Rac1b Regulates the Neurotrophin-3 Mediated Neuronal Commitment of Bone Marrow Derived MIAMI Cells

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RAC1B REGULATES THE NEUROTROPHIN-3 MEDIATED NEURONAL COMMITMENT OF BONE MARROW DERIVED MIAMI CELLS

By

Kevin M. Curtis

A DISSERTATION

Submitted to the Faculty of the University of Miami in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Coral Gables, Florida

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RAC1B REGULATES THE NEUROTROPHIN-3 MEDIATED NEURONAL COMMITMENT OF BONE MARROW DERIVED MIAMI CELLS

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Emerging trends in cell-therapy based tissue repair have focused on the renewable source of adult stem cells including human bone marrow-derived mesenchymal stromal cells (hMSCs). Due to immunomodulatory properties as well as a potential to differentiate into cells characteristic of all three germ layers, hMSCs provide a source of immature cells for utilization in cell-therapy based treatments. Marrow isolated adult multilineage inducible (MIAMI) cells are a homogeneous sub-population of hMSCs which maintain self-renewal potential during ex vivo expansion, in addition to efficiently undergoing trans-differentiation into neuron-like cells in vitro. Even though hMSCs have the potential to be used for neural tissue repair, the molecular mechanisms by which they are stimulated to become neuron-like cells have not been fully characterized. Therefore the work described herein focuses on the molecular mechanisms by which MIAMI cells undergo NT-3 dependent neuronal commitment.

MIAMI cells express both the full length (FL-) and tyrosine kinase deficient (TKd-) isoforms of the NTRK3 receptor, the primary NT-3 receptor, at the protein level. NT-3 stimulation of MIAMI cells during neuronal commitment induced the phosphorylation of FL-NTRK3, degradation of TKd-NTRK3, downstream activation of the Mek1/2-Erk1/2 signaling cascade, and subsequent up-regulation of a limited number
of pro-neuronal genes. These findings were verified using chemical inhibitors to block NTRK autophosphorylation (K252a) and Erk1/2 activation (U0126).

TKd-NTRK3 is hypothesized to activate Rac1 upon NT-3 stimulation. Rac1 was found to suppress NT-3 stimulated Erk1/2 phosphorylation, as well as downstream gene expression, as determined using a Rac1 chemical inhibitor. Further characterization confirmed that Rac1b is the predominant Rac1 isoform in MIAMI cells. Rac1b siRNA mediated knock-down resulted in increased expression of the pro-neuronal genes NGN2, MAP2, NFH and NFL during NT-3 stimulation via regulation of Mek1/2-Erk1/2. Rac1b is also involved in NT-3 stimulated cell proliferation, as well as repression of CCND1 and CCNB1 mRNA expression.

In an attempt to enhance neuronal differentiation of MIAMI cells, EGF and bFGF were used to pretreat MIAMI cells prior to NT-3 stimulated neuronal commitment. EGF/bFGF pretreatment increased NTRK3 and NTRK1 protein levels along with NT-3 stimulated Erk1/2 phosphorylation. In addition, bFGF versus EGF/bFGF pretreatment restricted the expression of the pro-neuronal transcription factors Ngn2 and Prox1 versus the neural stem cells self-renewal transcription factor Musashi-1, respectively. The culmination of this work provides a model for the NT-3 induced neuronal commitment of MIAMI cells in vitro, as well as insight into the neurogenic potential of MSCs for future applications in cell-therapy based tissue repair.
ACKNOWLEDGMENTS

I would like to thank the faculty and staff of the Department of Biochemistry and Molecular Biology at the University of Miami, Miller School of Medicine for providing me not only with the opportunity to pursue a Ph.D., but also for their advice, training, and friendship throughout these years. I also want to thank the Veterans Affairs Medical Center along with all of the scientists and staff of the Geriatric Research Education Clinical Center for their funding, in addition to their unwavering support and guidance which helped me develop my skills as a scientist. Finally I would like to thank my mentor, Paul C. Schiller, along with my committee members; Dr. Zafar Nawaz, Dr. Karoline Briegel and Dr. Jacqueline Sagen for their outstanding guidance and mentorship.
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ACRONYMS

BM: bone marrow,
FL-NTRL3: full length-NTRK3
MIAMI: marrow isolated adult multilineage inducible,
MSCs: marrow stromal cells,
NSCs: neural stem cells, NT-3: neurotrophin-3,
NTRK3: neurotrophin tyrosine receptor kinase 3,
NT-3: neurotrophin 3
PTK: protein tyrosine kinase domain,
RT-qPCR: real time-quantitative polymerase chain reaction,
TKd-NTRK3: tyrosine kinase deficient-NTRK3
CHAPTER 1
INTRODUCTION

Marrow Stromal Cells (MSCs) were first described as fibroblast-like cells that reside in the bone marrow (BM) of vertebrate animals, including humans (Friedenstein, et al. 1970), capable of forming clonal colonies with osteogenic potential Marrow-derived human MSCs (hMSCs), were later referred to as mesenchymal stem cells (Caplan 1991) body a non-hematopoietic, heterogeneous population of uncommitted and lineage-committed adult progenitor stem cells which have the ability to differentiate into multiple cell lineages including osteoblasts, chondrocytes and adipocytes (Colter, et al. 2001, Minguell, et al. 2001).

hMSCs isolated from bone marrow have been shown to attach to the surface of culture dishes and to form colonies, thus allowing for the expansion of hMSCs ex vivo (Banfi, et al. 2000). The ability of hMSCs to be expanded ex vivo gave rise to the idea that they could be directed to differentiate into specific phenotypic cell lineages and ultimately be used in tissue repair via autologous cell transplantation (Bruder, et al. 1997, D'Ippolito, et al. 1999, Dezawa, et al. 2004). hMSCs expanded ex vivo represent a heterogeneous population of progenitor cells (adult stem cells) with self-renewal properties and a multilineage differentiation potential. Due to the heterogeneity of the results using dissimilar cells isolated in different fashion in labs around the world, and all being termed MSCs, the International Society for Cellular Therapy defined the term multipotent mesenchymal stromal cells in an effort to unify the criteria defining this heterogeneous cell population (Dominici, et al. 2006). These cell are characterized by
the expression of CD105, CD73 and CD90, and lack expression of CD45, CD34, CD14 or CD11b, CD79α or CD19 and HLA-DR surface molecules.

Various isolation, selection, and culture conditions have been used (Dominici, et al. 2009) in order to develop more homogeneous populations of immature primitive hMSCs; such as MIAMI cells (D’Ippolito, et al. 2004), MAPC, MASC (Beltrami, et al. 2007), SSEA-4+ MSC (Gang, et al. 2007), CD133+ Selected MSC (Pozzobon, et al. 2009), and RS-1 cells (Colter, et al. 2000), which make up only a sub-fraction of the total bone marrow cell population. These sub-populations of hMSCs are characterized by increased self-renewal potential and the ability to differentiate not only into mature cells found in mesodermally-derived tissues (SSEA-4+ MSC), but also into cells characteristic of ectodermally- as well as endodermally-derived tissues (MIAMI cells, MAPC, MACS, CD133+ Selected MSC). Cells with similar characteristics of bone marrow-derived hMSCs have also been identified in other tissues, including umbilical cord blood (Erices, et al. 2000), peripheral blood (review, (He, et al. 2007)), adipose tissue (Zuk, et al. 2001), skeletal muscle and dermis (Young, et al. 1999, Young, et al. 2001).

Due to the decreased or limited ability of somatic human tissue to regenerate because of age, trauma or disease-related injury, the use of hMSCs for cell-therapy based repair, particularly for neural tissue repair, is of interest (Uccelli, et al. 2008). Neural stem cells (NSCs) which proliferate and have self-renewal capacity are limited to the forebrain sub-ventricular zone (SVZ), subgranular zone (SGZ) of the hippocampus and suggested parts of the cortex in the human and primate adult brain (Gage 2000, Gould, et al. 1999). It has been shown in animal models that SVZ-NSCs can migrate to the olfactory bulb and are responsible for continued olfactory bulb neurogenesis. In addition
SVZ and SGZ-NSCs have been correlated with hippocampal-neurogenesis and hippocampal-dependent tasks such as learning and memory (Burns, et al. 2009, Drapeau, et al. 2007). Although repair of age-, disease-, or trauma-related brain damage via NSCs migration and neurogenesis does occur to a limited extent in the adult brain, it is not sufficient to over-come extensive neural damage or degeneration (review (Basak and Taylor 2009)). In this regard, hMSCs could provide a renewable source of immature or pre-differentiated cells for cell-therapy based repair of neural tissue.

**Neurogenic Potential of Marrow Stromal Cells**

The neuronal differentiation of hMSCs in vitro has been successfully induced using fibronectin-coated substrates, retinoic acid, growth factors, demethylating agents, antioxidants, agents which increase intracellular cAMP, bone morphogenic proteins (BMPs), and through the use of serum-free medium. In addition, chemical inducers, such as β-mercaptopethanol, isobutylmethylxantine and 5-azacytidine have also been used. A comparative study analyzed various differentiation protocols and questions the effectiveness of these chemical inducers to upregulate neuroectodermal markers corresponding to neural differentiation (Bertani, et al. 2005, Chen, et al. 2006, Hermann, et al. 2006, Neuhuber, et al. 2004). In order to determine if hMSCs are undergoing neuronal differentiation or only mimicking a neuronal phenotype, markers of early and late neurogenesis (Nestin, Neurogenin2, MBP, MAP2a/b, MAP1b) must be taken into account along with the loss of mesenchymal properties of the hMSCs (Hermann, et al. 2006). Human bone marrow-derived MAPCs, MSCs, and MIAMI cells have all been shown to differentiate into neuron-like cells with a characteristic neuronal phenotype.

MSC are of interest for cell-therapy based tissue repair, due to their capabilities not only to be expanded *ex vivo*, but also due to their ability to differentiate into neuronal lineages allowing for large numbers of neural-specific MSCs to be prepared for transplantation. *In vivo* studies have indicated that MSCs may decrease the neurological defects associated with experimentally induced stroke ((Ohtaki, *et al.* 2008, Zhao, *et al.* 2002) see review; (Dharmasarosha 2009, Shyu, *et al.* 2006)) or improve behavioral indices associated with a 6-hydroxydopamine rat model of Parkinson’s disease (Dezawa, *et al.* 2004). It is unclear as yet whether fully differentiated MSCs or pre-treated MSCs will be the most efficacious for *in vitro* transplantations. The answer is probably situation specific, and dependent on the type of cell required and/or the type of damage to be repaired (review, (Chen, *et al.* 2006, Parr, *et al.* 2007, Prockop 2009)). Since the beginning of MSC research in 1970 (Friedenstein, *et al.* 1970), the characterization, *in vitro* differentiation of MSCs and *in vivo* animal models have shown the potential of MSCs for cell-therapy based repair of neural tissue. Not only for age-related degenerative diseases such as Parkinson’s disease (PD), but also stroke-induced cerebral ischemia, head trauma, spinal cord injury, peripheral nerve damage, and the targeting of gliomas for gene therapy are a few examples under consideration for MSC-based repair of neural tissue.

**MIAMI cells**

Our research group has isolated a subpopulation of hMSC known as *marrow-isolated adult multilineage inducible* (MIAMI) cells (D'Ippolito, *et al.* 2004). MIAMI
cells are distinguished from other bone marrow-derived MSCs such as MAPCs, SPCs, and RS-1 cells by virtue of their expression of markers distinctive of totipotent embryonic stem cells (Oct-4, Sox2, Rex-1, Nanog and SSEA-4) (Colter, et al. 2000, D'Ippolito, et al. 2004, Gronthos and Simmons 1996, Pittenger, et al. 1999, Reyes, et al. 2001). MIAMI cells have been isolated from males and females (3 to 72 years of age) as early passage cells capable of extensive expansion in vitro; maintaining a remarkably consistent molecular profile independent of age and gender. MIAMI cells have the potential to generate mature cells derived from all three embryonic germ layers, as evidence of their primitive developmental state and broad differentiation potential (D'Ippolito, et al. 1999). The expansion of MIAMI cells ex vivo is done under low-oxygen tension (3% pO₂) in order to maintain the self-renewal capacity of the cells in an undifferentiated state (D'Ippolito, et al. 2006).

**Neurogenic Potential of MIAMI cells**

MIAMI cells maintain basal expression levels of several neuroectodermal lineage-specific genes (ntrk3, cnfr, bmpr, nse, pou4f1 transcription factor) during ex vivo expansion (D'Ippolito, et al. 2004). The neuronal differentiation of cadaveric vertebral body isolated MIAMI cells is done at low cell density on fibronectin-coated dishes using a three step cytokine-dependent protocol, under normoxic oxygen tension (21% pO₂). Neuronal specification (Step #1) exposes the cells to fibroblast growth factor 2 (bFGF) for 24 hours, in the presence of 3% fetal bovine serum (FBS). bFGF is known to strongly stimulate cell division and neuronal phenotype differentiation (Craig, et al. 1996, Kennea and Mehmet 2002, Kuhn, et al. 1997). After the end of Neuronal specification (Step #1), there is an up-regulation of nestin, neurotrophin tyrosine kinase 3 (NTRK3), neuron
specific enolase (NSE), and glial fibrillary acidic protein (GFAP), together with the appearance of a bipolar morphology consistent with neuronal precursors. MIAMI cells express basal levels of NTRK3, which is unregulated after Step #1, suggesting that MIAMI cells may respond to neurophin-3 in a ligand-dependent fashion. In addition, MIAMI cells do not express the other neurotrophin receptors; NTRK1, NTRK2 or p75NTR at the protein level under expansion conditions or after Step #1. Neuronal commitment (Step #2) was induced by exposing the cells to neurotrophin-3 (NT-3) for 48 hours, in serum free media. NT-3 signals through NTRK3 and is hypothesized to be responsible for the up-regulation of β-III Tubulin (TuJ1), NTRK1, and NTRK2, as seen after the completion of Step #2 (Tatard, et al. 2007). A stronger nestin expression was also observed along with the elongation of neurite-like processes after Step #2. Final neuronal differentiation (Step #3), is induced by treatment of the cells for 3-7 days with NT-3, BDNF, and NGF, which signal through NTRK3, NTRK2, and NTRK1 respectively, in media containing 0.5-1.0% FBS. After the total 6-10 day neuronal differentiation procedure, vertebral body isolated MIAMI cells express neuronal markers NeuN, NF-L, NF-M, NF160, and TuJ1, and show elongation of neurite-like out-growths (D'Ippolito, et al. 2004, Tatard, et al. 2007).

MIAMI cells can also be induced to a dopaminergic phenotype using the same neurotrophin-directed differentiation as described above, but with the addition of sonic hedgehog (SHH), fibroblast growth factor 8 (FGF8), and retinoic acid (RA) during Step #2. The inclusion of these three factors causes the activation of genes found in the SHH pathway (gli and ptch), and genes corresponding with a dopaminergic phenotype (nurr1 and tyrosine hydroxylase). Tyrosine hydroxylase is the rate-limiting enzyme involved in
dopamine synthesis. The MIAMI cell-derived neuronal cells also display a membrane potential within the range of -45 to -60mV, a capacitance in the order of 5-8 pF, and inward and outward currents upon depolarization which are all similar to those observed in voltage-gated ion channels found in mature neurons (Tatard, et al. 2007). Unfortunately action potentials were not observed, leading us to hypothesize that MIAMI cell-derived neuronal cells are more characteristic of immature neurons rather than of a mature neuronal phenotype.

**Scheme of Neuronal Differentiation of MIAMI cells:**

**Step 1: Specification**

MIAMI cell + bFGF (24 hr) \(\rightarrow\) Neuronal precursor

**Step 2: Commitment**

Neural precursor + NT-3 (2 days) \(\rightarrow\) Committed neuronal progenitor

**Step 3: Differentiation**

Committed neuronal progenitor + NT-3/NGF/BDNF (4-6 days) \(\rightarrow\) Neuronal cell

**Neurotrophin Signaling During MIAMI Cell Neuronal Differentiation**

Neuronal differentiation of MIAMI cells is dependent on NT-3. Omission of NT-3 results in a decrease in the number of viable cells by more than two-fold, a 50% decrease in the expression of TuJ1, and a significant loss of the resting membrane potential (Tatard, et al. 2007). This dependence on NT-3, underscores the importance of
understanding the molecular mechanism(s) by which NT-3 induces MIAMI cell neuronal differentiation.

NT-3 treatment of cells in Step #2 takes advantage of the basal and up-regulated levels of NTRK3, the receptor for NT-3. We hypothesize that NT-3 signals through NTRK3 in Step #2 to induce the observed up-regulation of the other two neurotrophin receptors: NTRK1 and NTRK2, along with the expression of nestin, TuJ1, and the elongation of neurite-like cellular processes. Step #3 then incorporates all three neurotrophin factors: NT-3, BDNF, and NGF, which have the highest binding affinities for NTRK3 (TrkC), NTRK2 (TrkB), and NTRK1 (TrkA), respectively, in order to further activate neurotrophin-directed neuronal differentiation (Tatard, et al. 2007).

**Neurotrophin Signaling**

Neurotrophins are comprised of a series of low-molecular weight proteins which act through neurotrophin tyrosine receptor kinases (NTRKs) in a retrograde mechanistic manner to promote cell growth and survival. There are four neurotrophins characterized in mammals. Brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), neurotrophin-3 (NT-3), and neurotrophin (NT-4) are derived from evolutionarily conserved genes with similar sequence and protein structure (Hallbook 1999). These neurotrophins directly bind and dimerize NTRKs, causing autophosphorylation of tyrosine residues found in their cytoplasmic protein tyrosine kinase (PTK) domains, leading to activation of downstream signaling cascades (Zweifel, et al. 2005). All of the neurotrophin (NT) ligands have distinct receptor specificities. NGF is specific for NTRK1 (TrkA), BDNF and NT-4 (avian) are specific for NTRK2 (TrkB), and NT-3 is
specific for NTRK3 (TrkC). Another neurotrophin binding receptor, p75NTR, has no sequence similarities with the NTRKs and is a member of the TNF receptor superfamily. p75NTR can bind all of the NTs with a similar low affinity (nM) or it can heterodimerize with the NTRKs changing their binding affinity or specificity for NTs (Rodriguez-Tebar, et al. 1991). Expression of p75NTR also increases the binding affinity of NGF for NTRK1. Additionally, the NTs have been found in some cases to bind NTRKs other than their primary NTRK receptor. NTRK2 has the highest affinity for BDNF, and a low affinity for NT-3/4 when over-expressed in 293T cells in vitro (15-20 fold decrease in binding affinity) (Urfer, et al. 1995). Contrary to this finding, NT-3 was found to only bind its primary receptor, NTRK3, and not NTRK2 in the vestibular and auditory systems of mice (Stenqvist, et al. 2005). In the presence of p75NTR, NTRK2 was also found to have an increased binding specificity for BDNF. During development p75NTR and the NTRKs can be expressed alone or in combination. In vitro and in vivo evidence also shows that NT activation of p75NTR alone can cause neuronal death via NFκB-induced apoptosis in the developing PNS and CNS (Casademunt, et al. 1999). Neurotrophin signaling through these receptors activates signaling cascades which control the switch between survival and differentiation in the developing nervous system, or programmed cell death via apoptosis. In the mature brain, neurotrophin signaling is also important in maintaining axon growth, dendrite pruning, proper innervations throughout the brain and the long term survival of neurons (Huang and Reichardt 2001).

**Neurotrophin Tyrosine Kinase Receptor 3**

NTRK3 is the first neurotrophin receptor expressed during early development in the neural tube and neural plate, suggesting its role in early development as well as
during neurogenesis, neuron maturation and survival (Bernd 2008). The human ntrk3 gene also has 98% sequence homology compared to rat and porcine, in addition to a highly conserved variant of NTRK3 found in invertebrate snails (LTRK) suggesting evolutionary conservation between vertebrates and invertebrates (van Kesteren and Geraerts 1998). In addition to being evolutionarily conserved there are four known splice variants of NTRK3 within humans and two of the major variants are: 1) full length NTRK3 (FL-NTRK3) which contains an active intracellular PTK domain (90-gp120/145kDa) and, 2) a truncated NTRK3 (TKd-NTRK3) which, due to splice variance and a translational frameshift, has a truncated inactive intracellular PTK domain (70-90/100kDa) (Beltaifa, et al. 2005, Elkabes, et al. 1995, McGregor, et al. 1994, Menn, et al. 1998, Quartu, et al. 2003, Shelton, et al. 1995, Tsoufas, et al. 1996). Studies of the human prefrontal cortex and cerebella tissues in embryos, pre-term newborns, postnatal infants, adolescents and adults have revealed the presence of 80kDa and 50kDa TKd-NTRK3 isoforms (Beltaifa, et al. 2005, Quartu, et al. 2003). Interestingly, NTRK2 also has various truncated isoforms due to alternative splicing. MIAMI cells only express basal levels of FL-NTRK3 and TKd-NTRK3, and the expression of NTRK2, NTRK1 and p75NTR are not detected during ex vivo long-term expansion and maintenance.

The extracellular domain of NTRK3 is made up of three leucine rich repeats and two IgG-like C2 domains which correspond to the binding site of NT-3 (Ultsch, et al. 1999, Urfer, et al. 1995). The intracellular domain contains a protein tyrosine kinase (PTK) domain (McInnes and Sykes 1997). Upon NT-3 ligand binding, NTRK3 homodimerizes, which initiates autophosphorylation of the PTK domains, inducing downstream activation of various intracellular signaling cascades. There are two main
signaling pathways which are activated via NT-3 mediated NTRK3 signaling that are important in early neuronal differentiation of neural stem cells. The Ras-Erk1/2 MAP kinase pathway mediates neuronal differentiation, while the PI3K-Akt pathway is involved in the survival of pre- and post-mitotic neuronal precursors and mature neurons (Bibel and Barde 2000, Kaplan and Miller 2000, Lim, et al. 2007).

The protein tyrosine kinase deficient (TKd-NTRK3) isoforms were classically thought to be dominant negative forms of NTRK3 involved in the quenching of NT-3 mediated signaling, as a function of NT-3 sequestering or preventing autocatalysis due to TKd-NTRK3/NTRK3 heterodimerization during neurogenesis (Biffo, et al. 1995, Palko, et al. 1999, Tsoufas, et al. 1993, Tsoufas, et al. 1996). It is now thought that TKd-NTRK3 may be involved in neuronal differentiation and plasticity (Hapner, et al. 1998, Menn, et al. 1998). TKd-NTRK3 has also been shown to maintain the neural differentiation potential of mouse derived neural progenitor cell line MB-G by maintaining the expression the pro-neural genes: nestin, TuJ1, MAP2, and NF-L (Matuszyk, et al. 2003). In addition TKd-NTRK3 was found to activate the Arf6-Rac1 pathway via the scaffold protein tamalin in an NT-3 ligand-dependent event in HEK293 cells (Esteban, et al. 2006). These studies provide evidence that TKd-NTRK3 may signal through distinctive signaling pathways other than the classical Mek-Erk, PI3K-Akt pathways activated via NT-3/FL-NTRK3 signaling.

