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GASTRIN-RELEASING PEPTIDE-MEDIATED NEUROBLASTOMA GROWTH: A ROLE FOR THE PI3K/AKT PATHWAY

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GASTRIN-RELEASING PEPTIDE-MEDIATED NEUROBLASTOMA GROWTH: A ROLE FOR THE PI3K/AKT PATHWAY

by

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Dedication

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Gastrin-Releasing Peptide-Mediated Neuroblastoma Growth: A Role for the PI3K/Akt Pathway

Publication No._____

Titilope Adenike Ishola, Ph.D.

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Neuroblastoma is the most common extracranial solid tumor in infants and children. Our lab and others have shown trophic actions of gastrin-releasing peptide (GRP), and its analogue bombesin (BBS), in neuroblastomas. Our lab also found that undifferentiated neuroblastomas express increased levels of GRP receptor (GRPR). Activation of the phosphatidylinositol-3-kinase (PI3K)/Akt pathway, a crucial regulator of cell survival, is associated with poor outcome in neuroblastomas and our lab's previous work has shown that GRPR regulates the expression of PI3K/Akt pathway components. However, the signaling mechanisms involved in this process are not clearly defined. Therefore, the objective of this project was to determine how GRP/GRPR, by way of PI3K pathway, regulates neuroblastoma growth.

GRP/BBS treatment rapidly increased phosphorylation of both Akt and GSK-3β in neuroblastoma cells. Antagonism or silencing of GRPR attenuated BBS-induced phosphorylation of Akt. PI3K inhibition also abrogated BBS-stimulated phosphorylated (p)-Akt as well as its cell cycle targets. GRP increased G1/S phase progression in SK-N-SH cells

and BBS-mediated BrdU incorporation was blocked with a PI3K inhibitor. These findings identify PI3K/Akt as an important signaling pathway for GRP-mediated neuroblastoma cell growth. In order to determine the *in vivo* significance of GRP/GRPR, the effects of BBS treatment in nude mice with human neuroblastoma xenografts were assessed. BBS treatment significantly increased the growth and mediators of angiogenesis of SK-N-SH and BE(2)-C tumors, as well as increased p-Akt levels. A GRPR antagonist reduced BBS-stimulated tumor growth and angiogenic markers *in vivo*. GRP or GRPR silencing inhibited the expressions of VEGF, p-Akt, and p-mTOR *in vitro*. GRPR knockdown induced cell morphology changes, reduced cell size, decreased cell proliferation, and inhibited DNA synthesis, which corresponded to G2/M cell cycle arrest. Activated Akt and its downstream regulators of protein synthesis and metabolism were also significantly downregulated by GRPR silencing. GRPR knockdown upregulated the expression of PTEN, the inhibitor of the PI3K/Akt pathway. Furthermore, silencing of GRPR or GRP suppressed anchorage-independent growth; while GRPR overexpression resulted in soft agar colony formation, which was inhibited by a GRP-blocking antibody. In conclusion, these findings demonstrate that GRP/GRPR signaling regulates the PI3K/Akt pathway and promotes neuroblastoma growth, angiogenesis, and oncogenic properties.

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CHAPTER 1

INTRODUCTION

1.1 NEUROBLASTOMA

1.1.1 INCIDENCE AND SIGNIFICANCE

Cancer is the second leading cause of death in children younger than 15 years of age [1]. Neuroblastoma is the most common pediatric extracranial solid tumor in infants and children, accounting for 7-10% of all childhood cancers [2, 3]. In the United States, 700 new cases are diagnosed annually, with an overall incidence estimated at about 1 case per 10,000 live births [2, 4]. A striking 90% of children with neuroblastoma present by 5 years of age, with half of all the cases found in children less than 24 months and the median age at diagnosis is 22 months [3, 5, 6]. This unpredictable tumor accounts for more than 15% of cancer related deaths in children [1, 7]. Considering the incidence of these tumors at such a young age, the potential successful treatment of neuroblastoma would, therefore, result in the preservation of significant life-span years. In the last several decades, there has been an improvement in the outcome of pediatric patients with solid tumors, largely due to advancement in the multiple modality treatment of surgery, radiotherapy and chemotherapy [8]. In fact, this combined modality therapy has resulted in remarkable cure rates for other solid tumors of childhood (e.g., Wilms' tumor, medulloblastoma); however, the prognosis for patients with advanced-stage neuroblastoma is very poor [8] and the overall mortality for all tumor stages remains at a striking 50% [9]. Furthermore, combination treatment modalities

for advanced tumors do not improve the grim prognosis [3, 10]. Neuroblastoma remains a complex medical challenge; therefore it is important to define the critical pathways linked with the aggressiveness of this tumor.

1.1.2 CLINICAL PRESENTATION

Due to their neural crest cell lineage, neuroblastomas may occur in the adrenal medulla (most common location) or anywhere along the sympathetic ganglia. Hence, patient presentation and symptoms are commonly a manifestation of tumor location [11, 12]. The intraabdominal neuroblastomas, most notably in the retroperitoneum and posterior mediastinum, tend to present as asymptomatic masses detected by parents and/or during routine clinic visits. Pelvic masses, although an atypical location for neuroblastomas, can compress the bladder or rectosigmoid colon and may produce urinary retention or constipation. Thoracic neuroblastomas usually present as incidental masses on chest radiographs. Cervical tumors can occasionally compromise sympathetic innervation leading to Horner syndrome. Early symptoms, when present, are frequently nonspecific such as general malaise, weight loss, and unexplained fever. However, the tumor may spontaneously hemorrhage, leading to an acute onset of abdominal pain with anemia-induced malaise. In general, severe symptoms do not occur until the tumor has reached a critical size and/or developed metastases. By the time of diagnosis, it is not uncommon for there to already be hematogenous metastases. Infants and children who present with protracted cervical masses (characteristically defined as lymphadenopathy), may have primary or metastatic neuroblastoma. Bone metastases present with bone pain as well as sudden distinct changes in activity level. Furthermore, if there is skull involvement, periorbital ecchymoses and/or

proptosis may ensue, at times mistaken for trauma-induced symptoms. Paraspinal tumors may extend through the vertebral foramina to compress the spinal cord, thereby generating motor deficits and progressive paraplegia. Infants can also present with painless, bluish subcutaneous nodules, called blueberry muffin syndrome. This is a common feature in stage 4S disease and indicates a favorable prognosis with potential for spontaneous tumor regression.

Paraneoplastic syndromes are rare, but distinguishable, occurrences in patients with neuroblastoma. Neuroblastomas that secrete vasoactive intestinal peptide classically develop intractable secretory diarrhea with resultant dehydration and hypokalemia [13]. This syndrome usually signifies a well-differentiated tumor and symptoms resolve after resection. Opsomyoclonus, another neuroblastoma-induced paraneoplastic syndrome, is characterized by involuntary jerking movements of the limbs and trunk along with rapid, conjugate eye movements. These symptoms are thought to be a consequence of cerebellar toxicity caused by antibodies against the neural tissue of the tumor. Although opsomyoclonus is associated with early-stage disease with favorable features, symptoms commonly persist despite successful treatment of tumor resulting in neuro-developmental delay [14].

1.1.3 Classification and Staging

The categorization of neuroblastomas is determined by the degree of cellular differentiation within the tumor. Poorly-differentiated tumors, with abundant neuroblasts, are classified as neuroblastomas, while well-differentiated benign tumors with mature ganglion cells, increased stroma compartments and sparse neuroblasts are described as ganglioneuromas [15]. Ganglioneuroblastomas are an intermediary category possessing

features of both the immature neuroblastomas and differentiated ganglioneuromas [16]. The Shimada classification, modified as the International Neuroblastoma Pathology Classification [17], has been widely used to describe and predict neuroblastoma behavior and prognosis. The system takes into consideration histologic features such as the degree of cellular differentiation, schwannian stroma and the mitosis-karyorrhexis index (MKI), in addition to the age of the patient [18, 19]. However, in terms of documenting the stage and extent of disease progression, both at the time of diagnosis and to establish appropriate treatment protocols, the generally accepted method is the International Neuroblastoma Staging System (**Table 1.1**) [20].

1.1.4 Prognostic Pathobiological Markers

Cytogenetics

Cytogenetic aberrations identified in neuroblastoma include chromosome losses, gains, and DNA index abnormalities. Loss of heterozygosity (LOH) on chromosome 1 (deletion of 1p36 region) occurs in >70% of tumors [21, 22], strongly correlating with unfavorable prognosis and poor patient outcome [20]. Deletions on chromosome 11q and 14q are also frequently detected in neuroblastoma [23]; in fact, unbalanced 11q LOH is also strongly associated with worse clinical prognosis [24]. Additionally, allelic gains on chromosome 17q have been shown to be clinically significant for neuroblastoma [25]. Furthermore, the DNA index of neuroblastomas has been shown to be associated with tumor chemosensitivity and prognosis [26]. Approximately two-thirds of advanced-stage neuroblastomas have diploid DNA content and are often chemoresistant. However,

Stage	Definition
1	Localized tumor with complete gross excision, with or without microscopic residual disease, negative ipsilateral lymph nodes
2A	Localized tumor with incomplete gross excision; negative ipsilateral nonadherent lymph nodes
2B	Localized tumor with or without complete gross excision, with positive ipsilateral nonadherent lymph nodes; negative contralateral lymph nodes
3	Unresectable unilateral tumor infiltrating across the midline, with or without regional lymph node involvement, or Localized unilateral tumor with contralateral regional lymph node involvement, or Midline tumor with bilateral extension by infiltration (unresectable) or by lymph node involvement
4	Any primary tumor with dissemination to distant lymph nodes, bone, bone marrow, liver, skin or other organs (except as defined for stage 4S)
4S	Localized primary tumor (as defined for stage 1, 2A or 2B) with dissemination limited to skin liver and bone marrow (limited to infants < 1 year age)

TABLE 1.1 International Neuroblastoma Staging System.

the presence of hyperdiploid DNA content is correlated with early disease stage and improved prognosis [26].

Serum Markers

Neuron-specific enolase (NSE) is nonspecific for neuroblastomas, however, increased levels of NSE (>100 ng/ml) have been shown to significantly correlate with advanced-stage neuroblastomas and poor patient survival rates [27]. Lactate dehydrogenase (LDH) is also not specific for neuroblastoma, but high levels (>1500 U/ml) are associated with rapid cellular turnover and poor prognosis [28, 29]. Several patients with neuroblastoma may have increased levels of the iron-binding protein ferritin. High ferritin levels (>142 ng/ml) are commonly seen in patients with advanced-stage disease and correlate with lower survival rates as well as unfavorable outcome [30].

MYCN amplification

Neuroblastoma is characterized by several molecular markers that correlate to various degrees of prognosis, including MYCN, cell surface glycoprotein CD44, tyrosine kinase receptors TrkA and TrkB, and their respective ligands NGF and BDNF. Of these markers, MYCN is the most important—amplified in 25% of primary neuroblastomas and strongly correlated to advanced-stage, rapid disease progression, and poor clinical outcome [31-33]. Tumors with MYCN amplification are typically characterized by florid vascularization [31-33]. Moreover, MYCN amplification has been associated with other poor prognostic indicators such as chromosome 1p deletion and increased expression of multidrug resistance-

associated protein (MRP) [34, 35]. We and others have shown that the expression of N-myc, the resultant oncogenic transcription factor, is regulated by the PI3K/Akt pathway [36, 37].

1.1.5 DIAGNOSIS AND TREATMENT

Diagnosis

Screening

The routine diagnostic screen, for new or recurrent disease, is to assess for elevated urinary catecholamine metabolites homovanillic acid (HVA) and vanillylmandelic acid (VMA) [38, 39]. Although this screening method has become a standard practice for all infants in Japan, it is only selectively utilized in other countries as indicated by the presence of associated signs and symptoms.

Imaging

Various imaging modalities are utilized to aid in the diagnosis and evaluation of disease progression and extension to adjacent and distant organs [11, 12]. Chest radiographs can help narrow the differential diagnoses and also detect any tumor calcification. Ultrasonography aids in determining the solid nature of the tumor, characteristically a heterogeneous echo pattern. Contrast-enhanced computed tomography, the gold-standard imaging study, provides analyses of tumor consistency, localized extension as well as distant organ involvement. Magnetic resonance imaging is particularly beneficial for distinguishing the degree of tumor extension into the spinal canal. Radiolabeled *meta*-iodobenzylguanidine (MIBG) scan is very specific and sensitive for assessing bone and bone marrow disease, since MIBG is taken up by most neuroblastomas but not normal bone [40]. In addition, a

technetium 99m (^{99m}Tc) methylene diphosphonate bone scan enhances the MIBG results by significantly reducing the number of false negatives [41]. Of course, bone marrow aspiration or biopsy of primary or secondary tumors is necessary to confirm a diagnosis of neuroblastoma. Tissue samples are assessed for cytogenetic abnormalities and tumor biomarkers, and histologic sections are analyzed with the Shimada criterion. Favorable prognostic features include differentiation, low MKI (defined as fewer than 100 mitotic or karyorrhectic cells per 5000 cells) and stroma-rich tumors [3, 42]. Other favorable prognostic indicators are <1 year of age, clinical stages 1, 2, 4S and MYCN nonamplification [3].

Treatment

Presently, therapy options for neuroblastoma comprise combined modality of surgery, chemotherapy and radiotherapy. This approach is individually modified depending on disease stage and patients' age at presentation.

Surgical approach

The main objectives of surgical intervention are complete resection of tumor, staging by examination, and biopsy of advanced-stage disease [11, 12]. Assessment of resectability is carefully determined from diagnostic imaging studies. There are several considerations of vital importance including, gauging the size of tumor, whether there is adherence or extension into adjacent structures such as vessels or spinal cord, and the likelihood of surgical cure. The foremost objective in surgery for early stage tumors (stages 1, 2A, 2B) is complete gross resection, with care taken to avoid spillage of tumor, injury to adjacent structures and/or massive blood loss. As with any cancer operation, the fundamental surgical techniques of vascular control and complete tumor resection are applied for early-stage

resectable tumors. On the other hand, preliminary surgical intervention for advanced-stage tumors (stages 3, 4) should be limited to an open biopsy. The biopsied tissue can then be analyzed for diagnosis, cytogenetic profile, and tumor biomarkers. Postponement of surgical resection after adjuvant therapy increases the degree of complete excision and has resulted in decreased morbidity of patients. In the case of infants with stage 4S disease, surgical resection of the primary tumor is frequently considered aggressive and not necessary, as many of these tumors may differentiate and spontaneously regress despite no specific treatment.

Chemotherapy

Combination chemotherapy has been successful in patients with advanced primary, refractory or metastatic neuroblastomas and includes various agents such as cyclophosphamide, vincristine, cisplatin, and melphalan [11, 12, 43]. For advanced-stage tumors, combined chemotherapy failed to effectively eradicate the disease; however, it often reduced the size of the primary tumors of advanced stage, allowing them to be resectable. Even though there is an improvement in long-term survival with more intense combination therapy, the toxicities remain a crucial problem. Bone marrow-ablative therapy with total body irradiation or melphalan with subsequent bone marrow transplant (BMT) has shown some success for patients with high-risk neuroblastomas [44]. In another study, transplantation of purged autologous bone marrow has shown to significantly improve event-free survival as compared with intensive chemotherapy [45]. A recent consortium report also demonstrated an effective combined therapy of ^{131}I-MIBG with myeloablative chemotherapy

for patients with neuroblastomas that are refractory to chemotherapy [46]. However, these are still extreme procedures with unfavorable consequences.

Radiation therapy

Radiation therapy has been shown to be successful in decreasing the local relapse rate for high-risk neuroblastoma [11, 12]. Whereas for early-stage tumors, even with local residual disease, the potential complications associated with radiotherapy significantly outweigh the potential benefits of the treatment. Radiation is also contraindicated for intraspinal tumors, because of associated vertebrae damage leading to growth arrest and scoliosis. However, for patients presenting with acute neurological symptoms due to intraspinal tumor compression of the cord, emergent radiation therapy has proven to be of value. Infants with stage 4S should also be spared from radiation therapy, unless hepatomegaly-induced respiratory symptoms exist. In these clinical situations, however, the radiation is localized to the liver alone. Radiation therapy has also been used in conjunction with chemotherapy in order to improve the resectability of advanced-stage disease. Radiotherapy is further utilized for total body irradiation preceding an autologous BMT. In Europe, targeted MIBG treatment is a principal treatment option for both advanced-stage and refractory neuroblastomas [47-49]; unfortunately, complications can include generation of secondary malignancy or thyroid dysfunction [50, 51]. Recently, it has been found that the combination of localized irradiation, myeloablative chemotherapy with stem cell rescue, and aggressive surgical resection effectively resulted in local tumor control in high-risk neuroblastomas [52].

1.1.6 CHALLENGES TO NEUROBLASTOMA THERAPY

Pediatric neuroblastomas are enigmatic, multifaceted tumors that continue to remain a clinical challenge. For over two decades, therapeutic advances have failed to significantly increase the 5-year survival rates of children with aggressive, advanced-stage neuroblastomas. Combination chemotherapy has been successful and is a mainstay for patients with advanced primary, refractory or metastatic neuroblastomas. However, chemotherapeutic regimens are usually unsuccessful at effectively eradicating the disease and furthermore, the toxicities remain a significant problem. Currently, the most common chemotherapeutic agents utilized are cyclophosphamide, iphosphamide, vincristine, doxorubicin (adriamycin), cisplatin, carboplatin, etoposide (VP-16) and melphalan [11, 12, 43]. The severe toxicities of the aforementioned include, but are not limited to: myelosuppression, gastrointestinal toxicity, nephrotoxicity and neurotoxicity [43]. Hence, additional adjuvants may be required to garner the benefits of chemotherapy while diminishing the harmful side effects. In addition, since heterogeneity is a hallmark of neuroblastoma, it is therefore important to focus on addressing the various pathogenic intricacies of this tumor. Understanding the various biological and molecular components regulating tumor progression in neuroblastoma is necessary to successfully improve survival rates and may lead to innovative agents for more effective combinational therapy.

1.2 GASTRIN-RELEASING PEPTIDE

1.2.1 STRUCTURE AND FUNCTION

Gastrin-releasing peptide (GRP) is a mammalian member of the bombesin-like peptide family including, amphibian equivalent bombesin (BBS) and mammalian neuromedin B (NMB) [53, 54]. GRP is a both a gut/neurohormone that is functional as a 27-amino acid (aa) peptide processed from preproGRP (148 aa) and proGRP (125 aa) [53, 54]. GRP was named after the hormone's first discovered activity of stimulating gastrin secretion from gastric tissue [53, 54]; however, it has been found to be released in GRP is found in the brain and gut with a stimulatory effect noted on the growth of various tissues [55, 56], in particular, the gastrointestinal (GI) mucosa [54, 55, 57, 58]. Our laboratory has shown that GRP is a potent mitogen for normal intestinal mucosa [57] and pancreas [59]. Our lab has shown that GRP enhances intestinal adaptation that occurs after bowel resection and prevents elemental diet-induced gut mucosal atrophy [57]. GRP can also induce secretion of other gut hormones [e.g., gastrin, neurotensin (NT)], which can then stimulate proliferation of normal GI tissues [60-62]. This further suggests that GRP can control GI cell growth indirectly by activating the release of other mitogenic peptides. GRP can also regulate cell growth directly [63] and is known to stimulate DNA synthesis and cell replication in many cell lines [61, 64].

1.2.2 GASTRIN-RELEASING PEPTIDE RECEPTOR

The GRP receptor (GRPR), a model G-protein coupled receptor (GPCR) with seven transmembrane regions, belongs to the BBS receptor family with subtypes NMBR (390 aa), GRPR (384 aa), BRS-3 (399 aa), and BB4-R (392 aa) [53, 54]. In regards to ligand affinity,

GRPR has high affinity for both GRP and BBS, and less for NMB; while NMBR has affinity for NMB and lower affinity for GRP [54]. BRS-3 has low affinity for all three peptides and there does not appear to be a mammalian ligand for BB4-R [54].

GRPR is expressed in the normal brain [65], the GI tract [66], pancreas [67], found transiently during the early stages of embryogenesis [54], and in some cancers of the breast [68], lung [69, 70], stomach [68], pancreas [71], prostate [72] and colon [73]. In fact, since activation of GPCR signaling is the most frequent manipulation of aggressive neoplasias [74], GRPR expression may be important for cancer biology on many levels. For instance, GRPR can function as a biomarker for disease progression and it can potentially serve as an effective target for anti-tumor therapy.

1.2.3 Effects on Cancer Growth

The endocrine control of tumorigenesis was first established at the turn of 20th century and currently it is accepted that hormones and growth factors are key players in the tumor milieu. Studies have shown that these stimulatory peptides promote tumor proliferation, motility, and metastatic behavior [74, 75]. Therefore, to achieve therapeutic success, it is imperative to target the tumor as well as the interacting factors in its microenvironment. The success of hormone-targeted therapy has been shown to be an important aspect in the treatment of hormone-responsive breast [76] and prostate cancers [77]. In addition, certain tumors of the GI tract (e.g., stomach, pancreas, and colon) possess receptors for GI hormones, and the growth of these cancers can be altered by hormone administration or receptor blockade. Gut hormones can act in an endocrine, paracrine or autocrine fashion to stimulate growth of various tissue types, including cancers.

Our lab and others have demonstrated the mitogenic effects of GRP and BBS in a host of cancers: prostate [78, 79], breast [68, 80], pancreas [81, 82], stomach [83-85], colon [86], and lung [59, 87]. Blockade of the GRP receptors has been shown to inhibit the growth of cancer cells [88, 89]; our lab has shown direct stimulation of breast [68] and gastric cancer cell growth with GRP that was blocked with selective GRPR antagonists [84, 85]. In fact two recent reviews emphasized the significance of GRP and its receptor in a variety of tumors [54] and the importance of targeting them for antiangiogenic and antitumor therapy [54, 90].

1.2.4 Role in Neuroblastoma Pathophysiology

Many neoplastic cells have the notable ability to respond to environmental signals in order to promote their survival. Neuroblastoma is an example of a tumor that can manipulate autocrine and paracrine growth signals to enhance its pathogenesis [58]. As a consequence of their neuroendocrine origin [91], neuroblastomas are, therefore, able to produce and respond to several different peptides and hormones, including GRP, serotonin, catecholamines, and vasoactive intestinal polypeptide [58]. The regulatory mechanisms that determine which clinical course the tumor pursues are unknown.

GRP is also expressed in neuroblastomas and has been determined to be an autocrine growth factor for neuroblastomas [92, 93] (**Fig. 1.1**). However, the molecular mechanisms involved in the GRP-induced growth regulation of neuroblastoma are not clearly defined. Our lab has shown that human neuroblastomas produce GRP and express GRPR [83, 92]. Increased GRP expression appears to correlate with more aggressive undifferentiated neuroendocrine tumors in children [94]; correspondingly, our lab has found that GRPR expression is increased in poorly differentiated human neuroblastoma samples in comparison

to the benign ganglioneuroma samples [92]. Several studies have correlated the frequency of GRPR to the biologic behavior of different tumor cell types such as prostate [95], breast [96, 97], and small-cell lung carcinoma [98]. However, the functional significance of GRP/GRPR expression in the proliferation and differentiation of human neuroblastomas remains relatively unknown. The growth of other tumors, which express GRPR, can be modulated by selective GRPR antagonists (e.g., BIM26226, H-2756). Therefore, I postulate that the growth of the more aggressive neuroblastomas, with increased GRPR expression, can be affected by blockade of the GRPR or other novel therapies, which target pathways regulated by GRPR expression.

The signaling cascades that take place after GRP binds to GRPR, in neuroblastoma are not fully understood. Our lab recently found that GRP treatment increased growth in neuroblastoma cell lines by inducing calcium influx into the cells [92]. This is consistent with the fact that GRP binding to GRPR can activate phospholipase C (PLC) and transduce MAPK signaling through protein kinase C (PKC) and calcium (**Fig.1.1**) [99, 100]. However, the GRP transduction cascade is inherently more complex, as our lab has also discovered a relationship between GRP signaling and the well-described phosphatidylinositol 3-kinase (PI3K) pathway [101]. A better understanding of the molecular mechanisms involved in GRP signaling is warranted in order to comprehend endocrine modulation of neuroblastoma growth and the regulation of GPCR-mediated signaling. Additionally, this information could result in the development of specific therapeutic targets for neuroblastomas and other hormone secreting tumors.

