FOCAL ADHESION KINASE MEDIATES GASTRIN-RELEASING PEPTIDE

RECEPTOR-INDUCED NEUROBLASTOMA PROGRESSION

By

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To my beloved parents and infinitely supportive husband, Kyungho Park.

Without them, there would be no beginning and no end of my Ph.D. degree.

사랑하는 엄마 아빠, 감사합니다!

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LIST OF ABBREVIATIONS

1,2,4,5-benzenetetraamine tetrahydrocholoride Y15

6-diamidino-2-phenylindole .... DAPI

Adenosine triphosphate .... ATP

Allophycocyanin .... APC

Analysis of variance .... ANOVA

Band 4.1, ezrin, radixin, moesin homology .... FERM

Bombesin .... BBS

Cellular retinoic acid-binding protein II .... CRABP-II

Epidermal growth factor .... EGF

Epidermal growth factor receptor .... EGFR

Extracellular matrix .... ECM

Extracellular signal-regulated protein kinases 1 and 2 .... ERK1/2

Focal adhesion kinase .... FAK

Focal adhesion-targeting .... FAT

G-protein-coupled receptors .... GPCRs

Gastrin-releasing peptide .... GRP

Gastrin-releasing peptide receptor .... GRPR

Glyceraldehyde 3-phosphate dehydrogenase .... GAPDH

Guanosine diphosphate .... GDP

Guanosine triphosphate .... GTP

Hank's balanced salt solution .... HBSS

Matrix metalloproteinase .... MMP

Mitogen-activated protein kinase .... MAPK

Mouse double minute 2 homolog MDM2

Multidrug resistance-associated protein .... MRP

Non-targeting control siRNA .... siNTC

Phosphatase and tensin homolog .... PTEN

Phosphate buffered saline .... PBS

CHAPTER I

INTRODUCTION

Neuroblastoma

Cancer is the second leading cause of death in pediatric population younger than 15 years of age [1]. Neuroblastoma represents 7~10% of children cancers and is the most common extra-cranial solid tumor in infants and children [2]. It accounts for greater than 15% of all pediatric cancer-related deaths [3]. The overall mortality for all stages of tumors remains significant at 50%; advanced-stage tumors are highly refractory to current treatment modalities [4]. Approximately 700 new cases are reported for neuroblastoma in the United States each year, nearly half of which present in toddlers < 2 years of age [5]. Considering the incidence of neuroblastoma at such a young age, the potential successful treatment would be able to result in the preservation of significant life-span years. Despite remarkable recent advancement in pediatric cancer therapy including surgery, radiotherapy and chemotherapy, the 5-year survival rate for patients with advanced disease is a dismal 50% and associated with severe long-term complications [6,7]. Importantly, two thirds of neuroblastoma patients present with metastases at the time of diagnosis and usually metastases occur in the bone marrow, liver, lymph nodes, skin and brain. In addition, most children in stage 4 that are treated for this disease will frequently experience a relapse of tumor growth. As a sympathetic neural crest derived tumors,

neuroblastoma can occur anywhere in the location of sympathetic nervous system; however, adrenal medulla is the most frequent primary site. One of the most unique aspects of neuroblastoma is its wide spectrum of clinical presentations, ranging from spontaneous remission to rapid tumor progression [8]. Infants with stage 4S disease can have a favorable prognosis with potential for spontaneous tumor regression [9,10,11].

Neuroblastoma is categorized by the degree of cellular differentiation of tumor cells; ganglioneuroma is well-differentiated benign tumors with mature ganglion cells, neuroblastoma is poorly-differentiated tumors with abundant neuroblast cells and ganglioneuroblastoma is an intermediary category possessing features of both the immature neuroblastomas and differentiated ganglioneuromas [12,13]. Neuroblastoma is also characterized by several molecular markers that correlate to various degrees of prognosis, for example, *MYCN*, cell surface glycoprotein CD44, and tyrosine kinase receptors TrkA and TrkB. In particular, *MYCN* is the most well known marker in neuroblastoma. Approximately 25% of primary neuroblastomas demonstrate *MYCN* amplification, which is strongly correlated with advanced-stage tumors and poor prognosis [14,15,16,17] (Table 1). *MYCN* amplification in neuroblastoma is typically characterized by florid vascularization [14,15,16]. Moreover, *MYCN* amplification has been associated with other poor prognostic indicators such as chromosome 1p deletion as well as enhanced multidrug resistance-associated protein (MRP) expression [18,19]. Additionally, our laboratory and others have shown that the MYCN expression is regulated by phosphatidylinositol 3-kinase (PI3K)/AKT pathway [20,21]. Thereby, PI3K/AKT pathway is a potent regulator of vascular endothelial growth factor (VEGF), which

Stage	Low	Intermediate	High
1	All	None	None
2A, B	<1 year, or 1–21 years and *MYCN* non-Amp, or 1–21 years and *MYCN* Amp, FH	None	1–21 years with *MYCN* Amp with UH
3	None	<1 year and *MYCN* non-Amp, or 1–21 years and *MYCN* non-Amp with FH	0–21 years and *MYCN* Amp, or 1–21 years and *MYCN* non-Amp with UH
4	None	<1 year and *MYCN* non-Amp	<1 year and *MYCN* Amp, or 1–21 years
4S	*MYCN* non-Amp; FH; hyperploid	*MYCN* non-Amp; UH; diploid	*MYCN* Amp

Amp, amplified; FH, favorable histology; UH, unfavorable histology.

Table 1. Neuroblastoma risk-group criteria [17]

correlates with unfavorable histology and aggressive neuroblastomas [22,23]. Our laboratory has evaluated the role of MYCN in the regulation of PI3K-mediated VEGF expression. We have found that MYCN plays an important role in PI3K-mediated VEGF regulation in neuroblastoma cells by targeting *MYCN* as a novel effector of PI3K-mediated VEGF [21]. Furthermore, other groups have demonstrated that MYCN binds to the promoter of focal adhesion kinase (FAK) and activates FAK expression in *MYCN*-amplified neuroblastoma cell lines [24,25], suggesting FAK expression is correlated with amplification of the *MYCN* oncogene in neuroblastoma cells. In addition, neuroblastomas have the notable ability to interact with their microenvironment in order to promote cell survival and that can manipulate autocrine and paracrine growth signals to enhance its pathogenesis [26]. Neuroblastomas are classified as amine precursor uptake decarboxylase tumors and hence, they secrete peptides and other substances including vasoactive intestinal polypeptide, catecholamines, and gastrin-releasing peptide (GRP) [26,27,28]. These peptides may be involved in the regulation of tumorigenesis and their expressions have been correlated with clinical tumor behavior.

Overall, combination therapy with surgical resection, chemotherapy and targeted radiation has been developed and is a mainstay for patients with advanced primary, refractory or metastatic neuroblastoma. However, these therapeutic advances have failed to significantly increase the 5-year survival rates and current chemotherapeutic regimens are usually unsuccessful at effectively eradicating the disease. Furthermore, the toxicities remain a significant problem. This is why pediatric neuroblastomas that are enigmatic, multifaceted tumors continue to remain a clinical challenge. Hence, development of additional adjuvants may be required for the benefits of chemotherapy while decreasing the harmful side effects. Moreover,

since neuroblastomas are highly heterogeneous, it is important to focus on addressing the various pathogenic intricacies of this tumor. Understanding the various biological and molecular components regulating neuroblastoma progression is necessary to successfully improve survival rates of pediatric patients with high-risk neuroblastoma and may lead to innovative therapeutic agents for more effective combinational therapy.

Gastrin-Releasing Peptide (GPR) and GRP-receptor (GRPR)

As a gut/neuropeptide, GRP is found in the gut and brain with a stimulatory effect on the growth of various tissues [29,30]. GRP induces the release of gastrin and other neurotransmitters (i.e., neurotensin, somatostatin), which can then regulate secretion and/or hormone release from the numerous endocrine organs [31,32,33]. Moreover, GRP can regulate cell growth directly by stimulating DNA synthesis and cell replication [32,34] and increase invasiveness of prostate cells through enhanced cell motility [35].

GRPR is a member of the family of G-protein-coupled receptors (GPCRs) that have seven transmembrane domains. A GPCR is a large family of cell surface receptors that is coupled to G-proteins. G proteins are a family of similar proteins located in the plasma membrane and are specialized proteins with the ability to bind the nucleotides, guanosine triphosphate (GTP) and guanosine diphosphate (GDP). When activated, they undergo conformational changes by binding or hydrolyzing GTP to modify channel gates in the cellular membrane. In this way, the receptors are coupled to intracellular responses. Specifically, GRPR is abnormally expressed in various cancers of the breast [36], lung [37], stomach [36], pancreas [38], prostate [39] and colon [40], indicating GRPR may function as a biomarker for cancer

progression and can potentially serve as an effective target for anti-cancer therapy. Likewise, cancer cells express GRPR and secrete GRP acting as an autocrine growth factor. As a neuroendocrine tumor, neuroblastoma frequently expresses and secretes gastrointestinal hormones, for example, GRP, vasoactive intestinal peptide, somatostatin, neurotensin, gastrin, and cholecystokinin. These peptides are found to exert various autocrine, paracrine, and endocrine functions in neuroblastoma cells [41,42]. However, their exact role in the regulation of cell proliferation is not yet fully elucidated.

Clearly, endocrine hormones, acting as mitogens for stimulation of tumor growth is important in terms of cellular function in neuroendocrine tumors. Similarly, it has become more evident that neuroendocrine peptides can represent an important tumor marker as well as a potential target for novel treatment strategies for neuroblastoma. Therefore it is important to discuss the mitogenic actions of a specific peptide, GRP, and the cellular mechanisms involved in GRP/GRPR-mediated neuroblastoma cell growth response. Our laboratory has discovered the increased expression of GRP and GRPR in human neuroblastoma tissue sections (Fig. 1) and the signaling cascades that take place after GRP binds to GRPR in neuroblastoma; GRP treatment increased growth in neuroblastoma cell lines by inducing calcium influx into the cells [43], which can activate phospholipase C (PLC) and transduce mitogen-activated protein kinase (MAPK) signaling through protein kinase C (PKC) and calcium. We have also discovered a relationship between GRP signaling and PI3K pathway [44]. Moreover, PI3K inhibition destabilizes the protein levels of neuroblastoma oncogene MYCN [20,21], and further we have found that PI3K regulates mediators of angiogenesis through MYCN-dependent pathways [21].

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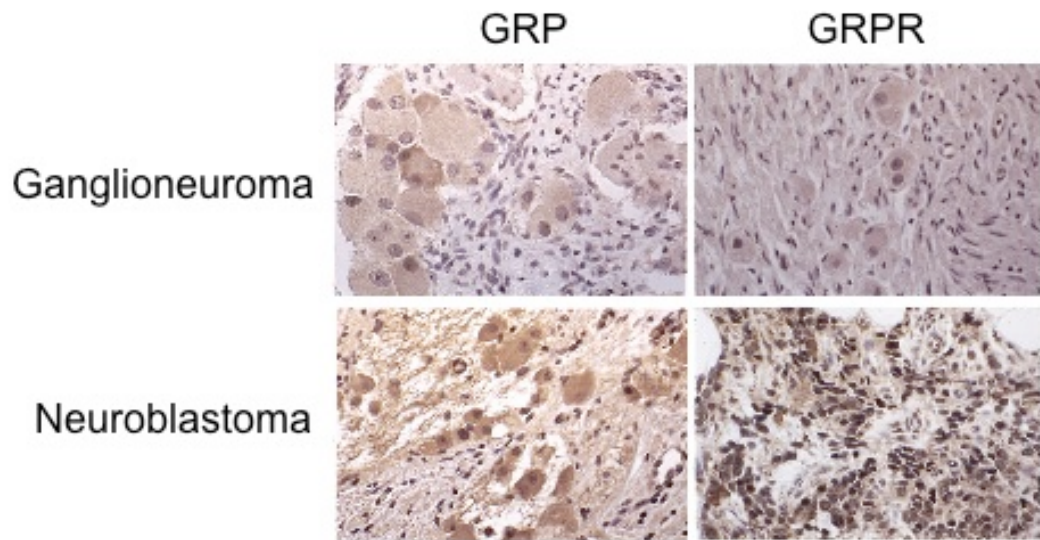
Figure 1. GRP/GRPR expression in human neuroblastoma sections. Representative images of two subtypes of human neuroblastomas showing differential GRP and GRPR expression by immunohistochemical staining (100X). Increased expression of GRP and GRPR protein (shown by the brown staining) is noted in undifferentiated neuroblastoma compared with well-differentiated ganglioneuroma [43].

In addition, we have reported that GRPR overexpression decreased phosphatase and tensin homolog (PTEN) expression and increased activated AKT expression in neuroblastoma cells [44]. However, still the exact molecular mechanisms by which GRP/GRPR signaling is involved in neuroblastoma growth and metastasis and their functional role in regulating neuroblastoma progression are not clearly elucidated. Moreover, GRP transduction cascade is inherently more complex and a relationship between GRPR and its downstream pathway has not been fully defined. A better understanding of the molecular mechanisms involved in GRP/GRPR signaling is necessary to understand neuroblastoma progression. This could result in the development of better specific therapeutic targets for neuroblastoma.

Its high expression in a large spectrum of human cancers, as well as the demonstration of its role as a tumor growth factor in various tumor models gives support to study GRP/GRPR antagonists as anticancer agents. GRP/GRPR antagonists have been developed as anticancer candidate compounds, exhibiting impressive antitumor activity in various murine and human tumors *in vitro* and *in vivo* [45,46]. In an animal model of chemical-induced oral cancer, a GRP antagonist has been shown to prevent the formation of malignant tumor lesions [47]. Furthermore, selective GRPR antagonists attenuated GRP-stimulated tumor growth and angiogenic markers *in vivo*. In addition, the interference with GRP/GRPR signaling was shown to have indirect effects upon other intracellular signaling pathways, which was already shown to have clinical importance in cancer therapy, such as the inhibition of VEGF-dependent tumor angiogenesis and epidermal growth factor (EGF)-dependent tumor growth [48,49,50,51,52,53].

Clinical trials with GRP/GRPR antagonists in cancer patients are in the initial phase, and as anticipated by animal toxicology studies, preliminary evaluation in humans did not indicate the existence of major toxic effects limiting to normal tissue [54]. A phase I trial was recently conducted in an institution to determine the safety and feasibility of RC-3095, a GRPR antagonist to 25 patients with advanced solid malignancies [55]. Despite of the fact that the promising results in the preclinical setting have been observed by targeting GRP/GRPR expression using monoclonal antibodies or their antagonists, still limited anti-tumor effects have been observed when these agents have been administered to cancer patients. Presently, efforts to identify the most suitable candidates and to improve the formulation of drug for human use are considered priorities [46].

Focal Adhesion Kinase (FAK)

FAK is a 125-kDa cytoplasmic protein tyrosine kinase that plays an essential role during embryonic development and in the pathogenesis of human disease, including cancer and cardiovascular disease [56,57,58]. FAK is expressed in most tissues and its sequence is highly conserved across species and its expression has increased in invasive and metastatic human cancers including breast cancer, colon cancer, ovarian cancer, thyroid cancer, melanoma, and sarcoma [59,60,61] compared to normal tissue [62]. In normal cells, FAK and adhesion signaling pathways are involved in important cellular processes, including development, vascular function, and repair while in cancer cells, FAK expression and activation play a wide variety of roles in cell survival, proliferation, migration and invasion that are crucial in the progression and malignancy of tumors. For these reasons, many studies have investigated the exact role of FAK in the regulation of these processes.

FAK is composed of three major domains: the N-terminal band 4.1, ezrin, radixin, moesin homology (FERM) domain, the central kinase domain, and the C-terminal focal adhesion-targeting (FAT) domain. Activation occurs when integrin interacts with the FERM domain, exposing the catalytic site, resulting in autophosphorylation of FAK at tyrosine 397 (Y397), which creates docking sites for proteins with Src homology 2 (SH2) domains, specifically Src, the p85 subunit of PI3K, Grb7, and PLC [63,64]. Phosphorylated FAK can recruit and bind the SH2 domain of several other molecules including Src and PI3K and FAK phosphorylates other tyrosine residues including residues Y576 and Y577 in the kinase activation loop resulting in further activation. These phosphorylation events are required for initiating a cascade of phosphorylation events and new protein-protein interactions to trigger numerous signaling pathways. FAK signaling pathways have shown to be important to regulate a variety of intracellular functions including cell spreading, migration, invasion, proliferation, survival, and apoptosis in both normal cell development and cancer cell progression (Fig. 2).