A number of studies have defined the role of NTRK3 in mouse central and peripheral nervous system (CNS / PNS) development. Moreover, in vivo studies conducted in mice have also shown that the overexpression of NTRK3 in the CNS increased survival of developing hippocampal neurons, as well as increased the synaptic
plasticity within the hippocampus (Sahun, et al. 2007). During development of the mouse PNS, expression of FL-NTRK3 and TKd-NTRK3 is restricted to neurons in the trigeminal ganglion (TG) and increases throughout development (Wyatt, et al. 1999). In \textit{nt3}+/- (or \textit{ntrk3}+/-) gene knock-out embryos, there is a decrease in NTRK3 and TKd-NTRK3 expression within the trigeminal ganglion, and in NT3-/- homozygous knock-out embryos there is also a marked decrease in the overall number of neurons within the TG. This shows that neural development of the TG requires both isoforms of NTRK3, and that NTRK3 expression is also regulated \textit{in vivo} by physiological levels of its own ligand NT-3 (Wyatt, et al. 1999). Overexpression of NTRK3 in transgenic mice showed increased catecholaminergic, tyrosine hydroxylase positive neurons within the CNS, which lead to an increased anxiety-like behavior and enhanced panic reaction (Dierssen, et al. 2006). Further characterization of these findings suggested that the symptoms are similar to panic disorders, characteristically found in humans (Amador-Arjona, et al. 2010). Finally it has also been suggested that in NTRK3 null mutant mice, NT-3 may signal through receptors other than NTRK3 during neuronal development and cardiogenesis (Tessarollo, et al. 1997).

During human development, NTRK receptors are expressed and co-localize with their ligands within the human cerebellum from prenatal to adult age, suggesting NTRKs participate not only in development and differentiation, but also in maintenance of the human cerebellum (Quartu, et al. 2003). One study found that FL-NTRK3 (150kDa) and TKd-NTRK3 (50kDa) were expressed in the prefrontal cortex during development and maturation at low and relatively abundant levels, respectively. TKd-NTRK3 levels increased early in development up through adolescence, and then both FL-NTRK3 and
TKd-NTRK3 were expressed at similar levels during post-natal life, with a subsequent decline during aging. Taken together these results suggest that FL-NTRK3 is required for the early proliferation of neural precursors, and then as TKd-NTRK3 increases during development it is responsible for increased neural differentiation toward a post-mitotic cell, and away from proliferating neural precursor cells (Beltaifa, et al. 2005).

TKd-NTRK3 has also been found to play a role in a variety of cancers. In neuroblastoma neural precursor-like cells, down-regulation of TKd-NTRK3 via endogenous miRNA increased cell proliferation, yet in medulloblastomas, the presence of NTRK3 isoforms resulted in a more favorable survival rate due to increased NTRK3-MAP/Erk5 kinase activation-induced apoptosis. These results indicates that NTRK3 isoforms in cancer induce a variety of effects and that TKd-NTRK3 may selectively increase the proliferation of neural precursor like cells (Segal et al., 1994; Sturla et al., 2005; Laneve et al., 2007). In contrast to these findings, a study involving avian neural crest cells, which express both FL-NTRK3 and TKd-NTRK3, found that TKd-NTRK3 in conjunction with p75NTR was significantly more effective than FL-NTRK3 at promoting neural differentiation but had no effect on cell proliferation. Whereas avian NTRK3 was found to be involved in both proliferation and differentiation of neural crest cells (Sharon et al., 1998).

Both NTRK3 isoforms (FL- & TKd-NTRK3) have been found in the developing central and peripheral nervous systems, have high sequence homology among mammals, signal through putative distinct signaling cascades (TKd-NTRK3), and are both expressed at the mRNA and protein levels in human bone marrow-derived MIAMI cells.
This raises the question of the role of TKd-NTRK3 and FL-NTRK3 during NT-3 mediated neuronal commitment of MIAMI cells.

**Role of Rac1 During Neural Development**

As stated above, TKd-NTRK3 reportedly has the potential to regulate neural differentiation in a mouse neural precursor cell line, as well as activation of the Arf6-Rac1 pathway in HEK293 cells. Hence the question is raised as to whether Rac1, in combination with the high expression level of TKd-NTRK3 in MIAMI cells, could play a role in the regulation of NT-3 mediated neuronal commitment. The small GTP-binding protein, Rac1 is known to have a high activation/turn-over rate via guanine nucleotide exchange factor-mediated activation by Arf6. The known downstream target of Rac1-GTP is PAK1, a serine/threonine p21-activated kinase, known to be involved in regulating cell motility and morphology. *In vitro*, in human fetal subventricular zone (SVZ) derived neurons (review: (Khodosevich and Monyer 2010)), and in PC12 and neuroblastoma cell lines Rac1 was found to control neurite formation, growth, and extension (Jeon, et al. 2010, Picard, et al. 2009). In cultured rat primary cortical and hippocampal neurons, as well as in the rat brain, Rac1 was found to regulate glutamate synapse formation as well as hippocampal dendritic spine formation respectively, via an miRNA and reactive oxygen species regulatory/activation mechanism (Hayashi-Takagi, et al. 2010, Impey, et al. 2010, Tsai, et al. 2009). *In vivo* studies in various mouse Rac1 knock-out models have shown that Rac1 is required for cortical projection (Kassai, et al. 2008), ventral telencephalic neuron formation (Chen, et al. 2007) and their migration across the midline commissure during development (Kassai, et al. 2008), proper axon
outgrowth and guidance (Briancon-Marjollet, et al. 2008), and myelin sheath formation in oligodendrocytes (Thurnherr, et al. 2006).

Rac1 has two major isoforms, Rac1a and Rac1b. The majority of research involving Rac1 has focused on knocking down the rac1 gene, which does not distinguish between the two Rac1 isoforms in the observed results as described above. Rac1a which undergoes normal G-protein GEF / GAP regulation is known to be involved in cytoskeletal organization and arrangement. In the context of neurons and neurite outgrowth, Rac1a is known to activate PAK1 leading to growth cone localized MEK/ERK activation and inhibition of stathmin/Op18, (microtubule destabilization proteins), allowing for microtubule assembly and extension (review (Pullikuth and Catling 2007)). Rac1b is the constitutively active isoform of Rac1. It contains a 19 amino acid insert near its binding domain/pocket that prevents the hydrolysis of GTP to GDP (Fiegen, et al. 2004), causing Rac1b to always be in an active form as Rac1b-GTP. Rac1b is known to bind PAK1 in some cases, as well as binding to other protein partners unique to Rac1b-GTP. These molecular studies, in addition to the above mentioned in vitro and in vivo studies, suggest a role for Rac1, either Rac1a or Rac1b, during normal CNS and PNS development. The known functions of Rac1 during neural development also raise the questions, 1.) Do MIAMI cells express Rac1, and 2.) Does NT-3 stimulation during Neuronal Commitment (Step #2) activate Rac1 to further promote the up-regulation of pro-neural genes?

**Rationale**

Emerging trends in age-, trauma-, and disease-related cell-therapy based tissue repair have focused on the renewable autologous or xenographic source of adult stem
cells found throughout the somatic tissues of the human body. Due to their immunomodulatory properties as well as their potential to differentiate into mature somatic tissues, adult stem cells, such as bone marrow-derived MSCs, provide a source of immature cells which can be expanded \textit{ex vivo} for utilization in cell-therapy based treatments (Uccelli, \textit{et al.} 2008). MIAMI cells are a homogeneous sub-population of bone marrow-derived hMSCs which maintain their self-renewal potential during \textit{ex vivo} expansion, in addition to efficiently undergoing trans-germinal differentiation into neuron-like cells \textit{in vitro} (Tatard, \textit{et al.} 2007). Additionally MIAMI cells are not burdened by ethical restrictions or problems such as partial vs. full epigenetic reprogramming, tumorigenicity potential, and the controversial clinical functionality associated with embryonic stem cells (ESC) and induced pluripotent stem (iPS) cells (Pozzobon, \textit{et al.} 2009, Smith, \textit{et al.} 2009).

Even though MSCs have the potential to be used in cell-therapy based approaches for the repair of age-, disease-, or trauma-related damage to the nervous system, the molecular mechanisms by which they undergo trans-germinal neuronal differentiation have not been fully characterized. Therefore the work described herein was formulated to determine the molecular mechanisms by which MIAMI cells undergo NT-3 dependent neuronal differentiation, in anticipation of their applied use in cell-therapy based repair of nervous system tissue. In addition, characterizing the similarities and / or differences between the molecular mechanisms of the trans-germinal neuronal differentiation of MSCs into neuron-like cells, compared with the known mechanism of neural development may provide insights into novel strategies for cell-based therapies.
As stated above, NT-3 signals through NTRK3, which is the first neurotrophin tyrosine receptor kinase expressed in the neural tube and plate during early development. MIAMI cells express both FL-NTRK3 and TKd-NTRK3 and undergo a three step NT-3 dependent neuronal differentiation protocol: specification, commitment, and differentiation. The neuronal differentiation of MIAMI cells is an NT-3 dependent event (Tatard, et al. 2007), therefore we hypothesized that NT-3 signaling through NTRK3 mediates this process, specifically during NT-3 mediated neuronal commitment of MIAMI cells. Moreover, we further hypothesize that NT-3 signaling via the NTRK3 receptor leads to the subsequent activation of the Ras-Erk1/2 pathway and the Arf6-Rac1 pathway, leading to the downstream activation of pro-neural genes and neuronal commitment of human MIAMI cells in vitro.
Figure #1.1: Schematic representation of the NTRK3 / NT-3 mediated signaling pathways involved in the upregulation of pro-neuronal genes during neuronal differentiation. FL-NTRK3 has a functional protein tyrosine (PTK) kinase intracellular domain which upon NT-3 stimulation undergoes homodimerization, autophosphorylation, and activation of the downstream Mek-Erk signaling cascade. TKd-NTRK3 has a truncated intracellular domain comprised of exons 13b/14b which do not have a known PTK function, but have been shown to recruit Tamalin leading to the activation of downstream Arf6-Rac1 via NT-3 stimulation.
**Specific Aims**

**AIM 1:** Establish the role of the Ras-Erk1/2 MAP kinase pathway on the NT-3 mediated activation of NTRK3 and TKd-NTRK3 during neuronal commitment (Step #2) of MIAMI cells *in vitro*.

**AIM 2:** Establish the role of the Arf6-Rac1 pathway on the NT-3 mediated activation of NTRK3 and TKd-NTRK3 during neuronal commitment (Step #2) of MIAMI cells *in vitro*.

**AIM 3:** Characterize the protein interactions and downstream signaling pathway(s) of TKd-NTRK3 involved in the neuronal commitment (Step #2) of MIAMI cells *in vitro*.

**Additional Supporting Data:** Establish the effect of bFGF or bFGF/EGF pretreatment on neurotrophin-3 signaling during neuronal commitment (Step #2) of MIAMI cells *in vitro*. 
MIAMI Cell Isolation

Whole bone marrow was obtained from the iliac crest of a 20 year old living male donor (Lonza Walkersville, MD; MIAMI #3515). As previously described (D'Ippolito, et al. 2004), isolated whole bone marrow cells were plated at a constant density of $1 \times 10^5$ cells/cm² in DMEM-low glucose media, containing 3% FBS (Hyclone Waltham, MA, Lot#30039), 20mM ascorbic acid (Fluka/Sigma St. Louis, MO, #49752), an essential fatty acid mixture (Sigma; 12.9nM arachidonic acid, (#A9673), 1.12µM cholesterol (#C3045), 290nM DL-alpha tocopherol-acetate (#T3376), 85.9nM myristic acid (#M3128), 69.4nM oleic acid (#01383), 76.5nM palmitic acid (#P5585), 77.1nM palmitoleic acid (P9417) and 68.9nM stearic acid (#S4751) (modified from (Ludwig, et al. 2006)) and antibiotics (100 U/mL penicillin, 0.1 mg/mL streptomycin) (Gibco Carlsbad, CA, #15140) on 10ng/ml fibronectin (Sigma, MO, #F2518) coated flasks (Nunclone Rochester, NY). Whole bone marrow cells, containing adherent and non-adherent cells, were incubated at 37°C under hypoxic conditions (3% O₂, 5% CO₂ and 92% N₂). Seven days later, half of the culture medium was replaced. Fourteen days after the initial plating, the non-adherent cells were removed. Pooled colonies of adherent cells were rinsed with PBS and plated at low density for expansion (100 cells/cm²) in 75cm² fibronectin coated flasks.
**MIAMI Cell Expansion and Culture Conditions**

MIAMI cells were grown in expansion media consisting of DMEM-low glucose in low oxygen conditions (3% O₂, 5% CO₂ and 92% N₂) as described above. Media was changed every 2-3 days and the cells were detached and pelleted using trypsin (Gibco, #25300) upon reaching ~60% confluency. Pelleted cells were resuspended in media and plated in 10ng/ml fibronectin (Sigma, MO, #F2518) coated flasks (Nunclon, Rochester, NY) at 100cells/cm². Prior to RNA isolation, adherent cells were rinsed twice with PBS.

**MIAMI Cell Induced Neuronal Commitment**

Expanded MIAMI cells were re-seeded at 3,000 cells/cm² for 24 hours at low oxygen (3% pO₂) prior to transfer to 21% pO₂ for induction of the neuronal differentiation program as described by (Tatard, et al. 2007). Neuronal specification (Step#1) was induced by treating the cells with DMEM-HG supplemented with 3% FBS, antibiotics, essential fatty acid solution and 10ng/ml bFGF (Peprotech, Rocky Hill, NJ: #AF-100-18B) for 24 hours. Neuronal commitment (Step#2) consists of treating the cells with DMEM-HG supplemented with antibiotics, essential fatty acid solution and 30ng/ml NT-3 (Peprotech: #AF-450-03) for 48 hours, under serum free conditions. Prior to Step #2 induction, the cells were washed three times with PBS and then incubated in serum free media for 12-24 hours to induce G0/G1 synchronization.
**MIAMI EGF and bFGF Pretreatment**

Epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) treatment of MIAMI cells was performed using 20, 50 or 100ng/mL each of EGF (#AF-100-15) and bFGF (Peprotech, #AF-100-18B) alone or in combination. After 5-days, the pre-treated cells were re-seeded, followed by a second 5 day pretreatment period. Pre-treated cells were grown in expansion media under expansion conditions (3% O₂, 5% CO₂ and 92% N₂). Media was changed every 2-3 days and the cells were removed using trypsin (Gibco, #25300) upon reaching ~60% confluency followed by re-seeding for induction of the neuronal differentiation program.

**Total RNA Sample Preparation and cDNA Synthesis**

MIAMI cells were detached (Trypsin) and centrifuged to form a cell pellet. RNA was isolated using the RNAqueous®-4PCR kit (Ambion, #AM1914) according to manufacturer’s directions. Total RNA was quantified on the Nanodrop ND-1000 Spectrophotometer (Nanodrop, Wilmington, DE). Reverse transcription of 2µg total RNA to cDNA was done with random hexamer primers using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, #4368814). The cDNA was diluted 1:20 with Nuclease-Free Water (Gibco#10977-015) to a final cDNA concentration of 5ng/µl, aliquoted, and stored at -20°C until used. Only RNA with a 260/280 ratio between 1.9-2.0 was used for PCR analysis.

**Quantitative real-time RT-PCR (RT-qPCR)**

Quantitative real-time PCR (RT-qPCR) was done using 10µl of 1:20 diluted cDNA (50ng) on the Mx3005P Multiplex Quantitative PCR System (Stratagene/Agilent
Technologies, Wilmington, DE) using RT-qPCR SYBR GREEN Reagents (Agilent Technologies, Brilliant® II SYBR® Green QPCR Master Mix) with ROX reference dye. Forward and reverse primer pairs were reconstituted in Nuclease-Free Water (Gibco, #10977-015). A 2µM stock solution containing both forward and reverse primer pairs was mixed and stored at -20°C (Table #1). A final concentration of 160nM forward and reverse primer pairs was used for each RT-PCR reaction. The cycling conditions were as follows: an initial 95°C for 10 minutes, followed by 40 cycles of 95°C for 30 sec, 58°C for 30 sec, 72°C for 15 sec. MxPro-Mx3005P v4.10 software was used to determine the CP for each amplification reaction. Results were exported to Microsoft Excel for analysis.

Normalization and Analysis of RT-qPCR Data

All of the corresponding RT-qPCR data were analyzed using the ∆∆CP method (Pfaffl 2001) and normalized against one negative control, and two-three reference genes (housekeeping genes) (Figure #2.2).

\[
\text{Fold Difference Ratio} = \frac{(E_{\text{target}})^{\Delta CP(\text{target gene}) (\text{Control-Sample})}}{(E_{\text{reference}})^{\Delta CP(\text{reference gene}) (\text{Control-Sample})}}
\]

The crossing point (CP) is defined as the point at which the fluorescence rises appreciably above the background fluorescence. The ‘Fit Point Method’ was used by the Mx3005P software to determine the CP for each reaction. The control sample is set to the value of “1” in all cases and error bars in the respective figures are displayed as standard deviation. The determination of each gene’s primer pair efficiency (E) for real-
time PCR was calculated using this equation: $E=10^{(-1/m)}$ (Rasmussen 2001). The slope ($m$) was calculated by plotting the cycle number crossing point (CP) calculated during the exponential phase of the amplification plot (PxPro-Mx3005P v4.10 software) against the total cDNA concentration. Concentrations of cDNA ranged from 50-1ng per reaction. The percent efficiency ($%E$) was also calculated: $%E=(E-1)*100$. Eight genes were tested as putative reference genes for normalization: [beta-actin ($ACTB$, NM_001101), beta-2-microglobulin ($B2M$, NM_004048), eukaryotic translational elongation factor 1 alpha ($EF1\alpha$, NM_001402), glyceraldehyde-3-phosphate dehydrogenase ($GAPDH$, NM_002046), hypoxanthine phosphoribosyltransferase 1 ($HPRT1$, NM_000194), ribosomal protein L13a ($RPL13a$, NM_01242), tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide variant 1 & 2 ($YWHAZ$, NM_003406 & NM_145690), and ubiquitin C ($UBC$, NM_021009)]. $EF1\alpha$, $RPL13a$, and $YWHAZ$ were found to be the three most stable “reliable” housekeeping genes suitable for the normalization of RT-qPCR data in hMSCs (Submitted: In Review: (Curtis KM 2010).

![Gene Stability: Expansion Conditions](image)

**Figure #2.2:** Determination of the gene stability of 8 potential reference “housekeeping” genes during the expansion of MIAMI cells at 3% pO$_2$. The gene stability was determined by comparing the average CP standard deviations for each gene between experiments. The average CP standard deviation was calculated by dividing the summation of the CP standard deviations of 8 independent experiments (2-3 data points per experiment) by N-1. N=8 independent experiments.
Primer Pairs used for RT-qPCR analysis

Primer pairs were ordered through Operon (Eurofins MWG Operon, Huntsville, AL) and were prepared salt-free and HPLC purified. All primer pairs were validated for amplicon size using gel-electrophoresis prior to use for RT-qPCR analysis. Isoform specific primer pairs were constructed by comparing the FASTA mRNA sequences for regions of similarity (>50bp). Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome) was used to construct primer pair sequences.

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*(Vandesompele, et al. 2002)*

**MIAMI Cell Transfection Conditions**

Transfection of MIAMI cells with siRNA or plasmids was performed using a Digital Bio/BTX Microporator with 10 or 100µl pipette tips (Digital Bio: #MPK-1096 or #MPK-10096, respectively). After optimization the final electroporation program
consisted of 1400V Pulse Voltage, 10ms Pulse Width with a total of 3 Pulses. Initial optimization was done using 10 µl tips for electroporation with 1-100µM siRNA for 1-5x10^5 cells respectively in a total volume of 10 µl or 1-5x10^6. SiGLO (Dharmacon, Lafayette, CO, D-001600-01) was used as a visual determinant of siRNA incorporation into MIAMI cells. Using a higher siRNA concentration (50µM) with a higher number of cells (5x10^5) in a total volume of 10ul produced ~30-40% cell death. Cells which adhered appeared healthy and did not have variations in normal housekeeping gene mRNA levels. Approximately 95% of all post transfection adherent cells were positive for siGLO. The use of larger (100 µl) transfection pipette tips which hold up to 5x10^6 cells in a total volume of 100 µl reduced cell death to ~5-10% with equal siGLO transfection efficiency (post-transfection adherent cells). Transfection solution (10 µl–100 µl) was tested to determine % efficiency and % cell death. Digital Bio/BTX Solution R was compared with STAUDT Transfection Reagents (modified from (Staudt, et al. 2007)). STAUDT transfection reagent reduced death by ~10%, and increased efficiency ~10-15%, and increased the visual intensity of siGLO per cell (Figure #2.3). STAUDT transfection reagent were prepared with fresh ATP, aliquoted and stored at -20°C (modified from Figure #2.3: MIAMI cells were transfected with siGLO using Solution R (Top Panel) or STAUDT transfection solution (Bottom Panel) for 7 days. siGLO (green) is shown here localized to the nucleus.
SiRNA was ordered from Dharmacon and used at a final concentration of 10-20µM for Rac1b and SMART pool NTRK3 siRNA (0.25-0.33fmol siRNA/cell) or from 10-50µM for FL- and TKd-NTRK3 either alone or in combination. Custom siRNA’s were designed using Dharmacon siDESIGN Center (http://www.dharmacon.com/designcenter/designcenterpage.aspx). Twenty-four hours post-transfection with siRNA or plasmid, MIAMI cells were washed with PBS and expanded with normal MIAMI expansion media.

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<th>% knock-down</th>
<th>Target</th>
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<td>TKd-NTRK3</td>
<td>AATAAGCCTTCCCCGACA</td>
<td>0% non-specific</td>
<td>Exon-exon</td>
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<td>ON-TARGET plus Control Pool</td>
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<td>*(Matos and Jordan 2008)</td>
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Table#2.2: siRNA sequences are listed. All siRNA was stored at 100µM stock concentration. % knock-down data represents N=3 independent experiments (1-2 samples per experiment).
**siRNA Transfection Program for MIAMI Cell Neuronal Commitment**

MIAMI cells were expanded as adherent cell cultures at 3% pO₂ followed by in suspension electroporation (Digital Bio/BTX Microporator) induced siRNA transfection. After transfection MIAMI cells were re-seeded at 3,000 cells/cm². Transfected MIAMI cells were incubated overnight at 3% pO₂, followed by 24 hours at 21% pO₂. Neuronal specification using bFGF (10ng/ml) was induced for 24 hours followed by overnight serum starvation (serum free media). If chemical inhibitors were used they were added 1 hour prior to the addition of NT-3 (30ng/ml) during Step#2 (neuronal commitment).

Protein lysates were collected at early time points for western blot analysis (signal transduction) and after 48 hours of NT-3 stimulation for RT-qPCR analysis (gene expression analysis).

**Over-expression of FL-NTRK3 and TKd-NTRK3**

The PrecisionShuttle Vector (#PS10001: pCM6-ENTRY) from OriGene (www.origene.com) was used for the overexpression of the ORF of FL-NTRK3 (#RC213333) or TKd-NTRK3 (#RC203781) under the control of the CMV promoter. The carboxyl terminus (internal domain) has a Myc or FLAG tag for western blot detection, immunoprecipitation, immunoflourescent detection, and / or purification via anti-Myc (#TA100010) or anti-FLAG/DKK (#TA100011) mouse monoclonal antibodies.
TurboFectin 8.0 Transfection Reagent (#TF81001) or Microporation (as described above) was used to transfect the MIAMI cells with the vectors. Stable transfectants were selected for using a high dose Puromycin (200µg/ml, G418 analogue) or a lower dose of 50-100µg/ml for maintenance. Restriction enzyme digests of the pCMV6 overexpression plasmids were used to check the orientation of the ORF (Mlu1 cleaves once in the multiple cloning region of the pCMV6 plasmid; Sca1 cuts once within the ORF of both FL-NTRK3 and TKd-NTRK3). Double digestion of FL-NTRK3 and TKd-NTRK3 produced bands of approximately 929 and 290bp, respectively, indicating that both plasmids have ORF in the correct orientation to the CMV promoter (Figure #2.5).