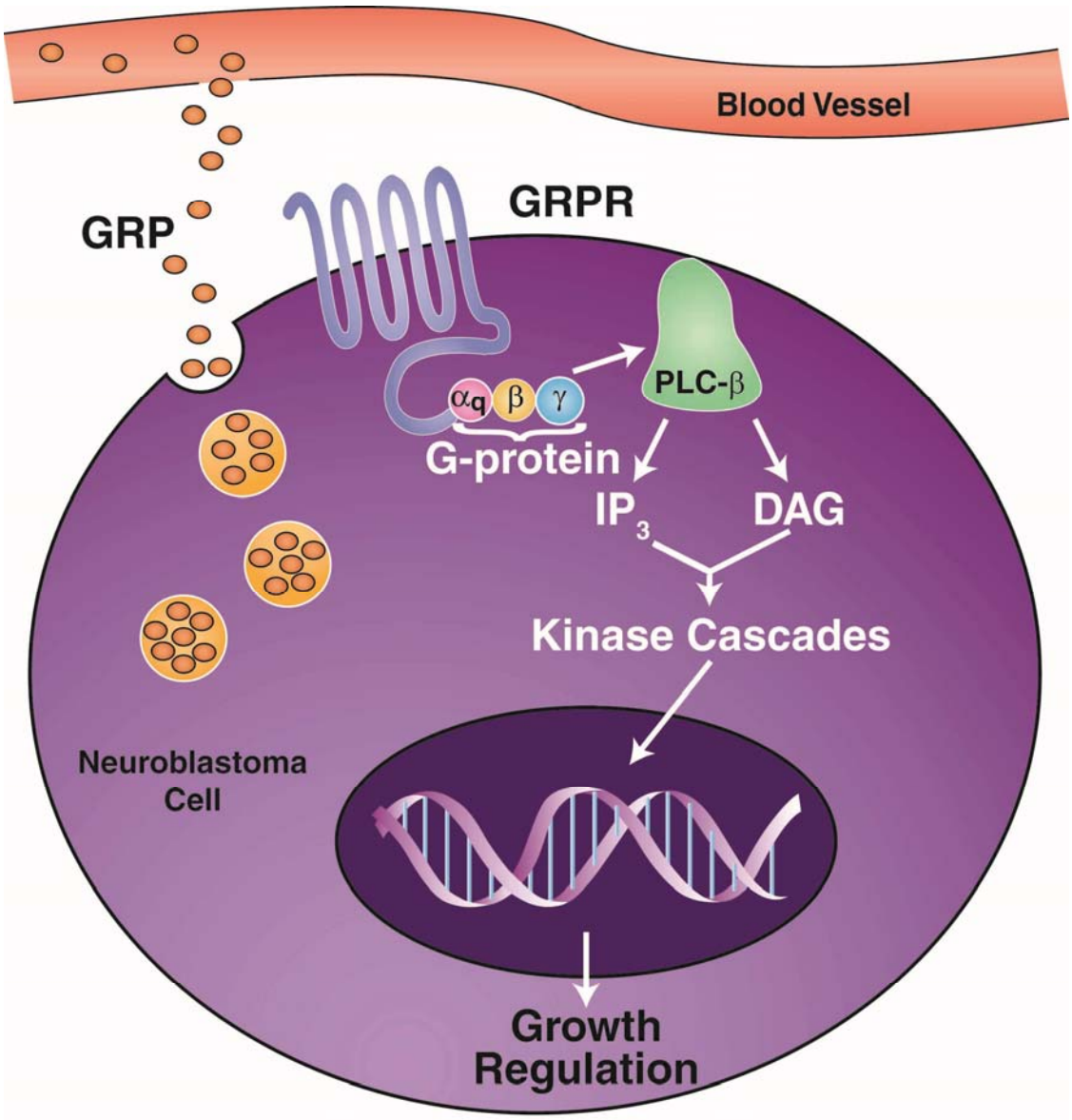

FIG. 1.1 GRP/GRPR SIGNALING IN NEUROBLASTOMA. GRP can stimulate neuroblastoma growth through autocrine, paracrine and/or endocrine mechanisms. GRP functionally couples to GRPR, a G-protein-coupled receptor containing seven trans-membrane domains, to activate intracellular signaling. GRPR antagonists (e.g., BIM 26226, H-2756) inhibit actions of GRP.

1.3 PHOSPHATIDYLINOSITOL 3-KINASE/AKT PATHWAY

1.3.1 COMPONENTS OF PATHWAY

PI3K, a ubiquitous lipid kinase, is composed of a Src homology 2 domain-containing regulatory subunit (p85) and a 110-kDa catalytic subunit (p110) [102, 103]. PI3K catalyzes the phosphorylation of the inositol ring at the D-3 position in a variety of phosphoinositide lipids, forming 3-phosphorylated phosphoinositide substrates [104-106]. The tumor suppressor gene PTEN (phosphate and tensin homologue deleted from chromosome 10) negatively regulates PI3K activity by dephosphorylating the phosphoinositide lipids (**Fig. 1.2**) [107, 108]. Although involved in receptor tyrosine kinase (RTK) signaling, PI3K can also regulated by GPCRs (**Fig. 1.2**) [109-111]. This attribute is dependent on the type of PI3K molecule. Class IA PI3K, made up of a p85 regulatory subunit and a p110 (α/β/δ) catalytic subunit, are usually activated by RTKs, but may also be activated by GPCRs; while Class IB PI3K, with p101 regulatory and p110γ catalytic subunits, are regulated by GPCRs [109, 110]. Interestingly, Class IB PI3K signaling has been determined in only a few cell types, namely the exocrine pancreas and leukocytes [109, 112].

PI3K and its downstream effector Akt, a serine/threonine protein kinase also known as protein kinase B (PKB), is involved in the regulation of normal cellular processes such as cellular growth, vesicular trafficking, intracellular vesicle transport, and insulin-regulated glucose uptake [113, 114]. The PI3K/Akt role in cell survival involves stimulating effectors of cell transcription and translation, inhibition and regulation of apoptotic proteins, and also by triggering cell-cycle promoters (**Fig. 1.2**) [105, 107, 110].

1.3.2 Effects on Cancer Growth

PI3K/Akt plays an important role in various cancers by regulating survival, mitogenesis, anti-apoptosis and cytoskeletal rearrangement [115-120]. PI3K has been shown to promote tumorigenesis in the GI tract and specific inhibitors of PI3K have demonstrated inhibition of tumor growth [121, 122]. Oncogenic activity of the PI3K pathway is not limited to GI tract and has been shown to be involved in formation of breast cancer cells [123, 124]. Moreover, components of the PI3K/Akt pathway are mutated, or stimulated by growth factors, in a multitude of cancers and familial syndromes; including colon cancer, breast cancer, prostate cancer, and Cowden Syndrome [105, 107, 108, 125, 126]. PI3K/Akt/PTEN aberrations also play an important role in neuroendocrine tumor progression, particularly in regards to angiogenesis [127].

1.3.3 Role in Neuroblastoma Pathophysiology

A potential role for the PI3K pathway in the regulation of neuroblastoma growth has also been studied. Our lab and others have demonstrated that the PI3K/Akt pathway is deregulated in undifferentiated neuroblastomas [101, 128] and that the inhibitors of the PI3K/Akt pathway (e.g., LY294002, wortmannin) result in decreased cell growth of neuroblastomas [37, 101, 129-131]. In fact, recently, activated Akt has been determined to be an indicator of poor prognosis in neuroblastoma [128]; and our lab previously found that poorly differentiated neuroblastomas have decreased expression of PTEN [101]. Of additional importance to neuroblastoma is the fact that PI3K inhibition destabilizes the protein levels of neuroblastoma oncogene N-myc [36, 37] and further, we have found that

PI3K regulates mediators of angiogenesis through N-myc-dependent pathways [37]. However, the molecular mechanisms involved in this process are not clearly elucidated.

Interestingly, our lab has found that GRPR overexpression down-regulated PTEN expression and increased activated Akt expression in neuroblastoma cells [101]. Hence, our lab's results suggest that PI3K is an important pathway in neuroblastoma growth regulation and may be involved in GRP-mediated signaling. However, the interaction of PI3K/Akt with the GRPR pathway in neuroblastomas is largely unknown.

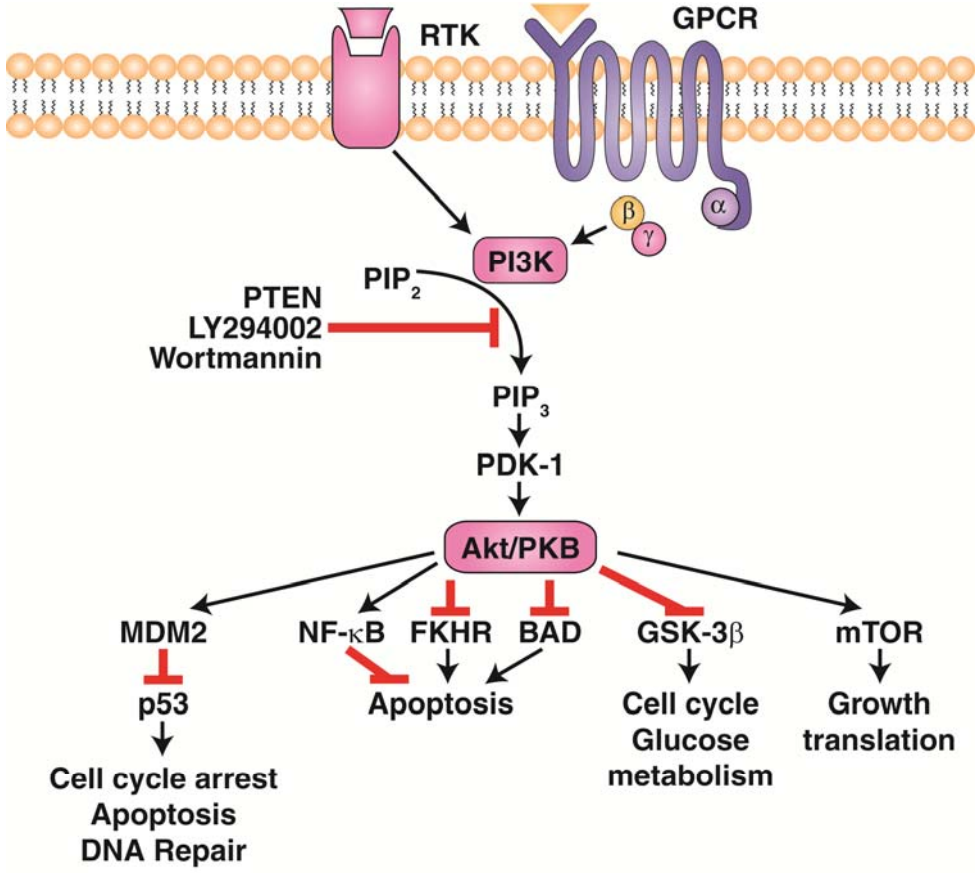

FIG. 1.2 THE PI3K/AKT PATHWAY. PI3K catalyzes the phosphorylation of a variety of phosphoinositide lipids to activate signal transduction. Akt, a crucial effector of PI3K, regulates various aspects of cell survival, such as cell cycle and apoptosis. Important target proteins include GSK-3β, mTOR, NF-κB, and MDM2. LY294002, wortmannin and PTEN tumor suppressor inhibit the actions of the PI3K/Akt pathway.

1.4 RATIONALE FOR THE STUDY

1.4.1 BACKGROUND OF PROBLEM

Neuroblastoma, the most common pediatric extracranial solid tumor in infants and children, accounts for approximately 10% of all childhood cancers [2, 3]. Greater than 15% of cancer-related deaths in children are attributed to neuroblastoma [1, 7]. The majority of neuroblastoma cases present under the age of 5 years, with 50% occurring in children under 2 years old [3, 5]. Despite current advances in multimodal therapy for neuroblastoma patients, the overall mortality for all stages of the tumor remains dismal [3, 10]. Therefore, in order to develop more effective therapeutic options, it is important to determine the signaling mechanisms associated with the aggressiveness of this tumor.

GRP, the mammalian equivalent of BBS, is a gut/neuropeptide that can stimulate the growth of both normal and neoplastic cells [54, 57, 83, 87]. As a neuroendocrine tumor, neuroblastoma has the capability to produce and respond to various gastrointestinal hormones, including GRP [58]. Our lab's previous findings demonstrated an increase of GRPR expression in undifferentiated neuroblastomas and a potential relationship in regulating the PI3K/Akt pathway [92]. However, the signaling cascades that take place after GRP binds to its G-protein coupled receptor, GRPR, in neuroblastoma remain largely unknown.

My studies will help in defining the role of GRP and its receptor in neuroblastomas. Understanding the cellular mechanisms and signaling pathways regulating GRP-induced neuroblastoma proliferation is clinically important because 1) GRP/GRPR could be a tumor

progression marker for neuroblastoma, and 2) this information could possibly result in the development of novel agents to enhance treatment of this fatal disease.

1.4.2 CENTRAL HYPOTHESIS

My overall hypothesis is that GRP/GRPR can regulate neuroblastoma growth by activation of the PI3K/Akt pathway. Specifically, I propose that: 1) GRP/GRPR is a crucial regulator of neuroblastoma tumorigenesis; and 2) PI3K/Akt pathway is an important effector of GRP/GRPR signaling. The central hypothesis was tested in the two following specific aims.

1.4.3 SPECIFIC AIMS

Specific Aim 1 tests hypothesis 1: GRP/GRPR is a crucial regulator of neuroblastoma tumorigenesis

Exp. 1 – To determine whether GRP stimulation induces cell cycle progression and modulates cell cycle regulators in neuroblastoma cells (**Chapter 3**)

Exp. 2 – To determine whether BBS treatment induces the growth of neuroblastoma xenografts (**Chapter 4**)

Exp. 2a – by determining the effect of systemic (intraperitoneal) administration of BBS on the growth of subcutaneous neuroblastoma xenografts in nude mice

Exp. 2b – by determining the effect of systemic (intraperitoneal) administration of BBS on the growth of orthotopic neuroblastoma xenografts in nude mice

Exp. 2c – by determining the effect of systemic (intraperitoneal) administration of BBS and/or RC-3095 (GRPR antagonist) on the growth of subcutaneous neuroblastoma xenografts in nude mice

Exp. 3 – To determine the importance of GRPR expression and GRPR silencing for neuroblastoma cell proliferative capacity and malignant potential (**Chapter 5**)

Exp. 3a – by determining the effect of GRPR silencing on neuroblastoma cell proliferation, DNA synthesis, and cell cycle progression

Exp. 3b – by determining the effect of GRPR silencing on neuroblastoma cell size

Exp. 3c – by determining the effect of GRPR silencing on neuroblastoma cell migration

Exp. 3d – by determining the effect of GRPR expression and silencing on neuroblastoma anchorage-independent growth

Specific Aim 2 tests hypothesis 2: PI3K/Akt pathway is an important effector of GRP/GRPR signaling

Exp. 1 – To determine whether GRP/GRPR activates the PI3K/Akt pathway in neuroblastoma cells (**Chapter 3**)

Exp. 1a – by determining the effect of GRP/BBS treatment on the phosphorylation of Akt and GSK-3β in neuroblastoma cells

Exp. 1b – by determining the effect of PI3K inhibition after GRPR stimulation in neuroblastoma cells

Exp. 1c – by determining the effect of GRPR antagonists or silencing on Akt activation in neuroblastoma cells

Exp. 2 – To determine whether BBS treatment induces PI3K/Akt pathway activation in neuroblastoma *in vitro* and *in vivo* (**Chapter 4**)

Exp. 2a – by determining the effect of BBS treatment on the activation Akt and expression VEGF in neuroblastoma cells *in vitro*

Exp. 2b – by determining the effect of systemic (intraperitoneal) administration of BBS on Akt phosphorylation and VEGF expression in neuroblastoma xenografts

Exp. 2c – by determining the effect of systemic (intraperitoneal) administration of BBS and/or RC-3095 on Akt activation and VEGF expression in neuroblastoma xenografts; and the effect on systemic circulation of VEGF

Exp. 2d – by determining the effect of GRPR or GRP silencing on phosphorylation of both Akt and p-mTOR, and VEGF expression in neuroblastoma cells *in vitro*

Exp. 3 – To determine whether the PI3K/Akt pathway components are regulated by GRPR silencing (**Chapter 5**)

Exp. 3a – by determining the effect of GRPR silencing on Akt phosphorylation and PTEN expression

Exp. 3b – by determining the effect of GRPR silencing on p70S6K and S6 expression

CHAPTER 2

MATERIALS AND METHODS

2.1 REAGENTS

GRP, BBS, GRP-H2756, and rabbit anti-GRP IgG (GRP-neutralizing antibody) were purchased from Bachem (Torrance, CA). BME was a gift from Dr. David H. Coy (Tulane University, New Orleans, LA). SB216763 and SB415286 were purchased from Tocris Bioscience (Ellisville, MO); both compounds are potent and selective GSK-3β inhibitors and function by competing with ATP [132]. LY294002 and antibodies against phosphorylated (p)-Akt (Ser473), total Akt, p-mTOR, p-GSK-3α/β, p-Rb, Rb, PTEN, ERK, p-p70S6K (Thr389), p-S6 (Ser 235/236 and Ser240/244), and S6 were purchased from Cell Signaling (Beverly, MA). Antibodies against GSK-3β, cyclin D, p21, and p27 were purchased from BD Biosciences (San Jose, CA). GRPR antibody was from Abcam, Inc. (Cambridge, MA) and p-ERK1/2 antibody was from Promega (Madison, WI). RC-3095, fetal bovine serum (FBS), and β-actin antibody were purchased from Sigma-Aldrich (St. Louis, MO). Antibody against VEGF, GRP, platelet endothelial cell adhesion molecule (PECAM)-1, and horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Cellular DNA Flow Cytometric Analysis and Cell Proliferation BrdU ELISA kits were obtained from Roche Applied Science (Indianapolis, IN). pEGFP (GFP) vector was obtained from Clontech Laboratories (Mountain View, CA). Non-targeting control siRNA (siNTC), siGRP and siGRPR were purchased from Dharmacon,

Inc. (Lafayette, CO). RNAqueous kit was obtained from Ambion, Inc. (Austin, TX) and agarose was from Cambrex Bio Science (East Rutherford, NJ). Lipofectamine 2000 and Hanks Balanced Salt Solution (HBSS) were from Invitrogen (Rockville, MD). Cell Counting Kit-8 (CCK-8) was obtained from Dojindo Molecular Technologies, Inc. (Gaithersburg, MD). Immunohistochemistry reagents were purchased from either Dako Corporation (Carpinteria, CA) or Richard-Allan Scientific (Kalamazoo, MI). Immunoblot polyvinylidene difluoride (PVDF) membrane was from Bio-Rad Laboratories (Hercules, CA). VEGF enzyme-linked immunosorbant assay (ELISA) kit was purchased from R&D Systems, Inc. (Minneapolis, MN). All cell culture related products were from Cellgro Mediatech, Inc. (Herndon, VA), unless otherwise specified.

2.2 CELL CULTURE, SIRNA, PLASMID TRANSFECTION

Human neuroblastoma cell lines display heterogeneous populations of cells akin to the complexity found in patient tumors [133]. There are three phenotypes described for these tumor-derived cell lines: (1) N-type cells are neuroblastic with neuroendocrine properties; (2) S-type cells are stromal and non-neuronal; and (3) I-type ("intermediate") cells are the most malignant with stem cell properties [133, 134]. **Table 2.1** highlights various characteristics of seven neuroblastoma cell lines, including phenotype and MYCN amplification status; while the protein expression of GRPR is shown in **Figure 2.1**. Even though studies have been performed in all the depicted cell lines, the majority of this work focuses on BE(2)-C and SK-N-SH, the cell lines with the highest and lowest GRPR levels, respectively (**Fig. 2.1**). BE(2)-C, IMR-32, SH-SY5Y, SK-N-MC and SK-N-SH were purchased from American Type Culture Collection (Manassas, VA). JF, a primary neuroblastoma cell line, was a gift

from Dr. Jason M. Shohet (Baylor College of Medicine, Houston, TX) and LAN-1 was a gift from Dr. Robert C. Seeger (University of Southern California, Los Angeles, CA). Cells were maintained in RPMI 1640 or EMEM medium with L-glutamine supplemented with 10% FBS. The cells were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

	BE(2)-C	IMR-32	JF	LAN-1	SH-SY5Y	SK-N-MC	SK-N-SH
Phenotype	I	N	N	N	N	S	N
Doubling Time (hrs)	18	20	U*	32	48	36	48
MYCN Amplification	High	High	High	High	Low	Low	Low
p53 Status	Mutant	Wild-type	Wild-type	Mutant	Wild-type	Mutant	Wild-type

TABLE 2.1 Characteristics of Neuroblastoma Cell Lines. Note that doubling time is variable and depends on factors such as methods and growth conditions; data from American Type Culture Collection and references [135-137]. *U = unknown.

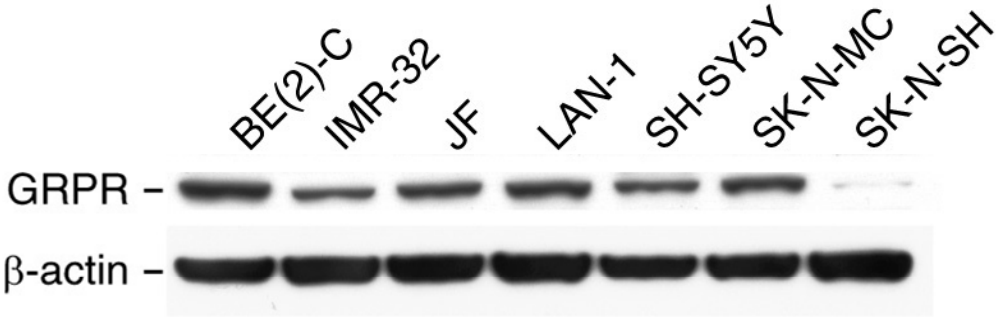

FIG. 2.1 GRPR expression in neuroblastoma cells. Western blot analysis of endogenous protein levels of GRPR in seven neuroblastoma cell lines. Cells were grown to confluence in RPMI 1640 or EMEM medium with L-glutamine supplemented with 10% FBS.

For siRNA transfection assays, 6-9 x 10^6 cells/400 μl (SK-N-SH, BE(2)-C) or 9 x 10^6 cells/200 μl (LAN-1) were transfected with siRNA by electroporation using Gene Pulser Xcell System (Bio-Rad, Hercules, CA) and seeded onto a 100mm dish. Setup conditions were 400V, 500 μF for BE(2)-C, SK-N-SH (250 V, 950 μF for some experiments); and 300V, 150 μF for LAN-1.

For experiments in Chapter 3: the day after transfection, the cells were replated onto a 6-well plate (2-5 x 10^5 cells/well). In order to clearly understand the effects of GRP on signaling transduction and to eliminate the effects of serum in neuroblastoma cells, all GRP and BBS treatments were performed using serum–free medium. The cells were seeded on culture plates and serum-starved overnight, then treated with GRP-H2756/BME (1 μM), LY294002 (20 μM), and/or GSK-3β inhibitors, SB216763 (10 μM) or SB415286 (30 μM), for 30 min prior to GRP/BBS (100 nM) stimulation for the indicated time periods. Cells were then harvested for immunoblot or cell cycle analysis.

For experiments in Chapter 4: the day following transfection, medium was replaced and the cells were replated onto 60 mm dishes (5-10 x 10^5 cells). Cells were harvested 2 and 3 days after transfection for various assays. For BBS treatment, the cells were seeded onto 6-well plates for 24 h and then maintained in serum-free conditions overnight. After BBS treatment, cells were harvested for immunoblotting. For GFP plasmid transfection, SK-N-SH cells were transfected with Lipofectamine 2000 according to the manufacturer's instructions. A stable cell line (GFP-SK-N-SH) was established with G418 treatment at a dose of 300 μg/ml for 2 weeks. The transfected clones were identified by FACS A219 cell sorter (BD Biosciences, San Jose, CA) on the basis of GFP fluorescence. GFP expression was ~70%, as

determined by fluorescence-activated cell sorting. GFP-SK-N-SH cells were then used for orthotopic xenografts.

