TARGETING GASTRIN-RELEASING PEPTIDE IN NEUROBLASTOMA

By

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To my gorgeous Ma who waits for me to come back home and to my beloved Bapi who encourages me to live my dreams.

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ALK	Anaplastic lymphoma kinase
ANOVA	Analysis of variance
ATRX	α-thalassemia/metal retardation X-linked
AURKA	Aurora kinase A
BBS	Bombesin
BCL2	B-cell lymphoma 2
BET	Bromodomain and extra-terminal
BMI1	B lymphoma Mo-MLV insertion region 1 homolog
BSA	Bovine serum albumin
CDK	Cyclin dependent kinase
COG	Children's Oncology Group
COX-2	Cycloxygenase-2
DAG	Diacylglycerol
DAPI	6-diamidino-2-phenylindole
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
EMT	Epithelial-to-mesenchymal transition
ERK1/2	Extracellular signal-regulated protein kinases 1 and 2
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GPCRs	G-protein-coupled receptors

GRP	Gastrin-releasing peptide
GRP-R	Gastrin-releasing peptide receptor
HBSS	Hank's balanced salt solution
HUVECs	Human umbilical vein endothelial cells
IGF	Insulin-like growth factor
IgG	Immunoglobulin G
IL-8	Interleukin-8
IP3	Inositol 1,4,5 trisphosphate
INSS	International Neuroblastoma Staging System
MAPK	Mitogen-activated protein kinase
MDM2	Mouse double minute 2 homolog
MIC	Metastasis-initiating cancer stem cells
MMPs	Matrix metalloproteinases
mTOR	Mammalian target of rapamycin
NFκB	Nuclear factor kappa B
NKT	Natural killer T cells
siNTC	Non-targeting control siRNA
PARP	Poly ADP ribose polymerase
PBS	Phosphate buffered saline
PHOX2B	Paired-like homeobox 2b
PI3K	Phosphatidylinositol 3-kinase
PIP2	Phosphatidylinositol-4,5-bisphosphate
PIP3	Phosphatidylinositol-3,4,5-bisphosphate
PKC	Protein kinase C

PLC-β	Phospholipase C-β
PTEN	Phosphatase and tensin homolog
RA	Retinoic Acid
RT	Room temperature
SCLC	Small-cell lung cancer
shRNA	Short hairpin ribonucleic acid
siRNA	Small interfering ribonucleic acid
SEM	Standard error of the mean
TIMP	Tissue inhibitor of metalloproteinase
TRK-B	Tropomysin recptor kinase B
VEGF	Vascular endothelial growth factor
Y397	Tyrosine 397

CHAPTER I

INTRODUCTION

Neuroblastoma

An embryonal tumor of the sympathetic nervous system, neuroblastoma arises from aberrantly committed neural crest cells [1]. Approximately 700 new cases are reported for neuroblastoma each year, making it the most common extracranial solid tumor in the pediatric population [2]. Arising from the cells of the sympathetic nervous system, neuroblastoma usually occurs along the sympathetic chain, but most frequently in the adrenal medulla (**Fig. 1**). Neuroblastoma typically metastasizes to the regional lymph nodes, bone and the liver [1]. In North America, the Children's Oncology Group (COG) has stratified neuroblastoma risk groups (low, intermediate and high) based on the stage of the tumor (as defined by the International Neuroblastoma Staging System), age at diagnosis, histopathology and tumor biology. Despite multimodality therapy, which can be compromised of chemotherapy, surgery, radiation, myeloablative therapy, autologous stem cell rescue and 13-cis retinoic acid (RA) treatment, the overall survival rate for patients with high-risk disease is a dismal 10-30% [3,4]. Minimal residual disease resulting in disease relapse remains a primary reason for this low event-free survival rate. Long-term remission is further hindered by neuroblastomas that remain refractory to standard treatment strategies. To make matters worse, two-thirds of neuroblastoma patients present with metastasis at the time of diagnosis and are considered advanced-stage disease, precluding the efficacy of current conventional regimes. Hence, the last decade has seen an increased attempt in developing targeted therapies based on the genomic and proteomic signatures of neuroblastoma patients

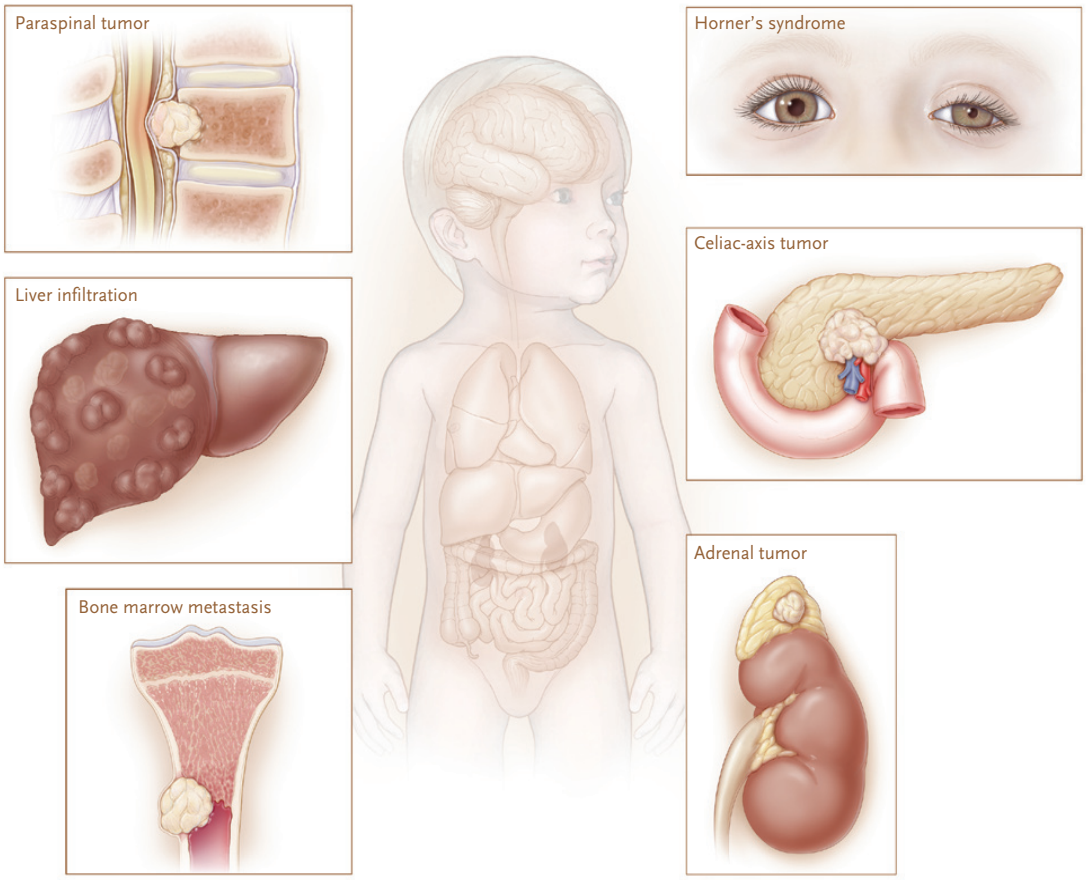

Figure 1. Clinical presentations of neuroblastoma (Maris, *NEJM*, 2010).

with aggressive, refractory phenotypes of the disease.

Current studies are heavily focused on the induction of disease remission at a molecular level using neuroblastoma-specific gene transcripts and aberrant signaling pathways (**Fig. 2**). *MYCN* amplification, with a prevalence of ~22%, is the most common genetic feature in neuroblastoma and correlates with poor prognosis and unfavorable patient outcomes [5,6]. Despite being extensively studied as a major oncogenic driver in neuroblastoma, there are currently no clinical trials targeting *MYCN* in neuroblastoma. Hence, recent drugs have focused on signaling pathways that regulate MYCN protein, such as aurora kinase A (AURKA) and the bromodomain and extra-terminal (BET) domain family of proteins [7,8]. Mutations in the anaplastic lymphoma kinase (*ALK*) and more recently, in α-thalassemia/mental retardation syndrome X-linked (*ATRX*) genes account for some of the other mutations observed in children with neuroblastoma [4]. However, there has been limited progress in successfully initiating clinical trials targeting these chromosomal aberrations. The other class of targeted therapy that is gaining significance in pediatric tumors after their successful implementation in adult cancers includes targeting growth factors, cell surface receptors and protein kinases downstream of such receptors. Targeting growth factors such as insulin-like growth factor (IGF), vascular endothelial growth factor (VEGF) and hepatocyte growth factors (HGF) demonstrated promising results in neuroblastoma preclinical trials [9,10,11]. Pharmacological inhibitors against protein kinases, AKT, ALK and AURKA, recently have entered clinical trials after their success in preclinical studies [12,13,14].

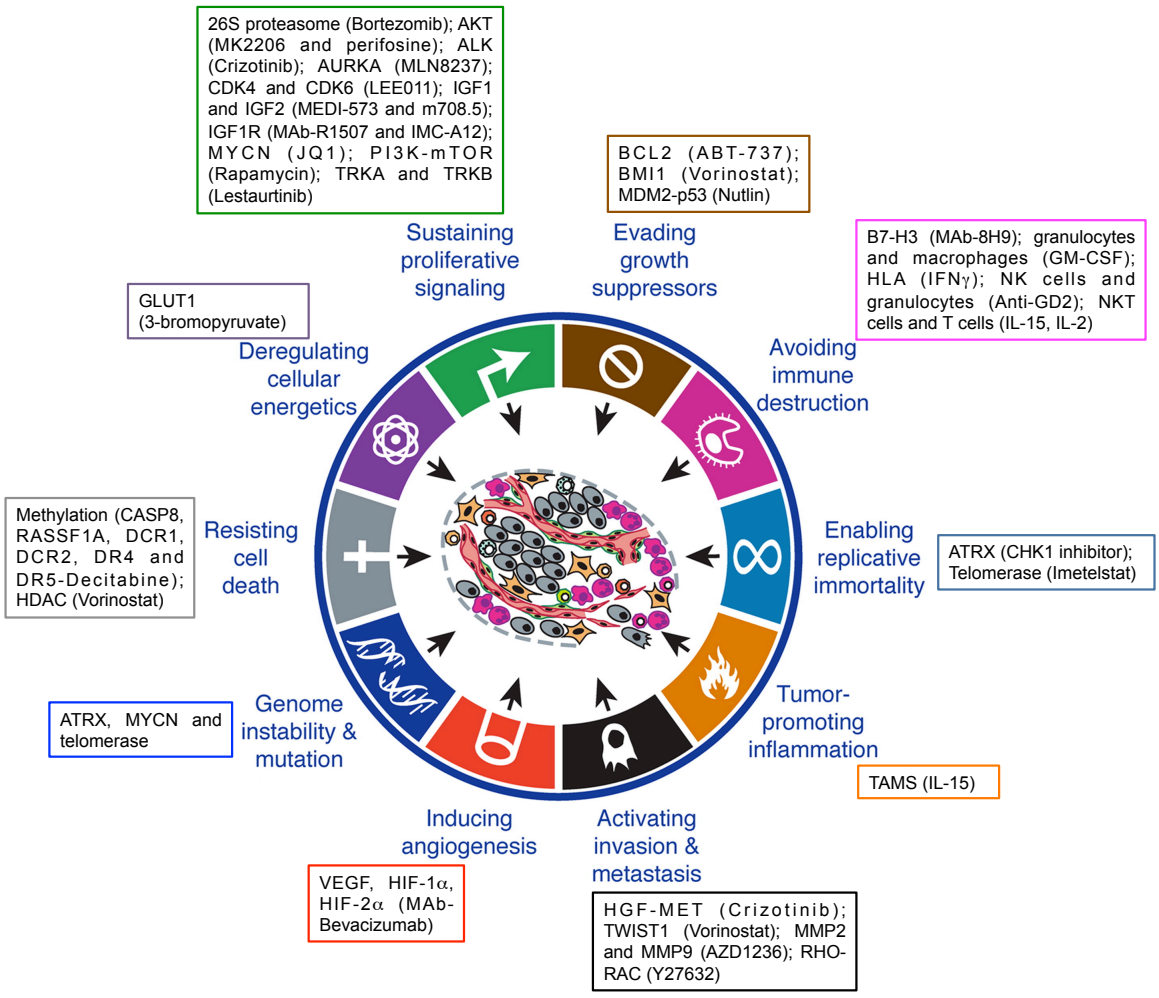

Figure 2. Hallmarks of high-risk neuroblastoma with druggable targets. Preclinical and clinical targets presently under investigation for patients with high-risk neuroblastoma. (Adapted from Cheung and Dyer, *Nature Reviews Cancer*, 2013; Hanahan and Weinberg, *Cell*, 2011).

However, the stark reality of (i) acquired drug resistance to efficacious drugs, and (ii) failure of clinical trials in patients with recurrent and progressive disease, inspite promising preclinical data, are concerns that need to be addressed. These concerns can be partly attributed to flawed preclinical studies where effects on tumor inhibition are investigated using subcutaneous murine tumor models, but clinically tested in cohorts of patients with refractory disease and/or metastasis. In such circumstances, it becomes pertinent to investigate novel therapeutic strategies using animal models that can mimic aggressive, metastatic disease.

Accounting for about 40% of all targeted therapies and representing the hub of drug development activities are members of the G-protein coupled receptor (GPCR) superfamily, which transmit chemical signals into a wide gamut of cell types. The Nobel Prize in Chemistry for 2012 provided further recognition to the importance of GPCRs and to the efforts of Drs. Lefkowitz and Kobilka. Our laboratory has previously shown that one such GPCR, called gastrin-releasing peptide receptor (GRP-R) and its ligand gastrin-releasing peptide (GRP), are notably increased in undifferentiated human neuroblastomas when compared to its benign phenotype, ganglioneuromas [15]. Moreover, we have also demonstrated that GRP acts as a growth factor for neuroblastoma cells *in vitro* and stimulates growth of subcutaneous tumors *in vivo* [15,16]. The next sections will provide a brief insight into the role of GRP under normal conditions and during tumorigenesis.

Gastrin-releasing Peptide

Initially isolated as the amphibian equivalent bombesin (BBS), it took almost a decade to identify and characterize the mammalian GRP [17]. The name for this peptide was derived from its first known activity as an inducer of gastrin secretion

from G cells in the gastric antrum. Cloning of the GRP cDNA [18] facilitated analysis of *GRP* mRNA expression in various tissues by Northern analysis and *in situ* hybridization. GRP was found in the brain, central nervous system, nerve fibers throughout the gastrointestinal tract and pancreas, lung, thymus, prostate, urethra and pregnant uterus [19].

Apart from gastrin secretion, GRP is also involved in the secretion of other gastrointestinal peptides such as somatostatin and cholecystokinin. Moreover, GRP stimulates exocrine secretion from the pancreas and smooth muscle contraction in the stomach and small intestine; along with neural effects regulating suppression of food intake, sensation of itch and the circadian system. Furthermore, BBS/GRP has the ability to stimulate the growth of gastrointestinal mucosa and pancreas [20,21]. Interestingly, BBS/GRP was observed to have protective functions in animal models of gastrointestinal injury [22]. Moreover, its mitogenic effects in normal tissues and regenerative/protective functions during wound healing are dependent on signaling pathways that are commonly activated during tumorigenesis [23]. Hence it was not surprising when Cuttitta and colleagues demonstrated that GRP acts as an autocrine growth factor in small-cell lung cancer (SCLC) [24].

Gastrin-releasing Peptide and the Hallmarks of Cancer

Since the initial observation made by Cuttitta and colleagues regarding its role as a novel mitogen, studies demonstrated the role of GRP as a potential morphogen and a pro-angiogenic molecule [25,26,27]. Some groups argue that the mitogenic properties of GRP is subordinate to its morphogenic property and that GRP essentially acts as an “onco-fetal antigen”, recapitulating its role in normal development, but in a dysfunctional manner. To truly understand the oncogenic

properties of GRP, studying its role with respect to multiple hallmarks of cancer simultaneously is becoming increasingly important. Some of the affected hallmarks have been outlined below:

1. Sustaining cell proliferation – As an autocrine/paracrine growth factor, a critical role for GRP with regard to this hallmark is predictable. Subcutaneous injection of an SCLC cell line followed by BBS treatment significantly increased tumor weight and DNA content [28]. In neuroblastoma, GRP/BBS stimulation enhanced tumor cell proliferation *in vitro* and subcutaneous tumor growth *in vivo* [15,16]. Conversely, GRP antagonists suppressed the growth of SCLC, prostate, gastric, pancreatic, breast and colorectal cancer cell lines *in vitro* and *in vivo* [29,30,31,32,33]. Martinez and colleagues reported that a specific GRP blocker 77427 completely reduced tumor volume in a xenograft model of lung cancer, but surprisingly, the tumors grew back at normal rates once the treatment was suspended [34]. This observation indicates a cytostatic role for GRP inhibition instead of a cytotoxic effect, highlighting the significance of developing combination therapies targeting GRP.