Optimization of the selection conditions was performed using a puromycin analog, G418 (Sigma #A1720), as well as the transfection reagent, Turbofectin 8.0 (Origene #TF81001). G418 concentrations greater then 100ng/ml reduced and or prevented cell
growth (Figure #2.6A). For stable transfection selection, 500ng/ml G418 killed the MIAMI cells, while 200ng/ml G418 was found to allow for puromycin resistant clones to grow. Turbofectin 8.0 (0.2-0.8µl/ml) was found to have minimal impact on MIAMI cells growth (Figure #2.6B).

![Figure#2.6](image)

**Figure#2.6** Optimization of the Neomycin analog (G418) (A) and the transfection reagent Turbofectin 8.0 (Origene) (B) were performed using MIAMI cells H3515(3). Total cell counts of adherent cells were performed every 24 hours for 5 days.

**Protein Preparation and Western Blot Analysis**

Cell pellets were collected via scraping of cells in PBS and spinning down at 1,500rpm for 5-10 minutes. The cell pellets were separated into Triton X-100 soluble and insoluble portions. The cell pellets were resuspended in buffer A (500mM TrisHCl pH6.8, 50mM EGTA, 1M KCl, 10% Triton X-100), triturated, vortexed and centrifugated at 13,000G for 20 minutes to pellet the Triton X-100 insoluble pellet. Buffer A containing the supernatant Triton X-100 soluble fraction was stored at -80°C. The Triton X-100 insoluble pellets were resuspended and vortexed in buffer B (500mM TrisHCl pH6.8, 50mM EGTA, 850mM sucrose, 10% Triton X-100), centrifugated at 13,000G for 20 minutes, and then rinsed twice with buffer B. Buffer B was removed from the Triton X-100 insoluble pellet and the pellets were reconstituted in Laemmli buffer (10%SDS, 500mM TrisHCl pH6.8, 4% glycerol, 1% β-ME, bromophenol blue), boiled for 5 minutes
and then stored at -80°C until used. Directly prior to collecting protein lysates, 5µl of Na₃VO₄ (100mM), 100µl of NaF (500mM) were added to 1ml of TritonX-100 lysis buffer. NP40 lysis buffer contained 50mM Tris, 1.0% NP40, 150mM NaCl, 2mM EGTA, 2mM EDTA, 50mM NaF, 0.1mM NaVO₄, with a final pH of 8.0. Protease inhibitor cocktail (Sigma, #P8340) was added (2µl / 1ml lysis buffer) to NP40 or TritonX-100 lysis buffer directly before protein extracts were collected. The protein concentration was determined using the BCA protein assay (Pierce, Rockford, IL). Antibodies used are listed in Table #2.3. Blocking solution consisted of either 2-5% BSA or milk in TBST (1-2.5% Tween 20).

Positive controls for p-cRaf1(ser621) were used in order to determine antibody specificity. These controls included HeLa cells (treated with 100ng/ml EGF, 15min), as well as whole cell lysates from HeLa, THP-1, and NIH3T3 cells (Figure #2.7).

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<td>Rockland (#600-401-993)</td>
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### Table #2.3

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<td>Santa Cruz (#sc-8316)</td>
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<td>Anti-Myc</td>
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### FACS Analysis & Propidium Iodide staining

MIAMI cells were detached (Trypsin), pelleted and rinsed twice with cold PBS prior to resuspension in 200µl PBS containing 2mM EDTA (PBS/EDTA). Drop-wise, 600µl of 70% cold ethanol (4°C) was added to the cells while vortexing at low speed. All ethanol-fixed cells were stored at 4°C until used. Directly prior to propidium iodide FACS analysis, the fixed cells were pelleted and resuspended in 0.5ml PBS/EDTA containing 10µl of RNase A (10mg/ml) and 10µl of propidium iodide (1mg/ml) followed by incubation at 37°C for 30 minutes. For Rac1b FACS analysis, anti-Rac1b (Millipore, Billerica, MA, #09-271) was used at a dilution of 1:50 and incubated for 1 hour at room temperature. AlexaFlour 549 (1:1000) was used as the secondary antibody. A general IgG antibody (1:50 dilution) was used as the negative control for gating. An Accuri C6 Flow Cytometer (Accuri, Ann Arbor, MI) was used for propidium iodide detection and Rac1b FACS analysis.

### Chemical Inhibitors and Inhibitory Antibodies

Inhibitors of NTRK3, Arf-Rac1, PI3K-Akt or Rac-Erk1/2 MAP kinase signaling were administered 30-60 minutes prior to treatment with NT-3 during neuronal
commitment (Step#2). All inhibitors were aliquoted and used once per experiment. Anti-NT3 antibody was used to bind and inhibit the effect of endogenous NT-3 secreted by MIAMI cells. 500ng of anti-NT3 was added to 2mls of media in a 6-well plate for 2 days. The inhibitor K252a derived from soil fungi (*Nocardiopsis* sp.) is known to inhibit all NTRK receptors, via their protein tyrosine kinase domain, as well as other protein tyrosine kinases (Tamura, *et al.* 2006, Tapley, *et al.* 1992). U0126 inhibits both Mek1 and Mek2 which are upstream activators of Erk kinase, thereby preventing Erk activation. The Rac1 inhibitor (Calbiochem,#553502) interferes with the Rac-specific GEF (guanine exchange factors) Trio and Tiam1 which are known to activate Rac1-GDP into Rac1-GTP.

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<td>(Tamura, <em>et al.</em> 2006)</td>
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<td>Erk1/2</td>
<td>Sigma:#U120 (U0126)</td>
<td>(10 µM)</td>
<td>DMSO</td>
<td>(Brambilla, <em>et al.</em> 2002)</td>
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<td>Rac1</td>
<td>Calbiochem: #553502 (Rac1Inhibitor)</td>
<td>50-100µM (100 µM)</td>
<td>H2O</td>
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<tr>
<td>Anti-NT-3</td>
<td>Chemicon: #AB1780SP</td>
<td>500ng</td>
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<td>(Freeman and Pierce 2002)</td>
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</table>

Table #2.4: Chemical Inhibitors

**Pulse-Chase Analysis**

MIAMI cells were deprived of methionine via incubation in DMEM-LG (-methionine, -cysteine) for 30 minutes. A pulse of radio-labeled methionine was induced
for 45 minutes using 50µCi 35S-methionine (PerkinElmer, Waltham, MA, EasyTag[TM]
35S-M) in methionine-free DMEM-LG for 4x10^5 cells per 6cm² culture dish. After the
pulse, the cells were rinsed twice in PBS, once in MIAMI expansion media supplemented
with 5mM L-methionine (Chase Media) (Sigma #M-9625), and then incubated for 24
hours in the Chase Media. The day 0 control sample was harvested after the two PBS
rinses. Protein lysates were collected using NP40 lysis buffer as described in the Protein
Prep and Western Blot analysis section above. 400µG of total protein lysate was used
for immunoprecipitation. Protein A/G Plus Agarose (Santa Cruz Biotechnology, Inc.,
Santa Cruz, CA., #sc-2003) was incubated with anti-NTRK3 antibody (Cell Signaling
Technology , Boston, MA., #MAB3731) for 2 hours at room temperature, and then rinsed
thrice with PBS. Protein lysates were added to the complexed agarose beads and rotated
overnight at 4°C. Protein lysates were removed and the agarose beads were rinsed thrice
with PBS. Western blot PAGE was performed as described previously. A BioRad Gel
Dryer (Model #583) was used to dry the gel for 1hr at 60°C. The column of molecular
weight bands were cut out of the gel and rehydrated in scintillation fluid (MP
Biomedical Inc, Solon, OH.) for 30 minutes prior to reading total radiation using a
liquid scintillation analyzer (Packard 2200CA). All DPMI counts were normalized
against total protein in each lane determined by coomassie blue staining of the gel prior
to analysis. D0 was set to 100%.

Immunoprecipitation

Immunoprecipitation analyses were performed using 25-50µl of reconstituted
Protein A/G Agarose (Santa Cruz Biotechnology, Inc., sc-2003) incubated with primary
antibody (2-4µg), specific for the protein of interest, 1 hour on a rotating carousel at room
temperature. The complexed agarose-antibody mixture was pelleted (3,000g) and rinsed thrice with PBS. 200-400µg of protein lysates (either NP40 or Triton X-100 soluble lysates) were added to the agarose-antibody complex and incubated overnight on a rotating carousel at 4°C. The non-bound lysate supernatant was removed and used for total protein verification (negative control for equal loading). Immunoprecipitated proteins with the agarose-antibody complex were rinsed thrice with PBS, all supernatant was removed and then 25µl of Laemmli Loading Buffer (described previously) was added. Prior to loading onto an agarose gel for PAGE analysis, the samples was heated at 95°C for 5 minutes. Protein A/G agarose beads complexed with general IgG antibody was also used as control.

**Mass Spectrometry: Sample Preparation and Analysis**

Immunoprecipitation using Protein A/G agarose beads was used to pull-down the NTRK3 receptor using 2µg of anti-NTRK3 antibody (Santa Cruz, #sc-14025). Immunoprecipitated proteins were separated using PAGE and silver stained for visualization. For silver staining, acrylamide gels were soaked in 7% acetic acid for 7 minutes followed by two incubation in 200ml of 50% methanol for 20 minutes each. After fixing the gel was rinsed twice in H2O for 10 minutes each. The silver stain was comprised of: Solution A: 0.8g silver nitrate + 4ml H2O; Solution B: 21 ml H2O +250µl of 30% NaOH + 1.4ml of 14.8M NH4OH. Solutions A and B were mixed drop-wise with stirring, and then 76ml of H2O was added. The gel was soaked in silver staining solution for 15 minutes followed by two rinses in H2O. After silver staining the gel was developed in a solution containing 200ml H2O + 1ml (1%) acetic acid + 100µl (37%) formaldehyde, for 2-15 minutes until bands were visible.
NTRK3 molecular weight bands of interest were verified via western blot analysis. The bands of interest were excised from either a silver stained polyacrylamide gel, or PVDF Immobilon-P membrane post western blot imaging. Excised bands were rinsed to remove detergents and non-adherent protein residue using the following solutions: ten times with 1ml dH2O, twice for 30min each with 1M NaCl, five times for 30 min each with 1ml dH2O, twice for 5min each with 0.1% TFA, ten times with 1ml dH2O. The digestion buffer (40mM Ammonium Acetate, 40% Acetonitrile pH8.0) was then added to each sample and the mixture was preheated to 95°C for 5 minutes. Trypsin-Gold (20µg/ml; Trypsin Gold-MS Grade; Promega, Madison, WI., #V528A) was added to each sample and the samples were then incubated at 37°C over night, 24 hours prior to mass spectrometry analysis. Thereafter, trifluoroacetic acid was added to the mixture at a final concentration of 2% to quench the reaction, and then the sample was analyzed or frozen at -20°C until analysis. The samples were direct injected, or run through a coupled HPLC (HypersilGold C18 column) before injection into the LTQ XL Linear Ion Trap Mass Spectrometer (Thermo Scientific). 3,000-7,000 volts were used for electrospray ionization (ESI) for sample injection into the mass spectrometer. Bovine serum albumin digested with trypsin was used as a control for sample preparation, HPLC optimization, mass spectroscopy analysis, and BioWorks 3.0 software analysis. BioWorks 3.0 in conjunction with Blastp (www.ncbi/blast.gov) were used to determine the theoretical peptide fragment sequence from the MS^3 data, as well as to match the sequence with known proteins. This work was done under the guidance and training of Dr. Joe Laurino, Full Professor, Department of Chemistry, University of Tampa, Tampa, FL.
**NT-3 ELISA**

The majority of reagents were from R&D Systems (Minneapolis, MN.) human NT-3 ELISA detection kit (#DY267). For 96-well plate ELISA preparation, 100µl of diluted NT-3 capture antibody (0.5µg/ml) (R&D Systems: #840210) was added per well and incubated overnight at room temperature. Each well was rinsed thrice using 400 µl wash buffer (0.05% Tween20 in PBS, pH 7.2-7.4), followed by blotting the 96-well plate dry between each rinse. The wells were blocked using 300µl of 1% BSA in PBS (reagent diluents) for 1 hour at room temperature followed by three rinses as described above. For NT-3 detection, 100µl of sample or standard NT-3 solution was added to each well and then the plates were incubated for 2 hours at room temperature, followed by rinsing as described. 100µl of biotinylated anti-human NT-3 detection antibody (200ng/ml) (R&D Systems, #840211) diluted in reagent diluents was added to each well, followed by incubation for 2 hours at room temperature, then followed by rinsing. 100µl of Streptavidin-HRP (R&D Systems, #890803) was added to each well and incubated for 20 minutes at room temperature, followed by rinsing. 100µl of substrate solution (R&D Systems #DY999) (20 min incubation at room temperature, rinsing) followed by 50µl of stop solution (R&D Systems #DY994) were added to each well. Optical density (OD) at 450nm was determined for each well using a microplate reader. OD readings at 540 and 570nm were also used to determine background of the plastic 96-well plates. MIAMI cells were expanded, washed, and incubated in serum-free media for 24-72 hours. Conditioned media was removed and immediately added to prepared NT-3/ELISA 96-well plates for analysis. NT-3 standard curves (30-2000pg/ml) were used to calculate NT-3 (pg/ml) concentrations in the media.
**Rac1/cdc24 PAK1-PBD Pulldown Assay**

A Rac1/cdc42 activity pull-down assay was purchased from Millipore (#14-325). Expanded MIAMI cells were lysed using MLB lysis buffer (25mM HEPES, pH 7.5; 150mM NaCl; 1% Igepal CA-630; 10% glycerol; 25mM NaF; 10mM MgCl2; 1mM EDTA; 1mM Sodium Orthovanadate; 10µg/ml leupeptin; 10µg/ml aprotinin). *Lysis buffer must contain MgCl2 to prevent dissociation of the Rac1-GTP / PAK1-PBD complex. 10µg (5µl) PAK1-PBD/agarose complex was added to 1ml MLB lysate sample. The mixture was gently agitated using a rocking plateform at 4°C for 60 minutes. The pull-down agarose complex was rinsed thrice in MLB lysis buffer, resuspended in Laemmlli sample buffer (described previously) and then boiled for 5 minutes. SDS-PAGE was used to separate / characterize the pulled-down proteins. Anti-Rac1 and anti-cdc42 antibodies were used to detect all Rac1/cdc42 which was pulled down. Theoretically only GTP-bound Rac1/cdc42 should be pulled down by the PAK1-PBD, which is a known downstream binding partner of both proteins *in vivo.*

**Immunocytochemistry**

MIAMI cells were cultured on glass coverslips at least 24 hours prior to immunocytochemical analysis. Adherent cells were rinsed thrice in PBS followed by paraformaldehyde fixation (4%) at 4°C overnight. The following day fixed cells were rinsed thrice in PBS. For antibodies that detect the intracellular domain of receptors (TKd-NTRK3;Rockland/Cambrex, Charles City, IA., #600-401-993), and or are specific for intracellular proteins, cell permeabilization was done using 0.2% Triton X-100 added to the cells with subsequent incubation at room temperature (RT) for 5-10 minutes. The
fixed cells were blocked in 10% normal goat serum for 1 hour at RT. Primary antibody was then added, with subsequent incubation overnight at 4\(^\circ\)C in PBS followed by three rinses in PBS. Secondary antibodies were added for 1 hour at RT. DAPI containing mounting media was used for mounting. AlexaFlour 549 (1:500) and Alexaflour 488 (1:500) were primarily used as fluorescent secondary antibodies for imaging. TKd-NTRK3 antibody (Rockland #600-401-993) was used at a dilution of 1:50-1:200. NTRK3 antibody (R&D Systems, MAB#3731) detects all NTRK3 protein isoforms and was used at 1:100-1:500 dilution.

**Statistical Analysis**

Only data sets containing $N \geq 2-3$ independent experiments (2-3 samples per condition per experiment) were used for statistical analysis. A One-way ANOVA followed by Tukey’s post-hoc analysis or a Student’s $t$-test was used to calculate statistical significance between conditions or treatment groups using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego, CA, [www.graphpad.com](http://www.graphpad.com). All error bars represent the standard deviation between experimental samples. $p \leq 0.05 (*)$, $p \leq 0.01 (**)$, $p \leq 0.001(***)$
CHAPTER 3

NT-3 INDUCED SHORT-TERM ERK1/2 PHOSPHORYLATION LEADING TO THE UPREGULATION OF PRO-NEURONAL GENES

The aim of this study was to determine the effect of NT-3 on neuronal differentiation of MIAMI cells into neuron-like cells (Tatard, et al. 2007). Iliac crest derived MIAMI cells express only one neurotrophin receptor, NTRK3, and do not express the other neurotrophin receptors (NTRK1, NTRK2 or p75NTR) at the protein level during long-term expansion or upon induction of neuronal specification. This leads to the hypothesis that NT-3 stimulates NTRK3, followed by upregulation of pro-neuronal genes during the neuronal commitment of MIAMI cells. This chapter focuses on the role of NT-3 signaling through NTRK3 leading to the activation of Erk1/2 and the up-regulation of pro-neuronal genes. Additionally, the presence of both full length NTRK3 (FL-NTRK3) and the tyrosine kinase deficient NTRK3 (TKd-NTRK3) isoforms were validated using RT-qPCR, western blot, and mass spectroscopy analyses.

**NT-3 induced Erk1/2 phosphorylation**

To elucidate the role of neurotrophin-3 (NT-3) during the neuronal commitment (Step #2) of MIAMI cells, MIAMI cells were treated with NT-3 for different time points (5, 10, 15, 30, 45, 60 min. and 24 & 48 hrs.). It is hypothesized that NT-3 stimulates the Erk1/2 signaling pathway in MIAMI cells which is the main signaling pathway involved in the pro-neuronal biological effect of NT-3 during neural development, as depicted in Figure #1.1, (Bibel and Barde 2000, Lim, et al. 2007). MIAMI cells were expanded and
induced toward neuronal specification followed by overnight serum starvation. NT-3 was added and protein lysates were collected at early (0, 5, 10, 15, 30, 60min) time points to assess Erk1/2 phosphorylation. NT-3 (30ng/ml) induced Erk1/2 phosphorylation around 5-10 minutes with a rapid decrease within 5 minutes (Figure #3.1A). Treatment with a higher concentration of NT-3 (100ng/ml) increased the duration of Erk1/2 phosphorylation till 30 minutes, and decreased to basal levels within 1 hour (Figure #3.1B). The reason for the sudden decrease in Erk1/2 phosphorylation might be attributable to the activation of mitogen-activated protein kinase phosphatase (MKP-3), which is known to dephosphorylate and inactivate selective Erk family mitogen-activated protein (MAP) kinases, such as Erk1/2 (Camps, et al. 1998, Muda, et al. 1998). Therefore, NT-3 stimulation of MIAMI cells during neuronal commitment induced short-term Erk1/2 phosphorylation.

**Figure #3.1:** MIAMI cells (H3515(3/4) were plated at a cell density of 3,000 cells/cm at 21% pO₂ prior to neural specification (Step #1) induction. Neural specification was induced followed by overnight serum starvation. Neuronal commitment was induced via addition of NT-3 (A: 30ng/ml, B: 100ng/ml). Protein lysates were collected for western blot analysis of Erk1/2. Coomassie blue stain was used for total protein.
**Profile of neurotrophin receptors and NT-3 in MIAMI cells**

NT-3 has the highest affinity for NTRK3. NT-3 is also known to bind NTRK2 *in vitro*, but with a lower affinity. Contradictory to the *in vitro* finding, *in vivo* studies have also shown that NT-3 does not bind NTRK2. In addition, p75NTR is known to bind all 3 neurotrophin receptors, changing their affinity for NT-3, as well as the other neurotrophin ligands. Due to the complicated nature of neurotrophin signaling, it is important to gain insight into the possible receptor(s) through which NT-3 stimulated downstream Erk1/2 phosphorylation. Real time-quantitative PCR (RT-qPCR) analysis was used to determine the mRNA expression profile of the neurotrophin receptors in MIAMI cells.

All three neurotrophin receptors (NTRK1, NTRK2, NTRK3), in addition to p75NTR were detected at the mRNA level using RT-qPCR analysis (Figure #3.2). NTRK1, NTRK2, and p75NTR were found to have comparable levels of mRNA expression. Primer pairs which detect all NTRK3 mRNA isoforms (NTRK3-ALL) showed that their expression was approximately 30-fold higher compared to NTRK1 which was set to the value of “1” for relative comparison (Figure #3.2A). Using primer pairs specific for only TKd-NTRK3 mRNA, RT-qPCR showed a 15-fold higher level compared to NTRK1 (Figure #3.2A). This suggests that approximately 50% of the fold increase seen using non-specific primer pairs for all NTRK3 isoforms (NTRK3-ALL) was due to TKd-NTRK3 mRNA expression. Using western blot analysis NTRK1, NTRK2, and p75NTR were not detected at the protein level during long-term expansion. Immunoprecipitation followed by SDS-PAGE analysis using antibodies specific for the intracellular domain of TKd-NTRK3, or specific for the extracellular domain of NTRK3, which is common to all isoforms (NTRK3-ALL) detected both the TKd-NTRK3 isoforms...
(70kDa & 100kDa) as well as the FL-NTRK3 isoforms (90kDa, 140/160kDa and 200kDa) (Figure #3.1B).

**Figure #3.2:** MIAMI cells (H3515) were expanded at 3% pO₂ at low density (100 cells/cm²). Protein lysates and mRNA were collected for RT-qPCR analysis of NTRK mRNA transcripts (A: N=3 independent experiments (2-3 samples per experiment), as well as for immunoprecipitation of NTRK3/Tkd-NTRK3 (B). Tkd-NTRK3 designated primer pairs or antibodies specific only for the tyrosine kinase deficient isoform of NTRK3. NTRK3-ALL designates primer pairs or antibodies that recognize all NTRK3 mRNA isoforms, or are specific for the extracellular domain of NTRK3, which both FL- and Tkd-NTRK3 share in common. (*) represents the FL-NTRK3 molecular weight band which is phosphorylated upon NT-3 stimulation (Figure #3.5). NTRK1, NTRK2 and p75NTR were not detected at the protein level.

MIAMI cells that were stimulated by NT-3 displayed induction of short-term Erk1/2 phosphorylation. Additionally, the cell expressed high mRNA expression levels for the NTRK3 receptor, and that was the only neurotrophin receptor detected at the protein level. To further characterize neurotrophin signaling in MIAMI cells, an ELISA assay was performed to determine if MIAMI cells secrete NT-3 during expansion at 3% pO₂ (low oxygen tension) or at 21% pO₂ (differentiation condition). MIAMI cells were expanded in serum-free media for 48 hours (conditioned media) in order to negate the effects of serum NT-3 levels and to determine if NT-3 was secreted by MIAMI cells. Standard curves were determined using recombinant human NT-3 (Figure #3.3A: 31-
2000pg/ml and Figure #3.3B: 31-62.5pg/ml). MIAMI cell serum-free conditioned media contained 6.35±0.92 and 6.43±0.47 pg/ml NT-3 at 3% and 21% pO₂, respectively (Figure #3.3C). This approximates to 48.77pM NT-3 produced by MIAMI cells at 3% pO₂. To test the effect of endogenous NT-3 and its potential autocrine stimulatory effect on MIAMI cells, an anti-NT-3 antibody was added to MIAMI cells expanded in serum-free media for 3 days. Compared to expansion using a general IgG antibody, there was a decrease in MIAMI cell growth over 2 days in the presence of the anti-NT3 antibody ($p$≤0.05, Figure #3.3).
Mass spectroscopy verification of NTRK3 isoforms in MIAMI cells

MIAMI cells express both the truncated (TKd-NTRK3) and full length (FL-NTRK3) NTRK3 receptors. Due to the high expression of the TKd-NTRK3 mRNA transcript (Figure #3.2A) and the detectable protein levels (Figure #3.2B), mass spectroscopy (MS) analysis was used to validate the identity of FL- & TKd-NTRK3 in MIAMI cells.

