tumors (ganglioneuroma/ganglioneuroblastoma) or neuroblastoma (NB) tumors. Mann-Whitney: ****\( p < 0.0001 \). Box plots are presented as median (horizontal bars) and interquartile range (boxes). C) Immunofluorescence in neuroblastoma specimens for HBEGF (green). DAPI nuclear stain in blue. 40X images, scale bar=50µM. Images are representative of seven independent early-stage and seven independent late-stage tissue samples.
Figure 8: HBEGF is decreased in late-stage, aggressive neuroblastomas and correlates with survival

A) Microarray analysis for HBEGF expression in neuroblastoma patients by stage. Kruskal-Wallis: p<0.0001, Mann-Whitney for intergroup comparisons: **p<0.01, ***p<0.001, ****p<0.0001. B) Microarray analysis for HBEGF expression in the GSE49710 dataset in non-amplified (NA) or MYCN-amplified (Amp) neuroblastoma tumors. Mann-Whitney: ****p<0.0001. Box plots are presented as median (horizontal bars) and interquartile range (boxes). C) Event-free survival in neuroblastoma patients with low (bottom 50%; red) and high (top 50%; blue) HBEGF expression in the GSE49710 dataset (left) or the NB Prognosis dataset (right).
Since MYCN amplification is associated with more aggressive tumors and worse survival, we compared HBEGF expression in MYCN-amplified versus non-amplified patients (Knelson et al. 2013, Munro et al. 2014, Su et al. 2014, Wang et al. 2014). Neuroblastoma patients with MYCN-amplified disease had significantly decreased HBEGF compared to those patients with non-amplified disease (Figure 8B). Further, patients with high HBEGF expression had a significant survival advantage over those with low HBEGF expression using two unique microarray datasets ((Wei et al. 2004, 2014, Munro et al. 2014, Su et al. 2014, Wang et al. 2014) and Figure 8C). These data support a role for HBEGF in neuroblastoma pathogenesis.

3.2.2 HBEGF promotes neuroblast differentiation

As HBEGF was highly expressed in more differentiated, early-stage tumor specimens (Figure 7C), we examined whether HBEGF could enhance neuroblast differentiation in widely used models of neuroblast differentiation: expression of neuron specific proteins including neurofilament 160kDa (NF160) and neuron growth-associated protein 43 (GAP43) in BE2, SK-N-AS, and 5Y cells (Scarpa et al. 1996, Encinas et al. 2000, Edsjo et al. 2007, Hahn et al. 2008, Monaghan et al. 2008, Knelson et al. 2014a), β3-tubulin in SHEP (Henrich et al. 2011, Knelson et al. 2014a), and neuron-specific enolase (NSE) in 5Y (Leli et al. 1992, Edsjo et al. 2007, Knelson et al. 2014a). Neuroblast differentiation markers were enhanced in a dose-dependent manner in BE2, SK-N-AS, and 5Y neuroblastoma cells (Figures 9A and B). The effects of HBEGF were specific, as
Figure 9: HBEGF promotes neuroblast differentiation in neuroblastoma cells

A) Western blot for differentiation markers after 72 hours of HBEGF treatment in BE2, SK-N-AS, and 5Y. Densitometry for NF160 normalized to β-actin is shown as the percentage of control. B) Quantification of NF160 densitometry normalized to β-actin from 3 independent Western blots (5Y, BE2) or 8 independent Western blots (SK-N-AS) after 72 hours of treatment with 1ng/mL HBEGF and presented as mean ± SEM. One-way ANOVA: p<0.001, 1-sample Student’s *t*-test: *p<0.05, **p<0.01, ****p<0.0001. C) Western blot for differentiation markers after 72 hours of HBEGF (0, 0.25, 0.5, 0.75, 1, 2ng/mL) and a neutralizing HBEGF antibody (nAb; 0.0075, 0.015, 0.03, 0.05, 0.1, or 0.5µg/mL). Densitometry for NF160 normalized to β-actin is shown as the percentage of control. D) Western blot for β3-tubulin and HBEGF in SHEP stably expressing a non-targeting control shRNA (NTC) or shRNA to HBEGF (shHBEGF #1, #2). Densitometry for β3-tubulin normalized to β-actin is shown as the percentage of control. E) Linear regression analyses for HBEGF and SOX10 expression using the microarray meta-dataset (left) or the GSE49710 dataset (right). Images in A, C, and D are representative of at least three independent experiments.
an HBEGF-neutralizing antibody inhibited HBEGF-induced differentiation in a dose-dependent manner (Figure 9C). Further, in SHEP cells, which are often used as a model of Schwannian stroma and display a differentiated phenotype (Ross et al. 2003), stable knockdown of HBEGF with two independent shRNAs led to a decrease in neuroblast differentiation as determined by β3-tubulin levels (Figure 9D). In our microarray meta-dataset and the GSE49710 dataset (Knelson et al. 2013, Consortium 2014, Munro et al. 2014, Su et al. 2014, Wang et al. 2014), HBEGF expression positively correlated with the neural crest differentiation marker SOX10 (Figure 9E) (Gershon et al. 2005, Kelsh 2006), suggesting that HBEGF may regulate neuroblast differentiation in vivo.

### 3.2.3 Soluble HBEGF derived from Schwannian stroma enhances neuroblast differentiation

We identified that HBEGF expression is increased in early-stage tumors that are often surrounded by an abundant Schwannian stroma (Figures 7C and 8A); therefore we investigated whether the Schwannian stroma was a source of HBEGF. Immunofluorescence on patient specimens revealed that HBEGF colocalized with stromal cells labeled with the Schwann cell marker, S100 (Liu et al. 2005, Du et al. 2008), with early-stage tumors displaying an increased number of stromal cells and thus more HBEGF expression compared to late-stage tumors (Figure 10). We also determined mRNA expression of EGF family ligands in a publicly available dataset that delineates tumors as stroma-poor or stroma-rich (GSE7529, (Albino et al. 2008)). In this dataset, the authors used tumor microdissection to identify a ranked list of genes whose expression
Figure 10: HBEGF co-localizes with the Schwannian stroma in early-stage neuroblastoma tissue specimens

A) Immunofluorescence in neuroblastoma specimens using HBEGF (green) and S100 Schwannian stroma (red) antibodies. DAPI nuclear stain in blue. 40X images, scale bar=50µM. Images are representative of seven early-stage and seven late-stage replicates.
Figure 11: Schwannian stroma-derived HBEGF promotes neuroblast differentiation

A) EGF family ligand expression in neuroblastic tumors based on stroma content in a publicly available microarray dataset (GSE7529). Kruskal-Wallis: p<0.0001, Mann-Whitney for intergroup comparisons: **p<0.01, ****p<0.0001. Bold text indicates heparan sulfate-binding ligands. Box plots are presented as median (horizontal bars) and interquartile range (boxes).

B) Western blot for differentiation markers in 5Y after 72 hours of co-culture or treatment with conditioned media from SHEP stably expressing a non-targeting control shRNA construct (shNTC) or shRNA to HBEGF (shHBEGF #1, #2). Densitometry for NF160 normalized to β-actin is shown as the percentage of control. Images in B are representative of three independent experiments.
was higher in the stromal cells compared to neuroblastic cells. Interestingly, HBEGF was in the top 40 genes that were more highly expressed in the stromal compartment of the isolated tumors. Our analysis also determined that HBEGF was increased in stroma-rich tumors as previously described (Albino et al. 2008, Knelson et al. 2014a), while other EGF-family ligands, including EGF, AREG, BTC, EREG, TGFα, NRG1, and NRG2, were unchanged (Figure 11A).

As HBEGF is highly expressed in the stroma and can promote neuroblast differentiation, we used direct co-culture assays to determine whether stromal-derived HBEGF could promote neuroblast differentiation of tumor cells. SHEP cells were used to model the Schwannian stroma (Ross et al. 2003) and were plated suspended above 5Y neuroblastoma cells. SHEP cells stably expressing a non-targeted control (NTC) promoted differentiation of 5Y, while stable HBEGF knockdown with two independent shRNAs abrogated this effect (Figure 11B). Similar results were obtained when conditioned media from SHEP cells was used. Conditioned media from SHEP cells with stable HBEGF knockdown failed to promote differentiation in 5Y to the same extent as SHEP NTC cells (Figure 11B). These data suggest that HBEGF is stromal-derived and its release from Schwannian stroma cells contributes to neuroblast differentiation in the neuroblastoma tumor microenvironment.
Figure 12: HSPGs promote HBEGF-mediated neuroblast differentiation

Western blots for differentiation markers after 72 hours of treatment with: A) 10ng/mL sTβRIII or sCD44 or 100ng/mL sGPC1, sGPC3, or sSDC3 in the absence or presence of 0.5ng/mL HBEGF in 5Y cells. Densitometry for NF160 normalized to β-actin is shown as the percentage of control, B) 10ng/mL sTβRIII or 100ng/mL sSDC3 or sGPC1 in the absence or presence of 0.5ng/mL HBEGF. Densitometry for NF160 normalized to β-actin is shown as the percentage of control, C) a dose course of HBEGF in SK-N-AS following 96 hour TβRIII knockdown. Densitometry for NF160 normalized to β-actin is shown as the percentage of control, D) 0.5µg/mL heparin, ODSH, 2DES, 6DES, or NDES heparin in the absence or presence of 0.5ng/mL HBEGF in SK-N-AS. Densitometry for NF160 normalized to β-actin is shown as the percentage of control. Images are representative of at least three independent experiments.
3.2.4 HSPGs and EGFR interact to promote HBEGF-induced neuroblast differentiation

HBEGF requires binding to HSPGs as well as its receptor, EGFR, to initiate signaling (Higashiyama et al. 1993, Aviezer and Yayon 1994, Shishido et al. 1995, Raab and Klagsbrun 1997). We have previously demonstrated a critical role for HSPGs in promoting neuroblastoma differentiation (Knelson et al. 2013, Knelson et al. 2014a). Therefore, we investigated whether HBEGF could potentiate HSPG-induced differentiation. Treatment with HBEGF enhanced differentiation compared to cells treated with soluble TβRIII, GPC1, GPC3, and SDC3 alone (Figures 12A and B). In contrast, the HSPG CD44 had no effect on HBEGF-mediated differentiation (Figure 12A). In a reciprocal manner, silencing of TβRIII expression blunted the differentiation response to HBEGF (Figure 12C). However, these cells were still able to undergo neuronal differentiation with HBEGF treatment, suggesting that other HSPGs may be compensating for the loss of TβRIII.