For experiments in Chapter 5: All stably-transfected cells were selected with G418 (Cellgro) at 300 μg/ml and/or zeocin at 50 μg/ml for 2 weeks. For GRPR overexpression and silencing, pEGFP-GRPR and pENTR^{TM}/H1/TO (Invitrogen) were used, respectively. The sequence targeting GRPR (NM_005314) is underlined in the following shRNA sequence: 5'-CACCGTAACGTGTGCTCCAGTGGACGAATCCACTGGAGCACACGTTA-3', the non-specific control shCON was: 5'-CACCGGGCGCGCTTTGTAGGATTCGCCGAAGCGAA TCCTACAAAGCGCGCC-3'.

2.3 CELL PROLIFERATION ANALYSIS

Cells were seeded onto 96-well plates (8-12 x 10^3 cells/well) in RPMI 1640 or EMEM culture medium with 10% FBS. Cell viability was assessed 3 days after treatment using CCK-8 assay kit. In this tetrazolium-based assay, the formazan dye produced by metabolically active cells was quantified by measuring the absorption of dye solution in a scanning multi-well spectrophotometer at 450 nm. The values, corresponding to the number of viable cells, were read at OD 450 nm.

2.4 BRDU INCORPORATION ASSAY

Cells were seeded in 96-well plates at a density of 1-1.2 x 10^4 cells per well and BrdU incorporation was determined by Cell Proliferation ELISA. BrdU was added to the cell culture for 4-16 h prior to detection and BrdU incorporation was measured according to the manufacturer's instruction. The values, corresponding to the amount of DNA synthesis, were

read at OD450 with EL808 Ultra Microplate Reader (BioTek Instrument Inc., Winooski, VT).

2.5 FLOW CYTOMETRY ANALYSES

For cell cycle analysis, 1 x 10^6 cells were trypsinized, washed once with PBS, and fixed in 70% ethanol. Fixed cells were washed with PBS, incubated with 100 μg/ml RNAase for 30 min at 37°C, stained with propidium iodide (50 μg/ml), and analyzed on a FACScan flow cytometer (Becton-Dickinson Instruments, San Jose, CA). The percentages of cells in different cell cycle phases were analyzed using Cell-FIT software (Becton-Dickinson Instruments).

For cell size determination, the average cell size in 100,000-cell samples was assessed using CellQuest v3.3 software (BD Biosciences), according to a previous report [138]. The resulting parameter, mean forward scatter height (FSC-H), is a measure of relative cell size.

2.6 SOFT AGAR COLONY FORMATION ASSAY

Cells were trypsinized and resuspended in RPMI 1640 containing 0.4% agarose and 5% FBS. Cells were overlaid onto a bottom layer of solidified 0.8% agarose in RPMI 1640 containing 10% FBS, at cell concentrations of 5 x 10^3 cells/well and 2.5 x 10^3 cells/well of a 6-well plate, and incubated for 4 and 3 weeks, respectively. Colonies were stained with 0.05% crystal violet, photographed, and quantified.

2.7 XENOGRAFT STUDIES

Male athymic nude mice (4-6 weeks old) were obtained from Harlan Sprague Dawley (Indianapolis, IN) and housed in sterile cages in a temperature-controlled pathogen-free room with an alternating 12 h light and dark cycle. The mice were fed autoclaved chow and tap water *ad libitum* and allowed to acclimate for 1 week. All studies were approved by the Institutional Animal Care and Use Committee at UTMB.

In one experiment, mice were anesthetized using halothane and xenografts were established by subcutaneous (s.c.) injection of 2 x 10^6 SK-N-SH cells/100 µl HBSS onto the bilateral flanks using a 26-gauge needle. After two days, the mice were randomized into three experimental groups: I (vehicle control; 0.01 M acetic acid in saline) injection only], II (BBS; 10 µg/kg/injection), and III (BBS; 20 µg/kg/injection). All injections were administered intraperitoneal (i.p.) q 8 h. In a second experiment, the mice were anesthetized using halothane and a small left flank incision was created under sterile conditions to expose the kidney. An inoculum of 2 x 10^6 SK-N-SH cells in 0.1 ml of HBSS was injected into the superior pole of the left kidney using a 27-gauge needle and then the abdominal wound was closed in one layer with staples. After ten days, the mice were randomized into two experimental groups: I (control) and II (BBS; 20 µg/kg/i.p. injection, q 8 h). In a third experiment, xenografts were initiated by s.c. injection of 5 x 10^5 BE(2)-C cells in 0.1 ml of HBSS onto bilateral flanks of mice using a 26-gauge needle. Two days after injection, the mice were randomized into four groups: I (control), II (BBS; 20 µg/kg/s.c. injection, t.i.d.), III (BBS/GRP antagonist, RC-3095; 10 µg/kg/s.c. injection, q 12 h), and IV (BBS plus RC-3095). For all *in vivo* experiments, tumor growth was assessed bi-weekly by measuring the

two greatest perpendicular tumor dimensions with vernier calipers (Mitutoyo, Tokyo, Japan) and body weights were recorded weekly. The tumor volumes were calculated as follows: tumor volume (mm^3) = [tumor length (mm) x tumor width $(mm)^2$]/2. At sacrifice, tumors were excised, weighed, and snap frozen in liquid nitrogen for storage at -80ºC until the assay. Blood was also collected for ELISA.

2.8 RNA ISOLATION AND REAL TIME RT-PCR

Total cellular RNA extraction was carried out using RNAqueous kit and real-time RT-PCR was performed by the method previously described [139]. Total RNA was isolated using RNAqueous™ kit (Ambion) according to the manufacturer's instructions. Isolated RNA was used to synthesize cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Applied Biosystems assays-on-demand 20x assay mix of primers and TaqMan MGB probes (FAM™ dye-labeled) for the target genes, human GRP (NM_002091, Hs00181852_m1), human GRPR, human VEGF (NM_003376, Hs00173626_m1), and pre-developed 18S rRNA (VIC™-dye labeled probe) TaqMan® assay reagent (P/N 4319413E) were utilized. Human GRPR and glyceraldehyde 3-phosphate dehydrogenase (GAPDH)-specific oligonucleotide primers were the same as described [140]. Amplification was performed for 40 cycles (30 cycles for GAPDH) of 2 min at 94°C, 30 sec at 55°C, and 40 sec at 72°C.

2.9 IMMUNOBLOTTING

Whole-cell lysates or tissues were prepared as previously described [139, 141]. Cell lysis buffer (Cell Signaling) containing 1 mM PMSF and protein inhibitors cocktail (Roche

Applied Science) was used to extract total protein. The protein concentrations were quantified using Bio-Rad Protein Assay kit. Equivalent amounts of protein (30-100 μg) were electrophoresed on NOVEX NuPAGE 4-12% Bis-Tris gels (Invitrogen, Carlsbad, CA), electro-transferred to PVDF membranes (Bio-Rad), and probed with primary rabbit, mouse or goat anti-human antibodies (1:200-1000 dilutions) for 3 h at room temperature (RT) or overnight at 4°C. The membranes were washed and incubated with secondary antibodies (1:5000 dilution) conjugated with HRP. Immune complexes were visualized using the enhanced chemiluminescence system. Equal protein loading was confirmed by blotting the same membrane with β-actin antibody.

2.10 IMMUNOHISTOCHEMISTRY

Neuroblastoma xenografts were fixed in formalin overnight and embedded in paraffin wax. Tumor sections (5 μm) were mounted on glass slides. Sections were de-paraffinized with xylene, rehydrated with ethanol, antigen retrieval performed with 10 mM sodium citrate buffer, and then blocked with blocking solution, for 20 min at RT. Slides were incubated with primary antibodies (VEGF or PECAM-1) overnight at 4ºC. They were washed with buffer three times for 5 min each and incubated with secondary antibodies for 30 min at RT. Sections were developed with DAB reagent. The reaction was terminated by immersing slides in dH_2O and sections were counterstained in hematoxylin. Slides were then dehydrated with ethanol and xylene. Coverslips were mounted and slides were left to dry.

2.11 VEGF ELISA

For *in vitro* studies, supernatant of cultured cells was collected 3 days after transfection. For *in vivo* studies, mice blood was collected into tubes with EDTA to prevent coagulation, centrifuged at 2 x 10^3 rpm at RT for 15 min, and plasma was stored at -80ºC. For assay, plasma samples were thawed, centrifuged, and VEGF levels were measured using human VEGF ELISA kit according to manufacturer's instructions.

2.12 STATISTICAL ANALYSES

For *in vitro* experiments, conditions were compared using Student's paired t test. One way analysis of variance (ANOVA) on the ranks for repeated measures was performed for multiple comparisons. Tumor size and body weight were analyzed using analysis of variance (ANOVA) for a two-factor experiment with repeated measures on time in SK-N-SH *in vivo* experiments. All effects were assessed at the 0.05 level of significance and all interactions of the effects were assessed at the 0.15 level of significance as the experiment-wise error rates. Fisher's least significant difference procedure was used for multiple comparisons with 0.005 as the comparison-wise error rate. Data analysis was conducted using PROC MIXED with LSMEANS option and Satterthwaite approximation for the denominator degrees of freedom in SAS®, Release 9.1 (SAS Institute Inc., Cary, NC). In BE(2)-C *in vivo* experiments, the *p* values were analyzed by one-way ANOVA for comparison among the treatment groups. Image J (NIH) was used to perform the densitometric analysis of protein expression from immunoblots. For all experiments, a p value of < 0.05 was considered significant.

CHAPTER 3

PHOSPHATIDYLINOSITOL 3-KINASE REGULATION OF GASTRIN-RELEASING PEPTIDE-INDUCED CELL CYCLE PROGRESSION IN NEUROBLASTOMA CELLS

3.1 ABSTRACT

GRP, the mammalian equivalent of BBS, is an autocrine growth factor for neuroblastoma; its receptor is up-regulated in undifferentiated neuroblastomas. PI3K is a critical cell survival pathway; it is negatively regulated by the PTEN tumor suppressor gene. Our lab has recently found that poorly-differentiated neuroblastomas express decreased PTEN protein levels. Moreover, overexpression of the GRP receptor, a member of GPCR family, downregulates PTEN expression, resulting in increased neuroblastoma cell growth. Therefore, I sought to determine whether GRP or BBS activates PI3K in neuroblastoma cells (BE(2)-C, LAN-1, SK-N-SH). GRP or BBS treatment rapidly increased phosphorylation of Akt and GSK-3β in neuroblastoma cells. Inhibition of GRP receptor, with antagonist GRP-H2756 or siRNA, attenuated BBS-induced phosphorylation of Akt. LY294002, a PI3K inhibitor, also abrogated BBS-stimulated phospho-Akt as well as its cell cycle targets. GRP increased G1/S phase progression in SK-N-SH cells. BBS-mediated BrdU incorporation was blocked by LY294002. These findings identify PI3K as an important signaling pathway for GRP-mediated neuroblastoma cell growth. A novel therapy targeted at GRP/GRP receptor may prove to be an effective treatment option to inhibit PI3K in neuroblastomas.

3.2 INTRODUCTION

Neuroblastoma is the most common extracranial solid tumor in infants and children, accounting for more than 8-10% of all childhood cancers [2, 3, 91]. Many advances have been made in understanding the biology of this enigmatic tumor; however, despite current multimodal therapy, the overall prognosis for all stages of tumor is still dismal. There have been numerous studies directed towards known prognostic factors, such as MYCN amplification. However, neuroblastoma is a heterogeneous tumor with an unpredictable course, in spite of prognostic factors [2, 3]. Therefore, in regards to therapeutic agents, it is necessary to target entities that are generalized to tumor progression. The programs that direct cell survival in tumors are common to a wide range of tumor tissues, including the well-described PI3K pathway [105].

The PI3K pathway regulates cell growth in normal and cancer cells by inducing phosphorylation of its downstream effector, Akt [142, 143]. Cell cycle progression is one of the many survival pathways modulated by PI3K; this regulation involves PI3K/Akt inhibition of GSK-3β leading to the rescue and nuclear accumulation of cyclin D, an inducer of G1/S phase progression [142, 143]. PI3K can also regulate tumor suppressors p21 and p27, two negative regulators of the cell cycle, by promoting their phosphorylation and translocation to the cytoplasm; additionally, PI3K regulates the degradation of p27 [105, 143]. Our lab has previously shown that PTEN, a negative regulator of the PI3K pathway, is down-regulated in poorly differentiated neuroblastomas [101], which may contribute to a malignant phenotype.

Since neuroblastomas are neuroendocrine tumors, they secrete and respond to various hormones including GRP [42, 58]. Our lab has found that GRP, the mammalian equivalent of

BBS, stimulates neuroblastoma cell growth by an autocrine and/or paracrine effect [92]. Our lab has also found that the GRP receptor, a member of the GPCR family, is significantly increased in more undifferentiated neuroblastomas [92], and that overexpression of the GRP receptor down-regulates PTEN expression, resulting in increased neuroblastoma cell growth [101]. However, the intracellular signaling mechanisms involved in these GRP-mediated proliferative processes are not clear.

In this study, I sought to elucidate the cell survival mechanisms involved in GRP-induced neuroblastoma cell growth and whether the PI3K pathway is involved. Since the PI3K pathway is a positive regulator of cell cycle progression, I wanted to determine whether GRP activates this pathway and its downstream cell cycle regulators, thereby, amplifying the pro-growth effects of GRP. My findings demonstrate that inhibition of the GRP receptor or PI3K leads to significant decreases in Akt phosphorylation and modulates G1/S phase regulators cyclin D, p21, and p27.

Please refer to CHAPTER 2 for the materials and methods used in this chapter.

3.3 RESULTS

3.3.1 GRP/BBS INDUCES CELL CYCLE PROGRESSION AND PI3K-REGULATED BRDU INCORPORATION

GRP is an autocrine growth factor for neuroblastomas [58]. The first experiment determined whether the effects of GRP are associated with cell cycle progression in neuroblastoma cells by flow cytometry analysis using SK-N-SH cells treated with GRP for

16 h (**Fig. 3.1A**). GRP treatment resulted in a significant increase in the S phase, compared to control cells. BBS treatment also increased BrdU incorporation in SK-N-SH cells (**Fig. 3.1B**); increase was statistically significant at 48 and 72 h timepoints. LY294002 treatment with or without BBS strongly reduced BrdU incorporation, in comparison to control cells (**Fig. 3.1B**). Interestingly, control cells showed marked decrease in BrdU incorporation over a time course, likely as a result of serum-starvation. These data are consistent with the findings that GRP/BBS stimulates neuroblastoma cell growth by regulation of the cell cycle.

3.3.2 PI3K/AKT/GSK-3B PATHWAY IS ACTIVATED BY BBS TREATMENT

PI3K is a potent regulator of the cell cycle [142, 143]. In order to determine whether there is interaction between GRP and PI3K signaling, cells were treated with BBS or GRP over a time course and immunoblots were performed to assess phosphorylation of Akt and GSK-3α/β, downstream effectors for the PI3K pathway. Treatment with BBS or GRP rapidly increased phosphorylation of Akt and GSK-3β (up to 5-fold); the maximal stimulation was noted at 5 min (SK-N-SH, BE(2)-C) (**Figs. 3.2, 3.3**) and 15 min (LAN-1) after treatment (**Fig. 3.3**). To further confirm GRP activation of PI3K, cells were pretreated with GRP receptor antagonists GRP-H2756 or BME, a PI3K inhibitor LY294002, or GSK-3β inhibitors SB216763 or SB415286, for 30 min prior to BBS stimulation. Akt phosphorylation was decreased by GRP receptor antagonists and completely abrogated by LY294002 in BE(2)-C and SK-N-SH cells (**Fig. 3.2**). BBS-induced GSK-3β phosphorylation was also inhibited by SB216763 and SB415286 in SK-N-SH cells (**Fig. 3.2B**). Collectively, these results demonstrate that GRP activates the PI3K pathway.

3.3.3 MODULATION OF G1/S-PHASE REGULATORS BY GRP-MEDIATED PI3K STIMULATION

To determine a possible mechanism of cross-talk between GRP and cell cycle regulation, I next examined the effects of GRP stimulation on G1/S-phase cell cycle regulators cyclin D, Rb, p21, and p27. As shown in **Figure 3.3A**, GRP treatment increased expression of cyclin D (approximately 2-fold) and phosphorylation of Rb (approximately 2-fold), and decreased expression of the cyclin-dependent kinase inhibitors p21 (2-60 min) and p27 (5 and 30 min) in SK-N-SH cells. Similarly, increased expression of cyclin D (2 and 5 min) and decreased expression of p27 (2, 15, and 60 min) was noted in LAN-1 cells after BBS treatment (**Fig. 3.3B**). These results are consistent with the GRP-mediated increase of the G1/S phase noted by flow cytometry and DNA synthesis assessed by BrdU incorporation (**Fig. 3.1**). BBS-induced increases of the G1/S-phase regulator cyclin D in BE(2)-C cells were blocked with LY294002 (**Fig. 3.3C**). These data further confirm the critical role of the PI3K pathway in GRP-mediated cell cycle changes in neuroblastoma cells.

3.3.4 INHIBITION OF THE PI3K PATHWAY BY GRP RECEPTOR KNOCKDOWN

GRP-mediated PI3K activation was further confirmed using siRNA targeted to the GRP receptor. BE(2)-C, SK-N-SH, and LAN-1 cells were transfected with siRNA directed to the GRP receptor (siGRPR) or a non-targeting control (siNTC). Protein was collected 72 h post-transfection, immunoblotted, and probed for phospho-Akt, Akt, and GRP receptor. β-actin was used as a loading control. GRP receptor siRNA decreased BBS-induced Akt phosphorylation in all three neuroblastoma cell lines (**Fig. 3.4**). These data further support the finding that PI3K is activated by GRP.

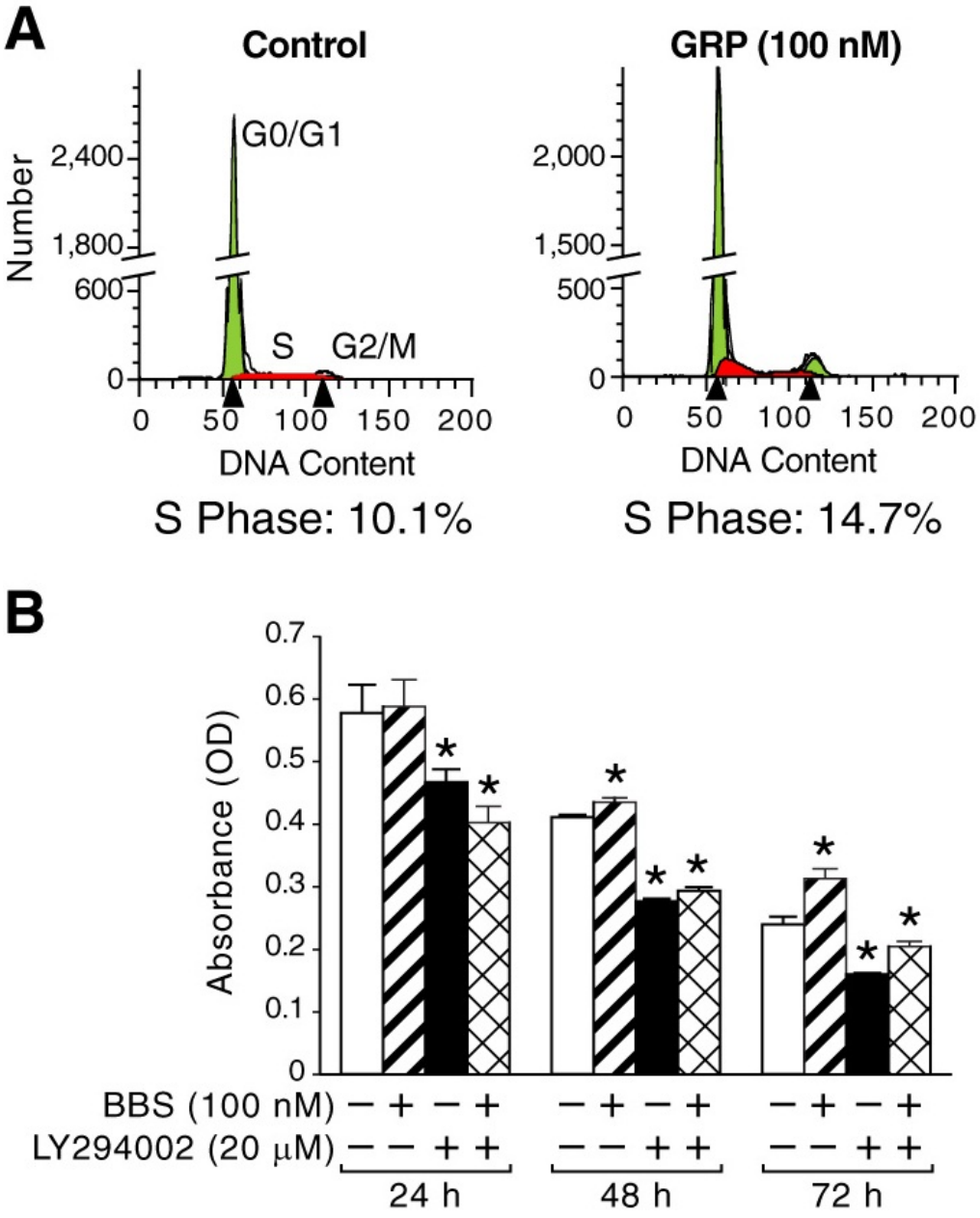

FIG. 3.1 GRP/BBS treatment induces S-phase progression and PI3K-regulated BrdU incorporation in neuroblastoma cells. (A) SK-N-SH cells were serum starved for 24 h prior to treatment with GRP for 16 h. GRP caused an increase in the S and G2/M phases, in comparison to control cells. **(B)** SK-N-SH cells were serum starved for 24 h prior to treatment with BBS and/or LY294002 over a time course (24-72 h). BBS induced BrdU incorporation at 48 and 72 h; this response was blocked significantly with LY294002. Data represent mean ± SEM; * $p < 0.05$ vs. control.

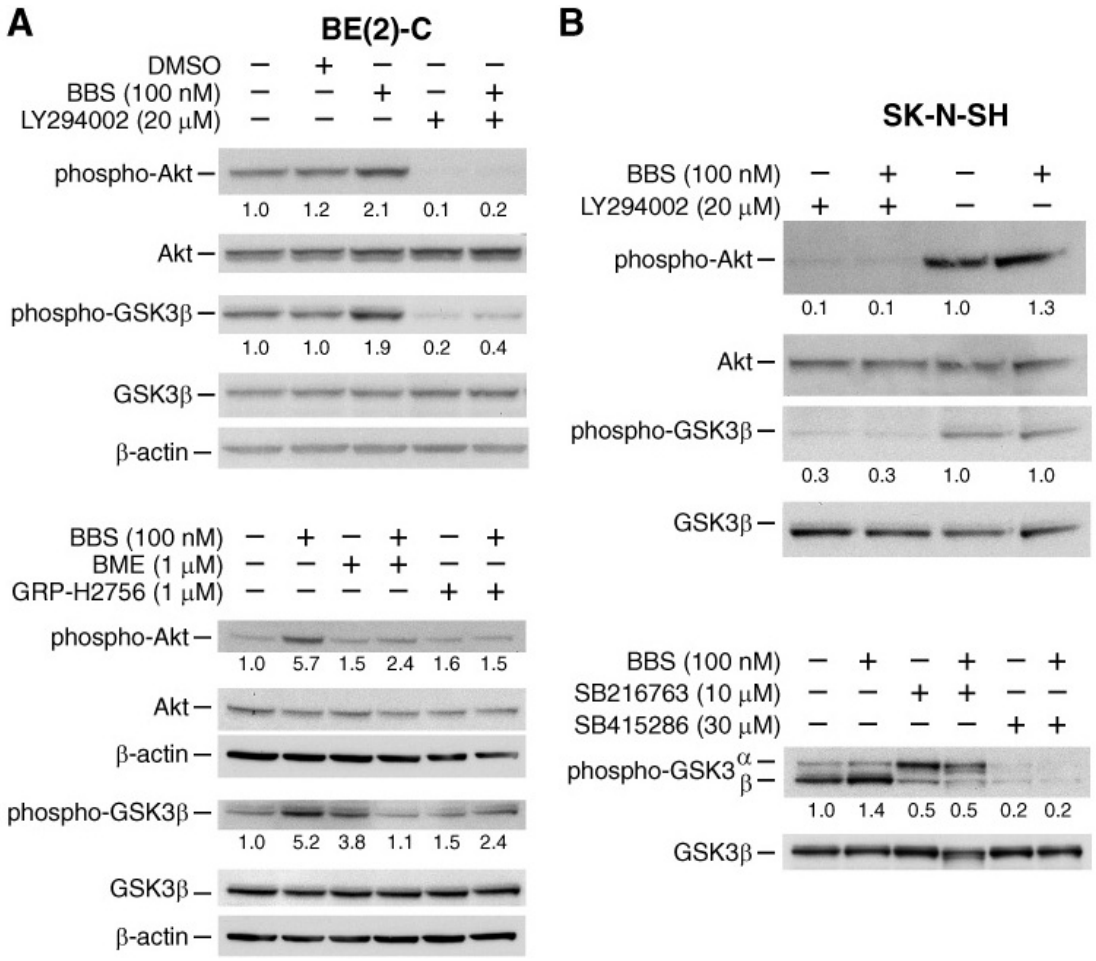

FIG. 3.2 BBS treatment causes activation of PI3K/Akt/GSK-3β pathway in neuroblastoma cells. (A, B) BBS treatment caused rapid phosphorylation of Akt and GSK-3β, which was decreased by GRP receptor antagonists and completely abrogated by LY294002. **(B)** BBS-induced GSK-3β phosphorylation was also inhibited by SB216763 and SB415286. Values under blots represent protein fold-change levels (relative to respective β-actin) in comparison to control lane.