In order to isolate the NTRK3 isoforms and post-translation molecular weight variants, immunoprecipitation using an antibody against the extracellular domain of NTRK3 was used to pull-down both FL-NTRK3 and TKd-NTRK3. Immunoprecipitated proteins were separated using SDS-PAGE and either transferred to PVDF Immobilon-P membrane for western blot analysis, or silver stained for band excision, purification, trypsin digestion and mass spectroscopy analysis. Initially western blot analysis was used to determine that FL-NTRK3 and TKd-NTRK3 molecular weight bands were immunoprecipitated. An antibody specific for TKd-NTRK3 was not available during the initial stages of this project, therefore the protein bands which corresponded to the molecular weight of TKd-NTRK (70, 90/100 kDa) (McGregor, et al. 1994, Shelton, et al. 1995) were isolated for MS analysis. The NTRK3 receptor also undergoes post-translational glycosylation which contributes an additional 30-60 kDa of mass, and is required for membrane insertion, neurotrophin dependent activation, and contributes to
neurotrophin ligand specificity (Urfer, et al. 1995, Watson, et al. 1999). Therefore each isoform of NTRK3 has 2-3 predicted molecular weight bands; TKd-NTRK3 (70, 90/100 kDa) and FL-NTRK3 (90, 145, 200+ kDa). With this in mind, it was difficult to determine which bands corresponded to each isoform relying solely on molecular weight.

To further characterize and identify the isoforms, protein bands were excised at 145kDa (FL-NTRK3) and 100kDa or 70kDa (Tkd-NTRK3) (Figure #3.4A) in preparation for mass spectroscopy analysis. After immunoprecipitation-based purification and excision from the acrylamide silver stained gel, the lyophilized protein was proteolytically digested using Trypsin producing 53 predicted fragments. Digested samples were frozen until transport to the University of Tampa for mass spectroscopy analysis. HPLC was used for protein fragment separation prior to direct injection in the Mass Spectrometer (LTQ XL Linear Ion Trap Mass Spectrometer). Solvent vaporization via electrospray ionization utilizing 3,000-7,000 volts was used for simple injection. (refer to example diagram: Figure 3.4B). A Quadrupole Ion Trap was used to isolate NTRK3 protein fragments in order to perform MS^N (N=2-3) via molecular bombardment fragmentation for protein fragment sequence determination.

Using BioWorks Browser software, only one fragment from the extracellular domain of NTRK3, which corresponds to the binding site of NT-3, was identified from MS^3 data (Figure #3.4C). Five fragments (16-20%) within the intracellular domain of FL-NTRK3, and one fragment within the intracellular domain of TKd-NTRK3 (Figure #3.4C) were identified. In addition, using blastp ((http://blast.ncbi.nlm.nih.gov), the one MS-fragment found which corresponded to the TKd-NTRK3 intracellular domain, did not correspond with the sequence of any other known human proteins.
Figure #3.4: Protein lysates were immunoprecipitated using agarose-antibody conjugated beads. An anti-NTRK3 antibody specific to the extracellular domain of NTRK3 was used to pull down both FL-NTRK3 and TKd-NTRK3. Silver staining of the PAGE separated NTRK3 isoforms was used to visualize each MW band (A: representative) for proteolytic digestion and HPLC (HypersilGold C18) coupled Mass Spectroscopy (MS) analysis (B). MS^n (2-3) spectrums were used to determine the hypothetical protein sequence for each protein fragment. BioWorks3.3 software was used to blast the MS-identified sequences against the known NTRK3 protein sequence (C: shown in red). SymGlycan220 software was used to determine hypothetical N-glycosylation sites (C: shown in blue).
It was hypothesized that only one fragment of the extracellular domain of NTRK3 was identified due to high levels of glycosylation of NTRK3. Out of 53 predicted tryptic fragments for NTRK3, 48 contained putative N-glycosylation sites (NetNGlyc 1.0 Server: http://www.cbs.dtu.dk/services/NetNGlyc/). If we assume that NTRK3 is highly glycosylated, there would be approximately 135,000 possible tryptic fragments from the extracellular domain of glycosylated NTRK3 alone. From the combined data showing the molecular weights of the excised bands, in addition to the mass spectroscopy data, we can conclude that FL-NTRK3 and TKd-NTRK3 are present in MIAMI cells.

**NT-3 stimulation of NTRK3 and inhibition of Rac1 during Erk1/2 phosphorylation**

During this series of studies, cell growth changes, Erk1/2 and NTRK3 phosphorylation, and total FL- & TKd-NTRK3 protein levels were examined after short-term NT-3 stimulation of MIAMI cells. Additionally, I collected mRNA in order to analyze the downstream effects of NT-3 signaling on genes involved in cell cycle regulation, self renewal, and neurogenesis.

During neuronal commitment (Step #2), after 15 minutes of NT-3 stimulation, protein lysates were collected and the NTRK3 receptor was immunoprecipitated in order to determine if any of the NTRK3 isoforms were phosphorylated. There was a detectable increase in FL-NTRK3 phosphorylation (2.74 fold increase) and a net decrease in Tk-NTRK3 (0.07 fold decrease) protein levels. In addition, phosphorylation was only detected on the 140 kDa molecular weight band, corresponding to FL-NTRK3 (Figure #3.5). There was no phosphorylation detected at any of the molecular weight bands corresponding to TKd-NTRK3 (70-100kDa).
In order to analyze the role of NT-3 induced Erk1/2 phosphorylation in downstream gene activation leading to neuronal commitment of MIAMI cells, a series of chemical inhibitors were used to inhibit Erk1/2 phosphorylation (U0126), NTRK protein tyrosine kinase phosphorylation (K252a), and Rac1 activation (Rac1 inhibitor). The chemical inhibitors were added one hour prior to NT-3 stimulation during neuronal commitment (Step #2). Protein lysates were collected after a short-term NT-3 stimulation period of 15 minutes in order to detect changes in Erk1/2 phosphorylation in the presence of the chemical inhibitors.

**Figure #3.5:** MIAMI cells were expanded at 3% pO2 and induced toward neuronal specification at 21% pO2 for 24 hours followed by overnight serum starvation. NT-3 (30ng/ml) was used to stimulate the MIAMI cells and protein lysates were collected after 15 minutes. Immunoprecipitation using an anti-NTRK3 antibody (2µg) specific for the extracellular domain complexed with Protein A/G agarose beads was used to pull-down both FL- & TKd-NTRK3 receptor isoforms (200 µg total protein lysate). Western blot analysis using an NTRK3 antibody or anti-phospho-NTRK antibody was used. The percent change in band intensity was calculated using ImageJ. Representative of 2 independent experiments.
Unfortunately there was not a significant increase in p-Erk1/2 after NT-3 stimulation during normal neuronal commitment (Step #2) conditions (Figure #3.6A). This could be a result of the short time span (10-15min.) during which Erk1/2 is phosphorylated after NT-3 (30ng/ml) treatment (Figure #3.6A) and/or mistiming of cell lysis for protein isolation. While Erk1/2 was not phosphorylated there was a significant increase in cell proliferation (Figure #3.6B) and in FL-NTRK3 phosphorylation (Figure #3.5).

Addition of the general protein tyrosine kinase inhibitor (K252a) caused p-Erk1/2 levels to decrease in the control sample and there was no observed increase after stimulation with NT-3 (Figure #3.6A). K252a has been shown to inhibit the protein tyrosine kinase domain of the NTRK receptors (Tapley, et al. 1992), therefore the observed decrease in basal Erk1/2 phosphorylation levels may be due to a decrease in endogenous NT-3 (Figure #3.3C) signaling through NTRK3.

Addition of the Erk1/2 inhibitor U0126 ablated the basal levels of p-Erk1/2 as well as inhibited the NT-3 induced levels of p-Erk1/2 (Figure #3.6A). Blocking the Erk1/2 pathway also produced a slight increase in cell proliferation after NT-3 treatment (Figure #3.6B).

Due to the high levels of TKd-NTRK3 in MIAMI cells, we hypothesized that NT-3 stimulation via TKd-NTRK3 may lead to Rac1 activation. The subsequent use of the Rac1 Inhibitor induced an increase in NT-3 stimulated Erk1/2 phosphorylation (Figure #3.6A), as well as an NT-3 dependent significant decrease in cell proliferation (Figure #3.6B). If we assume that the Rac1 chemical inhibitor blocked Rac1 activation and
downstream signaling, this would suggest that the Rac1 signaling pathway inhibits Erk1/2 phosphorylation, preventing the full extent of NT-3 induced Erk1/2 phosphorylation. It has been shown that TKd-NTRK3 activates Rac1 in NIH3T3 cells (Esteban, et al. 2006), but it is not clear whether Rac1 inhibition of NT-3 induced Erk1/2 phosphorylation is dependent or independent of NTRK3/NT-3 signaling. This data suggests a novel role for Rac1 inhibition of Erk1/2 phosphorylation due to NT-3 stimulation.

Figure #3.6: MIAMI cells were expanded at 3% pO2 and induced toward neuronal specification at 21% pO2 for 24 hours followed by overnight serum starvation. NT-3 (30ng/ml) was used to stimulate the MIAMI cells and protein lysates were collected after 15 minutes for Erk1/2 western blot analysis (A). All chemical inhibitors were added one hour prior to NT-3 stimulation: K252a (5nM), U0126 (10µM), Rac1 Inh. (100µM). MIAMI cells were plated at a cell density of 3,000 cells / cm² (350,000 cells total) for neuronal specification induction (Step #1). Total cell counts were completed after 48 hours of NT-3 stimulation (B). N=2 independent experiments. $p \leq 0.01$ (**)

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<tr>
<th>Treatment</th>
<th>p-Erk1/2</th>
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<tbody>
<tr>
<td>Expansion Only</td>
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<tr>
<td>Neuronal specification (Step #1)</td>
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<td>Erk1/2 Inh. (U0126)</td>
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<td>NTRK Inh. (K252a)</td>
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<td>NT-3 (30ng/ml) 15min.</td>
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A. p-Erk1/2

B. Cell Growth Analysis (48hr post treatment)
**NT-3 stimulates limited pro-neuronal gene expression due to Rac1 repression**

In order to determine the biological significance of NT-3 stimulation on NTRK3 activation and downstream Erk1/2 phosphorylation, RT-qPCR analysis was used to detect changes in mRNA transcript levels after 48 hours of NT-3 induced neuronal commitment of MIAMI cells. As previously described in Figure #3.6, chemical inhibitors specific for NTRK protein tyrosine kinase phosphorylation (K252a), Erk1/2 (U0126), and Rac1 were added one hour prior to NT-3 induced neuronal commitment of MIAMI cells. The relative fold change in gene expression levels was calculated comparing to untreated samples for each group, which was set to the value of “1”. Out of 40+ genes analyzed, only the genes which had significant changes ($p \leq 0.05$) compared to their untreated controls, as well as between treatment groups (pretreatment ± chemical inhibitor) are shown in Table #3.1.

Of the genes stimulated by NT-3 during normal neuronal commitment, 9 showed an increase (3 genes $\geq 1.5$ fold), and 6 showed a decrease (2 genes $\geq 1.5$ fold) (Table #3.1: Column 1). The two genes which decreased greater than 1.5 fold were transcription factor neurogenin 2 ($Ngn2$: -8.70±0.02 fold decrease) and the low molecular weight neurofilament ($NFL$: -5.02±1.35 fold decrease) after NT-3 stimulation of MIAMI cells. Two of the genes which increased above 1.5 fold were Nestin (2.39±0.23) and microtubule associated protein 2 ($MAP2$: 1.52±0.63). The third gene which increased was Calbindin-D28K ($CALB1$), a known calcium binding protein found mainly in post-mitotic functionally mature neurons *in vivo*. This gene showed a 2038±345 fold increase after NT-3 stimulation and was not detectable prior to NT-3 treatment. In order to re-verify the finding that NT-3 stimulates $CALB1$ expression, MIAMI cells treated with NT-
3 (neuronal commitment: Step #2) were compared with fetal neuroepithelial cells (FNE). The FNE cells had 100 fold more \textit{CALB1} mRNA transcript then the NT-3 stimulated MIAMI cells. Unfortunately there was no detected CALB1 protein found in MIAMI cells at any time point (data not shown).

Pretreatment with the NTRK inhibitor (K252a) during neuronal commitment induced a decrease in medium molecular weight neurofilament (\textit{NFM}: -1301±157 fold decrease), microtubule associated protein 2 (\textit{MAP2}: -4.14±1.59 fold decrease), and CyclinD1 (\textit{CCND1}: -4.97±0.86 fold decrease). The decrease in \textit{CCND1} mRNA transcript levels did not coincide with a decrease in NT-3 stimulated cell proliferation (Figure #3.6B). \textit{NFL} was the only gene to increase (4.41±1.12 fold increase) after treatment of the MIAMI cells with the protein tyrosine kinase (NTRK) inhibitor.

Pretreatment with the Erk1/2 inhibitor (U0126) prior to NT-3 stimulation did not have an upregulation of \textit{Nestin} or \textit{MAP2}, nor a downregulation of \textit{Ngn2} or \textit{NFL} as seen during normal neuronal commitment. This inhibition of NT-3 stimulated gene expression further supports the hypothesis of NT-3 stimulation of downstream gene expression via the Erk1/2 pathway.

In addition, the Rac1 inhibitor caused 12 genes to increase greater than 1.5 fold upon NT-3 stimulation. If we hypothesize that the Rac1 inhibitor did inhibit Rac1 activation and signaling, allowing for increased Erk1/2 phosphorylation via NT-3 (as suggested in: Figure #3.6A), this would indicate that Rac1 is repressing the NT-3 stimulation of downstream pro-neural genes via the Erk1/2 signaling cascade. Of the genes upregulated during neuronal commitment in the presence of the Rac1 inhibitor,
transcription factors Ngn2 and Oct4 had a 16,965±1016 and 37.43±5.80 fold increase, respectively. The intermediate filaments Nestin (45.76±8.15 fold increase), NFL (20,103±2487 fold increase), NFM (1,220±286 fold increase), NFH (7.65±1.28 fold increase), as well as the microtubule associated protein, MAP2 (191±33 fold increase) were also found to be up-regulated.

It is of interest to point out that the mRNA for neuron restrictive silencing element (NRSF (REST) 6.29±1.51 fold increase) as well as its co-protein (coREST: 4.78±1.73 fold increase) were up-regulated; as well, both of which are known to inhibit neuronal differentiation and promote the maintenance of neural precursors (review (Lunyak and Rosenfeld 2005)) and self-renewal transcription factors Oct4, Sox2 and Nanog (Singh, et al. 2008).

Also of note, MIAMI cells expressed basal levels of Hes1 and Hes5 mRNA, both transcription factors involved in the self-renewal, proliferation, and maintenance of neural stem cells. The pro-neuronal transcription factors Mash1, Math1 and Math5 were also expressed (see review; (Kageyama, et al. 2008). Hes3 mRNA expression was not detected. Egr-1, a transcription factor known to be activated by neurotrophin mediated signaling via the Mek1/2-Erk1/2 signaling cascade (Huang and Reichardt 2001) was also present at the mRNA level.
### Neuronal Commitment (Step #2): + NT3

<table>
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<tr>
<th>Transcription Factors</th>
<th>Normal Step #2</th>
<th>NT3 Inhibitor (K252a)</th>
<th>Erk Inhibitor (U0126)</th>
<th>Rac1 Inhibitor</th>
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<td>NFM</td>
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<td>-1301</td>
<td>-0.65</td>
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<th>Erk Inhibitor (U0126)</th>
<th>Rac1 Inhibitor</th>
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<tr>
<td>Oct4a</td>
<td>-0.30</td>
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<td>0.94</td>
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<table>
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<th>Other:</th>
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<th>NT3 Inhibitor (K252a)</th>
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<td>ND</td>
<td>ND</td>
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<td>REST</td>
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<td>6.29</td>
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<td>coREST</td>
<td></td>
<td>-0.36</td>
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<td>4.78</td>
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Table #3.1: MIAMI cells were expanded at 3% pO₂ and induced toward neuronal specification at 21% pO₂ for 24 hours followed by overnight serum starvation. NT-3 (30ng/ml) was used to stimulate the MIAMI cells and total RNA was collected after 48 hours for RT-qPCR analysis. Chemical inhibitors were added one hour prior to NT-3 stimulation: NTRK inhibitor (K252a, 5nM), Erk1/2 inhibitor (U0126:10µM), or the Rac1 Inhibitor (100µM). RT-qPCR data was normalized against 2 housekeeping genes: EF1a & RPL13a (Curtis KM 2010). Of 40+ genes analyzed, only the genes which were significantly different (p≤0.05) comparing NT-3 treated versus untreated (+ chemical inhibitor) are shown. All values are displayed as the fold change, relative to the control (untreated) set to the value of “1”. Blank boxes represent “no-change”, yellow boxes “decrease”, green boxes “increase”, in mRNA expression. N=2 independent experiments (3-4 samples per treatment group per experiment). A Student’s t-test was used to determine significance between samples. ND: no data.

A review of the data collected using chemical inhibitors to block the specific NTRK, Erk1/2 and Rac1 pathways suggests that the inhibition of Erk1/2 caused a
decrease in NT-3 stimulated genes, while inhibition of Rac1 caused an overall increase in NT-3 stimulated genes (Table #3.1). Although inconclusive as to the role of Rac1, it can be hypothesized that Rac1 regulates the phosphorylation of Erk1/2 (Figure #3.6A) and downstream gene expression (Table #3.1) during NT-3 stimulation.

**Increased NT-3 stimulated Erk1/2 phosphorylation during Rac1 inhibition**

In order to verify the observation that added NT-3 indeed stimulated Erk1/2 phosphorylation in the presence of Rac1b inhibition (Figure #3.6), MIAMI cells were pretreated with the Rac1 inhibitor prior to NT-3 stimulation. In the presence of the Rac1 inhibitor, there was an increase in NT-3 stimulated Erk1/2 phosphorylation (Figure #3.7A). Phase contrast microscopy also showed that the MIAMI cells maintained a similar morphology in the presence or absence of the Rac1 inhibitor. There was also a slight increase in the number of cells with neurite-like extensions after NT-3 stimulation (3-5% increase) (Figure #3.7B (*)).

**Figure #3.7:** MIAMI cells were seeded at 3,000 cells /cm² overnight prior to NT-3 induction. The Rac1 inhibitor (75µM) was added 1 hour prior to NT-3 (30ng/ml) induction of neuronal commitment (Step #2). Western blot analysis was used to determine Erk1/2 phosphorylation (A). This is a representative western blot of two independent experiments. Images were taken after 48 hours to ensure that the Rac1 Inhibitor did not kill the cells (B). Cells treated with Rac1 Inhibitor + NT-3 showed ~3-5% of the cells with an increased number of neurite-like extensions. Neurites are defined as a cell processes which exceeds twice the width of the cell, and were counted as such (*). 4 slides per treatment were counted for 2 independent experiments.
Over-expression of FL-NTRK3 and TKd-NTRK3

In order to verify the role of NTRK3 during the observed NT-3 stimulated Erk1/2 phosphorylation, as well as downstream up-regulation of pro-neuronal genes, the over-expression of both FL-NTRK3 and TKd-NTRK3 in MIAMI cells was attempted. Overexpression vectors (pCMV6-ENTRY) for the open reading frames (ORF) of FL-NTRK3 and TKd-NTRK3 contained Myc/Dkk(FLAG) tags for their internal domain (carboxyl terminus) to facilitate analysis of low receptor levels and isoform specific receptor localization. Transient transfections were carried out for 48-72 hours prior to harvesting for total RNA or whole cell lysates. Stable transfections were performed the same as transient transfections, except after 24 hours the cells were split 1/100 into
selective media containing G418 (200ng/ml: see methods for optimization). After 2-3 weeks of selection the stable transfectants were split and clones were isolated.

<table>
<thead>
<tr>
<th>Vector</th>
<th>Colonies</th>
<th>Growth</th>
<th>Passages Survived</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCMV6-Entry</td>
<td>22</td>
<td>Normal DT: 30-32 hrs</td>
<td>4 to 10</td>
</tr>
<tr>
<td>pCMV6-FL-NTRK3</td>
<td>10</td>
<td>Fast DT: 24-29 hrs</td>
<td>3</td>
</tr>
<tr>
<td>pCMV6-TKd-NTRK3</td>
<td>0 (5 cells)</td>
<td>None</td>
<td>1</td>
</tr>
</tbody>
</table>

Table #3.2: Stable transfections were done using 1ug pCMV6 plasmid / 2,000 MIAMI cells, containing either the FL-NTRK3 or TKd-NTRK3 ORF. After 2 weeks of G418 selection (200ng/ml), stable transfectants were split and cloned. Normal media was used for 1 week after cloning prior to continued selection with G418 (50-100ng/ml). Cell counts were performed using a hemocytometer to determine doubling time (DT). Long term expansion was done using a 1 week on, 1 week off method of selection with G418.

Stable clones transfected with the pCMV6-ENTRY empty vector survived 4+ passages and pCMV6-FL-NTRK3 clones survived 3 passages. After selection, the pCMV6-TKd-NTRK3 clones did not survive past 1 passage (5 cells: 0 clones) (Table #3.2). In addition, the pCMV6-FL-NTRK3 stable clones had a faster doubling time of 24-29 hours compared to normal MIAMI cells doubling time of 30-32 hours (Table #3.2). Due to the limited survival of the stable clones, there were not enough cells for future characterization or experiments to be performed.

Because of the limited survival of the stable clones, transient transfectants (4 days) were characterized using RT-qPCR and western blot analysis. RT-qPCR analysis showed an increase in all NTRK3 isoform mRNA levels, as well as an increase in TKd-NTRK3 specific mRNA (approximately 2.4 and 2.0 fold for pCMV6-FL-NTRK3 and pCMV6-TKd-NTRK3, respectively) (Figure #3.8). It is unclear as to why there was also
an increase in TKd-NTRK3 mRNA when FL-NTRK3 (pCMV6-FL-NTRK3) was over-expressed. The $4 \times 10^6$ fold increase shown in Figure #3.8A may be due to undigested plasmid present in the transiently transfected MIAMI cells that was not fully digested during DNaseI treatment of total RNA isolates.

