Role of Glia Maturation Factor beta (GMFβ) in Differentiation of the Childhood Cancer Neuroblastoma

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Background: Neuroblastoma (NB), the second most common solid tumor of the peripheral nervous system, accounts for 10% of pediatric cancers. Most children present with advanced stage disease with very poor survival rates. 13-cis retinoic acid (RA), a differentiating factor, is a component of NB treatment following surgery, radiation and chemotherapy. Glia Maturation Factor beta (GMFβ) was initially characterized as a factor that caused process extension and reduced cell proliferation when added to glioblastoma cells. GMFß as an actin depolymerizing factor (ADF) may modify cell morphology by interacting with the peripheral actin cytoskeleton localized within the distal axon. GMFß is highly expressed in neuronal tumor-derived cell lines, especially NB. **Results:** GMFβ phosphorylation was shown to decrease with RA treatment of NB cell lines. GMFβ was hypophosphorylated in adult mouse brain tissues. GMFβ transfection showed modest increases in differentiation in NB cell lines as demonstrated in a neurite extension assay. A possible GMF-β dimer is formed with dephosphorylation of NB cells and in more differentiated mouse tissues. A MYCN-amplified NB cell line has increased GMFβ expression. **Conclusions**: Our results are consistent with the role of GMFβ as an ADF, since ADFs function when dephosphorylated. These experiments support a relationship between GMFβ phosphorylation and differentiation of NB cell lines. Examination of the role of GMFß as an ADF in NB differentiation may ultimately contribute towards novel biological therapies directed against this highly malignant childhood tumor.

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Introduction

Neuroblastomas are defined as tumors of the sympathetic nervous system, with ~50% of primary tumors arising in the adrenal glands[1]. The tumors can spread via the lymphatic system or blood. The clinical behavior of neuroblastoma (NB) can vary greatly from cancers that spontaneously regress to very aggressive cancers. The majority of NB cases are in the under 10 age group. Overall 80% of patients achieve complete or partial remission. However, the 3 year event-free survival (EFS) rate for advanced stage disease is less than 15%. Neuroblastoma represents approximately 15% of all pediatric oncology deaths in patients under the age of 15 [2]. Although a presumed diagnosis can be made with urinary catecholamine testing, histological sampling is used to determine the level of differentiation of the NB cells; increased differentiation is more favorable [2].

The International Neuroblastoma Staging System (INSS) was developed to provide a uniform staging system for NB. Stage 1 is defined as a localized tumor that is completely excised, regardless of residual microscopic disease, and ipsilateral lymph nodes (LN) are negative (~100% overall survival, OS). Stage 2A is defined as a localized tumor that is not completely excised; the LN are negative (~80% OS). Stage 2B is defined as tumors that may or may not be totally excised but ipsilateral LN are positive, while contralateral LN are negative (~70% OS). Stage 3 is defined by three different scenarios: (i) an unresectable unilateral tumor that extends across the midline, regardless of LN status; (ii) an unresectable unilateral tumor with LN involvement; (iii) an unresectable midline tumor with bilateral extension by infiltration or LN involvement. OS with stage 3 is 40%. Stage 4, with ~15% OS, is defined as any primary tumor that has remote disease limited to bone, bone marrow, soft tissue or distant LN, unless the patient has Stage 4S. Stage 4S, with ~85% OS, is defined as infants (< 1 yr) with stage 1 or 2 and remote spread to the liver, skin, or bone marrow but not bone. Unfortunately, more than 80% of patients present with stage IV disease at diagnosis [3].

Amplification of the oncogene MYCN (on chromosome 2p) is associated with NB and is found in 90% of NB cell lines. The products of the MYCN gene are normally associated with fetal and neonatal rather than adult growth. MYCN gene amplification is associated with a less favorable EFS and poorer prognosis than non-amplified tumors. N-myc expression is decreased in retinoic acid (RA) treated NB cell lines [4]. Cytogenetic features may include deletions or loss of heterozygosity (LOH) of chromosome 1p (usually 1p36) and gain of chromosome 17q21-qter [5]. Activating mutations of ALK kinase have also been associated with NB [6].

NB results from a defect in neural crest development. The cells from which NB originate are mostly neuroblasts and Schwann cells. Neuroblasts are pluripotent cells that arise in the neural crest. NB cells can arise in a stem cell (SC) compartment that has the ability to self-renew. These cells ultimately differentiate to form many structures of the sympathetic nervous system including the sympathetic ganglia, chromaffin cells of the adrenal medulla, and paraganglia. Unsurprisingly, these sites are common origins for NB tumors. NB cells show commonalities with neural crest SC; some are able to grow on media that grows neural crest SC and when exposed to certain factors NB cells may differentiate into mature neurons. Some research suggests that many fetuses have tissue identical to NB as a result of normal development. Therefore, normally developing neuroblasts and NB may have very similar profiles. This suggests that NB tissue, or something similar, is normal during development but is abnormal after birth.

The Schwann cells found in NB are thought to be either non-malignant tissue or arise from the same pluripotent cell as neuroblasts. The fact that some studies show that Schwann cells have normal DNA supports the first theory [7]. Schwann cells produce myelin in the PNS and are known to secrete growth factors. Differentiating neuroblasts express growth factor receptors. There is interest in determining the role of Schwann cells in NB differentiation mediated through Schwann cell-NB tumor cell interactions. Schwann cell-conditioned culture medium promotes survival and differentiation of human NB cells [8].

Glia maturation factor (GMFβ) was first described as an activity that elicited process extension when added to malignant glial tumor cells [9]. GMF is a 17 kD single chain polypeptide without a signal peptide or nuclear localization signal. Initially, the 141 a.a. protein had no known homology except a tripeptide LRE motif [10]. GMF immunoreactivity (IR) was found in the cytoplasm of neurons, glia and Schwann cells [10]. When GMF was added to cultures, cells elongated, redistributed actin to the cell periphery, underwent contact inhibition and arrested at G1/G0 [11]. Human GMF cDNA encodes 699 bp and an mRNA transcript of 3.7 kb [12]. Recombinant human GMF (rhGMF) added to CNS tumor cells increased glial fibrillary acidic protein (GFAP) [13].