The anticoagulant heparin is structurally similar to the heparan sulfate modifications in HSPGs. Heparin and heparan sulfate consist of repeating disaccharide units of glucuronic acid linked to glucosamine. These moieties are variably sulfated at the 3-O, 6-O, and N sites on glucosamine, as well as the 2-O site on glucuronic acid (Whitelock and Iozzo 2005). Distinct sulfation patterns determine the binding affinity for different ligands, including HBEGF, though the critical sulfation sites required for HBEGF to interact with heparan sulfate are unknown (Ashikari-Hada et al. 2004,
Figure 13: EGFR is decreased in neuroblastoma, correlates with survival, and promotes neuroblast differentiation

A) Microrarray analysis for ERBB receptor expression in benign neuroblastic tumors (ganglioneuroma/ganglioneuroblastoma) or neuroblastoma (NB) tumors in the microarray meta-dataset (only GSE12460, GSE16237, GSE13141 were included in analysis due to unshared probe for EGFR), Kruskal-Wallis: p<0.0001, Mann-Whitney for intergroup comparisons: **p<0.01, ***p<0.001. Bold text indicates HBEGF receptor. B) EGFR expression in neuroblastoma patients by stage in the GSE49710 dataset. Kruskal-Wallis: p<0.0001, Mann-Whitney for intergroup comparisons: **p<0.01, ****p<0.0001. Box plots are presented as median (horizontal bars) and interquartile range (boxes). C) Event-free survival in neuroblastoma patients with low (bottom 50%; red) and high (top 50%; blue) EGFR expression in the GSE49710 dataset. D) Western blot for differentiation markers after 72 hours of treatment with EGFR inhibitors followed by 48 hours of 1ng/mL HBEGF treatment. Densitometry for NF160 normalized to β-actin is shown as the percentage of control. Image in D is representative of four independent experiments.
Whitelock and Iozzo 2005). We have previously demonstrated that heparin and its variably sulfated derivat ives can mimic the differentiating effects of HSPGs on neuroblastoma cells with varying degrees of efficacy (Knelson et al. 2014a). Therefore, we examined whether HBEGF could enhance neuroblast differentiation induced by heparan sulfate mimetics. Treatment with HBEGF in addition to low doses of heparin, a 2-O, 3-O desulfated heparin (ODSH), or a 2-O desulfated heparin (2DES) enhanced neuroblast differentiation, while a heparin derivative lacking N-sulfation (NDES) had no effect (Figure 12D). Treatment with 6-O desulfated heparin (6DES) had inconsistent effects on HBEGF-induced differentiation in our experiments. These data suggest that N- sulfation is critical for HBEGF-induced neuroblast differentiation and supports the use of heparan sulfate mimetics for neuroblastoma differentiation therapy.

HBEGF is known to actively bind its receptor, EGFR, in addition to HSPGs to mediate its downstream effects (Raab and Klagsbrun 1997). In our microarray meta-dataset (Knelson et al. 2013), we identified a decrease in EGFR expression in patients with neuroblastoma compared to those with benign tumors (Figure 13A). High EGFR expression was also correlated with decreased stage of disease and improved prognosis (Figures 13B and C). To investigate whether EGFR is involved in HBEGF-induced differentiation, we utilized three clinically available EGFR inhibitors. Erlotinib, gefitinib, or lapatinib treatment dose-dependently prevented HBEGF-induced differentiation (Figure 13D), suggesting a role for EGFR in enhancing neuroblast differentiation.
Figure 14: HSPGs and EGFR interact to promote HBEGF-mediated neuroblast differentiation

A) Co-immunoprecipitation in COS-7 overexpressing TβRIII and an empty-vector (EV) control or EGFR. Cells were either left untreated or were treated for 72 hours with HBEGF followed by an additional 5 minute HBEGF treatment prior to lysis. Image is representative of two independent experiments. B) In situ proximity ligation assay in SK-N-AS following 5 minutes of treatment with 1ng/mL HBEGF or 1ng/mL EGF. 40X images, scale bar=50µM. Normalized TβRIII/EGFR complexes per cell (75-100 cells/condition) from six independent experiments. One-way ANOVA: p<0.001, 1-sample Student’s t-test: *p<0.05, ****p<0.0001.
Since our data indicates that both EGFR and HSPGs are required for HBEGF-induced neuroblast differentiation, we investigated whether HSPGs act as an HBEGF coreceptor in neuroblastoma cells. Consistent with a coreceptor role, exogenous expression of TβRIII co-immunoprecipitated with exogenous EGFR in COS-7 cells and soluble HBEGF increased this interaction (Figure 14A). To determine whether endogenous TβRIII and EGFR form a complex, we performed proximity ligation assays in SK-N-AS neuroblastoma cells. We observed an interaction among these endogenous proteins that was enhanced when cells were treated with HBEGF ligand, but not with the closely related EGF ligand (Figure 14B). Using a publicly available microarray dataset (Consortium 2014, Munro et al. 2014, Su et al. 2014, Wang et al. 2014), sequential-stratification identified that patients with high HBEGF, TβRIII, and EGFR expression had excellent event-free-survival. This three-gene signature was comparable to MYCN oncogene amplification as a prognostic biomarker (Figure 15). Interestingly, HBEGF, TβRIII, and EGFR expression positively correlate in two unique microarray datasets (Figure 16) (Knelson et al. 2013, 2014, Knelson et al. 2014a, Munro et al. 2014, Su et al. 2014, Wang et al. 2014), suggesting these proteins may be co-regulated in neuroblastoma patients. These data demonstrate that HBEGF, HSPGs, and EGFR interact to potentiate neuroblast differentiation in the tumor microenvironment.
Figure 15: High HBEGF, TβRIII, and EGFR expression identify patients with excellent event-free survival and holds similar prognostic value to MYCN amplification

A) Analysis of event-free survival in the GSE49710 dataset stratified by the top and bottom 50% for HBEGF, then TGFBR3, then EGFR expression. B) Analysis of event-free survival in the GSE49710 dataset stratified by the top and bottom 12.5% for HBEGF, then TGFBR3, then EGFR expression. C) Analysis of event-free survival in the GSE49710 dataset stratified by MYCN amplification status. NA, non-amplified.

3.2.5 HBEGF promotes differentiation via ERK/STAT3 signaling and ID1 upregulation

To investigate which signaling pathways are critical for HBEGF-induced differentiation, we treated neuroblastoma cells with recombinant HBEGF over 72 hours to induce differentiation. As expected, HBEGF treatment enhanced phosphorylation of ERK, AKT, and STAT3 (Figure 17). The inhibitor of DNA binding 1 (ID1) transcription factor has been shown to act downstream of ERK in neuroblast differentiation and its expression was recently revealed to be regulated by STAT3 signaling (Passiatore et al. 2011, Knelson et al. 2013, Knelson et al. 2014a, Yu et al. 2014). Consistent with these data, we identified that HBEGF promoted ID1 expression at concentrations that were also
Figure 16: HBEGF, TβRIII, and EGFR expression correlate in neuroblastoma patients

Linear regression analyses using the GSE49710 dataset or the microarray meta-dataset for A) HBEGF and EGFR, B) TGFB3 and EGFR, and C) HBEGF and TGFB3.

found to promote differentiation in neuroblastoma cells (Figure 18A). Conversely, SHEP neuroblastoma cells that are basally differentiated expressed high levels of ID1, which was decreased with stable shRNA-mediated HBEGF knockdown (Figure 18B). Knockdown of ID1 with siRNA in neuroblastoma cells also attenuated the differentiating effects of HBEGF (Figure 18C). In two microarray datasets, HBEGF and
Figure 17: HBEGF enhances ERK and STAT3 signaling

A) Western blot for signaling markers in BE2 and 5Y treated 72 hours with a dose course of HBEGF. Densitometry for phosphorylated proteins normalized to total protein is shown as percentage of control. B) Western blot in SK-N-AS for phosphorylated and total STAT3 or ERK1/2 after treatment for 72 hours with 1ng/mL HBEGF. Densitometry for phosphorylated proteins normalized to total protein is shown as the percentage of control. Images are representative of at least three independent experiments.