Meanwhile, the abundant expression of both FAK mRNA and protein in aggressive human neuroblastoma tumors has been reported [24]. In addition, it has been demonstrated that MYCN regulates the expression of FAK through its promoter in human neuroblastoma cells so that FAK is overexpressed in *MYCN* amplified neuroblastoma cells compared to *MYCN* non-amplified cells [25]. Moreover, inhibition of FAK has resulted in decreased cellular attachment and increased cellular apoptosis in these cells [65]. Furthermore, use of small molecule inhibitors in human neuroblastoma has also been reported. FAK inhibition with TAE226 treatment of *MYCN* amplified neuroblastoma cells results in significantly decreased cell viability with minimal effects upon *MYCN* non-amplified cells [66]. Additionally,
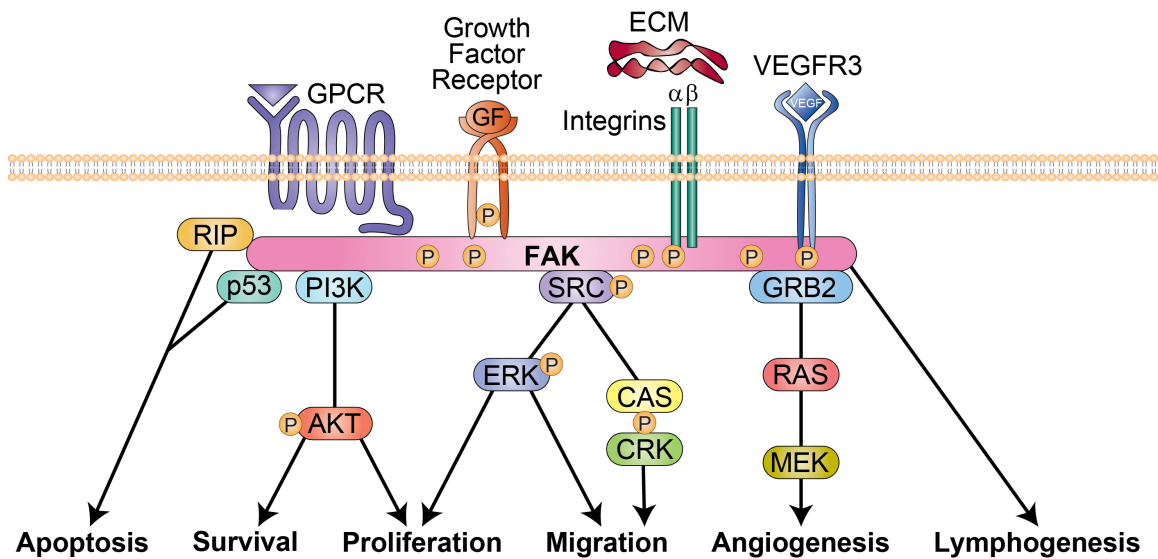
Figure 2. FAK signaling pathways. FAK is an important mediator of interacting various proteins and signaling cascades that are involved in apoptosis, cell survival, proliferation, migration, angiogenesis and lymphogenesis. Interaction with integrins and growth-factor receptors as well as intracellular kinases causes a conformational change in FAK and induces activation of FAK. This initiates a cascade of phosphorylation events of other downstream proteins and new protein-protein interactions to trigger numerous signaling pathways.

treatment with 1, 2, 4, 5-benzenetetraamine tetrahydrochloride (Y15) resulted in decreased cellular attachment and increased apoptosis *in vitro*, and decreased neuroblastoma tumor growth *in vivo*. These effects were more pronounced and sensitive in the *MYCN* amplified neuroblastoma cell lines [67]. Y15 is known to specifically and directly block phosphorylation of Y397 site of FAK in a dose and time dependent manner [68]. As mentioned above, Y397 is an autophosphorylation site of FAK and is a critical site for activating a variety of downstream targets. As a main phosphorylation site Y397 is also a site of binding of PI3K and Src family kinases. In addition, treatment of Y15 in normal ganglion cells had no effect upon the viability of these non-cancer cells.

There are several FAK inhibitors currently under investigation in clinical trials. Recently, it has been reported that an adenosine triphosphate (ATP) competitive reversible inhibitor of FAK has bioavailability suitable for preclinical animal and human studies. PF-562271 is a diaminopyrimindine-type compound that inhibits FAK and exhibits high degree of selectivity in the inhibition of protein tyrosine kinases [69]. PF-562271 exhibited >100 fold selectivity for FAK and showed a dose-dependent decrease in FAK phosphorylation at the Y397 site in cancer cells. PF-562271 is currently in phase II clinical trials. In preclinical testing, PF-00562271 was found to slow tumor growth in prostate and glioma xenograft models and promote regression of bone tumors in a xenograft model of metastasis with minimal weight loss or mortality [69,70]. Phase I trials with thirty two patients receiving from 5 mg up to 105 mg twice a day of this drug in patients with advanced solid malignancy has been performed in two places in the United States and each one place in Canada and Australia. Overall, phase I trials in adult patients with solid tumors demonstrated

this particular drug to have good tolerability with favorable pharmacokinetics and pharmacodynamics and prolonged disease stabilization [71].

Integrins

Integrins are heterodimeric glycoprotein cell surface receptors comprised of α and β subunits and are the major metazoan mediators of cell-cell adhesions and cell-extracellular matrix (ECM) interactions [72]. These interactions make transmembrane connections to the cytoskeleton and activate multiple intracellular responses including adhesion and migration [73]. Additionally, ECM, which is the defining feature of connective tissue in animals, provides structural support to cells in addition to performing various other important functions such as regulating intercellular communication and/or cell's dynamic behavior [74]. The integrin family comprises of 18 α and 8 β subunits that combine to form at least 24 distinct heterodimers in mammals [72]. Integrins bind cell surface and ECM components such as collagen, fibronectin, laminin, and vitronectin, and their ligand specificity is determined by the specific combination of α and β subunits [75]. Integrins are known to participate in many aspects of tumorigenesis [76]. Although integrins alone are not oncogenic, recent data have shown that integrin signaling is required for some oncogenes to initiate their ability of tumor growth and invasion [77].

Aggregation of FAK with integrins and cytoskeletal proteins in focal contacts has been proposed to be responsible for FAK activation in cell adhesion [78]. For this association, the cytoplasmic domain of integrin β subunits is important [79]. Autophosphorylation of FAK at Y397 by integrins leads to its association with Src, resulting in the activation of both kinases and potential substrates, tensin, paxillin and p130cas [80]. A number of studies have shown that integrin signaling through

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FAK leads to an increase in cell migration as well as potentially regulating cell proliferation in cancers [81,82].

In neuroblastoma cells, several studies were reported that integrin expression correlates with neuroblastoma tumorigenesis; neuroblastomas with a good prognosis express several integrin heterodimers, whereas those with a poor prognosis lack integrin expression [76,83]. Moreover, increased integrin expression resulted in increased neuroblastoma cell adhesion and differentiation [84]. More importantly, Tanaka *et al*. have shown that MYCN overexpression inhibits cell adhesion to the ECM and promotes cell migration by downregulating expression of integrin in neuroblastoma cells [85]. These findings suggest that MYCN may be exerting a negative regulatory effect on transcription of integrin, and further MYCN promotes metastasis by downregulating integrin, representing one mechanism that promotes increased neuroblastoma metastasis. Therefore, these data also suggest that integrin is an important regulator of neuroblastoma progression. However, it is unknown whether integrin expression correlates with other membrane receptors, specifically with GRPR signaling, and further whether there is any association between GRPR signaling and integrin on FAK activation thereby regulating neuroblastoma cell migration. My study expanded our laboratory's investigations on the role of integrin expression and GRPR signaling in neuroblastoma cells by examining cell migration.

Integrin targeted therapeutics using targeted antibodies for specific integrin subunits or heterodimers, peptide-based drugs for competing against integrin ligands and/or small molecule, peptidomimetic integrin inhibitors are currently under preclinical development and clinical evaluation for the treatment of cancer [86].

Role of FAK in interacting with GRP/GRPR signaling and integrins

A large number of studies suggest that FAK is an important regulator in tumorigenesis and metastasis. The mechanisms by which FAK mediates the different processes involved in tumor progression are likely to be numerous. In the development of tumors, FAK overexpression seems to be important for the signal transduction initiated at attachment sites of cell. In fact, FAK is a key regulatory factor of the cell signaling cascade initiated either at the site of cell attachment or at growth factor receptors. FAK plays an essential role in cell adhesion and migration mediating the intracellular signaling of integrin receptors [87]. Cell-cell interactions and binding of the ECM trigger integrin clustering and the formation of focal adhesions within the cell. This allows bidirectional signaling across the plasma membrane and regulates adhesion, migration, survival, growth and differentiation in normal cells.

Meanwhile, the effect of GRP/GRPR activation on tyrosine kinase cascades has been extensively studied in other cancers. In fact, binding of GRP to GRPR causes the tyrosine phosphorylation of FAK and paxillin, which links to the integrity of cell cytoskeleton and increases in cell motility [88]. GRPR itself does not have tyrosine kinase activity but interacts with G proteins, which activates downstream signal cascades and phosphorylates various protein substrates including FAK. Besides the signals from GRPR, FAK also integrates inputs from a variety of cell surface-associated receptors. Integrin and other growth factor receptors can facilitate FAK activation as measured by increased tyrosine phosphorylations at Y397, Y576, Y577, Y861, and Y925 to regulate cell growth, survival, and migration [89,90,91,92]. FAK indirectly localizes to sites of integrin clustering through C-terminal-domain-mediated interactions [93] with integrin-associated proteins such as

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paxillin [94,95] and talin [96]. Previous studies by others have shown that integrin and growth factor receptor signaling can interact through either membrane-proximal clustering of the two receptors or the activation of common downstream signaling pathways [97,98,99,100,101]. Although there is a wealth of knowledge regarding the signaling pathways activated by both integrin and growth factor receptors, little is known about how these signals are integrated by cells and whether there are common receptor-proximal control points that synchronize the execution of biological functions such as cell motility. Sieg *et al*. have demonstrated that FAK acts as a receptor-proximal bridging protein that links growth factor receptor through its N-terminal domain and integrin through its C-terminal domain [101].

Statement of Problems and Hypothesis

Despite advances in multi-modality treatment for pediatric solid tumors, patients with advanced-stage neuroblastoma, particularly in older children (> 1 yr), have a dismal prognosis. Novel therapeutic strategies are required to address the issue of tumor metastasis. Moreover, it is critical to attain a better understanding of the cellular and molecular mechanisms involved in neuroblastoma cell growth, migration, invasion and metastasis.

Our laboratory previously reported that GRP and GRPR are overexpressed in aggressive neuroblastoma cell lines as well as in tumor sections from neuroblastoma patients when compared to benign type of cells and tissues. Further research demonstrated that the mitogenic effects of GRP are mediated by activation of the PI3K pathway followed by phosphorylation of AKT. Moreover, GRP also induces activation of FAK at Y397 site. The activation of FAK is involved in various intracellular pathways, including GRP-mediated cell signaling [102,103]. Additionally,

high levels of GRPR and FAK have been reported in human prostatic tissues from patients with advanced disease and in tumorigenic cell lines [104,105,106]. Likewise, many accumulating results suggest that GRP/GRPR signaling and FAK are associated and they contribute to tumor growth. Nevertheless, it is unknown whether there is a functional and physiological relationship between GRP/GRPR signaling and FAK, and consequently, whether GRP/GRPR regulates FAK in modulating cell morphology, growth, motility, and metastasis in neuroblastoma. Therefore, these evidences led me to hypothesize that as a critical downstream target, FAK has critical functions during GRP/GRPR-mediated neuroblastoma progression. Furthermore, I sought to determine whether GRPR interacts with integrins to regulate FAK activation in neuroblastoma cells, and whether there is a critical role of integrins to regulate cell migration in GRPR expression-modulated neuroblastoma cells. For this study, I investigated the expression level of FAK and GRPR in neuroblastoma patient samples. Moreover, I determined the role of FAK in GRP/GRPR-mediated neuroblastoma cell growth, migration and invasion *in vitro* and *in vivo*. Furthermore, I delineated the mechanism by which crosstalk between GRPR and integrin regulates FAK activation and metastasis. In the long term, a better understanding of the crosstalk between signaling pathways in neuroblastoma could lead to novel therapeutic strategies to be used as adjuvant treatment options in this clinically aggressive cancer.

CHAPTER II

MATERIALS AND METHODS

Antibodies and reagents

Primary antibodies used include GRPR from Abcam, p-FAK, FAK and matrigel from BD Biosciences, MMP-2 from Calbiochem, TIMP-1 from Bethyl Laboratories, β-actin, Tubulin from Sigma-Aldrich, MYCN, MMP-9, p-AKT, AKT, p-ERK1/2, ERK1/2 from Cell Signaling Technology, integrin α3 and β1 from Santa Cruz Biotechnology, Alexa Fluor 568 and 488 from Molecular Probes. FAK (4.47) antibody for immunohistochemistry was from Millipore. All secondary antibodies against mouse and rabbit IgG were purchased from Santa Cruz Biotechnology. GRP and BBS from TOCRIS Bioscience and Bachem, Y15 ($C_6H_{10}N_4\cdot4ClH$, 1,2,4,5-benzenetetraamine tetrahydrochloride) from Sigma-Aldrich, and LY294002, U0126 from Cell Signaling Technology were used. cDNA GEArray® Microarray and Flow Cytometric Analysis kits were obtained from SuperArray Bioscience Corporation. Agarose (SeaPlaque®) was from Cambrex Bio Science. Cell Counting Kit-8 (CCK-8) was from Dojindo Molecular Technologies. Immunohistochemistry reagents were from Dako Corporation. siRNAs against FAK, integrin α3 and β1 were from Dharmacon, along with non-targeting scrambled sequences that were used as controls.

Cell culture and transfection

Human neuroblastoma cell lines, SK-N-SH and BE(2)-C, were purchased from American Type Culture Collection. Cells were maintained in RPMI 1640 media with L-glutamine (Cellgro Mediatech) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich). Cells were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Plasmids for GRPR overexpression and silencing have been used as previously described [44], and pCMV6-PTK2 from OriGene was used for transient FAK overexpression. shFAK and pCDH-FAK plasmids for *in vivo* study were kindly provided by Dr. S.T. Lim (University of South Alabama). pMSCV-LucSh, which contains a luciferase and zeocin-resistance fusion gene was also kindly provided by Dr. Andrew M. Davidoff (St. Jude Children's Research Hospital). For plasmid transfection, both cell lines were transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Stably-transfected cells for GRPR were established by selection with G418 at 300 µg/ml and/or zeocin at 50 µg/ml for 2 weeks. Stable populations of shFAK cells were obtained by lentiviral infection and puromycin selection at 2.5 µg/ml. Luciferase-expressing cells were established by selection with zeocin at 50 µg/ml. Cells were passed twice a week using trypsin 0.25% with EDTA 0.1% (Cellgro Mediatech, Inc.). GRP was administered after serum-starvation overnight to synchronize cells prior to stimulation. Cells were treated with LY294002 (20 µM), U0126 (10 µM) or Y15 (10 µM), for 30 min prior to GRP (100 nM) stimulation for 5 min [67,107]. Experiments were repeated on 3 separate occasions.

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Inducible knockdown system

For the knockdown of our target genes, human GRP and GRPR, we used BLOCK-iT Inducible H1 Lentiviral RNAi System (Invitrogen). The sequence targeting GRPR (NM_005314) is underlined in the following shRNA (shGRPR) sequence: 5'-CACCG<u>TAACGTGTGCTCCAGTGGACG</u>AATC<u>CACTGGAGCACACGTTA</u>-3'; the sequence targeting GRP (NM_002091) is underlined in the shRNA (shGRP) sequence: 5'-CACCA<u>GCAATCAGCAGCCTTCGTGGGA</u>CGAA<u>TCCCACGAAGG CTGCTGATTGC</u>-3'; the nonspecific control shRNA (shCON) is: 5'-CACCGGGCGCGCTTTGT AGGATTCGCCGAAGCGAATCCTACAAAGCGCGCC-3'. shRNA sequences were cloned into the BLOCK-iT Inducible H1 RNAi Entry Vector (pENTRTM/H1/TO). Then shRNA was inserted into lentiviral vector pLenti4/BLOCK-iT-DEST by LR recombination between pENTRTM/H1/TO entry and pLenti4/BLOCK-iT expression constructs. Inducible shRNA expression cells were established by transfecting BE(2)-C cells with both pLenti6/TR and pLenti4/BLOCK-iT-DEST, or by introducing the vectors with the lentiviral-mediated delivery system. Production of lentivirus was performed in 293FT cells. Stable cell lines BE(2)-C/tet/shCON, BE(2)-C/tet/shGRP, and BE(2)-C/tet/shGRPR were established by selecting with blastatin at 8 ug/ml and zerocin at 50 ug/ml post lentiviral transductions.