GMF and Signal Transduction: GMF is phosphorylated *in vitro* upon NGF withdrawal and following the application of phorbol esters, protein kinases A (PKA) and C (PKC). *In vitro,* rhGMF, when activated by PKA, is a potent inhibitor of the MAP kinases ERK1 and ERK2 and stimulates p38, another MAP kinase[14].

GMF Expression Studies: Dr. Eisenstat cloned the mouse GMF homolog, mGMF; mGMF has 98% homology to hGMF. During development, mGMF is preferentially expressed in the developing CNS. GMF is highly expressed in neocortex, hippocampus, deep cerebellar nuclei and the spinal cord. He designed and characterized specific polyclonal antibodies to two distinct GMF. Immunohistochemistry (IHC) demonstrates GMF IR in neurons, glia, oligodendrocytes, and Schwann cells. GMF is localized to the cytoplasm and may be polarized in some cells. In primary cultures, GMF IR is found in the soma (cell body) and the proximal axon, but with maturation or process extension *in vitro*, GMF is expressed in the distal axon and can extend to the elaborated growth cone. This has been replicated in ATRA (all-trans - RA) treated SY5Y NB cell cultures and suggests a role for GMF-directed process outgrowth *in vivo* [16].

GMF is an Actin Binding Protein and Actin Depolymerizing Factor Family Member**:** GMF has significant structural identity to members of the actin-binding protein family, specifically the ADFs, including cofilin [17]. The 3-D structure of Destrin, a relative of cofilin, confirms the predicted structure of GMF as a member of the Cofilin/ADF Superfamily [18]. ADFs are inactive when phosphorylated, whereas dephosphorylation restores their activity. ADF/cofilin phosphorylation occurs almost exclusively on serine residues [17, 18]. Drebrin, related to cofilin, causes neurite extension and plays a role in axonal transport [19]. Co-immunoprecipation (co-IP) experiments from the Eisenstat laboratory confirm that GMF and actin form protein-protein complexes *in vivo* in adult mouse brain. Interruption of the actin cytoskeleton with cytochalasin D disrupts GMF IR and actin localization in the periphery of the cell, whereas interference of microtubule networks with colchicine has no effect (Eisenstat et al, in preparation). GMF and actin also co-localize in the distal axon and elaborated growth cone using the ATRA treated NB

cell lines. Knowing that GMF binds to actin *in vivo* provides us with the first evidence towards determining its unique biological function.

Materials and Methods

Cell culture - Human Neuroblastoma SK-N-BE (2) and SY5Y cells were grown in DMEM/F12 1:1 media with 10% FBS and 1% Penicillin Streptomycin at 37°C in a 5% CO_2 humidified incubator. Cells were trypsinized and passaged 1:12 every 7 days. Media was changed every 2-3 days.

Mouse tissues - Flash frozen embryonic and adult mouse tissues were lysed in a protein lysis buffer (RIPA solution [1% triton, 158 mMNaCl, 10 mMTris-HCl ph 7.6, 0.1% SDS, 1% Na-deoxycholate, 1 mM EDTA], 1X protease inhibitor cocktail, 50 mMNaF, 1 mM sodium vanadate, 1 mM PMSF) for 30 mins on ice, centrifuged at 12,000 rpm for 4mins and the supernatant was stored at -80°.All animal protocols were approved by the University of Manitoba animal care committee.

Retinoic acid treatment - Cells were treated with 10mM 13-cis retinoic acid (Sigma), dissolved in 100% EtOH mixed into basic media at a dilution of 1:1,000. Controls were incubated in basic media mixed with 100% EtOH at a dilution of1:1,000. Media was changed everyday 24hrs. Cells were harvested for protein on day 0, 1, 3 and 5. Cells were fixed on days 1,3 and 5 in 3.7% formaldehyde for 30 mins, washed 3x10 mins with PBS and stored at 4°C in PBS.

Plasmid transfection - SK-N-BE(2) cells were plated at 2.4 x 10^4 cells/cm^2 and incubated at 37°C in normal growth media 24hrs prior to transfection. Cells were washed with PBS for 5 mins. Cells were transfected in Opti-MEM reduced serum media (Invitrogen) with a DNA Lipofectamine 2000 (Invitrogen) ratio of 1:4. An amount of 1.6μg of DNA was used per well of 12 well plate, 4.0μg was used per well of a 6 well plate. The plasmids I used included: GMF-GFP, mutant actin binding domain (ΔABD)-GFP, and GFP (pEGFP-C). Controls using Lipofectamine 2000 alone and untreated cells were also used. The DNA and Lipofectamine 2000 solutions were incubated for 30 mins at RT prior to transfection. Cells were incubated in Opti-MEM reduced serum media for 24 hrs at 37°C, then media was changed to normal growth media. Transfection efficiency was assessed qualitatively by fluorescent microscopy. Cells grown on coverslips fixed for immunofluorescence (IF), lysates collected for western immunoblotting and cells for qPCR, were all collected 48hrs after transfection.

Small interfering RNA (siRNA) - SK-N-BE(2) cells were plated at 2.4 x 10^4 cells/cm^2 and incubated at 37°C in basic growth media 24hrs prior to transfection. Cells were rinsed once with PBS. Cells were transfected with siRNA (Santa Cruz) directed against GMF-β and control siRNA A (Santa Cruz). 0.625μg of siRNA was used per well of a 6 well plate. A ratio of siRNA (μg): Transfection reagent (μl) (Santa Cruz) was used. Cells were incubated at 37°C for 6hrs in siRNA transfection media (Santa Cruz). Media was changed to 2x normal growth media, DMEM/F12 1:1 media with 20% FBS and 2% penicillin streptomycin. Transfection efficiency was assessed qualitatively by fluorescent microscopy. Cells were incubated a further 24hrs then media changed to normal growth

media. Cells grown on coverslips were fixed for IF, lysate for western immunoblotting and cells for qPCR were all collected 48hrs after transfection.