2. Evasion of cell death – Most studies have focused on the role of GRP as a mitogen and observed decreased cancer cell proliferation after GRP antagonist treatment. But whether these antagonists induce a concomitant cancer cell death remains to be studied. A recent report suggests that GRP stimulates the growth of a hepatocellular carcinoma cell line, HepG2, by blocking endoplasmic reticulum stress-mediated apoptosis [35]. Interestingly, our laboratory has recently demonstrated that GRP stimulation can potentially inhibit autophagy-mediated cell death of human umbilical vein endothelial cells (HUVECs) [26]. Whether inhibiting GRP signaling can induce neuroblastoma cell death and the plausible mechanism(s) involved remain to be elucidated.

3. Inducing angiogenesis – More than a decade ago the proangiogenic effects of BBS/GRP had been established when Levine and colleagues demonstrated that BBS stimulation induced NFκB activation and the expression of proangiogenic molecules in prostate cancer [36]. Since then studies have reported that GRP antagonists block angiogenesis in cancers of the lung, breast and kidney [34,37,38]. Neuroblastoma is characterized by florid vascularization and our laboratory has previously demonstrated that GRP treatment stimulates vascular endothelial cell proliferation and *in vitro* tubule formation [26]. Hence, it will be pertinent to study whether targeting GRP directly affects neuroblastoma cell-mediated angiogenesis.

4. Invasion and metastasis – "Unless tumor cells are able to invade, that is, push into surrounding tissue, they cannot force their way into blood vessels. Therefore, without invasion metastasis cannot occur…" [39]. The importance of tissue invasion and metastasis had been established as early as the 19th century, but a role for GRP in this hallmark of cancer has only recently been identified. GRP stimulation increased the invasiveness of prostate cancer cell lines *in vitro* [40]. Similarly, Zhang and colleagues demonstrated that combination treatment of GRP receptor (GRP-R) and epidermal growth factor receptor (EGFR) inhibitors decreased head and neck squamous cell carcinoma invasion [41]. Moreover, BBS treatment enhanced the incidence of peritoneal metastasis from gastric cancers induced *in vivo* [42]. Interestingly, survival of gastric cancer patients positive for GRP-R was not significantly different from patients negative for GRP-R expression [43]. In contrast, breast cancer patients with lymph node metastasis had a lower survival rate when positive for GRP [44]. Using an *in vivo* metastasis model our laboratory demonstrated that silencing GRP-R inhibited the formation of secondary lesions in

the liver [45]; however, we have yet to investigate the effect of inhibiting GRP in neuroblastoma metastasis.

Additionally, recent studies have implicated a role for GRP/GRP-R in deregulating cellular metabolism and in tumor-associated inflammation [46,47]. Hence, targeting GRP will potentially have multi-faceted effects on neuroblastoma tumor initiation and progression and needs to be investigated extensively. As an autocrine/paracrine growth factor, GRP and its receptor, GRP-R, activate various signal transduction pathways, so the next section will briefly discuss various signaling pathways mediating the oncogenic effects of GRP/GRP-R in cancer.

Signaling Pathways Activated by Gastrin-releasing Peptide in Cancer

GRP-R, the G-protein coupled receptor for GRP, couples to G proteins triggering downstream signal transduction pathways critical for cancer cell proliferation, survival, angiogenesis and metastasis. Typically upon ligand binding, GPCRs via $G\alpha q$ protein stimulate phospholipase C-β (PLC-β) resulting in the production of second messengers inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG), increased free cytosolic Ca^{2+}, and the activation of protein kinase C (PKC) and mitogen-activated protein kinase (MAPK) pathways. In addition, $G\alpha q$ also activates AKT pathway, thereby, modulating mTOR and NFκB signaling. GPCRs via $G\alpha s$ proteins initiate PKA- and MAPK-dependent signaling; $G\alpha i$ activates SRC, PI3K, ERK and Rho-mediated signals. Moreover, crosstalk with growth factor receptors allows GPCRs to regulate ERK, PI3K and JAK-STAT signaling pathways [48,49]. Differential activation of downstream signaling pathways by GPCRs, termed

"functional selectivity", determines the biological outcome in tumor cells and provides a potential explanation regarding tumor heterogeneity.

Studies in prostate cancer have identified that BBS/GRP stimulation activates MAPK pathway during tumor cell proliferation [50]; GRP enhances interleukin-8 (IL-8) and VEGF expression in prostate cancer cells [36] via NFκB [47]. Similarly, Chao and colleagues have shown that in breast cancer cells GRP-R synergizes with EGFR to regulate cell migration and IL-8 expression, but not cell proliferation, and ectopic expression of GRP-R alone was sufficient in eliciting similar responses [51]; BBS/GRP stimulated hepatocellular cancer cell proliferation via an EGFR-independent MAPK pathway activation [35]. In colon cancer, a proteomic based approach identified that heat shock protein (HSP), heterochromatin protein 1 (HP1), intercellular adhesion molecule 1 (ICAM-1) and acetyl-coenzyme A acyltransferase (ACAT) were upregulated after GRP-R overexpression and contributed to the aggressiveness of this disease [52]. These studies clearly underline the complexity of GRP/GRP-R signaling within solid tumors and the necessity to study disease-specific downstream signaling to effectively understand GRP/GRP-R-dependent tumor pathophysiology and pathobiology.

In the context of neuroblastoma, our laboratory has identified multiple pathways that mediate the oncogenic effects of GRP/GRP-R (**Fig. 3**). GRP can induce rapid short term VEGF secretion in neuroblastoma cells by activating the PKC pathway [53]. GRP/GRP-R signaling can also induce neuroblastoma cell motility by activating focal adhesion kinase (FAK) – a phenomenon observed in adult solid tumors as well [54]. Higher levels of VEGF or FAK correlate with unfavorable histology and aggressive tumor behavior in neuroblastoma [54,55]. The observation

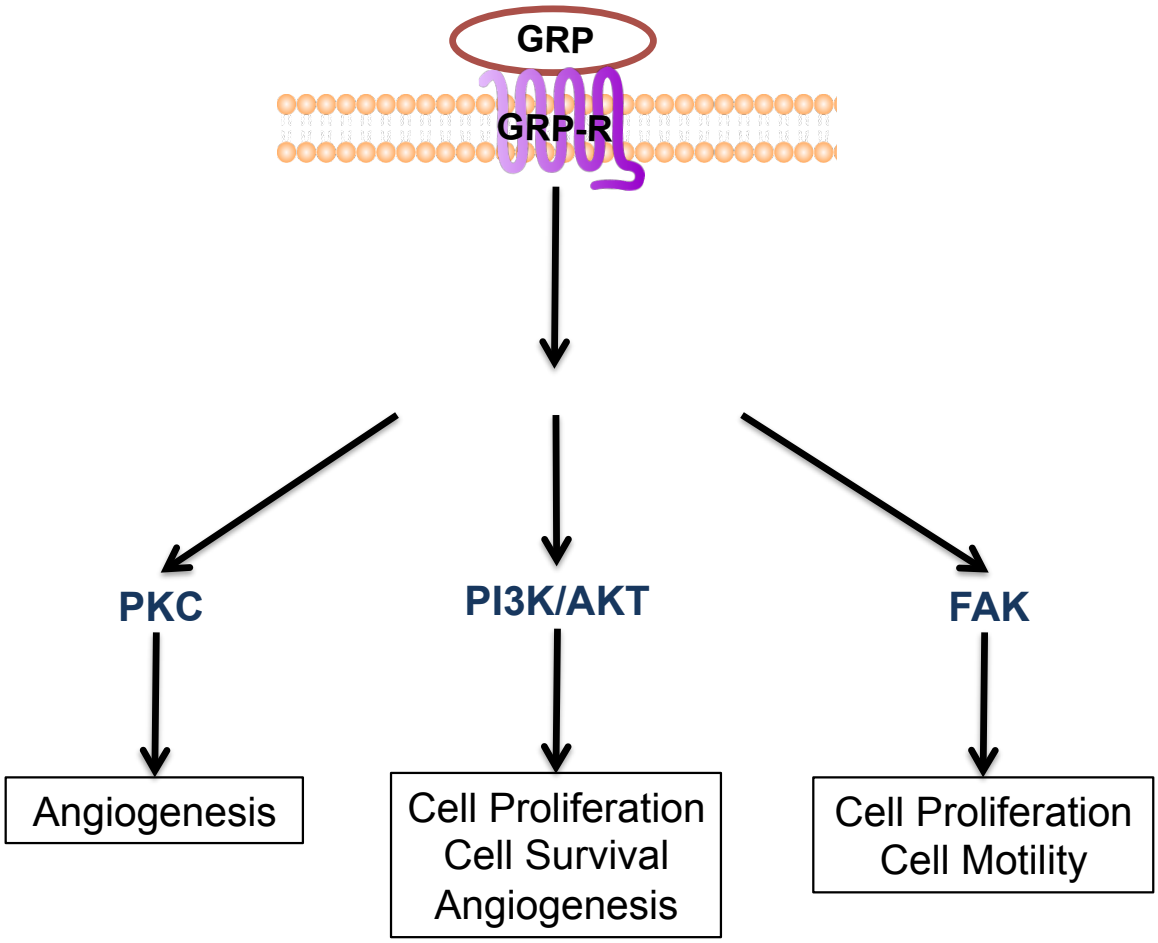

Figure 3. GRP/GRP-R activates multiple downstream signaling pathways to induce neuroblastoma cell survival, proliferation, motility and tumor cell-mediated angiogenesis.

that GRP/GRP-R stimulated neuroblastoma cell-mediated angiogenesis and motility provides a strong lead regarding the potential efficacy of inhibiting neuroblastoma progression by targeting GRP [16]. But from these studies, the pathway that stands out to be critical in mediating GRP/GRP-R regulated hallmarks of neuroblastoma is the AKT signaling. AKT activation by GPCRs had been first identified nearly 15 years ago, when two seminal studies reported that GPCRs induce phosphorylation of AKT at Ser473 in a PI3K-dependent manner [56,57]. We have demonstrated that GRP stimulation induces neuroblastoma cell cycle progression via AKT pathway [58]. Furthermore, GRP-R silencing downregulated the activation of AKT and S6 kinase in neuroblastoma cells [45], whereas, GRP-R overexpression suppressed the mRNA and protein expression of PTEN [59]. The next section will briefly highlight how PI3K/AKT pathway has proven to be critical and positioned AKT as a key determinant in the biological aggressiveness of neuroblastomas.

AKT/PTEN Axis in Neuroblastoma

From inducing resistance to chemotherapy to facilitating MYCN-mediated oncogenic effects, PI3K/AKT has garnered considerable interest in neuroblastoma studies. Most importantly, activation of AKT signaling correlates with poor prognosis in neuroblastoma patients [60]. PI3K/AKT signaling is required for mediating the actions of oncogenes like IGF-1R, ALK and TRK-B in neuroblastoma cell proliferation, evasion of apoptosis and chemoresistance [61,62,63]. Studies have also implicated a role for AKT in mediating CD133-regulated chemoresistance and inhibition of neuroblastoma cell differentiation [64,65]. Activation of PI3K/AKT/mTOR signaling appears to be critical for MYCN protein stabilization and MYCN-dependent angiogenesis in neuroblastoma [66,67,68,69].

Based on neuroblastoma preclinical studies, targeting AKT and its downstream target, mTOR, has achieved immense significance. AKT inhibition alone or in combination with rapamycin, a specific mTOR inhibitor, suppressed neuroblastoma growth *in vitro* and *in vivo* [12]. NVP-BEZ235, a novel dual inhibitor of PI3K and mTOR, decreased angiogenesis and increased overall survival in a primary xenograft model of neuroblastoma [69]. Preclinical studies demonstrating sensitivity to mTOR inhibitors led to the initiation of clinical trials using temsirolimus in neuroblastoma patients with relapsed/refractory disease, but failed to have any significant antitumor activity in such patients [70]. Interestingly, a specific AKT antagonist significantly inhibited VEGF production in rapamycin-refractory neuroblastoma cell lines [71]. This indicates that as a central molecule and a merging point for multiple signaling nodes AKT is a more promising target than individual membrane receptors or downstream AKT targets, like mTOR, in treating aggressive, metastatic neuroblastomas.

Phosphatase and tensin homolog (PTEN), a negative regulator of AKT (**Fig. 4**), acts as tumor suppressor and is frequently mutated in cancers. Early work on PTEN in neuroblastoma suggested that only a small number of cell lines harbor mutations in this gene that could contribute to oncogenesis and malignant tumor progression [72,73]. These initial studies seemed to indicate that significance of PTEN is, at best, marginal and that its role as a tumor suppressor may not be critical in neuroblastoma. There is a dearth of studies examining the role of PTEN as a negative regulator of AKT, and thereby, in the aggressiveness of neuroblastomas. Interestingly, a differential expression in PTEN and phosphorylated AKT (pAKT) levels was observed in neuroblastoma patients; a higher PTEN expression was noted in

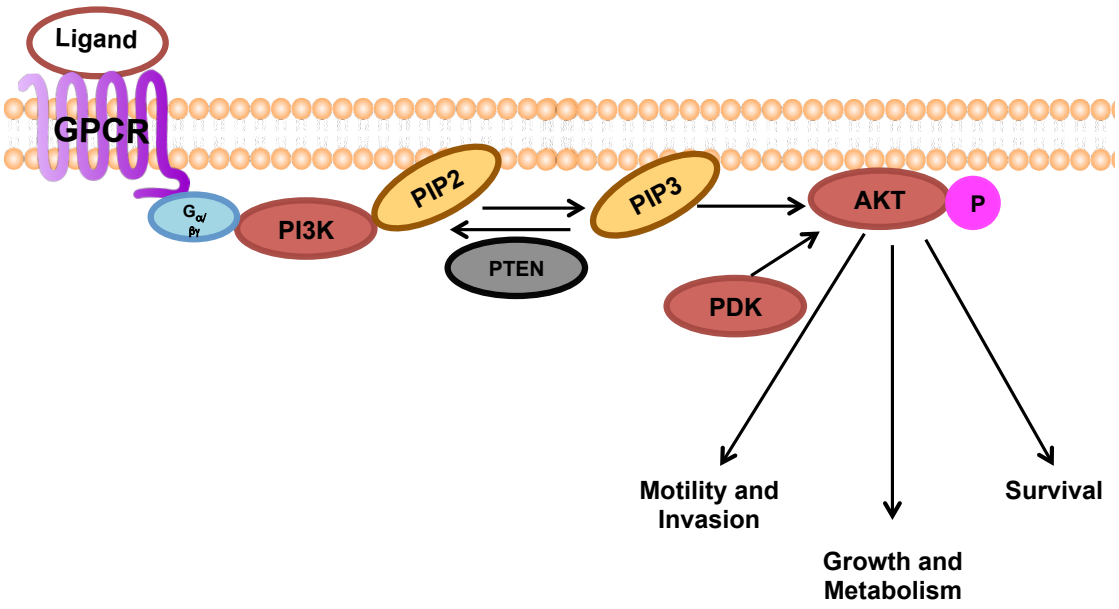

Figure 4. GPCR-mediated PI3K/AKT/PTEN signaling in cancer. Generation of PIP3 after PI3K activation recruits AKT to the cell membrane where PDK1 phosphorylates AKT on Thr308 and at Ser473 by PDK2. Activated AKT, via downstream signaling molecules, enables various hallmarks of cancer. PTEN acts as a negative regulator of AKT, by converting PIP3 to PIP2.

differentiated ganglioneuroblastomas in comparison to undifferentiated neuroblastoma [59]. There was no significant difference in pAKT levels based on the differentiation status of these tumors [59]. But this observation does not discount a potential role for AKT in neuroblastoma progression. Moreover, based on this work, the association between PTEN and a more undifferentiated neuroblastoma phenotype suggests that PTEN could potentially regulate molecular pathways associated with invasion and neuroblastoma metastasis. Observations in adult solid tumors where PTEN regulates cell migration, raises such a possibility [64-66]. To date only one study has reported that PTEN overexpression enhanced the inhibitory effect of a c-Met antagonist on neuroblastoma cell proliferation and migration [74]. There is a huge gap in the current literature regarding the role of PTEN in neuroblastoma metastasis and the mechanism(s) by which PTEN/AKT axis can modulate GRP-mediated neuroblastoma progression. Furthermore, if PTEN/AKT axis indeed plays a critical role in neuroblastoma metastasis, then it would be important to study the expression of these proteins in neuroblastoma patients with respect to aggressiveness of the disease.

Statement of Problem

Despite recent advances in multi-modality therapy, the overall survival for neuroblastoma remains dismally low. The two major problems in treating this heterogeneous cancer are - 1) frequent resistance to standard chemo- and radiation therapy resulting in relapse and/or refractory tumors, and 2) a majority of the patients present with metastasis at the time of diagnosis. We need to address the concern of not only reducing the recurrence and metastasis of these tumors, but also decrease chemotherapy-mediated complications by allowing usage of lower drug doses in

children affected by this disease. Moreover, based on the genomic and proteomic signatures of patients, we need to identify novel therapeutic strategies which when used in combination to current treatment regimes will provide potential options for neuroblastoma patients with aggressive, metastatic disease who are refractory to current therapeutic modalities alone.