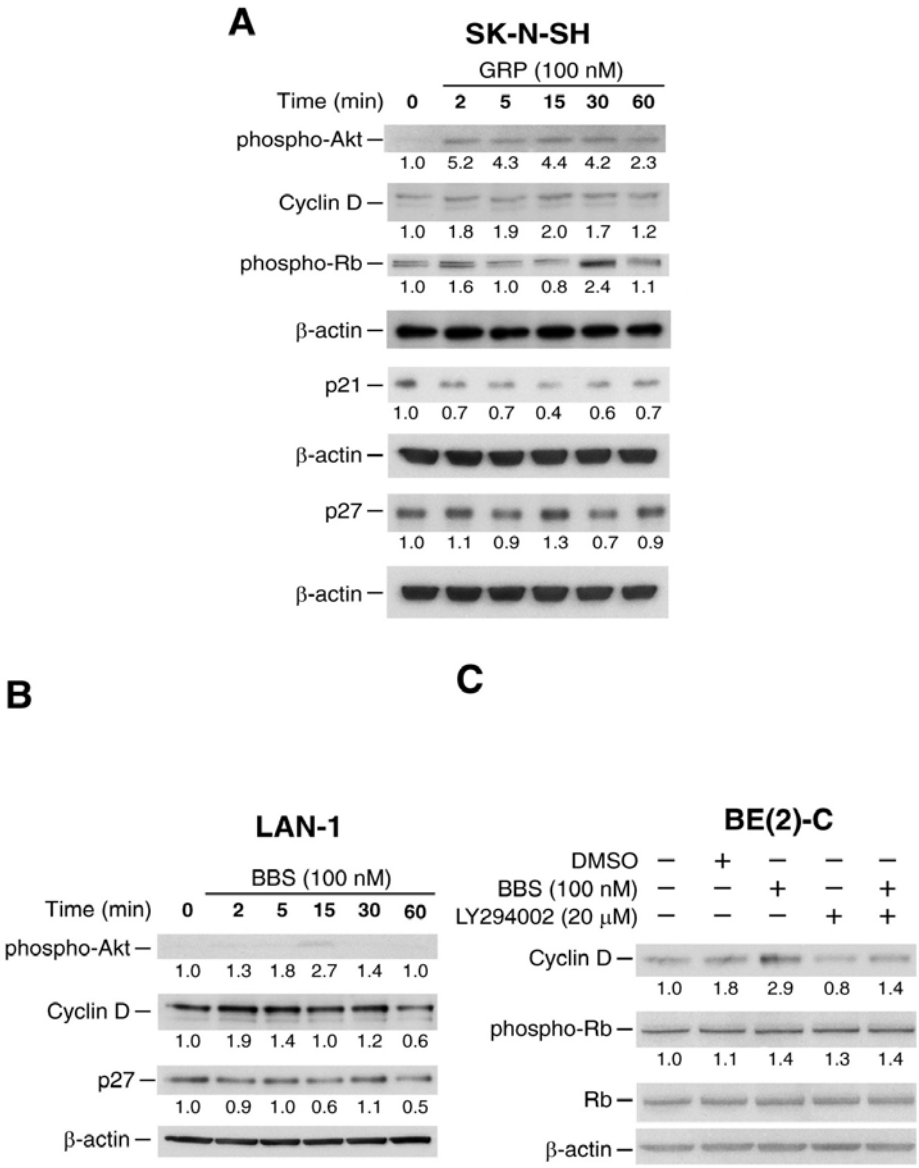

FIG. 3.3 GRP/BBS modulates G1/S-phase regulators in neuroblastoma cells by the PI3K pathway. (A) SK-N-SH cells demonstrated increased phospho-Akt, cyclin D and phospho-Rb expression, while displaying decreased p21, and p27, with GRP treatment. **(B)** LAN-1 cells exhibited increased phospho-Akt and cyclin D and decreased p27 with BBS treatment. **(C)** BBS-induced changes to G1/S-phase regulator cyclin D in BE(2)-C cells were blocked with LY294002 (membrane in Figure 3.2A was stripped and reprobed). Values under blots represent protein fold-change levels (relative to respective β-actin) in comparison to control lane.

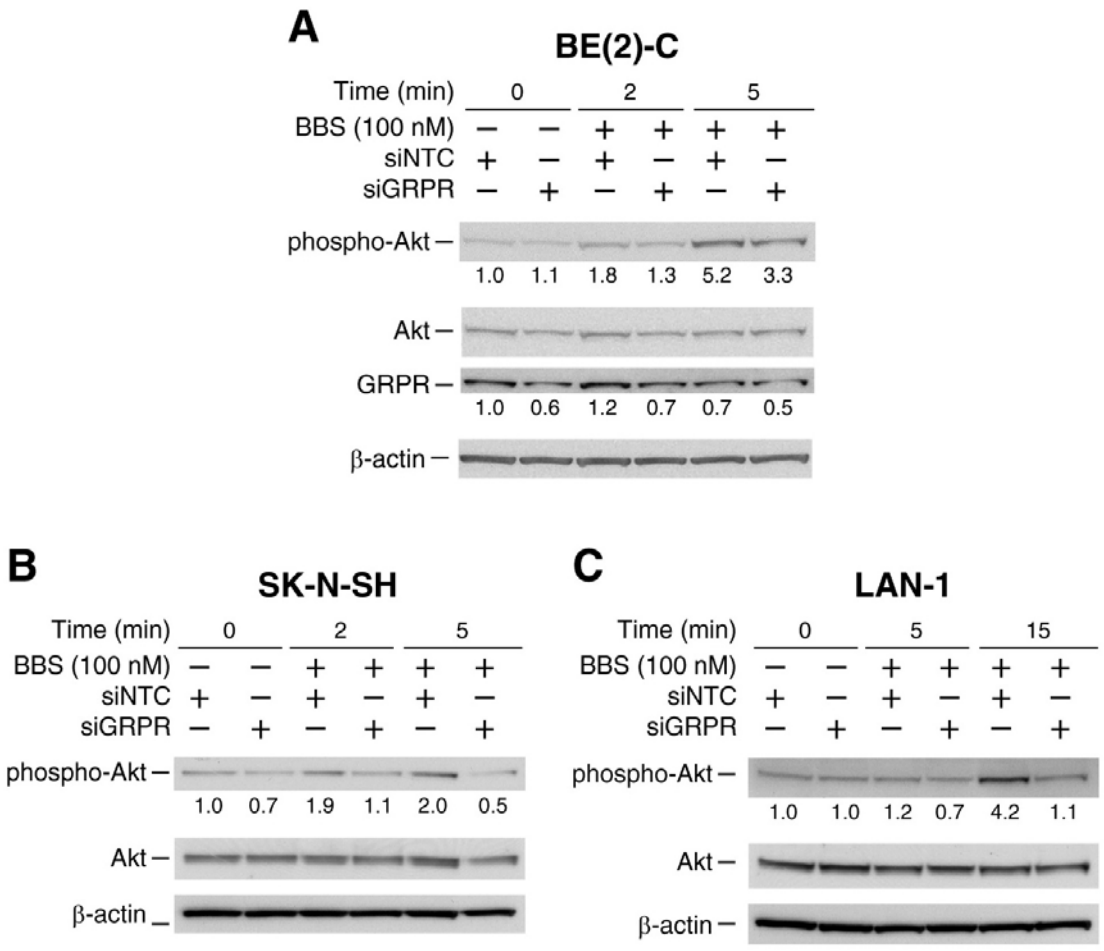

FIG. 3.4 Knockdown of GRP receptor inhibits PI3K activation. (A) BE(2)-C cells were transfected with siGRPR or siNTC. Protein was collected 72 h post-transfection. Knockdown of GRP receptor caused a decrease in BBS-induced Akt phosphorylation. **(B, C)** SK-N-SH and LAN-1 cells also demonstrated decrease in phospho-Akt by siGRPR. Values under blots represent protein fold-change levels (relative to respective β-actin) in comparison to control lane.

3.4 DISCUSSION

The signaling mechanisms involved in GRP-induced neuroblastoma cell growth are not fully understood. Based on our lab's previous study demonstrating a relationship between undifferentiated neuroblastomas and deregulation of both GRP and PI3K pathways, I sought to determine whether these two pathways converge to regulate the cell cycle in neuroblastoma cells. I found that GRP activates the PI3K pathway to induce cell cycle progression by modulating G1/S-phase regulators (**Fig. 3.5**). Moreover, inhibition of PI3K blocked the GRP/BBS-mediated alterations to cell cycle proteins and inhibited BBS-induced BrdU incorporation. Therefore, the growth stimulatory properties of GRP may be due, in part, to PI3K regulation of the cell cycle.

PI3K pathway regulation of the G1/S-phase is well established [144]; therefore, I analyzed the effect of PI3K activation on the expression of cyclin D, p21, and p27. Upon phosphorylation by GSK-3β, cyclin D translocates to the cytosol where it undergoes protein degradation [105, 143]. PI3K-mediated phosphorylation of GSK-3β, and resultant inhibition, leads to the nuclear accumulation of cyclin D which promotes cell cycle progression [143]. PI3K/Akt has also been shown to be important in regulating cyclin D gene transcription and translation [143]. My results confirmed that cyclin D protein expression is increased with PI3K activation by GRP treatment and decreased upon addition of its pathway inhibitor (**Fig. 3.3**). Akt phosphorylation of cyclin-dependent kinase inhibitors p21 and p27 also induces the progression of G1/S phase. Once phosphorylated, p21 and p27 are translocated and sequestered in the cytoplasm; p27 may also undergo proteolysis by indirect Akt stimulation [105, 143]. Here, I show that GRP/BBS stimulation decreased levels of p21 and p27 in SK-

N-SH cells and decreased p27 in BE(2)-C and LAN-1 cells (**Fig. 3.3**). These findings were further corroborated by the increase in G1/S phase (**Fig. 3.1A**), as recorded by flow cytometry, and BrdU incorporation (**Fig. 3.1B**), a marker for DNA synthesis. Notably, levels of p21 could not be detected in BE(2)-C and LAN-1 cells; I speculate that this is most likely due to their $p53^{-/-}$ genotype [145, 146]. Since p53 regulates p21 gene transcription [147, 148]; the absence of p53 in these cell lines may explain the lack of p21 expression. LY294002 inhibition of BrdU incorporation (**Fig. 3.1B**) reinforces the role of PI3K in this GRP-mediated process.

I have shown that GRP promotes rapid activation of the PI3K pathway by Akt phosphorylation (**Fig. 3.2**). The GRP receptor is a member of the GPCR family and has been associated with phospholipase C and Ca^{2+} second messenger signaling [92, 149]. In lymphocytes, PI3K is coupled to GPCRs through direct interaction with the $G_{\beta\gamma}$ protein subunits [109]. It is unclear whether the GRP receptor activates the PI3K pathway directly or through indirect cross-talk mechanisms involving other signal transduction pathways. In a study of head and neck squamous cancer cells, Lui et al. [150] found that the stimulatory effects of GRP were mediated by the epidermal growth factor receptor (EGFR)/MAPK pathway. Sumitomo et al. [151] also found that the mitogenic effects of BBS could be attributed to the insulin-like growth factor receptor (IGFR) leading to PI3K activation in prostate cancer cells. This implicates GRP signaling in the transactivation and indirect stimulation of major proliferation and survival pathways. Neuroblastoma cells also express EGFR and IGFR [152, 153]. Further studies of GRP receptor signaling would help delineate

the downstream targets regulating neuroblastoma growth; I believe that both direct and indirect mechanisms are possible.

In summary, I have shown that GRP/BBS rapidly activated the PI3K pathway; this was associated with cell cycle progression. Inhibition of the GRP receptor prevented BBS-induced activation of the PI3K pathway, further suggesting that PI3K is an important signaling mechanism for GPCR-mediated stimulation of neuroblastoma cell growth. A novel therapy targeted to the GRP receptor may prove to be an effective combinational treatment option to inhibit PI3K activation in neuroblastomas.

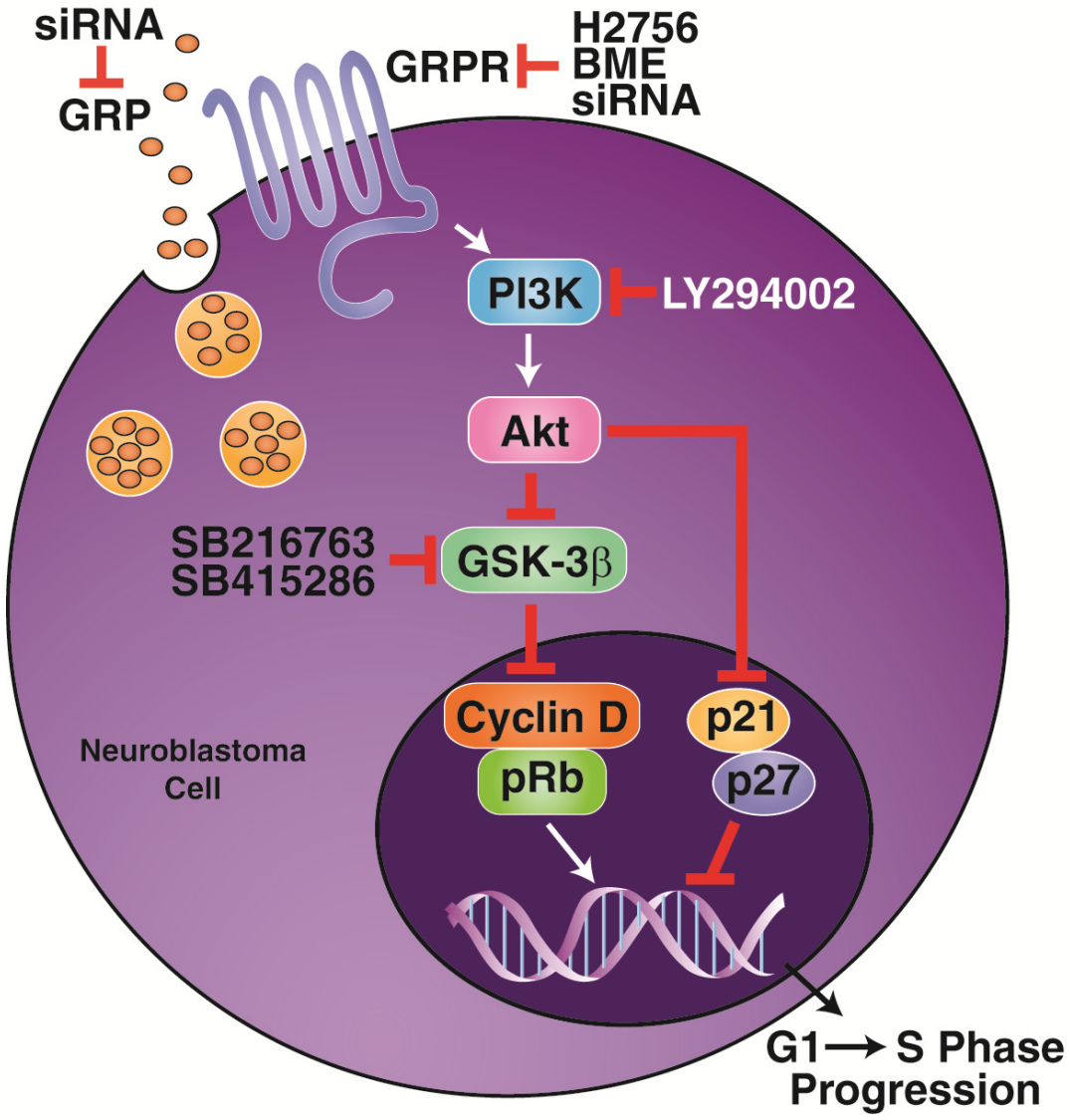

FIG. 3.5 PROPOSED MECHANISM OF GRP/GRPR-MEDIATED CELL CYCLE PROGRESSION IN NEUROBLASTOMA CELLS.

CHAPTER 4

BOMBESIN INDUCES ANGIOGENESIS AND NEUROBLASTOMA GROWTH

4.1 ABSTRACT

GRP, the mammalian equivalent of BBS, is a trophic factor for highly vascular neuroblastomas; its mechanisms of action *in vivo* are unknown. This study sought to determine the effects of BBS on the growth of neuroblastoma xenografts and on angiogenesis. BBS significantly increased the growth of SK-N-SH and BE(2)-C human neuroblastomas; tumors demonstrated increased expression of angiogenic markers, PECAM-1 and VEGF, as well as phosphorylated (p)-Akt levels. RC-3095, a BBS/GRP antagonist, attenuated BBS-stimulated tumor growth and angiogenic markers *in vivo*. GRP or GRPR silencing significantly inhibited VEGF expression as well as phosphorylation of Akt and mTOR *in vitro*. Our findings demonstrate that BBS stimulates neuroblastoma growth and the expression of angiogenic markers. Importantly, these findings suggest that novel therapeutic agents, targeting BBS-mediated angiogenesis, may be useful adjuncts in patients with advanced-stage neuroblastomas.

4.2 INTRODUCTION

Neuroblastoma is the most common extracranial solid tumor in infants and children, accounting for more than 8-10% of all childhood cancers [2]. In particular, patients older

than one year of age often present with more aggressive disease and have a dismal prognosis [2, 42]. This subset of advanced-stage tumors, and the resultant metastases, are characterized by florid vascularization, which contributes to a rapid tumor progression [154, 155]. Vascular endothelial growth factor (VEGF) is a key mediator of carcinogenic neovascularization, and has been associated with poor prognosis in solid tumors [155-159]. VEGF and its receptors are also expressed in human neuroblastoma tumors and cell lines [155, 160, 161]. Higher levels of VEGF in neuroblastomas correlate with unfavorable histology and aggressive tumor behavior [155, 161]. However, the exact cellular mechanisms involved in stimulation of angiogenesis and tumor progression in neuroblastomas are relatively unknown.

Derived from neuroendocrine precursor cells, neuroblastomas produce and respond to various hormones, including GRP [58]. Our lab has previously shown that GRP stimulates neuroblastoma cell growth by an autocrine and/or paracrine mechanism [92]. Our lab also found that the expression of GRPR, a member of the GPCR family, is notably increased in poorly-differentiated neuroblastomas [92]. BBS, the amphibian analogue of GRP, also binds to GRPR with high affinity to stimulate growth [54, 79, 162, 163]. However, the intracellular signaling mechanisms involved in these peptide-mediated proliferative processes are unclear.

This study sought to determine the trophic effects of BBS on the growth of neuroblastoma xenografts and to further elucidate BBS-mediated mechanisms of angiogenesis. It was determined that exogenous BBS treatment enhances neuroblastoma growth and stimulates mediators of the angiogenic pathway, whereas, inhibition of BBS/GRP with an antagonist, RC-3095, suppresses tumor progression and vascularization. Moreover,

small interfering RNA against GRP (siGRP) or GRPR (siGRPR) decreased VEGF expression and secretion in neuroblastoma cells.

Please refer to CHAPTER 2 for the materials and methods used in this chapter.

4.3 RESULTS

4.3.1 BOMBESIN INCREASES SK-N-SH CELL PROLIFERATION AND VEGF EXPRESSION

BBS is a trophic factor for a host of cancers, including breast, colon, and prostate [54]; its role in angiogenesis in neuroblastoma is not well understood. This experiment sought to determine the trophic and angiogenic effects of BBS on human neuroblastoma cells. BBS treatment for 3 days significantly increased cell viability by 15% in SK-N-SH cells (**Fig. 4.1A**). As shown in **Figure 4.1B**, this increase was associated with a nearly 5 fold upregulation of VEGF expression. In addition, p-Akt, a known upstream regulator of VEGF [164], was rapidly increased by 7 fold at 5 min after BBS treatment (**Fig. 4.1C**). Similar to SK-N-SH, another human neuroblastoma cell line, BE(2)-C, also showed a significant increase in cell viability after BBS treatment (data not shown). These results demonstrate that BBS is an important stimulator of the angiogenic factors and proliferation in neuroblastoma cells.

4.3.2 BOMBESIN STIMULATES GROWTH OF SK-N-SH XENOGRAFTS

To further assess the growth stimulatory actions of BBS on neuroblastoma, the next experiment examined the effects of BBS on neuroblastoma xenografts. SK-N-SH cells were injected onto bilateral flanks subcutaneously in athymic nude mice and then mice were

randomized to receive either vehicle or BBS. Tumor volume was measured twice weekly and the xenografts were observed daily until sacrifice. As shown in **Figure 4.2A**, SK-N-SH tumor volume was significantly increased by treatment day 40 in mice receiving BBS injections at a dosage of 20 μg/kg q 8 h when compared to the control group receiving vehicle alone. Mice receiving BBS at a lower dosage of 10 μg/kg/injection also showed appreciably enhanced tumor volume by treatment day 40; this significant growth stimulatory effect of BBS was delayed to day 43 when compared to the higher dosage of BBS. The trophic actions of BBS were sustained until the end of treatment at day 45. Interestingly, BBS stimulation of SK-N-SH tumor growth was similar with either 10 or 20 μg/kg dosage after 43 days of treatment. At sacrifice, tumor weight in mice receiving BBS correlated with tumor volume data; however, a statistical difference in tumor weight was noted only in the higher BBS dosage group (20 μg/kg) due to considerable tumor size variability (**Fig. 4.2B**). The next experiement was to further examine the trophic actions of BBS in orthotopically-established SK-N-SH tumors. Based on our previous experiment, the BBS dosage of 20 μg/kg/injection was chosen. Similar to the heterotopic xenograft study, significantly increased growth was noted in the orthotopic xenografts of mice treated with BBS at 6 weeks (**Figs. 4.2C, D**), further confirming the role of BBS as a trophic factor for neuroblastoma growth *in vivo*.

4.3.3 BOMBESIN INDUCES THE EXPRESSION OF ANGIOGENIC MARKERS IN SK-N-SH *IN VIVO*

In order to elucidate the effects of BBS on angiogenesis *in vivo*, SK-N-SH heterotopic tumor sections were analyzed for VEGF, an important signaling protein involved in both

vasculogenesis and angiogenesis, and PECAM-1, a marker for microvessels. As shown in **Figure 4.3A**, BBS-treated tumors showed enhanced expression of VEGF. Moreover, BBS-treated tumors also showed increased expression of PECAM-1 (**Fig. 4.3B**). For both immunohistochemical studies, VEGF and PECAM-1 appeared as dark brown staining in blood vessels and adjacent tissues. In addition, there was also an enhanced phosphorylation of Akt by nearly 2-fold in SK-N-SH tumors as noted by Western blot analysis at sacrifice following BBS treatment (**Fig. 4.3C**). These data confirm our *in vitro* findings and further support our hypothesis that BBS regulates angiogenic markers in neuroblastoma.