Western blot analysis - Cells were scraped from the plate and the cell pellet isolated by centrifuging at 12,000rpm for 4 mins and the lysate stored at -80°. The pellet was then lysed with RIPA buffer for 30 mins on ice, centrifuged for 20 mins at 12,000 rmp at 4°C and the supernatant stored at -80°. Tissue from animals was prepared as discussed above. Protein concentration was determined using a BCA assay and absorbance measured using Spectra Max 190 instrument (Molecular Devices). Samples were prepared with protein lysate or ladder (Fermentas), 1X SDS (sodium dodecyl sulfate) Lysis Buffer (4X: 0.25M Tris-HCl pH 6.8, 8% SDS, 10% 2-mercapto-βethanol, 30% Glycerol, 0.02% Bromophenol blue), and PBS to ensure an equal volume of 25µl per lane. Samples were boiled for 5 mins to denature protein. Varying amounts of protein were loaded per well (30-100µg of protein to optimize western blotting. Samples were run on a 12-15% SDS polyacrylamide running gel, and a 4% SDS stacking gel at 160V for ~1.5hrs. A running buffer of 0.025M Tris, 0.19 M glycine and 0.1% SDS at pH 8.3 was used. The samples were transferred onto a 0.2µm nitrocellulose membrane (BioRad) at 30V overnight in a transfer buffer of 0.19M glycine, 0.025 M Tris, 173 mM SDS and 20% methanol by volume. Membranes were blocked for 1hr at RT in 5% non-fat powdered milk in TBS-T buffer at pH 7.4. The primary antibody was incubated overnight at 4°C in 1% milk TBS-T, or 1hr at RT in the case of anti-β-actin primary antibodies. The membranes were washed 3x10 mins in TBS-T. Next, the secondary antibody was incubated for 2 hrs at room temperature. After a further 3x10 min washes in TBS-T the membrane was treated with ECL for 5 mins and developed using ECL plus film (GE Healthcare Life Sciences).

Total GMF-β, phosphorylated GMF-β and hypophosphorylated GMF- β were measured with densitometry and standardized to β-actin. Values for RA treated cells are reported compared to the appropriate untreated samples.

Table 1 Primary antibodies used for western blotting

Primary Antibody	**Dilution**	**Source**
Goat polyclonal anti- *GMF β*(C-17)	1:500	Santa Cruz
Mouse monoclonal anti- *GMFβ*(SP-61)	1:500	Santa Cruz
Mouse monoclonal anti-β-actin	1:10,000	Sigma

Table 2 Secondary antibodies used for western blotting

Secondary Antibody	**Dilution**	**Source**
Rabbit anti-goat HRP	1:5,000	Santa Cruz
Goat anti-mouse HRP	1:5,000	Santa Cruz

λ-Phosphatase Treatment - Protein lysate were prepared as described above with the addition 1µl of λ-phosphatase (New England Biolabs) per 15µg of protein, 1X NEBuffer (50 mM HEPES, 100 mM NaCl, 2 mM DTT, 0.01 % 35, pH 7.5 (New England Biolabs)) and $MnCl_2$ (New England Biolabs) to a total volume of 25 µl per lane. These samples were incubated for 3 hrs at 30°C as well as control samples without the addition of λ-

phosphatase. The 1X SDS Lysis Buffer was not added until after the incubation. Samples could then be stored at -80°C or run on an SDS gel immediately.

Immunofluorescence - Cells were grown on round coverslips (Fisher) fit into the well of 24 well cell culture plates. Cells were fixed with 3.7% formaldehyde for 30 mins, washed 3x10 mins with PBS and stored at 4°C in PBS. Each coverslip was placed with blocking buffer (0.1% BSA, 0.2% Triton-X 100, 0.2% Sodium Azide, 5% Horse Serum in 1XPBS pH 7.4) for 1 hr at RT. The primary antibody was diluted in prepared blocking buffer and incubated overnight at 4°C. The following day, slides were washed 3x5mins in PBS-T. Coverslips were incubated with the secondary antibody for 2 hrs at RT in the dark. After washing again, coverslips were incubated in Phalloidin (Invitrogen) diluted in PBS at 1:200 for 20 min at RT in the dark. Coverslips were washed then inverted onto 10µl of VectaShield with DAPI (Vector Labs) onto SuperFrost Plus slides (Fisher).

Table 3 Primary antibodies used for immunofluorescence

Primary Antibody	**Dilution**	**Source**
Goat polyclonal anti- *GMF- β*(C-17)	1:100	Santa Cruz

Table 4 Secondary antibodies used for immunofluorescence

Secondary Antibody	**Dilution**	**Source**
Donkey anti-goat Alexafluor 488	1:200	Molecular Probes
Donkey anti-mouse Alexafluor 488	1:200	Molecular Probes
Biotinylated horse anti-goat	1:200	Vector Laboratories Inc.

Table 5 Tertiary antibodies used for immunofluorescence

Tertiary Antibody	**Dilution**	**Source**
Streptavidin conjugated Alexafluor 488	1:200	Molecular Probes

Immunohistochemistry - Formalin fixed paraffin-embedded (FFPE) NB slides were baked for 20 mins at 60°C to melt paraffin, slides were then placed in Xylene twice for 5 mins each, then rehydrated through an ethanol gradient (50%, 75%, 85%, 95%, 100%, 100% and H_2O for 5 mins each). Antigen presentation was completed by heating the slides in a pressure cooker filled with citrate buffer (10mM citric acid monohydrate pH 6.0) for 20 mins. Slides were cooled to room temperature and washed 3x5min with 1xPBS. Slides were blocked with prepared blocking solution (0.1% BSA, 0.2% Triton-X 100, 0.2% Sodium Azide, 5% Horse Serum in 1XPBS pH 7.4) for 1 hr at RT. The primary antibody was diluted in prepared blocking buffer and incubated overnight at 4 °C. The following day, slides were washed 3x5mins in 1xPBS-T. The slides were incubated with the secondary antibody for 2 hrs at RT. Slides were incubated in 0.3% H_2O_2 for 30mins at RT. Slides were washed again then incubated for 30 min with Elite ABC solution (Vector) at RT. Following another round of washing, slides were developed using DAB (Vector) until staining was detected and was stopped with the addition of water after 7-10 mins. Slides were dehydrated through an ethanol gradient (50-100%, Xylene for 5 mins each) and mounted with coverslips (Fisher) and Permount mounting medium (Daigger).