ID1 positively correlated in patient samples, supporting the clinical relevance of our in vitro studies (Figure 18D) (Knelson et al. 2013, 2014, Knelson et al. 2014a, Munro et al. 2014, Su et al. 2014, Wang et al. 2014). As ERK signaling has been implicated in neuronal differentiation in response to α-lipoic acid and retinoic acid (Wang et al. 2011, Qiao et al. 2012) and is the critical pathway for FGF2-induced differentiation (Knelson et al. 2013,
Figure 18: HBEGF induces neuroblast differentiation via upregulation of ID1

A) Western blot for ID1 in BE2, SK-N-AS, and 5Y after treatment for 72 hours with a dose course of HBEGF. Densitometry for ID1 normalized to β-actin is shown as the percentage of control. B) Western blot for ID1 normalized to β-actin is shown as the percentage of control. C) Western blot in SK-N-AS after 96 hour ID1 siRNA-mediated (siID1) knockdown and 72 hour treatment with 1ng/mL HBEGF. NTC=non-targeting control. Densitometry for NF160 normalized to β-actin is shown as the percentage of control. D) Linear regression analyses for ID1 and HBEGF using the microarray meta-dataset (left) or the GSE49710 dataset (right). Images in A-C are representative of at least three independent experiments.
Knelson et al. 2014a), we sought to determine the contribution of the ERK pathway to HBEGF-induced differentiation. Pharmacologic inhibition of MEK/ERK with U0126 and CI-1040 abrogated HBEGF-induced differentiation and ID1 upregulation (Figures 19A and B). Previous data has implicated STAT3 activation in the differentiating process downstream of TGFα, an EGFR ligand, and ERα, a nuclear hormone receptor, in neuroblastoma cells (Ciana et al. 2003). Similarly, inhibition of STAT3 signaling with a dominant-negative STAT3 and pharmacologic inhibitor treatment also reduced differentiation and ID1 upregulation in the presence of HBEGF (Figure 19C). These data support a role for the STAT3/ID1 axis in HBEGF-induced differentiation and further implicate the ERK/ID1 signaling pathway in neuroblast differentiation.

### 3.2.6 Soluble HBEGF promotes differentiation to suppress neuroblast proliferation

Since neuroblast differentiation is often associated with cell-cycle arrest and decreased tumor growth (Pahlman et al. 1981, Leli et al. 1992, Scarpa et al. 1996, Encinas et al. 2000, Voigt et al. 2000, Edsjo et al. 2007, Hahn et al. 2008, Knelson et al. 2013, Knelson et al. 2014a), we examined whether HBEGF treatment could reduce proliferation. Indeed, treatment with HBEGF led to a decrease in proliferation in three neuroblastoma cell lines (Figure 20A). As increased p21 expression has been implicated in neuronal differentiation downstream of ERK (Wang et al. 2011, Qiao et al. 2012, Knelson et al. 2013, Knelson et al. 2014a), we determined whether HBEGF expression
Figure 19: HBEGF induces neuroblast differentiation via ERK and STAT3 signaling and upregulation of ID1

A) Western blot for differentiation and signaling markers in 5Y following 24 hour co-treatment with U0126 or CI-1040 and 1ng/mL HBEGF. Densitometry for NF160 normalized to β-actin is shown as the percentage of control. B) Western blot for differentiation markers and ID1 following 24 hour co-treatment with 1ng/mL HBEGF and the indicated doses of U0126 or CI-1040. Densitometry for NF160 normalized to β-actin is shown as the percentage of control. C) Western blot for differentiation markers following 72 hour expression of an empty-vector control (EV) or a dominant negative STAT3 (DN STAT3) construct and 48 hour treatment with 1ng/mL HBEGF, or 48 hour treatment with ruxolitinib and 24 hour treatment with 1ng/mL HBEGF. Densitometry for NF160 normalized to β-actin is shown as the percentage of control. Images are representative of at least three independent experiments.
altered p21 levels. Indeed, HBEGF led to enhanced expression of the cell cycle inhibitor p21 (Figure 20B). In patient microarray data, CDKN1A (p21) positively correlated with HBEGF expression, while there was a negative correlation with the marker of proliferation MKI67 (Figure 20C).

Our data demonstrate for the first time that HBEGF forms a complex with EGFR and TβRIII to promote neuroblast differentiation, leading to a decrease in neuroblastoma cell proliferation.

3.3 Discussion

Here, we have utilized clinical data and in vitro experiments to identify a novel member of the pro-differentiating stroma secretome. As the stroma has been implicated in promoting neuroblast differentiation (Kwiatkowski et al. 1998, Liu et al. 2005) and differentiation is a validated treatment strategy in neuroblastoma, it is imperative to understand the differentiating factors secreted by the stroma in an effort to reveal new therapeutic strategies for neuroblastoma patients.

Our data demonstrate that HBEGF-induced neuronal differentiation requires HSPGs and EGFR to promote neuroblast differentiation (Figures 12C and 13D). This is consistent with previous reports demonstrating a role for HSPGs (Knelson et al. 2013, Knelson et al. 2014a) and EGFR (Evangelopoulos et al. 2005, Evangelopoulos et al. 2009, Zhou and Besner 2010) in promoting neurite outgrowth and enhancement of differentiation markers. Previous research has also determined that HBEGF interacts
Figure 20: HBEGF suppresses neuroblast proliferation

A) Proliferation index from three (5Y) or four (SK-N-AS, BE2) independent replicates (mean ± SEM) of thymidine incorporation following HBEGF treatment for 24 hours (SK-N-AS), 48 hours (5Y), or 72 hours (BE2), normalized to untreated control. One-way ANOVA: p<0.0001, 1-sample Student’s t-test: **p<0.01, ***p<0.001. B) Western blot for p21 after 72 hours of HBEGF treatment in BE2, SK-N-AS, and 5Y. Densitometry for p21 normalized to β-actin is shown as the percentage of control. Images are representative of three independent experiments. C) HBEGF expression in high (top 50%) or low (bottom 50%) CDKN1A or MKI67-expressing neuroblastic tumors using the microarray meta-dataset (left) or the GSE49710 dataset (right). Kruskal-Wallis: p<0.0001, Mann-Whitney for intergroup comparisons: ****p<0.0001. Box plots are presented as median (horizontal bars) and interquartile range (boxes).
with SDC3 (Hienola et al. 2006) and CD44 (Bennett et al. 1995), while GPC1 (Kleeff et al. 1998, Matsuda et al. 2001) and SDC1 (Celie et al. 2012) can mediate HBEGF activity. As demonstrated here, HBEGF is able to potentiate GPC1, GPC3, SDC3, and TβRIII-induced differentiation (Figures 12A and B), suggesting a possible interaction between the heparin-binding domain of HBEGF and the heparan sulfate modifications attached to these receptors. Previous work has also demonstrated an interaction between CD44 and HBEGF (Bennett et al. 1995); however, CD44 does not promote HBEGF-induced differentiation in our studies (Figure 12A). This observation may be due to the requirement for an alternatively spliced exon V3 that lends CD44 accessible to heparan sulfate modifications (Brown et al. 1991, Bennett et al. 1995, Jackson et al. 1995).

Treatment with variably sulfated forms of heparin also reveal that N-sulfation is required to mediate HBEGF binding to heparan sulfate chains, while the 6-O site appears to be dispensable in some experiments (Figure 12D). This is in contrast to reports that 6-O sulfation is required for HBEGF signaling in ovarian cancer cells (Cole et al. 2014). The sulfation requirements are likely cell context specific and may be determined by the relative amounts of functional heparan sulfate and competing heparin-binding ligands present in the tumor microenvironment (Chu et al. 2005).

Previous studies have identified HBEGF and ERBB receptors as potential targets for neuroblastoma treatment due to their effects on proliferation and angiogenesis (Ho et al. 2005, Tamura et al. 2007, Richards et al. 2010, Nam et al. 2015). Our data
demonstrate that HBEGF and EGFR expression is often suppressed in neuroblastoma
tumors, decreases with advanced stage of disease, and is correlated with increased
survival. HBEGF is also highly expressed in the stromal compartment of tumors, which
also correlates with an improved prognosis (Figures 7, 8, 10, 11, 13). These data suggest
that HBEGF and EGFR are not likely driving a pro-proliferative or pro-angiogenic
phenotype in the majority of neuroblastomas. Further, we demonstrate that these
proteins are involved in promoting neuronal differentiation and decreasing cell
proliferation. While inhibition of HBEGF and ERBB receptors has been shown to induce
apoptosis (Tamura et al. 2007, Richards et al. 2010), it is possible that the doses required
to promote apoptosis in a clinical setting may be too high for children with
neuroblastoma, leading to an increased toxicity profile. Further, the antitumor effect
may not be sustained due to a propensity for tumor cells to adapt and become resistant,
particularly in response to apoptotic stimuli. Our data caution against the therapeutic
use of non-specific tyrosine kinase inhibitors and neutralizing antibodies that are aimed
at further reducing HBEGF and EGFR in the tumor microenvironment, which may lead
to inhibition of differentiation and tumor resistance.

We have identified a three-gene gene signature that may identify patients who
will benefit from differentiation therapy aimed at enhancing expression of pro-
differentiating proteins including TβRIII and HBEGF (Figure 15). We have previously
determined that TβRIII expression is epigenetically suppressed by direct N-Myc binding
to Sp-1 sites on the TβRIII promoter (Figure 2) (Knelson et al. 2013). Our data demonstrate that HBEGF expression is decreased in MYCN-amplified neuroblastomas (Figure 8B) and its expression positively correlates with TBRIII expression (Figure 16C), suggesting a similar mechanism may contribute to the decreased HBEGF expression seen in neuroblastoma patients. Further, HBEGF also contains Sp-1 binding sites that may contribute to its regulation (Edwards et al. 2009, Miyata et al. 2014). Restoring expression of this important differentiation pathway using epigenetically targeted therapies may be necessary to re-establish a functional differentiation program.