Western blot analysis using antibodies specific for the extracellular domain of NTRK3 (detects both FL- & TKd-NTRK3) did not detect any over-expression of NTRK3 (Figure #3.9A). Probing the western blot using an anti-Dkk epitope antibody detected faint bands for pCMV6-FL-NTRK3 only (arrow), which signifies a slight over-expression after 4-days of transient transfection (Figure #3.9B). In the positive control lysates for pCMV6-TKd-NTRK3 (Origene: HeLa cells) was detected as very high levels using the anti-Dkk antibody (Figure #3.9B). Due to the low expression levels of pCMV6-FL-NTRK3, and the lack of expression of pCMV6-TKd-NTRK3, in addition to the inability to make stable cell lines which could be expanded ex vivo, it was not possible to use the over-expression of NTRK3 for future experiments.
**siRNA mediated knock-down of NTRK3**

To determine the relationship between NT-3 induced Erk1/2 phosphorylation and the NTRK3 receptor, a general chemical inhibitor (K252a) was used to inhibit the protein tyrosine kinase domain of the NTRK receptors. The data reflected a loss of basal and NT-3 induced Erk1/2 phosphorylation (Figure #3.6), as well as changes in downstream gene regulation (Table #3.1). Due to the off-target effects of chemical inhibitors, siRNA was used to specifically target NTRK3 mRNA transcripts to confirm the previous results.
Four different siRNAs, specific for TKd-NTRK3 mRNA transcripts’ 3’untranslated region (3’-UTR) or exon-exon junctions, were used individually or combined. In addition, siRNA transfection conditions for MIAMI cell were compared using lipid based transfection reagents (Fugene, Turbofectin 8.0, Lipofectamine) versus electroporation using a Digital Bio/BTX Microporator. The use of electroporation instead of lipid based transfection reagents decreased cell death from 50-60% down to 30-40% as well as increased experiment reproducibility. Transfection efficiency increased from ~40% to ~90-100% (adherent cells) with the use of electroporation versus lipid based reagents.

In the case of siRNA targeted against TKd-NTRK3 mRNA transcripts, all 4 siRNA’s were used for microporation induced transfection of MIAMI cells. There was an observed ~25-30% reduction in mRNA, after transfection for 1-4 days, but no reduction in protein levels at day 4 (Figure #3.10). In addition Dhharmacon smart pool siRNA targeted against all NTRK3 mRNA isoform transcripts resulted in an 80% reduction in mRNA levels from 2-5 days. There was a slight reduction (~20-30%) after 7-8 days in TKd-NTRK3 protein levels, but no significant decrease in total NTRK3 protein levels between 5-10 days post siRNA transfection was observed (Figure #3.11).
Figure #3.10: MIAMI cells were transfected using microporation with 4 siRNA’s (10µM siRNA/100µl/3-4x10⁶) against TKd-NTRK3 (siRNA-TKd) mRNA transcript and cultured for 4-days under MIAMI cell expansion conditions. Whole cell lysates were collected using NP40 lysis buffer. Western blot analysis using a TKd-NTRK3 specific antibody was used, only the 70kDa band is shown. α-tubulin and total protein were used as loading controls.

Figure #3.11: MIAMI cells were transfected using microporation with Dharmacon smart pool siRNA targeted against all NTRK3 mRNA transcripts (siNTRK3), or with a scrambled control (siC); (10µM siRNA/100µl/3-4x10⁶). Whole cell lysates were collected using NP40 lysis buffer. Western blot analysis using a TKd-NTRK3 specific antibody (top) or an antibody specific for the extracellular domain of NTRK3 (NTRK3-ALL) common to all NTRK3 isoforms was used. Total protein was used for normalization during densitometry analysis (ImageJ). 10* indicates MIAMI cells transfected two times with siRNA, Day0 and subsequently on Day5 prior to harvesting protein on Day10.
NTRK3 mRNA levels were found to decrease upon siRNA transfection after 2-5 days, but overall NTRK3 protein levels modestly decreased after 5-10 days. This suggests that NTRK3 has a low turn-over rate and the limited duration of siRNA mediated mRNA knock-down is not long enough to see a decrease in protein levels. In order to determine the optimum length of time required for TKd-NTRK3, as well as FL-NTRK3 protein levels to degrade once there is a reduction in their mRNA transcript levels; a pulse-chase experiment was performed using radioactive methionine ($^{35}$-methionine) to determine the turn-over rate of endogenous NTRK3 protein. MIAMI expansion media containing $^{35}$-methionine (50µCi) was added for a 45 minute pulse period. Following the pulse, the MIAMI cells were chased using MIAMI expansion media containing 5mM cold methionine. The day 0 pulse-control sample was collected directly after the pulse-period. SDS-PAGE analysis was used to separate the total protein lysates (Figure #3.12B). Each lane corresponding to 24 hour times points (Day 0-4) were excised, rehydrated in scintillation fluid and the total radioactivity (DPMI) was counted using a liquid scintillation counter (Figure #3.12A).
Figure #3.12: Radioactive methionine (S<sup>35</sup>-methionine: 50µCi) was added to MIAMI cells in culture for a 45 minutes pulse period. Chase media containing 5mM cold methionine was added for 24 hours. Day 0 pulse-control was harvested for total protein directly after the pulse period. Protein lysates (400µg) were separated using PAGE and stained using coomassie blue (B). Each lane was excised, re-hydrated in scintillation fluid, and radioactivity determined (DPMI) using a liquid scintillation counter (A). DPMI counts were normalized against total protein using densitometric analysis to account for loading error.

\[ y = -16.885x + 101.55 \]
\[ R^2 = 0.839 \]

Figure #3.13: Radioactive methionine (S<sup>35</sup>-methionine: 50µCi) was added to MIAMI cells in culture for a 45 minutes pulse period. Chase media containing 5mM cold methionine was added for 24 hours post pulse. Total protein lysates (400 µg) were immunoprecipitated using Protein A/G agarose beads complexed with an anti-NTRK3 antibody specific for the extracellular domain of NTRK3. Immunoprecipitated NTRK3 protein was separated using PAGE (B). Each lane was excised, re-hydrated in scintillation fluid, and radioactivity quantified (DPMI) using a liquid scintillation counter (A). DPMI counts were normalized against total protein using densitometric analysis to account for protein error. Linear regression analysis was used to calculate the average rate of decrease in DPMI / Day (m). The dashed line represents background DPMI counts using the MW ladder of the acrylamide gel (A).
In order to determine the rate of endogenous NTRK3 protein turnover, immunoprecipitation was used to pull-down all NTRK3 isoforms (Figure #3.13 B) during the pulse-chase experiment as described. As stated above, the total radioactive counts (DPMI) were determined for each corresponding lane, representative of each time point (Day0-4) (Figure #3.13A). The average rate of decrease in radioactivity (DPMI) versus the number of days post-pulse was calculated using linear regression analysis. The average decrease in endogenous NTRK3 protein levels was determined to be approximately 17% per day (Figure #3.13A). Therefore in order to detect a noticeable decrease in NTRK3 protein levels after siRNA mediated knock-down of mRNA levels (2-5 days), it would take an estimated additional 3-7 days in order to see ≥50% decrease in protein levels.

**Nuclear localization of TKd-NTRK3**

Upon reviewing the literature it became clear that the unique intracellular domain of TKd-NTRK3 has an almost unknown function. The TKd-NTRK3 receptor is known to heterodimerize with FL-NTRK3, thereby preventing the autophosphorylation of FL-NTRK3 via NT-3 stimulation (Huang and Reichardt 2001). This is a result of the truncated tyrosine kinase deficient intracellular domain of TKd-NTRK3, but not a direct function due to activation of TKd-NTRK3 alone. Bioinformatics was used to determine whether the truncated intracellular domain of TKd-NTRK3 shares sequence or structural homology with any known proteins. The truncated intracellular domain of TKd-NTRK3 was found to share 98% amino acid sequence homology with mouse, rat, and monkey TKd-NTRK3 receptor (Blastx: http://blast.ncbi.nlm.nih.gov/). Upon examination of its structural properties using theoretical comparative modeling techniques, there were no
reported structurally significant similarities within the mammalian proteome (ModWeb: https://modbase.compbio.ucsf.edu/scgi/modweb.cgi, MODELLAR: http://www.salilab.org/modeller/). Only one DNA binding repair protein, RadA, in *Syntrophus aciditrophicus* (anaerobic bacteria), was found to share 36% sequence homology with the intracellular domain of TKd-NTRK3 (Blastp: http://blast.ncbi.nlm.nih.gov/). Other protein tyrosine kinase receptors, such as the EGF receptor, have been shown to enter the nucleus upon stimulation resulting in direct transcriptional regulation and DNA binding (Dittmann, *et al.* 2010). In order to test the hypothesis that the intracellular domain of TKd-NTRK3 enters the nucleus, similar to the EGF receptor, immunocytochemistry was used to determine the intracellular localization of TKd-NTRK3 in MIAMI cells.

**Figure #3.14:** MIAMI cells were expanded at 3% pO₂ prior to fixation in paraformaldehyde. Permeabilization was done using Triton X-100 (TX-100 labeled images). A general IgG primary antibody was used as a control, along with un-permeabilized cells stained with TKd-NTRK3 specific antibody. An antibody specific for the extracellular domain of NTRK3 was also used to determine total cell NTRK3 localization. Cell nuclei were stained with DAPI for better visualization. All images were captured at 20X magnification.
Immunocytochemistry was done using an antibody specific for the extracellular domain of NTRK3 (NTRK3 ex. dom.), common to both FL-NTRK3 and TKd-NTRK3, as well as an antibody unique to the intracellular domain of TKd-NTRK3. Staining the MIAMI cells with an antibody specific for the extracellular domain of NTRK3 showed a general dispersal of NTRK3 throughout the cells (Figure #3.6). TKd-NTRK3 was found to be localized to a large extent within the nucleus of MIAMI cells, with decreased staining throughout the remainder of the cell (Figure #3.14). These results support the conclusion that although there was specific TKd-NTRK3 localization during neuronal commitment (Step #2), there no change in this localization upon NT-3 stimulation.

Summary

The data shown here focused on the role of NT-3 stimulation of MIAMI cells and its effect on Erk1/2 phosphorylation, NTRK3 activation and degradation, as well the downstream upregulation of various pro-neural genes. In addition the presence of TKd-NTRK3 in MIAMI cells was validated using both mass spectrometry analysis and immunocytochemistry (AIM #3). NT-3 was found to induce short-term Erk1/2 phosphorylation which is blocked upon addition of an Erk1/2 inhibitor and or protein tyrosine kinase (NTRK) inhibitor during neuronal commitment. The inclusion of both these inhibitor also changed the NT-3 stimulation of downstream gene expression. TKd-NTRK3 was found to undergo rapid degradation after NT-3 stimulation, as well as the phosphorylation of the FL-NTRK3 receptor. These results suggest that both NTRK3 receptor isoforms may be activated via NT-3. Thus it can be concluded that FL-NTRK3 phosphorylation is responsible for downstream Erk1/2 phosphorylation and gene regulation due to the inhibition of this process with the NTRK inhibitor which inhibits
NTRK protein tyrosine kinase autophosphorylation. TKd-NTRK3 was also found to be localized within the nucleus of MIAMI cells but there was no change after NT-3 stimulation, further suggesting an alternative yet unknown role for TKd-NTRK3 in MIAMI cells.

The over-expression and or siRNA-mediated knockdown of NTRK3 was also attempted. Stable clones of FL-NTRK3 exhibited increased cell growth rates but did not survive longer than 3 passages. The TKd-NTRK3 clones did not proliferate and died after 1 passage. siRNA was found to rapidly decrease both isoforms of NTRK3 mRNA after 1-5 days, but there was no decrease in NTRK3 protein levels seen after 5-10 days. Further analysis of the turn-over rate of NTRK3 using a pulse-chase experiment showed a slow turnover rate of approximately 18% per day. This suggests that a longer period of mRNA knock-down, such as with the use of shRNA viral vectors, must be used in order to see a significant change.

As previously stated chemical inhibitors of Erk1/2 and protein tyrosine kinase receptors (NTRK) were used during the NT-3 stimulated neuronal commitment (Step #2) of MIAMI cells. A Rac1 inhibitor was also used to explore the hypothesis that NT-3 stimulation of TKd-NTRK3 can activate Rac1 leading to the downstream up-regulation of pro-neuronal genes. Surprisingly, in the presence of the Rac1 inhibitor, there was an increase in NT-3 stimulated Erk1/2 phosphorylation as well as an increase in downstream gene regulation. Because the over-expression and or siRNA knock-down of NTRK3 did not work, it is unclear if Rac1 is linked to TKd-NTRK3 as previously suggested. It is concluded from this data that NT-3 phosphorylated the FL-NTRK3 receptor leading to the downstream activation of Erk1/2 and regulation of gene expression (AIM #1).
Additionally is it also tentatively concluded that the NT-3 induced Erk1/2 phosphorylation and neuronal commitment of MIAMI cells is regulated via Rac1 activity (AIM2).
CHAPTER 4

RAC1B REGULATES NT-3 INDUCED ERK1/2 PHOSPHORYLATION AND
STIMULATION OF DOWNSTREAM GENE EXPRESSION

The following experiments focus on the role of Rac1 during NT-3 stimulated neuronal commitment of MIAMI cells. Specifically, what is the role of Rac1 in MIAMI cells regarding NT-3 stimulated Erk1/2 phosphorylation and downstream gene regulation? It is hypothesized that the Rac1 protein is regulating the extent of NT-3 induced Erk1/2 phosphorylation, and thus repressing the up-regulation of downstream genes.

Main Questions:

1. Does the Rac1 protein become activated (Rac1-GTP) upon NT-3 stimulation?

2. Will the knock-down of Rac1 using siRNA allow for an increased NT-3 induced Erk1/2 phosphorylation, in addition to the up-regulation of the 12 previously described genes which appear to be repressed?
Activation of Rac1 upon NT-3 stimulation

The previously reported findings suggest that Rac1 is regulating Erk1/2 phosphorylation. Therefore, it is of interest to determine if Rac1 is activated in an NT-3 dependent mechanism. Rac1 is a small GTP-binding protein known to have a high activation/turn-over rate between its GDP-bound inactive and GTP-bound active form. This process is mediated via guanine nucleotide exchange factors, such as Arf6, which was found to activate Rac1 upon NT-3 stimulation of TKd-NTRK3 in HEK293 cells (Esteban, et al. 2006). A known downstream target of active Rac1-GTP is PAK1, a serine/threonine p21-activated kinase, known to be involved in regulating cell motility and morphology.

In order to determine the amount of active Rac1-GTP present in cells, a pull-down assay using the protein binding domain (PBD) of PAK1 conjugated to Protein A/G agarose beads (PAK1-PBD) was employed. Controls lysates for active (GTP-bound) and inactive (GDP-bound) Rac1 protein lysates were incubated with a non-hydrolyzable form of GTP (GTPγS) or GDP prior to using the PAK1-PBD pull-down assay (Figure #4.1A). MIAMI cells were treated with NT-3 from 5-120 minutes during neuronal commitment (Step #2). There was an inconsistent pull-down of Rac1 via PAK1-PBD during NT-3 stimulation (Figure #4.1B), as seen by alternating Rac1 pull-down in the untreated controls. This experiment was repeated 3 times, resulting in a different pattern of PAK1-PBD pull-down of active Rac1 over the course of NT-3 stimulation for each experiment. Another GTP-binding protein known to bind PAK1, cyclin division control protein (cdc42), also showed the same inconsistent pull-down during NT-3 stimulation (Figure #4.1A). In order to verify that the NT-3 used was bioactive, the total protein lysates from
each pull-down assay were probed for Erk1/2, showing NT-3 stimulated Erk1/2 phosphorylation (Figure #4.1B). In addition, the presence of magnesium is known to stabilize the Rac1-GTP / PAK1 interaction and was used in all rinse and lysis buffers throughout these experiments. Hence, at this time point we have no definite answer as to why this assay was inconsistent.

**Figure #4.1:** MIAMI cells were lysed using a magnesium-containing MLB lysis buffer. GDP and GTPγS treated lysates were used as positive (Rac1-GTP) and negative (Rac1-GDP) controls for the PAK1-PBD pull-down assay (A). MIAMI cells were stimulated with NT-3 (100ng/ml) during neuronal commitment (Step #2). Protein lysates were collected in MLB lysis buffer and subjected to PAK1-PBD pull-down. Precipitated proteins were separated using SDS-PAGE prior to probing for Rac1 and cdc42 (B). Total cells lysates were also probed for Erk1/2 phosphorylation to show bioactivity of NT-3 stimulation (B). Coomassie blue was used for total protein staining.

**Rac1b is the predominant isoform of Rac1 present in MIAMI cells**

Due to the inconsistent results for the pull-down assay of active Rac1-GTP during NT-3 stimulation, it is unclear if Rac1 is involved in this process and hence, needs to be
further characterized. RT-qPCR analysis was used to measure the levels of both Rac1 isoforms, *Rac1a* and *Rac1b*, along with *Rac2* and *Rac3* mRNA transcripts. *Rac1b*, which is the constitutively active splice-variant of *Rac1a*, was found to be the predominant Rac1 mRNA isoform in MIAMI cells, regardless of oxygen tension (Figure #4.2A). To follow up on this finding, the MIAMI cells (20yr old male) used throughout this study were compared with another isolate of iliac crest derived MIAMI cells from a 15 yr old male. In both cases there was approximately a 30 fold increase in the ratio of *Rac1b* to *Rac1a* mRNA level at 3% pO$_2$ under expansion conditions (Figure #4.2B).

![Graph A](image1.png) ![Graph B](image2.png)

**Figure #4.2:** Real time quantitative PCR (RT-qPCR) analysis of Rac mRNA expression was normalized against two housekeeping genes; *EF1α* and *RPL13a*. *Rac1a* was used as the relative control sample and was set to the value of “1”. Isoform-specific primer pairs were used to detect the Rac1 isoform mRNA transcripts; *Rac1a* or *Rac1b*, under varied oxygen tension (A). Examination of MIAMI cells isolated from a 20 and 15 yr old male also show that *Rac1b* is the predominant Rac1 isoform (B) N=3 independent experiments (2-3 sample per condition). $p<0.01**$, $p<0.001***$

MIAMI cells were positive for the Rac1b protein under expansion conditions, and there was also an increase in Rac1b protein levels upon pretreatment with EGF and bFGF (Figure #4.3A). 92% of adherent MIAMI cells were also found to be positive for Rac1b using FACS analysis (Figure #4.3B).
Western blot analysis using an antibody specific for Rac1b showed expression of Rac1b protein under normal MIAMI cell expansion conditions, as well as an increase after pretreatment with 50ng/ml EGF and bFGF for 10 days (A). Flow cytometry analyses using an anti-Rac1b antibody showed 92% of gated cells were Rac1b positive (*), compared with an IgG negative control (B). Alexaflour 569 was used for secondary antibody fluorescent detection (FL2). N=2 (showing one representative experiment for each).

**Rac1b knock-down via siRNA**

These data shown in the previous section support preliminary conclusions, as well as raise questions relating to the inhibitory role of Rac1 in the NT-3 stimulated Erk1/2 pathway. Rac1 activation was found to be inconsistent or random, which may be due to the fact that constitutively active Rac1b was found to be the predominant isoform of Rac1 in MIAMI cells. Therefore, it can be hypothesized that Rac1a activation via an NT-3 / TKd-NTRK3 mediated mechanism as shown previously in HEK293T cells, does not account for the observed inhibitory role of Rac1 during neuronal commitment. (Esteban, *et al.* 2006). This raises the question as to whether Rac1b regulates NT-3 stimulated
Erk1/2 phosphorylation, as well as downstream gene regulation during neuronal commitment of MIAMI cells?

In order to confirm the inhibitory effect of Rac1 and to determine the role of Rac1b in MIAMI cells during NT-3 stimulated neuronal commitment, transfection of MIAMI cells with siRNA specific for Rac1b (siRac1b) was used. Two Rac1b specific siRNAs were used that have been previously described in detail (Esufali, et al. 2007, Matos, et al. 2008, Radisky, et al. 2005). After a 3 day transfection Rac1b protein levels (0.38) (Figure #4.4A) as well as the specific Rac1b mRNA transcript levels decreased (-0.05±0.01; p≤0.001).

**Figure #4.4:** Two siRNAs (Matos, et al. 2008) specific for the Rac1b isoform (siRac1b) of Rac1 were used to decrease the levels of Rac1b protein (A) and mRNA (B) levels over a 3 day period in MIAMI cells (H3515(3)). A non-targeting scrambled control siRNA was also used (siC). Quantification of Rac1b protein levels was done using densitometry (ImageJ Software) and are normalized against both α-tubulin and total protein (Coomassie blue stain). All RT-qPCR results are normalized against both EF1α and RPL13a. The fold decrease in (siRac1b) Rac1b mRNA levels compared against siC was significant (p≤0.001,***). Error bars are displayed as standard deviation. A: N=2. B: N=3 (3).
**Rac1b knockdown alters NT-3 stimulated Mek1/2 and Erk1/2 phosphorylation**

As described previously, NT-3 induces short-term Erk1/2 phosphorylation (Figure #3.1) and in the presence of the chemical Rac1 Inhibitor, NT-3 induced Erk1/2 phosphorylation is increased (Figure #3.6 & 3.8). To determine the role of Rac1b during NT-3 induced neuronal commitment, MIAMI cells were transfected with siRNA specific for Rac1b (siRac1b) 2-days prior to NT-3 stimulation (see Figure #2.4). In MIAMI cells transfected with siRac1b and stimulated with NT-3, there was a significant decrease in the magnitude ($p \leq 0.001$) but an increase in duration ($p \leq 0.05$) of Erk1/2 phosphorylation (Figure #4.5C). Because of its known role in Erk1/2 activation, Mek1/2 was next analyzed. Mek1/2 phosphorylation decreased under Rac1b knock-down conditions after

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**Figure #4.5:** MIAMI cells H3515(3) were transfected with siRac1b or a scrambled siRNA control (siControl) for 2-days followed by induction of neuronal specification (Step #1) and an overnight period of serum starvation (see Methods). Neuronal commitment (Step #2) was induced via stimulation with NT-3 (30ng/ml) in serum free media. Western blot analysis was used to probe for phosphorylated and total levels of cRaf1 (A), Mek1/2 (B) and Erk1/2 (B). α-tubulin was not used for normalization because it was found to increase in cells transfected with siRac1b and treated with NT-3 (data not shown). Quantification was performed for 2 independent experiments comparing band intensity (ImageJ) of the phosphorylated target protein normalized against unphosphorylated protein and total protein (TP: Coomassie Blue Staining). (*) in column A designates the correct molecular weight of cRaf1. N=2
NT-3 stimulation (Figure #4.5B). Raf1 is a known upstream activator of Mek1/2-Erk1/2 during NT-3 stimulation of early neural stem cell neuronal differentiation throughout development (Lim, et al. 2007). There was no increase in basal Raf1 phosphorylation levels under normal neuronal commitment (Step #2) conditions, nor during Rac1b knock-down (Figure #4.5A). These results in conjunction with the results found using the chemical Rac1 Inhibitor confirm that Rac1, specifically the constitutively active Rac1b isoform, is regulating Mek1/2-Erk1/2 phosphorylation during NT-3 induced neuronal commitment of Miami cells.

**Rac1b regulates NT-3 stimulated gene expression via Erk1/2**

Previously 12 different genes were observed to increase when Rac1 was inhibited (Rac1 Chemical Inhibitor) during NT-3 stimulated neuronal commitment (Step #2) (Table #3.1). These genes included important pro-neural transcription factors (NGN2), neurofilaments (NFH, NFM, NFL, Nestin, MAP2), neurotrophin ligands (NT-3), and genes involved in self-renewal and maintenance of neural precursor cells (OCT4a, REST, coREST). In order to test if Rac1b is responsible for the aforementioned regulation of NT-3 induced gene expression, MIAMI cells were transfected with siRNA specific for Rac1b (siRac1b) prior to neuronal commitment, with or without the presence of the Erk1/2 inhibitor (U0126). In MIAMI cells transfected with siRac1b treated with NT-3 during neuronal commitment, there was a significant ($p \leq 0.05$) increase in the pro-neural transcription factor Neurogenin 2 (NGN2: 3.02±0.91) (Figure #4.6A). In addition, important proteins involved in neurite outgrowth and stabilization were also up-regulated, including low (NFL: 4.28±1.68) and high (NFH: 3.33±1.32) molecular weight neurofilaments and microtubule associated protein 2 (MAP2: 3.91±1.24) (Figure #4.6B).
The structural protein Nestin, as well as the medium molecular weight neurofilament NFM increased in the presence of NT-3, but were not affected by Rac1b knock-downs (Figure #4.6B). The RS-1 silencing transcription factor (REST), which is a transcriptional repressor of neuronal genes in non-neuronal tissue was also up-regulated (6.82±3.2) (Figure 4.7C).