4.3.4 GRP ANTAGONIST INHIBITS BE(2)-C GROWTH AND VEGF EXPRESSION *IN VIVO*

BBS upregulation of angiogenic markers was further confirmed using an antagonist to BBS/GRP *in vivo*. Human neuroblastoma BE(2)-C cells were implanted subcutaneously in athymic nude mice. Mice were then randomized to receive vehicle, BBS, and/or the BBS/GRP antagonist, RC-3095. As shown in **Figure 4.4A**, RC-3095 treatment significantly suppressed BE(2)-C neuroblastoma growth when compared to BBS treatment alone or control group. Additionally, RC-3095, in combination with BBS, also attenuated tumor growth comparable to control group (**Fig. 4.4A**). Western blot analysis showed induction of VEGF (3.2-fold) protein expression and increased phosphorylation of Akt (2.2-fold) in BBS-treated mice when compared to vehicle treated mice (**Fig. 4.4B**). Correlative to tumor volume inhibition, RC-3095 treatment also blocked BBS-mediated VEGF expression and Akt phosphorylation (**Fig. 4.4B**). When tumor sections were examined for VEGF protein expression using immunohistochemistry, RC-3095 treatment significantly attenuated BBS-induced increases in VEGF expression in BE(2)-C xenografts (**Fig. 4.4C**), further suggesting

an important role of BBS as an inducer of angiogenesis in neuroblastomas. As shown in **Figure 4.4D**, ELISA analysis of plasma VEGF levels also clearly demonstrated significantly suppressed levels of plasma VEGF with RC-3095 treatment, alone and/or in combination with BBS. This further suggests BBS as a pro-angiogenic factor for neuroblastomas *in vivo*. In all three *in vivo* experiments, mice tolerated the injections of either BBS or RC-3095 without any detectable systemic toxicity.

4.3.5 GRP/GRPR SILENCING INHIBITS ANGIOGENIC EFFECTORS IN NEUROBLASTOMA CELLS

The next experiment assessed the effects of endogenous GRP or GRPR knockdown on VEGF expression. SK-N-SH and BE(2)-C cells were transfected with either siGRP, siGRPR or siNTC. Efficacy of siGRP transfection in SK-N-SH cells was confirmed by quantitative RT-PCR, which showed significantly decreased GRP mRNA levels (**Fig. 4.5A**). Transfection efficacy of siGRP in BE(2)-C cells was confirmed by Western blotting (**Fig. 4.5B**). Western blot analysis showed a significant decrease in VEGF protein expression with GRP silencing when compared to siNTC in both SK-N-SH and BE(2)-C cells (**Fig. 4.5A, B**). This was also associated with a decrease in upstream regulators of the angiogenic pathway, p-Akt and p-mTOR [164] (**Fig. 4.5A, B**). As shown in **Figure 4.5C**, VEGF mRNA levels were significantly decreased in BE(2)-C cells transfected with siGRP, suggesting regulation of VEGF at the transcription level. This decrease in mRNA levels correlated with marked reduction in VEGF secretion into cell culture media with GRP knockdown (**Fig. 4.5C**). Since GRP binds with high affinity to its receptor GRPR, BE(2)-C cells were transfected with siGRPR to confirm GRP/GRPR-mediated angiogenic effects in neuroblastoma cells.

Transfection efficacy of siGRPR in BE(2)-C cells was confirmed by Western blotting. There was also a significant decrease in VEGF protein expression with GRPR silencing when compared to siNTC-transfected cells (**Fig. 4.5D**). Consistent with the effects of GRP silencing on VEGF mRNA and secretion, GRPR knockdown in BE(2)-C cells also showed a reduction of VEGF mRNA as well as its peptide secretion into the cell culture media (**Fig. 4.5D**), further confirming that specific GRP binding to its cell surface receptor, GRPR, is critical to induce VEGF activation in neuroblastoma cells. In addition, similar to our *in vivo* findings with RC-3095, GRP-silenced neuroblastoma cells showed a significant decrease in cell viability in comparison to control cells (data not shown). These results further suggest an angiogenic role for GRP in neuroblastoma progression.

4.4 DISCUSSION

In this study, BBS treatment enhances tumor growth and stimulates the expression of angiogenic markers in neuroblastoma, both *in vivo* and *in vitro*. Inhibition of BBS/GRP with an antagonist, RC-3095, or siRNA significantly suppresses tumor progression and vascularization. Moreover, BBS induces activation of Akt, a major effector of PI3K, suggesting an important role for this signaling pathway in the regulation of angiogenesis and growth in neuroblastoma.

Neuroblastomas are archetypical solid tumors, where vascular proliferation is a hallmark and corresponds to tumor prognosis [154, 155]. The significance of neovascularization in neuroblastomas is supported by numerous studies on anti-angiogenic

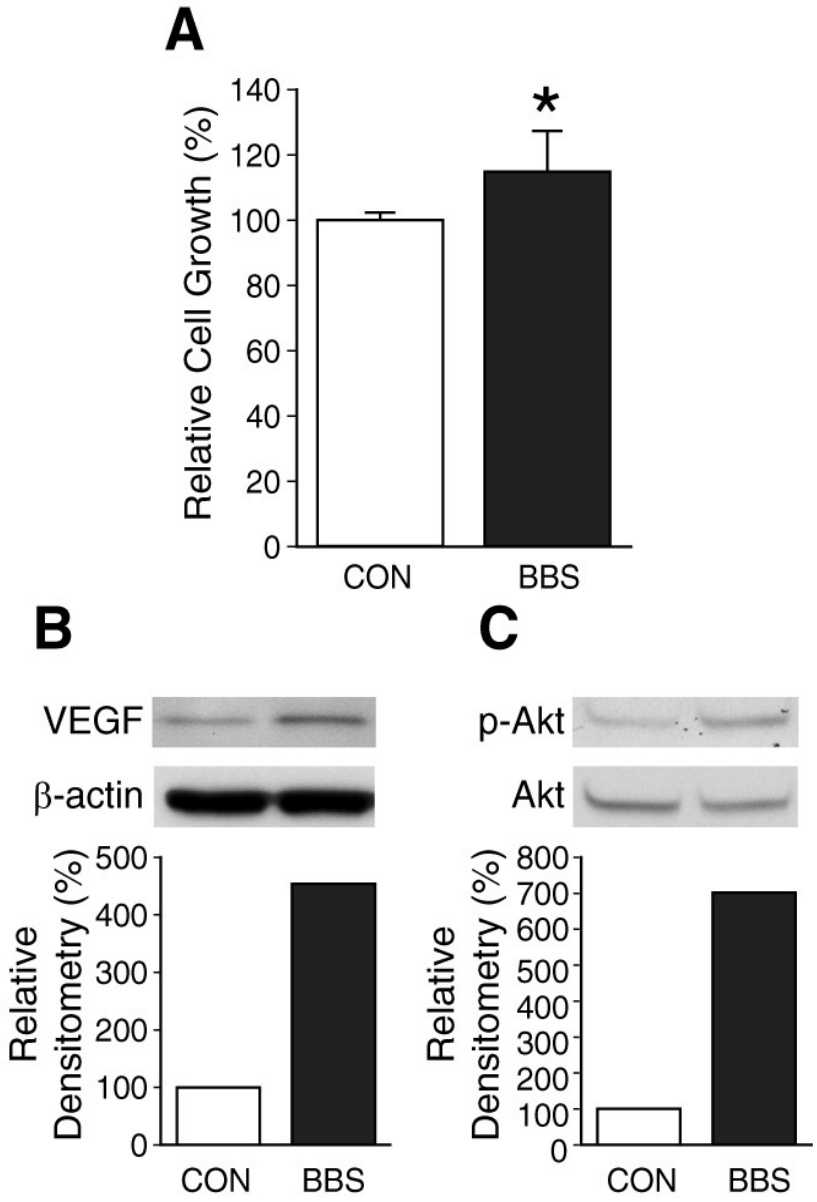

FIG. 4.1 BBS stimulates cell proliferation, VEGF and p-Akt expression in SK-N-SH cells. (A) Effects of BBS (10^{-7} M) on SK-N-SH cell viability were determined using CCK-8 kit (Data represent mean ± SEM values of eight replicate experiments; * $p <$ 0.05 vs. control). **(B)** SK-N-SH cells were treated with BBS (10^{-7} M) for one day after overnight serum-free conditions. Cell lysates were prepared and analyzed by Western blot for VEGF. **(C)** SK-N-SH cells were treated with BBS (10^{-7} M) for 5 min after overnight serum-free condition. Cell lysates were prepared and analyzed by Western blot for p-Akt and total Akt. Experiments were repeated on two separate occasions.

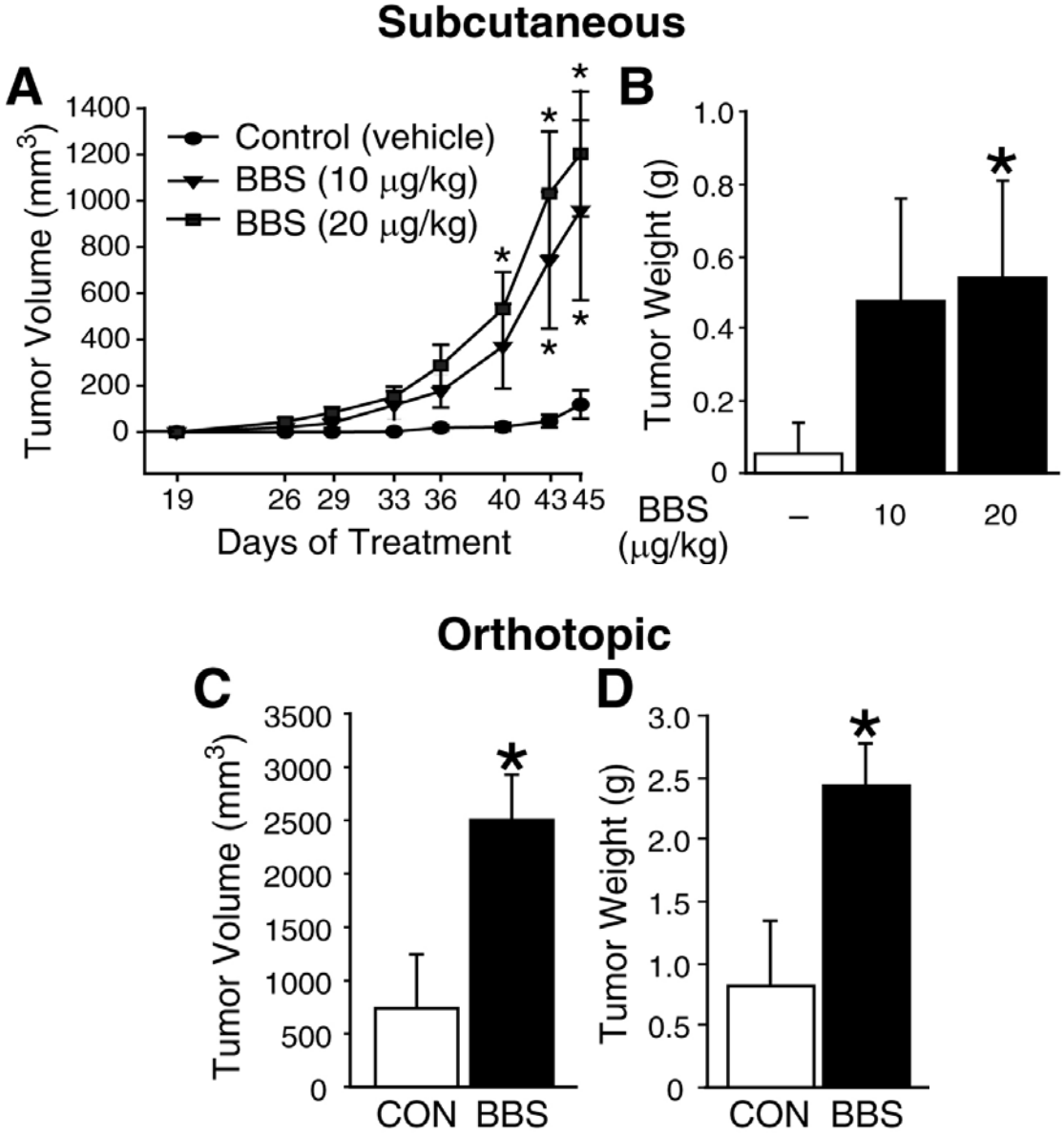

FIG. 4.2 BBS promotes SK-N-SH growth *in vivo*. (A, B) Tumor volumes and tumor weights of subscapular SK-N-SH xenografts in mice treated with vehicle or BBS (i.p.) for 45 days as described in "Materials and Methods" (4-6 mice/group). **(C, D)** Tumor volumes and tumor weights of orthotopic SK-N-SH xenografts at sacrifice, established by injecting cells into left kidney of mice and treated with vehicle or BBS (20 μg/kg/injection; i.p.) for 43 days as described in "Materials and Methods" (5-6 mice/group). Data from all figures represent mean ± SEM; * $p < 0.05$ vs. control.

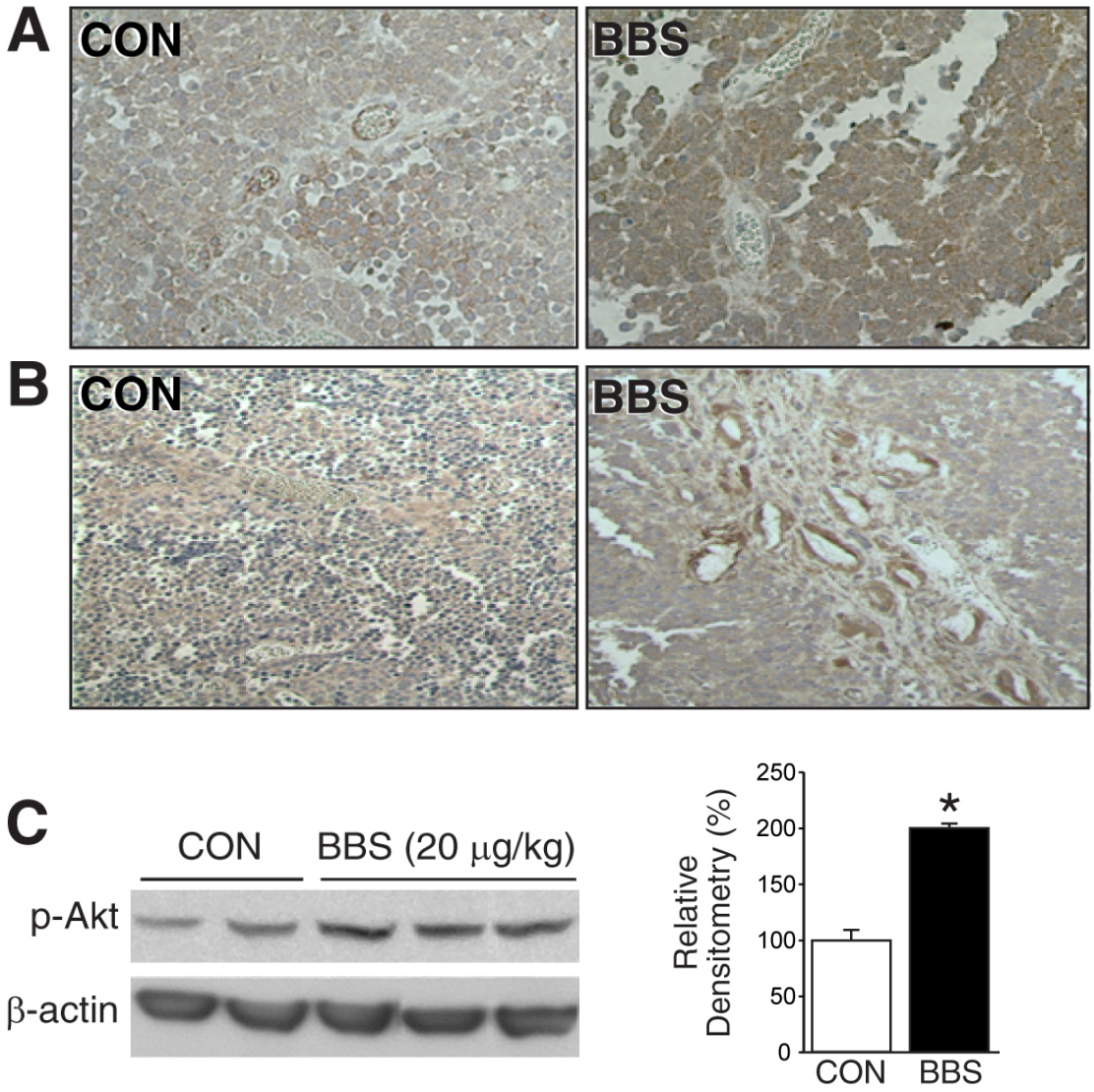

FIG. 4.3 BBS induces angiogenesis in SK-N-SH xenografts. (A) Representative sections of SK-N-SH tumors from vehicle- or BBS-treated mice stained with anti-human VEGF antibody (brown); magnification, X400. **(B)** Representative sections of SK-N-SH tumors from vehicle- or BBS-treated mice stained with anti-human PECAM-1 antibody (brown); magnification X200. **(C)** Expression of p-Akt in SK-N-SH tumor tissue samples from mice treated with BBS or vehicle; five representative tumors are shown (Data represent mean ± SEM; * $p < 0.05$ vs. control).

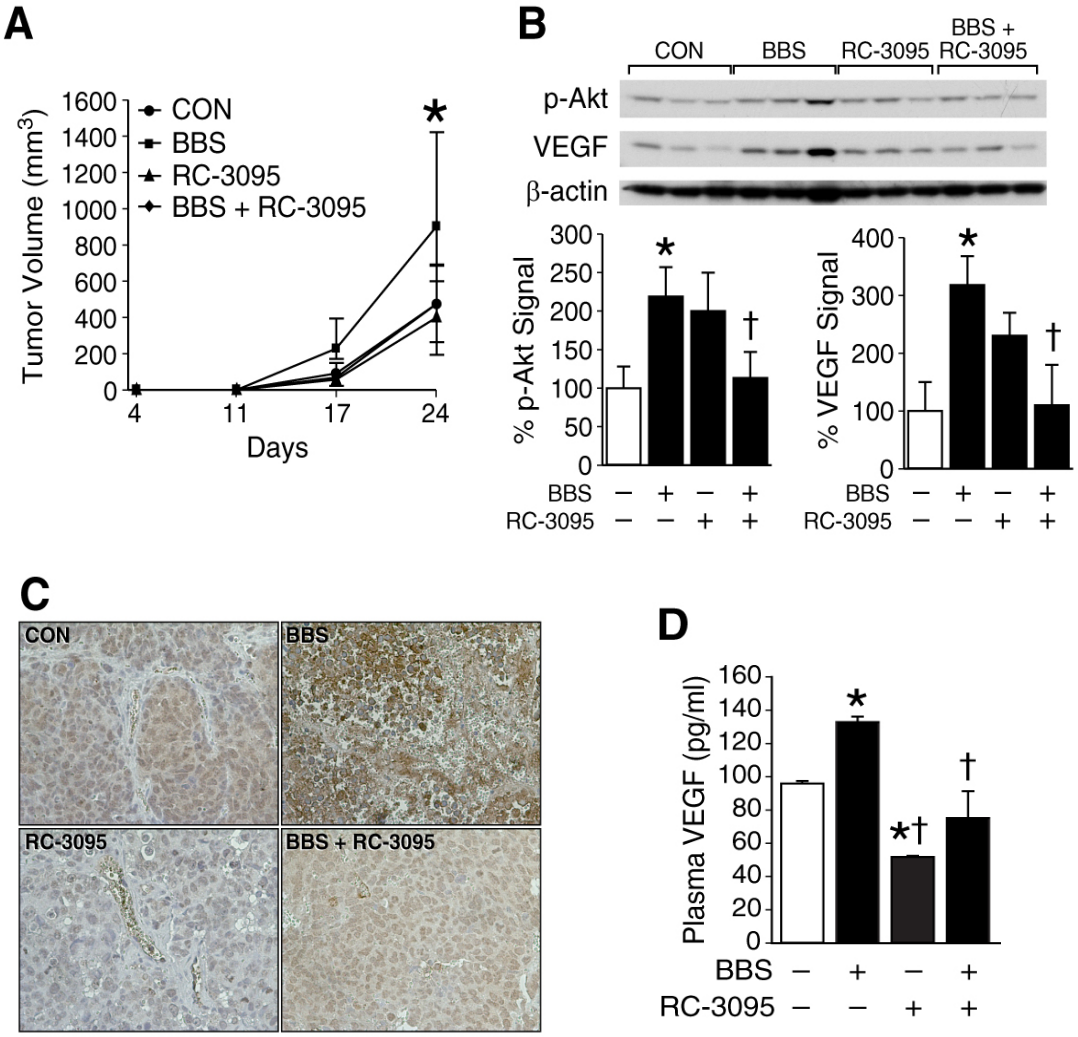

FIG. 4.4 GRP antagonist inhibits BE(2)-C tumor growth and angiogenesis. **(A)** Tumor volumes in nude mice with BE(2)-C cells treated with vehicle, BBS (20 µg/kg/injection; s.c., t.i.d.), and/or RC-3095 (10 µg/kg/injection; s.c., q 12 h), as described in "Materials and Methods" (5-6 mice/group). **(B)** Expression of p-Akt and VEGF in BE(2)-C tumor tissue samples (three representative tumor samples from each group are shown). **(C)** Representative sections of BE(2)-C tumors stained with anti-human VEGF antibody (brown); magnification, X400. **(D)** VEGF plasma levels from mice detected by ELISA. Data from all figures represent mean ± SEM; * $p < 0.05$ vs. control. † $p < 0.05$ vs. BBS alone.

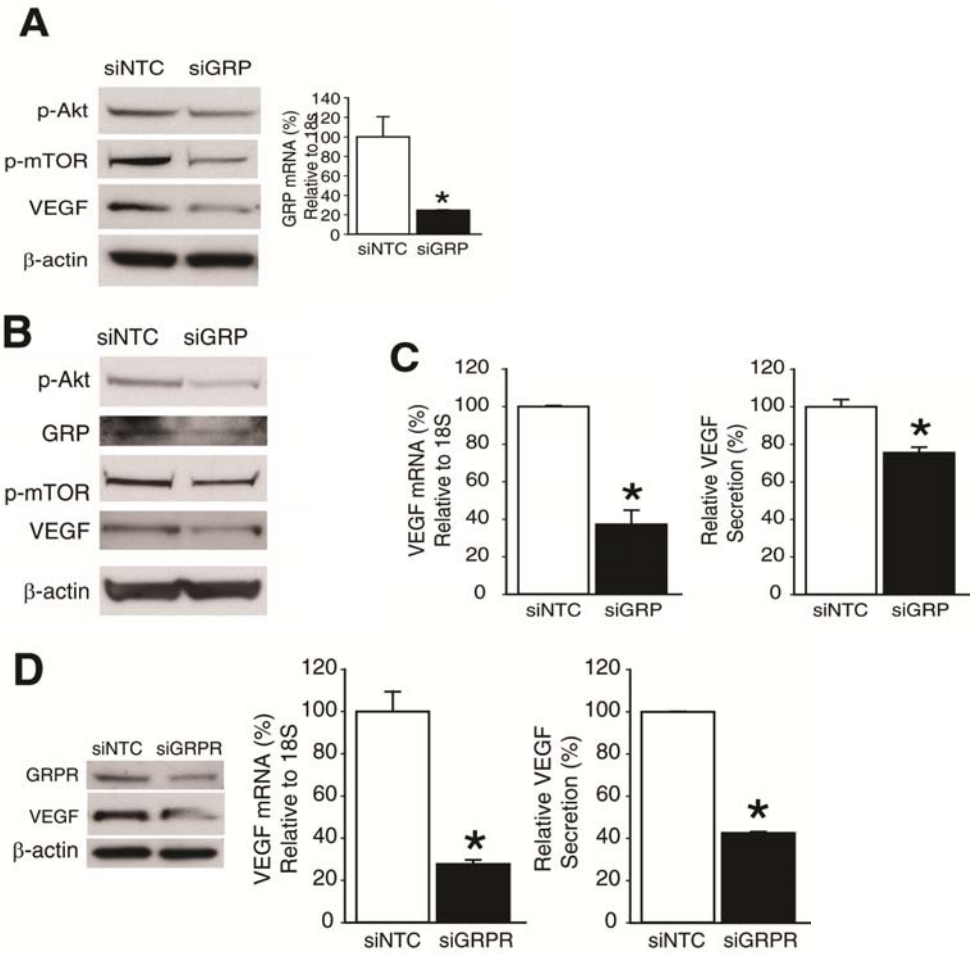

FIG. 4.5 GRP/GRPR siRNA attenuates angiogenesis in human neuroblastoma cells. **(A)** Western blot analysis of p-Akt, p-mTOR and VEGF protein expression in SK-N-SH cells at 3 d post-transfection with siNTC or siGRP (*left panel*). Quantitative RT-PCR for GRP mRNA levels in SK-N-SH cells at 2 d post-transfection with siNTC or siGRP (*right panel*). **(B)** Western blot analysis of p-Akt, GRP, p-mTOR and VEGF protein expression in BE(2)-C cells at 3 d post-transfection with siNTC or siGRP. **(C)** Quantitative RT-PCR analysis for VEGF mRNA level in BE(2)-C cells at 2 d post-transfection with siNTC or siGRP (*left panel*). VEGF levels in BE(2)-C using human VEGF ELISA kit. Cell culture supernatants were harvested at 3 d post-transfection with siNTC or siGRP (*right panel*). **(D)** BE(2)-C cells were transfected with siNTC or siGRPR for various assays. GRPR and VEGF protein expression was determined by Western blot analysis at 3 d post-transfection (*left panel*). VEGF mRNA levels were assessed by quantitative RT-PCR at 2 d post-transfection (*middle panel*) and VEGF levels in cell culture supernatants were measured by ELISA at 3 d post-transfection (*right panel*). Data from all figures represent mean ± SEM; * $p < 0.05$ vs. control.

strategies [165-168]. In particular, specific blockade of VEGF resulted in the inhibition of tumor growth and decreased recruitment of preexisting blood vessels in neuroblastoma *in vivo* models [166-168]. This study has shown that BBS increases angiogenic markers, VEGF and PECAM-1, in neuroblastomas *in vivo* and/or *in vitro*.