Central Hypothesis and Specific Aims

GRP acts as an autocrine and paracrine growth factor in neuroblastoma [15,58]. But much remains unknown in GRP-induced evasion of neuroblastoma cell death and the downstream signaling pathways associated with its oncogenic functions. It becomes pertinent to study whether targeting GRP can inhibit tumorigenesis by inducing apoptosis in neuroblastoma cells. This would support the use of GRP antagonists as an adjuvant to current chemotherapeutic regimes in treating refractory neuroblastomas. Moreover, we have identified PTEN/AKT axis as a critical mediator of the mitogenic potential of GRP/GRP-R signaling; but the role of this axis in regulating GRP-mediated neuroblastoma progression remains unknown. Hence, the central hypothesis of this study is that GRP-mediated AKT activation and PTEN inhibition drives neuroblastoma progression by regulating multiple aspects of tumorigenesis. To examine and confirm this hypothesis, two specific aims had been designed:

Aim 1: Determine the role of GRP in cell cycle progression and modulating the cytotoxic effects of standard chemotherapeutic agents. This study aimed to examine whether silencing GRP enhanced apoptosis in neuroblastoma cells. Moreover, we also wanted to investigate whether targeting GRP could potentiate the

cytotoxic effects of some commonly used chemotherapeutic drugs in neuroblastoma. GRP antagonists like RC-3095 and 2A11 (monoclonal antibody) have been previously used in preclinical and clinical studies in adult solid tumors and demonstrated no toxic effects. Thus, this study can provide a potential rationale for the use of GRP antagonists in combination with standard chemotherapeutic regimes in treating neuroblastoma patients with aggressive, refractory disease.

Aim 2: Determine the role of GRP and PTEN/AKT axis in regulating neuroblastoma progression. This study was designed to examine the role of GRP in the multi-step invasion-metastasis cascade in neuroblastoma. We also wanted to determine the downstream signaling pathways that facilitate GRP-mediated neuroblastoma progression. Thus, this study can provide a rationale for targeting GRP as a therapeutic approach in advanced-stage neuroblastoma patients by potentially regulating multiple aspects of tumor progression.

CHAPTER II

MATERIALS AND METHODS

Reagents

Primary antibodies used include cleaved caspase-3, cleaved PARP, p53, p21 pERK1/2, pAKT (S473), AKT, PTEN, pmTOR, mTOR, TWIST from Cell Signaling Technology; pFAK and FAK from BD Biosciences; β-actin from Sigma-Aldrich; Alexa Fluor 568 and 488 from Molecular Probes. Horseradish peroxidase (HRP)-conjugated secondary antibodies against mouse and rabbit IgG were obtained from Santa Cruz Biotechnology, Inc. Enhanced chemiluminescence (ECL) HRP substrates were purchased from Millipore (Immobilon Western) and Perkin Elmer (Western Lightning). Primers for *GRP*, *MYCN*, *FAK* and *TWIST* were designed using Primer-BLAST and ordered from Sigma-Aldrich. GRP was purchased from Bachem. Propidium Iodide (0.5mg/mL) was obtained from Roche Diagnostics. Agarose (SeaPlaque®) was from Cambrex Bio Science. Doxycycline was purchased from Sigma-Aldrich Cell Counting Kit-8 (CCK-8) was from Dojindo Molecular Technologies. Immunohistochemistry reagents were from Dako Corporation. siRNA pool against GRP was purchased from Dharmacon, along with non-targeting scrambled sequences that were used as controls.

Cell culture, plasmids and transfection

Human neuroblastoma cell lines, SK-N-SH, SH-SY5Y and BE(2)-C, were purchased from American Type Culture Collection. JF cell line was a kind gift from Dr. Jason Shohet (Baylor College of Medicine). Cells were maintained in RPMI 1640 medium with L-glutamine (Cellgro Mediatech) supplemented with 10% fetal bovine

serum (FBS; Sigma-Aldrich). The cells were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2. For transfection, cells were plated in 6-well plates and were transfected with plasmid (4μg) or siRNA (100nM) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. All GRP (100nM) treatments were performed after serum-starved overnight to clearly understand the effects of GRP on signaling pathways. Experiments were repeated on 3 separate occasions. Vectors pBP_2 and pBP_2-HA-PTEN were gifts from Dr. Webster Cavenee (Univ. of California, San Diego, CA).

Inducible knockdown system

For knockdown of our target gene, human GRP, we used BLOCK-iT Inducible H1 Lentiviral RNAi System (Life Technologies, Invitrogen, Grand Island, NY). The sequence targeting GRP (NM_002091) is underlined in the shRNA (shGRP) sequence: 5'-CACCAGCAATCAGCAGCCTTCGTGGGACGAATCCCACGAAGG CTGCTGATTGC-3'; the nonspecific control shCON is: 5'-CACCGGGCGCGCTTTGT AGGATTCGCCG AAGCGAATCCTACAAAGCGCGCC-3'. shRNA sequences were cloned into the BLOCK-iT Inducible H1 RNAi Entry Vector (pENTR™/H1/TO), and then shRNA was inserted into Lentiviral vector pLenti4/BLOCK-iT-DEST by LR recombination between pENTR™/H1/TO entry and pLenti4/BLOCK-iT expression constructs. Inducible shRNA expression cells were established by transfecting cells with both pLenti6/TR and pLenti4/BLOCK-iT-DEST, or by introducing the vectors with the lentiviral-mediated delivery system. Production of lentivirus was performed in 293FT cells. Stable cell lines BE(2)-C/Tet/shCON, BE(2)-C/Tet/shGRP, SH-SY5Y/Tet/shCON and SH-SY5Y/Tet/shGRP were established by selecting with

Blasticidin at 8 µg/ml and Zeocin at 50 µg/ml post lentiviral transductions.

RNA isolation and reverse transcription

Total RNA was isolated from neuroblastoma cells using Trizol® (Life Technologies). Cells were incubated in Trizol® reagent. Chloroform was added and mixed by vortexing. Cells were then centrifuged at 12000x g for 15 min. The clear supernatant was transferred to a new tube, incubated with isopropanalol for 10 min and centrifuged. The pellet obtained was washed twice with 70% ethanol and resuspended in DNAse, RNAse free water. The concentration of RNA (OD260:280) was measured using a FlexStation 3 (Molecular Devices). Isolated RNA (1 µg) was used to synthesize cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to manufacturer's instruction.

Quantitative and semi-quantitative PCR

Semi-quantitative PCR was performed using a Peltier Thermal Cycler (PTC-200) using specific 3'and 5' primers for GRP was final product visualized on 1% agarose gel using a Gel Doc (BioRad). Quantitative PCR was performed using a Bio-Rad Thermocycler CFX96. SsoFAST EvaGreen Supermix, cDNA and specific 3' and 5' primers were incubated together using the manufacturer's protocol (Bio-Rad). The reactions were set up at 20 µl with 1 µl cDNA template, 10 µl Sso Fast™ EvaGreen Supermix, 1 µl of each primer (5 µmol/l), and 7 µl distilled water. The reactions were programmed with an initial denaturation step of 2 min at 98°C, followed by 40 temperature cycles for 5 s at 98°C and 5 s at 60°C. At the end of amplification, the melting curve analysis was performed for the PCR products to ensure the amplification specificity. All measurements were produced in duplicate.

Relative mRNA levels were calculated based on ratios of the initial cDNA quantity of housekeeping control. GAPDH and β-actin were used as housekeeping controls. Primers: GAPDH forward primer 5′-TCCTCTGACTTCAACAGCGACACC-3′, GAPDH reverse primer 5′-TCTCTCTTCCTCTTGTGCTCTTGG-3′; β-actin forward primer 5'-ACCGAGCGCGGCTACAG-3', β-actin reverse primer 5'-CTTAATGTCACGCACGATTTCC; GRP forward primer 5'-GCTGGGTCTCATAGAAGCAAAG-3', GRP reverse primer 5'-TGGAGCAGAGAGTCTACCAAC-3'; MYCN forward primer 5'-GCTTCTACCCGGACGAAGATG-3', MYCN reverse primer 5'-CAGCTCGTTCTCAAGCAGCAT-3'; FAK forward primer 5'-TTATTGGCCACTGTGGATGA-3', FAK reverse primer 5' TACTCTTGCTGGAGGCTGGT-3'; TWIST forward primer 5'-GGAGTCCGCAGTCTTACGAG-3', TWIST reverse primer 5'-TCTGGAGGACCTGGTAGAGG-3'.

Immunohistochemistry

Tissues were fixed in formalin for 3 days and embedded in paraffin wax. Paraffin-embedded sections (5 μm) were deparaffinized in three xylene washes followed by a graded alcohol series, antigen retrieval performed with 10 mM sodium citrate buffer, and then blocked with blocking solution for 1 h at RT. Sections were incubated with primary antibodies (PTEN or pAKT) overnight at 4°C, washed with PBS, incubated with secondary antibodies for 30 min at RT, and developed with DAB reagent. All sections were counterstained with hematoxylin, and then

dehydrated with ethanol and xylene. Coverslips were mounted and slides observed by light microscopy.

Immunoblotting

Whole cell lysates were collected using cell lysis buffer (20 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.1% SDS, 1% sodium deoxycholate, 1% Triton X-100, aprotinin, leupeptin, and 1 mM sodium orthovanadate) supplemented with proteinase inhibitors (Roche). PMSF (1 mM) was added immediately prior to use. Protein (30 µg) was run on a SDS-PAGE gel, transferred onto a PVDF membrane, and probed with antibodies. Blots were developed using an enhanced chemiluminescence system (Amersham Biosciences). Image J (NIH) was used to perform the densitometric analysis of protein expression from immunoblots.

Cell cycle analysis by flow cytometry

Cell cycle distribution was analyzed using flow cytometry. Cells were trypsinized, washed once with PBS, and fixed in 70% ethanol. Fixed cells were washed with PBS, incubated with 100 mg/ml RNAse for 30 min at 37ºC, stained with Propidium Iodide (5 mg/ml) and approximately 1 x 10^6 cells were analyzed on a 5-laser BD LSRII. The percentages of cells in different cell cycle phases were analyzed using FACSDiva version 6.1.3.

Cell viability assay, anchorage-independent growth and clonogenic assay

JF and SK-N-SH cells were seeded at a density of 3 x 10^3 cells in a 96-well plate and grown for up to 4 days after transfection. Cell numbers were assessed using CCK-8 daily. Each assay was performed in triplicate, and the experiment was

repeated three times for each cell line. The values, corresponding to the number of viable cells, were read at OD450 with a FlexStation 3 (Molecular Device). For anchorage-independent growth, BE(2)-C and SH-SY5Y cells were trypsinized and resuspended in media containing 0.4% agarose and 7.5% FBS and then overlaid onto a bottom layer of solidified 0.8% agarose in 5% serum media. Cells were plated at varying concentrations and incubated for 5 weeks. Colonies were stained with 0.05% Crystal Violet, photographed using a Gel Doc^{TM} XR+ system (BioRad) and quantified using a colony counting software Quantity One (4.6.9, BioRad). For clonogenic assay, JF cells were seeded at low concentrations (1 X 10^3) in 6-well plates and allowed to form colonies. Colonies were counted in a manner similar to the anchorage-independent growth assay.

DNA fragmentation ELISA

Cells (100 µl; 5-10 x 10^3 cells/well) were plated in triplicate 24 h before transfection. Cells were then treated with siRNA for control (NTC) or GRP for 48 and 72 h. Cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes) were detected using a Cell Death Detection ELISAplus kit according to manufacturer's recommended protocol. The experiments were repeated on at least three separate occasions.

Migration assay

For transwell migration assay, polycarbonate transwell filters (8 µm; Corning Inc., Corning, NY) were coated on the lower side with 5 µg/ml collagen type I (BD Biosciences) overnight and then blocked with 2.5% BSA in PBS for 1 h. 1 × 10^5 cells

in 500 µl of serum-free media were added to the transwell and allowed to migrate for 4 h at 37 °C under tissue culture conditions. Media with 1% FBS or 100 nM GRP was added to the lower chamber. Cells that failed to migrate through the filter after incubation were scraped out using a sterile cotton swab. Cells that migrated to the bottom surface of the filter were fixed with 4% paraformaldehyde, stained with DAPI, and counted. Each substrate was repeated in duplicate wells, and within each well counting was done in five randomly selected microscopic fields (200X magnification).

In vitro tubule formation assay

HUVECs grown to ~70% confluence were trypsinized, counted, and seeded with various conditioned media at 48 X 10^3 cells per well in 24-well plates coated with 300 µl of Matrigel (BD Biosciences) and treated with cell culture supernatant from transfected BE(2)-C or SH-SY5Y cells. HUVECs were periodically observed by microscope as they differentiated into capillary-like tubule structures. After 6 h, cells were stained with hematoxylin & eosin (H&E) and photographed via microscope. The average number of tubules was calculated from examining three separate microscopic fields (200X) and representative photographs obtained.

Tissue microarray and pathological scoring

For preparation of the neuroblastoma tissue microarray, the surgical pathology specimen database at Vanderbilt Medical Center was searched for neuroblastoma diagnosis from 1992 to 2011 (Vanderbilt IRB protocol #111723). A Beecher Instruments Manual Tissue Arrayer was used to prepare tissue cores from selected regions of archival tissue blocks. Four 1 mm cores were prepared for each

tumor case. In general, tissue biopsies were obtained from the adrenal medulla or paraspinal mass for Stage 1-3 patients without metastasis, and from the lung, lymph node or liver for Stage 4 patients with metastasis. Blinded scoring (0-3) was performed by a pathologist with expertise in neuroblastoma (Dr. Hernan Correa).

In vivo studies

Male athymic nude mice (4–6 weeks old) were maintained as previously described [16]. All studies were approved by the Institutional Animal Care and Use Committee at Vanderbilt University and were conducted in accordance with NIH guidelines. BE(2)-C cells stably transfected with Tet/shCON or Tet/shGRP was used for animal experiments. Mice were anesthetized with isofluorane/oxygen mixture, and a small left flank incision was made to isolate and exteriorize the spleen. A total of 1×10^6 cells in 50 µl of HBSS was injected into the splenic capsule using a 27-gauge needle. Abdominal wall was closed with metal wound clips. Mice were randomized to 3 groups: (1) vector-control group BE(2)-C/Tet/shCON (n=3) was allowed to drink autoclaved water mixed with sucrose (3%) and doxycycline (2 mg/mL), (2) inducible-control group BE(2)-C/Tet/shGRP (n=4) was given sucrose (3%) alone without doxycycline, and (3) inducible-treatment group BE(2)-C/Tet/shGRP (n=4) was given sucrose (3%) and doxycycline (2 mg/mL). Mice were weighed weekly and tumor growth was assessed biweekly. At about 4 weeks mice were sacrificed, spleens and livers were harvested, weighed and fixed in formalin for analyses.

Statistical analysis

Scoring index was expressed as means ± SEM for both *in vitro* and *in vivo* experiments; statistical analyses were performed using Student's t-test for *in vitro* and *in vivo* experiments and Kruskal-Wallis one-way analysis of variance by ranks for comparisons between the treatment groups *in vivo*. For immunohistochemistry, quantification was based on blinded scoring by a pediatric pathologist across serial sections from multiple animals or patient samples. Scores were analyzed by Student's t-test for statistical significance. Data analysis was conducted using GraphPad InStat3 (GraphPad Software). For all experiments, a p value of < 0.05 was considered statistically significant.

CHAPTER III

GASTRIN-RELEASING PEPTIDE AS A NEOADJUVANT IN REFRACTORY NEUROBLASTOMAS

Introduction

Despite advances in multi-modality therapy in neuroblastoma, survival rates for all stages remain a dismal 50%, and therefore, novel therapeutic options are needed to improve patient outcomes. Acquisition of chemo-resistance represents a significant issue concerning the failure to achieve long-term survival in the treatment of neuroblastoma [75]. Failure to respond to conventional chemotherapy may indicate a shift to the malignant phenotype of the disease, and may impose altered molecular regulation involving apoptosis and cell cycle regulation signaling pathways [76]. Hence, novel molecular approaches that upregulate apoptotic pathways in neuroblastoma cells may potentiate the effect of existing anticancer drugs, such as vincristine and etoposide; this would allow for use of lower dosages, thus minimizing serious complications associated with chemotherapeutic agents.

Vincristine is a vinca alkaloid that disrupts microtubule assembly and arrests cells in metaphase, preventing cell replication. It is part of an arsenal of chemotherapeutic agents commonly used to treat solid tumors based on its mechanism of action to induce apoptosis, which in part, is mediated by the inactivation of Raf1/MEK/ERK cascade [77]. Some of the unwarranted side effects of vincristine include neuropathy [78]. Etoposide, an epipodophyllotoxin, interferes with topoisomerase II activity and arrests cell division in the late S-G2 phase of the cell cycle; it induces a caspase-3-dependent apoptosis in neuroblastoma cells [79].