To test whether the observed increased in gene expression was due to Rac1b regulation of the Mek1/2-Erk1/2 signaling pathway, the Erk1/2 chemical inhibitor U0126 was added 1 hour prior to NT-3 induction of neuronal commitment in MIAMI cells.
transfected with siRac1b. NGN2, NFL, NFH, MAP2, had decreased NT-3 stimulation in the presence of the Erk1/2 inhibitor U0126. This result verifies that the observed Rac1b repression of NT-3 stimulated genes is through Erk1/2 regulation (Figure #4.6B/C).

**Rac1b knock-down decreases NT-3 stimulated MIAMI cell proliferation but does not retard cell-cycle progression**

During NT-3 mediated neuronal commitment, there is an increase in cell proliferation after NT-3 stimulation (Figure #3.6). Rac1b has been implicated in the regulation of cell proliferation and control of Cyclin check point proteins such as CyclinD1(CCND1) and CyclinB1(CCNB1) in colorectal tumor cells (Matos and Jordan 2008). Therefore, the role of Rac1b in the observed NT-3 stimulated increase in cell proliferation, cell-cycle progression, and morphological changes in MIAMI cells was analyzed.

MIAMI cells transfected with siRac1b showed a decrease in NT-3 stimulated cell proliferation from a 3.44±0.54 fold increase, down to only a 1.34±0.2 fold increase, compared to non-NT-3 treated cells (Figure #4.8B). There was also a small increase (20-30%) in the number of neurite-like extensions in MIAMI cells transfected with siRac1b, after NT-3 stimulation (Figure #4.7A). The presence of the Erk1/2 inhibitor had no effect on siRac1b-mediated changes in cell proliferation (Figure #4.7B). The basal mRNA expression of CCND1 (1.71±0.24) and CCNB1 (1.63±0.29) significantly increased in MIAMI cells transfected with siRac1b, but did not increase further upon NT-3 stimulation (Figure #4.7C). Incorporation of the Erk inhibitor U0126 further increased the basal mRNA expression levels of CCND1 (2.43±0.41) and CCNB1 (2.38±0.39). The
mRNA expression levels of the cyclin-dependent kinase inhibitors p21 and p27, as well as p53 did not change.

**Figure #4.7:** MIAMI cells were transfected with siRNA for Rac1b (siRac1b) or non-specific siRNA control (siC). The Erk1/2 inhibitor (U0126, 10µM) was added 1 hour prior to NT-3 stimulation. NT-3 treated MIAMI cells were counted 48 hours after NT-3 induction of neuronal commitment (A/B). 3 random areas per slide were counted for total cell number and compared to their respective non-NT-3 treated controls (+NT3 normalized against −NT3). For comparison siC was set to the value of “1” (B). RT-qPCR was used to determine changes in CyclinD1 (CCND1) and CyclinB1 (CCNB1) mRNA levels. All values were normalized against the 2 housekeeping genes, EF1a and RPL13a and the Normal (N) control was set to the value of “1”. Neurite-like extensions were counted (30 cells / 3-random fields / slide) and are defined as: a cellular extension which is ≥ twice the diameter of the cell body. Error bars are shown as standard deviation. A: N=2 experiments (3 slides / treatment / experiment). C: N=2 independent experiment (2-3 samples per experiment).

The percent of MIAMI cells in the G0/G1 or G2/M phase of the cell-cycle was determined by propidium iodide staining and FACS analysis. During MIAMI cell expansion at 3% pO2, 64.3% of the cells were in G0/G1 while only 28.6% were in G2/M phase of the cell cycle (Figure #4.8). These percentages did not change after 24 hour
neuronal specification (Step #1). After overnight serum starvation, 87% of the cells were synchronized in G0/G1 and only 10.8% in G2/M. S-phase was also very low or non-existent after serum starvation. After 48-hours of NT-3 stimulation during neuronal commitment (Step #2), there was no significant change in the percentage of cells in G0/G1 or G2/M, suggesting that there is a limited number of cell-cycle progressions ending in renewed G0/G1 synchronization (87%). MIAMI cells which were transfected with siRac1b did not exhibit significant changes in cell-cycle progression throughout neuronal commitment (Figure #4.8). There was also no change when the Erk1/2 inhibitor U0126 was added to siRac1b transfected MIAMI cells.

**Figure #4.8:** Propidium iodide (PI) FACS analysis was used to determine cell-cycle progression: G0/G1, S, G2-M. Gating was used to exclude dead cells and debris. Heat inactivated RNaseH was used as a negative control for background PI staining. MIAMI cells were collected for FACS analysis after expansion at 3% pO2, neuronal specification (Step #1), 16 hours serum-starvation (SFM), neuronal commitment (Step #2: ±NT-3 (30ng/ml). MIAMI cells were transfected with siRNA against Rac1b 48 hours prior to FACS analysis (Expansion, Step#1, or Initiation of Step#2 ±NT-3). N=1 (2 samples per group).
Summary

The data showing an inconsistent or random pull-down of active Rac1-GTP via PAK1-PBD in MIAMI cells suggests that Rac1 was not being activated via NT-3 stimulation, and may be a part of some other regulatory process(es). Following up on this result, Rac1b was found to be the predominant isoform of Rac1 in MIAMI cells, regardless of oxygen tension and confirmed in two young adult male donors. Due to the constitutive active nature of Rac1b and its controversial role in PAK1 interaction, this may help explain the previous results showing random active Rac1 pull-down. The predominance of Rac1b in MIAMI cells also suggests that Rac1b is responsible for the observed regulatory role of Rac1 in NT-3 stimulated Erk1/2 phosphorylation and downstream gene regulation.

To determine the role of Rac1b in MIAMI cells, transfection of siRNA specific for Rac1b was used to decrease both Rac1b mRNA and protein levels during NT-3 stimulated neuronal commitment. Knocking down Rac1b in MIAMI cells resulted in a decrease in Mek1/2 and Erk1/2 phosphorylation. There was also an increase in the duration of Erk1/2 phosphorylation. These results confirm the previous findings suggesting that Rac1 regulation of NT-3 induced Erk1/2 phosphorylation. In addition, cRaf-1, which is known to activate Mek1/2-Erk1/2 was not stimulated via NT-3.

Rac1b was also found to repress the expression of NGN2, MAP2, NFH, NFL, and REST mRNAs during NT-3 stimulated neuronal commitment. Addition of the Erk1/2 inhibitor reconfirmed that Rac1b is regulating this process via Erk1/2. Rac1b was also found to be responsible for the observed NT-3 stimulated increase in cell proliferation, as
well as repression of *CCND1* and *CCNB1* mRNA expression. Finally, propidium iodide FACS analysis showed that Rac1b knock-down does not cause retardation or inhibition of cell-cycle progression, suggesting that Rac1b is involved in the rate of the cell-cycle, but not progression past cell-cycle checkpoints.
Chapter 5

EGF/bFGF Pretreatment of MIAMI Cells Enhances NT-3 Stimulated Neuronal Commitment

Epidermal growth factor (EGF) and fibroblast growth factor (bFGF) are known neural stem cell mitogens found throughout development of the CNS. FGF-2 (bFGF) is well known to strongly stimulate cell division and promote neuronal differentiation (Craig, et al. 1996, Kennea and Mehmet 2002, Kuhn, et al. 1997). EGF has also been reported to act as a neural determinant of precursor cells (review, (Weiss, et al. 1996). We hypothesized that bFGF/EGF pretreatment of MIAMI cells would increase the efficiency of NT-3 stimulated neuronal commitment (Step #2). bFGF alone or EGF/bFGF in combination were used to pretreat MIAMI cells under expansion conditions for two 5-day periods prior to induction of neuronal specification (Step #1), overnight serum-starvation and NT-3 stimulated neuronal commitment (Step #2).

Scheme of Neuronal Differentiation of MIAMI cells:

**EGF/bFGF Pretreatment:**
MIAMI cells + EGF/bFGF or bFGF (2X 5-day)

**Step 1: Specification**
MIAMI cell + bFGF (24 hr) → Neuronal precursor

**Step 2: Commitment**
Neural precursor + NT-3 (2 days) → Committed neuronal progenitor
**EGF/bFGF pretreatment up-regulated NTRK1 and NTRK3 protein levels**

Analysis of the NT3 receptor, neurotrophin tyrosine receptor kinase 3 (NTRK3), revealed that both the full length isoform (gp145, FL-NTRK3) and the tyrosine kinase deficient isoform (gp90, TKd-NTRK3) of NTRK3 were up-regulated along with NTRK1, after EGF-bFGF treatment (Figure #5.1). Lower concentrations of EGF/bFGF (5, 10, 20ng/ml) or bFGF alone showed no significant changes in NTRK1 or NTRK3 protein levels. NTRK2 and p75NTR were not detected during normal expansion or with EGF/bFGF pretreatment.

**EGF/bFGF pretreatment increases the duration of NT-3 stimulated Erk1/2 phosphorylation**

The effect of EGF/bFGF pretreatment on NT-3 stimulated neuronal commitment (Step #2) was next determined as an extension of the finding that EGF/bFGF pretreatment increased the NTRK3 receptor. NT-3 stimulation (30 ng/ml) during normal neuronal commitment induced Erk1/2 phosphorylation (p42/p44), which peaked after 5 min, decreased after 10 min and was not detectable after 15 min of stimulation (Figure #5.2). EGF-bFGF pretreatment resulted in a small but significant increase in sustained Erk1/2 phosphorylation (p42 and p44, \( p \leq 0.05 \)), which was still present at 10 min of NT3 stimulation.
stimulation compared to non-treated cells (Figure #5.2). The observed increase in the NT-3 receptor, NTRK3, may explain the observed increase in downstream Erk1/2 phosphorylation.

![Western blot analysis of Erk1/2 phosphorylation](image)

**Figure #5.2:** MIAMI cells were pretreated with EGF/bFGF (50ng/ml) for two 5-day periods. After pretreatment, the MIAMI cells were trypsinized and re-plated for normal neuronal specification (Step 1) induction. Neuronal commitment (Step #2) was induced using 30ng/ml of NT-3. Western blot analysis was used to determine the levels of phosphorylated and total Erk1/2. Densitometry analysis was used to determine the fold increase in p-Erk1/2 normalized against total Erk1/2 and total protein (Coomassie blue stain) (ImageJ). A one-way ANOVA was used to compare NT-3 stimulated Erk1/2 phosphorylation at 5-15 minutes between normal and EGF/bFGF pretreatment MIAMI cells. ($p \leq 0.05$) N=2 (representative western blot).

**EGF/bFGF or bFGF pretreatment reduced NT-3 stimulated cell growth**

As shown above (Figure #3.6), NT-3 stimulation during neuronal commitment induces cell proliferation. During neuronal commitment, MIAMI cells which were pretreated with either bFGF or EGF/bFGF in combination (20-50ng/ml) proliferated significantly less than normal MIAMI Cells (Figure #5.3). There was also no appreciable increase in floating or dead cells in bFGF or EGF/bFGF pretreated MIAMI cells during neuronal commitment. Additionally, there was no change in *CyclinD1, p21, p27 or p53* mRNA expression (data not shown). *CyclinB1*, which is required for the G2 transition into mitosis, had a 4.31±0.94 fold decrease in mRNA expression levels in EGF/bFGF.
(50ng/ml) pretreated MIAMI cells during neuronal commitment. The decrease in *CyclinB1* may account for the observed decrease in NT-3 stimulated cell proliferation.

**Figure #5.3:** MIAMI cells were pretreated with bFGF or EGF/bFGF for two 5-day periods. After pretreatment, the MIAMI cells were trypsinized and replated for normal neuronal specification (Step #1) induction. Neuronal commitment (Step #2) was induced using 30ng/ml of NT-3. Total cell numbers determined 48 hours after NT-3 stimulation and were normalized to the non-NT-3 treated MIAMI cells. For statistical significance the pretreated MIAMI cells were compared with the normal MIAMI cells (0). ***p≤0.001

**EGF/bFGF or bFGF pretreatment stimulates NT-3 induced gene expression of Ngn2, Prox1, MSI1 and the neurofilaments**

MIAMI cells pretreated with the EGF/bFGF (20-50ng/ml) combination showed significant increase in the mRNA expression of the pro-neural RNA-binding protein *Musashi-1 (MSI1)* (Okano, et al. 2005) during neuronal commitment (20ng/ml: 2.72±0.29; 50ng/ml: 2.86±0.61 fold increase), compared to the 1.26±0.38 fold increase in non-pretreated MIAMI cells (Figure #5.4A). Pretreatment with bFGF alone did not cause the same NT-3 dependent increase in *MSI1* mRNA expression as seen with EGF/bFGF in combination. There was a significant difference between the increase seen with EGF/bFGF versus that seen with bFGF pretreatment, implicating a cooperative effect of EGF/bFGF in combination. This result may also be due to EGF pretreatment alone, but
cannot be determined from these experiments and will have to be followed up in the future.

**Figure #5.4:** MIAMI cells were pretreated with EGF/bFGF for two 5-day periods. After pretreatment, the MIAMI cells were trypsinized and re-plated for normal neuronal specification (Step #1) induction. Neuronal commitment (Step #2) was induced using 30ng/ml of NT-3 for 48 hours followed by total RNA isolation and RT-qPCR analysis. All RT-qPCR data was normalized against two housekeeping genes; EF1α and RPL13a. The relative fold change was determined in comparison with non-NT3 treated controls for each pretreatment group (0, 20-50bFGF, 20-50 EGF/bFGF) during neuronal commitment. Non-NT3 treated controls are indicated by the dashed line set to the value of “1”. A one-way ANOVA followed by Tukey’s post hoc test was used to determine statistical significance between treatment groups treated with NT-3. Symbols were used to identify the comparison between treatment groups (i.e. #, &, $, *). D: The symbol (*) designates comparison of all NF’s to normal NT-3 treated MIAMI cells (0). N=2 independent experiment (2-3 samples per treatment group per experiment) $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***)
Neurogenin 2 (Ngn2), a transcription factor which promotes the maintenance and survival of neural precursor cells (Yi, et al. 2008), decreased (-0.04±0.012) in EGF/bFGF (50ng/ml) pretreated MIAMI cells, similar to non-pretreated MIAMI cells. Conversely, bFGF pretreatment alone caused an increased in Ngn2 mRNA (1.13±0.08) after NT-3 stimulation (Figure #5.4B). There was a significant difference in Ngn2 mRNA expression between normal MIAMI cells, bFGF, and EGF/bFGF treated (50ng/ml) MIAMI cells, suggesting that bFGF pretreatment enhanced NT-3 stimulated Ngn2 mRNA expression.

The transcription factor Prox1 is known to promote neurogenesis of neural precursor cells of the forebrain as well interneurons in the spinal cord. It has also been suggested that the expression of Prox1 is controlled in an Ngn2-dependent manor (Misra, et al. 2008). Prox1 had an mRNA expression profile similar to Ngn2 with a significant increase (2.26±0.17) in expression with only bFGF (50ng/ml) pretreatment and not EGF/bFGF in combination (Figure #5.4C). There was also the same significant difference in Prox1 mRNA expression between normal MIAMI cells, bFGF, and EGF/bFGF treated (50ng/ml) MIAMI cells, suggesting that bFGF pretreatment enhanced NT-3 stimulated Prox1 mRNA expression.

Analysis of the low molecular weight neurofilament (NFL) showed a significant increase with bFGF (20ng/ml, 8.65±1.21; 50ng/ml, 13.41±4.67 fold increase) or EGF/bFGF pretreatment (20ng/ml, 23.23±3.97; 50ng/ml, 32.76±1.93 fold increase) in a concentration-dependent trend (Figure #5.4D). Medium molecular weight neurofilament (NFM) showed a similar, but reduced increase with both bFGF (20ng/ml, 4.2±0.76; 50ng/ml, 5.98±0.75 fold increase) and EGF/bFGF (20ng/ml, 6.2±0.18; 50ng/ml,
8.22±0.52 fold increase) pretreatment, except with no difference between concentrations and or pretreatment groups (Figure #5.4D). High molecular weight neurofilament (NFH), which is known to increase in post-mitotic neurons with maturing neurite and axonal processes (review: Lariviere Julien 2003), increased only with EGF/bFGF (20ng/ml, 2.09±0.28; 50ng/ml, 2.71±0.20 fold increase), and not bFGF pretreatment during NT-3 stimulated neuronal commitment (Figure #5.4D).

**Summary**

Pretreatment of MIAMI cells with EGF/bFGF in combination at higher concentrations (50-100ng/ml) up-regulated the expression of the neurotrophin receptors; NTRK1, and the NTRK3 receptors FL-NTRK3 and TKd-NTRK3. In addition, there was an increase in the duration of phosphorylation of Erk1/2 after NT-3 stimulation during neuronal commitment of MIAMI cells pretreated with EGF/bFGF (50ng/ml). In both of these cases, the use of bFGF alone or the combination of EGF/bFGF at lower concentrations (5, 10, 20ng/ml) did not induce a change in NTRK receptor levels or NT-3 stimulated Erk1/2 phosphorylation. There was, however, a significant NT-3 stimulated increase in cell proliferation of MIAMI cells pretreated with bFGF alone or EGF/bFGF in combination during neuronal commitment.

Analysis of MSII showed an increased mRNA expression only with the combination EGF/bFGF pretreatment during neuronal commitment. Ngn2 and Prox1, both of which are involved in the progression of NPCs toward neuronal differentiation, had increased mRNA expression only with bFGF and not with EGF/bFGF pretreatment of MIAMI cells.
Additional analysis of the neuronal intermediate filaments NFL, NFM and NFH showed a concentration-dependent increase in NFL mRNA expression. Both NFL and NFM showed an increase with EGF/bFGF in combination, compared to bFGF pretreatment alone. NFH had the lowest, albeit significant increase in mRNA expression in MIAMI cells pretreated in combination with EGF/bFGF.

Analysis of these pro-neural transcription factors and neurofilament mRNA expressions shows a cooperative effect of EGF/bFGF pretreatment toward a hypothesized neural precursor cell phenotype after NT-3 stimulation. Whereas, bFGF pretreatment alone appears to push the transcriptional regulation toward a more pro-neuronal differentiation phenotype.
CHAPTER 6
DISCUSSION

Since the first isolation of MSCs from hamsters in 1970 (Friedenstein, et al. 1970), the field of hMSC research has expanded, illuminating their potential as human adult stem cells with the ability to transdifferentiate into cell lineages characteristic of all three germ layers. In addition, this unique ability of MSC-derived adult stem cells to be used as an autologous source of renewable cells, which can be expanded en mass \textit{ex vivo} and differentiated into various tissue types, shows their potential for cell-therapy based treatments. The potential clinical applications of MSC are diverse, including the cell-therapy based repair of injured tissue, treatment of autoimmune diseases and chronic degenerative diseases (Prockop 2009, Uccelli, et al. 2008).

Human bone marrow-derived adult stem cells, MAPCs, MSCs, RS-1 and MIAMI cells, have all been shown to differentiate into neuron-like cells with a characteristic neuronal phenotype \textit{in vitro} (Cho, et al. 2005, Tao, et al. 2005, Tatard, et al. 2007, Trzaska, et al. 2007). Even though MSCs have been characterized as being able to transdifferentiate into neuron-like cells, the mechanism(s) driving differentiation and their comparison with the developmental program of neural stem cells (NSCs) has not been reported. Therefore, it is important to understand how MSCs, such as MIAMI cells are differentiated into neuron-like cells in order to determine their potential and/or limitation(s) for repair of neural tissue in humans. The focus of this research was to characterize and determine the functional mechanism(s) for the NT-3 induced neuronal commitment of bone-marrow derived MIAMI cells via the receptor NTRK3.
**NT-3 INDUCED SHORT-TERM ERK1/2 PHOSPHORYLATION LEADING TO THE UP-REGULATION OF PRO-NEURONAL GENES**

*Neurotrophin signaling characterization in MIAMI cells during neuronal commitment*

Neurotrophins (NT-3/4, NGF, BDNF) act through neurotrophin tyrosine receptor kinases (NTRKs) to promote cell growth, differentiation, and survival (Hallbook 1999). We found that MIAMI cells stimulated with NT-3 during neuronal commitment demonstrated a short-term induction of Erk1/2 phosphorylation. This is very similar to the described duration of Erk1/2 phosphorylation throughout development of the nervous system, via neurotrophin activation of the neurotrophin tyrosine receptor kinase (NTRK), inducing the up-regulation of pro-neuronal genes leading to neurogenesis and neuronal maturation (Bibel and Barde 2000). In addition, NTRK3 was found to be the only neurotrophin receptor in MIAMI cells expressed at the protein level (Figure #3.2), suggesting that NT-3 stimulation of Erk1/2 is through its primary receptor, NTRK3. Similar neurotrophin receptor expression profiles have been reported during early development in the neural plate and neural tube where the only neurotrophin receptor expressed is NTRK3 and its ligand NT-3, prior to the later up-regulation of NTRK2, NTRK1 and p75NTR (Bernd 2008). This would suggest that MIAMI cells are similar to early neural epithelial precursor cells with respect to the expression profile of NTRK3, as well as to NT-3 induction of Erk1/2 phosphorylation.

NT-3 is implicated not only in neurogenesis and differentiation but also in the proliferation of NSCs throughout development in the adult brain (Hallbook 1999), as well as in other somatic cells such as epithelial cells (Botchkarev, *et al.* 2004). Therefore it was of interest that MIAMI cells secreted low levels of NT-3 during expansion, which
upon blocking with an inhibitory antibody, decreased cell proliferation (Figure #3.3). Addition of exogenous NT-3 also stimulated cell proliferation during MIAMI cells neuronal commitment (Figure #3.6). This would suggest that NT-3 plays a role in the proliferation of MSCs, such as MIAMI cells. In addition, over-expression of the full length isoforms of NTRK3 (FL-NTRK3) reduced the doubling time of MIAMI cells by almost 6-7 hours (Figure 3.9), further supporting the role of NT-3 and its receptor FL-NTRK3 in proliferation of MSCs. The initial characterization of neurotrophin signaling in MIAMI cells suggests a central role for exogenous and endogenous NT-3 stimulation, potentially via its primary receptor NTRK3, which is also the sole neurotrophin receptor expressed at the protein level in MIAMI cells.

Further characterization of NTRK3 in MIAMI cells identified the expression of both FL-NTRK3 and TKd-NTRK3 mRNAs as well as the proteins corresponding to FL-NTRK3 (90, 140, 200+kDa) and TKd-NTRK3 (70, 100kDa) (Figure #3.2) (McGregor, et al. 1994). Due to the varied molecular weights of FL- and TKd-NTRK3 (McGregor, et al. 1994, Shelton, et al. 1995) reported between in vitro and in vivo studies, mass spectroscopy (MS) analysis was used to verify the NTRK3 isoforms in MIAMI cells. MS analysis identified one fragment from the extracellular domain of NTRK3, as well as 16-20% sequence coverage (5-6 fragments) and one fragment from the intracellular domains of FL- and TKd-NTRK3, respectively (Figure #3.4). The extracellular domain of NTRK3 is known to undergo glycosylation (+30-60kDa) (Urfer, et al. 1995, Watson, et al. 1999) and contains 48 putative N-glycosylation sites (Figure #3.4). Therefore, tryptic digest of NTRK3 could theoretically produce ≥135,000 fragments, making MS analysis difficult due to the possible combination of glycosylation length in addition to
the number of putative sites. This may explain the low sequence coverage determined for the extracellular domain of NTRK3 via MS analysis, as well as the 200+kDa band observed during western blot analysis (Figure #3.2). The presence of FL-NTRK3 as well as TKd-NTRK3, both of which are highly conserved in vertebrates (McGregor, et al. 1994), raises the question as to the function of the NTRK3 isoforms during the observed NT-3 stimulation of MIAMI cells.