Table 6 Primary antibodies used for IHC

Primary Antibody	Dilution	Source
Goat polyclonal anti- *GMF- β*(C-17)	1:100	Santa Cruz

Table 7 secondary antibodies used for IHC

Secondary Antibody	Dilution	Source
Biotinylated horse anti-goat	1:200	Vector Laboratories Inc.

Real-time quantitative and reverse transcriptase PCR analysis - Cell pellets were obtained from cell culture and stored at -80°C. RNA was extracted from SK-N-BE(2) cells with TRIzol (Invitrogen), and treated with DNase (Sigma) prior to reverse transcription. RNA (1μg) was used to synthesize cDNA with SuperScript II reverse transcriptase (Invitrogen). For each sample, 1μl cDNA was then used as a template for GMF-β amplification. Real-time qPCR was performed using the iCycleriQTM Multi-color real-time PCR detection system (Bio-RAD). mRNA copy numbers were calculated utilizing standard curves generated by using plasmids containing the target sequences. Gapdh was used as a control compare to GMF- β expression levels.

Table 8 Primers

Primer	Sequence
f *GMF- β* (forward)	(25) 5'-CCGGAAGGAAATGAGTGAG-3' (44)
r GMF- β (reverse)	(310) 5'-CTTACATCCAACAGGACTGG-3' (330)

Cell counting - After transfection, cells were visualized using the IF protocol described. Cells were characterized as either "A", "B" or "C" type based on the length of neurite extension as a measure of differentiation (figure 10). At least 200 cells were counted per coverslip. The enumerator was blinded to the treatment condition while counting to eliminate bias.

Imaging - Images of slides were obtained using an Olympus BX51 microscope with either a SPOT 1.3.0 digital CCD camera (Diagnostic Instruments Inc., Sterling Heights, MI) for brightfield images or an Olympus DP70 digital camera for fluorescence images. Images were captured with DP controller v2.3.1.231 and DP Manager v2.2.1.95 software. Images were colour corrected with Adobe Photoshop CS3.

Statistics – all p values were calculated with a two-tailed t-test. A value of <0.05 was deemed statistically significant.

Hypotheses:

1) GMFβ is a member of the actin depolymerizing family.
2) GMFβ plays a role in neuroblastoma differentiation by modifying the actin cytoskeleton.

Results

GMFß *expression in neuroblastoma cell lines*- Western blotting was optimized using two commercial antibodies (C-17 and SP-61, Santa Cruz). IF and IHC were attempted. IF with the C-17 antibody shows GMFß location in the cytoplasm of the cell (Figure 1).

Retinoic acid (RA) treatment - RA potently differentiates neural cells; treated cells showed a significant change in morphology as compared to the relative controls. Cells demonstrated significant process extension (Figure 2). Western blot analysis of RA treated cells showed a notable difference in the proportion of phosphorylated GMFß to total GMFß in RA treated samples as compared to untreated samples (Figure 3g). This difference approached statistical significance (Figure 3h) with a p value of 0.08. As well, an overall decrease in phosphorylated GMFß (Figure 3f) was noted. The amount of hypophosphorylated GMFß decreased (Figure 3e). Total GMFß remained much the same, showing a small decrease (Figure 3d). Treatment with λ-Phosphatase (Figure 4) confirms that the observed upper band is in fact a phosphorylated form of GMFß.

GMFß *and MYCN amplification* - SY5Y cells showed a relative decrease in GMFß levels using western blotting as compared to SK-N-BE(2) cells (Figure 5).

*Mouse brain GMF*ß *expression* - E18.5 brain showed decreased GMFß expression compare to adult brain (Figure 6) However, neither tissue showed a phosphorylated band.

Plasmid transfection - SK-N-BE(2) cells were transfected with both GMFß -GFP and mABD-GFP (GMFß with a mutant actin binding domain) developed by Eisenstat et al (Figure 7). Transfection efficiency of cells was visualized using IF staining for GFP. Transfection efficiency was approximately 10%. By western blotting, no appreciable increase in GMFß could be noted (Figure 8). RT-PCR showed a PCR product of ~300bp with the expected product using these specific primers is 311bp (Figure 9). qRT-PCR analysis of GMFß mRNA (Table 10) showed no appreciable increase in GMFß in GMFß-GFP transfected cells, whereas mABD transfected cells showed more consistent results, indicating that GMFß levels remained similar to controls (Table 10) using these cell lines.

Cells were fixed and stained for Phalloidin and DAPI to evaluate cell morphology. Process extension was evaluated using cell counting (Figure 11). A modest increase in differentiation was seen in the GMFß-GFP transfected cells (Figure 11), with more "B" cells compared to the mutants and controls. Cells transfected with mABD showed no increase in differentiation (Figure 11).

siRNA - siRNA transfection of SK-BE(2) cell lines was used to explore whether interfering with post-transcriptional GMFß expression would decrease neurite extension. Cell toxicity and lack of transfection of cells with siRNA were problems encountered with this experiment, so there is no data to show.

IHC- IHC using FFPE human neuroblastoma tumor samples with C-17 antibody (Santa Cruz) did not yield any appreciable staining (refer to discussion).

Other observations- A western blot band at ~25kDa was observed in λ - phosphatase treated cell lines (SY5Y and SK-N-BE(2)) and E18.5 mouse brain (Figures 4 and 6). This band was not observed in adult brain tissue or cell lines not treated with λ - phosphatase (Figures 4 and 6).