Although etoposide is highly cytotoxic for neuroblastoma, the side-effects as a result of myelosuppression makes it dose-limiting in the treatment of this childhood cancer.

Suppression of GRP activity with cell surface receptor antagonists or neutralizing antibodies has been shown to inhibit tumor growth [16,80]; however, the molecular mechanisms involved in the inhibition of neuroblastoma cell proliferation upon GRP down-regulation are not known. Furthermore, combining conventional chemotherapy and targeted antagonism of GRP-R appears to significantly enhance cancer cell death by a mechanism termed as “receptor enhanced chemosensitivity”. Therefore, the purpose of our current investigation was to demonstrate and elucidate, in broader detail, the mechanism by which GRP inhibition induces neuroblastoma cell death and potentiates the cytotoxic effects of chemotherapeutic drugs in the treatment of aggressive, refractory neuroblastomas.

In this study, we report that silencing GRP induced apoptosis in neuroblastoma cell lines, JF and SK-N-SH, when administered alone or in combination with chemotherapeutic drugs, vincristine or etoposide. Moreover, GRP silencing decreased cell proliferation and induced cell cycle exit of neuroblastoma cells. We also observed, at the molecular level, that p53 and its downstream target p21 are upregulated by GRP knockdown, leading to a decreased activation of cell proliferation regulator, ERK. Moreover, GRP or GRP-R silencing in a neuroblastoma cell line deficient in p53 activity, enhanced the expression of another tumor suppressor, PTEN. Our findings demonstrate that silencing GRP promotes apoptosis in neuroblastoma cells and enhances the cytotoxic effects of chemotherapeutic agents by the potential activation of tumor suppressors.

Results

GRP silencing induced apoptosis in neuroblastoma cells

Using quantitative and semi-quantitative PCR, we first examined the level of *GRP* mRNA in a panel of neuroblastoma cell lines available in our laboratory and reported the differential expression of *GRP* in these cell lines (**Fig. 5A**). To examine the effects of GRP siRNA on *GRP* mRNA expression, we used constitutively GRP-overexpressing (JF and SK-N-SH) human neuroblastoma cell lines. As assessed by quantitative PCR, siGRP resulted in significant *GRP* mRNA reduction of approximately 80-90% in both JF and SK-N-SH cells after 48 h treatment indicating the specificity of siGRP in our study (**Fig. 5B**). We next examined the effect of GRP inhibition on neuroblastoma cell death. Using a clonogenic assay, we demonstrated that GRP silencing decreased colony formation by JF and SK-N-SH cells (**Fig. 5D**). Moreover, a significant increase in apoptosis was detected in both human neuroblastoma cell line, JF and SK-N-SH, at 48 h after GRP silencing, as measured by levels of DNA fragmentation, a hallmark of apoptosis (**Fig. 5E**). Increases in apoptosis were noted to a maximum of 2.5 fold change. Conversely, targeting GRP also notably decreased neuroblastoma cell proliferation, as measured by the Cell Counting Kit-8 (CCK-8) (**Fig. 5F**), thus demonstrating dual cellular effects of GRP silencing on proapoptotic as well as anti-proliferative responses in neuroblastoma cells.

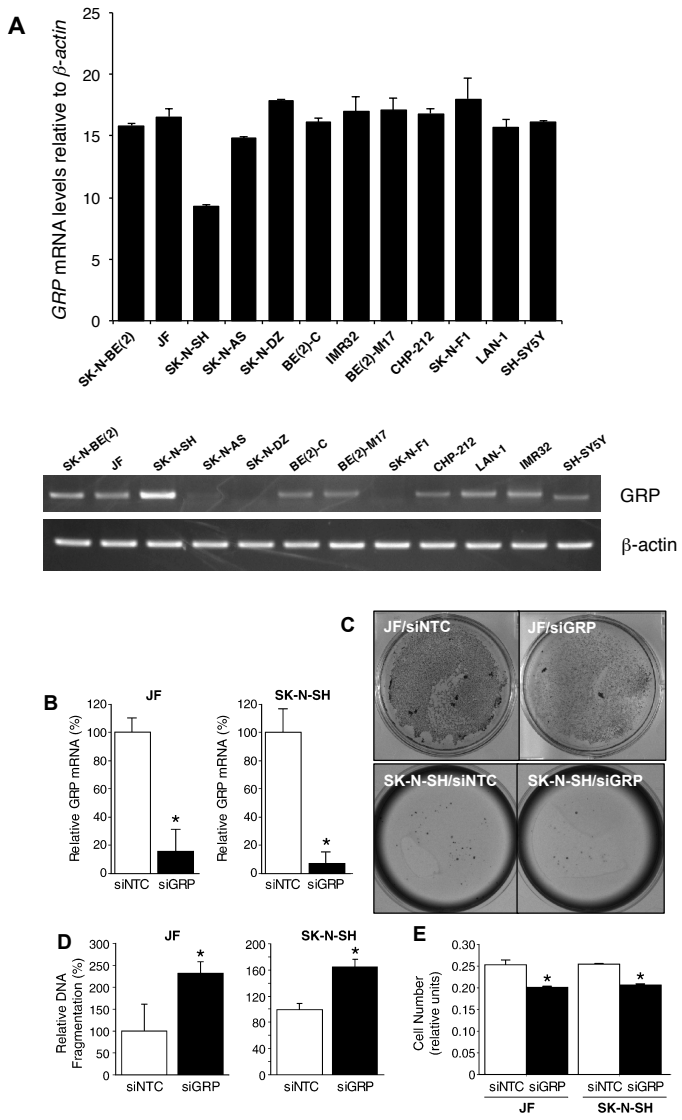

Figure 5. Silencing GRP induced apoptosis in neuroblastoma cells. (A) Differential expression of *GRP* mRNA levels in a panel of 12 neuroblastoma cell lines. **(B)** JF and SK-N-SH cells were transfected with siGRP or siNTC, and gene expression was analyzed using quantitative PCR. GRP expression was effectively silenced with siGRP when compared to controls (siNTC) in both cell lines examined. **(C)** Clonogenic assay using JF cells demonstrated decreased colony formation after GRP silencing. **(D)** Cells treated with siGRP exhibited an increase in apoptosis in comparison to control cells (siNTC). **(E)** GRP silencing (siGRP) resulted in a significant decrease in cell proliferation when compared to control cells. Apoptosis and cell proliferation were analyzed using Cell Death ELISA and CCK-8, respectively (mean ± SEM; * = $p < 0.05$). Relative DNA Fragmentation indicates DNA fragmentation of treated cells relative to control cells, where control cells have been assumed to have 100% DNA fragmentation.

Chemotherapy-induced apoptosis in neuroblastoma cells

Chemotherapeutic drugs exert lethality on tumor cells by induction of apoptosis. So, we determined apoptotic dose-response curves of two commonly used chemotherapeutic agents, vincristine and etoposide. Human neuroblastoma cell lines, JF and SK-N-SH, were treated for 48 h with varying dosages of vincristine and etoposide. Treatment with chemotherapeutic drugs resulted in significant cell death of JF and SK-N-SH cells in a dose-dependent manner. The lowest dosages of vincristine that produced significant apoptosis were determined to be 25 nM and 1 nM for JF and SK-N-SH cells, respectively (**Fig. 6A**); whereas, these values for etoposide were 2 μM for JF cells and 0.1 μM for SK-N-SH cells (**Fig. 6B**).

GRP knockdown enhanced chemotherapy-induced apoptosis

Individually, GRP inhibition and administration of chemotherapeutic drugs appeared to have the same end point – apoptosis of human neuroblastoma cells. Therefore, it is conceivable that GRP silencing could serve as an adjuvant therapy to chemotherapy. Hence, we combined the two approaches and measured the level of apoptosis in human neuroblastoma cell lines. JF and SK-N-SH cells were transfected with siGRP, and then subsequently exposed to the lowest dosages of either vincristine or etoposide that produced apoptosis. A significant augmentation in apoptosis was observed for the combination treatment using siGRP and either vincristine (**Fig. 7A**) or etoposide (**Fig. 7B**) when compared to chemotherapeutic agent alone. These findings suggest an important role for siGRP as an effective adjuvant therapy to be used in combination with lower concentrations of current

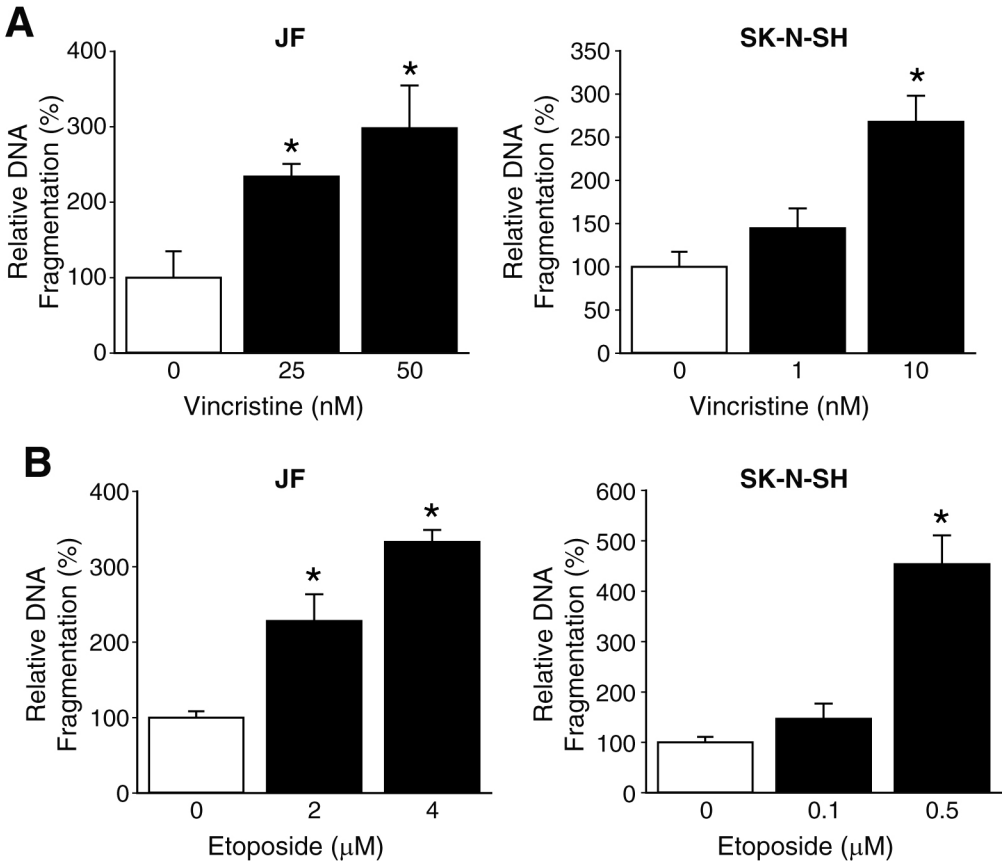

Figure 6. Chemotherapy treatment induced apoptosis in neuroblastoma cells. **(A)** A dose-response curve for the effects of chemotherapeutic agents on apoptosis was assessed using Cell Death ELISA for JF and SK-N-SH cells at 48 h time point. (A) The lowest effective dosage of vincristine was determined as 25 nM for JF cells and 1 nM for SK-N-SH cells. **(B)** The lowest effective dosage of etoposide was determined as 2 μM for JF cells and 0.1 μM for SK-N-SH cells (mean ± SEM; * = $p <$ 0.05).

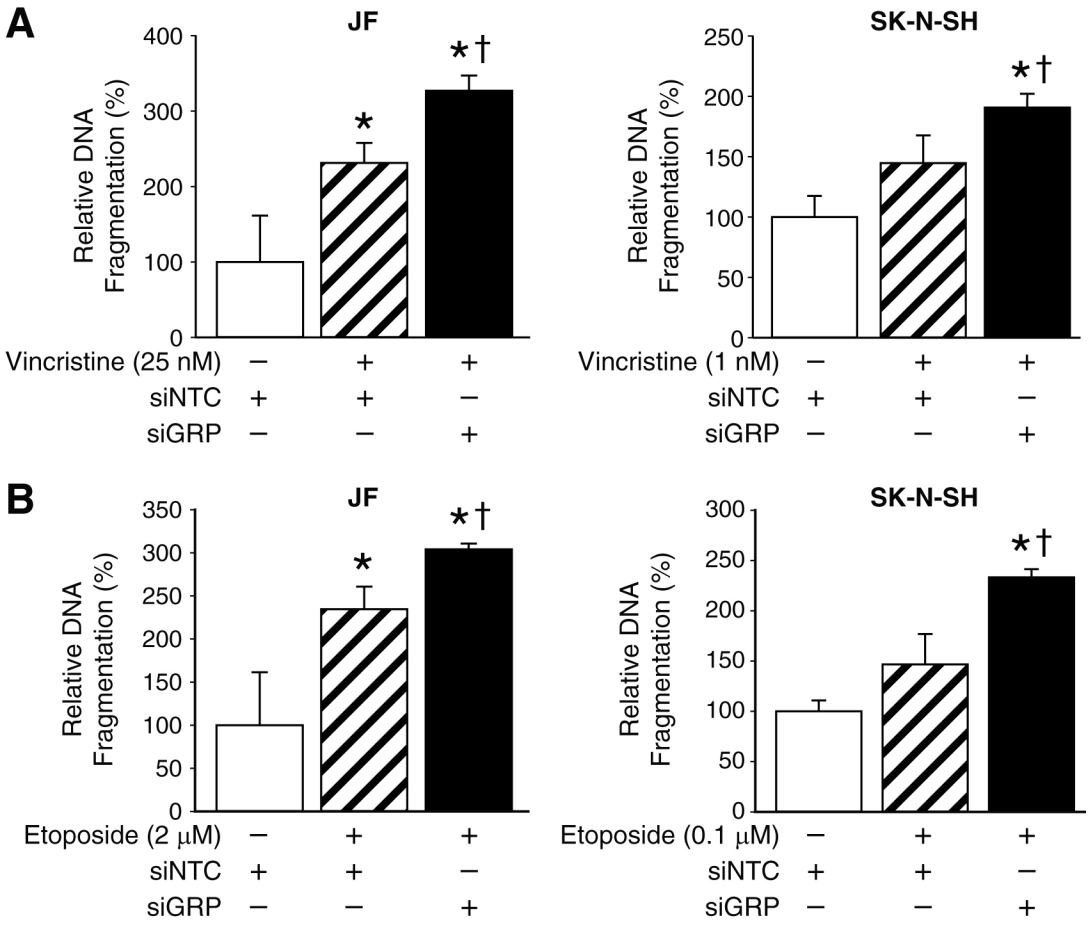

Figure 7. GRP knockdown enhances chemotherapy-induced apoptosis. (A) Combination treatment with vincristine and siGRP resulted in augmentation of apoptosis in JF and SK-N-SH cells when compared to vincristine alone. **(B)** GRP silencing, in addition to treatment with etoposide, resulted in an increase in apoptosis in comparison to drug treatment alone in both JF and SK-N-SH cells (mean ± SEM; * = $p < 0.05$ vs. siNTC alone, †= $p < 0.05$ vs. siNTC plus drug).

chemotherapeutic regimen, thus potentially decreasing the incidence of chemotherapy-associated complications.

Silencing GRP induced PARP and caspase-3 cleavage

To further validate our hypothesis that GRP silencing results in human neuroblastoma cell death via an apoptotic pathway, we measured cleavage of PARP and caspase-3, as markers of apoptosis. SK-N-SH cells were transfected with siGRP, and then treated to varying concentration of either vincristine or etoposide for 48 h. siNTC transfected cells served as controls. Dose-dependent increases in cleaved PARP protein levels were observed with both chemotherapeutic drugs; these effects were further enhanced when combined with GRP silencing (**Fig. 8**). Similarly, siGRP also produced additive effects on caspase-3 activity, but only with higher dosages of vincristine and etoposide.