Our data demonstrate a critical role for the STAT3 pathway in neuroblast differentiation (Figures 17 and 19C). Prior research suggests the use of STAT3 inhibitors in neuroblastoma patients, noting their effectiveness in decreasing tumor growth in mouse models (Yan et al. 2013); however, the doses utilized may not be clinically achievable in children, as seen previously with the aurora kinase A inhibitor, MLN8237 (Maris et al. 2010, Mosse et al. 2012). As demonstrated here, the importance of STAT3 signaling in promoting neuroblast differentiation urges consideration when designing clinical trials aimed at inhibiting this critical differentiating pathway.

In conclusion, we have identified a novel and clinically relevant differentiating complex consisting of HBEGF, HSPGs, and EGFR. Our work cautions against targeting this important pro-differentiation pathway and instead provides rationale for the use of HBEGF and heparin derivatives for neuroblastoma differentiation therapy.
4. Targeted therapies cooperate to differentiate neuroblasts and suppress neuroblastoma tumor growth

Neuroblastoma arises from undifferentiated sympathoadrenal neural crest precursor cells. Survival rates are devastatingly low for patients with late-stage disease compared to those with early-stage disease, urging the identification of novel therapeutic options for late-stage patients. Here we identify a combination therapy that promotes neuroblast differentiation while decreasing proliferation and tumor growth. Single-agent treatment with inhibitors of aurora kinase A (AurkA), DNA methyltransferases (DNMT), and histone deacetylases (HDAC) induce neuroblast differentiation and decrease proliferation in a diverse range of neuroblastoma cell lines. The combination of AurkA and DNMT inhibitors at clinically achievable doses dramatically improves the differentiating effects compared to either agent alone both in vitro and in vivo. These data support the clinical development of this combination therapy for neuroblastoma.

4.1 Introduction

The pediatric tumor neuroblastoma is diagnosed in about 700 new patients in the United States every year (Brodeur 2003). This tumor arises from neuroblasts and often presents in the adrenal gland of children. Approximately 60% of patients are diagnosed with late-stage disease when the cancer has already spread to other parts of the body. These patients have less than a 50% chance of survival, with existing therapies being only modestly effective (Maris 2010). Several targeted agents in clinical trials, including
inhibitors of AurkA, DNMTs, and HDACs have shown modest benefit in Phase I/II clinical trials for aggressive, late-stage neuroblastoma; however the excessive doses necessary for this single agent treatment to be effective in children are often prohibitive due to adverse side effects (Table 2) (Maris 2010, He et al. 2013). The potential for these agents to be used at lower doses in combinatorial therapies as well as their ability to promote neuroblast differentiation remains unclear.

Inhibitors of AurkA, DNMTs, and HDACs function to regulate the epigenetic state of neuroblasts; however, their effects on differentiation are unknown. AurkA is known to protect N-Myc from proteasomal degradation and promotes colony formation and angiogenesis in MYCN-amplified neuroblastoma (Otto et al. 2009, Romain et al. 2014). The AurkA inhibitor MLN8237 is effective in decreasing cell growth in preclinical models of neuroblastoma (Maris et al. 2010, Carol et al. 2011, Brockmann et al. 2013) and is currently in Phase I/II clinical trials for recurrent or resistant neuroblastoma (Table 2) (Mosse et al. 2012). MLN8237 suppressed proliferation in a diverse panel of neuroblastoma cell lines (Maris et al. 2010), suggesting AurkA may have a role independent of MYCN amplification.

Aberrant DNA methylation is also an important mediator of neuroblastoma pathogenesis (Hoebeeck et al. 2009). The DNMT inhibitor decitabine is currently in Phase I clinical trials in patients with relapsed or refractory neuroblastoma (Table 2) (George et al. 2010). Previous work has suggested that decitabine promotes neuroblast
differentiation to suppress proliferation (Bartolucci et al. 1989a, Bartolucci et al. 1989b), however its specific mechanism of action is not understood.

In addition to methylation, de-acetylation is associated with suppressed transcription (West and Johnstone 2014). HDAC inhibitors, including valproic acid, have been used in clinical trials for a variety of cancers, with greatest success when used in combination with other treatments (Shabason et al. 2010). Vorinostat is currently being tested in neuroblastoma patients in combination with the differentiating agent, 13-cis-retinoic acid (Table 2) (Fouladi et al. 2010). Here we investigate the effects of targeted therapies on neuroblastoma differentiation and tumor growth.

4.2 Results

4.2.1 Pro-differentiating factors negatively correlate with N-Myc

We have previously identified members of the pro-differentiating secretome, including TβRIII, GPC1, SDC3, HBEGF, and EGFR, that are suppressed in neuroblastomas compared to benign tumors (Chapter 3) (Knelson et al. 2013, Knelson et al. 2014a). Recent studies from our lab demonstrated a potential mechanism for this suppression. We established that the pro-differentiating HSPG, TβRIII, is decreased in neuroblastoma tumors harboring MYCN amplification (Figure 5) (Knelson et al. 2013). We further demonstrated that N-Myc binds to Sp-1 sites on the TβRIII promoter to suppress its expression (Figure 2) (Knelson et al. 2013). To determine if other pro-differentiating factors might be regulated in a similar way by N-Myc, we compared
Figure 21: MYCN is negatively correlated with pro-differentiating HSPGs, receptors, and ligands

A) Microarray analysis for TGFBR3, GPC1, EGFR, and HBEGF expression in the GSE49710 dataset in non-amplified (NA) or MYCN-amplified (Amp) neuroblastoma tumors. Kruskal-Wallis: p<0.0001, Mann-Whitney: ****p<0.0001. B) Analysis for SDC3 in the microarray meta-dataset in non-amplified (NA) or MYCN-amplified (Amp) neuroblastoma tumors. Mann-Whitney: ***p<0.001. Box plots are presented as median (horizontal bars) and interquartile range (boxes). C) Linear regression analyses using the GSE49710 dataset for MYCN and TGFBR3, GPC1, EGFR, or HBEGF. D) Linear regression analysis using the microarray meta-dataset for MYCN and SDC3.
expression of several heparan sulfate signaling components in neuroblastoma patients with MYCN amplified disease or non-amplified disease. In the publicly available GSE49710 dataset, TβRIII was decreased in MYCN-amplified disease as demonstrated previously in another microarray dataset (Figure 5) (Knelson et al. 2013). Similarly, GPC1, EGFR, and HBEGF were also decreased in MYCN-amplified disease compared to non-amplified tumors (Figure 21A). As SDC3 was not analyzed in the GSE49710 dataset, we utilized our microarray meta-dataset and found that this pro-differentiating HSPG was also decreased in MYCN-amplified disease (Figure 21B). We then performed linear regression analysis to compare MYCN expression levels to TβRIII, GPC1, EGFR, HBEGF, and SDC3 expression in neuroblastoma patients. Interestingly, these proteins all negatively correlated with MYCN expression (Figures 21C and D).

To determine whether N-Myc protein may be a regulator of these pro-differentiating factors, we used two model systems where N-Myc expression could be induced with 4-hydroxytamoxifen (4OHT; SK-N-AS-N-MycER) or repressed by addition of doxycycline (Dox; SHEP-21N) (Valentijn et al. 2005). Induction of N-Myc expression with 4OHT led to a dose-dependent reduction in the expression levels of the differentiating HSPGs, GPC1 and SDC3 in the SK-N-AS-N-MycER cell line. Interestingly, the neuroblast differentiation marker β3-tubulin paralleled the decrease in GPC1 and SDC3 levels, potentially linking neuronal differentiation to MYCN-mediated HSPG suppression (Figure 22A). Conversely, N-Myc reduction with Dox treatment led
to an increase in GPC1, SDC3, and HBEGF expression in the SHEP-21N model system (Figure 22B). Together, these data suggest that N-Myc may be a potential global mediator of the pro-differentiating secretome.

Figure 22: N-Myc suppresses HSPG and HBEGF expression

A) Western blot for GPC1, SDC3, β3-tubulin, and N-Myc expression in the SK-N-AS-N-MycER cell line following induction of N-Myc with a dose course of 4-hydroxytamoxifen (4OHT) over 72 hours. B) Western blot for GPC1, SDC3, and HBEGF in SHEP-21N cells following repression of N-Myc with a time course of 50ng/mL doxycycline (Dox). Images are representative of two independent experiments.
4.2.2 Targeted therapies promote neuroblast differentiation to suppress proliferation

Since we observed a negative correlation between N-Myc and proteins involved in neuroblast differentiation (Figures 21 and 22), we sought to determine whether inhibition of N-Myc with targeted agents in clinical trials for neuroblastoma could promote neuroblast differentiation in a variety of cell lines representing common subtypes of neuroblastoma, including MYCN-amplified, non-amplified, p53 wild-type, p53 non-functional, retinoic acid-sensitive, and multidrug-resistant models (Appendix A: Table 3). Indeed, treatment with the Aurka inhibitor MLN8237 (Figure 23A), the DNMT inhibitor decitabine (Figure 23B), and the HDAC inhibitor vorinostat (Figure 23C) enhanced well-established markers of neuroblast differentiation: expression of neuron specific proteins including neurofilament 160kDa (NF160) and neuron growth-associated protein 43 (GAP43) in BE2, SK-N-AS, and 5Y cells (Scarpa et al. 1996, Encinas et al. 2000, Edsjo et al. 2007, Hahn et al. 2008, Monaghan et al. 2008, Knelson et al. 2014a), β3-tubulin in IMR32 and SK-N-AS (Henrich et al. 2011, Knelson et al. 2014a), and neuron-specific enolase (NSE) in 5Y (Leli et al. 1992, Edsjo et al. 2007, Knelson et al. 2014a).