Discussion

GMFß expression in neuroblastoma cell lines- A major difficulty was the small size of GMFß (17kDa); this contributed to the failure of many blots most likely due to the improper transfer of GMFß. Ultimately, the percentage of methanol in transfer buffer, pore size of the membrane and transfer voltage and times were adjusted to successfully detect GMFß . Another problem faced was the isolation of sufficient amounts of protein from tissues; for example, E18.5 mouse adrenal glands did not yield sufficient protein. Also, the close proximity of the phosphorylated and hypophosphorylated bands caused poor separation when using a 10% SDS-PAGE gel; 15% SDS-PAGE gels led to better visualization of the bands. Only after many attempts did the antibodies (C-17 and SP-61) reliably show GMFß using IF.

Retinoic acid (RA) treatment- By treating with RA, the role of GMFß in actin binding, subsequent rearrangement of the cytoskeleton and process extension can be explored. Specifically, we predicted that with the addition of RA, GMFß and actin co-localization to the growth cone in partially differentiated NB cells could be visualized using available antibodies to GMFß and phalloidin that binds to actin polymers (F-actin). This was not possible due to difficulty with IF protocols using the C-17 and SP-61 antibodies (Santa Cruz). Eisenstat et al. had previously isolated two polyclonal anti-peptide GMFß antibodies (PO1 and PO2). These antibodies have been successfully used for western blotting, IF and IHC. In the future re-synthesis of these antibodies will successfully visualize GMFß in cells differentiated using RA.

The trend observed in this experiment supports a role for RA in the activation of GMFß, possibly describing a role of GMFß in the mechanism of RA treatment. This is consistent with the finding that GMFß was also hypophosphorylated in fetal (E18.5) and adult mouse brain (Figure 6), as these are more differentiated neural tissues than embryonic tissues and NB cell lines. Our results supports the hypothesis that hypophosphorylation of GMFß is related to differentiation of neural cells. Other members of the ADF/cofilin family are activated by dephosphorylation [20].

The enzyme used for λ-Phosphatase treatment was an older reagent and dephosphorylation was modest. Probing for Phospho-Histone H3 (used as a positive control) was done to confirm the efficacy of treatment with λ-Phosphatase. However, the antibody used was not effective and this experiment will be repeated.

It is possible that GMFß phosphoryation could be used as a marker for NB staging and clinical decision making. In the future, comparison of patient tumor samples of different stages to examine the phosphorylation of GMFß could provide more insight into the relationship of GMFß phosphorylation to differentiation. Phosphorylation status in mouse embryos of different gestational ages could provide more information regarding

the role of GMFß in neural development. Primary cell culture could also be used to further explore the phosphorylation of GMFß in different tissues and discover the relevant kinases and key phosphorylated residues. The MYCN mouse model of NB [21] and human NB tissue microarrays could be used to assess whether phosphorylation status of GMFß correlates with tumor staging and N-MYC status.

GMFß and MYCN amplification- SK-N-BE(2) cells are MYCN-amplified whereas SY5Y cells are not MYCN-amplified. Perhaps GMFß expression is increased in NB cell lines that are MYCN-amplified. However, this observation requires further study in other cell lines and tumor samples.

Mouse brain GMFß expression - It is possible that embryonic tissue earlier in gestation would show the phosphorylated form of GMFß since GMFß is first expressed at gastrulation (E7.5). A comparison of tissue from embryos of different ages could demonstrate the time period at which GMFß becomes hypophosphorylated and thus deactivated. This would provide insight into the role of GMFß in the development of neural tissues. Other tissues of interest are embryonic and adult adrenal glands, the most common primary site of NB.

Plasmid transfection -SK-N-BE(2) cells express GMFß at a high level, so using other NB cell lines that express a lower endogenous level of GMFß, for example, SY5Y cells, could be helpful when quantifying GMFß expression following transfection as any difference in GMFß would be more appreciable. The successful RT-PCR demonstrates that the primers designed effectively amplify GMFß cDNA. However, the lack of consistent qRT-PCR is possibly due to the low transfection efficiency. The more consistent qRT-PCR results with the mABD transfected cells lack significance since the transfection efficiency was so low.

Increased transfection efficiency would solve the problem of visualizing the small differences in differentiation at such a low transfection efficiency. Another challenge with transfection was maintaining sufficient confluency of cells to transfect without cell toxicity while maintaining a low confluency levels to visualize the neurites of cells. In repeating these experiments, different plasmid: Lipofectamine 2000 ratios would be used, as well as quantity of plasmid.

Precisely measuring neurite extension length may aid in enumerating cells that were exhibiting some neurite outgrowth but not enough to be classified as a B or C cell. Also, quantifying the length of each neurite would enable further statistical analysis. Cells could be treated with RA to hypophosphorylate GMFß, whereas cells with over-expression of GMFß may show more differentiation. Also, transfecting with a control such as β-galactoside would allow for a more quantitative measure of transfection efficiency.

siRNA

Different transfection reagents, DNA:transfection reagent ratios, and cell confluency would be the first step in optimizing the experiment. GMFß protein and mRNA levels would be confirmed with western blotting and qRT-PCR. In the future, IF and cell counting analogous to that used in plasmid transfections would be used to determine

whether decreased GMFß expression reduces the number of cells with significant neural processes, thus determining if GMFß is necessary for NB cell differentiation. Cells could also be treated with RA and siRNA to GMFß, demonstrating if RA mediated differentiation can occur in the absence of GMFß.

IHC - GMFß has been shown to localize to Schwann cells and differentiated portions of the neuroblastoma tumor (Eisenstat, preliminary data). IHC for GMFß using FFPE human NB tumor slides was carried out to confirm the cell type expressing GMFß as well as cellular location. Eisenstat et al. have shown that GMFß phosphorylation status is inversely related to tumour stage in glioblastoma (GBM). IHC using the antibodies made by Eisenstat et al. could detect the phosphorylated form of GMFß in neuroblastoma, to be added to the GBM data. This could further support the relationship between GMFß phosphorylation and tumor differentiation.