Quantitative real-time PCR (QRT-PCR)

Total RNA was isolated from neuroblastoma cells using the RNAqueous^{TM} (Ambion) according to the manufacturer's instructions. Isolated RNA (1 µg) was used to synthesize cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Primers were designed to amplify a 158-bp FAK fragment

(BC028733.2): forward primer 5'-TTATTGGCCACTGTGGATGA-3'; reverse primer 5'-TACTCTTGCTGGAGGCTGGT-3'. Primers for GRPR: forward primer 5'-ATTTGGCAGGATTGGCTGC-3'; reverse primer 5'-TGAGGCAGATCTTCATCAG-3' and primers for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a control: forward primer 5'-TCCTCTGACTTCAACAGCGACACC-3'; reverse primer 5'-TCTCTCTTCCTCTTGTGCTCTTGG-3' were the same as published [53]. QRT-PCR was performed in the CFX96™ Real-Time PCR Detection Systems using SsoFast™ EvaGreen Supermix (Bio-Rad). The reactions were set up at 20 µl with 1 µl cDNA template, 10 µl Sso Fast™ EvaGreen Supermix, 1 µl of each primer (5 µmol/l), and 7 µl distilled water. The reactions were programmed with an initial denaturation step of 2 min at 98°C, followed by 40 temperature cycles for 5 s at 98°C and 5 s at 60°C. At the end of amplification, the melting curve analysis was performed for the PCR products to ensure the amplification specificity. All measurements were produced in duplicate. Relative FAK mRNA levels were calculated based on ratios of the initial cDNA quantity of FAK/GAPDH.

Immunofluorescence

Cells were plated onto glass coverslips in 24-well plates. The following day, cells were fixed with 4% paraformaldehyde for 20 min at room temperature (RT). After three washes with PBS, the cells were permeabilized with 0.1% Triton X-100 for 15 min and blocked with 1% BSA/PBS for 30 min. Cells were incubated with primary antibodies (1:100) for 1 h at RT, washed five times with PBS and then incubated with secondary antibodies (1:500) for 30 min at RT. Nuclei were counterstained with vectashield mounting medium containing 6-diamidino-2-phenylindole (DAPI) (Vector Laboratories). Mouse IgG was used as a non-specific staining control, and

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secondary antibody, without primary antibody, was used for background staining. The coverslips were washed with PBS five times before being mounted on microscope slides. Immunofluorescence signal was observed under a fluorescent microscope (Nikon Eclipse E600).

Immunohistochemistry

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

Immunoblotting

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

Flow cytometry

Cells were trypsinized, washed once by adding 0.1% BSA/PBS and spun down. Cell pellets were resuspended in 0.1% BSA/PBS and adjusted to 1 x 10^6 cells/mL. Integrin antibody (1 µg) was added to 1 mL of cell solution and incubated for 1 h at 4°C on a shaker, then the cells were washed with 0.1% BSA/PBS three time by centrifugation at 300g for 3 min. Allophycocyanin (APC)-conjugated secondary anti-mouse antibody (1:250) was added and incubated for 30 min at 4°C (excitation at 633 nm). Cells were washed three times and resuspended for flow cytometry analysis. Cells without primary antibody incubation was used as negative control. Flow cytometry was performed using a FACSCalibur System (BD Bioscience), and data were collected for viable cells according to side and forward scatter. Integrin expression on the cell surface was quantitated with fluorescence intensity.

Cell viability assay

Cells were seeded at a density of 3 x 10^3 cells/well in a 96-well plate and grown for up to 4 days after transfection. Cell numbers were assessed using CCK-8 daily. Each assay was performed in triplicate, and the experiment was repeated three times for each cell line. The values, corresponding to the number of viable cells, were read at OD450 with the EL808 Ultra Microplate Reader (Bio Tek Instrument).

Soft agar colony formation assay

Cells were trypsinized and resuspended in media containing 0.4% agarose and 7.5% FBS and then overlaid onto a bottom layer of solidified 0.8% agarose in 5% serum media. SK-N-SH and BE(2)-C were plated at concentrations of 5x10^3

cells/well and $3\text{x}10^3$ cells/well of a 12-well plate and incubated for 5 and 3 weeks, respectively. Colonies were stained with 0.05% Crystal Violet, photographed and quantified.

Migration assay

For wound healing assay, a confluent monolayer of cells in 12-well plate were wounded with a sterile plastic 200 µl micropipette tip. Cells in media containing 1% FBS for control and plus GRP (100 nM) were incubated and observed microscopically at 24 to 72 h. The percentage of wound filling was calculated by measuring the remaining gap space. For transwell migration assay, polycarbonate transwell filters (8 µm; Corning) were coated on the lower side with 5 µg/ml collagen type I (BD Biosciences) overnight and then blocked with 2.5% BSA/PBS for 1 h. 1×10^5 cells in serum-free media added to the upper and allowed to migrate for 4 h at 37°C under tissue culture conditions. Cells that failed to migrate through the filter after incubation were scraped out using a sterile cotton swab. Cells that migrated to the bottom surface of the filter were fixed with 4% paraformaldehyde, stained with DAPI, and counted. Assay was performed in duplicates, and counting was from five randomly selected microscopic fields (200X magnification).

In vivo assay

Male athymic nude mice (4–6 weeks old) were maintained as described [108]. All studies were approved by the Institutional Animal Care and Use Committee at Vanderbilt University and were conducted in accordance with guidelines issued by the National Institutes of Health. BE(2)-C cells were transfected with luciferase alone or stably-transfected with plasmids (shCON, shGRPR, shFAK and/or pCDH, pCDH-

FAK). Mice were anesthetized with isofluorane, and a small left flank incision was made to isolate and exteriorize the spleen; a total of 1×10^6 cells in 50 µl of Hank's balanced salt solution (HBSS) were injected into the splenic capsule using a 27-gauge needle. Mice were treated daily with 50 µl of control vehicle (phosphate buffered saline-PBS) or Y15 (30 mg/kg/day) and/or BBS (20 µg/kg/t.i.d.). Previous findings with various doses and dosing schedules of the compound proved this to be the optimal, nontoxic dose [68,108]. Tumor growth was observed either by measuring luciferase signal with bioluminescence imaging system (IVIS Lumina II, Xenogen, Caliper Life Sciences) or by measuring body weight weekly. To perform tumor imaging, mice were injected with D-luciferin (OZ Biosciences) subcutaneously (1 mg/mouse in 100 µl of HBSS) before being anesthetized with isofluorane. Measurement of total flux (photons/sec) of the emitted light reflects the relative number of viable cells in the tumor. Data were analyzed using Xenogen Living Image software (version 4.1). At sacrifice, spleens and livers were excised, weighed and fixed in formalin for further analyses.

Statistical analysis

In vitro data represent the means ± standard deviation (SD). Statistical analyses were performed using a Student's paired *t*-test. One-way analysis of variance (ANOVA) on the ranks for repeated measures was performed for multiple comparisons. *In vivo* data represent the means ± standard error of the mean (SEM). Tumor size and body weight were analyzed by one-way ANOVA for comparison among the treatment groups or Mann Whitney test with repeated measures on time. Data analysis was conducted using GraphPad InStat3 (GraphPad Software). For all experiments, a *p* value of < 0.05 was considered statistically significant.

CHAPTER III

FAK MEDIATES GRPR-INDUCED NEUROBLASTOMA PROGRESSION

Introduction

Neuroblastoma is highly aggressive with frequent metastases, which contributes to overall significant morbidity and mortality [109]. Our laboratory has shown that gastrin-releasing peptide receptor (GRPR), a G-protein coupled receptor, is involved in neuroblastoma cell survival, invasive potential and metastasis [44,110]. Our laboratory reported that the upregulation of GRPR increases the binding capacity for its ligand GRP, resulting in a faster constitutive neuroblastoma cellular growth rate [44]. Conversely, downregulation of GRPR reversed the aggressive cell phenotype and inhibited liver metastases *in vivo* [110]. Therefore, GRPR-mediated signaling plays critical roles in tumorigenesis and metastasis in neuroblastoma. However, we have yet to clearly define the molecular mechanisms responsible for GRPR-mediated tumorigenicity.

Focal adhesion kinase (FAK), a 125-kDa cytoplasmic non-receptor protein tyrosine kinase, plays an essential role in cell adhesion and migration [87]. FAK is comprised of a central catalytic domain flanked by large N- and C-terminal non-catalytic domains. The N-terminal domain of FAK binds to sequences in the cytoplasmic domain of β-integrin subunits, thereby functioning as an important

member of the integrin signaling pathway. The C-terminal region of FAK is rich in protein-protein interaction sites, directing FAK to newly-formed and existing adhesion complexes [87]. Cancers are known to express FAK, which is responsible for stimulated cell motility, invasiveness and proliferation [59,105,111]. FAK activation is involved in various intracellular pathways, including GRP-mediated cell signaling [102,103].

High levels of GRPR and FAK have been reported in prostatic tissues from patients with advanced cancer and in tumorigenic cell lines [59]. One report showed that expression of FAK and phosphorylated (p)-FAK (Y397) correlates with the degree of colon cancer cell differentiation as well as to GRP/GRPR co-expression [112]. BBS, an amphibian equivalent of GRP, induces PC-3 cell motility through FAK activation [113]. Our laboratory and others have shown that GRP and BBS bind to GRPR with high affinity to stimulate neuroblastoma cell growth in an autocrine and/or paracrine fashion [43,113]. However, the intracellular signaling mechanisms involved in GRP/GRPR-mediated FAK activation and subsequent neuroblastoma cell growth, motility and metastasis remain unclear.

Results

GRPR and FAK correlated with malignant potential of neuroblastoma.

Our laboratory reported that an increased GRP and GRPR expression is found in more undifferentiated neuroblastoma [43]. FAK expression has been correlated with advanced-stage neuroblastoma [24]. In this study, we wanted to

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determine whether GRPR expression is associated with FAK in neuroblastoma. Firstly, I performed immunohistochemistry to assess GRPR and FAK expression in seven paraffin-embedded tumor sections consisting of five undifferentiated neuroblastomas and two ganglioneuromas. As expected, increased FAK expression was noted in undifferentiated neuroblastomas when compared to more benign phenotype of ganglioneuromas; its expression also correlated with GRPR (Fig. 3A). But due to the limited sample size, I could not determine a correlation of these two protein markers with clinical disease staging. Next, when grown on soft agar, BE(2)-C cells exhibited significantly more colony formation (Fig. 3B), which indicates malignant potential. Consistent with GRPR protein levels, we found higher levels of FAK protein (Fig. 3B) as well as mRNA (Fig. 3C) in BE(2)-C when compared to SK-N-SH cells. Immunofluorescence also demonstrated that BE(2)-C cells show more intense GRPR and FAK expression when compared to SK-N-SH cells (Fig. 3D). These results show that FAK expression correlates with malignant potential induced by increased GRPR expression in neuroblastoma.

GRP induced FAK activation (Y397) and cell migration in BE(2)-C cells.

FAK activation at Y397 site by GRP is well established in 293 HEK cells and various other cancer types [102,113,114]. When GRP was exogenously administered for 5 min, an increase in p-FAK (Y397) was noted in a dose-dependent manner in BE(2)-C cells, whereas SK-N-SH cells did not (Fig. 4A). Moreover, I also found that GRP treatment induces significant BE(2)-C cell migration in both transwell migration assay and wound healing assay (Fig. 4B, C). This results support for the important role of FAK as a mediator of GRP/GRPR signaling, and further validate GRP as an inducer of cell migration in neuroblastoma.
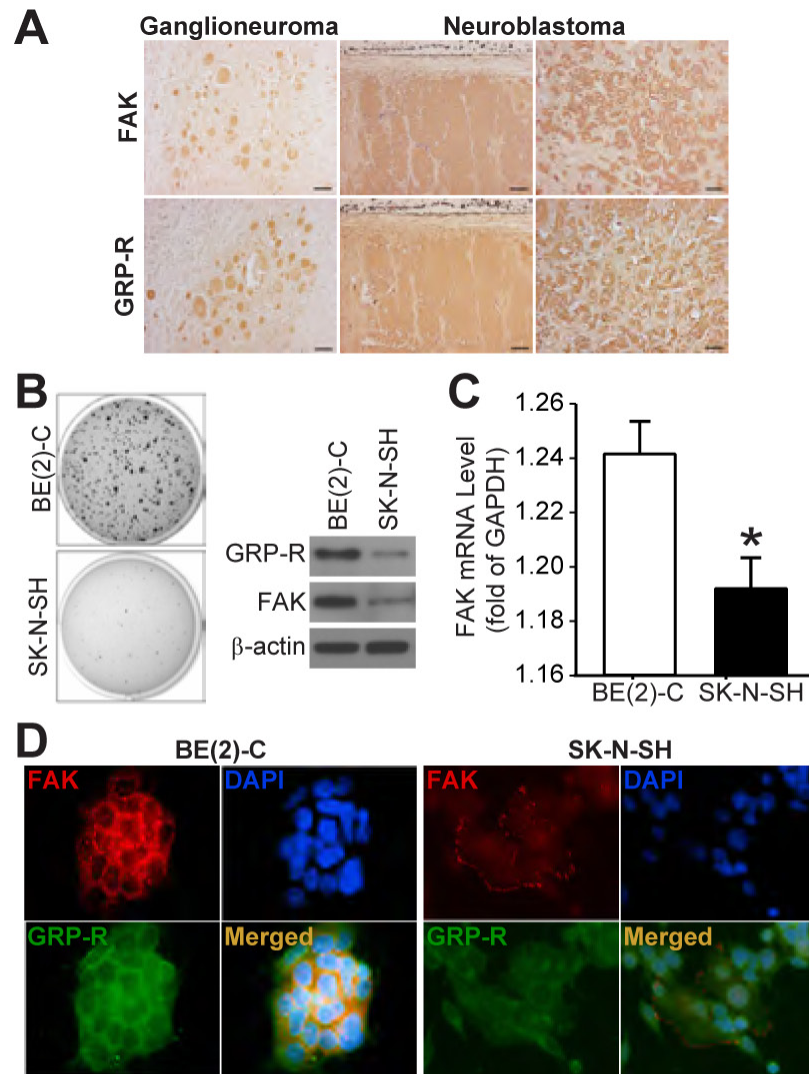
Figure 3. GRPR and FAK expressions correlate to neuroblastoma malignancy. (A) Representative histological sections from human ganglioneuroma and undifferentiated neuroblastomas showed similar FAK (*top row*) and GRPR (*bottom row*) expressions by immunohistochemistry (100X magnification, 20 μm bar). (B) Increased number of colony formation was observed in BE(2)-C cells. BE(2)-C cells demonstrated higher constitutive GRPR and FAK protein levels when compared to SK-N-SH cells by immunoblotting. β-actin showed relatively equal loading. (C) A higher basal level of FAK mRNA was also observed in BE(2)-C cells. FAK mRNA level was expressed as relative copies of FAK/GAPDH. Bars represent the averages of three independent experiments. (*= *p* <0.05 vs. BE(2)-C). (D) More intense immunofluorescence of FAK and GRPR were observed in BE(2)-C cells when compared to SK-N-SH cells (600X magnification).
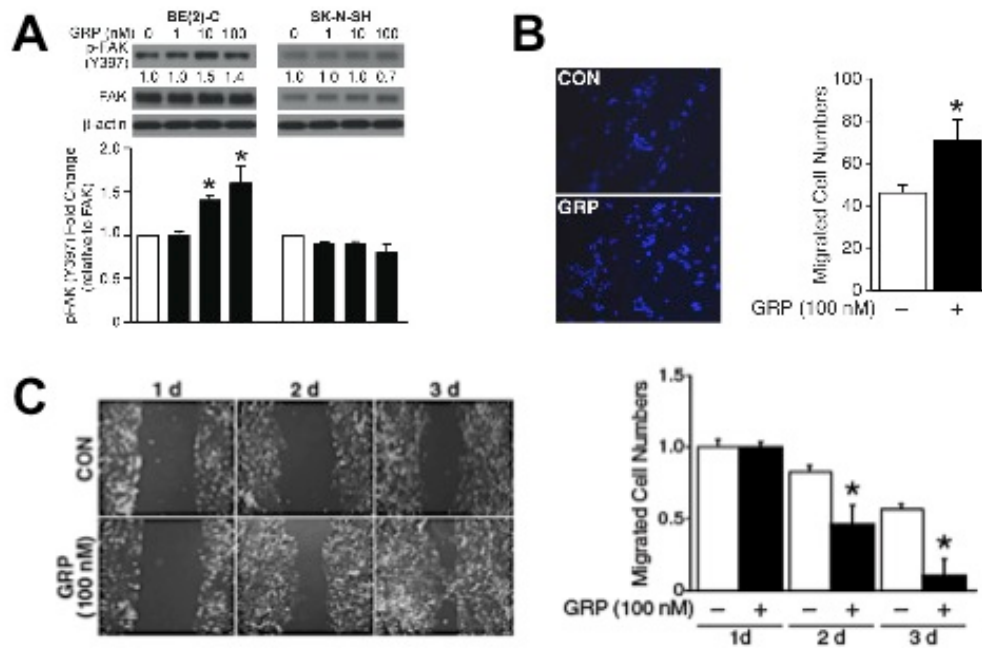
Figure 4. GRP induces FAK activation (Y397) and migration. (A) Exogenous GRP for 5 min after overnight serum starvation increased p-FAK (Y397) as measured by immunoblotting; increased p-FAK by 10 nM and 100 nM of GRP in BE(2)-C was observed when compared to SK-N-SH cells (*= p <0.05 vs. no treated control). (B) GRP-stimulated cell migration was performed using transwell plates in BE(E)-C cells. Values are expressed using migrated cell numbers from two experiments performed in duplicate. Representative images of DAPI staining for counting were shown (200X magnification) (*= p <0.05 vs. CON). (C) Wound closure was measured from microscopic images at 24, 48, and 72 h after wounding (100X magnification). Data are representative of the mean distance of unclosure from three independent experiments (*= p <0.05 vs. CON).