Since neuroblast differentiation often correlates with a decrease in proliferation (Pahlman et al. 1981, Leli et al. 1992, Scarpa et al. 1996, Encinas et al. 2000, Voigt et al. 2000, Edsjo et al. 2007, Hahn et al. 2008), we examined whether these targeted therapies could also suppress proliferation. Treatment with MLN8237, decitabine, and vorinostat
Figure 23: Targeted therapies promote neuroblast differentiation

Western blot for differentiation markers in BE2, SK-N-AS, IMR32, and 5Y following treatment for 72 hours with a dose course of A) MLN8237, B) decitabine, or C) vorinostat. Images are representative of at least three independent experiments for BE2, SK-N-AS, and 5Y cells. Images for IMR32 cells are representative of one independent experiment.
Figure 24: Targeted therapies suppress proliferation in neuroblastoma cells

Thymidine incorporation in A) BE2 and B) 5Y following treatment for 48 hours with MLN8237 (left), decitabine (middle), or vorinostat (right). One-way ANOVA: p<0.05 for MLN8237 in BE2 and 5Y and for decitabine in BE2, p<0.001 for vorinostat in BE2, and p<0.0001 for vorinostat in 5Y. 1-sample Student’s t-test: *p<0.05, **p<0.01, ***p<0.001. Images are representative of at least three independent experiments for A (all treatments) and B (MLN8237 and vorinostat) and two independent experiments for B (decitabine). Jasmine Nee helped to generate the data in this figure.
resulted in a dose-dependent decrease in proliferation in MYCN-amplified BE2 cells (Figure 24A) and non-amplified 5Y cells (Figure 24B). These data demonstrate that targeted therapies that inhibit N-Myc expression and function can promote neuroblast differentiation to suppress proliferation in a variety of neuroblastoma cell lines.

4.2.3 Targeted agents enhance heparan sulfate signaling components

We have previously demonstrated that HDAC inhibition restores TβRIII expression in neuroblastoma cell lines leading to an increase in neuroblast differentiation (Knelson et al. 2013). Our *in silico* and *in vitro* studies have also suggested that N-Myc may negatively regulate members of the heparan sulfate signaling complex, potentially contributing to an undifferentiated tumor state (Figures 21 and 22). Therefore, we sought to determine if MLN8237, decitabine, and vorinostat could restore expression of pro-differentiating factors in neuroblastoma cell lines. MLN8237 treatment led to increased TβRIII, GPC1, SDC3, HBEGF, and FGF2 in cell lines that had low basal expression of these proteins (Figure 25A). Similarly, treatment with decitabine or vorinostat led to enhanced TβRIII, GPC1, SDC3, and HBEGF in cell models that displayed low basal heparan sulfate component expression (Figures 25B and C). Conversely, we observed some conditions where basal expression of HSPGs or ligands was high. In these cases, targeted therapy treatment resulted in decreased pro-differentiating factor expression, possibly due to other regulatory mechanisms contributing to heparan sulfate signaling component expression.
Figure 25: Targeted therapies enhance HSPG co-receptor and ligand expression

Western blots for heparan sulfate signaling components in: A) BE2, SK-N-AS, IMR32, or 5Y following a dose course of MLN8237 for 72 hours (BE2, SK-N-AS) or 48 hours (IMR32, 5Y), B) BE2 following decitabine treatment at the indicated doses for 72 hours, and C) SK-N-AS following a dose course of vorinostat for 48 hours. Images are representative of two independent experiments for A (BE2, SK-N-AS) and C and one independent experiment for A (IMR32, 5Y) and B.
Western blots for heparan sulfate signaling components in: A) BE2 and 5Y following a dose course of MLN8237 for 72 hours or 48 hours, respectively, B) SK-N-AS and 5Y following decitabine treatment at the indicated doses for 72 hours, and C) SK-N-AS following a dose course of vorinostat for 48 hours. Images in A (5Y) and C are representative of two independent experiments and images in A (BE2) and B are representative of one independent experiment.

Figure 26: Targeted therapies enhance heparan sulfate signaling
We and others have previously identified a requirement for FGF signaling and downstream ERK activation in neuroblast differentiation (Chapter 3) (Wang et al. 2011, Qiao et al. 2012, Knelson et al. 2013, Knelson et al. 2014a). To determine whether targeted therapies may function to enhance activation of FGF signaling and downstream ERK1/2 signaling, we performed Western blot analysis to examine phosphorylation of FGFR1 and ERK1/2 in response to targeted therapies. Treatment with MLN8237 promoted FGFR1 and ERK1/2 phosphorylation in two different cell lines (Figure 26A). Similarly, decitabine and vorinostat also resulted in ERK1/2 activation, further supporting a role for these pathways in neuroblast differentiation (Figures 26B and C).

To examine whether the differentiating effects we observed with targeted therapies occurs broadly in neuroblastoma models, we utilized two cell lines generated from a genetic mouse model of MYCN-amplified neuroblastoma (Weiss et al. 1997). The 9464D and 975A2 cell lines are derived from these genetically altered mice and represent a transplantable syngeneic tumor model of neuroblastoma (Kroesen et al. 2014). These cell lines exhibit N-Myc levels similar to human MYCN-amplified cell models (Figure 27A). Interestingly, 9464D and 975A2 have diverse morphological characteristics, with the 9464D cell line displaying neurite-like processes typical of differentiated cells, while the 975A2 cells appear to be more rounded and neuroblastic-like (Figure 27B). Interestingly, Western blot analysis revealed high expression of the differentiating markers β3-tubulin and GAP43, in addition to high HSPG and ligand expression, and
Figure 27: Targeted therapies promote heparan sulfate signaling to enhance neuroblast differentiation and suppress proliferation in two transplantable syngeneic mouse cell line models

A) Western blot analysis for basal N-Myc expression in cell lines derived from the tyrosine hydroxylase MYCN mouse model (TH-MYCN), MYCN-amplified human cell lines, or non-amplified human cell lines. Amp=amplified, NA=non-amplified. B) Images from 9464D or 975A2 cells in culture. 40X images. C) Western blot analysis for differentiating components after a dose course of MLN8237 for 72 hours. Images in C are representative of two independent experiments. D) Trypan blue exclusion results from four independent experiments following 48 hours of treatment with vehicle control (DMSO), 1nM MLN8237, 1µM decitabine, or 0.5µM vorinostat. One-way ANOVA: p<0.0001, 1-sample Student’s t-test: *p<0.05, **p<0.01.
FGF and ERK signaling component expression (Figure 27C). Treatment with MLN8237 had no differentiating effects in this cell line, as differentiation was basally very high. Conversely, the 975A2 cell line had low basal differentiating marker expression and relatively low HSPG, ligand, and signaling pathway expression. MLN8237 treatment enhanced HSPG and ligand expression leading to an increase in FGFR1 and ERK1/2 signaling (Figure 27C). We also observed a concomitant increase in differentiation marker expression, supporting the importance of these pro-differentiating factors in neuroblast differentiation (Figure 27C). As expected, treatment with MLN8237, decitabine, and vorinostat suppressed proliferation in the 975A2 cell line to a similar extent as seen in human cell lines (Figures 27D and 24). These data suggest that targeted therapies may enhance the heparan sulfate signaling pathway to increase neuroblast differentiation in a cell context specific manner.

4.2.4 MLN8237, ODSH, and decitabine combine to enhance neuroblastoma differentiation to decrease proliferation

MLN8237, decitabine, and vorinostat have shown very modest efficacy as single agents in Phase I/II clinical trials for aggressive, late-stage neuroblastoma. This lack of effect is partially due to the inability to reach tolerable dose levels necessary for this single-agent treatment to be effective in children (Maris 2010, He et al. 2013). Therefore, we explored the potential for these agents to be used as a combination therapy at low, clinically achievable doses.

We initially tested whether the targeted therapies could cooperate with the
Figure 28: Targeted therapies combine to enhance neuroblast differentiation

Western blot for differentiation markers in SK-N-AS following treatment for 72 hours with: A) a dose course of MLN8237 and 0.25µg/mL ODSH, B) a dose course of ODSH with 0.25nM MLN8237, or C) 0.25nM MLN8237 and/or 0.25µM decitabine. Images are representative of two independent experiments. Jasmine Nee helped to generate the data in this figure.
Figure 29: Targeted therapies synergize to suppress proliferation

Thymidine incorporation assays representative of two independent experiments for: A) SK-N-AS or B) BE2 following 24 hours of treatment with vehicle control (DMSO), 0.25nM MLN8237 (MLN), 0.25µM decitabine (Dec), or both 0.25nM MLN8237 and 0.25µM decitabine. For SK-N-AS, one-way ANOVA: p<0.001, 1-sample Student’s t-test: **p<0.01. For BE2, one-way ANOVA: p<0.001, 1-sample Student’s t-test: *p<0.05, ***p<0.001. Kevin Anderson helped to generate the data in this figure.

heparin derivative, ODSH. Our recent data has demonstrated that ODSH is a potent differentiating agent with antitumor and antimetastatic activity in orthotopic xenograft mouse models (Knelson et al. 2014a). The combination of MLN8237 and ODSH at low doses led to an increase in differentiation compared to either agent alone (Figures 28A and B). Addition of decitabine or vorinostat to ODSH did not produce any reliable enhancements in differentiation in our hands.
We next examined whether targeted therapies might cooperate with each other to promote neuroblast differentiation in the SK-N-AS cell line, which is known to be insensitive to the differentiating agent used as the standard of care in neuroblastoma, retinoic acid. Very low doses of vorinostat were extremely potent as a single-agent; therefore we did not see any combinatorial effects when vorinostat was added to other targeted therapies (data not shown). Interestingly, the combination of low dose MLN8237 and decitabine dramatically enhanced neuroblast differentiation compared to either agent alone (Figure 28C).