Since human neuroblastomas actively secrete GRP [58], the mammalian analogue of BBS, these observations further underscore the relevance of the growth factor properties of GRP. Levine et al. [169], noted that GRP treatment in prostate cancer cells stimulated pro-angiogenic factors NF-κB, IL-8, and VEGF. Our lab has also observed an increase in IL-8 gene transcription in neuroblastoma cells upon treatment with GRP [170]. Recently, it was reported that GRP can directly stimulate endothelial cell migration and cord formation *in vitro* and tumor angiogenesis *in vivo*; these effects were reversed upon treatment with a GRP antagonist [171]. The pro-angiogenic property of BBS/GRP was also confirmed in studies in which antagonists decreased angiogenesis in breast cancer, renal cell carcinoma, and glioblastoma models [172-174]. Likewise, there was a reduction in neuroblastoma tumor size and VEGF expression *in vivo* with RC-3095 (**Fig. 4.4**). The antagonist, RC-3095, shows potential for treatment of a number of solid malignancies; however a current phase 1 clinical trial provided promising, yet inconclusive results due to pharmacokinetic concerns [175]. In addition to pharmacologic inhibition, GRP silencing in neuroblastoma cells significantly reduced VEGF expression and secretion, while also decreasing phosphorylation of Akt and mTOR, upstream regulators of VEGF (**Fig. 4.5**). Therefore, I propose that targeting BBS/GRP may prove to be useful as adjuvant treatment in highly vascularized neuroblastomas.

These studies show that BBS activates the PI3K pathway *in vivo* and *in vitro*. Phosphorylation of Akt, a PI3K downstream effector, regulates cellular signals that are critical for cell survival and angiogenesis [105, 176]. Tan et al. [164], showed that PI3K activation up-regulated VEGF expression in prostate cancer cells and that activated Akt stimulates mTOR by phosphorylation and promotes VEGF transcription [164]. The studies also demonstrate the activation of Akt and mTOR in our model, with subsequent VEGF upregulation. In conclusion, these studies have, for the first time, demonstrated that BBS stimulates neuroblastoma growth and the expression of angiogenic markers *in vivo,* and have also elucidated possible molecular mechanisms through regulation of PI3K and VEGF signaling pathways (**Fig. 4.6**). Novel strategies targeting GRP-mediated tumor growth may be beneficial in patients with highly vascularized, advanced-stage neuroblastomas.

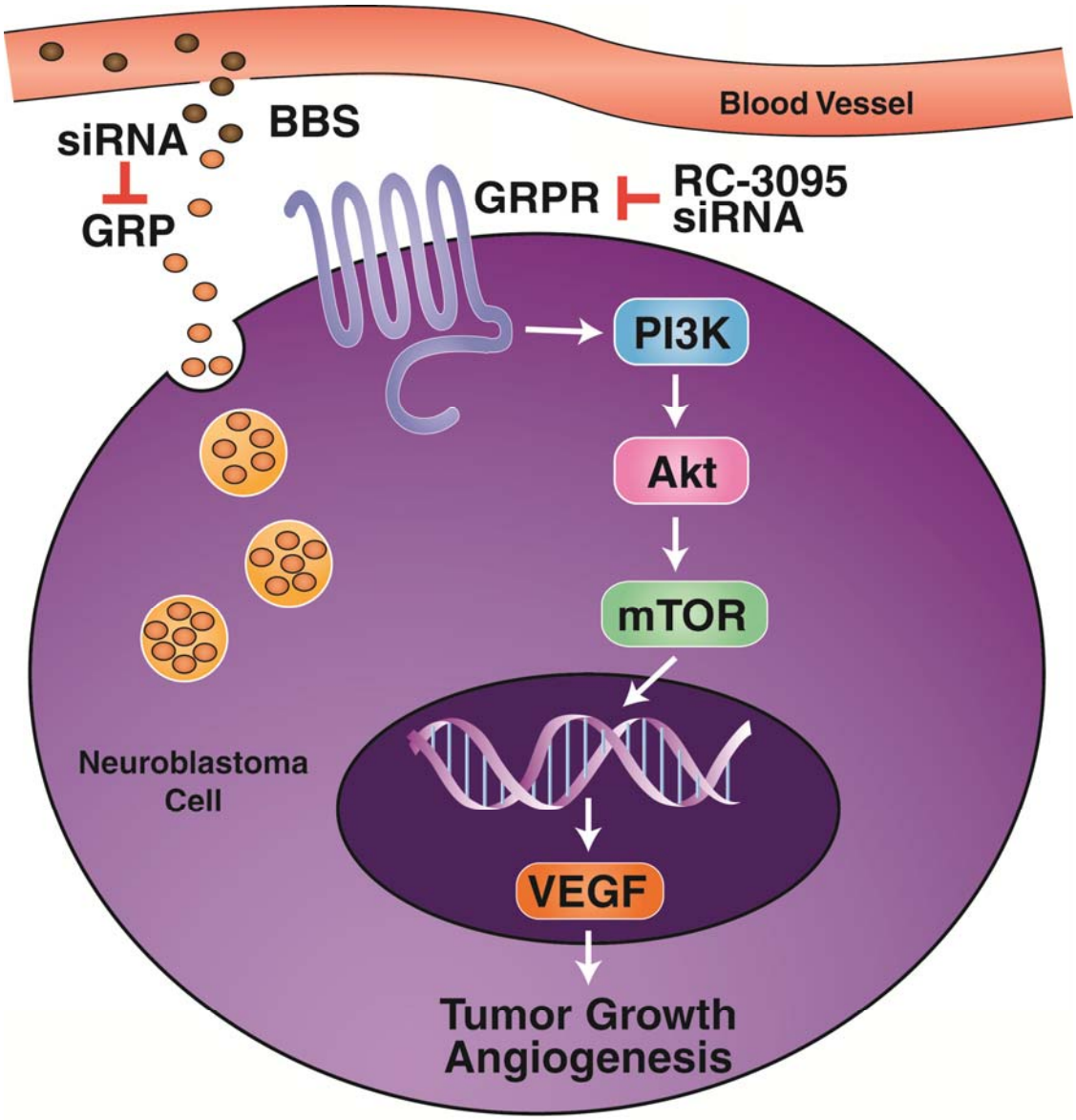

FIG. 4.6 PROPOSED MECHANISM OF GRPR-MEDIATED VEGF EXPRESSION IN NEUROBLASTOMA CELLS.

CHAPTER 5

GASTRIN-RELEASING PEPTIDE RECEPTOR SILENCING SUPPRESSES NEUROBLASTOMA TUMORIGENESIS

5.1 ABSTRACT

Neuroblastoma accounts for nearly 15% of all pediatric cancer-related deaths. Our lab has previously shown that GRP stimulates neuroblastoma growth and that its cell surface receptor, GRP-R, is overexpressed in advanced-stage human neuroblastomas; however, the effects of GRP/GRP-R on tumorigenesis and metastasis *in vivo* are not clearly elucidated. In the present study, GRP-R knockdown in the aggressive cell line BE(2)-C induced cell morphology changes, reduced cell size, decreased cell proliferation, and inhibited DNA synthesis, corresponding to cell cycle arrest at G2/M phase. Activated Akt, a crucial regulator of cell survival and metastasis, was downregulated by GRP-R silencing. In addition, phosphorylation of p70S6K and its downstream target molecule S6, key regulators of protein synthesis and cell metabolism, were also significantly decreased by GRP-R silencing. GRP-R knockdown also upregulated the expression of tumor suppressor PTEN, the inhibitor of the PI3K/Akt pathway. Furthermore, silencing GRP-R as well as GRP in BE(2)-C cells suppressed anchorage-independent growth *in vitro*. Conversely, overexpression of GRP-R in less aggressive SK-N-SH neuroblastoma cells resulted in soft agar colony formation, which was inhibited by a GRP-blocking antibody. These findings demonstrate that GRP and GRP-R have important oncogenic properties beyond their established mitogenic functions.

Therefore, GRP-R may be an ideal therapeutic target for the treatment of aggressive neuroblastomas.

5.2 INTRODUCTION

Advanced-stage neuroblastoma in children remains highly lethal with mortality rates exceeding 50% [177]. Due to their neuroendocrine lineage, neuroblastomas can produce a variety of peptides that contribute to the classic clinical symptoms and are related to tumor prognosis, including GRP [58]. GRP and its receptor, GRP-R, are known to be upregulated in various cancers, including undifferentiated neuroblastoma [92], small cell lung carcinoma [178], and prostate cancer [179]. Our lab have previously shown that GRP is secreted by neuroblastoma cells and acts as an autocrine growth factor to promote proliferation [92]. Our lab has also found that stable transfection of GRP-R, a member of the G-protein coupled receptor family, causes an increase in the binding capacity for its ligand GRP to stimulate a constitutive cellular growth rate in SK-N-SH neuroblastoma cells [101].

GRP/GRP-R signaling functionally correlates with aberrations in neuroblastoma behavior including cell cycle progression and angiogenesis. We have found that GRP treatment induces G1-S phase progression [130, 180]. We have also shown that BBS, the amphibian equivalent of GRP, increases the vascularization of neuroblastoma xenografts *in vivo* by the upregulation of vascular endothelial growth factor [130, 180]. These processes were shown to be mediated in part by the PI3K/Akt survival pathway [130, 180]. Correspondingly, GRP-R overexpression upregulates Akt activation in neuroblastoma cells and our lab also found that the ratio of Akt, in comparison to its negative regulator PTEN,

was increased in human malignant neuroblastomas [101]. This finding is especially relevant since a recent study has shown that Akt activation correlates with poor prognosis in primary neuroblastoma [128].

The mitogenic actions of GRP in tumor cells have been well-established; however, another less known property of GRP/GRP-R is its morphogenic capability [54]. Morphogenesis is an important step for cell motility during the development of the invasive nature of various cancers, including breast and colon [181, 182]. In addition to its growth factor functions, our lab has also noted morphological alterations in neuroblastoma cells that overexpress GRP-R [101]. Therefore, GRP/GRP-R may be involved in regulating multiple steps of tumorigenesis. The molecular mechanisms responsible for GRP-mediated tumor aggressiveness and metastatic potential are not clearly defined. The purpose of this current investigation was to elucidate, in broader detail, the oncogenic effects of GRP-R in relation to neuroblastoma survival, invasive potential, and metastasis development.

In this study, downregulation of GRP-R reversed the aggressive phenotype of human neuroblastoma cell line BE(2)-C, decreased cell proliferation, inhibited DNA synthesis, and induced cell cycle arrest at G2/M phase *in vitro*. GRP-R silencing also significantly blocked neuroblastoma tumorigenicity by reducing colony formation *in vitro*, and inhibiting xenograft growth and liver metastasis *in vivo*. Additionally, it was observed at the molecular level, that cell survival mediator Akt and its downstream targets p70S6K and S6 are regulated by GRP-R in human neuroblastoma. These results further demonstrate that GRP-R is a clinically relevant therapeutic target in human neuroblastomas.

Please refer to CHAPTER 2 for the materials and methods used in this chapter.

5.3 RESULTS

5.3.1 STABLE KNOCKDOWN OF GRPR INHIBITS NEUROBLASTOMA CELL GROWTH AND DOWNREGULATES THE PI3K/AKT PATHWAY

Our lab has shown that GRP acts as an autocrine growth factor for neuroblastoma cells [92]; this effect appears to be dependent on ligand binding to GRP-R [92, 180]. To confirm the importance of GRP-R in mediating neuroblastoma growth, the effect of GRP-R silencing on the cell proliferative capacity was measured. Short hairpin RNA (shRNA) vectors were used to establish stable knockdown of GRP-R in BE(2)-C cells and knockdown was confirmed with reverse transcription-polymerase chain reaction (RT-PCR) and Western blot analysis (**Fig. 5.1E**). Over a time course, the BE(2)-C cells expressing GRP-R shRNA (shGRP-R) displayed stagnant growth and significantly reduced proliferation, in comparison to control shRNA (shCON) cells, whose growth rate steadily increased between each time point (**Fig. 5.1A**). In addition, the amount of BrdU incorporation in shGRP-R cells was measured and it was found that DNA synthesis was decreased to 57% compared to control cells (**Fig. 5.1B**). This reduction corresponded with a 37% decrease of shGRP-R cells in S phase of the cell cycle and an increase of cells arrested in G2/M phase, in comparison to shCON cells (**Fig. 5.1C**). **Figure 5.2** confirms that the transfected control cells did not change the endogenous protein levels of GRP-R as well as the proliferative capacity when compared to native BE(2)-C cells. These data show that GRP-R plays a vital role in maintaining neuroblastoma cellular proliferation.

Our previous studies found that GRP/GRP-R could activate the PI3K/Akt survival pathway [130, 180]. In this study, it was also found that GRP-R overexpression enhanced the

levels of p-Akt, and decreased the levels of PTEN, a tumor suppressor that negatively regulates the PI3K/Akt pathway [101]. Furthermore, our lab had previously determined that the ratio of Akt/PTEN was increased in poorly differentiated human neuroblastoma tissue samples [101]. Since, Akt activation regulates numerous oncogenic processes and correlates with poor prognosis in primary human neuroblastomas [128]; the next experiment assessed the role of GRP-R knockdown on Akt phosphorylation and PTEN expression. shGRP-R significantly decreased p-Akt (Ser473) without notably affecting the levels of total Akt (**Fig. 5.1D**). Additionally, PTEN was upregulated in cells expressing shGRP-R. Therefore, PI3K pathway activation appears to be regulated by GRP-R; on the other hand, activated ERK1/2 protein levels remained relatively unchanged with shGRP-R expression (**Fig. 5.1D**).

5.3.2 GRPR SILENCING NEGATIVELY REGULATES CELL SIZE AND DECREASES P-P70S6K AND S6 EXPRESSION

Cell viability has been associated with cell size and metabolic activity [183, 184]. Therefore, the next experiments delineated the effects of GRP-R inhibition on tumor cell size. The expression of shGRP-R in BE(2)-C cells resulted in physiologically smaller cells, when compared to shCON cells, as assessed by phase-contrast microscopy (**Fig. 5.3A**). The cell sizes were quantitatively determined by FACS analysis. Despite the variability due to the wide distribution of size, an analysis of over 100,000 cells per group confirmed that shGRP-R cells were significantly smaller than control cells (**Fig. 5.3B**). Similar results were also shown in transiently transfected cells with GRP-R small interfering RNA (siRNA) (data not shown). The next experiment investigated the expression of two downstream targets of Akt that regulate cell size – p70S6K and S6 [184, 185]. When activated, the kinase p70S6K

phosphorylates ribosomal protein S6, which in turn initiates translation of essential proteins [185]. As shown in **Figure 5.3C**, both p-p70S6K and p-S6 were appreciably decreased in shGRP-R cells; interestingly, there was also a significant decrease in total S6 levels. These results demonstrate that regulators of cell size are dependent upon GRP-R expression. Furthermore, the typical BE(2)-C cell morphology of a flat and aggregated appearance (shCON) changed to a round and more polarized shape with GRP-R silencing (**Fig. 5.3A**). Thus, in addition to its mitogenic properties, GRP/GRP-R might also function as a morphogen for human neuroblastoma cells and thereby, regulate their invasive properties. As an initial *in vitro* measure of GRP-R-mediated invasiveness, wound-healing assay was performed and it was found that siGRP-R significantly prevented wound closure in BE(2)-C cells (**Fig. 5.4**), adding credence to the overall hypothesis.

5.3.3 SILENCING GRPR OR GRP DECREASES ANCHORAGE-INDEPENDENCE *IN VITRO*

Anchorage-independent growth in soft agar is another well-established property of *in vitro* tumorigenicity, reflecting the malignant potential of cells *in vivo* [186]. In order to evaluate whether GRP-R is critical for anchorage-independent neuroblastoma growth, the next experiment assessed the ability of BE(2)-C cells to grow in soft agar. Cells with stable expression of shGRP-R developed 60% fewer soft agar colonies than control cells (**Fig. 5.5A**). To confirm, the expression of GRP-R in BE(2)-C cells was decreased by transient transfection with siRNA. Both the size and number of colonies were significantly decreased with GRP-R siRNA (siGRP-R) (47% of control; **Fig. 5.5B, *left, middle***). In addition, colonies also formed more slowly than the control colonies transfected with a non-targeting control (siNTC). The efficiency of transfection was confirmed by Western blotting (**Fig.**

5.5B, *right*). In order to determine whether these effects were mediated by the binding of GRP ligand to GRP-R, and not just a result of receptor manipulation, BE(2)-C cells were transfected with GRP siRNA (siGRP), prior to soft agar analysis. Transfection with siGRP blocked the colony growth of BE(2)-C cells by 54% when compared to cells transfected with siNTC (**Fig. 5.5C, *left, middle***). Effective GRP knockdown was confirmed by RT-PCR (**Fig. 5.5C, *right***). Therefore, GRP binding to its cell surface receptor is an important step in the process of GRP-R-induced colony formation.

5.3.4 GRPR EXPRESSION LEVEL CORRELATES WITH MALIGNANT POTENTIAL IN HUMAN NEUROBLASTOMA CELLS

In order to examine whether endogenous GRP-R expression correlates with anchorage-independence in neuroblastoma cells and to eliminate cell-line specific effects, the next experiment compared the colony formation of BE(2)-C to another human neuroblastoma cell line, SK-N-SH. The findings were that BE(2)-C cells, which behave aggressively in an *in vivo* xenograft model (data not shown), exhibited significantly increased malignant potential by formation of soft agar colonies when compared to SK-N-SH cells (**Fig. 5.6A, *left***). Furthermore, BE(2)-C cells expressed higher levels of GRP-R than SK-N-SH cells by Western blotting (**Fig. 5.6A, *right***). This finding is consistent with our previous studies, in which more abundant GRP-R expression was noted in poorly differentiated, more aggressive neuroblastomas [92].

In order to further examine the role of GRP-R on neuroblastoma malignant potential, SK-N-SH cells were stably-transfected with either pEGFP or pEGFP-GRP-R plasmid, and then incubated the cells in soft agar for four weeks. GRP-R overexpressing SK-N-SH

neuroblastoma cells demonstrated a significantly increased number of colonies in soft agar, indicating stimulation of anchorage-independent cell growth by increased GRP-R expression (**Fig. 5.6B, *left***). In contrast, cells transfected with control vector, pEGFP, showed only a few smaller, isolated soft agar colonies. The number of colonies induced by GRP-R overexpression was markedly increased by nearly 8-fold (**Fig. 5.6B, *middle***). The expression of GFP and GFP-tagged GRP-R was confirmed by fluorescent microscopy, where intense green fluorescence was noted in GRP-R overexpressing SK-N-SH cells (**Fig. 5.6B, *right***). Similar results were obtained by transient transfection of SK-N-SH cells with a pEGFP-GRP-R plasmid (data not shown). Our findings suggest that the cell surface receptor, GRP-R, is tumorigenic in human neuroblastoma SK-N-SH cells. To further confirm the role of the ligand GRP in neuroblastoma colony formation, a specific GRP-neutralizing antibody was added to the culture media. The presence of the GRP-specific antibody significantly inhibited colony formation in SK-N-SH cells overexpressing GRP-R (**Fig. 5.6C, *left***). The number of colonies was significantly decreased to 25% of control (**Fig. 5.6C, *right***). These results further demonstrate that GRP, secreted by neuroblastoma cells, binds to GRP-R to act as a growth factor in soft agar assay, suggesting that the mitogenic actions of GRP may be an important mechanism in enhancing anchorage-independent growth.

5.4 DISCUSSION

We previously demonstrated the growth-stimulatory function of GRP in neuroblastoma cells and its correlation to PI3K/Akt pathway activation [92, 101, 130]. In the present study, GRP-R overexpression led to anchorage-independent growth in soft agar,

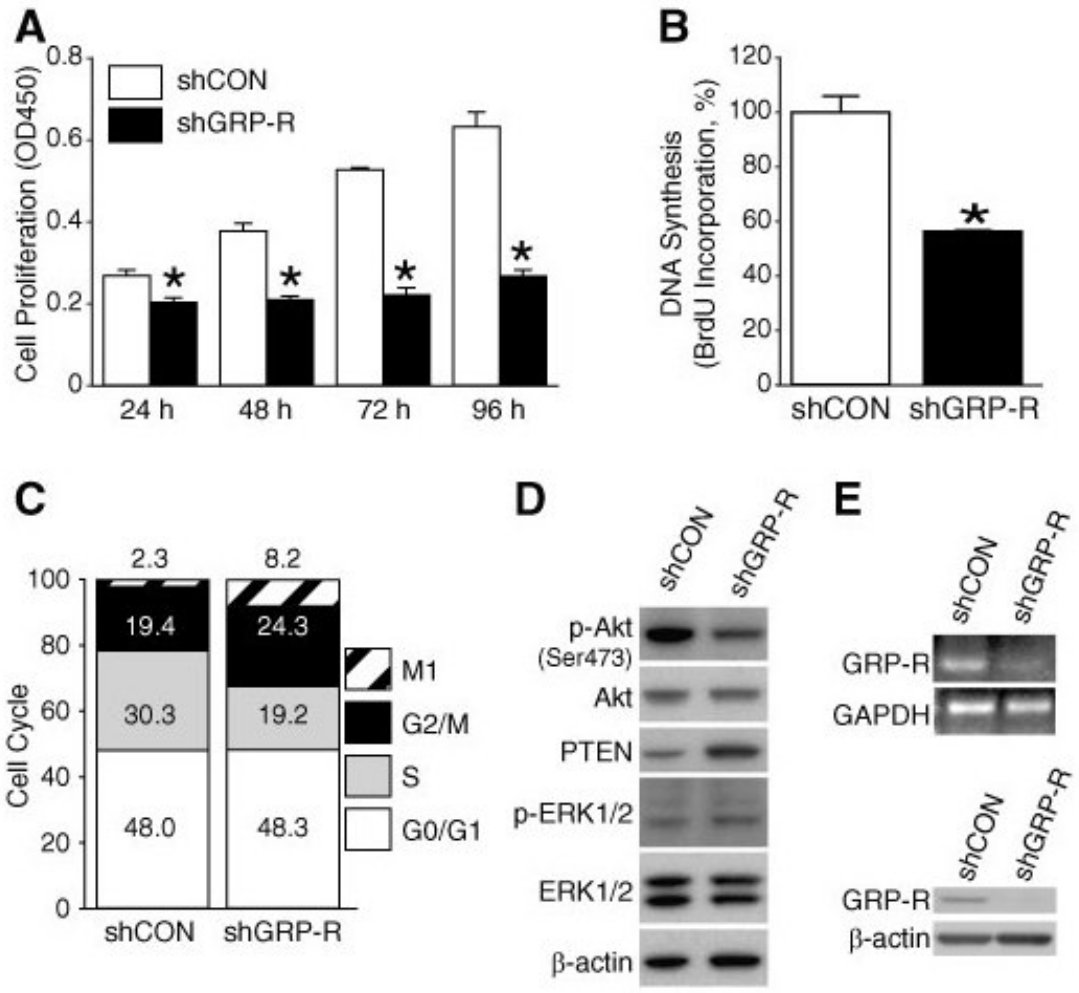

FIG. 5.1 GRP-R silencing inhibits BE(2)-C cell proliferation and downregulates the PI3-K/Akt pathway. (A) BE(2)-C cells expressing either shGRP-R or control vector shCON were plated 1 x 10^4 cells/well and cell proliferation was measured (mean ± SEM; *=p< 0.004 vs. shCON). (B) DNA synthesis was analyzed by measuring the BrdU incorporation (mean ± SEM; *=p< 0.0001 vs. shCON). (C) Stably-transfected cells (1 x 10^6 cells/well) were plated and analyzed for the percentage of cells in different cell cycle phases. (D) Western blot analysis was performed with the indicated antibodies in BE(2)-C cells after stable transfection (*left*). (E) GRP-R knockdown was confirmed with RT-PCR and Western blot analyses.