GRPR overexpression increased FAK and integrins expression, which promoted cell migration in SK-N-SH cells.

To understand the positive relationship between FAK and GRPR in neuroblastoma cells, we next performed studies using a GRPR overexpressing SK-N-SH cell line established in our laboratory [44]. In figure 5A, GRPR overexpressing SK-N-SH cells showed increased GRPR mRNA expression but no significant increase of FAK mRNA (*= p <0.05 vs. SK/CON). However, immunoblotting showed that FAK protein is upregulated in GRPR overexpressing SK-N-SH cells when compared to controls (Fig. 5B). Interestingly, integrin α3 and β1 expressions were also significantly upregulated in GRPR overexpressing cells (Fig. 5B). To confirm whether increased FAK activation in GRPR overexpressing cells is dependent on these upregualted integrin expressions, I next performed dual silencing of integrin α3 and β1 (siIntegrin α3β1) in GRPR overexpressing SK-N-SH cells and found that siIntegrin α3β1 significantly decreased p-FAK expression (Fig. 5C). Additionally, to validate these findings and to localize FAK expression, I next performed immunofluorescence study. GRPR overexpressing SK-N-SH cells, which have an altered cell morphology exhibiting a flatter shape with broad lamellipodial projections, showed significantly enhanced FAK expression at the leading edges of cells (Fig. 5D). Furthermore, GRPR overexpressing SK-N-SH cells exhibited increased cell migration in the transwell plates coated with collagen type I (Fig. 5E). These results indicate that GRPR regulates FAK levels post-transcriptionally and FAK activation is regulated in part by integrin expressions in GRPR overexpressing SK-N-SH cells.
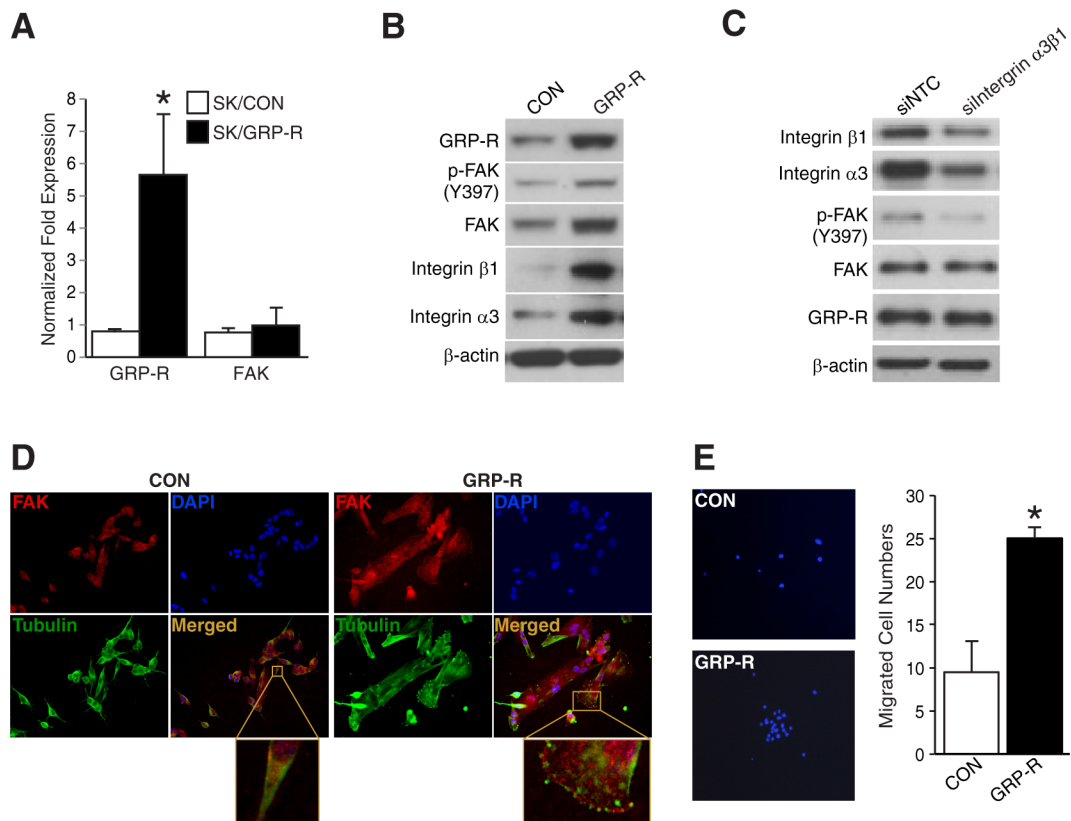
Figure 5. GRPR overexpression increases FAK and cell migration in SK-N-SH cells. (A) Increased levels of GRPR mRNA but no significant increase of FAK mRNA were observed in GRPR overexpressing SK-N-SH cells. Bars represent the averages of three independent experiments. (*= p <0.05 vs. SK/CON). (B) Increased FAK expression as well as integrin α3 and β1 levels in GRPR overexpressing SK-N-SH cells were confirmed by immunoblotting. β-actin demonstrated relatively equal loading. (C) Dual silencing of integrin α3 and β1 (siIntegrin α3β1) in GRPR overexpressing SK-N-SH cells decreased expression of p-FAK (Y397). β-actin demonstrated relatively equal loading. (D) GRPR overexpressing SK-N-SH cells demonstrated altered cellular morphology to a flatter appearance along with significantly more intense FAK immunofluorescence (*red color*). Merged image indicates FAK, Tubulin (*green color*) and nuclei (400X magnification). A representative higher magnification image (*enlarged box*) showed intense FAK localization at the leading edges. (E) GRPR overexpressing SK-N-SH cells migrated more in collagen type I-coated transwell plates when compared to the control. Values are expressed using migrated cell numbers from two experiments performed in duplicate. Representative images of DAPI staining for counting were shown (200X magnification) (*= p <0.05 vs. CON).

GRPR silencing decreased FAK and cell migration in BE(2)-C cells.

Next, to further validate the correlation between GRPR and FAK, I used stably-transfected GRPR silenced BE(2)-C cells (shGRPR) established in our laboratory [110]. In figure 6A, GRPR silenced BE(2)-C cells showed decreases in both GRPR as well as FAK mRNA levels (*= p <0.05 vs. BE/shCON). Furthermore, I found that both phosphorylated and total FAK protein levels were decreased in shGRPR (Fig. 6B). Interestingly, I also found that shGRPR cells downregulated MYCN (Fig. 6B), which is a well-known transcription factor of FAK in neuroblastoma [115,116]. Consistent with these findings, immunofluorescence showed significantly weaker FAK expression in shGRPR when compared to control cells (shCON) (Fig. 6C). Moreover, the appearance of BE(2)-C cells changed from typical flat and aggregated morphology to small and rounder shape after transfection with silencing of GRPR. Furthermore, shGRPR cells exhibited decreased cell migration in the transwell plates coated with collagen type I (Fig. 6D). Hence, my results support a positive correlation between GRPR and FAK, indicating that GRPR is important for a cellular function of cell morphology and migration in neuroblastoma cells by regulating FAK.

Downregulation of GRP/GRPR reduced expression of MYCN.

MYCN, a strong predictor of poor outcomes in patients with neuroblastoma, is a downstream target of PI3K/AKT pathway [21,117,118]. Our laboratory has previously reported that MYCN mediated PI3K-dependent regulation of VEGF expression in neuroblastoma cells [21]. Recent study further demonstrated that anti-angiogenic efficacy of NVP-BEZ235, which is a dual inhibitor of PI3K and mTOR, depended critically on *MYCN in vitro* and *in vivo* [119]. Interestingly, our laboratory's
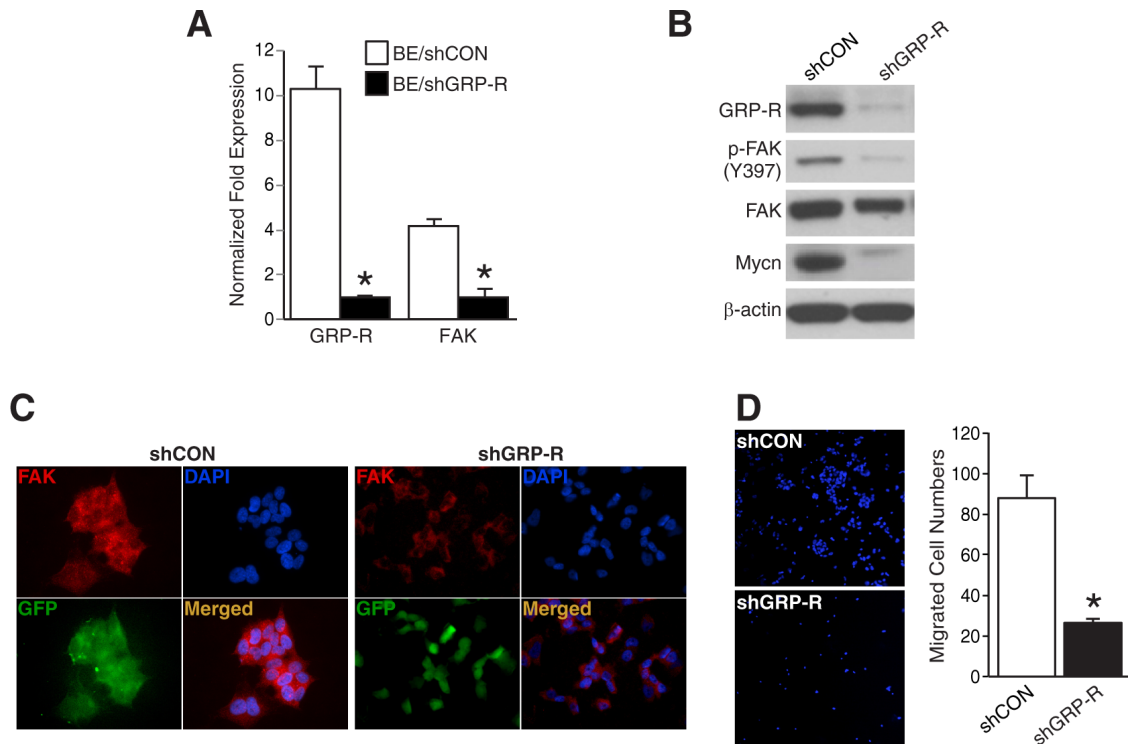
Figure 6. GRPR silencing decreases FAK and cell migration in BE(2)-C cells. (A) Decreases in both GRPR mRNA as well as FAK mRNA expression were in GRPR silenced BE(2)-C cells. Bars represent the averages of three independent experiments. (*= p <0.05 vs. shCON). (B) Decreased FAK and MYCN expressions in GRPR silenced BE(2)-C cells (shGRPR) was confirmed by immunoblotting. β-actin demonstrated relatively equal loading. (C) shGRPR demonstrated a rounder, smaller morphology and showed weaker FAK immunofluorescence (*red color*) when compared to control cells (shCON). Merged image indicates FAK and nuclei (400X magnification). (D) shGRPR migrated less in collagen type I-coated transwell plates when compared to shCON. Values are expressed using migrated cell numbers from two experiments performed in duplicate. Representative images of DAPI staining for counting were shown (200X magnification) (*= p <0.05 vs. shCON).

previous study demonstrated that GRPR is an important regulator for neuroblastoma progression and metastasis, and that the PI3K pathway is significantly downregulated in GRPR silenced BE(2)-C cells [110]. We examined whether GRP/GRPR signaling regulates MYCN expression in *MYCN*-amplified BE(2)-C cells. In order to exclude the effects of MYCN expression by cell cycle, the cells were synchronized by serum-starvation for 24 h, then re-fed in RPMI medium with 10% FBS. The expression of MYCN proteins in GRPR silenced BE(2)-C cells was significantly decreased in comparison to shCON cells at 0 h, and completely degraded after 2 h (Fig. 7A). However, the mRNA levels of *MYCN*, as measured by QRT-PCR, were not appreciably affected by GRPR silencing (Fig. 7B). Our results suggest that GRPR activation of cell signaling regulates endogenous MYCN expression at a post-transcriptional level. In order to exclude any specific effect of stable GRPR silencing, we also used a doxycycline-inducible silencing system in BE(2)-C cells (Tet/shGRPR), in which GRPR can be conditionally downregulated by doxycycline treatment. MYCN expression was significantly decreased in a dose-dependent manner after doxycycline-induced GRPR silencing (Fig. 7C). Furthermore, using another doxycycline-inducible system for silencing GRP (Tet/shGRP), we assessed the exact effects of GRP silencing on MYCN expression in BE(2)-C cells. This demonstrated similar results to the shGRPR inducible system, in which MYCN expression decreased following doxycycline treatment for 48 h (Fig. 7D). Hence, this finding indicate that GRP/GRPR signaling regulates MYCN expression, which may be at a post-transcriptional level in neuroblastoma cells.
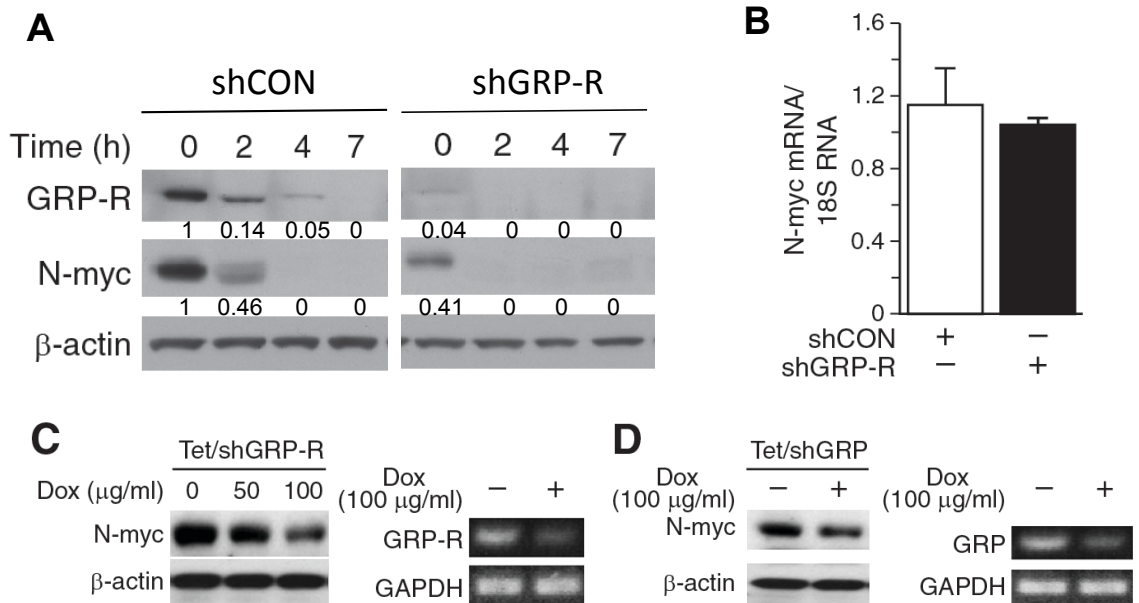
Figure 7. GRP/GRPR regulates MYCN expression. (A) MYCN expression was measured in control and GRPR silenced BE(2)-C cells by Western blotting. Cells were serum-starved for 24 h and were then re-plated in fresh RPMI media with 10% FBS. As expected, GRPR expression lower in shGRPR cells than in shCON cells. Interestingly, MYCN expression was decreased in shGRPR cells at 0 and 2 h. (B) Cells were serum-starved for 24 h, and then total RNA was extracted for real-time QRT-PCR at 24 h transfections. *MYCN* mRNA levels remained relatively unchanged. Bars represent the averages of three independent experiments. (C) Inducible GRPR silencing BE(2)-C/Tet/shGRPR cells were treated with doxycyclin for 48 h, and then the expression of MYCN were analyzed by Western blotting. MYCN expression level was correspondingly decreased with GRPR silencing (4C, *left*); Decreased GRPR mRNA was confirmed with RT-PCR (4C, *right*). (D) Similar to Fig. 4C, inducible GRP silencing BE(2)-C/Tet/shGRP cells were treated with doxycyclin for 48 h. The expression of MYCN was confirmed with Western blotting (4D, *left*). Inducible knockdown of GRP mRNA was confirmed with RT-PCR (4D, *right*). Levels of protein expression were quantified by densitometry analysis and the values were labeled under the bands.