Other observations - As described above, GMFß has been found to be hypophosphorylated in RA differentiated cell lines; therefore treatment with λ-phosphatase would then be expected to increase amounts of activated GMFß. The presence of this band in adult but not E18.5 mouse brain tissue suggests that it is associated with a more differentiated state. GMFß may bind to the Arp2/3 complex [20] but this protein is 224kd and too large to represent the additional band as is actin at 42 kd. However, this observed band could represent a dimer of GMFß. Co-immunoprecipitation experiments and/or pull-down experiments could more convincingly demonstrate GMFß dimers.

Conclusion

GMFß expression in neuroblastoma cell lines has been confirmed by RT-PCR, western blotting and IF in neuroblastoma cells lines. The capability of a commercial antibody to detect phosphorylated GMFß has been confirmed. The hypophosphorylation of GMFß was observed as neuroblastoma cell lines were differentiated with RA. The expression and phosphorylation status of GMFß in mouse tissue has been compared to less differentiated neuroblastoma cell lines. A possible GMFß dimer was observed in samples that had been dephosphorylated.

Further investigating GMFß as an ADF in NB differentiation may ultimately contribute towards new treatment of neuroblastoma.

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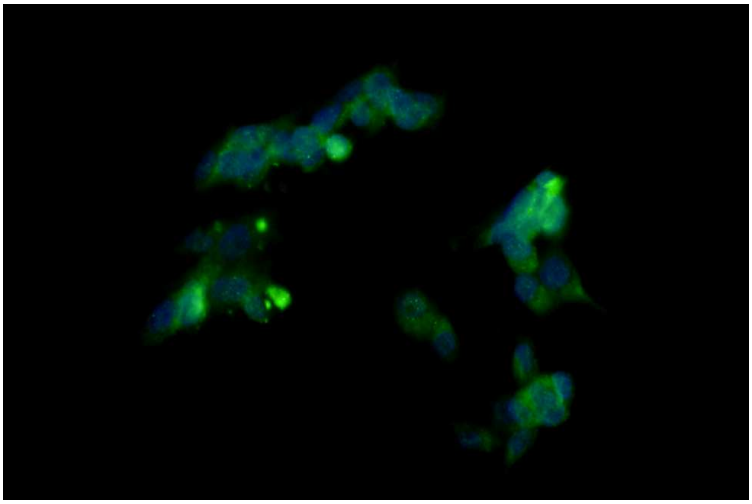


Figure 1 – GMFβ cytoplasmic expression in NB cell culture (SK-N-BE(2) cells). IF was performed using C-17 antibody. DAPI outlines the nuclei (blue).

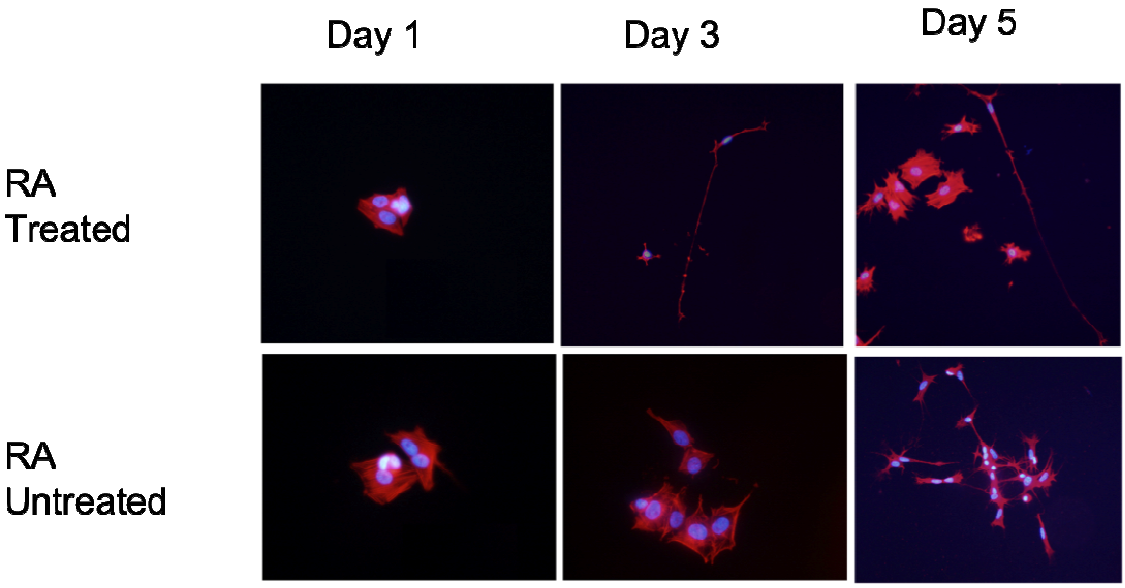
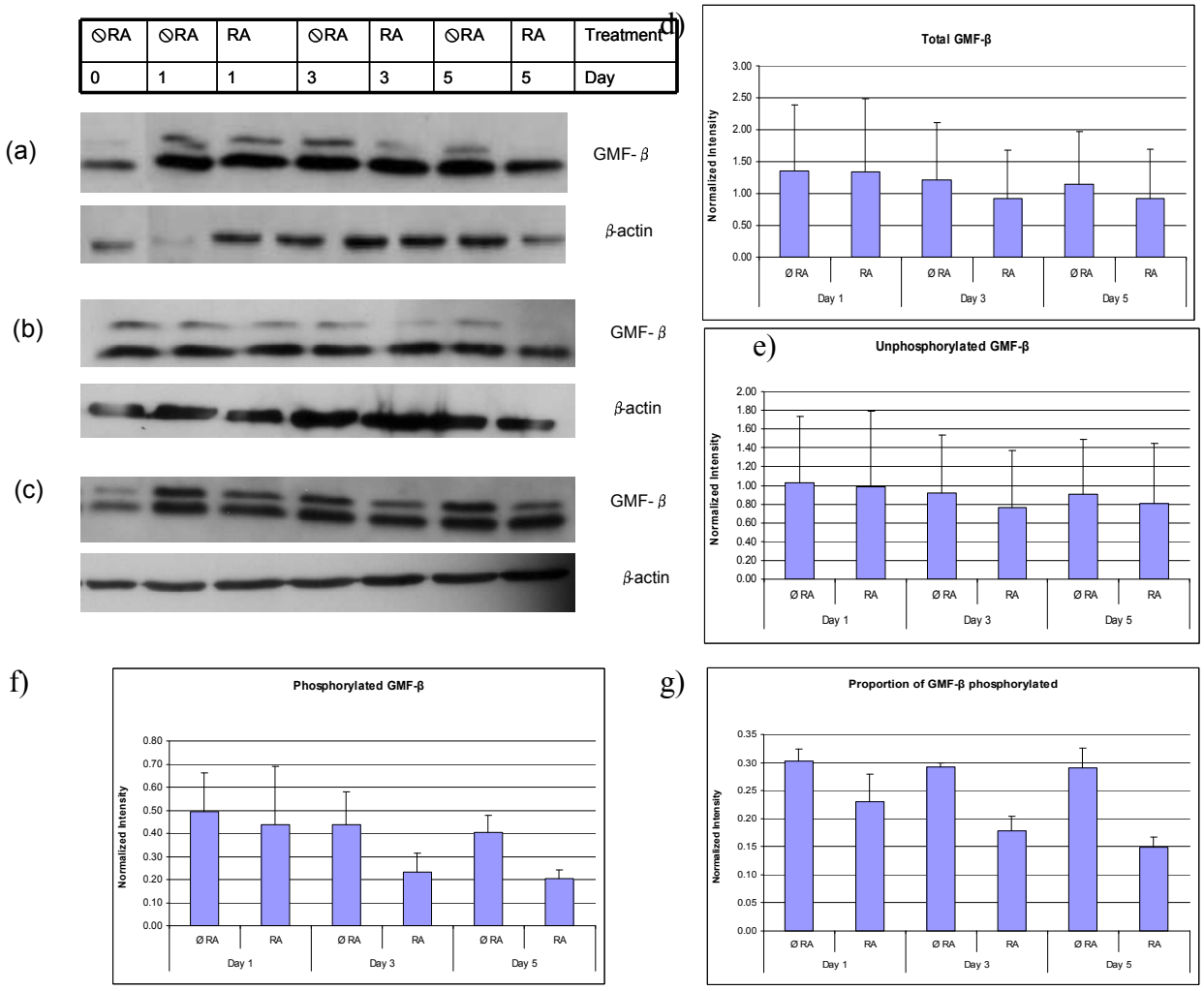