FL-NTRK3 contains a functional protein tyrosine kinase (PTK) domain (McInnes and Sykes 1997), and upon NT-3 binding undergoes dimerization. This leads to the autophosphorylation of PTK inducing downstream signaling cascades, such as Mek1/2-Erk1/2 phosphorylation (Kaplan and Miller 2000, Lim, et al. 2007). TKd-NTRK3 has a truncated intracellular domain which, due to a frame shift, does not exhibit any of the PTK properties of FL-NTRK3 (Tsoulfas, et al. 1996) and has been shown to heterodimerize with FL-NTRK3, blocking autophosphorylation via NT-3 binding (Bibel and Barde 2000, Biffo, et al. 1995, Palko, et al. 1999). In addition, TKd-NTRK3 has been shown to be activated via NT-3 stimulation leading to downstream activation of the Rac1 pathway (Esteban, et al. 2006), as well as being implicated in a unique role, independent of FL-NTRK3, in neuronal differentiation and plasticity (Hapner, et al. 1998, Menn, et al. 1998). NT-3 stimulation of MIAMI cells was found to induce the phosphorylation of FL-NTRK3 (140kDa), as well as the rapid degradation of TKd-NTRK3 (70kDa) (Figure #3.5). This proves that NT-3 induces FL-NTRK3 phosphorylation in MIAMI cells, similar to NT-3 induced neuronal differentiation of NSCs (Lim, et al. 2007). The degradation of TKd-NTRK3 due to NT-3 stimulation does not conclusively demonstrate “activation”. It is known that upon NT-3 binding TKd-
NTRK3 is rapidly (5-10 minutes) cleaved via MMP-3 (Mateos, et al. 2003). This activity could explain the rapid decrease in TKd-NTRK3 (70kDa) after NT-3 stimulation, suggesting that NT-3 stimulated FL-NTRK3 as well as TKd-NTRK3 in MIAMI cells. In addition, the over-expression of TKd-NTRK3 in MIAMI cells prevented cell proliferation, purportedly due to cell-cycle inhibition or deleterious effects of abnormal TKd-NTRK3 levels (Figure #3.9). This further supports the hypothesis that TKd-NTRK3 has a function, yet unknown, in MIAMI cells.

In an effort to further characterize NT-3 stimulated Erk1/2 phosphorylation and the possible role of FL-NTRK3 during neuronal commitment, an Erk1/2 inhibitor (U0126) and protein tyrosine kinase (NTRK) inhibitor (K252a) were added to MIAMI cells prior to neuronal commitment. Both the Erk and NTRK inhibitors blocked NT-3 induced Erk1/2 phosphorylation (Figure #3.6). K252a has been shown to inhibit the protein tyrosine kinase domain of the NTRK receptors (Tapley, et al. 1992), which would suggest that K252a should inhibit the autophosphorylation of FL-NTRK3 in MIAMI cells. Therefore, the observed decrease in basal and NT-3 stimulated Erk1/2 phosphorylation levels is due most likely to a decrease in NT-3 (Figure #3.3C) signaling via FL-NTRK3. We could hypothesize that if NT-3 stimulated Erk1/2 phosphorylation via TKd-NTRK3, there would have been no observed effect upon addition of K252a, which was not the case. These results indicate that the inhibition of FL-NTRK (contains PTK domain) receptor in MIAMI cells blocks NT-3 stimulated Erk1/2 phosphorylation. Therefore we can conclude that that NT-3 signals through FL-NTRK3 (Figure #3.2B) leading to Erk1/2 phosphorylation in MIAMI cells, similar to NT-3 stimulation of NSCs during neurogenesis (Bibel and Barde 2000)
NT-3 mediated regulation of gene expression during neuronal commitment

Neurotrophin induced neuronal differentiation is not only characterized by rapid signal transduction, but also by the long term up-regulation of pro-neuronal genes leading to characteristic morphological changes, such as the initiation and extension of neurite processing (Bibel and Barde 2000, Kennea and Mehmet 2002, Singh, et al. 2008). During the neuronal differentiation of NSCs, there is an up-regulation of pro-neuronal transcription factors (Math1, Mash1, Prox1) (Parras, et al. 2002, Yi, et al. 2008), neurofilaments (Nestin, NFL, NFM, NFH, TuJ1) and microtubule associated proteins (MAP2, MAP1B) involved in neurite formation (Dehmelt, et al. 2003, Lariviere and Julien 2004). Conversely there is a decrease in transcription factors involved in NSCs maintenance, proliferation and survival (HES1, HES3, HES5, MSI1, Ngn2) (Kageyama, et al. 2008, Okano, et al. 2005, Yi, et al. 2008).

Of the genes stimulated by NT-3 during neuronal commitment Ngn2 and NFL decreased (≥1.5 fold), and three of the genes which increased (≥1.5 fold) were Nestin, microtubule associated protein 2 (MAP2), and a slight increase in CCND1. Another gene which increased was Calbindin (CALB1), a known calcium binding protein found mainly in post-mitotic functionally mature dopaminergic neurons (Thompson, et al. 2005) that has been implicated in neuronal differentiation and neurite outgrowth in hippocampal progenitor cells (Kim, et al. 2006).

The downstream NT-3 stimulated up-regulation of pro-neuronal genes such as MAP2 and Nestin, which are found in NSCs and increase during neuronal differentiation (Dehmelt, et al. 2003), as well as CALB1, imply that NT-3 is directing the MIAMI cells
toward the neuronal lineage. The decrease in \textit{Ngn2} and \textit{NFL} suggests a decrease in self-renewal and proliferation as seen in NSCs (Yi, \textit{et al.} 2008), as well as a decrease in an early-neural intermediate filament (NFL) which is present during neurite-initiation, but decreases during neurite-extension (Lariviere and Julien 2004). Even though there is a decrease in the self-renewal marker \textit{Ngn2}, in addition to \textit{Oct4a}, there is still a slight increase in \textit{CCND1} in addition to an observed NT-3 stimulated cell proliferation. This suggests that NT-3 stimulated MIAMI cells remained in a proliferative state and are not progressing toward withdrawal from the cell cycle, as is required for NSC maturation into an immature post-mitotic neuron (Demir, \textit{et al.} 2009, Politis, \textit{et al.} 2008).

During NSCs differentiation toward an immature neuron, NFL is known to decrease, while MAP2, NFM and NFH increase driving a hallmark characteristic of neurons, neurite-extension and maturation (Lariviire and Julien 2004). NT-3 stimulation increased \textit{MAP2} and decreased \textit{NFL}, but no change was seen in \textit{NFM} or \textit{NFH} expression. Therefore, NT-3 stimulation during neuronal commitment of MIAMI cells induces transcriptional events only partially characteristic of a neural precursor cell becoming committed to the neuronal lineage.

Additional evidence for NT-3 stimulation of downstream gene expression via FL-NTRK3 was further verified by the decrease in \textit{NFM}, \textit{MAP2}, and \textit{CCND1} that was observed when the MIAMI cells were pretreated with the NTRK inhibitor (K252a). There was also a lack of an NT-3 induced increase in \textit{Nestin} and \textit{MAP2} or a decrease in \textit{Ngn2} and \textit{NFL}, in the presence of the Erk1/2 inhibitor during neuronal commitment. The opposite stimulatory pattern was seen in NT-3 stimulated expression profiles for \textit{CCND1}, \textit{Oct4a}, \textit{MAP2}, \textit{NFL} and \textit{NFM}, indicating that 1) FL-NTRK phosphorylation is required
for maintaining gene expression and/or 2) NT-3 is inducing altered gene expression via an NTRK receptor which does not have a PTK domain and is not inhibited via K252a, such as TKd-NTRK3. Taking into account the decrease in basal and NT-3 stimulated Erk1/2 phosphorylation in the presence of the NTRK inhibitor (Figure #3.6), these results provide direct evidence for an NT-3 stimulated mechanism of downstream gene regulation via FL-NTRK3 and Erk1/2 phosphorylation in MIAMI cells.

**Limited neuronal commitment potential: Implication for Hes genes and Notch signaling during in vitro neuronal differentiation**

MIAMI cells exhibited a limited NT-3 stimulatory potential compared to NT-3 stimulated NSCs. Therefore, we examined early transcription factors involved in and classified as “master repressors or activators” of neurogenesis.

MIAMI cells expressed mRNA levels for the repressor bHLH (basic helix-loop-helix) transcription factors *Hes1* and *Hes5*, but not *Hes3*, as well as the activator “pro-neuronal” transcription factors *Mash1, Math1, Math5* and *Ngn2*. *Hes1* and *Hes5* govern NPC proliferation, maintenance and self-renewal as well as repress the neuronal commitment of NSCs via repression of *Ngn2, Nestin, TuJ1, NeuN* (Kageyama, *et al.* 2008, Nakamura, *et al.* 2000), and were unchanged during neuronal commitment of MIAMI cells. Contrary to this finding there was still an increase in *Ngn2, Nestin*, and *CALB1*. In addition the only activator or pro-neuronal master transcription factor found to increase during NT-3 stimulation was *Ngn2*, but not *Math1, Math5* or *Mash1*. The observed profile of NT-3 stimulated gene expression is contradictory to the developmental program of NSCs as they progress toward a committed neuronal cell, in

Further evaluation of MIAMI cells has also shown that they express and have active stimulation of the Notch signaling cascade and Notch intracellular domain (Rios 2010), which is known to transcriptionally up-regulate *Hes1* and *Hes5* mRNA expression. In addition, *Mash1*, *Math1* and *Ngn2* expression is inhibited in NSCs via transcriptional repression by *Hes1* and *Hes5*. *Mash1* and *Ngn2* are also known to induce Notch activation thereby inhibiting the neuronal differentiation of neighboring cells (Castro, *et al.* 2006). Therefore in the developing brain, NSCs which have been stimulated to undergo neurogenesis have decreased *Hes1* and *Hes5* levels, resulting in an increase in *Mash1* and *Ngn2*, leading to Notch activation and the inhibition of neurogenesis in neighboring cells via *Hes1/Hes5* up-regulation. This process is known as lateral inhibition and is important in the development of the human brain and segregation of neurons and neural precursor cells (Artavanis-Tsakonas 1999).

Therefore, if MIAMI cells have active Notch signaling occurring during NT-3 stimulation, we could hypothesize that there would be mixed pro- and anti-neuronal stimuli occurring simultaneously in a continual feedback loop. This may explain the limited potential of MSC such as MIAMI cells to be differentiated into mature functional neuron-like cells *ex vivo*, as well as explain the lack of transcriptional regulation seen with the *Hes1* and *Hes5* genes as well as *Mash1*, *Math1*, *Math5* and *Ngn2*. As indicated above, MIAMI cells active Notch signaling, which has been suggested to play a role in their self-renewal capabilities (Rios 2010). Therefore it would be of interest to inhibit
Notch signaling in MIAMI cells during NT-3 stimulated neuronal commitment and determine if there is a subsequent decrease in *Hes1* and *Hes5* as well as an increase in *Mash1, Math1, Math5, and Ngn2*, allowing for increased progression toward neuronal commitment. This would elucidate the potential role for the master neuronal repressor (*Hes1 /Hes5*) and activator (*Math1, Mash1, Math5, Ngn2*) transcription factors, as well as the similarity between NSCs transcriptional regulation and MIAMI cells during neuronal commitment. In addition this might explain the increased NT-3 stimulated pro-neuronal gene expression compared with the limited expression profile seen during normal neuronal commitment of MIAMI cells as compared with NT-3 stimulation of NSCs during development (Bibel and Barde 2000).

*Characterization and other possible roles for NTRK3 in MIAMI cells*

The NTRK3 receptor also appears to be very stable in MIAMI cells due to the inability to decrease NTRK3 protein levels via siRNA ablation (Figure #3.12 & #3.13), as well as the low rate (17%) of NTRK3 protein turnover (Figure #3.15). One hypothesis is that membrane-bound NTRK3 is recycled or remains on the cell surface due to a lack of receptor activation over time. This phenomenon has been described in CNS neurons in which NTRK receptors are internalized into intracellular vesicles in the absence of signal. The NTRK receptors are then re-inserted into the cell surface by exocytosis upon electrical activity, cAMP or increase Ca2+ stimulation (Du, *et al.* 2000, Meyer-Franke, *et al.* 1998). This may be the case in MIAMI cells. Even though we see low levels of endogenous NT-3 secretion and basal Erk1/2 phosphorylation levels, it may be that the majority of cell membrane bound NTRK3 is inactive and recycled. This does not explain
why continuous basal levels of NTRK3 mRNA are observed, as this would be inefficient for the cell to continue mRNA production while recycling the NTRK3 protein.

TKd-NTRK3 was found to localize to the nucleus of MIAMI cells, whereas staining with an antibody which recognizes all NTRK3 isoforms showed a more homogeneous distribution (Figure #3.16). The intracellular domain of TKd-NTRK3 shares 36% sequence homology with the DNA binding domain of RadA (S. aciditrophicus), further suggesting a role of TKd-NTRK3 in the nucleus. It is also unclear whether the entire TKd-NTRK3 receptor or only the intracellular TKd-domain is localized to the nucleus. The nuclear localization of a protein tyrosine kinase receptor is not unheard of. The EGF receptor, EGFR, has been shown to enter the nucleus and play a direct role in transcriptional regulation (Dittmann, et al. 2010). Future work will have to be done to re-confirm this exciting finding suggesting a role for TKd-NTRK3 localization in the nucleus.

Variations between vertebral and iliac crest bone marrow-derived MIAMI cells

MIAMI cells were first isolated from the vertebral bodies of cadaveric donors (VB-MIAMI) (D'Ippolito, et al. 2004) and characterized as being able to differentiate into multiple cells lineages (D'Ippolito, et al. 2006), including neuron-like cells (Tatard, et al. 2007). The 3-step neuronal differentiation program for the trans-differentiation of MIAMI cells into neuron-like cells was based on the molecular profile of VB-MIAMI (3 yr old male) cells which expressed similar NTRK3 protein and mRNA levels, but also expressed low levels of NTRK1 and NTRK2 protein during expansion and neuronal specification (Step #1). This is in comparison to iliac crest derived MIAMI cells (IC-
MIAMI) (20 yr old male) which express NTRK3, NTRK2 and NTRK1 mRNA, but only NTRK3 protein. In addition, VB-MIAMI cells treated with NT-3 during neuronal commitment had an increase in Nestin, TuJ1, NTRK1, NFM, NeuN, and NSE (Tatard, et al. 2007). We report here that IC-MIAMI cells stimulated with NT-3 have an increase in Nestin, MAP2, and CALB1, and a decrease in Ngn2 and NFL. There was no increase in TuJ1, NFM, NeuN, or NSE in NT-3 stimulated IC-MIAMI cells compared to that reported in VB-MIAMI cells. There was also only a slight increase in NTRK1 in IC-MIAMI cells, compared to a 40-50% increase in NTRK1 after NT-3 stimulation of VB-MIAMI cells. One similarity between the two cell types is the observed increase in neurite-like extension after neuronal commitment (Step #2) in both IC and VB-MIAMI cells. Comparing these two isolations of MIAMI cells induced toward neuronal commitment (Step #2) using identical protocols, suggests apparent differences between MIAMI cells derived from different skeletal locations and/or different donors.

The neuronal differentiation (Step #3) of MIAMI cells incorporates all three neurotrophin factors NT-3, BDNF and NGF which bind primarily NTRK3, NTRK2 and NTRK1, respectively (Urfer, et al. 1995). The addition of NT-3, BDNF and NGF during neuronal differentiation (Step #3) was determined as the optimum conditions to push VB-MIAMI cells toward a neuron-like cell due to their basal expression levels of NTRKs in addition to the high expression of NTRK3 and up-regulation of NTRK1 during neuronal commitment (Step #2). Again the results described here display the different profiles and effects of NT-3 stimulation on two different MIAMI cell isolates. Therefore, the neuronal differentiation (Step #3) of IC-MIAMI cells should respond differently compared to VB-MIAMI cells when stimulated with NT-3, NGF and BDNF. Future
studies directing bone marrow-derived MIAMI cells toward neuron-like cells for implementation into autologous cell-therapy based procedures will have to take into account varied neurotrophin receptor profile(s) in addition to secretion of the neurotrophin ligands, prior to *ex vivo* expansion and differentiation.

*Rac1 regulates NT-3 induced gene expression via Erk1/2*

We have shown that NT-3 stimulation of MIAMI cells during neuronal commitment induces short-term Erk1/2 phosphorylation, as well as up-regulation of a limited number of pro-neuronal genes linked to the activation of FL-NTRK3. We tested the hypothesized that TKd-NTRK3 activates Rac1 upon NT-3 stimulation as shown previously in HEK293T cells (Esteban, *et al.* 2006), via the addition of a chemical Rac1 inhibitor during neuronal commitment (Step #2). NT-3 stimulation of MIAMI cells in the presence of a Rac1 inhibitor caused an increase in Erk1/2 phosphorylation (Figure #3.6 and #3.8) and up-regulation of pro-neuronal genes, as well as a decrease in cell proliferation (Figure #3.7). These results indicate that Rac1 regulates the extent of NT-3 stimulated Erk1/2 phosphorylation, thereby mediating the downstream regulation of gene expression. It is unclear from this data whether Rac1 inhibition of NT-3 induced Erk1/2 phosphorylation is dependent or independent of TKd-NTRK3/NT-3 signaling.

If we hypothesize that the Rac1 inhibitor did inhibit Rac1 activation and signaling, allowing for increased Erk1/2 phosphorylation via NT-3 (Figure #3.6A), then Rac1 is repressing the NT-3 stimulation of downstream pro-neuronal genes via the Erk1/2 signaling cascade. Of the genes up-regulated during neuronal commitment in the presence of the Rac1 inhibitor, *Ngn2, Oct4a, REST* and *coREST* are important
transcription factors known for their role in the self-renewal and maintenance of neural precursor cells (Yi, et al. 2008), as well as the repression of pro-neuronal gene transcription via chromatin remodeling (Lunyak and Rosenfeld 2005). Nestin, NFL, NFM, NFH, and MAP2 were also found to be up-regulated and are indicative of neurogenesis, specifically neurite initiation and extension (Dehmelt, et al. 2003).

Rac1 appears to regulate NT-3 stimulated pro-neuronal genes during neuronal commitment, in addition to the regulation of genes involved in self-renewal and maintenance of NSCs and stem cells. These results fit the known role of Rac1 in axonal formation within the hippocampus (Corbetta, et al. 2009), formation of the midline commissures and migration of hippocampal neurons and axon formation (Chen, et al. 2007), in addition to the direct role of Rac1 in lamellipodia and neurite-initiation (de Curtis 2008, Pullikuth and Catling 2007). Rac1 is also known to accumulate in the nucleus during the G2 phase of the cell cycle promoting cell division (Michaelson, et al. 2008), which fits with the decrease in NT-3 stimulation of cell proliferation upon Rac1 inhibition. Therefore, Rac1 appears to play a central role in MIAMI cell neuronal commitment (Step #2) via the regulation of Erk1/2 phosphorylation, downstream gene expression, and cell proliferation. This might also explain the limited stimulation of pro-neuronal genes during NT-3 stimulation as seen during normal neuronal commitment.

The studies reported here indicate the potential of MIAMI cells to be stimulated by NT-3 inducing the limited up-regulation of pro-neuronal genes, as well as stimulating cell proliferation and the formation of neurite-like processes. NT-3 stimulation of FL-NTRK3 induced phosphorylation was shown to regulate downstream gene expression via Erk1/2 activation, using the chemical inhibitor K252a and U0126, and thus providing a
direct mechanism for NT-3 directed neuronal commitment of MIAMI cells. In addition, Rac1 was found to repress NT-3 stimulated Erk1/2 phosphorylation, thereby repressing downstream pro-neuronal gene expression as well as cell proliferation. The findings described here for the first time present a mechanistic model for the NT-3 stimulated neuronal commitment of human bone marrow-derived MIAMI cells (Figure #6.1).

**Figure#6.1:** Model of the NTRK3 / NT-3 mediated signaling pathways involved in the regulation of signal transduction and downstream gene regulation during the neuronal commitment of MIAMI cells. Black solid arrows or text indicate data driven conclusions. Grey dashed arrows or text depict hypothetical activation pathways or protein interactions. FL-NTRK3 has a functional protein tyrosine (PTK) kinase intracellular domain and was found to be phosphorylated upon NT-3 stimulation, inducing Erk1/2 phosphorylation and downstream gene regulation. TKd-NTRK3 is truncated containing a tyrosine kinase deficient intracellular domain and was found to be rapidly degraded upon NT-3 stimulation, but it is unclear as to its role during neuronal commitment and or Rac1 activation. Rac1 was found to regulate NT-3 stimulated Erk1/2 phosphorylation, as well as cell proliferation and repression of downstream gene expression.
Repressed via Rac1:
Nestin, MAP2, NFL, NFM, NFH
NTRK2, p75NTR
NT3
REST, coREST, NGN2
RAC1B REGULATES NT-3 INDUCED ERK1/2 PHOSPHORYLATION AND STIMULATION OF DOWNSTREAM GENE EXPRESSION

In view of the previously reported activation of Rac1 via NT-3 stimulation of TKd-NTRK3 (Esteban, et al. 2006), as well as the observed Rac1 regulation of Erk1/2 phosphorylation in MIAMI cells, the question is raised as to the role of Rac1 in MIAMI cells during NT-3 mediated neuronal commitment.

Rac1 is a small G-protein which undergoes normal GEF / GAP regulation in which its active form is Rac1-GTP, while its inactive form is Rac1-GDP. In addition one of the main downstream partners of Rac1-GTP is PAK1. MIAMI cells stimulated with NT-3 during neuronal commitment had inconsistent activation of Rac1 via pull-down of active Rac1-GTP via a PAK1 pull-down assay (Figure #4.2).

Rac1 has two major isoforms, Rac1a and Rac1b, and upon further characterization of MIAMI cells, the Rac1b isoform was found to be predominant (Figure #4.3), as well as expressed homogeneously throughout MIAMI cell cultures (Figure #4.4). There were also no distinguishable double bands corresponding to both Rac1a and Rac1b protein, suggesting that only Rac1b is expressed at the protein level. This result is not absolutely clear due to the small (0.2kDa) difference between the molecular weights of the two isoforms that would prove difficult to distinguish via western blot analysis. Although, others have described being able to distinguish between the two isoforms in a colorectal cancer cell line (Caco-2) (Matos, et al. 2008).

The predominance of Rac1b in MIAMI cells suggests that the previously reported Rac1 regulation of NT-3 stimulated neuronal commitment is due to the Rac1b isoform. Rac1b is the constitutively active isoform of Rac1. It contains a 19 amino acid insert
near its binding domain/pocket that prevents the hydrolysis of GTP to GDP (Fiegen, et al. 2004), causing Rac1b to always be in an active form as Rac1b-GTP. The presence of Rac1b in MIAMI cells causes confusion regarding its hypothesized activation via NT-3 stimulation, due to the fact that Rac1b has been reported to interact with the PAK1 GTPase-binding domain, but not with full length PAK1 (Fiegen, et al. 2004). Yet in another report, Rac1a-GTP but not Rac1b-GTP was found to interact with PAK1 (Lozano, et al. 2008). Therefore, it is unclear as to the specificity of the PAK1 pull-down assay for Rac1b, as well as the activation state of Rac1 in MIAMI cells.