FAK regulated neuroblastoma cell growth in vitro and in vivo.

In order to examine the critical role of FAK on neuroblastoma malignant potential, I transiently transfected SK-N-SH and BE(2)-C cells with FAK plasmid (Fig. 8A) and siRNA against FAK (siFAK) (Fig. 9A, B), respectively. To validate the effects of downstream signaling pathways on modulating FAK expression in each cell line, phosphorylated and total expressions of AKT and extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) were examined. Interestingly, modulation of FAK expression led to differential expression of p-AKT and p-ERK (Figs. 8A, 9B). FAK overexpression stimulated phosphorylation of AKT and ERK. Conversely, FAK silencing decreased p-AKT and p-ERK levels, hence suggesting a mechanism of FAK activation of PI3K and MEK pathways in neuroblastoma cells. I used soft agar colony assay to assess for anchorage-independent cell growth, which is a well-established indicator of tumorigenicity of cancer cells *in vitro* [120]. FAK overexpression showed an increased number of colonies by > 2.5-fold, and quite interestingly, it also resulted in formation of larger colonies (Fig. 8B). Furthermore, cell viability also increased over a time course with most significant increase at 96 h (Fig. 8C). In contrast, siFAK showed decreased number of colonies by 35%; siFAK resulted in fewer and smaller colonies when compared to non-targeting control siRNA (siNTC) (Fig. 9C). siFAK also significantly decreased BE(2)-C cell viability (Fig. 9D). To validate these findings *in vivo* and to further examine the metastatic potential of BE(2)-C cells stably-transfected with shRNA against either control (shCON) or FAK (shFAK), I performed intrasplenic injections of neuroblastoma cells in nude mice. Our laboratory established the murine model to investigate neuroblastoma metastasis, and reported that GRPR silencing inhibited tumor growth
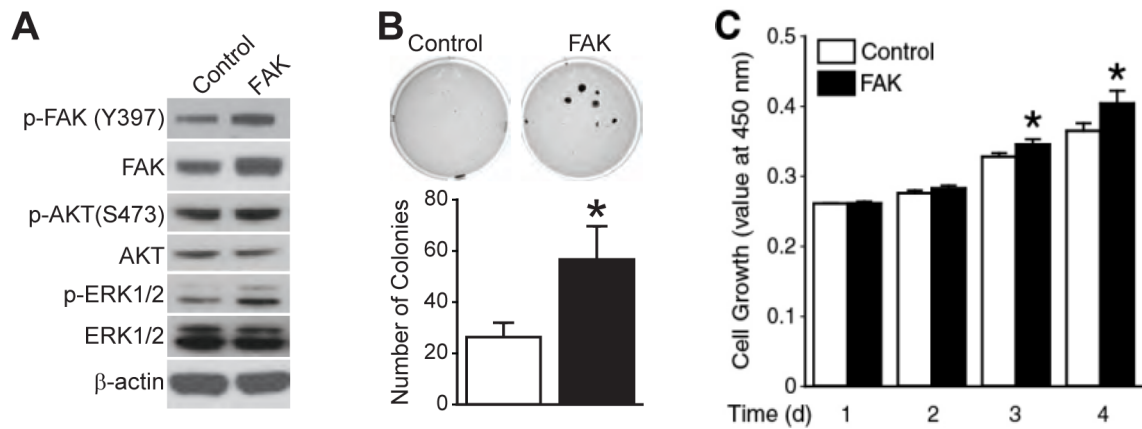
Figure 8. FAK overexpression upregulates SK-N-SH neuroblastoma cell growth. (A) Immunoblotting demonstrated FAK overexpression in SK-N-SH cells. pCMV6-XL4 was used for control. Phosphorylated and total protein levels of AKT and ERK1/2 were tested in the cells. β-actin demonstrated relatively equal loading. (B) FAK overexpression increased soft agar colonies by nearly 2.5-fold (*= p <0.05 vs. CON). (C) FAK overexpression increased SK-N-SH cell viability after 72 h. Bars represent the averages of three independent experiments. (*= p <0.05 vs. CON).
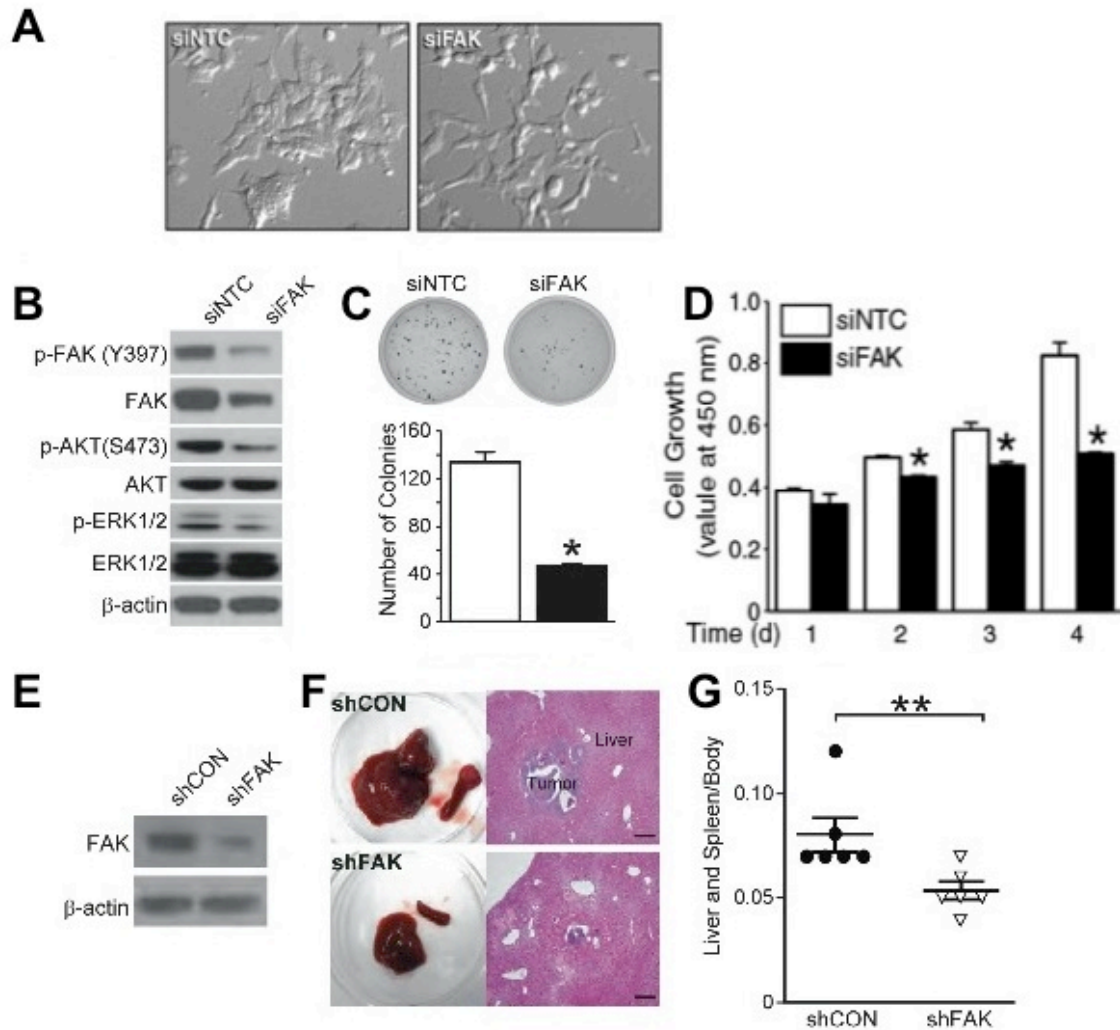
Figure 9. FAK silencing inhibits BE(2)-C neuroblastoma cell growth *in vitro* and *in vivo*. (A) Cells after 48h transfection of siRNA against FAK. (B) Immunoblotting demonstrated transient FAK silencing (siFAK) in BE(2)-C cells. siFAK decreased phosphorylated protein levels of AKT and ERK1/2. β-actin demonstrated relatively equal loading. (C) siFAK inhibited colony formation by 35% compared to control (siNTC) (*= *p* <0.05 vs. siNTC). (D) siFAK significantly inhibited BE(2)-C cell viability after 48 h. Bars represent the averages of three independent experiments. (*= *p* <0.05 vs. siNTC). (E) Immunoblotting demonstrated stable FAK silencing (shFAK) in BE(2)-C cells. β-actin demonstrated relatively equal loading. (F) Representative gross images of tumor from mice and H&E staining of liver sections (200X magnification, 50 µm bar) (G) Spleen and liver weight relative to body weight (*n* = 6 mice in each group; **= *p* <0.005 vs. shCON; Mann Whitney test).

and liver metastases *in vivo* [110]. Specificity of FAK silencing was demonstrated by immunoblotting (Fig. 9E). Six weeks after injections, tumor volume of spleens and livers were examined. While shCON formed numerous large liver metastases, shFAK developed very few liver lesions (Fig. 9F). The average spleen and liver weight in the shFAK group was approximately 62% of the shCON group (mean value 0.08 for shCON vs. 0.05 for shFAK) (Fig. 9G). These results indicate that FAK silencing decreases malignant potential of neuroblastoma cells, thus providing further support for the importance of FAK as a regulator of neuroblastoma malignancy.

FAK overexpression rescued GRPR silencing-mediated inhibition of cell growth in vitro and in vivo.

To assess whether FAK is a downstream target of GRPR-mediated cell signaling, I next performed rescue experiments using a FAK plasmid in shGRPR cells in order to test whether FAK overexpression recovers the inhibitory effect of shGRPR. Immunoblotting showed knockdown of GRPR and overexpression of FAK after transfections (Fig. 10A). As previously reported [110], shGRPR exhibited significantly reduced number of soft agar colony formation. FAK overexpression resulted in increased colony formation in both shCON and shGRPR cells (Fig. 10B). FAK overexpression in shGRPR cells rescued their ability to form colonies to a similar value to that of shCON. Correlative to soft agar colony formation, cell viability assays demonstrated restored cell growth after FAK overexpression in shGRPR cells (Fig. 10C). Furthermore, to demonstrate these observations *in vivo*, I used a murine model after stably-transfecting BE(2)-C cells with a lentiviral system of FAK
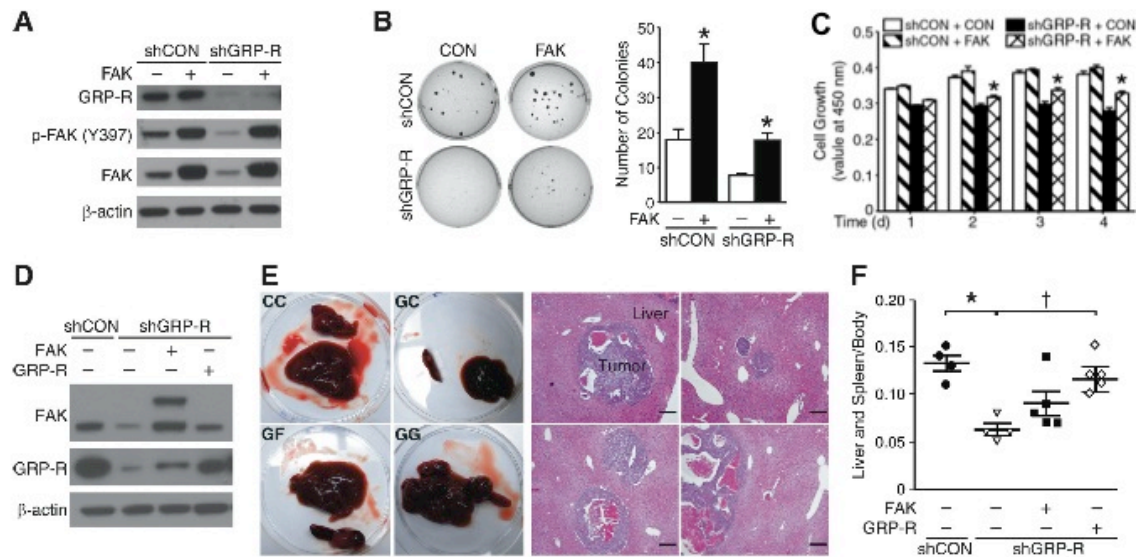
Figure 10. FAK overexpression in GRPR silencing restores neuroblastoma growth.
(A) Immunoblotting confirmed transient FAK overexpression (pCMV6-PTK2) in shGRPR. β-actin demonstrated relatively equal loading. (B) FAK overexpression increased soft agar colonies in shCON and shGRPR when compared to controls of FAK overexpression (*= p <0.05 vs. FAK vector control). (C) FAK overexpression in shGRPR restored cell viability. Bars represent the averages of three independent experiments. (*= p <0.05 vs. FAK vector control). (D) Immunoblotting confirmed stable FAK overexpression (pCDH-FAK) in shGRPR. β-actin demonstrated relatively equal loading. (E) Representative gross images of tumor from mice (shCON/pCDH = CC, shGRPR/pCDH = GC, shGRPR/pCDH-FAK = GF, shGRPR/pCDH-GRPR = GG) and H&E staining of liver sections (200X magnification, 50 µm bar) (F) Spleen and liver weight relative to body weight (n = 4-5 mice in each group; *= p <0.05 vs. CC, †= p <0.05 vs. GC; Mann Whitney test).

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plasmid. Specificity of each protein expression was demonstrated by immunoblotting (Fig. 10D). BE(2)-C cells with GRPR knockdown (GC) induced fewer metastatic lesions in the liver when compared to control (CC), and reintroducing GRPR (GG) into cells rescued the inhibitory effect of GC (Fig. 10E). Although FAK overexpression in GC did not show statistically significant effects on liver metastasis, the mean weight of spleen and liver in these mice increased by ~1.5-fold of GC (mean value 0.06 for GC vs. 0.09 for GF) (Fig. 10F). These findings suggest that FAK plays a critical role in GRPR-induced anchorage-independent growth, and as a downstream target of GRPR, FAK expression, in part, compensates for the inhibitory effect of GRPR silencing in neuroblastoma. Taken together, my results demonstrate that FAK is an important mediator in the GRP/GRPR signaling pathway.

FAK inhibition reduced GRP-induced colony growth and cell migration in vitro.