Figure 2 - Differentiation of NB cell lines using RA. With increasing length of treatment time, neurite processes lengthen. Phalloidin stains for actin and DAPI outlines the nuclei (blue).



Days of RA treatment	**Total GMF-β**	p-value	**Unphosphorylated GMF-β**	p-value	**Phosphorylated GMF-β**	p-value	**Proportion of GMF-β phosphorylated**	p-value
Day 1	0.98	0.87	0.96	0.76	0.89	0.63	0.76	0.50
Day 3	0.76	0.29	0.83	0.05	0.53	0.18	0.61	0.18
Day 5	0.81	0.17	0.89	0.34	0.51	0.13	0.52	0.08

Figure 3 - Decreased GMF-β phosphorylation with RA treatment

Western blots for multiple repeats of treating with RA (a,b,c). Total GMFβ, phosphorylated GMF-β and hypophosphorylated GMFβ bands measured with densitometry and standardized to β-actin. Values represent the ratio as compared to the untreated samples for that day. P–values were calculated using a two-tailed paired t-test.

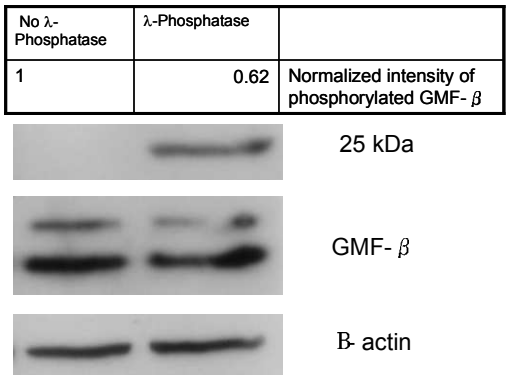


Figure 4 - λ-phosphatase treatment of NB SK-N-BE(2) cells. Densitometry shows a significant decrease in phosphorylated GMFβ in the λ-phosphatase treated cells. A band is noted at ~25kDa that may be a GMFβ dimer.

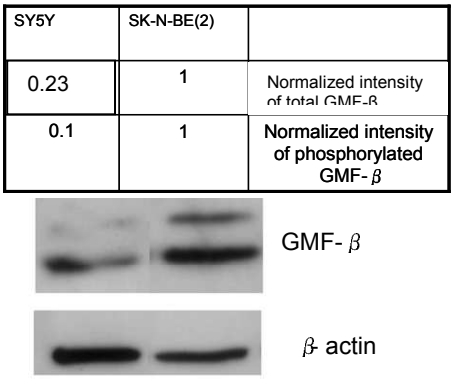


Figure 5 - Decreased phosphorylation and expression of GMFβ in SY5Y cells as compared to SK-N-BE(2) cells. Using densitometry standardized to actin, a significant decrease in both total and phosphorylated GMFβ is observed.

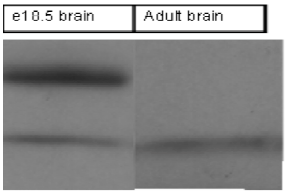


Figure 6 - Hypophosphorylation of GMFβ in e18.5 and adult mouse brain tissue. A band is noted at ~25kDa that may be to a GMFβ. dimer.