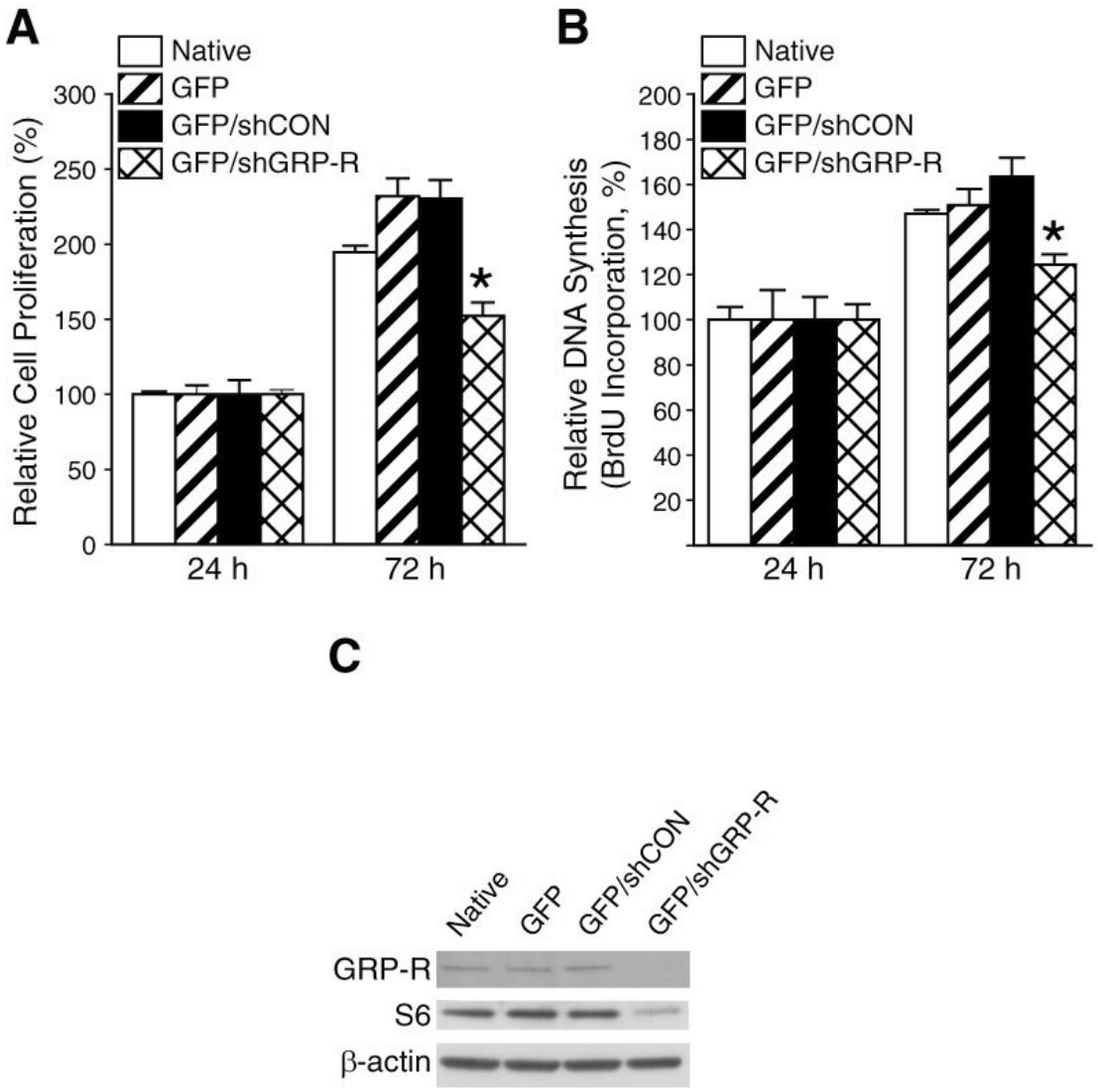

FIG. 5.2 Comparison of transfected versus native BE(2)-C cells. (A) BE(2)-C cells – native, expressing GFP, shGRP-R, or shCON – were plated 4 x 10^3 cells/well and cell proliferation was measured (data represent mean ± SEM; *=p< 0.02 vs. native, GFP or shCON). (B) DNA synthesis was analyzed by measuring the BrdU incorporation (data represent mean ± SEM; *=p< 0.02 vs. native, GFP or shCON). (C) Western blot analysis was performed with GRP-R and S6 antibodies; β-actin was used as a loading control.

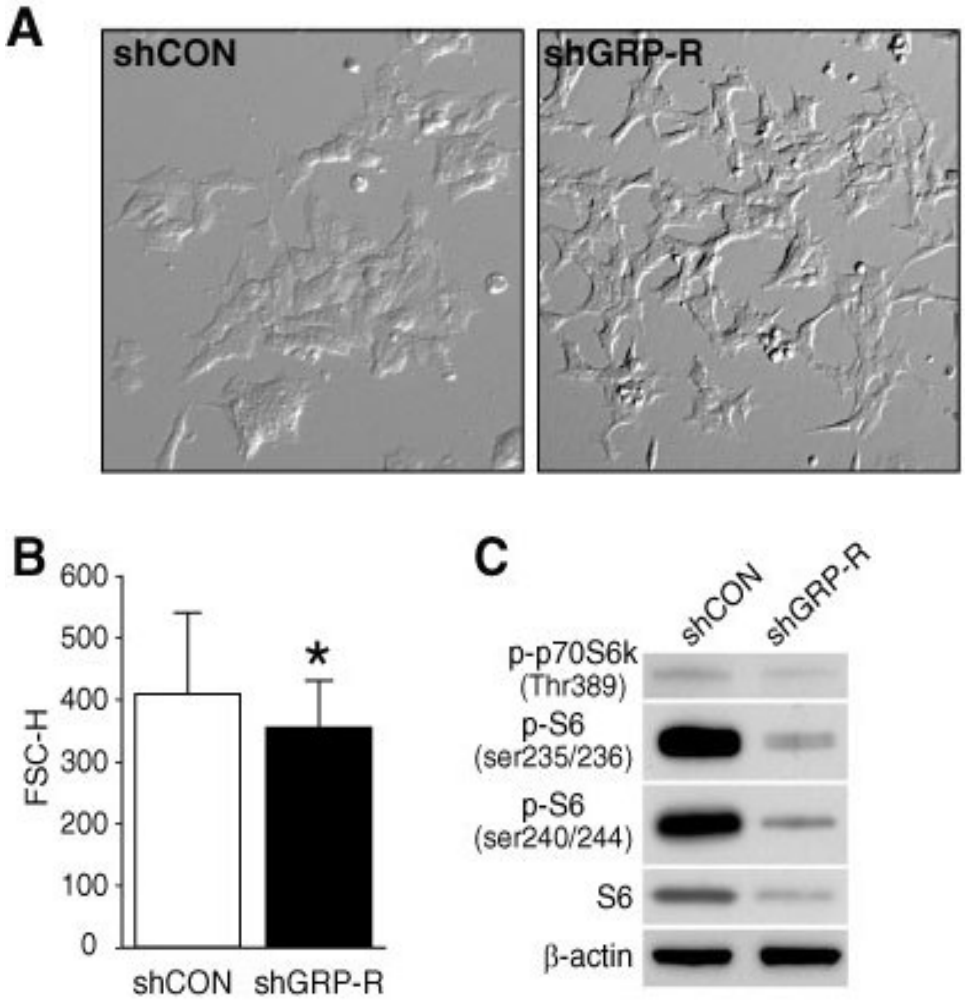

FIG. 5.3 GRP-R silencing induces changes in cell morphology, reduces cell size and decreases p-p70S6K and S6 expression. (A) The morphology of BE(2)-C cells transfected with either shGRP-R or shCON were revealed by phase contrast microscopy (magnification 100X). (B) The size of cells expressing shGRP-R and shCON was determined as described in the Materials and Methods. The average size is represented as the mean FSC-H ± SD of the counted cells (*=p< 0.0001 vs. shCON). (C) Western blot analysis of p-p70S6K, p-S6, and S6.

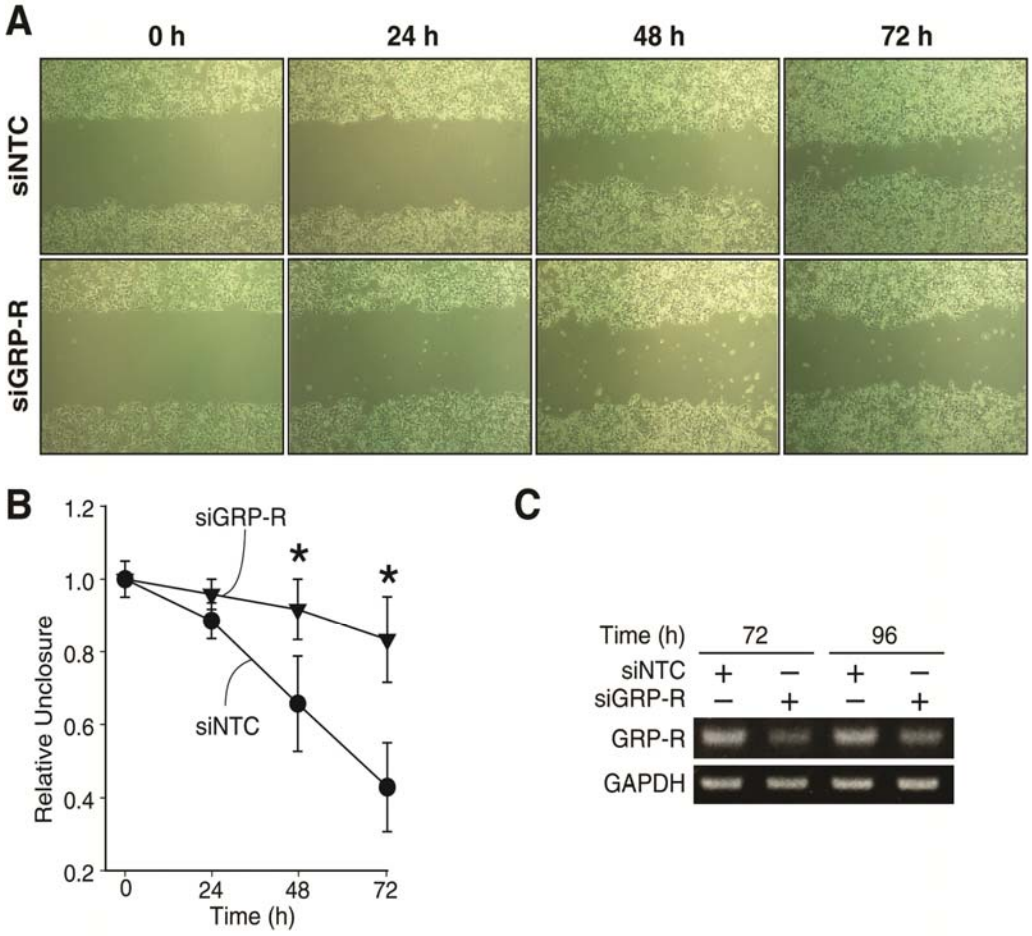

FIG. 5.4 GRP-R silencing inhibits wound closure. (A) BE(2)-C cells transfected with siNTC or siGRP-R were grown to confluence on 6-well plates in complete culture media and then serum-starved overnight. Wounds, or defects, were generated in confluent cell monolayers using a modified rubber scraper at 48 h after transfection. Serum-free medium was changed to 1% FBS/RPMI 1640 after wounding. Representative photographs of wound closure are shown for the indicated time points. Data are representative of the mean unclosure distance of three independent experiments. (B) Quantitative analysis of wound-healing assay (*=$p< 0.05$ vs. siNTC). (C) GRP-R knockdown was confirmed by RT-PCR analysis 72-96 h after transfection.

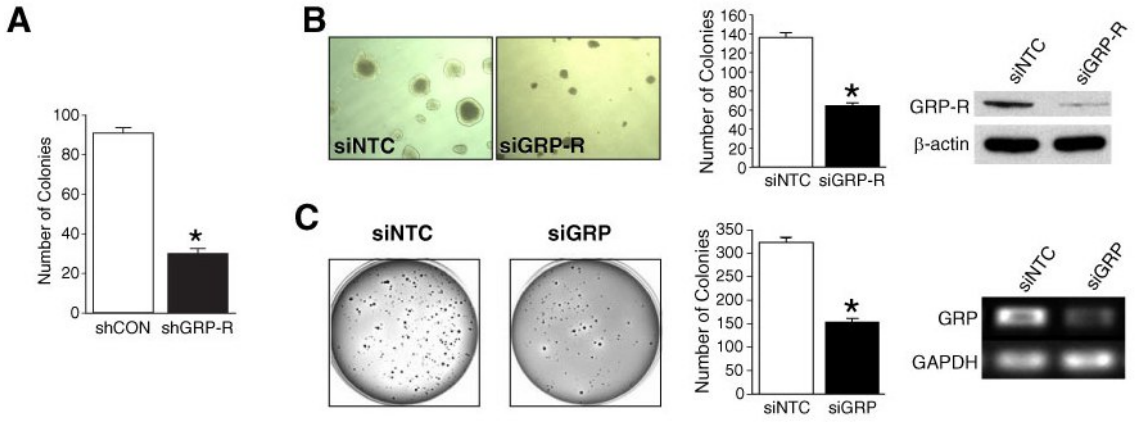

FIG. 5.5 Knockdown of GRP-R or GRP inhibits soft agar colony formation. (A) BE(2)-C cells expressing either shGRP-R or shCON were plated in soft agar (2.5 x 10^3 cells/well) for 3 weeks and colony formation was quantitatively assessed (*=p< 0.0001 vs. shCON). (B) BE(2)-C cells were transfected with siGRP-R or siNTC for 48 h and then plated in soft agar (2.5 x 10^3 cells/well) for 3 weeks (live cells, magnification 40X; *left*). Bar graph represents the quantitative assessment (*=p< 0.05 vs. siNTC; *middle*). Western blotting confirmed inhibition of GRP-R protein levels by siRNA (*right*). (C) BE(2)-C cells were transfected with siGRP or siNTC for 48 h, incubated in soft agar at 2.5 x 10^3 cells/well for 3 weeks, and then photographed after staining (*left*). Bar graph represents the quantitative assessment of colony growth (*=p< 0.05 vs. siNTC; *middle*). The knockdown of GRP mRNA by siRNA was confirmed with RT-PCR (*right*).

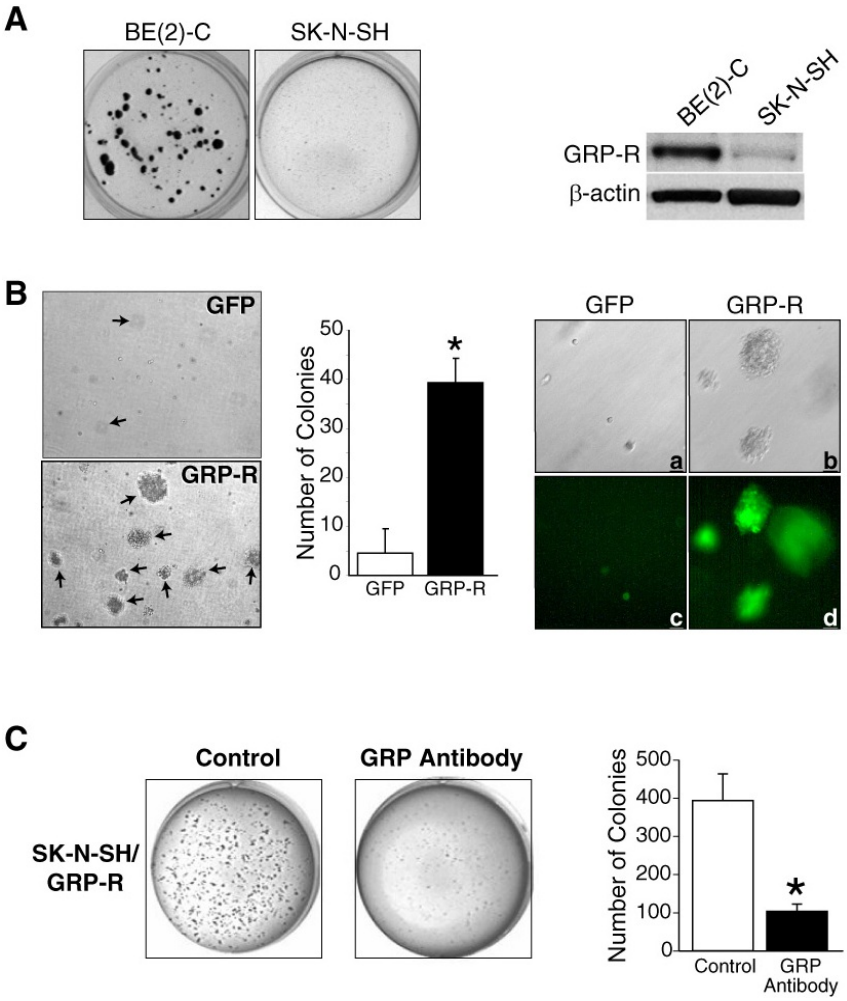

FIG. 5.6 Constitutive expressions of GRP-R correlate to anchorage-independent growth in human neuroblastoma cells. (A) BE(2)-C and SK-N-SH cells were incubated in soft agar at 2.5 x 10^3 cells/well in a 6-well plate for 3 weeks and 5 x 10^3 cells/well for 4 weeks, respectively, and then photographed after staining (*left*). Western blot analysis of endogenous levels of GRP-R in BE(2)-C and SK-N-SH cells (*right*). (B) Soft agar analysis of SK-N-SH cells stably-transfected to overexpress GFP or GFP-tagged GRP-R; arrows indicate colonies (live cells, magnification 40X; *left*). Quantitative analysis of soft agar assay (*=$p< 0.05$ vs. GFP control cells; *middle*). GFP and GFP-tagged GRP-R were expressed in SK-N-SH cells stably-transfected with pEGFP and pEGFP-GRP-R plasmids (*right*). (C) GRP-R stably-transfected SK-N-SH cells (5 x 10^3 cells/well) were incubated in soft agar for 4 weeks. GRP-neutralizing antibody (1 ng/ml) was applied to the top of the soft agar and colonies were photographed after staining (*left*). Quantitative analysis of soft agar assay (*=$p< 0.05$ vs. without antibody; *right*).

which is considered a criterion for cell transformation and malignant potential *in vivo*. Conversely, GRP-R knockdown with siRNA and shRNA inhibited colony growth in soft agar. These results show, for the first time, that GRP/GRP-R plays a role in the oncogenic process of anchorage-independence and they suggest a critical role for tumor growth and metastasis in human neuroblastoma. These findings further corroborate the multiple mitogenic functions of GRP, which are known to be associated with various malignant and aggressive human tumors [66, 150, 178, 179].

GRP, and its equivalent BBS, acts as an autocrine and/or paracrine growth factor to promote cell proliferation in cancer cells [56, 59]. In accordance with this, overexpression of the BBS peptide receptor has been shown to promote cell proliferation in Rat-1 fibroblasts [187]. Similarly, our lab has previously shown that GRP-R overexpression in SK-N-SH neuroblastoma cells increased the proliferative capacity of the cells [101]. In addition, we have found that exogenous BBS promotes neuroblastoma growth and that its antagonist suppresses tumor progression *in vivo* [180]. In this study, GRP-R overexpression induces anchorage-independent growth that requires the GRP ligand, since a neutralizing antibody reversed the effects. It was also determined that cell proliferation, DNA synthesis, and cell cycle progression are intricately related to GRP-R expression, as silencing GRP-R inhibited each process. GRP has also been thought to be a morphogen, since it is capable of altering cell morphology in colon cancer cells [73]. In this study, GRP-R silencing induced a round, polarized shape in BE(2)-C cells, which normally exist in flatter, densely-packed formations. Dynamic cytoskeletal modifications are a function of cell motility and, thus, characteristic of invasive cells [188]. This is consistent with our results since GRP-R knockdown inhibited

metastatic potential *in vitro*, correlating to the loss of the anchorage-independent phenotype and suggesting that in addition to proliferation, GRP-R signaling is also an effective regulator of malignant transformation in neuroblastoma cells.

PI3K/Akt pathway activation is frequently observed in human cancers; specifically in neuroblastoma, activation of Akt predicts poor outcome [128]. Akt contributes to diverse cellular roles, which include cell survival, growth, proliferation, angiogenesis, metabolism, and migration [189]. One of the best-conserved functions of Akt is its role in promoting cell growth and increasing cellular mass by regulating nutrient uptake and metabolism [190]. The Akt-regulated mediators of this process include mTOR and its target S6K [184, 185, 191]. In this study, GRP-R silencing downregulated the phosphorylation of Akt and its downstream effectors p70S6K and S6, in addition to reducing cell size. Interestingly, there were not significant changes in mTOR (data not shown), suggesting that Akt may additionally regulate p70S6K and S6 independent of mTOR. This is consistent with many studies in which Akt functions in both mTOR-dependent and -independent pathways [189]. Akt also plays a role in the regulation of cell cycle G2/M phase transition [192] and correspondingly, these results demonstrated that GRP-R knockdown resulted in a decrease in activated Akt and further caused a decrease in DNA synthesis and cell cycle arrest at G2/M phase. GRP-R knockdown also appears to indirectly inhibit Akt activation through PTEN, the endogenous inhibitor of PI3K/Akt, whose expression was increased with GRP-R shRNA. Therefore, downregulation of Akt activity is most likely an important factor coordinating tumor growth inhibition subsequent to GRP-R silencing.

In conclusion, our study demonstrates that GRP/GRP-R is a crucial regulator of neuroblastoma cell growth and transformation, and that Akt may be an important downstream effector of GRP/GRP-R-mediated oncogenic properties (**Fig. 5.7**). These results are consistent with previous histological findings in which GRP and GRP-R expression were increased in poorly-differentiated, aggressive human tumor samples [92]. In a recent review [54], the biological importance of GRP and GRP-R in relation to various cancers, was discussed. However, it also emphasized the need for further investigation in regards to cancer-specific strategies. This study clearly demonstrates the roles of GRP-R in neuroblastoma tumorigenesis. These findings are clinically relevant because advanced-stage neuroblastomas are refractory to current treatment modalities; hence, understanding GRP/GRP-R regulation of tumor metastatic potential could provide a novel therapeutic adjunct for aggressive, undifferentiated neuroblastomas.