Previously, our laboratory has demonstrated that GRP treatment activated PI3K by showing the increase of phosphorylation of AKT and GSK3α/β, downstream effectors for the PI3K pathway [107]. Moreover, the activation at Y397 of FAK by GRP treatment has been reported by many other studies. In order to examine the effect of FAK inhibition on GRP-induced activation of downstream signal pathway, I performed combination treatment in soft agar colony formation and migration assay. First of all, I found that pre-treatment with Y15 compound (10 μM), a FAK-specific inhibitor, for 30 min failed to induce FAK activation by GRP for 5 min after overnight serum starvation at Y397 in BE(2)-C cells (Fig. 11A). GRP alone induced activation of AKT and ERK1/2, whereas Y15 treatment attenuated GRP-induced increases in p-AKT and p-ERK (Fig. 11A). Additionally, I found that pretreatment of PI3K-specific inhibitor (LY249002) or ERK-specific inhibitor (U0126) did not block FAK activation
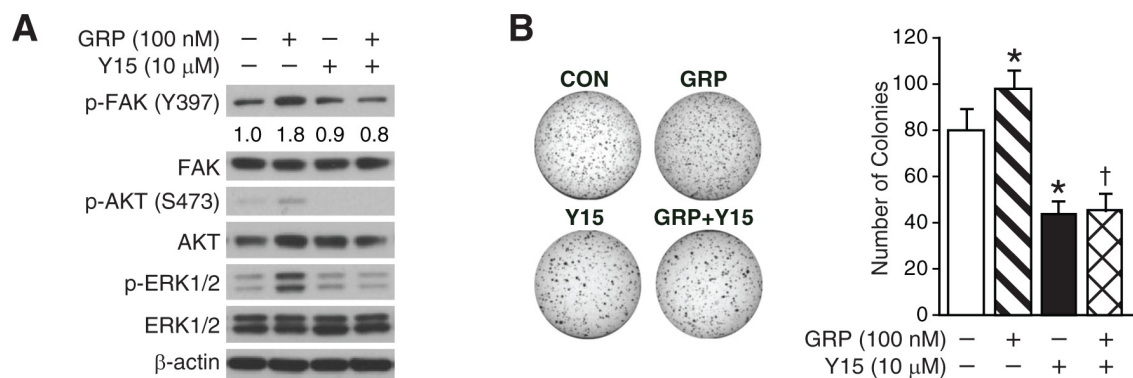
Figure 11. Y15, a FAK inhibitor blocks GRP-induced neuroblastoma growth *in vitro*.
(A) Immunoblotting confirmed that GRP-induced FAK activation in BE(2)-C cells was blocked by pre-treatment of Y15 (10 μM) for 30 min. Y15 treatment attenuated GRP-induced activation of AKT and ERK1/2. (B) GRP (100 nM) increased colony formation by ~1.2-fold whereas Y15 (10 μM) inhibited it by 45% compared to control; similarly Y15 also inhibited GRP-induced colony formation. Bars represent the averages of three independent experiments. (*= p <0.05 vs. CON, †= p <0.05 vs. GRP). GRP and Y15 were added into serum media on solidified soft agar.
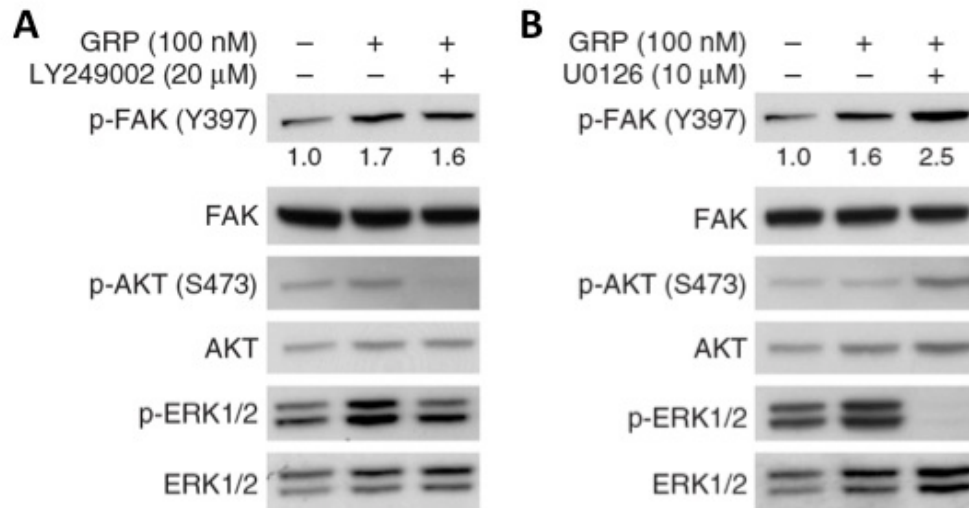
Figure 12. FAK activation by GRP treatment combined with other inhibitors. GRP plus LY249002 (A) or U0126 (B) induced phosphorylation of FAK (Y397). Neither LY249002 nor U0126 altered GRP-induced phosphorylation of FAK (Y397). Immunoblotting confirmed the specific inhibitory effects of LY249002 or U0126 on AKT and ERK, respectively. Cells were pretreated with each inhibitor for 30 min after overnight serum starvation, and stimulated with GRP (100 nM) for 5 min.
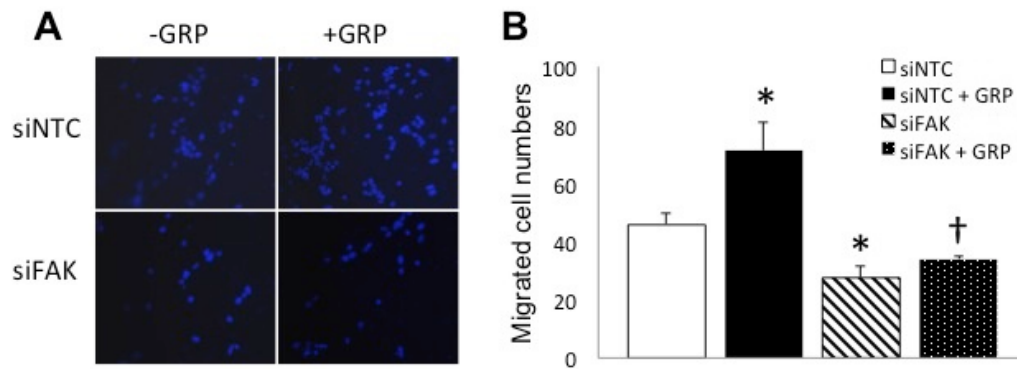
Figure 13. FAK silencing decreases GRP-induced neuroblastoma cell migration. (A) Representative images of DAPI staining for counting were shown (200X magnification). (B) GRP-stimulated cell migration in siNTC and siFAK was performed using transwell plates in BE(E)-C cells. siFAK inhibited cell migration by 40% compared to siNTC. GRP (100 nM) increased cell migration by ~1.2-fold in siNTC cells whereas GRP did not induce cell migration in siFAK cells (*= p <0.05 vs. siNTC, †= p <0.05 vs. siNTC+GRP). GRP were added into the bottom well of transwell plates. Values are expressed using migrated cell numbers from two experiments performed in duplicate.

at Y397 when GRP was treated following those inhibitors (Fig. 12A, B). These data suggest that FAK activation by GRP is independent of AKT and ERK pathways, and further it indicates that FAK is upstream target of AKT and ERK. Interestingly, I also found that the Y15 treatment also significantly inhibited GRP-induced colony formation in soft agar by > 2-fold (Fig. 11B). Furthermore, GRP did not induce increased cell migration when FAK was downregulated by siRNA against FAK (Fig. 13 A, B). These findings strongly suggest that FAK is a critical mediator of GRP-induced neuroblastoma cell growth and migration. FAK inhibition by either a compound or an antisense oligonucleotide can effectively block GRP stimulation in neuroblastoma cells.

FAK inhibition reduced BBS-induced tumor growth and metastasis in vivo.

Previously, our laboratory has reported that BBS, an amphibian equivalent of GRP, promotes neuroblastoma tumor growth *in vivo*, and is an important stimulator of angiogenic pathway [21,108]. Additionally, Y15 has shown to decrease neuroblastoma growth *in vivo* [67,68]. Based on all these findings, I wanted to determine whether FAK inhibition could block BBS-induced tumor growth and metastases *in vivo*. 1×10^6 of luciferase-expressing BE(2)-C cells were injected into spleen in athymic nude mice. After three days, they were randomized into four groups: control (PBS vehicle), BBS (20 μg/kg/i.p./t.i.d.), Y15 (30 mg/kg/i.p./day), and BBS plus Y15. Bioluminescence imaging system was used to monitor for primary tumor growth in spleen as well as liver metastasis. As shown in Figure 14A, signals exhibited relatively equal intensities on day 1; however, by day 20, BBS significantly increased primary splenic tumor growth as well as liver metastases while a combination treatment with Y15 showed remarkable reduction in tumor growth and

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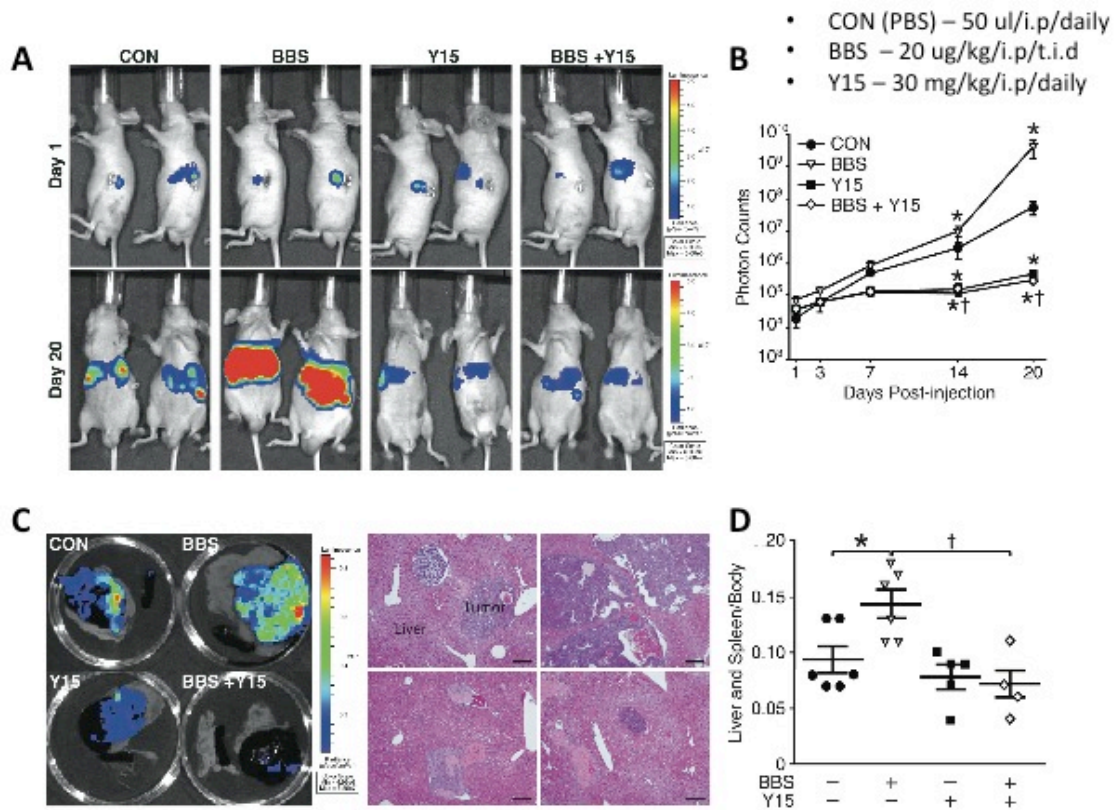
Figure 14. Y15 blocks BBS-induced neuroblastoma growth and metastasis *in vivo*.
(A) Bioluminescence images showing luciferase-expressing BE(2)-C cells in an intrasplenic injection murine model. Pseudocolor images from each group were adjusted to the same threshold of the day. At day 1, mice were imaged on their left side to detect relatively equal intensity of luminescent signal of spleen. At day 20, BBS (20 µg/kg/i.p./t.i.d.) promoted tumor growth and liver metastases, whereas Y15 (30 mg/kg/i.p./day) showed the opposite effect, and further inhibited BBS-induced tumor growth and liver metastases. (B) Quantitative values of bioluminescence were measured as photon counts. Results were given as the mean ± SEM (*n* = 5-6 mice in each group; *= *p* <0.05 vs. CON, †= *p* <0.05 vs. BBS). (C) Representative bioluminescence images of tumor tissue extracted from the mice of each group and representative images of H&E staining to confirm metastatic foci of neuroblastoma cells in liver lesions (200X magnification, 50 µm bar) (D) Spleen and liver weight relative to body weight (*n* = 5-6 mice in each group; *= *p* <0.05 vs. CON, †= *p* <0.05 vs. BBS; Mann Whitney test).

metastases. The inhibitory effects of Y15 in BBS-induced tumor growth were confirmed by luciferase activity of luciferase-expressing BE(2)-C cells (Fig. 14A, B) as well as by spleen and liver weight (Fig. 14C, D). Differences in luciferase activity at day 20 correlated with tumor weights in the liver and spleen. BBS increased luciferase activity by > 80-fold as compared to controls (mean activity 5.30×10^7 photons/s for controls vs. 4.27×10^9 photons/s for BBS) whereas Y15 decreased the activity by < 110-fold as compared to controls (mean activity 5.30×10^7 photons/s for controls vs. 4.78×10^5 photons/s for Y15). More importantly, BBS plus Y15 combination significantly reduced luciferase activity when compared to BBS alone (mean activity 4.27×10^9 photons/s for BBS vs. 2.65×10^5 photons/s for BBS plus Y15). Thus, our *in vivo* data corroborate *in vitro* findings, and further suggest that FAK is an important regulator of the GRP/GRPR signaling pathway and tumor metastasis in neuroblastoma.

In this study, I showed that GRPR and FAK expressions in human neuroblastoma tissues and cell lines correlate with tumor malignancy. Exogenous GRP induced FAK activation at Y397 and enhanced cell migration. Interestingly, GRPR overexpression increased FAK, integrin expressions as well as cell migration in SK-N-SH cells. Conversely, GRPR silencing resulted in decreased FAK and MYCN proteins in BE(2)-C cells while FAK overexpression in GRPR silenced BE(2)-C cells rescued cell growth. Moreover, FAK overexpression alone led to an increase in soft agar colony formation in SK-N-SH cells, whereas FAK silencing resulted in decreased colony formation in BE(2)-C cells. I also found that FAK silencing in BE(2)-C cells suppressed tumorigenesis and metastasis *in vivo.* Furthermore, using an intrasplenic murine model and bioluminescence imaging system, I confirmed that treatment with Y15, a FAK inhibitor, blocks BBS-induced neuroblastoma growth and

liver metastases *in vivo*. My results demonstrated that FAK correlates with GRPR, and that it exerts oncogenic effects in neuroblastoma as a mediator of GRPR signaling pathway. Hence, FAK may be a clinically important therapeutic target in the treatment of neuroblastomas.

Discussion

Our laboratory previously demonstrated that GRPR is overexpressed in malignant, advanced-stage neuroblastomas, and that GRPR silencing suppresses tumorigenesis and metastasis *in vivo* [43,110]. However, the exact molecular mechanisms of GPR/GRPR regulation of tumor progression are yet to be delineated. In this study, I provide compelling evidence from cell and animal studies that FAK is an important mediator of GRP/GRPR signaling-induced neuroblastoma growth and metastasis. I investigated the relationship between GRPR and FAK, as well as the effect of FAK silencing using transfected cells and/or a pharmacologic agent on tumor growth *in vitro* and *in vivo*. GRP/GRPR regulated FAK activation and expression, and further inhibition of FAK repressed GRP/GRPR signaling involved in neuroblastoma progression. These results suggested that FAK is a critical downstream target of GRP/GRPR, and therefore may be a promising therapeutic target for malignant neuroblastomas.

GRP, the mammalian analogue of BBS, is a growth factor that promotes cell proliferation in cancer cells [30,33]. Specifically, GRP induces activation of FAK at Y397 site, which is known to be critical for promoting both integrin- and/or growth

factor- stimulated cell migration, and is a high-affinity binding site for Src homology 2 (SH2) of the Src family kinases [121], which creates the possibility for interaction with a number of different signaling and adaptor proteins. In the present study, I found that GRP/GRPR signaling regulates FAK activation and expression. I also found that GRPR expression correlates with integrin expressions. Thus, our findings further support published results that integrin and growth factor receptor signaling can interact through either membrane-proximal clustering of the two receptor types or the activation of common downstream signaling pathways [98,101].

Our findings corroborate the multiple tumorigenic functions of FAK, which are associated with various malignant and aggressive tumors, including ductal carcinomas of the breast [122], primary colorectal tumors and metastases [123], and endometrial carcinomas [124]. FAK has been used as a prognostic indicator as well as a marker for malignant transformation in breast carcinoma [125]. Additionally, FAK has been established as a significant component in the BBS signaling pathways in prostate cancer [102,103,113] and described to play a critical role in GRP-mediated morphogenesis of colon cancer [114]. Glover *et al*. [102] showed that the FAK Y397 site is critical for GRP-induced morphogenesis in 293 HEK cells, and we have shown that BBS promotes tumor growth and angiogenesis in neuroblastoma *in vivo* [21,108]. Here, I show that GRP or BBS stimulates neuroblastoma cell migration as well as liver metastases, further supporting a critical tumorigenic role of GRP or BBS in neuroblastoma. More importantly, our results, for the first time, show that FAK inhibition is critical to block BBS-induced tumorigenesis and metastasis *in vivo*, and thus, further indicating FAK as an important therapeutic target in the treatment of neuroblastomas.

Y15 compound, 1,2,4,5-benzenetetraamine tetrahydrochloride, has been shown to block phosphorylation of FAK at Y397 in neuroblastoma cell lines as well as inhibit growth of *MYCN*-amplified neuroblastoma tumors *in vivo* [67]. In my study, we used *MYCN*-amplified I-type BE(2)-C and *MYCN* single copy N-type SK-N-SH neuroblastoma cell lines. Since, MYCN regulates FAK expression at the promoter level in neuroblastoma cells [25], I speculated that GRPR signaling would affect FAK by regulating MYCN. This is supported by the results that showed reduced level of FAK mRNA in GRPR silenced BE(2)-C cells. However, the molecular mechanism of GRP/GRPR signaling-dependent regulation of MYCN and subsequently FAK in *MYCN*-amplified neuroblastoma cells or *MYCN* single copy neuroblastoma cells remain to be defined.

Our laboratory previously reported that the aberrant activation of PI3K/AKT pathway regulates GRP/GRPR-mediated oncogenic functions [43,44,107,110]. We specifically found that of all three AKT isoforms, only AKT2 was downregulated with GRPR silencing in neuroblastoma cells [110]. Specifically, AKT2 silencing decreased MYCN expression, anchorage-independent cell growth, and liver metastases *in vivo*. Conversely, exogenously overexpression of AKT2 increased MYCN expression in BE(2)-C cells (data not shown here). Of the three AKT isoforms, AKT2 has been implicated more frequently in numerous cancers [126,127,128]. Consistent with other cancer cell types, our data showed that AKT2 is the most critical isoform responsible for progression of neuroblastoma. Therefore, we believe that activation of AKT signaling is critical for GRPR-mediated regulation on MYCN expression, which might be through post-transcriptional modulation of the stability of MYCN in neuroblastoma cells. A recent study has shown that *MYCN* contributes to tumorigenesis, in part, by repressing miR-184, and increasing AKT2 expression, a

direct target of miR-184 [129], and thereby indicating that AKT2 is a downstream target of MYCN. Taken together, it suggests that a positive regulatory loop exists between two oncogenic proteins, AKT signaling and MYCN in human neuroblastoma cells, which contributes crucially to the development of tumorigenicity. Collectively, we think that there is a GRPR/AKT/MYCN axis and in this study, I showed that GRPR silencing decreases MYCN expression, which affects on FAK expression in *MYCN*-amplified human neuroblastoma cells.