Since neuroblast differentiation often coincides with a decrease in proliferation (Pahlman et al. 1981, Leli et al. 1992, Scarpa et al. 1996, Encinas et al. 2000, Voigt et al. 2000, Edsjo et al. 2007, Hahn et al. 2008, Knelson et al. 2013, Knelson et al. 2014a), we tested our most potent combination of MLN8237 and decitabine in a thymidine incorporation assay. In two neuroblastoma cell lines, this combination therapy reduced proliferation by at least 50%, while the low dose single-agent treatments had no effect (Figure 29). Together, these data identify a novel combination therapy with differentiating properties and antiproliferative effects.

### 4.2.5 AurkA and DNMT inhibitors cooperate to decrease tumor growth in an orthotopic neuroblastoma xenograft model

Since we observed an increase in differentiation and an associated decrease in proliferation in neuroblastoma cell lines in vitro following treatment with MLN8237 and decitabine, we examined whether this combination therapy could be effective as an
antitumor treatment in a mouse model of retinoic acid-resistant neuroblastoma. We cautiously selected doses that were similar to amounts tolerated by children clinically (George et al. 2010, Mosse et al. 2012, Krishnadas et al. 2015). In an orthotopic xenograft mouse model of neuroblastoma, 10mg/kg MLN8237 and 0.4mg/kg decitabine dramatically reduced tumor growth compared to either agent alone (Figure 30A). Unexpectedly, we observed decitabine-induced toxicity that resulted in unanticipated deaths in our mouse cohort. Upon autopsy, no obvious reason for death was revealed. Interestingly, two out of three mice that were treated with the combination therapy presented with nearly undetectable tumor burden (Figure 30B). The third mouse in the combination cohort had a slightly larger tumor that was much less vascularized than tumors from the mice left untreated or treated with only a single agent, suggesting a potential anti-angiogenic effect of this combination treatment (Figure 30B, last panel on bottom right).

Due to the unexpected toxicity observed with decitabine treatment, we performed another in vivo orthotopic xenograft experiment to determine if we could reduce toxicity seen in our previous study. Reducing the doses of decitabine and MLN8237 by half to 0.2mg/kg and 5mg/kg respectively resulted in reduced toxicity and extension of life by several weeks. Similar to our previous results, we saw that combination treatment reduced tumor growth compared to mice left untreated or
Figure 30: Targeted therapies cooperate to decrease tumor growth

SK-N-AS orthotopic xenograft. A) Tumor radiance was measured after 21 days of tumor growth using luciferase in vivo imaging (photons/s/cm²/steradian). Radiance was measured again after 7 and 14 days of treatment with 10mg/kg MLN8237 (MLN) and/or 0.4mg/kg decitabine (Dec). Fold change in tumor radiance following 7 (blue) or 14 (red) days of treatment. UT=untreated. B) Tumor images are representative of each group (n=5) at humane endpoints.
Figure 31: Targeted therapies cooperate to decrease tumor growth at clinically achievable doses

SK-N-AS orthotopic xenograft. A) Tumor radiance was measured after 21 days of tumor growth using luciferase in vivo imaging (photons/s/cm2/steradian, denoted as Day 0). Radiance was measured again after 7, 14 and 21 days of treatment with 5mg/kg MLN8237 and/or 0.2mg/kg decitabine. Fold change in tumor radiance following 7 (blue), 14 (red), or 21 (black) days of treatment. UT=untreated. B) Tumor weights at humane endpoints for three mice per condition. C) Representative bioluminescence images from cage mates are shown for each cohort (n=3).
treated with single agents (Figure 31). Interestingly, one mouse appeared to have a complete response as no luciferase expression was detected after 21 days of treatment (Figure 31C). We did observe some toxicity at this low dose of decitabine as well, though life was extended compared to our previous experiment. Ongoing studies will seek to further reduce the decitabine dose in hopes of eliminating toxic side effects in the mice. We will also characterize the differentiating status of the tumors through analysis of tumor lysates for differentiation markers and examine whether the combination therapy has any effect on metastasis using histological analysis.

These data demonstrate that N-Myc is a potential regulator of the pro-differentiating secretome, informing the use of inhibitors of AurkA, DNMTs, and HDACs for neuroblastoma treatment. These therapies cooperate to promote neuroblast differentiation to suppress proliferation and tumor growth, revealing a novel combination therapy with potential efficacy for neuroblastoma treatment.

4.3 Discussion

Here we identify a novel combination therapy with the potential to promote neuroblast differentiation and suppress tumor growth at clinically achievable doses. As many therapies demonstrate promise in preclinical studies, but fail in clinical trials due to the inability to obtain an effective dose in patients or dose-limiting toxicities (Reynolds et al. 2003), it is imperative to identify therapeutics that will overcome these difficulties.
The AurkA inhibitor, MLN8237, in conjunction with the DNMT inhibitor, decitabine represented the most reliable and effective combination therapy in our hands. Decitabine in conjunction with chemotherapeutics has recently been tested in a clinical study. This study found that decitabine levels necessary to achieve clinically relevant biological effects in this combination therapy was not well tolerated by children with neuroblastoma (George et al. 2010). In other tumor contexts, such as acute lymphoblastic leukemia, the combination of decitabine with the HDAC inhibitor, vorinostat, provided clinical benefit to patients with relapsed disease (Burke et al. 2014), however, the dose tolerated by adults in this study was three times higher than the dose tolerated by children in the neuroblastoma study (George et al. 2010). This data reveals the need to identify combination therapies that may demonstrate efficacy at low doses to limit toxicities and underscores the need to test these agents in children rather than relying on clinical tolerability data from adult clinical trials.

AurkA, DNMTs, and HDACs can function by mediating N-Myc activity; however, it is unclear if this is the major mechanism by which these agents alter neuroblast differentiation. We have previously demonstrated that HDAC inhibitors can rescue expression of TβRIII, a pro-differentiating HSPG (Knelson et al. 2013). In addition, our data indicates that HSPG and ligand expression positively correlates with each other (Figure 16, Chapter 3) and is negatively correlated with MYCN amplification and expression (Figures 21 and 22) (Knelson et al. 2013). This common regulatory
mechanism displayed by several members of the pro-differentiating secretome suggests that N-Myc is a major player in mediating HSPG-induced neuroblast differentiation. Future studies will determine whether the major effects of AurkA, DNMT, and HDAC inhibitors is due to N-Myc inhibition and subsequent up-regulation of N-Myc target genes involved in differentiation, including HSPGs and their ligands.

In our in vivo studies, we established that the combination of low dose MLN8237 with decitabine could be effective in reducing tumor growth (Figures 30 and 31). Neuroblastoma is a very heterogeneous disease and patients with this disease often respond differently to therapeutic strategies. Similarly, we also found that some mice responded better than others to combination treatment in our study. Future studies to characterize the tumor profile of these mice before and after treatment could be useful in determining common features which may identify likely responders prior to therapy. These types of studies would limit the unnecessary toxicities neuroblastoma patients are often subjected to due to a lack of useful biomarkers or indicators of efficacy (Brodeur 2003, Janoueix-Lerosey et al. 2010, Maris 2010).

In conclusion, we have identified a novel combination therapy for use in promoting neuroblastoma differentiation and decreasing tumor growth in patients. These data emphasize the importance of understanding N-Myc regulation of neuroblastoma pathogenesis to inform the use of targeted therapies for neuroblastoma treatment. Our work also provides proof-of-principle that combining agents at clinically
achievable doses can have antitumor effects in neuroblastoma models and sets a precedent for future studies to perform pre-clinical research with doses that can be attained in children.
5. Conclusions and Significance

5.1 Stromal-derived HBEGF promotes neuroblast differentiation to suppress proliferation

The Schwannian stroma has been linked to neuroblast differentiation for almost 30 years, however, the critical factors secreted by the stroma have remained largely unknown (Shimada et al. 1984, Kwiatkowski et al. 1998, Liu et al. 2005). Our previous research has shed light on some soluble proteins that are stromal-derived and can promote differentiation, including TβRIII, GPC1, GPC3, and FGF2 (Knelson et al. 2013, Knelson et al. 2014a). We have demonstrated that the heparin-binding ligand HBEGF is decreased in neuroblastoma specimens and can be secreted by the stroma to promote neuroblast differentiation and suppress proliferation (Figures 9-12, 18-20), adding another signaling pathway to the differentiating stroma secretome. Ongoing studies aim to examine HBEGF expression in neuroblastoma patient serum compared to healthy controls to support our data demonstrating the importance of this protein in neuroblastoma pathogenesis.

Other heparin-binding ligands may also have similar effects to HBEGF and FGF2. Indeed, we have identified IGF-1 as a heparin-binding ligand (Table 1) that is increased in stroma-rich tumors compared to stroma-poor tumors, with high expression associated with increased survival in neuroblastoma patients (data not shown). Understanding the growth factors and receptors that play a role in this critical differentiating pathway will provide insight into how neuroblastomas develop and...
Our data supports the concept that enhanced differentiation correlates with decreased proliferation in neuroblastoma (Pahlman et al. 1981, Leli et al. 1992, Scarpa et al. 1996, Encinas et al. 2000, Voigt et al. 2000, Edsjo et al. 2007, Hahn et al. 2008, Knelson et al. 2013, Knelson et al. 2014a). HBEGF treatment is able to enhance differentiation and suppress proliferation in the retinoic-insensitive cell line, SK-N-AS, demonstrating the potency of this differentiation pathway and offering an alternative strategy to induce differentiation in neuroblastoma cells (Figures 9, 12, 18-20). Whether this approach will benefit other neuroendocrine-derived cancers, such as pancreatic neuroendocrine tumors, sarcomas (Desai and Jambhekar 2010), and small cell lung cancers (Park et al. 2011), remains unknown. The understanding that many neoplastic cells display reversible deficiencies in their differentiation capacity suggests that terminal differentiation as a therapeutic approach may demonstrate promise for many human cancers, however, further insight into the mechanisms responsible for differentiation blocks are sorely needed before this strategy can be broadly applied in the clinic (Yan and Liu 2016).