GRP inhibition induced cell cycle arrest in neuroblastoma cells

The mechanisms of action for the chemotherapeutic drugs, vincristine and etoposide, have been well elucidated. The cytotoxic effects of vincristine are associated with a cell cycle arrest in the G2/M phase and induction of apoptosis in target cells [77]. On the other hand, studies have reported that etoposide induces apoptosis in neuroblastoma cells in caspase-dependent fashion and upregulation of p21 [79,81]. In order to investigate the mechanism by which GRP silencing enhances the capacity of these drugs to induce apoptosis, we next examined cell cycle regulators, namely, p53, p21, and pERK, after siGRP treatment alone. As expected, GRP silencing led to increased expression of p53 and its transcriptional

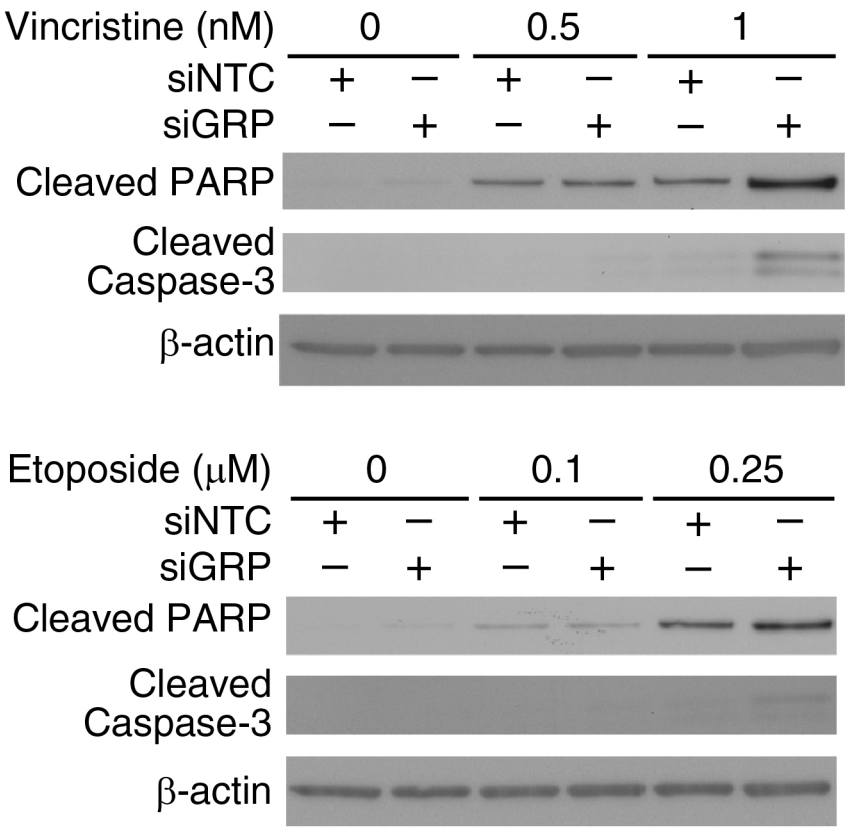

Figure 8. Activation of apoptotic pathway after GRP silencing. Combination treatment of SK-N-SH cells with chemotherapeutic drugs and siGRP resulted in increased cleavage of PARP and caspase-3 suggesting activation of apoptosis as the mechanism of cell death by GRP silencing. β-actin levels indicate equal sample loading.

target p21 at 48 h after treatment in SK-N-SH cells (**Fig. 9A**). A delayed yet significant decrease in the expression of pERK was observed at 72 h after treatment. In order to determine the effects of GRP silencing on cell cycle progression, we also analyzed SK-N-SH cells using flow cytometry after siGRP treatment. An increase in the percentage of cells in apoptotic sub G0/G1 phase was observed when compared to control cells (**Fig. 9B**). This indicates that upon GRP silencing neuroblastoma cells probably undergo cell cycle exit, and subsequent apoptosis.

GRP/GRP-R inhibition enhanced PTEN expression

We have previously shown GRP-R overexpression decreases PTEN levels in SK-N-SH cells [59]. Others have demonstrated that tumor suppressor PTEN and its downstream target p27 cooperate to inhibit cell cycle progression in prostate cancer cells [82,83], potentially by directly interacting in the cytoplasm [84]. PTEN can modulate the expression of p21 and p27, thereby, regulating neuroblastoma cell cycle arrest [85]. As another potential mechanism for cell cycle arrest, we analyzed the levels of PTEN in the cell line, BE(2)-C, that has mutant p53 and, probably, does not utilize p53 signaling to inhibit neuroblastoma cell cycle progression or modulate apoptosis. PTEN expression increased after doxycycline-induced GRP silencing in BE(2)-C cells (**Fig. 10A**). Similarly, doxycycline-induced GRP-R silencing increased PTEN levels in BE(2)-C cells **(Fig. 10B)**. Thus, our data indicate that targeting GRP/GRP-R enhanced the expression of PTEN in neuroblastoma cells, providing a potential p53-independent pathway in inducing neuroblastoma cell death.

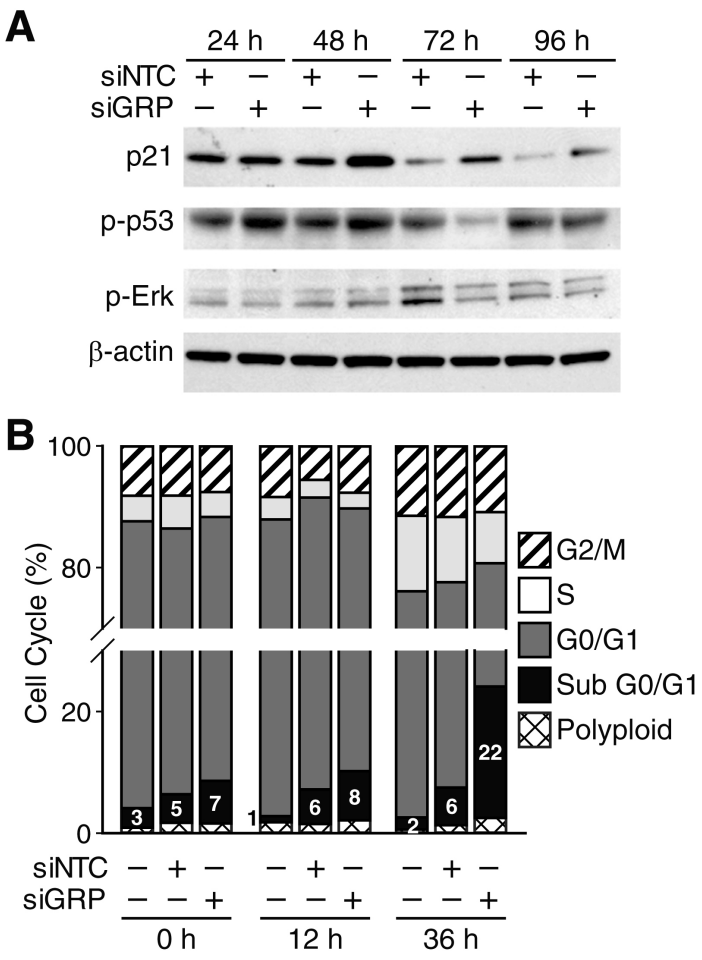

Figure 9. GRP silencing-induced apoptosis is mediated by p53 and G0-G1 cell cycle arrest. (A) Treatment of SK-N-SH cells with siGRP over a time course (24-96 h) resulted in an increase in phosphorylation of p53 and its downstream target p21. A delayed decrease in phospho-ERK was observed at 72 and 96 h time points. **(B)** siGRP or siNTC transfected SK-N-SH cells (1x 10^6 cells/well) were plated and analyzed for cells in different phases of the cell cycle using flow cytometry. The percentage of cells in the apoptotic sub G0/G1 phase showed a significant increase after siGRP treatment. The term sub G0/G1 refers to the neuroblastoma cells gated during cell cycle analysis by flow cytometry in a manner where they represent apoptotic cells as assessed by propidium iodide staining.

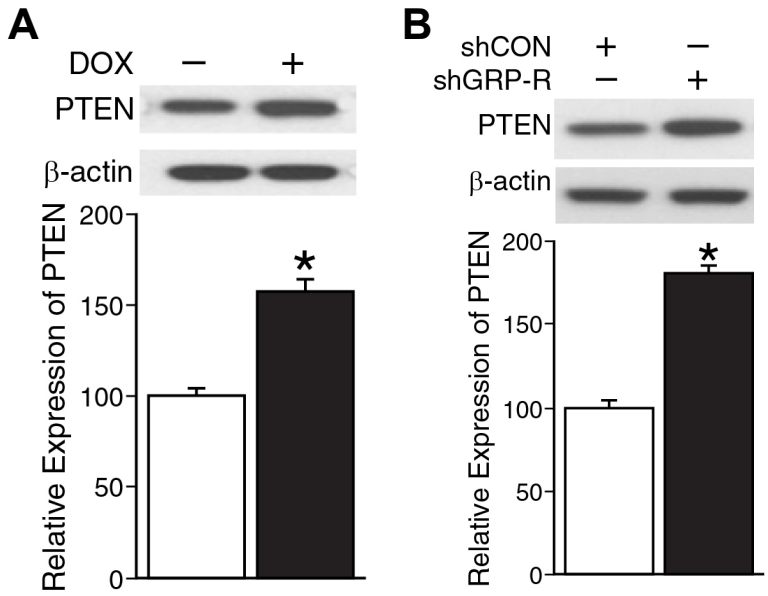

Figure 10. GRP silencing induced PTEN expression. (**A**) PTEN expression was enhanced by doxycycline-induced GRP silenced BE(2)-C cells. **(B)** PTEN expression was enhanced by doxycycline-induced GRP-R silenced BE(2)-C cells (mean ± SEM; *=p<0.05).

Discussion

In this study, we show that specific, selective silencing of GRP by siRNA leads to increased apoptosis in human neuroblastoma cells that constitutively express high levels of GRP; cell death was strongly associated with attenuation of cell proliferation, and cell cycle arrest in the G0-G1 phase. Moreover, GRP knockdown enhanced the cytotoxic effects of chemotherapeutic drugs routinely used in the treatment of neuroblastomas. Increases in caspase-3 activation and p53/PTEN expression after GRP silencing further validated activation of apoptotic pathways. These observations underscore the significance of targeting GRP, and suggest a potential novel combinational treatment for refractory, chemoresistance neuroblastomas.

GRP and its equivalent BBS act as autocrine growth factor to promote cell proliferation in various cancer cell types [28,86]; these mitogenic effects of GRP on tumor cells have been well established. Similarly, we have previously shown that overexpression of GRP-R increases the proliferative capacity of SK-N-SH human neuroblastoma cells [59]. In this study, we found that silencing of GRP in JF and SK-N-SH cell lines induced significant apoptosis. We also showed that cell proliferation and cell cycle progression are intricately related to GRP expression, as silencing of GRP led to inhibition of both of these processes. Our findings are consistent with reports published by others, where inhibition of GRP using GRP/BBS analogs inhibited cell proliferation in a variety of cancer cell types [41,87,88].

Evaluating drug toxicities and mechanism of action becomes extremely important when designing treatment regimens; this is especially critical for patients with advanced-stage neuroblastoma with a potential to develop drug resistance to conventional chemotherapy. Microtubule-damaging agents, such as vincristine,

induce apoptosis in cancer cells via inhibition of ERK/MAPK pathway. On the other hand, topoisomerase II inhibitors like etoposide activate a p53-dependent cell death mechanism in cancer cells [81]. In the current study, we report that both of these chemotherapeutic agents induced apoptosis in human neuroblastoma cell lines with wild-type p53. When the lowest effective dosages of either drug were combined with siGRP treatment, the level of apoptosis was enhanced in both cell lines examined in comparison to either drug alone. Targeting neuroblastoma cells with simultaneous use of sublethal dose of chemotherapeutic drugs and GRP silencing has not been reported previously. Thus, our findings in this study suggest a potential novel regimen to reduce chemotherapy-mediated side effects in neuroblastoma patients, thereby, improving the quality of their lives considerably.

Failure to activate apoptotic pathways in response to drug treatment as a result of development of chemoresistance has been one of the major hurdles in anticancer therapies. Previous studies have reported a correlation between silencing of caspase activation with that of chemoresistance in neuroblastoma patients with unfavorable outcomes [89,90]. Here, we demonstrated the activation of caspase-3 and PARP cleavage when JF and SK-N-SH cell lines were subjected to a combination treatment of chemotherapeutic drugs and GRP silencing, indicating additive effects on activation of apoptotic pathway. Consequently, induction of apoptosis via activation of caspase cascade upon treatment with siGRP would allow for potential bypassing of chemoresistance, and thus provide significant therapeutic benefit to patients with advanced-stage neuroblastomas.

Previous reports suggest that the presence of a wild-type p53 gene in neuroblastomas and the functional competence of this tumor suppressor protein in this form of pediatric cancer is controversial [91]. It has been suggested that

sequestration of p53 in the cytoplasm leads to an attenuated DNA-damage induced G1 arrest in neuroblastomas [92]. Our data demonstrated that silencing GRP leads to a plausible stabilization of p53 protein in neuroblastoma cells as assessed by the levels of phosphorylated p53 (stable form of p53), and thus leading to activation of p21, a transcriptional target of p53. This finding is in agreement with reports by others on reactivation of p53 function in neuroblastomas [91,93]. Moreover, our cell cycle analysis data suggests an enhancement in cell cycle arrest followed by apoptosis in SK-N-SH cells after GRP silencing. Hence, we postulate that GRP inhibition acts in parallel with the chemotherapeutic drugs to enhance G1-arrest in neuroblastoma cells, thereby leading to the induction of apoptosis in these cells.

We have previously demonstrated that GRP-R silencing inactivated the PI3K/AKT pathway and markedly increased PTEN expression [45]. To further examine other tumor suppressors downstream of GRP/GRP-R signaling that can potentially induce neuroblastoma cell death, we examined the expression of PTEN. Cancer cells typically have low nuclear to cytoplasmic ratio of PTEN. In the nucleus, PTEN can mediate cell cycle arrest and growth inhibition via downregulation of MAPK and cyclin D1, while in the cytoplasm, PTEN can downregulate AKT activity and upregulate p27 resulting in caspase-mediated apoptosis [94]. In this study, we further demonstrated that silencing GRP, the specific ligand for GRP-R, resulted in a similar increase in PTEN expression, thus indicating that PTEN is a key negative regulator of GRP/GRP-R signaling in neuroblastoma tumorigenesis. Thus, inhibition of GRP/GRP-R signaling increased PTEN, thereby, potentially inducing cell death in neuroblastoma under conditions of mutant p53.

In summary, chemoresistance in patients with refractory neuroblastomas and toxicities associated with conventional chemotherapeutic drugs necessitates the

need for novel therapeutics in advanced-stage neuroblastomas. Our findings from this study indicate that silencing GRP, an autocrine growth factor for neuroblastoma, induces significant apoptosis, allowing for chemosensitization. This could potentially allow for use of lower, safer doses of conventional chemotherapeutic drugs in multimodality treatment for neuroblastomas.

CHAPTER IV

GASTRIN-RELEASING PEPTIDE IN NEUROBLASTOMA PROGRESSION

Introduction

In the previous chapter we demonstrated the role of targeting GRP in inducing apoptosis-mediated neuroblastoma cell death, potentially through the activation of tumor suppressors like p53 and PTEN. Also, we have previously reported that GRP-stimulated neuroblastoma cell proliferation and cell survival is via AKT activation [15,58]. We also showed that BBS/GRP increases angiogenesis and primary neuroblastoma growth *in vivo* [16] and stimulates neuroblastoma cell migration *in vitro* [95]. While we have demonstrated the importance of GRP in the establishment of neuroblastoma at its primary site, its role in tumor progression and metastasis via regulation of the PTEN/AKT axis remains to be investigated.

Metastasis – the spread of cancer cells from the primary tumor to distant sites - is typically a hallmark of a more aggressive and chemoresistant phenotype for various cancers, and neuroblastoma is no exception. The process of metastasis requires tumor cell proliferation, angiogenesis and invasion into the local lymphatic and capillary network. Further, metastatic cells must detach and embolize into the systemic circulation, extravasate and arrest into distant organs and, finally, “seed” and survive at distant sites [96]. A key aspect in the transition from primary tumor growth to invasion and metastasis is acquisition of anchorage-independence [97]. Hence, tumor cells must acquire resistance to anoikis, a form of apoptosis induced in cells that become detached from the extracellular matrix [98]. The ability to evade anoikis early on sets the stage for cancer progression and eventual metastasis to other organs.

The patients >18 months of age presenting with metastatic neuroblastoma at diagnosis remain difficult to treat and cure [99]. Therefore, understanding the process of dissemination and invasion-metastasis cascade is critical to developing targeted therapeutic strategies that could prevent tumor progression. GRP is known to increase invasiveness of prostate cells through enhanced motility [100]. However, whether GRP is involved in promoting metastasis and by what mechanism this may occur in neuroblastoma has not been answered. Given its crucial function in primary neoplasm growth, we sought to determine the role of GRP in neuroblastoma invasion and metastasis.

In this report, we show that silencing of GRP signaling has a negative effect on the invasion-metastasis cascade in neuroblastoma cells. Our results demonstrate that GRP silencing leads to upregulation of phosphatase and tensin homologue (PTEN), a negative regulator of the PI3K/AKT pathway, with a simultaneous decrease in the expression of phosphorylated AKT (pAKT) and mTOR (pmTOR). We also identified new downstream targets of GRP in neuroblastoma that are known to be responsible for tumor progression. Furthermore, *in vitro* migration of cancer cells and tubule formation by human umbilical vein endothelial cells (HUVECs) demonstrate that PTEN overexpression decreased GRP-mediated motility and angiogenesis in neuroblastoma potentially through decreased activation of FAK and/or AKT. Importantly, using a tissue microarray we observed an inverse correlation between PTEN expression and AKT activation in metastatic lesions from liver or bone marrow when compared to localized disease. Finally, we demonstrate that GRP silencing inhibited liver metastasis in our *in vivo* tumor-metastasis model. Taken together, our findings illustrate the significance of GRP in promoting tumor

progression and make it a promising target in preventing a more aggressive, metastatic neuroblastoma phenotype.