Tilghman *et al*. [130] noted that loss of FAK results in marked changes in cell morphology and defects in leading edge formation. Similar to their findings, I also found that silencing of FAK by either cell transfection or chemical compound induced morphological changes analogous to the effects of silencing of GRPR, and further inhibited colony formation. Hence, I infer that GRPR and FAK are essential for regulating cell morphology, which can associate with the oncogenic properties such as highly migratory, anchorage-independent phenotype in neuroblastoma. Therefore, a better understanding of GRPR and FAK regulation may not only be of biological significance, but may also provide a molecular basis for potential clinical applications.

In conclusion, my study demonstrates that FAK is a crucial regulator of GRP/GRPR signaling in neuroblastoma. GRP/GRPR regulates neuroblastoma cell growth, transformation and migration by correlative regulation of FAK. Furthermore, my results suggest that targeting FAK can inhibit GRP/GRPR-mediated oncogenic properties. Moreover, this study demonstrates the roles of FAK in neuroblastoma tumorigenesis and metastasis *in vitro* and *in vivo*. These findings are clinically relevant because advanced-stage neuroblastoma is oftentimes refractory to current multi-modality treatment protocols, and effective novel therapeutic target(s) are highly desirable. Hence, a better understanding of the mechanisms involved in

GRPR/FAK-induced metastatic potential could provide insights into development of novel strategies in the treatment of aggressive, undifferentiated neuroblastoma.

Most data shown in this chapter were published in *Oncotarget*. 2012 Dec;3(12):1576-87. FAK is a critical regulator of neuroblastoma liver metastasis. Lee S, Qiao J, Paul P, O'Connor KL, Evers BM, Chung DH [131].

INTEGRIN β1 PLAYS A ROLE IN GRP/GRPR-INDUCED CELL MIGRATION

Introduction

Neuroblastoma, a neural crest cell-derived tumor, is the most common extra-cranial solid tumor of infants and children, accounting for approximately 10% of all childhood-related death. [132]. Since roughly 50% of patients will have metastatic disease at the time of diagnosis [133], there have been considerable efforts aimed at delineating the cellular mechanisms that contribute to metastatic progression in neuroblastoma, leading to poor overall survival. Clinicopathologic data has shown that age greater than 12 months at diagnosis, *MYCN* amplification, unfavorable tumor histopathologic evaluation, and the presence of systemic metastatic disease are factors that portend a 'high-risk' classification and are each associated with poor prognosis [134]. The poor survival seen in patients with metastatic or refractory neuroblastoma underscores the need for improved understanding of the molecular mechanisms that govern tumor cell migration and invasion.

Gastrin-releasing peptide (GRP) is a gut neuropeptide with mitogenic properties that is secreted by neuroblastoma in an autocrine/paracrine fashion [26]. We have previously shown that GRP binds to its cell surface receptor, GRPR, to stimulate neuroblastoma growth, and that GRPR overexpression increases

tumorigenicity and metastatic potential in neuroblastoma cells [43,44]. Further, increased expression of GRPR is found in more undifferentiated neuroblastoma that are frequently associated with metastatic disease and dismal patient outcomes. Conversely, our laboratory has recently shown that targeted silencing of GRPR through stable transfection can inhibit tumor growth and metastasis *in vivo* [110]; however, the exact cellular mechanisms responsible for distant organ metastasis in neuroblastoma are not clearly defined.

The capacity for tumor cell migration and invasion through the extracellular matrix (ECM) is one of the key components of the metastatic process. A multitude of intra- and extra- cellular signaling pathways coordinate to exert molecular changes that ultimately allow cancer cells to progress beyond the primary tumor. Integrins are a group of heterodimeric transmembrane surface glycoproteins that play a key role in the regulation of cell-to-cell and cell-to-ECM attachments, yet also possess the capacity to transmit information from the extracellular milieu that can regulate cellular growth, survival, and migration [135]. As key mediators of tumor progression, integrins allow cells to adapt to changing microenvironments during metastatic progression, and it is not surprising that changes in membrane integrin expression have been identified in various types of cancer [136]. The association between integrin expression and GRP/GRPR signaling in neuroblastoma is heretofore undefined.

Results

GRP stimulated neuroblastoma cell migration and invasion.

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To assess the ability of GRP to stimulate cell motility, serum-starved SK-N-SH cells were cultured with or without GRP. Cell migration and invasion assays were independently performed on each cell line using transwell system and relative values were calculated, as well as cell count quantification to measure invasion. GRP treatment significantly increased SK-N-SH cell migration (Fig. 15A). Furthermore, cell counts after GRP-induced invasion were significantly higher when compared to controls (Fig. 15B). Given the important role of matrix metalloproteinase (MMP) and tissue inhibitor of metalloproteinase (TIMP) in modulating the ECM during cell movement, I next wanted to determine whether GRP stimulation leads to altered expression of MMP or TIMP [137]. GRP significantly upregulated MMP-2 expression in SK-N-SH cells when compared to controls, while the expression of TIMP-1, a known inhibitor of MMP-2, was decreased after GRP treatment (Fig. 15C). Taken together, these results suggest a critical role for GRP-mediated neuroblastoma cell migration and invasion.

GRPR overexpression increased integrin α2, α3, and β1 expression in SK-N-SH.

GRP overexpression is known to stimulate neuroblastoma cell growth and proliferation [108]; however, its relationship to tumor progression and cell motility is less defined. In order to better understand the cellular mechanisms underlying GRP/GRPR mediated neuroblastoma cell motility, we established stably transfected GRPR overexpressing SK-N-SH cells. We then performed gene expression analysis using a cDNA GEArray® Microarray kit to identify target genes that may be altered as a result of GRPR signaling. We found that GRPR overexpressing SK-N-SH human neuroblastoma cells showed increased mRNA levels of integrin α2, α3, and β1 (Fig. 16A). Correlative to mRNA, integrin α2, α3, and β1 protein levels were also
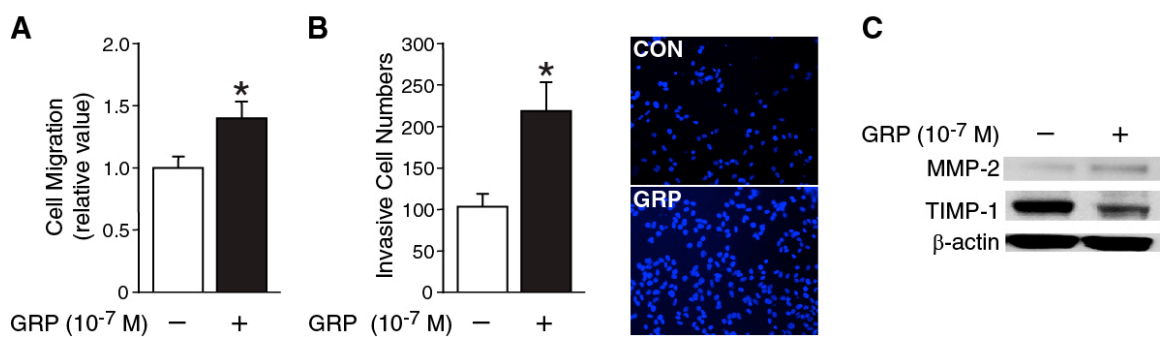
Figure 15. GRP increases cell migration and invasion by differential expression of MMP-2 and TIMP1. (A) GRP treatment (10^{-7} M) for 24 h increased SK-N-SH cell migration in transwell plates. Values are expressed using migrated cell numbers from two experiments performed in duplicate. (*= p <0.05 vs. control). (B) GRP also stimulated the number of SK-N-SH cells in matrigel-coated transwell plates. Bars represent the averages of two experiments performed in duplicate. (*= p <0.05 vs. control). (C) Increased MMP-2 but decreased TIMP-1 expressions were observed in SK-N-SH cells after GRP treatment.
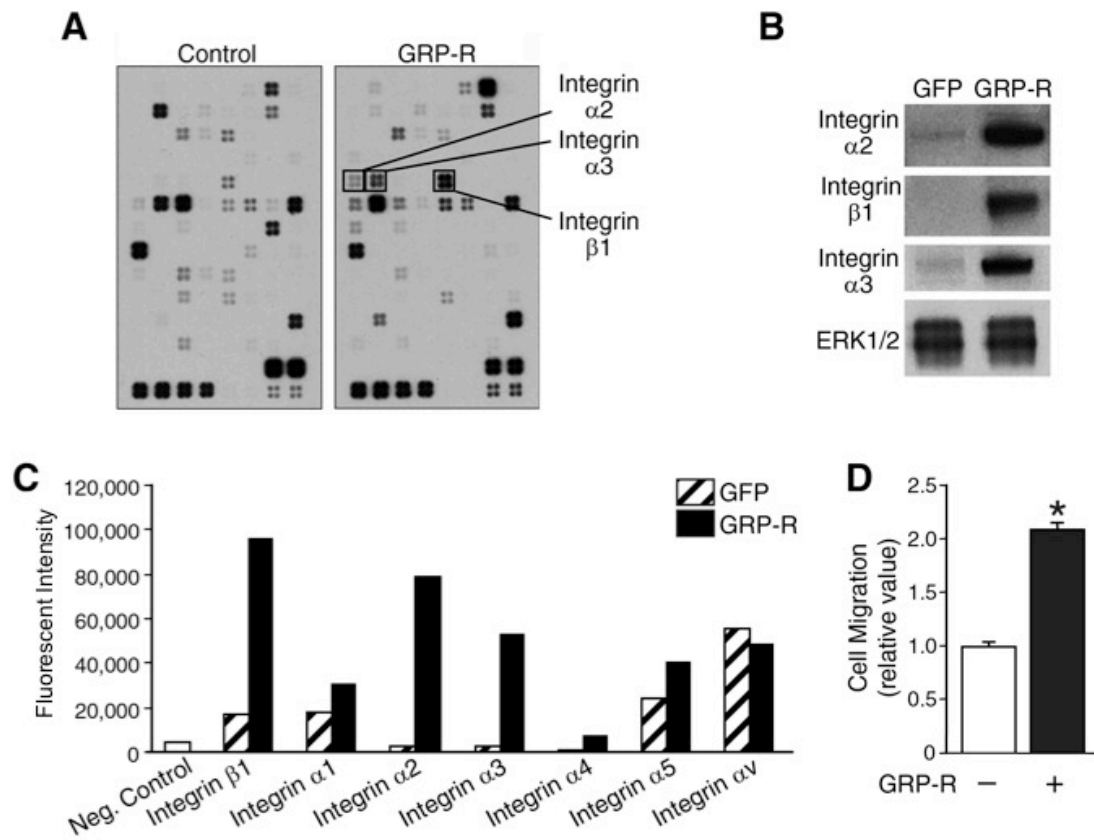
Figure 16. GRPR overexpression upregulates integrin α2, α3, and β1 expression in SK-N-SH cells and stimulated cell migration. (A) GRPR overexpression resulted in increased mRNA levels of integrin α2, α3, and β1 (*boxed areas*) as measured by SuperArray. (B) Western blot analysis was performed with indicated antibodies in GRPR overexpressing cells. Total ERK1/2 expression was probed to demonstrate equal protein loading. (C) Increased levels of integrin α2, α3, and β1 membrane expression in GRPR overexpressing cells as measured by flow cytometry. (D) GRPR overexpressing cells with stimulated cell migration rate. Values are expressed using migrated cell numbers from two experiments performed in duplicate. (*= p <0.05 vs. control).

increased as measured by Western blotting when compared to controls (Fig. 16B). In addition, these increased expression of integrin α2, α3, and β1 in GRPR overexpressing SK-N-SH cells were further confirmed using flow cytometry (Fig. 16C). Consistent with GRP-induced cell migration in Figure 1A, stable transfection of GRPR overexpressing SK-N-SH cells also resulted in a concomitant increase in cell migration (Fig. 16D). Hence, my results support a positive correlation between GRPR and integrin expression, indicating that GRPR is important for a cellular function of cell migration in neuroblastoma cells by regulating integrin.

Integrin β1 regulates SK-N-SH cell migration.

Since GRPR overexpression induced SK-N-SH cell migration and increased integrin expression, we sought to better characterize this cellular process. First, I used small interfering (si) RNA to silence integrin α2, α3, and β1 expression in SK-N-SH cells, then performed wound healing assays at 24, 48, and 72 h to determine the effects on cell migration (Fig. 17A). Wound healing assays have been used to measure cell migration, cell proliferation and wound closure. The process of wound healing begins by polarizing cells toward the wound, initiating protrusion, migrating and closing the wound area. Wound unclosure was measured microscopically and relative values were determined. Targeted silencing of integrin β1 (siIntegrin β1) significantly decreased cell migration after wounding at 72 h when compared to siRNA targeted at integrin α2 and α3 (Fig. 17B). Western blot analysis was used to confirm the specificity of siRNA (Fig. 17C).
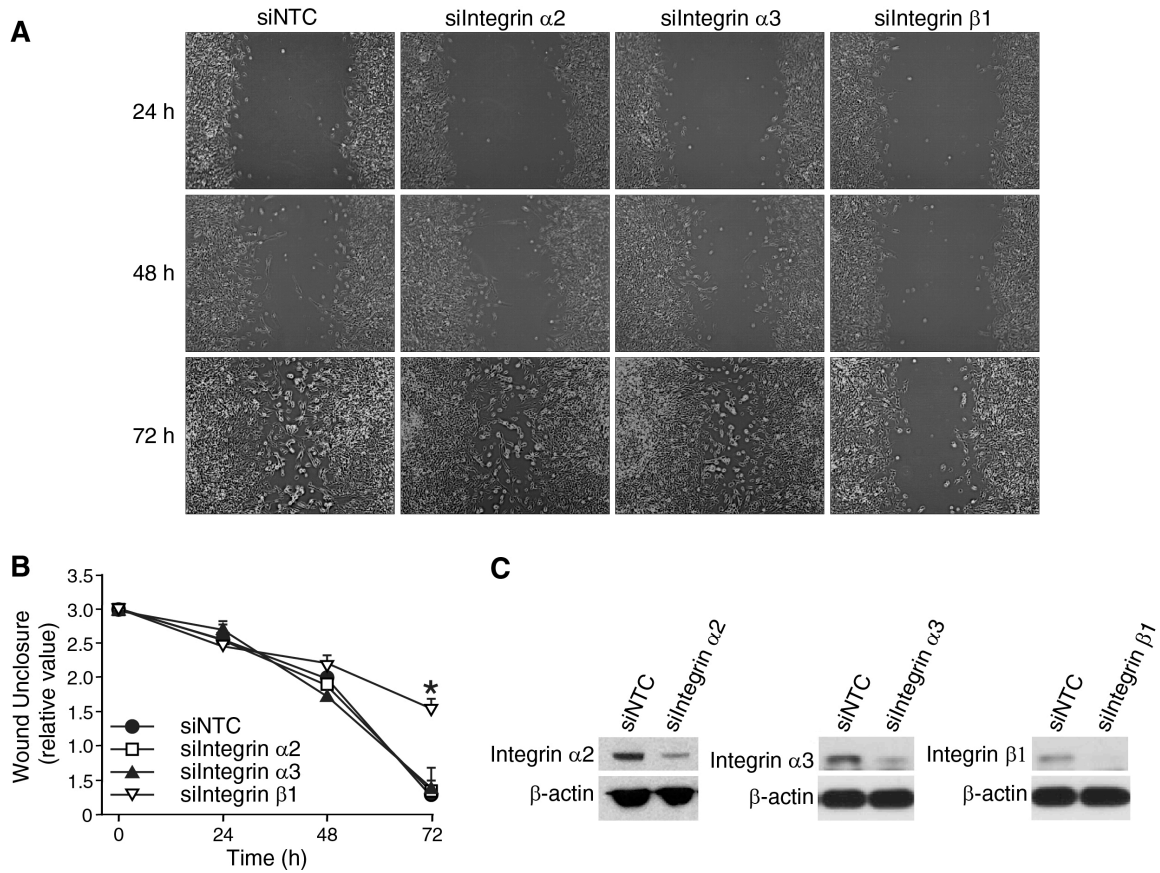
Figure 17. Silencing of integrin α2, α3, and β1 in SK-N-SH cells. (A) SK-N-SH cells were transfected with siRNA against integrin α2, α3, and β1. After 48 h, scratches were made using 200 µl tips. Wound closure was measured from microscopic images at 24, 48, and 72 h after wounding (100X magnification). (B) Data are representative of the mean distance of unclosure from three independent experiments (*= p <0.05 vs. siNTC). (C) Western blot analysis was performed with indicated antibodies in the cells. β-actin was used to demonstrate equal protein loading.