5.2 HSPGs, EGFR, ERK, and STAT3 are required for HBEGF-induced differentiation

We identified the central mechanism for HBEGF-induced differentiation to involve HSPGs and EGFR, which activated downstream ERK and STAT3 signaling to enhance ID1 transcription factor expression (Chapter 3). We utilized three clinically
available EGFR inhibitors to demonstrate the importance of EGFR activity in neuroblastoma differentiation (Figure 13D). Many of these EGFR inhibitors are in clinical trials for neuroblastoma due to their ability to promote apoptosis, but have only achieved modest results (Tamura et al. 2007, Jakacki et al. 2008, Furman et al. 2012). The importance of EGFR in neuroblast differentiation begs consideration when designing clinical trials targeted this pathway.

While it is known that HBEGF requires binding to HSPGs for its ability to bind and activate EGFR (Higashiyama et al. 1993, Aviezer and Yayon 1994, Shishido et al. 1995, Raab and Klagsbrun 1997), only SDC3 (Hienola et al. 2006) and CD44 variant 3 (Bennett et al. 1995) specifically have been shown to enhance an interaction between HBEGF and its receptor. Using co-immunoprecipitation studies to explore exogenous interactions and proximity ligation assays to detect endogenous complexes, we demonstrated for the first time a potential interaction between TβRIII and EGFR (Figure 14). Our work adds to the current knowledge of the specific HSPGs which are able to mediate HBEGF and EGFR activation. As our studies are highly suggestive of an interaction, they are not definitive. Additional studies using radiolabeled HBEGF to identify a direct interaction between these proteins is warranted. Furthermore, It remains unclear whether the interaction between EGFR and TβRIII may also have implications in other cancer settings, either through mediation of the EGFR pathway or changes in TGF-β superfamily signaling.
Our studies support prior research from our lab and others implicating the ERK signaling pathway as a major downstream mediator of neuroblastoma differentiation which converges on upregulation of ID1 (Lavenius et al. 1995, Kim et al. 2004, Passiatore et al. 2011, Wang et al. 2011, Qiao et al. 2012, Knelson et al. 2013, Knelson et al. 2014a). We utilized pharmacologic inhibitors and siRNA-mediated knockdown to demonstrate a critical role for this pathway in HBEGF-induced differentiation (Figure 18).

Interestingly, we also identified the STAT3 signaling pathway as a critical mediator of HBEGF-induced differentiation. Using a clinically available pharmacologic inhibitor and a dominant-negative construct, we demonstrated a requirement for STAT3 in HBEGF-induced differentiation (Figure 19). Our work supports the findings from another study where STAT3 was shown to be required for TGFα-induced neuroblastoma differentiation (Ciana et al. 2003). Downstream, ID1 also appears to be regulated by STAT3, as we observed decreased ID1 expression with STAT3 inhibition. Previously, ID1 has been shown to be a STAT3 target in colorectal cancer (Yu et al. 2014), demonstrating a broader role for STAT3 in ID1 regulation. While others have suggested STAT3 as a potential target for neuroblastoma treatment (Yan et al. 2013), these data warn against the use of STAT3 inhibitors as this strategy may lead to a block in differentiation.
5.3 Targeted agents cooperate to promote differentiation and suppress proliferation at clinically achievable doses

We performed microarray analysis as well as utilized chromatin immunoprecipitation and inducible and repressible cell line systems to uncover N-Myc as a potential master regulator of heparan sulfate signaling components (Figures 2, 5, 21, and 22). Using our comprehensive understanding of the differentiating stroma secretome and the evidence from our current studies revealing a common mechanism of regulation for this critical differentiating pathway, we identified candidate therapeutic agents that may counteract N-Myc to promote differentiation and suppress proliferation. Clinical data, in vitro experiments, and in vivo studies cooperate to identify MLN8237, decitabine, and vorinostat as agents with differentiating capabilities. These therapies may act via suppression of N-Myc and subsequent upregulation of heparan sulfate signaling factors, as we demonstrate increased HSPG, FGF2, and HBEGF expression, as well as increases in ERK signaling (Figures 25 and 26). Future studies will characterize whether these proteins could potentially function as biomarkers to indicate which patients might benefit from differentiating therapy and/or identify patients who are displaying a positive differentiating response to these treatments.

Many preclinical studies identify potential therapies for human disease; however, most of these agents fail when they get to the clinical trial stage. Only 8% of preclinical animal studies translate to successful clinical cancer trials in humans (Mak et al. 2014). This lack in equivalence can be attributed to many discrepancies between
animal studies and human studies, including the inability for animal studies to fully recapitulate the extremely complex process of human cancer initiation and progression and the failure to utilize drug doses that will be feasible for human patients (Reynolds et al. 2003, Schuh 2004).

Our studies have identified a combination therapy with efficacy in an aggressive orthotopic xenograft neuroblastoma mouse model. This combination consists of two targeted agents currently in clinical trials for neuroblastoma and other cancers, decitabine and MLN8237 (Table 2). We carefully selected a dosing strategy that was similar to concentrations tolerated by children in clinical trials (Figures 30 and 31) (George et al. 2010, Mosse et al. 2012, Krishnadas et al. 2015, DuBois et al. 2016). This is in contrast to the much higher doses used in most preclinical neuroblastoma studies for MLN8237 (Maris et al. 2010, Brockmann et al. 2013, Ham et al. 2016) or decitabine (Yang et al. 2003). Consideration needs to be taken when making efficacy claims for targeted agents based on non-translatable preclinical data. While our combination therapy demonstrated antitumor effects at clinically tolerable doses, our studies also have some limitations that may affect their translatability. First, the animals we utilized were adults, though most neuroblastomas occur in children. The long-term effects of this combination treatment on infants and children undergoing physiologic development are not known. Second, we employed a neuroblastoma cell line that was cultured in vitro for our orthotopic xenograft studies. While this method is widely accepted in the research
community, it does not best recapitulate human tumors. Studies to further confirm our results could test this therapy on patient-derived xenografts and genetic mouse models of neuroblastoma. A more comprehensive efficacy profile of this differentiating cocktail is warranted and may provide insight into its applicability as a neuroblastoma treatment.

5.4 Implications and Future Directions

In these studies we have identified a novel member of the pro-differentiating secretome to advance our understanding of how the neuroblastoma tumor stroma can promote neuroblast differentiation, developed the rationale and proof of principle for the use of the HSPG mimetic ODSH for neuroblastoma treatment, and provided a rationale for not using EGFR or STAT3 inhibitors in patients with neuroblastoma. In addition, our work on targeted agents could inform the use of combinatorial therapies to promote neuroblast differentiation and reduce tumor growth in children with late-stage, therapy-resistant disease. This research has the potential to lead to more successful treatment options for neuroblastoma patients, by both decreasing adverse side effects and increasing effectiveness of therapy.

Our work has alluded to the identification of potential biomarkers, including HBEGF, which may be useful in informing treatment choices for neuroblastoma patients. Futures studies will measure HBEGF levels in human serum and correlate this expression to tumor stage and stroma content. HBEGF serum levels could be used to
select patients who are likely to benefit from differentiation therapy. Ongoing studies will also determine which targeted differentiating agents are able to enhance HBEGF expression, as well as levels of other members of the pro-differentiating secretome.

Our current studies have suggested that N-Myc might be a critical regulator of pro-differentiating factors, including HSPGs, HBEGF, and FGF2. Our work will further characterize how these proteins are regulated in neuroblastoma cells. Many therapies in clinical trials act via mechanisms to suppress N-Myc levels and function (Table 2) and future studies will determine if the differentiating effects we observed with therapy treatment (Figures 23 and 28) can be attributed to enhanced heparan sulfate signaling.

We have identified a combination therapy that may be beneficial for patients with neuroblastoma, though it remains unknown if these findings will translate clinically. We will continue to work to identify therapies with potential pharmacologic synergy with heparin derivatives or with already recognized differentiating agents, such as 13-cis-retinoic acid or fenretinide. Combined with genetic and genomic screening, as well as the use of predictive and pharmacokinetic biomarkers, the identification of a potent differentiating combination therapy will potentially increase overall survival in these very young cancer patients.
# Appendix A: Cell line panel