Results

Silencing GRP inhibited neuroblastoma tumorigenicity in vitro

Tumor progression requires local migration and invasion, the ability to evade anoikis-induced cell death while disseminating through lymphatic and hematogenous systems to establish tumors at distant sites. Using a doxycycline-inducible system to silence GRP in human neuroblastoma BE(2)-C and SH-SY5Y cells, we examined the effects of targeting GRP in neuroblastoma progression. Here, we demonstrate that GRP silencing decreased the anchorage-independent growth of neuroblastoma cells, which indicates enhanced anoikis-induced cell death *in vitro*. The number of soft agar colonies after doxycycline treatment-induced GRP silencing was significantly reduced when compared to doxycycline-untreated BE(2)-C/Tet/shGRP cells or doxycycline-treated BE(2)-C/Tet/shCON cells (**Fig. 11A**). Moreover, doxycycline-induced silencing of GRP also significantly decreased transwell migration of BE(2)-C/Tet/shGRP cells when compared to controls (**Fig. 11B**). Consistent with decreases in soft agar colony formation and cell migration, HUVECs grown in cell culture supernatant from doxycycline treated BE(2)-C/Tet/shGRP also demonstrated visibly reduced tubule formation in comparison to untreated BE(2)-C/Tet/shGRP cells and BE(2)-C/Tet/shCON cells (**Fig. 11C**). Similar observations were made with the SH-SY5Y cells transfected with Tet/shCON or Tet/shGRP (**Fig. 11D-F**). GRP silencing was confirmed by semi-quantitative and quantitative PCR (**Fig. 11G and 11H, respectively**). Taken together, these data indicate that targeting GRP affects multiple steps of the invasion-metastasis cascade.

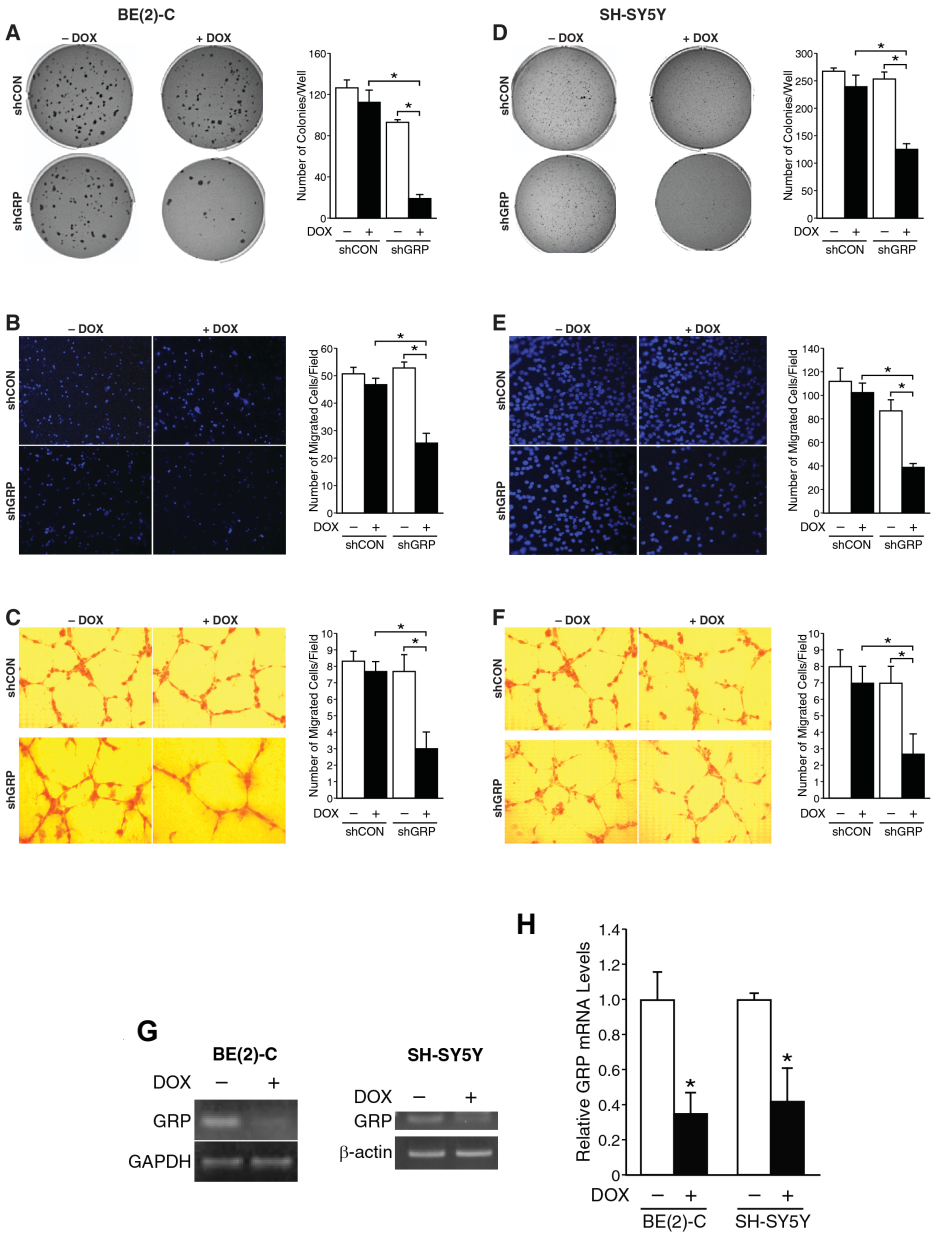

Figure 11. Targeted GRP silencing inhibited neuroblastoma progression. (A, D) BE(2)-C/Tet/shGRP (+DOX) cells and SH-SY5Y/Tet/shGRP (+DOX) cells, respectively, demonstrated a decrease in soft agar colony formation in comparison to Tet/shGRP (-DOX) cells or Tet/shCON (+DOX) cells. **(B, E)** GRP silencing in BE(2)-C/Tet/shGRP (+DOX) and SH-SY5Y/Tet/shGRP (+DOX) cells, respectively, decreased cell migration in a transwell assay in comparison to controls. **(C, F)** HUVECs cultured in cell culture supernatant from GRP silenced cells resulted in decreased tubule formation than when grown in supernatant from control cells (mean ± SEM; *=p<0.05). **(G, H)** Semi-quantitative and quantitative PCR analysis, respectively, confirmed GRP silencing after doxycycline treatment (mean ± SEM; *=p<0.05).

Silencing GRP downregulated AKT/mTOR signaling

Gastrin-releasing peptide receptor (GRP-R) overexpression downregulated PTEN transcription [59] and GRP treatment induces neuroblastoma cell cycle progression via PI3K/AKT [58]. Much is unknown about the downstream signaling pathways and target genes involved in GRP-mediated neuroblastoma progression. Similar to studies of GRP-R silencing [45], silencing of GRP using doxycycline inducible system increased PTEN expression with a concomitant decrease in pAKT expression and its downstream effector, pmTOR (**Fig. 12A**). Interestingly, GRP silencing in BE(2)-C/Tet/shGRP cells suppressed the transcription of critical oncogenes involved in neuroblastoma progression such as *MYCN*, *TWIST* and *FAK* (**Fig. 12B**). Correlative to downregulation at the transcriptional level, GRP silencing also decreased protein levels of MYCN, TWIST and FAK (**Fig. 12C**). SH-SY5Y, a *MYCN*-nonamplified cell line, did not demonstrate any appreciable change in FAK levels, but had lowered TWIST expression (**Fig. 12B and 12C**). Hence, our data indicate that GRP may modulate both the transcription of oncogenes as well as signaling pathways implicated in neuroblastoma progression.

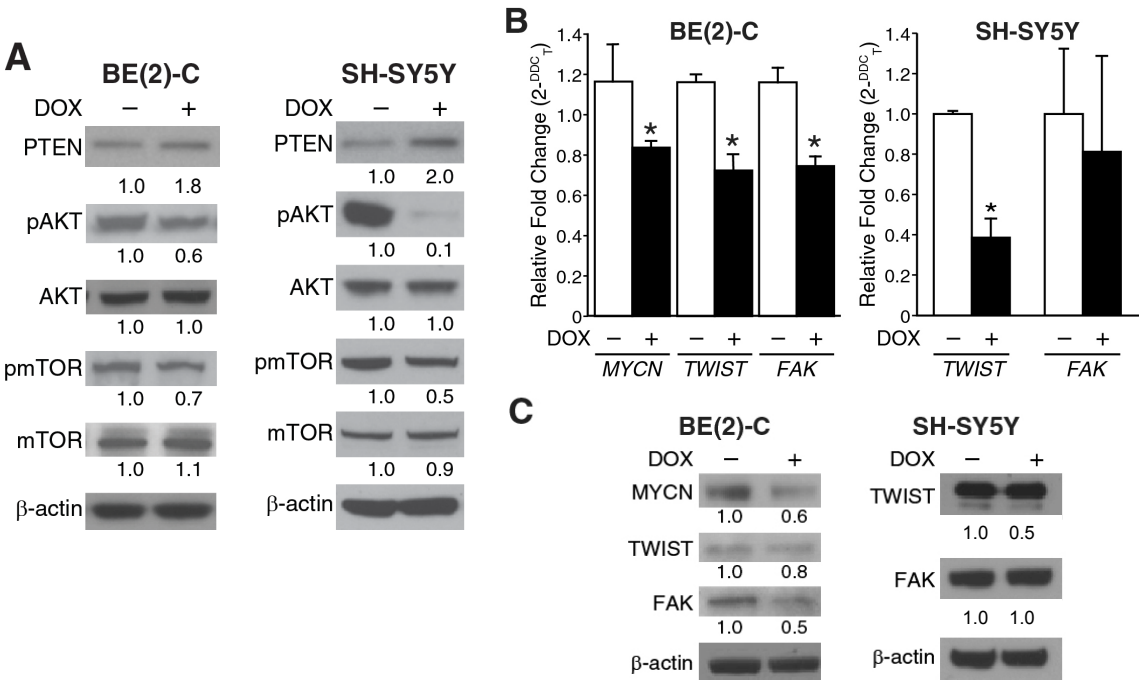

Figure 12. GRP silencing on PTEN/AKT/mTOR signaling. (A) BE(2)-C/Tet/shGRP (+DOX) cells and SH-SY5Y/Tet/shGRP (+DOX) cells had an increase in PTEN expression along with correlative decreases in pAKT and pmTOR expression when compared to control cells (without doxycycline; -DOX). **(B)** GRP silencing decreased the mRNA levels of *MYCN*, *TWIST* and *FAK* by ~50-60% in BE(2)-C/Tet/shGRP cells, but only *TWIST* was significantly decreased in SH-SY5Y/Tet/shGRP cells (mean ± SEM; *=p<0.05 vs. without DOX). **(C)** Immunoblotting confirmed the decreases in the protein levels of MYCN, TWIST and FAK after GRP silencing (+DOX) in comparison to untreated cells (-DOX) in BE(2)-C/Tet/shGRP cells, and TWIST expression in SH-SY5Y/Tet/shGRP cells. β-actin was used as a loading control.

PTEN overexpression decreased GRP-mediated neuroblastoma progression

PTEN negatively regulates cancer cell migration by suppressing the tyrosine phosphorylation of FAK or $p130^{CAS}$ [101]. Therefore, we next examined the role of PTEN overexpression in neuroblastoma cell migration using pBP_2-HA-PTEN overexpression plasmid or the control vector, pBP_2. BE(2)-C/HA-PTEN cell migration was significantly reduced when compared to BE(2)-C/CON cells when subjected to media containing 1% FBS in the lower chamber (**Fig. 13A**). In order to ascertain the role of PTEN in inhibiting GRP-mediated migration, we added media containing 1% FBS and 100 nM GRP in the lower chamber. As expected, under reduced serum conditions GRP treatment increased the migratory capacity of BE(2)-C/CON cells in comparison to BE(2)-C/CON cells without GRP (**Fig. 13A**). Interestingly, the number of migrated BE(2)-C/HA-PTEN cells with GRP treatment was significantly lower than BE(2)-C/CON cells with or without GRP (**Fig. 13A**). Furthermore, PTEN overexpression had a similar inhibitory effect on *in vitro* tubule formation indicating a novel role for PTEN in tumor progression (**Fig. 13B**). PTEN overexpression could completely block GRP-mediated increase in tubule formation by HUVECs (**Fig. 13B**). Similar observations were made with SH-SY5Y cells transfected with control vector or PTEN overexpression vector, and subsequently treated with or without GRP (**Fig. 13D and 13E**). PTEN acts a lipid phosphatase and converts PIP3 to PIP2, thereby, decreasing AKT activation, as assessed by phosphorylation of Ser473. PTEN can also act as a protein phosphatase and dephosphorylate FAK at Y397 directly. Immunoblotting demonstrated that PTEN overexpression decreased pAKT (Ser473) and pFAK (Y397) expression in BE(2)-C and SH-SY5Y cells (**Fig. 13C and 13F, respectively**). This set of novel observations indicated that PTEN overexpression could potentially inhibit GRP-induced neuroblastoma progression.

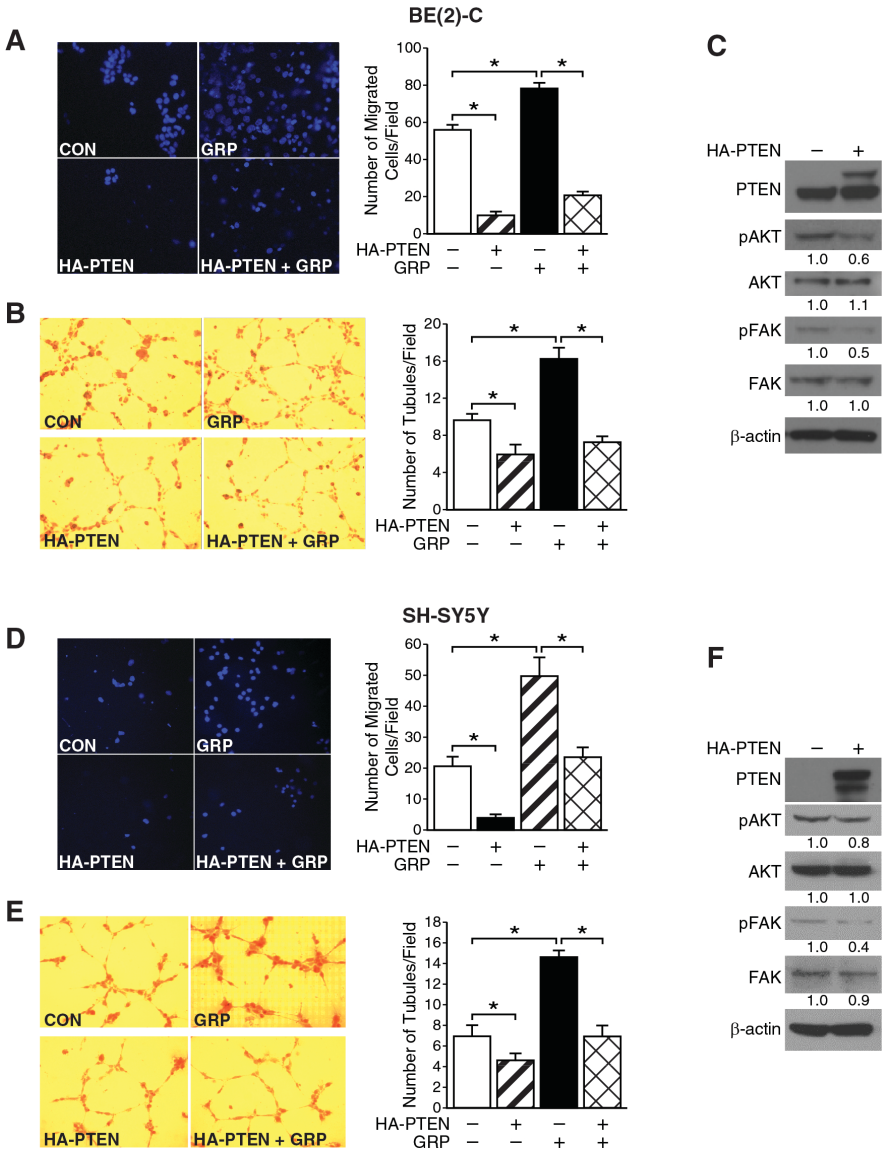

Figure 13. PTEN overexpression inhibited GRP-mediated neuroblastoma progression. (A, D) PTEN overexpression (HA-PTEN) decreased BE(2)-C and SH-SY5Y cell migration in comparison to vector control (CON) as assessed by transwell migration assay. GRP treatment increased BE(2)-C and SH-SY5Y cell migration; this was attenuated by PTEN overexpression (HA-PTEN+GRP). **(B, E)** *In vitro* tubule formation by HUVECs grown in the supernatant of PTEN overexpressing BE(2)-C or SH-SY5Y cells (HA-PTEN) was markedly reduced in number in comparison vector control (CON). GRP-mediated (GRP) increase in HUVEC tubule formation was inhibited when grown in supernatant from PTEN overexpressing BE(2)-C or SH-SY5Y cells (HA-PTEN+GRP). **(C, F)** PTEN overexpression in BE(2)-C and SH-SY5Y cells decreased pAKT and pFAK as assessed by immunoblotting. β-actin was used as a loading control (mean ± SEM; *=p<0.05).