There are a few other possibilities for irregular fluctuations in Rac1 activity. Rac1 along with cdc42 are known to regulate not only cytoskeletal changes (de Curtis 2008), but also G1 cell cycle progression independent of PAK1 (Lamarche, et al. 1996). One possibility to explain the fluctuations in activity is that the timing of cell cycle progression throughout the experiments was not identical, resulting in varied Rac1-GTP / PAK1 pull-down levels. However, this is unlikely since MIAMI cells are serum starved after neuronal specification (Step #1), inducing 80-90% of the cells to synchronize in G1 (Figure #2.1). It is also possible that NT-3 does not stimulate Rac1 activation and the observed results are due to activation via other unrelated cellular events.

With respect to NTRK3 mediated Rac1 activation, the previously reported research showing that TKd-NTRK3 activated Arf6-Rac1 was performed in HEK293T cells over-expressing TKd-NTRK3, and which do not express endogenous NTRK3 (Esteban, et al. 2006). In addition, HEK293T cells only express Rac1a, not Rac1b (Esufali et al., 2007). Hence, the results in these two reports indicating that TKd-NTRK3 activates Rac1 in HEK293T cells must have been due to activation of Rac1a not Rac1b.
Although the data presented neither support nor reject TKd-NTRK3 activation of Rac1a, due to the high levels of constitutively active Rac1b, Rac1a activation might not be detected or have biological significance. In that Rac1b is the predominant Rac1 isoform found in MIAMI cells (Figure #4.3), in addition to the inconsistent pull-down of active Rac1 via PAK1-PBD (Figure #4.2), it is hypothesized that NT-3 stimulation via TKd-NTRK3 could not activate Rac1a in MIAMI cells. This also suggests that the previously observed Rac1 regulation of NT-3 stimulated Erk1/2 phosphorylation is independent of TKd-NTRK3.

In order to determine the role of Rac1b in MIAMI cells, siRNA mediated knockdown was used to decrease Rac1b during induction of neuronal commitment (Step #2) (Figure #4.5). Rac1b was found to regulate the magnitude of Mek1/2 and Erk1/2 phosphorylation levels as well as the duration of Erk1/2 phosphorylation (Figure #4.6). Unlike the increase in NT-3 stimulated Erk1/2 phosphorylation when using the chemical Rac1 inhibitor (Figure #3.6 & #3.8), specific knockdown of Rac1b induced a decrease in the magnitude of Erk1/2. These results in conjunction with the results obtained using the chemical Rac1 Inhibitor confirm that Rac1, specifically the constitutively active Rac1b isoform, is regulating Mek1/2-Erk1/2 phosphorylation during NT-3 induced neuronal commitment of MIAMI cells. It is interesting to note that cRaf1 was not phosphorylated due to NT-3 stimulation (Figure #4.6), which is in contrast to the known Raf1-Mek1/2-Erk1/2 signaling cascade activated via neurotrophin signaling in NSCs during neurogenesis (Bibel and Barde 2000, Lim, et al. 2007).

The results described above suggest possible alternative Mek1/2-Erk1/2 activation mechanisms in view of the novel finding that cRaf1 is not involved during Rac1b
regulation of NT-3 mediated Mek1/2-Erk1/2 activation in MIAMI cells. There are other reported mechanisms of Mek1/2-Erk1/2 activation including 1) Rac1/PAK1 binding to MEK1 priming MEK1 for cRaf1 induced phosphorylation (Coles and Shaw 2002), 2) Rac1-Pak1-Mek1-Erk2 signaling cascade (no cRaf1 involvement) (Eblen, et al. 2002) and 3) Reactive Oxygen Species (ROS) induced phosphorylation of Erk1/2 (no cRaf1 involvement) (Mehdi, et al. 2005). Due to the lack of any NT-3 stimulated increase in cRaf1 phosphorylation (Figure #4.6), the direct Rac1-Pak1-Mek1-Erk2 (2) signaling cascade or the indirect ROS mediated activation of Erk1/2 (3) might explain the mechanism of regulating Mek1/2-Erk1/2 phosphorylation. In addition, Rac1b is known to produce ROS (Radisky, et al. 2005), providing further evidence that Rac1b may be indirectly regulating the extent of Mek1/2-Erk1/2 phosphorylation via a Rac1-ROS mechanism.

Analysis of the previously identified Rac1 regulated genes also showed that ablation of Rac1b via siRNA (Figure #4.5) increased the NT-3 stimulated expression of the Ngn2, MAP2, NFH, NFL and REST. The observed increase was inhibited in the presence of the Erk1/2 inhibitor (U0126) (Figure #4.7), verifying the regulation of gene expression via Rac1b regulation of Erk1/2 phosphorylation. In addition there was also a 20-30% increase in neurite-like extensions in MIAMI cells during Rac1b ablation (Figure #4.8), further supporting the known role of the up-regulated genes MAP2, NFH and NFL in neurite initiation and extension (Dehmelt, et al. 2003, Lariviere and Julien 2004) during MAIMI cell neuronal commitment (Step #2). These results verify that the NT-3 stimulated increase of pro-neuronal genes involved in neurite formation in MIAMI cells is repressed via Rac1b regulation of the Mek1/2-Erk1/2 pathway.
Additional questions arise as to the purpose and role of Rac1b in an hMSC population such as MIAMI cells, since the discovery of the Rac1b isoform in colorectal cancer cells gave birth to its nickname as the “Tumor-Specific Splice Variant” of Rac1 (Esufali, et al. 2007). Regarding the tumorogenic role of Rac1b, Rac1b was discovered as a splice variant of Rac1 over-expressed in malignant colorectal cancer cells (Jordan, et al. 1999), and has also been shown to activate NFκβ activation leading to EMT induction and ROS formation in mouse mammary carcinoma cells (SCp2 cells)(Radisky, et al. 2005), as well as increase tumor cell survival in colorectal cells (Caco-2) via transcriptional activation of CyclinD1 and the canonical Wnt pathway along with other mechanisms (Esufali, et al. 2007, Matos and Jordan 2005). In addition the concentration of Rac1b protein is known to increase in the cell because it does not undergo normal proteosomal cycling as is the case with Rac1a protein, which is ubiquitinated leading to degradation and turn-over (Visvikis, et al. 2008).

In MIAMI cells Rac1b ablation was found to decrease NT-3 stimulated cell proliferation (Figure #4.8), independent of Erk1/2 activation, suggesting its role in the rate or check-point progression of the cell-cycle separate from its Mek1/2-Erk1/2 regulatory role. Contrary to the findings of Matos and Jordan (2005), as well as those of Esufali (2007), CyclinD1 as well as CyclinB1 mRNA expression levels increased when Rac1b levels were decreased via siRNA (Figure #4.8), but did not change after NT-3 stimulation. In addition, the cyclin-dependent kinase inhibitors p21 and p27 as well as p53 mRNA expression levels did not change during Rac1b ablation.

Following up on the role of Rac1b in cell-cycle progression, flow cytometry analysis showed that in MIAMI cells with decreased Rac1b levels, there was no
difference in the profile of MIAMI cells in G0/G1 or G2/M during expansion, neuronal specification or commitment, as well as after serum starvation (Figure #4.9), indicating that Rac1b is not required for cell-cycle progression. The increase in CyclinD1 as well as CyclinB1, which are involved in the progression of G1-S and G2-M during the cell cycle, does not fit with the observed decrease in NT-3 stimulated cell proliferation when Rac1b levels are decreased, suggesting two independent roles for Rac1b in the cell cycle. The data shows that Rac1b ablation decreases NT-3 stimulated cell growth, yet increases CyclinD1 and CyclinB1 mRNA expression. Additionally, there is no change in the percent of cells in G0/G1, S or G2/M phase of the cell cycle in normal or Rac1b ablated MIAMI cells. Therefore, the effect of Rac1b on MIAMI cell proliferation does not appear to be due to transcriptional or Erk1/2 regulation, nor due to cell-cycle checkpoint regulation. It could be speculated that Rac1b is regulating the rate of cell-cycle progression, therefore when Rac1b protein levels are reduced, there is decreased NT-3 stimulated cell growth.

The scope of this analysis is limited to the role Rac1b plays in the transcription of cell-cycle regulatory proteins, and does not account for changes in protein levels and/or activation of cyclins and cyclin dependent kinases, which may explain the observed decrease in NT-3 stimulated cell proliferation. Rac1 has also been reported to accumulate in the nucleus during the G2 phase of the cell cycle, thereby promoting the G2-M transition (Michaelson, et al. 2008). This study was done via over-expression of RAC1 and did not distinguish between Rac1a or Rac1b protein. It may be that the observed NT-3 stimulation of cell proliferation is due to direct nuclear localization of Rac1b, thus increasing the rate of the G2-M transition. This could be determined in the
future using flow cytometry analysis with short time points following NT-3 stimulation to determine the rate of cell cycle progression in MIAMI cells with decreased Rac1b levels, as well as using immunocytochemistry to determine Rac1b localization.

Another role of Rac1 which has not been investigated in MIAMI cells is the direct role of the Rac1a protein in modulating neurite extension and growth cone formation via protein-protein interactions with microtubule associated proteins, neurofilaments and F-actin (Grabham, et al. 2003). In addition, Rac1a is known to activate PAK1 leading to growth cone localized MEK/ERK activation and inhibition of stathmin/Op18, (microtubule destabilization proteins), allowing for microtubule assembly and extension (review (Pullikuth and Catling 2007)). Both of these reports show that Rac1a plays a direct role in neurite extension and growth cone formation, and provide another hypothesis for the observed regulation of NT-3 stimulated Mek1/2-Erk1/2 phosphorylation via Rac1b. It is also possible that Rac1b functions in a fashion similar to Rac1a in modulating direct neurite formation.

Future work will have to be done to determine the role Rac1b plays in hMSC, such as MIAMI cells, to determine if its role in proliferation can be generalized to all MSC populations, or has been selected for during MIAMI cell isolation. In addition, the observed NT-3 stimulation of MIAMI cells, as well as the limited expression of pro-neuronal genes are both attributed to the presence of Rac1b. Taking into account the previously discussed role of Rac1 during CNS development and neurite formation, in combination with the Rac1b repression of pro-neuronal genes involved in neurite formation in MIAMI cells, it could be hypothesized that Rac1b is responsible for the role of \( \text{RAC1} \) seen during CNS development.
Another possible hypothesis is that the splicing of Rac1 between Rac1a and Rac1b is a mechanism to promote, and/or repress the expression of proneuronal genes as well as cell proliferation, thereby repressing MSC or other somatic tissues from transdifferentiation into neuron-like cells. A similar mechanism is seen with the transcriptional repressor NRSF (REST) which represses neuronal gene transcription in non-neuronal tissues via heterochromatin formation, and is known as a “master negative regulator of neurogenesis” (Lunyak and Rosenfeld 2005, Singh, et al. 2008).

The studies reported here have described novel roles for Rac1b in MIAMI cells including 1) the regulation of NT-3 stimulated Mek1/2-Erk1/2 phosphorylation and regulation of downstream pro-neuronal genes involved in neurite formation, 2) repression of *CyclinD1* and *CyclinB1* mRNA expression, and 3) its direct involvement in the observed and previously documented (Tatard, et al. 2007) NT-3 stimulated cell proliferation of MIAMI cells during neuronal commitment. In addition, the culmination of this work provides a model for the NT-3 induced neuronal commitment of MIAMI cells *in vitro*, as well as insight as to the neurogenic potential of hMSCs for future applications in cell therapy based tissue repair.
Figure 6.2: Model representing the neurotrophin mediated neuronal commitment of MIAMI cells *in vitro*. Rac1b was found to regulate the NT-3 stimulation of Mek1/2-Erk1/2 phosphorylation, as well as the repression of pro-neuronal genes involved in neurite formation. In addition, Rac1b was found to repress the levels of *CyclinD1* and *CyclinB1*, in contrast to its role in NT-3 stimulated cell proliferation.
EGF/bFGF PRETREATMENT OF MIAMI CELLS ENHANCES NT-3 STIMULATED NEURONAL COMMITMENT


Previous reports have shown that in MSCs, the expression of Nestin, a marker expressed by NSC (Gilyarov 2008), was up-regulated after EGF/bFGF exposure under adherent (Song, et al. 2007) or non-adherent conditions prior to in vitro neurogenesis (Hermann, et al. 2004, Kim, et al. 2006, Yang, et al. 2008). Furthermore, studies have also described the differentiation of MSCs, after a first-step pretreatment with EGF/bFGF, into dopamine producing cells leading to a functional improvement in a rat model of Parkinson’s disease (Barzilay, et al. 2008, Levy, et al. 2008). However, a precise characterization of the impact of an EGF/bFGF pretreatment, prior to MSC neuronal differentiation is still lacking (Hermann, et al. 2004, Kim, et al. 2006, Long, et al. 2005, Song, et al. 2007, Yang, et al. 2008). Therefore MIAMI cells pretreated with EGF/bFGF in combination or bFGF alone were characterized upon NT-3 stimulated neuronal commitment.
Analysis of the NT-3 receptor, NTRK3 revealed that both the full length (FL-NTRK3) and the tyrosine kinase deficient isoform (TKd-NTRK3) of NTRK3 were up-regulated along with the NGF receptor, NTRK1, after EGF-bFGF pre-treatment using higher concentrations (50-100ng/ml) (Figure #5.1), but were not observed using bFGF pre-treatment alone or at lower concentrations. In addition, NT-3 stimulation during neuronal commitment of EGF/bFGF pre-treated MIAMI cells increased the duration of Erk1/2 phosphorylation (Figure #5.2), as well as decreased NT-3 stimulated cell proliferation (Figure #5.3), presumably due to the observed decrease in the G2/M specific cell cycle regulatory protein CyclinB1. Both events are similar to the previously observed regulation of NT-3 stimulated cell proliferation and Erk1/2 phosphorylation via Rac1b. In addition, Rac1b was also found to increase after EGF/bFGF pre-treatment (Figure #4.4), providing suggestive evidence for the role for EGF/bFGF in modulating Rac1b regulation of MIAMI cells during NT-3 mediated neuronal commitment. These results show that MIAMI cells are responsive to EGF/bFGF, inducing an increased potential for neurotrophin mediated stimulation during neuronal differentiation.

Additional analysis during NT-3 stimulated neuronal commitment on downstream gene expression suggests that EGF/bFGF cooperatively enhance NT-3 induced neuronal commitment in vitro. The effect of EGF/bFGF pretreatment on NT-3 stimulated gene expression was cooperative in some cases (Prox1, Ngn2, MSI1) and additive in others (NFH, NFM, NFL) (Figure #5.2).

Mash1 and Ngn2 are two transcription factors commonly found in NPC (Parras, et al. 2002), and are known to stimulate the expression of the pro-neuronal transcription factor Prox1(Yamamoto, et al. 2001). Here we see that pre-treatment with bFGF alone
caused an NT-3 stimulated increase in both \textit{Ngn2} and its downstream target \textit{Prox1}. \textit{Prox1} is also known to regulate interneuron neurogenesis in the spinal cord by pushing the NPC out of the cell cycle, thereby initiating the expression of pro-neuronal genes and a post-mitotic phenotype (Misra, \textit{et al.} 2008). In addition, there was an observed decreasing trend in the pro-neural RNA binding protein, \textit{Musashi-1} (\textit{MSI1}) which is known to regulate NPC self-renewal and proliferation (MacNicol, \textit{et al.} 2008, Okano, \textit{et al.} 2005). This would suggest that bFGF pre-treatment of MIAMI cells enhances the NT-3 stimulated response of MIAMI cells pushing transcriptional regulation toward the neuronal lineage.

In contrast, pre-treatment with both EGF/bFGF prior to neuronal commitment caused an increase in \textit{MSI1}. Moreover, there was no observed stimulation of \textit{Ngn2} or \textit{Prox1} expression. These results show a cooperative stimulatory effect of EGF/bFGF pre-treatment on MIAMI cells, in addition to the up-regulation of genes involved in NPC self-renewal and proliferation (Okano, \textit{et al.} 2005). The combined data suggests that the cooperative effect of EGF/bFGF pre-treatment on NT-3 stimulation leads to the maintenance of a neural precursor-like phenotype, while bFGF alone enhances NT-3 induced neuronal commitment.

One finding which is contrary to this hypothesis was the additive and concentration-dependent increase in \textit{NFL}, \textit{NFM} and \textit{NFH} expression upon pretreatment with bFGF alone or EGF/bFGF in combination (Figure #5.2). This result fits with the previously discussed decrease in NT-3 stimulated cell proliferation after pretreatment with bFGF or EGF/bFGF in combination. It may be that MIAMI cells have two
populations of bFGF or EGF/bFGF responsive cells, which cannot be distinguished via RT-qPCR analysis of a cDNA library.

In early rodent embryos, it is known that CNS stem cells are responsive to only bFGF. After a few cell divisions, a sub-population then becomes responsive to EGF, in addition to bFGF (Ciccolini and Svendsen 1998). One model in human embryonic stem (ES) cells suggests that ES cells give rise directly to bFGF responsive cells which lead to the formation of the neural tube, followed by rise in EGF or EGF/bFGF responsive neural stem cells leading to neurogenesis (Tropepe, et al. 1999). In adult rodent models there is also evidence for a bimodal population of neural stem cells which maintain either exclusive bFGF responsiveness or are responsive to both EGF and bFGF (Tropepe, et al. 1999).

Taking into account the known models for bFGF versus EGF/bFGF responsiveness, it suggests the possibility that a fraction of MIAMI cells are responding to only bFGF, in addition to a sub-fraction becoming responsive to both EGF/bFGF. Or it may be that all MIAMI cells are uniformly responsive to both EGF/bFGF and there are distinct mechanisms controlling the transcriptional expression of neurofilaments compared to the transcription factors *MSII, Ngn2,* and *Prox1.*

Here we have shown that MIAMI cells are responsive to EGF/bFGF or bFGF alone similar to ES and NPC cells. One limitation of this study is the absence of pretreatment with EGF alone, which would have illuminated the role of EGF as seen during EGF/bFGF pretreatment. In addition, pretreatment of MIAMI cells can be utilized in the future to further enhance their NT-3 stimulated neuronal commitment, pushing
toward a self-renewing neural precursor like phenotype, or towards a more pro-neuronal phenotype. This scenario should allow for the pre-enrichment of MIAMI cells prior to use in cell-therapy based repair of neural tissue in the future.

**FINAL CONCLUSIONS AND REMARKS**

The studies presented here represent the systematic analysis of neurotrophin signaling and the role of Rac1b regulation and EGF/bFGF stimulation in order to determine a mechanism for the NT-3 stimulated neuronal commitment of MIAMI cells *in vitro*. We were able to determine that similar to NSCs stimulation via NT-3, FL-NTRK3 is phosphorylated, thus inducing downstream Mek1/2-Erk1/2 phosphorylation. In addition, cRaf1 was not stimulated via NT-3, suggesting an alternate mechanisms for FL-NTRK3 mediated activation of Mek1/2-Erk1/2. The data support a Rac1b-ROS or Rac1b-PAK1 mediated mechanism. The analysis of NT-3 mediated gene expression also showed that NT-3 stimulates a limited number of pro-neuronal genes during the neuronal commitment of MIAMI cells, due to Rac1b regulation, in addition to the suggested inhibitory role of Notch due to *ex vivo* microenvironment limitations.

An exciting result also indicates that the constitutively active splice variant of Rac1, Rac1b, regulates NT-3 stimulated Mek1/2-Erk1/2 phosphorylation as well as is responsible for the repression of pro-neuronal genes, as seen by the limited number of genes upregulated via NT-3 stimulation. We also observed that Rac1b plays a role in NT-3 stimulated cell proliferation, as well as the transcriptional regulation of cell-cycle check point proteins. Interestingly, EGF/bFGF pretreatment was also found to increase
NTRK3 and Rac1b protein levels, as well as modulate the expression of transcription factors involved in NPC self-renewal and maintenance, and in promoting neurogenesis.

Rac1b is known to produce reactive oxygen species (ROS) (Radisky, et al. 2005), which are known to regulate Mek1/2-Erk1/2 phosphorylation (Mehdi, et al. 2005). N-acetylcystine (NAC) acts as a precursor for glutathione synthesis and has been used to increase cellular glutathione concentrations thereby reducing total ROS levels (Wu, et al. 2004). If we hypothesize that Rac1b is regulating Mek1/2-Erk1/2 phosphorylation via ROS, then treatment of MIAMI cells with NAC prior to NT-3 stimulation during neuronal commitment should mimic the enhancement of pro-neuronal gene expression seen during Rac1b ablation. This may serve as a method to enhance the neuronal differentiation program of hMSC such as MIAMI cells by taking into account the role of Rac1b in repressing NT-3 stimulated neuronal commitment. This would be of interest to study not only for its possible role in MSC differentiation into neuron-like cells, but also to determine the physiological role of Rac1b expression in bone marrow derived MSC.

In regards to neurotrophin signaling in NSCs, MIAMI cells have shown similar responses to NT-3, as well as EGF/bFGF stimulation commonly seen in NSCs throughout neuronal differentiation and CNS development. These studies represent the initial findings concerning neurotrophin signaling in MIAMI cells and can be built upon in the future for in-depth analyses into transcriptional regulation, as well as neurite initiation and formation.

The work described herein does not determine if TKd-NTRK3 binds NT-3, or if there is heterodimerization between FL-NTRK3 / TKd-NTRK3 resulting in the observed
decrease in TKd-NTRK3 protein levels. In addition, the novel data showing the nuclear localization of TKd-NTRK3, regardless of NT-3 stimulation, shows an additional role for TKd-NTRK3 in MSC. Therefore, future studies would be of interest to determine the binding of NT-3 to TKd-NTRK3, as well as the nuclear localization of the entire receptor or of the internal TKd-domain. While the overexpression and/or siRNA knockdown of NTRK3 was unsuccessful, this may have been due to a slow NTRK3 turnover rate and/or due to the death of cells with overexpressed or decreased NTRK3 levels. Inducible overexpression and/or shRNA vectors would be advantageous for future studies to create a stable cell line prior to induction of NTRK3 overexpression or knockdown.

The focus of these studies was on the early NT-3 stimulatory events, such as the Mek1/2-Erk1/2 signaling cascade, as well as the initial regulation of gene expression after 48 hours of neuronal commitment. These initial events have illuminated the role of NT-3 stimulation of MIAMI cells, as well as characterized the expression of transcription factors which have been shown to be involved in NPC self-renewal (Hes1, Hes5, Ngn2, Musashi) as well as neurogenesis (Ngn2, Prox1, Math1, Math5, Mash1, Egr-1). In addition, we observed an increase in the genes involved in neurite formation such as MAP2, NFL, NFM and NFH, as well as a morphological increase in neurite-like extensions. Future work focusing on the nuclear localization, activation, and regulation of pro-neuronal genes via these transcription factors, along with the analysis of neurite formation via neurofilament polymerisation, phosphorylation and F-actin mediated growth cone formation (Lariviere and Julien 2004) will be important.

Future studies will build upon the work presented here and provide an in-depth analysis of the transcriptional regulatory mechanisms, as well as the functional
characterization of neurite formation in MSC. This data will provide an increased understanding of the similarities, differences, and limitations between MSC and their ability to mimic the neuronal differentiation program of NPC, allowing for the potential use of MSC-derived neuronal precursor cells and-or immature neuron-like cells in cell therapy based procedures of tissue repair.
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