Table 3: Cell line panel

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Symbol</th>
<th>Origin</th>
<th>Source</th>
<th>MYCN amplified</th>
</tr>
</thead>
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<tr>
<td>SK-N-BE2</td>
<td>BE2</td>
<td>Human</td>
<td>ATCC</td>
<td>Yes</td>
</tr>
<tr>
<td>IMR-32</td>
<td>IMR32</td>
<td>Human</td>
<td>ATCC</td>
<td>Yes</td>
</tr>
<tr>
<td>SK-N-AS</td>
<td>SK-N-AS</td>
<td>Human</td>
<td>ATCC</td>
<td>No</td>
</tr>
<tr>
<td>SH-SY5Y</td>
<td>5Y</td>
<td>Human</td>
<td>ATCC</td>
<td>No</td>
</tr>
<tr>
<td>SK-N-SH-SHEP</td>
<td>SHEP</td>
<td>Human</td>
<td>Michael Armstrong, Duke University</td>
<td>No</td>
</tr>
<tr>
<td>SK-N-AS-N-MycER</td>
<td>SK-N-AS-N-MycER</td>
<td>Human</td>
<td>Linda Valentijn, University of Amsterdam</td>
<td>MYCN-inducible with 4OHT</td>
</tr>
<tr>
<td>SHEP-21N</td>
<td>SHEP-21N</td>
<td>Human</td>
<td>Linda Valentijn, University of Amsterdam</td>
<td>MYCN-repressible with Dox</td>
</tr>
<tr>
<td>9464D</td>
<td>9464D</td>
<td>TH-MYCN Mouse</td>
<td>Rimas, Orentas, NCI</td>
<td>Yes</td>
</tr>
<tr>
<td>975A2</td>
<td>975A2</td>
<td>TH-MYCN Mouse</td>
<td>Rimas, Orentas, NCI</td>
<td>Yes</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Symbol</th>
<th>p53 status</th>
<th>Drug sensitive</th>
<th>Retinoic acid sensitive</th>
<th>1p loss</th>
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<tbody>
<tr>
<td>SK-N-BE2</td>
<td>BE2</td>
<td>Mutant</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>IMR-32</td>
<td>IMR32</td>
<td>Wild-type</td>
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<td>No</td>
<td>Yes</td>
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<tr>
<td>SK-N-AS</td>
<td>SK-N-AS</td>
<td>Mutant</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>SH-SY5Y</td>
<td>5Y</td>
<td>Wild-type</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>SK-N-SH-SHEP</td>
<td>SHEP</td>
<td>Wild-type</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
## Appendix B: Affymetrix probe list

Table 4: Affymetrix probe list

All affymetrix probes listed are from the HG-U133 Plus 2.0 platform. The GSE49710 probes listed are from the Agilent-020382 Human Custom Microarray platform.

<table>
<thead>
<tr>
<th>GENE</th>
<th>HBEGF</th>
<th>EGF</th>
<th>AREG</th>
<th>BTC</th>
<th>EREG</th>
<th>TGFα</th>
<th>NRG1</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSE49710 Probe ID</td>
<td>A_24_ P140608</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Affymetrix Probe ID</td>
<td>38037_ at</td>
<td>206254_ at</td>
<td>1557285_ at</td>
<td>207326_ at</td>
<td>205767_ at</td>
<td>205015_ s_at</td>
<td>208230_ s_at</td>
</tr>
<tr>
<td>Oncogenomics Probe ID</td>
<td>NB prognosis (35828)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GENE</td>
<td>NRG2</td>
<td>NRG4</td>
<td>MYCN</td>
<td>SDC3</td>
<td>GPC1</td>
<td>TGFR3</td>
<td>EGFR</td>
</tr>
<tr>
<td>GSE49710 Probe ID</td>
<td></td>
<td></td>
<td>A_24_ P94402</td>
<td></td>
<td>A_23_ P209904</td>
<td>A_23_ P200780</td>
<td>A_23_ P215790</td>
</tr>
<tr>
<td>Affymetrix Probe ID</td>
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<td>242426_ at</td>
<td>209757_ s_at</td>
<td>2082898_ at</td>
<td>204731_ at</td>
<td>201983_ s_at</td>
<td></td>
</tr>
<tr>
<td>GENE</td>
<td>HER2</td>
<td>HER3</td>
<td>HER4</td>
<td>ID1</td>
<td>MKI67</td>
<td>CDKN1A</td>
<td>SOX10</td>
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<tr>
<td>GSE49710 Probe ID</td>
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<td></td>
<td></td>
<td>A_23_ P252306</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Affymetrix Probe ID</td>
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<td>202454_ s_at</td>
<td>214053_ at</td>
<td>208937_ s_at</td>
<td>212022_ s_at</td>
<td>202284_ s_at</td>
<td>209843_ s_at</td>
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</table>

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Appendix C: HBEGF shRNA sequences

Table 5: HBEGF shRNA sequences

HBEGF shRNA (62203, 62204) knockdown constructs (Sigma Mission TRC1) are from the Duke University RNAi core facility.

<table>
<thead>
<tr>
<th>Construct</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>HBEGF shRNA #1</td>
<td>CCGGGAGGAGGTATGATGTTGGAAGACACGTGTCCATACATCATAACCTCTCTTTTTG</td>
</tr>
<tr>
<td>HBEGF shRNA #2</td>
<td>CCGGCCCATGTCTTCCGAAATACAAACTCGAGTTGATTTCCGAAAGACATGGGTTTTTG</td>
</tr>
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</table>
Appendix D: TβRIII ChIP primers

Table 6: TβRIII ChIP primers

Primers for chromatin immunoprecipitation (ChIP) were designed toward Sp-1 sites in the TβRIII promoter. Sp-1 site locations listed are relative to the TβRIII transcriptional start site.

<table>
<thead>
<tr>
<th>Human TβRIII Sp-1 site</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>+1kB</td>
<td>GCAGCAAGTTGGAGGAAAGC</td>
<td>GTCCGGATGGCGTAGTTTTG</td>
</tr>
<tr>
<td>+3kB</td>
<td>TCCTTTAACTGACACAACTGGCAGTG</td>
<td>AGGAAACAGCTGGGGTTTG</td>
</tr>
<tr>
<td>+5kB</td>
<td>TACATAATATGGGCGGCGGC</td>
<td>GTAGAGACGGGGTTCACCTG</td>
</tr>
<tr>
<td>+7kB</td>
<td>TCAACATAAAGAACCCACCACCA</td>
<td>ACAAGAGCAGCCAGAACCAGATG</td>
</tr>
<tr>
<td>-2kB</td>
<td>CTGACAAATGCCACCACGC</td>
<td>AGGCCAGGCAATCTCTTGAG</td>
</tr>
<tr>
<td>-20kB</td>
<td>AGATAATTCTGGACGCGGC</td>
<td>TGGTGGCCAGAACAGTCTCG</td>
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<tr>
<td>90kB (negative control)</td>
<td>TGTCCCTGAATCTCCGCACCTG</td>
<td>GTGGTGATGTTGAACCTGAG</td>
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</table>
# Appendix E: List of non-standard abbreviations

## Table 7: List of non-standard abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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</thead>
<tbody>
<tr>
<td>13-cis-RA</td>
<td>13-cis-retinoic acid</td>
</tr>
<tr>
<td>MKi67</td>
<td>Marker of proliferation Ki-67</td>
</tr>
<tr>
<td>NDES</td>
<td>N desulfated heparin</td>
</tr>
<tr>
<td>NF160</td>
<td>Neurofilament 160kD</td>
</tr>
<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
</tr>
<tr>
<td>NRG</td>
<td>Neuregulin</td>
</tr>
<tr>
<td>NDDES</td>
<td>N desulfated heparin</td>
</tr>
<tr>
<td>NSE</td>
<td>Neuron specific enolase</td>
</tr>
<tr>
<td>ODSH</td>
<td>2-O, 3-O desulfated heparin</td>
</tr>
<tr>
<td>NRG</td>
<td>Neuregulin</td>
</tr>
<tr>
<td>sHBEGF</td>
<td>soluble heparin-binding epidermal growth factor like growth factor</td>
</tr>
<tr>
<td>sTβRIII</td>
<td>soluble type III TGF-β receptor</td>
</tr>
<tr>
<td>TGFα</td>
<td>Transforming growth factor-alpha</td>
</tr>
<tr>
<td>TH</td>
<td>Tyrosine hydroxylase</td>
</tr>
<tr>
<td>TRKA</td>
<td>Tropomyosin receptor kinase A</td>
</tr>
<tr>
<td>TβRIII</td>
<td>Type III TGF-β receptor</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>EPGN</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EREG</td>
<td>Epiregulin</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
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<td>FGFR</td>
<td>Fibroblast growth factor receptor</td>
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<tr>
<td>GAP43</td>
<td>Growth-associated protein 43</td>
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<td>GPC</td>
<td>Glypican</td>
</tr>
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<td>HBEGF</td>
<td>Heparin-binding epidermal growth factor like growth factor</td>
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<td>Histone deacetylases</td>
</tr>
<tr>
<td>HSPG</td>
<td>Heparan sulfate proteoglycan</td>
</tr>
<tr>
<td>ID1</td>
<td>Inhibitor of DNA binding 1</td>
</tr>
</tbody>
</table>
References


to myc cellular oncogene is shared by human neuroblastoma cell lines and a neuroblastoma tumour." *Nature*, 305(5931): 245-248.


Wei, J. S., Greer, B. T., Westermann, F., Steinberg, S. M., Son, C. G., Chen, Q. R., Whiteford, C. C., Bilke, S., Krasnoselsky, A. L., Cenacchi, N., Catchpoole, D., Berthold,


Biography

Angela Lynn Gaviglio

Angela was born to Terry Lee and Mary Teresa Gaviglio on January 17, 1987 in Brainerd, Minnesota. She graduated in 2009 from the University of Minnesota in Minneapolis with a Bachelor of Arts degree in Physiology. She is scheduled to receive her Doctor of Philosophy in Molecular Cancer Biology degree from Duke University in Durham, North Carolina on October 6, 2016.

Publications


Posters


Presentations


Targeting TGF-β co-receptors in neuroblastoma. SURPH Student Presentation. June 24, 2015.


Activities and Awards

• Ruth L. Kirschstein National Research Service Award for Individual Predoctoral Fellows (F31) from the National Institute of Health (2015-2017)
• Duke Scholars in Molecular Medicine, Oncology track (2015-2016)
• Fitzgerald Academic Achievement Award (2015)
• Fitzgerald Retreat Poster Award (2015)
• Molecular Cancer Biology Representative to the Graduate and Professional Student Council (2014-2015)