PTEN and pAKT expression in human neuroblastoma sections

Activation of AKT has been correlated with poor prognosis in neuroblastoma patients and indicates disease progression [60]. We have previously identified an inverse correlation between PTEN expression and AKT activation with respect to differentiation in human neuroblastoma samples [59]. To further delve into how PTEN correlates with activation of AKT during neuroblastoma progression, we used an *in vivo* metastasis model established in our laboratory [45] for our study. Human neuroblastoma BE(2)-C cells were injected intrasplenically into mice and liver metastasis occurred in ~4 weeks. Primary tumors from the spleen as well as liver metastases were harvested, fixed and immunohistochemistry was performed in paraffin-embedded sections. Primary splenic tumors showed a comparatively higher expression of PTEN than the secondary liver lesions (**Fig. 14A, *left panels***). Interestingly, there was an increased expression of pAKT in the secondary lesions in the liver in comparison to the primary spleen tumor (**Fig. 14A*, right panels***).

To further confirm this inverse correlation of PTEN and pAKT expression in liver metastases from mice study, we next assessed the expression of PTEN and pAKT by immunohistochemistry using a tissue microarray containing human neuroblastoma sections from primary tumor or metastatic lesions at distant organs (**Fig. 14B**). Neuroblastoma sections obtained from 13 patients at the time of biopsy and/or resection with or without metastasis were chosen for further analyses. Immunohistochemistry analysis of patients with respect to multiple parameters and expression of PTEN/pAKT is summarized in **Table 1**. We found that there was an inverse correlation of PTEN and pAKT in more advanced-stage disease (i.e., stages 3 or 4), with pAKT expression being relatively higher. Specifically, two thirds of patients (4/6) who had higher PTEN expression were also characterized as having

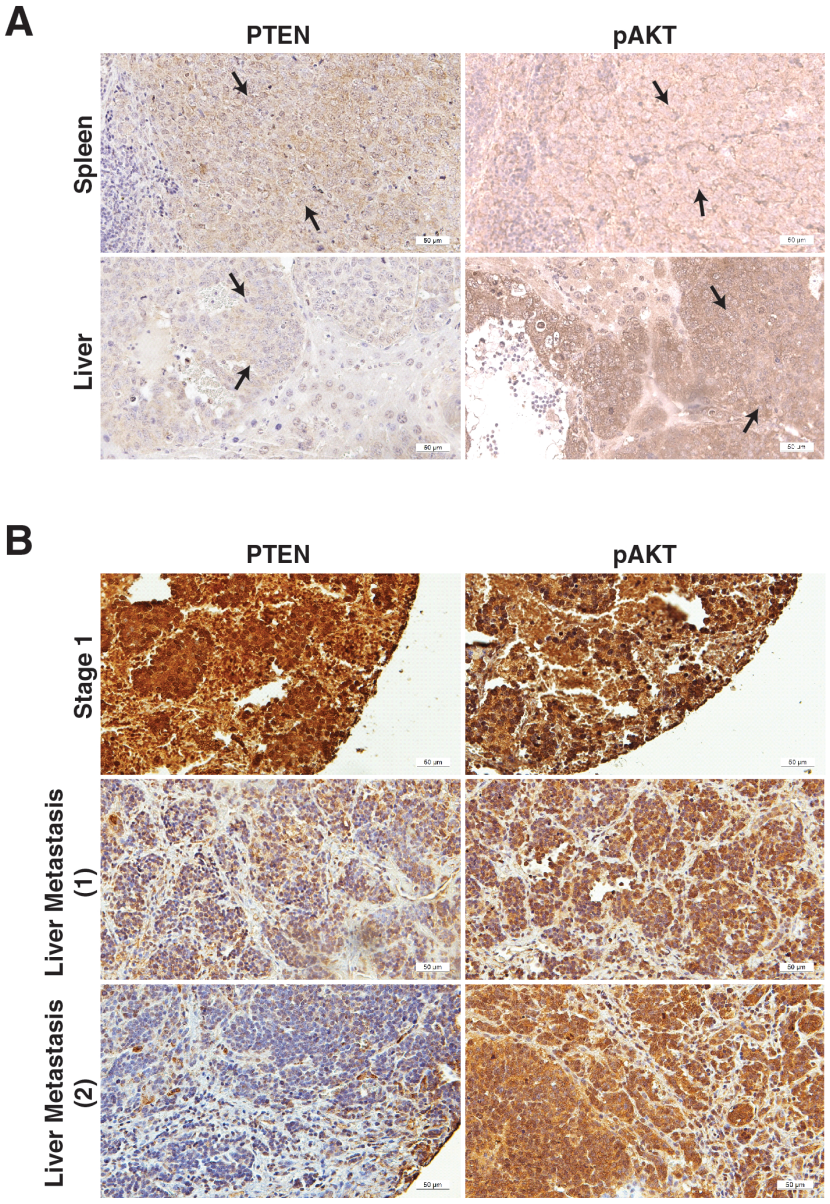

Figure 14. Inverse pattern of PTEN and pAKT expression in metastatic lesions. **(A)** Immunohistochemistry demonstrated increased pAKT expression in liver lesions (*brown staining; bottom right*) from our *in vivo* spleen-liver metastasis model in comparison to primary splenic tumors, whereas, PTEN expression was slightly decreased in metastatic liver foci when compared to primary splenic tumors (*brown staining; top left*) (black arrows indicate tumors). **(B)** PTEN expression was lower in liver metastatic sections from human neuroblastoma samples in comparison to sections from primary localized tumors. pAKT expression was comparatively higher in patients with liver metastasis.

Stage at diagnosis	*MYCN* status	Risk	Relapse	Metastasis	Status	PTEN	pAKT
4	No		Refractory	Lung	Deceased	+++	+
4S	No	Low	No		Alive	+++	+
4	No			Lymph node	Alive	+++	++
4S	Yes		Yes	Liver	Deceased	+	++
4	Yes	High	No	Liver	Alive	+	+++
4	Yes	High	Yes	Liver	Deceased	+	+++
3	No	Intermediate	No		Alive	+	++
1	No	Low	No		Alive	+++	+
4	No		No	Bone marrow	Deceased	+	+++
4	Yes	High	Refractory	Bone marrow	Deceased	-	++
3	Yes	High	Yes		Deceased	+	++
1	No	Low	No		Alive	+++	++
1	No	Low	Yes		Alive	++	+++

Table 1. PTEN and pAKT expression patterns in human neuroblastoma sections.

early-stage disease, suggesting that expression of this gene may be a positive prognostic indicator. Interestingly, the other two patients with high PTEN expression were both characterized as having stage 4 diseases with metastases to the lung and lymph nodes. The patients with stage 1 disease, as identified by the INSS, had similar expression pattern of PTEN and pAKT (**Fig. 14B; *top row***). Similar to our *in vivo* murine metastasis model, expression of pAKT was markedly higher in stage 4 patients with metastasis to the liver compared to PTEN expression (**Fig. 14B, *middle and bottom rows***). Taken together, PTEN and pAKT appear to be inversely correlated during neuroblastoma progression.

Silencing GRP inhibited liver metastasis

We next wanted to determine the effects of silencing GRP on neuroblastoma tumor growth and metastasis using our murine metastasis model. Mice intrasplenically injected with BE(2)-C/Tet/shCON (vector-control group) received water containing doxycycline and sucrose. Mice intrasplenically injected with BE(2)-C/Tet/shGRP were further randomized into two groups: (A) Inducible-treatment group receiving doxycycline and sucrose in drinking water, and (B) the inducible-control group receiving sucrose alone. Silencing GRP did not significantly reduce growth of primary tumors in murine spleen in comparison to mice in control groups (**Fig. 15A**). Interestingly, large liver lesions were observed in mice from the vector control group receiving doxycycline and inducible-control group without doxycycline compared to inducible-treatment group receiving doxycycline, indicating that silencing GRP inhibits establishment of macrometastases in the liver (**Fig. 15A**). Statistical analyses indicated a significant decrease in the tumor burden in mice injected with BE(2)-C/Tet/shGRP and receiving doxycycline in drinking water in

comparison to the controls (**Fig. 15B**). These data demonstrate the critical role of GRP in metastasis to secondary sites and a potential use of targeting GRP in treating aggressive, advanced-stage neuroblastomas.

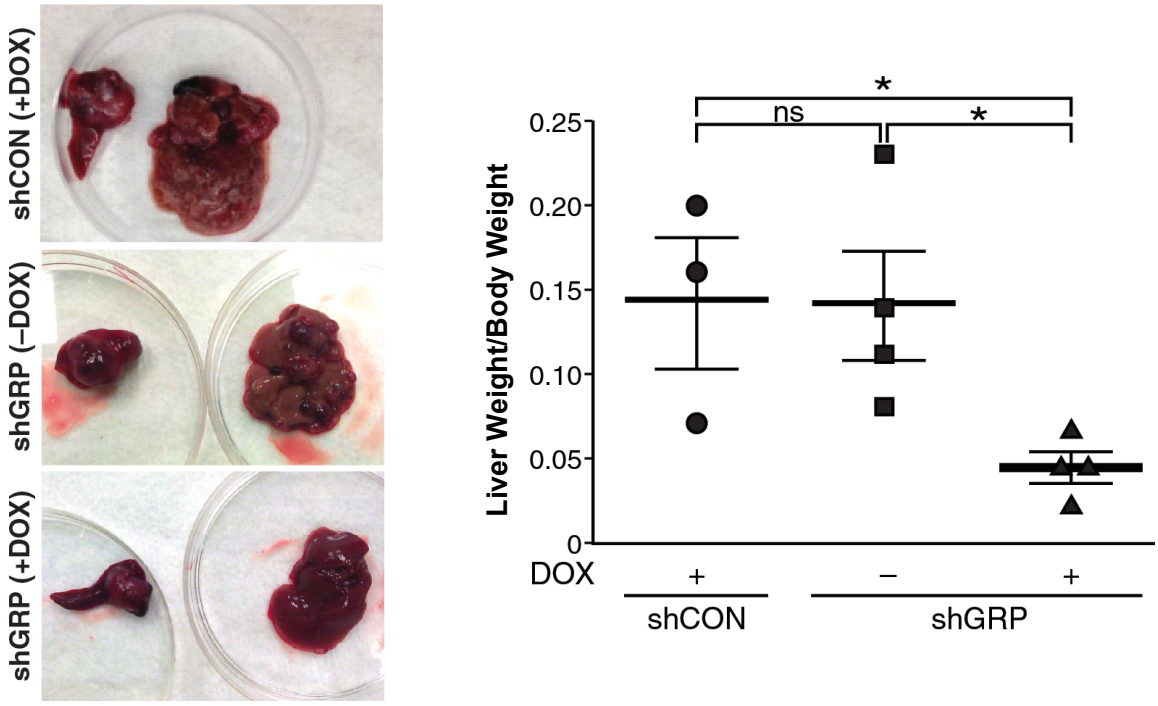

Figure 15. Targeted silencing of GRP inhibited liver metastasis. (A) Large gross tumors were observed in the spleen and liver of mice injected with BE(2)-C/Tet/shCON and subjected to drinking water with doxycycline (2mg/mL) and 3% sucrose [shCON (+DOX)] or injected with BE(2)-C/Tet/shGRP and subjected to drinking water with 3% sucrose alone [shGRP (-DOX)], whereas, a near complete inhibition of hepatic metastatic lesions was observed in mice subjected to drinking water with doxycycline (2 mg/mL) and 3% sucrose [shGRP (+DOX)]. **(B)** Liver weights of mice injected with BE(2)-C/Tet/shGRP and receiving doxycycline in drinking water significantly decreased in comparison to control groups (mean ± SEM; *=p<0.05).

Discussion

The presence of metastatic disease is a harbinger of poor clinical outcome and, unfortunately, decreased survival. Because of this fact, the importance of determining the mechanisms by which cancer cells undergo hematogenous and/or lymphatic dissemination and become metastatic cannot be understated. In the present study, we identify that key oncogenic properties, such as anchorage-independence, migration and angiogenesis, required for tumor invasion and metastasis, were all negatively affected by GRP silencing.

Due to our prior knowledge that *PTEN* mRNA and protein expression is negatively impacted by GRP-R overexpression [59] and that GRP treatment stimulates the PI3K/AKT pathway in neuroblastoma cells [58], we sought to determine whether GRP-mediated signaling regulates PTEN expression. Inhibition of GRP led to increased expression of PTEN. Furthermore, targeted inhibition of GRP suppressed the activation of the AKT/mTOR signaling cascade and transcription of critical oncogenes involved in neuroblastoma progression, specifically TWIST in both BE(2)-C and SH-SY5Y cell lines. Interestingly, in conjunction with *MYCN* amplification, the transcription factor TWIST has been shown to prevent the apoptotic response by inhibiting the ARF-p53 pathway in neuroblastoma [102]. Transcriptional regulation of oncogenes by GRP highlights its role in promoting metastasis.

A diverse group of “molecular sensors”, including cell adhesion molecules, integrins and ligands act in concert with one another to regulate anoikis [103]. These cellular molecules initiate signaling cascades that maintain a pro-apoptotic balance when cellular detachment occurs. Consequently, molecular targets, which can suppress aberrant cell signaling pathways that promote resistance to anoikis, have

become the focus of many investigations. Interestingly, PTEN plays a critical role in regulating anoikis [104] and overexpression of this gene inhibits cell migration and invasion in many different cell lines [101,105]. In concert with its inhibitory role in migration and invasion, restoration of the cellular function of PTEN has been shown to induce anoikis in glioma cell lines via suppression of AKT phosphorylation [106]. Independent of the PI3K/AKT pathway, phosphatase activity of PTEN has also been shown to act on FAK, by dephosphorylating this kinase at tyrosine 397 position (Y397) [101,105], which also acts as an autophosphorylation site for FAK, and initiate anoikis [107]. FAK, a nonreceptor protein kinase, has a significant role in many cellular pathways including cellular adhesion and migration [108], especially in neuroblastoma [54]. In a similar fashion, we demonstrated that PTEN overexpression reduced neuroblastoma cell migration and tumor-mediated angiogenesis in BE(2)-C and SH-SY5Y cell lines with concomitant suppression of pAKT and pFAK protein expression. Our findings suggest that PTEN has a crucial role in neuroblastoma, specifically directed at inhibiting cellular processes that promote resistance to anoikis and a pro-metastatic phenotype. Most importantly, PTEN overexpression blocked GRP-mediated tumor progression as assessed by *in vitro* functional assays, thereby, demonstrating the critical role of PTEN in reversing the oncogenic roles of GRP in neuroblastoma.

Our results demonstrated that silencing GRP has a negative effect on the development of characteristics necessary for invasion and metastasis. Previous studies ascertaining the efficacy of GRP antagonists in cancer have focused on its mitogenic property with the aid of subcutaneous xenograft models [16,54]. To our best knowledge, this is the first report of targeted inhibition of GRP with respect to metastatic disease *in vivo.* Using our above mentioned metastasis model, we were

able to demonstrate that targeting GRP inhibited tumor metastasis. This inhibition can also be attributed to delayed cell proliferation after GRP silencing, though only a marginal reduction in the proliferative capacity of neuroblastoma cells was observed after GRP silencing [109]. The result of these *in vivo* experiments illustrates the inhibition of several key features in the invasion-metastasis cascade. Taken together, our results are significant because it identifies a rationale for targeted therapy against GRP to modulate signaling pathways that contribute to neuroblastoma metastasis.

Focused efforts are needed to improve the clinical outcomes of children with advanced-stage, aggressive neuroblastomas and create specific therapeutic treatments that block molecular pathways contributing to resistant and metastatic disease. In this study we have identified that GRP silencing can negatively impact neuroblastoma progression in several ways. Functionally, it appears to inhibit critical steps that are required for metastasis including, anchorage-independence, migration, and angiogenesis. Mechanistically, GRP silencing resulted in upregulation of tumor suppressor PTEN with subsequent downregulation of critical oncogenes and proliferation/survival pathways implicated in neuroblastoma progression. Combination therapies using cytotoxic chemotherapeutic agents and GRP antagonists to targeting metastatic disease would be of significance in treating aggressive neuroblastoma in the future.