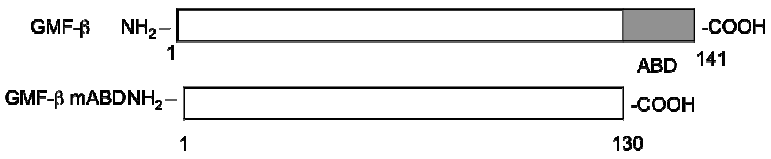


Figure 7 – GMFβ wild-type and mABD mutation NB cell lines were transfected with both wild type and mABD plasmids to visualize any differences in neurite outgrowth.

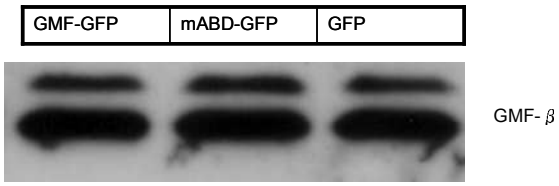


Figure 8 – Western blot of GMFβ wild-type and mABD transfected cells. Cells were transfected with both the wild type and mABD mutated plasmids. No appreciable difference in GMFβ expresssion is observed.

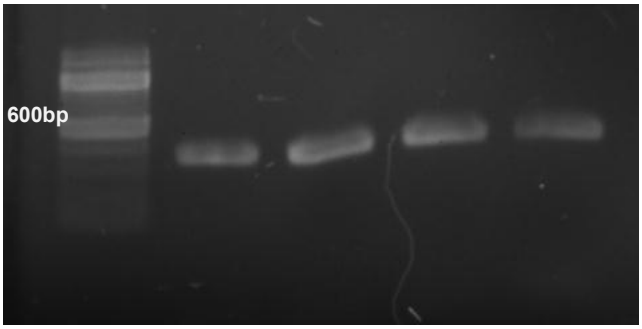
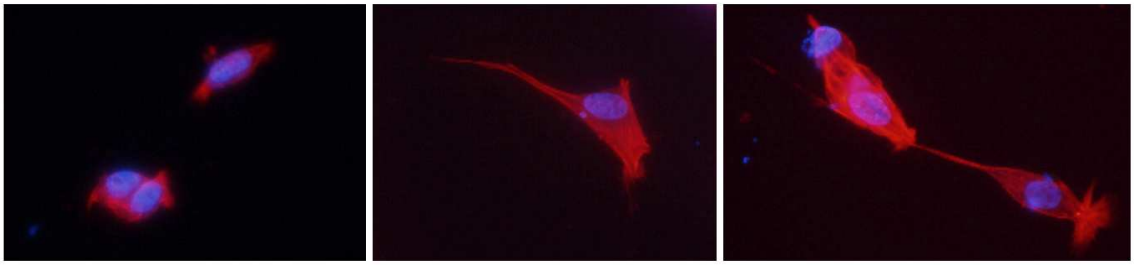


Figure 9 – RT-PCR analysis of transfected neuroblastoma tissue Primers were designed with an expected PCR product of 311kb, the product isolated is approximately 300kb. cDNA was generated from mRNA isolated from GMF, mABD and GFP transfected cell lines.

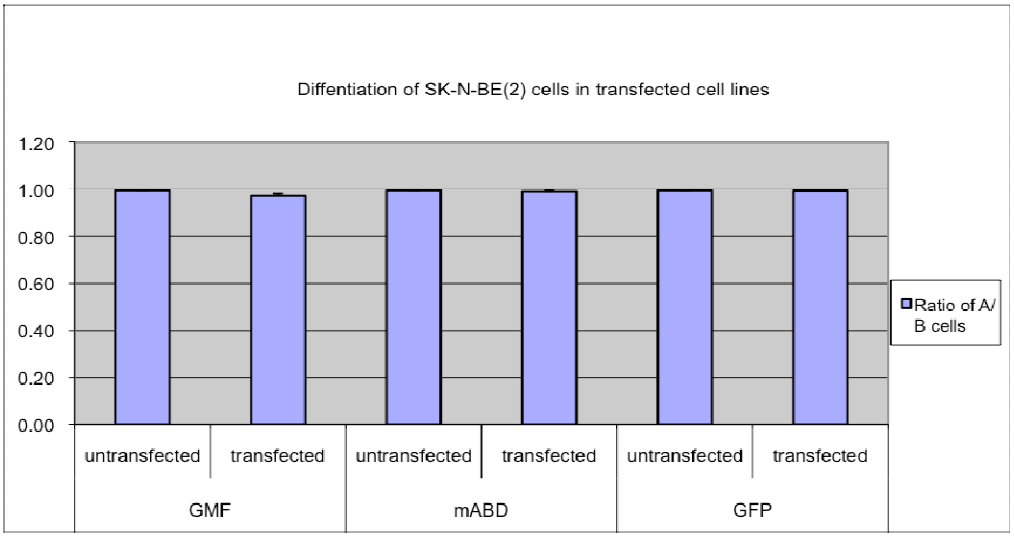


"A" cell Neurite< Soma	"B" cell Neurite≥ Soma	"C" cell Neurite≥ 2x Soma

Figure 10 - Classification of cell differentiation SK-N-BE(2) cell lines transfected with plasmids. Cells were classified as either A, B or C cell types after transfection with GMF, mABD and GFP. This method is used to measure differentiation of the NB cells.

	Trial #1	Trial #2		Trial #1	Trial #2
GFP	1	0.969232	GFP	1.00	1.00
GMF	0.006	1.000	mABD	0.6	0.05

Table 10 – qRT-PCR measurements of GMFβ transcript levels in GMFβ and mABD transfected cells. The levels of GMFβ were very inconsistent over the two trials of qRT-PCR, whereas mABD levels were more consistent.



	GMFß	mABD	GFP
p values	0.07	0.06	0.64

Figure 11 – Differentiation of neuroblastoma cells with GMFβ, mABD and GFP transfection. Values represent the average ratio of A to B cells for that condition. P-values were calculated using a two-tailed paired t-test. A very modest decrease in the ratio of "A" type to "B" type cells was observed in GMFβ transfected cells.