Integrin β1 is a critical regulator of cell migration in GRPR overexpressing SK-N-SH.

Given the measurable result of integrin β1 silencing on cell migration in SK-N-SH cells, I then sought to identify the effect of integrin β1 knockdown in GRPR overexpressing SK-N-SH cells. Scratch assays were established with siIntegrin β1 in GRPR overexpressing cells and measured at 24, 48, and 72 h for wound unclosure as a function of cell migration (Fig. 18A). siRNA inhibition of integrin β1 decreased cell migration in GRPR overexpressing SK-N-SH cells after wounding at 72 h compared with the non-targeted control GRPR overexpressing cells (Fig. 18B). Western blot analysis was used to confirm the specificity of siIntegrin β1 (Fig. 18C).

In this study, I examined the effects of GRP/GRPR signaling on neuroblastoma cell migration and invasion, and determine the role of integrin as it relates to GRPR-mediated neuroblastoma cell motility. GRP treatment led to increased migration and invasion of neuroblastoma cells. GRPR overexpression not only stimulated neuroblastoma cell migration, but also led to the upregulation of mRNA, protein, and membrane expression of integrin subunits α2, α3, and β1. Moreover, silencing of integrin β1 led to significant inhibition of cell migration suggesting its role as a potential key regulator of GRPR-mediated neuroblastoma cell migration and invasion.

Discussion

As a neuroendocrine tumor, neuroblastoma can exhibit mitogenic behavior in response to autocrine and paracrine stimulation, particularly in response to GRP. In fact, GRP/GRPR signaling has been shown to significantly induce neuroblastoma
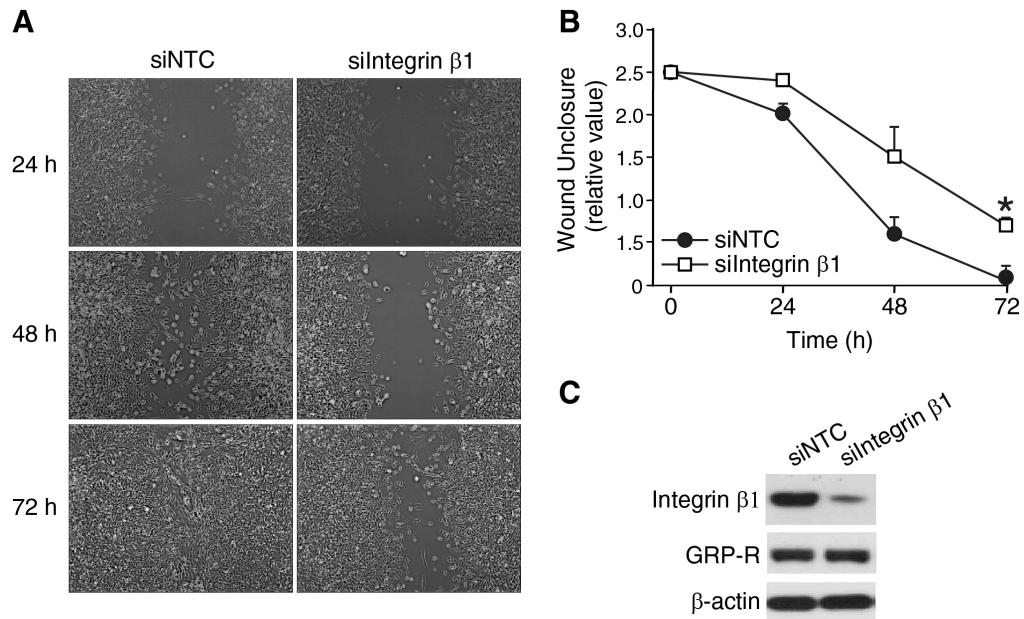
Figure 18. **Silencing of integrin β1 decreases cell migration in GRPR overexpressing SK-N-SH cells.** (A) GRPR overexpressing SK-N-SH cells were transfected with siRNA against integrin β1. After 48 h, scratches were made using 200 µl tips. Wound closure was measured from microscopic images at 24, 48, and 72 h after wounding (100X magnification) (B) Data are representative of the mean distance of unclosure from three independent experiments (*= p <0.05 vs. siNTC). (C) Western blot analysis was performed with indicated antibodies in the cells. β-actin was used to demonstrate equal protein loading.

cell growth through the PI3K/Akt pathway [107]. Advances in the treatment of high-risk neuroblastoma have focused on abrogating the progression of metastatic and recurrent disease, but with less than satisfactory results. Hence, a better knowledge of the molecular mechanisms involved in GRPR-mediated neuroblastoma cell motility may provide potential novel therapeutic targets. In this study I demonstrate that signaling through the GRP/GRPR axis results in neuroblastoma cell migration and invasion *in vitro*, and that GRPR signaling mediates its effects on neuroblastoma cell motility through upregulation of α2, α3, and β1 integrin subunits. Furthermore, I have shown that integrin β1 expression is particularly critical to GRPR-mediated cell migration as evidenced by the significant decrease observed in neuroblastoma cell migration upon inhibition of integrin β1 with siRNA.

It was demonstrated that GRP can stimulate cell migration in prostate [138] and breast cancers [139] through binding to its cognate receptor, GRPR. Similar studies using the GRP analog, BBS, have shown that BBS/GRPR signaling enhances the migratory and invasive capacity of colorectal carcinoma cells in a dose-dependent fashion [140], albeit the exact molecular mechanisms still remain unclear. While the role of GRPR signaling in neuroblastoma tumorigenesis has been established [110], the effects of such signaling events as they relate to neuroblastoma cell migration and invasion have not been examined. In the present study, our findings show that both GRP treatment and GRPR overexpression lead to increased motility of SK-N-SH neuroblastoma cells. The ability of a cancer cell to penetrate the ECM is paramount for tumor progression. Neoplastic cells take advantage of enzymatic digestion of the ECM by MMPs to accomplish these tasks. Interestingly, treatment of SK-N-SH cells with GRP led to increased expression of MMP-2 while expression of the MMP-inhibiting enzyme TIMP-1 was downregulated,

suggesting that GRP may play a secondary role in modulating the extracellular environment to allow for tumor cell motility through the ECM. In fact, it is known that MMPs play a crucial role in tumor progression through regulation of the tumor microenvironment [141], and that dysregulation of the balance between MMP proteolysis and TIMP expression is linked to cancer cell invasion [142]. Further studies examining the relationship between GRP signaling and regulation of the MMP/TIMP balance in neuroblastoma tumor progression will be needed.

Since cell migration and invasion, as well as intravasation and extravasation, are fundamental requirements of cancer cells during metastasis, we sought to further characterize changes in gene expression that may reflect cellular changes in tumor progression. As such, gene expression profiles between GRPR overexpressing cells and control cells were created using cDNA microarray analysis in order to identify gene targets. Comparative gene expression analysis showed that GRPR overexpression led to the upregulation of integrin α2, α3, and β1 mRNA. These findings were further corroborated with Western blot and flow cytometric analysis. Collectively, these findings show that GRPR overexpression increases the expression of integrin α2, α3, and β1 in SK-N-SH cells, and outlines a potential relationship between GRP/GRPR-induced cell migration/invasion and integrin expression, and emphasize the role of neuroendocrine signaling in neuroblastoma tumor progression, which underscore the notion that cell-ECM interactions can regulate molecular signaling in response to mitogenic factors.

As the prominent receptor for the ECM, integrins function beyond simple cell adhesion and can influence numerous cellular functions in response to signals from the environment. Previous studies have suggested that changes in integrin expression by tumor cells can modulate growth and survival, as well as cell adhesion

and migration [143]. Of interest in the present study is the relationship of GRP/GRPR induced integrin expression to cell migration and invasion. I first wanted to test which integrin subunit plays a role in regulating cell migration in SK-N-SH cells. I performed the knockdown of integrin α2, α3, and β1 subunits using siRNA against each integrin subunit and cell migration assay in SK-N-SH cells. In fact, the decrease in cell movement seen on scratch assay was significantly greater after integrin β1 silencing when compared to integrin α2 and α3 silencing. These effects were not rescued with GRPR overexpression, suggesting the crucial role of integrin β1 in GRPR-mediated cell motility. The dysregulation of cell adhesion molecules, particularly integrins, is well established in human cancers, and overexpression of the integrin β1 subunit is associated with increased metastatic potential in breast [144] and lung cancers [145]. Although previous reports have suggested that decreased integrin β1 expression in neuroblastoma cell lines leads to increased cell migration *in vivo* [146], our studies do not corroborate this finding.

In conclusion, the present study demonstrates that GRP and its associated receptor, GRPR, provide a significant molecular access point for the induction and stimulation of neuroblastoma tumor cell motility through cellular mechanisms that likely involve the modulation of integrin subunit expression, particularly integrin β1. The role of β1 integrin expression in cell attachment and invasion has been implicated in other cancers [147]. Further studies will be needed to identify the molecular mechanisms underlying GRP-mediated integrin expression and how these interactions contribute to the metastatic potential of neuroblastoma.

CHAPTER V

SUMMARY AND FUTURE DIRECTIONS

SUMMARY

The significant findings from these studies are described here. The findings that lead to the conclusion that FAK mediates GRP/GRPR-induced neuroblastoma progression are: 1) FAK expression correlates with GRPR expression, which corresponds to tumor malignancy; higher expression in more aggressive type of tumor; 2) GRPR overexpression increased FAK expression and cell migration whereas GRPR silencing decreased FAK and MYCN expression, cell viability and colony formation; 3) FAK 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; 4) BBS treatment significantly enhances the metastasis potential in human neuroblastoma xenografts in nude mice and effects can be reversed with a FAK inhibitor. Furthermore, the findings that lead to the conclusion that integrin β1 plays a role in GRP/GRPR-induced cell migration are: 1) GRP increased neuroblastoma cell migration and invasion in *MYCN* single copy, SK-N-SH cells, which do not increase FAK activity by GRP treatment. 2) GRPR overexpression stimulated cell migration and upregulated integrin α2, α3, and β1 protein as well as mRNA expression. 3) Targeted silencing of integrin β1 in GRPR overexpressing SK-N-SH cells inhibited cell migration. Altogether, these findings

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indicate that FAK and integrin play a functionally important role in mediating the stimulatory effects of GRP/GRPR during the progression of neuroblastoma pathogenesis.

FUTURE DIRECTIONS

Invasion and metastasis are critical hallmarks in cancer [148]. Metastatic lesions, which are often implicated in resistance to adjuvant therapy, remain as one of the major causes for high mortality from cancer [149]. In neuroblastoma, >50% of patients have metastatic disease at the time of diagnosis, thus creating major challenges for treatment. Moreover, 'high-risk' group of neuroblastomas often relapse despite initial response to therapies. Frequently, tumors acquire drug resistance or aggressive phenotypes through the selection of rare resistant clones from heterogeneous tumor environment, which can result in major clinical obstacles in the treatment of neuroblastoma [150]. Thus, a better understanding of the mechanisms and signaling pathways that contribute to metastasis will be valuable in the development of novel therapies.

Mechanism of FAK-mediated inhibition of cell growth

My studies have shown that FAK expression is crucial for various factors regulating tumorigenesis in neuroblastoma. In particular, FAK silencing significantly inhibited cell growth and anchorage-independent growth *in vitro* and *in vivo*. Proposed *in vitro* experiments would evaluate the effects of FAK silencing on the cell cycle (flow cytometry, BrdU incorporation, protein expression of cell cycle 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 agent is critical to diminish the harmful side effects seen with current neuroblastoma treatments. These also would be helpful to develop the potential synergistic benefits of multi-targeted therapy.

Mechanism of upregulated expression of focal adhesion proteins in GRPR overexpressing cells

My studies have shown that focal adhesion proteins including FAK and integrin are highly expressed in GRPR overexpressing SK-N-SH cells. In particular, the mRNA level of FAK did not increase but the protein level increased, indicating the modulation of FAK protein in GRPR overexpression is a post-transcriptional regulation process. According to immunofluorescence staining data, the protein expression of GRPR and FAK at the focal adhesions is prominent. Enhanced expression of integrin may show in a similar manner in GRPR overexpressing cells. If so, proposed *in vitro* experiments would evaluate the effects of GRPR overexpression on the protein stability of focal adhesion proteins (membrane protein extraction, Cycloheximide/MG132 treatment, protein half-life, protein degradation assay). These studies would provide evidence of how GRPR plays a role to interact with other membrane proteins and could further emphasize the importance of targeting GRPR in GRPR overexpressing cancer cells.

Mechanism of GRP/GRPR regulation of MYCN

The most widely associated feature with neuroblastoma prognosis is the level of *MYCN* amplification. Occurring in up to 25% of primary tumors, *MYCN* is strongly correlated with advanced-stage disease and treatment failure [14,15]. MYCN is an oncogenic transcription factor which functions in the regulation of proliferation, differentiation, transformation and apoptosis [151,152]. MYCN has been found to directly regulate the transcriptional expression of mouse double minute 2 homolog (MDM2), the inhibitor of p53 and FAK, and cellular retinoic acid-binding protein II (CRABP-II), which appears to be related cell motility [153]. However, because it is a nuclear transcription factor, it is difficult to directly target MYCN with small-molecule inhibitors.

Our laboratory and others have found that siRNA targeted to MYCN resulted in increased differentiation and apoptosis, with concurrent growth inhibition [154,155]. Other mechanisms of inhibiting MYCN are aimed at crucial upstream regulators of the oncogene, such as the PI3K/AKT survival pathway. It has been demonstrated that inhibition of PI3K/AKT pathway components resulted in MYCN destabilization via a GSK3β-regulated mechanism [20]. It is unknown exactly how GRPR targeting can regulate MYCN in neuroblastoma, therefore it will be interesting to investigate whether GRPR expression correlates with MYCN activity. In particular, since MYCN regulates the expression of FAK by binding to a *FAK* promoter region in neuroblastoma, it will be intriguing if there is a potential axis of GRPR/MYCN/FAK. In order to study this, I propose an experimental design in which *MYCN*-luciferase promoter constructs are transfected in neuroblastoma cells with high versus low levels of GRPR. Moreover, the stability and degradation of MYCN expression could be examined using Cycloheximide/MG132 treatment and ubiquitination assay. I also

propose that these future experiments should utilize inducible expression systems. This will help determine whether GRPR expression plays a role in mediating the activity of MYCN, and will further determine how it affects the regulation of FAK expression. 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 the mechanism and an effective target for this important subset of advanced, *MYCN*-amplified neuroblastoma.

Identification of the mediators between GRPR and FAK

My studies have shown the correlation between GRPR and FAK in neuroblastoma. In particular, I have provided data for the molecular, functional and physiological roles of FAK in GRPR-mediated neuroblastoma growth and metastasis *in vitro* and *in vivo*. However, still I have not answered how GRP/GRPR signaling actually interacts with FAK. Long *et al.* have demonstrated that SRC3Δ4 is an important mediator between EGF receptor (EGFR) and FAK to promote cell migration [156]. Furthermore, they found that overexpression of SRC3Δ4 promoted breast tumor metastasis, coordinating the signaling between EGFR and FAK while knockdown of SRC3Δ4 expression significantly decreases EGF-induced FAK activation and cell migration. They identified SRC3Δ4 as the missing adaptor protein that bridges the interaction between EGFR and FAK upon EGF stimulation. This finding suggests that SRC3Δ4 represents another potential therapeutic target for counter acting cancer metastasis. Src is a highly possible candidate for mediating signaling between GRPR and FAK because GRP can induce Src activity, which can act as an initiator of FAK activation. Identifying of the mediator and its roles would be able to provide another new therapeutic target for better neuroblastoma treatments.

Combined targeting of GRPR and FAK in neuroblastoma

My studies have shown the role of FAK in GRPR-mediated neuroblastoma progression. In particular, I have shown the FAK overexpression can rescue malignant potentials in GRPR silencing cells while FAK inhibition blocks GRP-induced cell growth and metastasis. I have not demonstrated the combined effects of targeting both GRPR and FAK because silencing of either of each already showed a great impact on inhibition of tumor growth *in vivo*. However, it might be difficult to show the similar effects in the actual neuroblastoma patients, especially in patients who harbor more aggressive type of neuroblastoma, receiving a combination therapy. Moreover, even though I suggested that FAK is a critical downstream target of GRP/GRPR signaling pathway, since both GRPR and FAK are such an upstream-acting, intermediate protein in cells, many signals are overlapped by them but not all. By testing the combination cytotoxic effects of targeting GRPR and FAK together, we may expect a better therapeutic strategy in neuroblastoma treatments, overcoming drug resistance in heterogeneous tumors, and by taking advantage of tumor growth kinetics with increasing the dose-density of combination treatments. The overall goal is to improve clinical efficacy with acceptable toxicity.

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