CHAPTER V

CONCLUSION

Summary

The National Cancer Institute has a number of active clinical trials for neuroblastoma patients, but only one such trial has progressed to Phase III, underlining the importance of investigating novel therapeutic strategies for refractory and/or metastatic neuroblastomas. Moreover, the overall survival for neuroblastoma remains dismal, in part due to the emergence of resistance to chemotherapeutic drugs resulting in aggressive, refractory disease. One of the hallmarks of neuroblastoma is increased cell proliferation; this is partly attributed to the synthesis and response to various growth factors and cytokines. As a neuroendocrine tumor, neuroblastomas secrete a number of peptides; one such being the gastrin-releasing peptide (GRP) [15].

We have previously demonstrated that GRP, a neuro- and gut peptide secreted by neuroblastoma, acts as an autocrine growth factor and stimulates neuroblastoma cell proliferation [15]. GRP antagonists have been used to inhibit the proliferation of glioblastoma, pancreatic and breast cancer cells. But whether there is a concomitant induction of cell death upon GRP inhibition and the plausible mechanism(s) involved required further investigation. Hence, for the first part of this project, my goal was to study whether GRP silencing could induce apoptosis in neuroblastoma cells and potentiate the cytotoxic effects of chemotherapeutic agents. We observed that GRP silencing induced apoptosis in neuroblastoma cells and, in combination, allowed the usage of sublethal doses of chemotherapeutic drugs to elicit responses similar to lethal doses of the same chemotherapeutic drugs when

used alone. Increase in the proteins levels of activated pro-apoptotic proteins, cleaved caspase 3 and cleaved PARP, further corroborated the efficacy of combination therapy in inducing apoptosis-mediated neuroblastoma cell death. Moreover, targeting GRP enhanced the percentage of neuroblastoma cells arrested in the subG0/G1 phase of the cell cycle, indicating an increase in apoptosis-mediated neuroblastoma cell death. These observations have two implications – 1) a potential reduction in the dose of commonly used chemotherapeutic agents in neuroblastoma and 2) a plausible diminution in the side effects associated with exposure to such drugs.

After examining the significance of targeting GRP in neuroblastoma cell death, my next goal was to establish the effects of targeting GRP in neuroblastoma metastasis. This study included understanding the significance of the PTEN/AKT signaling axis that GRP potentially utilizes in the invasion-metastasis cascade. Using a doxycycline inducible system, we demonstrated that silencing GRP suppressed anchorage-independent growth, migration and neuroblastoma cell-mediated angiogenesis *in vitro* and liver metastasis *in vivo*. GRP silencing activated PTEN signaling with a simultaneous inhibition of AKT/mTOR/FAK activation in neuroblastoma cells. Similarly, PTEN overexpression inhibited GRP-mediated neuroblastoma cell migration and tumor cell-mediated angiogenesis *in vitro*, and downregulated FAK and AKT activation at the molecular level. This placed PTEN as a critical negative regulator of the oncogenic effects of GRP in neuroblastoma. Using an animal *in vivo* metastasis model and neuroblastoma patient samples, we demonstrated an inverse correlation between the expression of pAKT and PTEN in metastatic lesions in the liver, thereby, implicating a role for GRP/AKT in neuroblastoma progression. These two studies provide a rationale for the use of

GRP antagonists in conjunction with conventional chemotherapeutic regimes in neuroblastoma patients with aggressive, refractory forms of this disease by mechanisms depicted in **Fig. 16**.

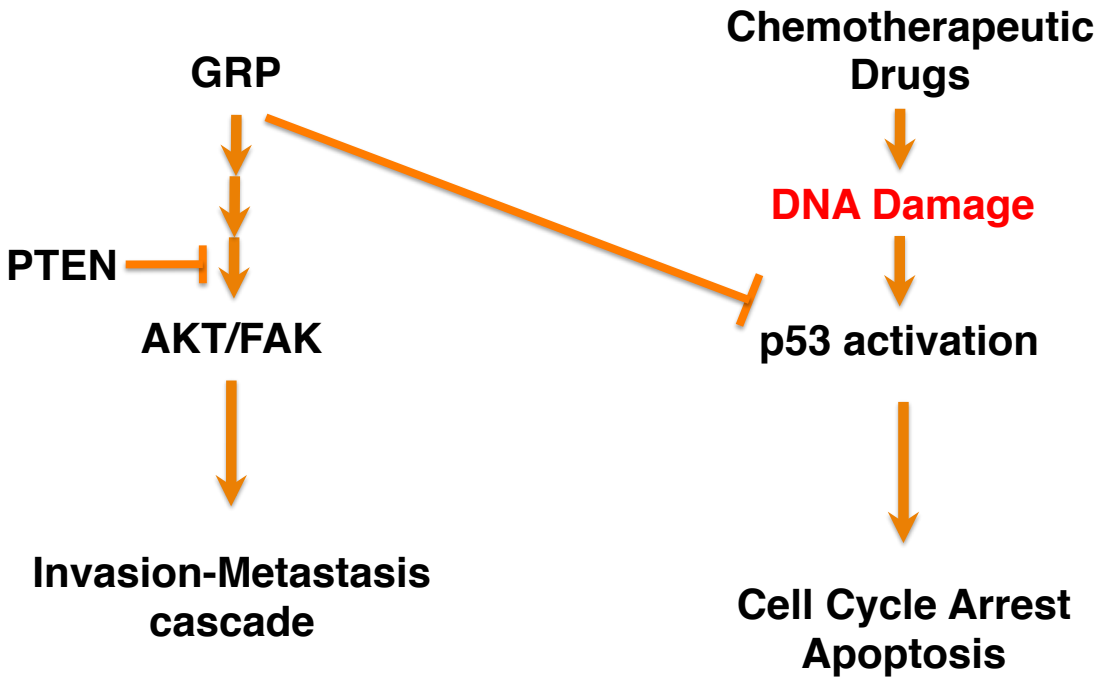

Figure 16. Schematic representation of the oncogenic effects of GRP in neuroblastoma. Combination treatment of GRP silencing and chemotherapeutic drugs can induce DNA damage in neuroblastoma cells, resulting in the activation of tumor suppressors, like p53, with subsequent neuroblastoma cell cycle arrest and apoptosis. GRP also regulates multiple aspects of the invasion-metastasis cascade in neuroblastoma, potentially, via activation of AKT/FAK and PTEN negatively regulates this oncogenic function of GRP in neuroblastoma progression.

Future Directions

Low- and intermediate-risk group neuroblastoma patients usually have a favorable outcome and >80% event-free survival rate [4]. It is the high-risk group, which requires our immediate attention with regards to improved novel targeted therapeutics. From my studies, a critical role for GRP in neuroblastoma initiation and progression can be predicted. Preliminary results from these studies merit further discussions and raise additional exciting questions.

Combination therapy using GRP antagonists and chemotherapy *in vivo*

A GRP specific antagonist reduced the tumor burden in an animal model of SCLC; but this effect was reversible and tumors reappeared when the treatment was stopped [34]. This study identified that blocking GRP might be cytostatic and not necessarily cytotoxic, arguing for the use of cytotoxic agents in combination with GRP antagonists. Keeping that in mind, I demonstrated that a combination of GRP inhibition and commonly used chemotherapeutic drugs enhances apoptosis-mediated neuroblastoma cell death in comparison to drugs alone [109]. It would be of significant importance to simulate the *in vitro* studies in an *in vivo* setting. Since, siRNA-mediated gene silencing is not yet suitable for *in vivo* studies, I will be using a specific GRP antagonist, RC-3940-II, which has been successfully used in preclinical studies involving benign prostatic hyperplasia and hepatic cancer [47,110]. Also RC-3940-II had a synergistic effect on colon cancer inhibition when used in combination with cytotoxic drugs [111]. This study further provides a rationale for the use of combination therapy involving RC-3940-II and vincristine/etoposide for preclinical studies in neuroblastoma. Successful completion of *in vitro* studies will facilitate

animal studies using this combination therapy using dosage of RC-3940-II as suggested by previous studies [47,111].

GRP antagonists and chemotherapy against acquired resistance

A number of neuroblastoma patients, who initially respond quite well to current therapeutic regimes, acquire resistance to conventional treatments – hypothesized about 15 years back as potentially due to expansion of a sub-population of neuroblastoma cells with alterations that confer drug resistance [112]. Today, the presence of stem cells as "alterations" in tumors has been examined extensively. Our laboratory has isolated clones of neuroblastoma cells that demonstrated resistance to high doses of chemo- or radiation therapy. These clones demonstrated increased neurosphere formation under stem cell-promoting conditions. I will use such refractory clones to mimic the phenomenon of acquired drug/radiation resistance and aid in *in vivo* examination of the efficacy of combination therapy using GRP antagonist and metronomic chemotherapy. Completion of such studies will place us one step towards initiating clinical trials for neuroblastoma patients with disease relapse due to acquired resistance.

GRP/GRP-R in neuroblastoma cell extravasation and formation of metastatic lesions

Investigations in the area of tumor metastasis should not be limited to understanding the tumor alone, but also the tumor microenvironment. In the second part of my studies, GRP inhibition suppressed formation of secondary lesions in the liver without significantly altering primary tumor burden in the spleen [113]. Tumor cell dissemination and the final establishment of secondary lesions in distant organs

involves multiple aspects including survival during circulation, extravasation at secondary sites and establishment of colonies amidst a new microenvironment [114].

Targeting GRP significantly, but not completely, inhibited anchorage-independent growth of neuroblastoma cells [113] – an assay that was used to determine the ability of GRP silenced cells to evade anoikis-mediated cell death and their capacity to form micro- or macrometastasis in the liver. But, this assay does not take into account the ability of GRP silenced cells to extravasate through the endothelial cells in the liver and the role of the hepatic microenvironment in aiding the formation of secondary tumors. Extending this to our *in vivo* observations, I hypothesize that extravasation of GRP silenced cells into the liver would behave as a rate-limiting step. Moreover, whether targeting GRP reduces the expression of molecules required for interacting with the hepatic microenvironment, thereby, preventing formation of metastatic lesions need to be investigated. Interestingly, we have recently demonstrated that silencing GRP-R upregulated expression of miRNAs that can play critical roles in inhibiting neuroblastoma progression [115]. Moreover, targeting GRP-R, or its downstream effector AKT2 [116], downregulated the mRNA expression of integrins, angiogenic factors and matrix metalloproteinases – key groups of molecules regulating extravasation of cancer cells and formation of tumor colonies in a new microenvironment. Using *in vitro* co-culture assays, I will test the ability of GRP silenced cells to attach, degrade extracellular matrix, migrate through and survive in the presence of hepatocytes or conditioned medium from hepatocytes.

Cancer stem cells are becoming increasingly important in the successful establishment of metastatic lesions. Hence, the role of metastasis-initiating cancer stem cells (MICs) is becoming a focal point of studies examining tumor metastasis

[117]. Identifying a role for GRP in MICs would be of great relevance to my study, as it would provide a potential explanation for reduced liver metastasis upon GRP silencing in neuroblastoma cells. Interestingly, GRP-R overexpression increased expression of stem cell marker, CD44, conversely, GRP-R silencing decreased the potential the neuroblastoma cells to form neurospheres under stem cell-promoting conditions. Using *in vitro* assays like neurosphere formation assay, ALDH fluorescence, limiting dilution assay, I will study the capacity of GRP/GRP-R in maintaining self-renewal of neuroblastoma cells and identify potential cancer stem cell markers through which GRP/GRP-R maintains stem cell-like properties of neuroblastoma cells.

GRP/GRP-R-mediated regulation of AKT2 via TWIST in neuroblastoma metastasis

In the second part of my studies, I demonstrated that GRP inhibition suppressed TWIST expression in both *MYCN*-amplified and nonamplified cell lines. We have also observed that GRP treatment enhanced TWIST luciferase activity in neuroblastoma cells. TWIST is a transcription factor involved in the transcription of *AKT2* in breast cancer cells [118]. Interestingly, silencing GRP-R downregulated AKT2 expression in neuroblastoma cells [116]. Hence, a potential role for TWIST as a mediator of GRP/GRP-R-mediated regulation of AKT2 needs to be examined. Using GRP stimulation and modulation of TWIST/AKT2 expression, I will perform luciferase-based studies to determine the GRP-dependent regulation of AKT2 by TWIST in neuroblastoma cells. Moreover, using *in vitro* functional assays I will determine the specific hallmarks of cancer regulated by GRP/AKT2 and critically dependent on TWIST. A critical role for TWIST in epithelial-to-mesenchymal

transition (EMT) and metastasis has been established in epithelial cancers [119]. Being a neuroendocrine tumor, neuroblastoma does not undergo this transition, but patients with advanced-stage neuroblastomas usually have metastasis to the bone, bone marrow and liver. Hence, it will be pertinent to identify the exact role of TWIST in neuroblastoma progression and precisely how GRP/TWIST induces neuroblastoma metastasis without EMT.

PTEN/AKT signaling axis in early- versus advanced-stage neuroblastoma

Subcutaneous and orthotopic models are confined to studying primary tumor growth. Using an *in vivo* model established in our laboratory to study liver metastasis, I have reported the significance of GRP in aiding the formation of metastatic lesions in the liver. Also, I have identified a differential expression of AKT/PTEN axis in primary neuroblastoma tumor and secondary liver lesions. But currently there is no animal model that mimics aggressiveness of this disease. Hence, using an *in vivo* selection model, I have isolated a sub-population of aggressive neuroblastoma cells from secondary liver lesions after two cycles of liver metastasis. This sub-population of cells had a higher rate of proliferation, anchorage-independent growth and neurosphere formation in comparison to the parental neuroblastoma cell line. Interestingly, this aggressive sub-population of neuroblastoma cells had a higher expression of pAKT and pFAK when compared to parental cells. Moreover, this aggressive sub-population demonstrated tumor burden in the spleen and liver metastasis while the parental cells had only established primary tumors in the spleen; providing a potential model to study early- versus advanced-stages of neuroblastoma. My studies do not extensively examine whether both the lipid phosphatase and/or the protein phosphatase function of PTEN is critical in inhibiting

GRP-mediated neuroblastoma progression. Hence, I will mutate PTEN in the lipid/protein phosphatase domains to study the role of this tumor suppressor in neuroblastoma progression and also use the *in vivo* selection model to examine how PTEN/AKT/FAK axis can be used for neuroblastoma prognosis and a potential novel tool for detecting early-stage neuroblastoma versus advanced-stage disease.

Differential role of AKT isoforms in neuroblastoma initiation and progression

Though, activation of AKT indicates poor prognosis and unfavorable outcome in neuroblastoma patients [60], isoform-based studies are lacking in neuroblastoma. A recent study from our laboratory demonstrated a role for AKT2 in neuroblastoma cell migration, invasion, VEGF secretion and anchorage-independent growth *in vitro*, and metastasis *in vivo* [116,120]. This is not surprising, as isoform-specific studies in cancer places AKT1 as the isoform involved in survival and proliferation of cancer cells, AKT2 in motility and invasion, and, AKT3 in inducing hormone-independence in cancers [121]. Silencing AKT1 or AKT3 had similar inhibitory effects on anchorage-independent growth of neuroblastoma cells *in vitro*, but only AKT1 and not AKT3 inhibition demonstrated reduced VEGF expression similar to AKT2. This leaves a scope for further studies examining the differential role of AKT1 and AKT2 with respect to neuroblastoma tumor initiation and progression as observed in adult solid tumors like breast, ovarian and colorectal cancer [122,123]. Moreover, AKT2 silencing, but not AKT1 or AKT3, inhibited MYCN expression and suppressed IGF-1 stimulated MYCN protein levels in neuroblastoma cells, indicating that receptor tyrosine kinases expressed on neuroblastoma cells might preferably use AKT2 for downstream effects, over AKT1 and AKT3 [116].

Using *in vitro* assays, I will determine the role of AKT1 versus AKT2 in different hallmarks of cancer and examine whether an isoform switch indeed occurs during the transition from primary tumor to metastatic disease. Immunohistochemistry-based detection of pAKT expression in the second part of my studies provided no significant difference in neuroblastoma patients with or without metastasis. Hence, isoform-based detection of protein expression might enable me to observe a difference in pAKT1 and pAKT2 levels, thus, confirming a role for a specific AKT isoform in neuroblastoma metastasis and a plausible AKT isoform switch during neuroblastoma progression.

Concluding Remarks

The results reported in this study demonstrate a crucial role for GRP in neuroblastoma progression. Silencing GRP in conjunction with chemotherapeutic drugs can enhance neuroblastoma cell death. Furthermore, inhibiting GRP expression suppressed tumorigenic properties of neuroblastoma cells *in vitro*. Using our *in vivo* metastasis model, we demonstrated the critical role of GRP in neuroblastoma metastasis, as GRP silenced cells failed to metastasize to the liver inspite of forming primary tumors in the spleen. I also reported the significance of the PTEN/AKT axis in regulating GRP-mediated oncogenic effects in neuroblastoma and report a novel role for PTEN in inhibiting GRP-dependent neuroblastoma cell migration and angiogenesis *in vitro.* A more comprehensive understanding of GRP/PTEN/AKT axis in neuroblastoma progression will help identify crucial steps in the invasion-metastasis cascade that this axis regulates and allow induction of successful clinical trials for children with aggressive, refractory neuroblastomas.

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