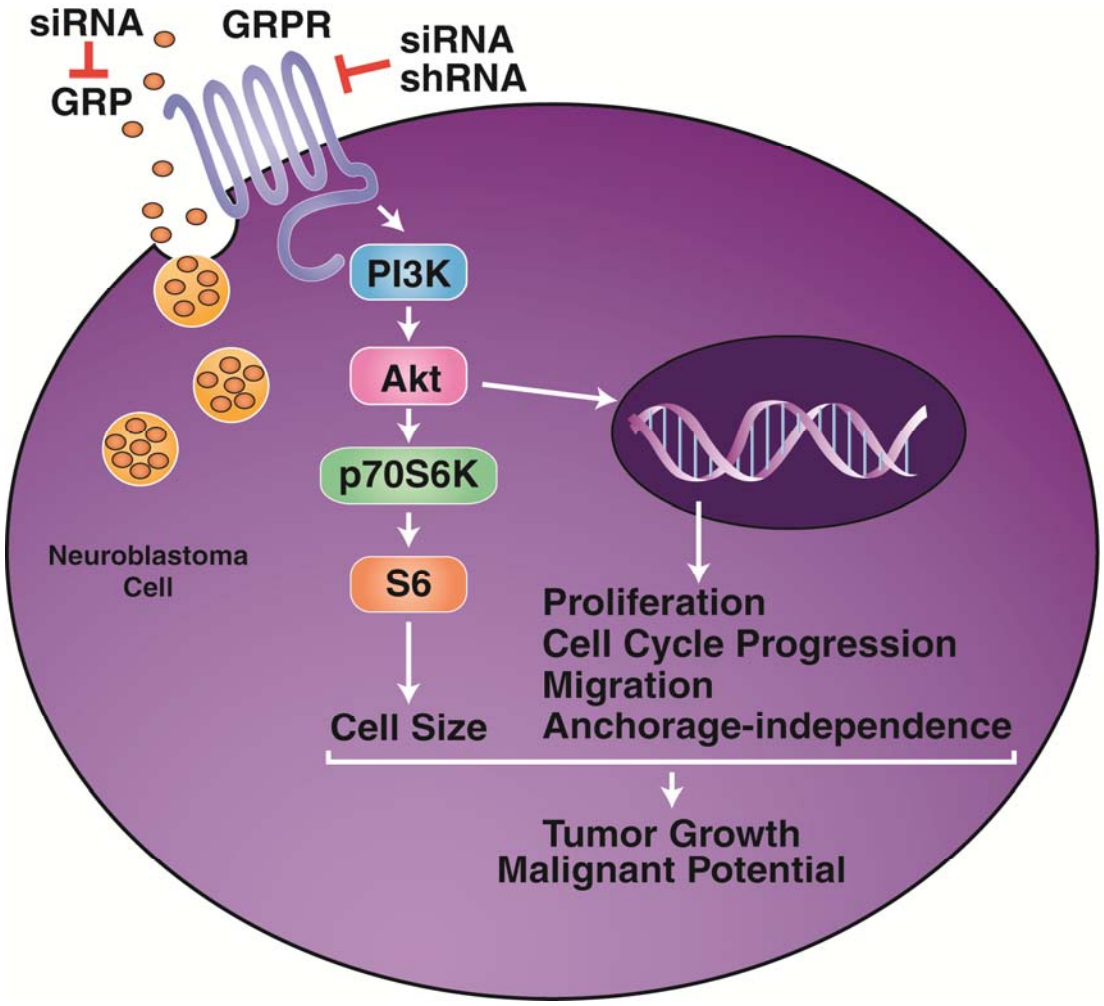

FIG. 5.7 PROPOSED MECHANISM OF GRPR/PI3K/AKT REGULATION OF NEUROBLASTOMA TUMORIGENESIS.

CHAPTER 6

SUMMARY AND FUTURE DIRECTIONS

6.1 A MECHANISM OF GRPR SIGNALING INVOLVING PI3K/AKT-MEDIATED PATHWAYS DURING NEUROBLASTOMA GROWTH REGULATION

The major points of interest emerging from these studies are described here. The findings that lead to the conclusion that GRP/GRPR is a crucial regulator of neuroblastoma tumorigenesis are: 1) GRP stimulation is able to induce G1-S phase cell cycle progression in neuroblastoma cells, which corresponds to changes in the expression levels of cell cycle regulators cyclin D, pRb, p21, and p27; 2) BBS treatment significantly enhances the growth of human neuroblastoma xenografts in nude mice and effects can be reversed with a GRPR antagonist; 3) GRPR expression is important for the regulation of neuroblastoma proliferative capabilities, cell size and anchorage-independent growth, which are a key features of tumor malignant potential.

The findings that lead to the conclusion that the PI3K/Akt pathway is an important effector of GRP/GRPR signaling are: 1) GRP/GRPR is able to rapidly activate effectors of the PI3K/Akt pathway (p-Akt and GSK-3β) – this activation is attenuated by PI3K inhibition, GRPR antagonists, or GRPR silencing; 2) GRPR stimulation leads to the activation of Akt and increased VEGF expression *in vitro* (in contrast to GRP or GRPR knockdown) and *in vivo* treatment with a GRPR antagonist results in a reversal of BBS-stimulated p-Akt and VEGF expression levels; 3) GRPR silencing regulates the expression of key components of the PI3K/Akt pathway, namely p-Akt, PTEN, p70S6K, and S6. Altogether, these findings

indicate that PI3K plays a functionally important role in mediating the stimulatory effects of GRP/GRPR during the progression of neuroblastoma pathogenesis. Here I propose a model whereby GRP/GRPR activates the PI3K/Akt pathway in neuroblastoma cells to mediate proliferation, angiogenesis, and malignant potential (**Fig. 6.1**).

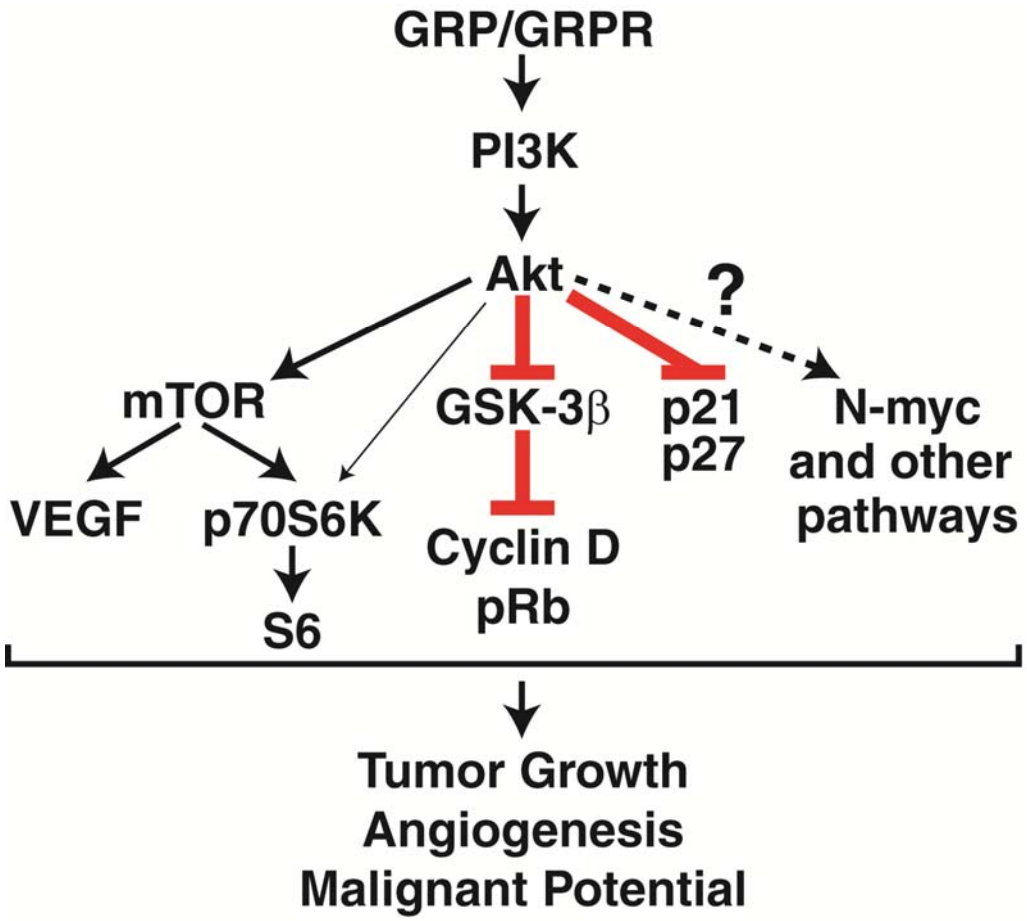

FIG. 6.1 THE IMPORTANCE OF PI3K/AKT-MEDIATED PATHWAYS IN GRPR REGULATION OF NEUROBLASTOMA GROWTH. GRP/GRPR regulates neuroblastoma growth through various PI3K/Akt-mediated mechanisms, including: cell cycle progression (GSK-3β, p21, p27), cell size (p70S6K, S6), angiogenesis (mTOR, VEGF), and other yet to be defined intermediaries such as N-myc. The net result is an increase in neuroblastoma growth, angiogenesis, and malignant potential – key features of poor prognosis and aggressive disease.

6.2 FUTURE DIRECTIONS

Neuroblastomas are complex, enigmatic tumors that predominantly affect children under 5 years of age. Advances in multimodality therapy continue to fall short in regards to making a significant impact on the clinical outcome of children with aggressive, refractory neuroblastomas. As heterogeneity is a hallmark of neuroblastoma, current biomedical research is focused on addressing the various pathologic intricacies of this tumor. Understanding the various biological and molecular components regulating tumor progression in neuroblastoma is necessary to successfully improve survival rates.

Neuroblastomas are classified as amine precursor uptake decarboxylase tumors and hence, they secrete peptides and other substances including vasoactive intestinal polypeptide (VIP), catecholamines, serotonin, acetylcholine, and GRP [42, 58, 193]. These peptides may be involved in the regulation of tumor growth and differentiation and the concentrations of peptides have been correlated with clinical behavior of neuroblastomas. For instance, VIP is generally a marker of differentiated neuroblastomas and usually signifies a favorable prognosis [194]. In contrast, our lab has found that GRP/GRPR is increased in undifferentiated, aggressive neuroblastomas [92] and hence, may be a potential marker of poor prognosis.

GRPR is a classic member of the GPCR family, and a recent review [74] has shed incredible light into the complexities of GPCR signaling in mediating cancer proliferation, inflammation, angiogenesis, and metastasis. Interestingly, it was noted that endocrine tumors were the most likely group of cancers to harbor an aberration in GPCR signaling [74]. These studies have provided evidence of the significance of GRP/GRPR-stimulated molecular

mechanisms in neuroblastomas; of note, are the vital contributions of the oncogenic PI3K/Akt pathway. However, there are important questions that remain concerning the role of PI3K/Akt signaling during GRP/GRPR-mediated neuroblastoma growth; for instance: 1) Does GRPR/PI3K/Akt signaling correlate with other known markers of neuroblastoma prognosis, in particular N-myc? 2) In regards to neuroblastoma angiogenesis, what is the distinct role of the PI3K/Akt pathway? 3) Is GRP/GRPR an effective neuroendocrine target for combinational therapy in neuroblastomas?

6.2.1 The Potential Role of N-myc in GRPR/PI3K/Akt Signaling

One of the features most widely associated with neuroblastoma prognosis is the level of MYCN amplification. Occurring in up to 25% of primary tumors, MYCN is considered the most important neuroblastoma prognostic marker, strongly correlating to advanced-stage disease and treatment failure [31, 32]. Expression of the amplified MYCN oncogene is associated with increased proliferation and enhanced malignant potential [195-198]. MYCN is an oncogenic transcription factor which functions in the regulation of proliferation, differentiation, transformation and apoptosis [91, 199]. Breakthroughs have been made in understanding the pathogenesis of MYCN in neuroblastoma with the recent discoveries of novel targets. For instance, MYCN has been found to directly regulate the transcriptional expression of MDM2, the inhibitor of p53 and crucial regulator of the cell-cycle [200], focal adhesion kinase, a potent regulator of cell survival [201], and cellular RA-binding protein II, which appears to be related cell motility [202]. However, because it is a nuclear transcription factor, it is difficult to directly target MYCN with small-molecule inhibitors. On the other hand, gene silencing is an effective method to downregulate MYCN activity. Antisense

inhibition of MYCN expression has shown to decrease proliferation and induce differentiation in neuroblastoma cell lines [203, 204]. RNA interference has also confirmed the importance of MYCN as a promoter of neuroblastoma tumorigenesis. We and others have found that siRNA targeted to MYCN resulted in increased differentiation and apoptosis, with concurrent growth inhibition [139, 205]. Paradoxically, due to its regulation of the cell-cycle, MYCN may additionally serve as a chemotherapeutic Achilles' heel for neuroblastoma. A recent *in vitro* study determined that cells with induced MYCN expression were more susceptible to the effects of current neuroblastoma chemotherapy drugs, such as paclitaxel and vincristine [206]. The MYCN-on cells exhibited increased apoptosis in contrast to MYCN-off cells [206]. In another study, low-dose hydroxyurea was found to reduce DNA synthesis and induce senescence in MYCN-amplified neuroblastoma cell lines, as opposed to nonamplified cell lines, which were unresponsive to the hydroxyurea treatment [207].

Other mechanisms of inhibiting MYCN are aimed at crucial upstream regulators of the oncogene, such as the PI3K/Akt survival pathway. Recently, activated Akt has been shown to be a prognostic indicator of poor outcome in patients with neuroblastoma [128] and was found to be specifically associated with late-stage and high-grade tumors. Furthermore, it has been demonstrated that inhibition of PI3K/Akt pathway components resulted in N-myc (MYCN resultant protein) destabilization via a GSK-3β-regulated mechanism [36]. It is unknown whether GRPR targeting can regulate N-myc in neuroblastomas, therefore it will be interesting to investigate whether GRPR expression correlates with N-myc activity. In particular, since GRP stimulation can activate PI3K/Akt signaling (**Chapter 3**), it will be intriguing if PI3K/Akt is a mediator of this potential relationship. In order to study this, I

propose an experimental design in which MYCN-luciferase promoter constructs are co-transfected with Akt wild-type and dominant negative adenoviral vectors in neuroblastoma cells with high versus low levels of GRPR. More importantly, I propose that these future experiments should utilize inducible expression systems (for both silencing and overexpression) which can be regulated by either tetracycline (overexpression) or lentiviruses (silencing). This will help determine whether GRPR and Akt are interdependent and whether GRPR expression plays a role in mediating the activity of N-myc. In regards to future therapies, an affiliation of this magnitude would not only define GRP/GRPR as a specific neuroblastoma prognostic marker but would potentially provide an effective target for this important subset of advanced, MYCN-amplified neuroblastomas.

6.2.2 GRPR-MEDIATED ANGIOGENESIS: DEFINING ROLES FOR MTOR AND N-MYC

Highly vascular in nature, neuroblastomas display increased tumor angiogenesis, which is also related to poor patient outcomes [154, 167]. VEGF is an important regulator of vascularization in neuroblastoma and its expression correlates with unfavorable histology and increased aggressive behavior [155, 161]. Moreover, recent preclinical studies performed with the anti-VEGF antibody bevacizumab, as a single agent and in combination with chemotherapy, demonstrated reduced vessel density and decreased growth of neuroblastoma xenografts [208, 209]. Regulation of VEGF expression involves multiple oncogenic pathways including the PI3K/Akt pathway. The PI3K/Akt pathway is a potent mediator of angiogenesis in both normal and cancer tissues [105, 210]; mTOR is one of the downstream effectors of this process [164, 211]. Correspondingly, our previous results utilizing rapamycin, the mTOR inhibitor, confirmed that PI3K/Akt acts through mTOR and HIF-1α to

regulate VEGF in neuroblastoma [37]. However, interestingly, we noted that in the studied cell lines, rapamycin-induced inhibition of VEGF was significant only after stimulation by IGF-1. Kurmasheva et al. postulated that VEGF expression in unstimulated neuroblastoma cells in standard medium conditions (with 10% serum) depends on PI3K/Akt-driven signaling that most likely bypasses mTOR [212]. Furthermore, in another study, Akt siRNA, but not rapamycin or mTOR siRNA, inhibited HIF-1α expression in glioblastoma and prostate cancer cells maintained in 10% serum conditions [213]. However, in that particular study, rapamycin inhibition was effective in the presence of lower serum concentrations. This corresponded with our rapamycin studies carried out in serum-free conditions [37]. The mechanism is still uncertain; however, it is likely that mTOR plays a more significant role in PI3K-mediated angiogenesis during nutrient depletion. Furthermore, our study also suggested that PI3K stimulation by growth factors may enhance the sensitivity of the mTOR-regulated VEGF pathway to rapamycin.

Not surprisingly, MYCN amplification correlates with enhanced angiogenic capability in human neuroblastoma tissue biopsies and cell lines [33]. In addition, overexpression of MYCN has been shown to downregulate inhibitors of angiogenesis [214, 215]. We have recently found, for the first time, that PI3K/Akt regulates VEGF via MYCN-dependent mechanisms. Specifically, we found that PI3K/Akt activation increased N-myc protein levels; this PI3K-induced N-myc stabilization was associated with an increase in VEGF secretion [37]. Therefore, N-myc represents a critical target for VEGF regulation and MYCN amplification may play a critical role in regulating PI3K-stimulated VEGF levels in neuroblastoma cells. Importantly, our findings suggest a distinct functional relationship

between two major factors associated with poor outcome in neuroblastoma—N-myc and Akt activation—and their role in directing critical angiogenic pathways. Our studies also suggest that N-myc regulation of PI3K-mediated VEGF expression in neuroblastoma may operate via pathways distinctive from mTOR [37]. If these pathways are indeed separate, it may be that under normal or nutrient-depleted growth conditions, PI3K can regulate N-myc to induce VEGF expression in neuroblastoma cells. However, it is likely that PI3K/Akt may subsequently activate mTOR-regulated mechanisms as a failsafe for angiogenic stability when neuroblastoma cells are subjected to inadequate nourishment, but further investigation is essential in order to fully understand these processes. In order to study this, cells should be evaluated in both serum-supplemented and serum-free media (or subjected to hypoxic stress) in a time course; then, the expression profile of Akt, mTOR, N-myc, HIF-1, and VEGF should be evaluated. Correspondingly, I propose to compare HIF-1 and VEGF expression during either MYCN or mTOR silencing. Co-transfection of the VEGF luciferase constructs with MYCN overexpressing or silenced cells can also shed light on the role of N-myc in the regulation of angiogenic markers.

Vascularization has also been found to be promoted by the autocrine and paracrine hormone stimulation. A distinguishing feature in neuroblastomas is their neuroendocrine properties, allowing them to secrete and respond to various hormones [58]. For instance, Neuropeptide Y (NPY) is expressed in neuroblastomas and associated with unfavorable clinical outcome [216]. In relation to angiogenesis, it has been demonstrated that NPY secreted from neuroblastoma cells stimulated the growth of microvascular endothelial cells *in vitro* and significantly increased neuroblastoma growth and vascularization *in vivo* [217].

GRP is also secreted by neuroblastomas and its expression is correlated with poor prognosis [58]. Our lab has found that GRP significantly induces the transcription, expression, and secretion of interleukin-8, a potent angiogenic cytokine [170]. This work also determined that BBS, the GRP analogue, increased tumor growth and vascular density of neuroblastoma xenografts and induced the expression of VEGF, with corresponding Akt activation, both *in vivo* and *in vitro* (**Chapter 4**). Further studies are necessary in order to determine whether this novel PI3K/Akt/N-myc regulation of angiogenesis exists under GRP/GRPR stimulation. I propose experiments analyzing VEGF expression and secretion (VEGF ELISA) utilizing the MYCN-luciferase constructs from above with addition of GRPR-inducible systems. Further, I propose studying the effects of sequential and combination silencing of GRPR, Akt, and MYCN and compare the production of VEGF and other angiogenic markers such as PECAM-1. I also propose the use of tubulogenesis assays as a functional determination of vascular development *in vitro*. These studies will lead to a better understanding of the interdependent relationship of predictors of unfavorable prognosis in neuroblastoma.

6.2.3 TARGETING GRP/GRPR AS A POTENTIAL ADJUVANT TO CHEMOTHERAPY

Despite the current advances in treatment options, clinical prognosis of aggressive neuroblastomas, especially in older patients, remains dismal [2]. Treatment of neuroblastoma is based on the International Neuroblastoma Staging System and is variable depending on age and presentation. Therefore, treatment can consist of a wide range of options, from no treatment to surgical resection only, to the full gamut of surgical resection, chemotherapy, radiation therapy, bone marrow transplant, and immunotherapy. Despite current advances in surgery, chemotherapy and radiation for the treatment of neuroblastoma, only 10 to 15% of

stage IV patients experience long-term survival. In fact, overall mortality for all stages of the cancer remains at a dismal 50% [9]. Unlike the majority of pediatric tumors, chemotherapy has not yet made a significant impact on the survival of children with neuroblastoma; additionally, the available regimens are not well-tolerated due to various toxicities. Therefore it is crucial to develop adjunct therapy that targets signaling pathways responsible for the aggressive nature of this tumor.

Our studies have shown that GRPR expression is crucial for various factors regulating tumorigenesis in neuroblastoma. In particular, GRPR silencing significantly inhibited proliferation, cell cycle progression, and anchorage-independent growth (**Chapter 5**). In addition to the use of knockdown techniques, I believe it would be necessary to evaluate the effects of GRPR overexpression under *in vitro* and *in vivo* systems; and even further in inducible GRPR expression systems. In order to support the hypothesis of GRPR-induced tumorigenesis, further studies in the areas of cell motility and migration should also be studied with wound-healing assays, invasion transwell chamber cultures, and protein assessment of integrin and Rho family proteins. Additionally, to understand anchorage-independence and malignancy with greater physiological application, I would propose the use of 3-D culture assays.

Moreover, GRP/GRPR targeting may be a novel avenue to increase neuroblastoma sensitivity to low-dose chemotherapy. *In vitro* experiments and eventual *in vivo* studies are essential to delineate the level of cooperation between the cytotoxic effects of chemotherapy and the inhibited pro-growth effects of GRPR expression on neuroblastoma cells. Proposed *in vitro* experiments would evaluate the effects of combining GRPR silencing with different

dosages of chemotherapy on the cell cycle (flow cytometry, BrdU incorporation, protein expression of regulators such as cyclin D, cyclin B1, and histone H3 phosphorylation) and apoptosis (TUNEL assay, Cell Death ELISA, protein expression of apoptotic markers such as Bcl-2 family proteins, caspase 3 and cleaved PARP). These studies are important because an effective adjuvant is critical to diminish the harmful side effects seen with current neuroblastoma treatment protocols, while providing the potential synergistic benefits of multi-targeted therapy.

6.2.4 CLINICAL SIGNIFICANCE

Despite an ever-increasing ability to reduce cancer-related mortalities in the pediatric population, neuroblastomas remain an enigma in that advances in treatment have failed to significantly increase patient survival rates. As neuroendocrine tumors, specific targeting of stimulatory GI-hormones, such as GRP, may prove to be beneficial for effective adjuvant therapy for neuroblastomas. The long-term goals of these studies are to understand the effects of neuroendocrine GPCRs, such as GRPR, on the growth of tumors, particularly neuroblastoma. These receptors and/or their ligands may be able to serve as disease progression markers and GRP/GRPR modulation may provide a unique target for therapeutic options in neuroblastoma. These studies will also enhance our knowledge of hormone-regulated cancer pathogenesis and help elucidate the complex signaling pathways involved.

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VITA

Titilope Adenike Ishola was born on May 25, 1981 in Lagos, Nigeria to Mrs. Elizabeth I. Ishola and Rev. Dr. Solomon A. Ishola. Moving to Fort Worth, Texas, Titilope graduated with honors from R. L. Paschal High School in 1999 and subsequently attended the University of Texas at Arlington (UTA). In the summer of 2001, she worked in the lab of Dr. Keith McDowell, assisting in the development of a computer program to simulate ion neutralization in order to examine the behavior of ions colliding on a linear chain and the effects of stochastic noise. From 2001-2003, she performed electrophysiological studies on proton-gated ion channels in C-6 rat glioma cells for her undergraduate honors thesis in the lab of Dr. Malgosia Wilk-Blaszczak. She received an Honors Bachelor of Science in 2003 from UTA, with a major in Biology and minor in Chemistry. Following graduation from UTA, Titilope entered the M.D./Ph.D. Combined Degree Program at the University of Texas Medical Branch (UTMB) in Galveston, Texas in 2003. While at UTMB, Titilope participated in various research experiences. In 2003, she examined the effects of oxidative stress on hepatitis C-transfected human hepatoma and replicon cells in the lab of Dr. Steven Weinman. In 2004, she studied the effects of protein kinase A and protein kinase C inhibitors on the synaptic plasticity of the central nucleus of the rat amygdale in the lab of Dr. Volker Neugebauer. Her work on this project was presented in poster format at the 2004 Society for Neuroscience meeting and published in the peer-reviewed journal *Molecular Pain* in 2008. Titilope joined the laboratory of Dr. Dai H. Chung in June 2005. In 2007, she was awarded an F31 Predoctoral Fellowship from the National Institutes of Health. She conducted her dissertation research focusing on the effects of gastrin-releasing peptide and the phosphatidylinositol-3 kinase pathway on neuroblastoma cell growth. Her work has been presented at the Advances in Neuroblastoma Research meeting, won the Annual M.D./Ph.D. Abstract Competition at UTMB, and won Best Student Poster at the UTMB Comprehensive Cancer Center Day. Her studies have also contributed to publications in the peer-reviewed journals *Biochimica et Biophysica Acta*, *Cancer Letters*, *Oncogene*, and *PNAS*. Following the completion of her UTMB education, Titilope will begin her pediatric residency training at Cincinnati Children's Hospital Medical Center. She plans to continue her scientific training after residency.

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Publications

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