A Study of RET Dependent Transcriptional Changes of Cell Signaling Proteins in Neuroblastoma Cells

Elizabeth Yvonne Flores

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ABSTRACT

The ret proto-oncogene codes for a receptor tyrosine kinase involved in the pathogenesis of numerous developmental defects, particularly those in neural crest-derived structures leading to the regulation of cell proliferation, migration, differentiation and survival during embryogenesis.

Constitutive activation or inactivation of the RET receptor results in the cancer syndrome Multiple Endocrine Neoplasia or Hirschsprung's disease, respectively. The importance of RET in these disease states has been well established. However, while many proteins associated with the RET signaling pathway have been identified, the transcriptional changes induced by the wild type receptor, particularly with respect to these signaling proteins in neuroblastoma cells, is less clear. In order to better understand the transcriptional changes that occur in response to RET activation, SK-N-SH neuroblastoma cells were treated with 100 ng/ml GDNF and the RNA isolated. Focusing on the changes in transcription of specific genes that occur in response to the activation of RET, including Etv4, Spry1 and Shp2, primers for these genes were designed and the genes characterized using standard RT-PCR and then further analyzed using real-time PCR. Some of these genes promote and control branching in kidney morphology while others, when mutated, are associated with several human diseases. Analyzing the expression levels of each gene in response to the activation of RET will aid in elucidating RET-mediated mechanisms that contribute to these diseases and understanding the role of RET on downstream signaling and cellular function.

These findings show quantitatively minimal effect on Spry1, Shp2 and Etv4 expression in response to 4-hour GDNF treatment in neuroblastoma cells by real-time PCR. Because the fold changes observed were not remarkable, the data suggests that expression regulation of these proteins may not be dependent on RET activation. Future studies include analyzing transcriptional changes of these signaling proteins in response to RET activation with longer GDNF treatments.
or by combination of different ligand stimulation to help further elucidate the possible role of RET on these signaling proteins.
MONTCLAIR STATE UNIVERSITY

A STUDY OF RET DEPENDENT TRANSCRIPTIONAL CHANGES OF CELL SIGNALING PROTEINS IN NEUROBLASTOMA CELLS/

by

Elizabeth Yvonne Flores

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INTRODUCTION

STRUCTURE of RECEPTOR TYROSINE KINASES

Cells have the ability to sense cues in their environment, process these cues and respond accordingly. Such processes are mediated through a diverse group of cell surface receptors, including a group called receptor tyrosine kinases (RTKs). RTKs represent a family of proteins present in all metazoans that have been conserved over evolutionary time because of their ability to contribute to the fitness of the organism in which they are expressed. The conservation of RTKs in mammals suggest that each is responsible for cellular communication to regulate different cellular processes including proliferation, differentiation, migration, survival, protein synthesis, metabolism and other cellular outcomes. The study of orthologs of human proteins in the nematode worm Caenorhabditis elegans elude to their respective functions in both species. The structure of RTKs is highly conserved from the C. elegans, and is described as an extracellular domain containing a ligand-binding site, a single hydrophobic transmembrane domain and an intracellular catalytic domain with protein tyrosine kinase activity. The kinase gene in C. elegans describes the mechanism by which RTKs have driven their evolution by gene duplication and thus, the mechanisms that influence evolution (Popovici et al., 1999).

Gene duplication highlights one of the main reasons for the extensive RTKs identified in the human genome (Yarden and Wheeler, 2015). In gene duplication, two identical copies exist for a particular function that was originally fulfilled by one copy. Consequently, only one copy is considered functional while the other is considered dispensable. The dispensable copy can either acquire mutations that may impair its function, or evolve an acquired function. The acquired function will remain as a paralogue of the original gene and may be expressed in different cell types, may be activated by different ligands or become involved in related signaling pathways. Otherwise, the dispensable copy and the functional copy can share the function of the ancestral gene.
The gene sequence similarities across these three possible outcomes give rise to the RTK gene families.

Takahashi et al., first identified the c-ret protooncogene as part of an oncogenic fusion protein resulting from a translocation assay *in vitro* (Takahashi et al., 1985). The gene, named rearranged during transfection (ret) was found to encode a structurally unique transmembrane receptor tyrosine kinase. Characterization of the gene, on human chromosome 10q11.2, revealed the primary structure of the RET protein (figure 1): an extracellular region consisting of four cadherin-like domains including a calcium binding site, the cysteine-rich extracellular domain that mediates co-receptor and ligand binding, a hydrophobic transmembrane domain and the intracellular catalytic tyrosine kinase domain with two flanking regulatory regions, the junxtamembrane and C-tail with tyrosine residue phosphorylation sites (Takahashi et al. 1988; Anders et al. 2001). Alternative splicing of the protein gives rise to two functionally distinct isoforms of RET that differ in their respective C-terminus tail, named RET 9 and RET 51 (Tahira et al., 1990).

The RET proto-oncogene represent a receptor tyrosine kinase. There are 90 tyrosine kinase genes in the human genome, of which 58 encode receptor tyrosine kinases (Robinson et al., 2000). The catalytic tyrosine kinase domain is the most conserved part of RET with 90 percent sequence similarity to vertebrate lineages and, to a lesser extent, the extracellular domain with 40 percent sequence similarity with RET vertebrate species (Hahn and Bishop, 2001). The cysteine rich domain (CRD) found within the extracellular domain of RET represents a homologous structural motif used to identify RET in lower organisms (Takahashi, 2001). Fourteen of the twenty-eight cysteines are conserved between human, zebrafish and Drosophila species (Hahn and Bishop, 2001). Studies have traced the evolutionary conservation of the RET protein sequence and its expression in development across different organisms. Expression of endogenous RET has been
described in cells originating from the neural ectoderm termed the neural crest. In *D. melanogaster*, the fruit fly, minimal amounts of RET are found in embryonic, larval and pupal stages (Sugaya et al., 1994). RET is also expressed in the late embryo in subpopulations of the central nervous system and peripheral nervous system (Sugaya et al., 1994). In *B. ranio*, the zebrafish, RET is detected in the nervous system and excretory system. In the nervous system, RET is found in primary and motor neurons as well as neural crest cells and ganglion cells. In the excretory system, RET is present in the developing functional part of the kidney, termed the nephron duct (Marcos-Gutierrez et al., 1997).

The two isoforms of the RET protein are expressed in higher organisms such as the chicken, mouse and human. The catalytic domain of the chicken is 91 percent similar to the human RET protein while the extracellular domain is 68 percent similar to human RET (Marcos-Gutierrez et al., 1997). The cysteine residues present in the extracellular domain of the chicken RET protein suggest the residues represent a conserved structure. During chicken embryogenesis, RET is expressed in sites of the nervous system and excretory system (Schuchardt et al., 1995). In addition, RET is found in the development of the ventral roots of the spinal cord, the mesenchymal cells and adult testes (Schuchardt et al., 1995), eluding to the role of RET in early development. Further, the extracellular and catalytic domains are similar in structure in both the mouse and human RET proteins. The nucleotide sequence of the mouse RET protein is 83 percent similar to the human RET protein (Iwamoto et al., 1993). The similarities in RET expression between higher and lower organisms suggest the function of RET has been conserved during evolutionary time. The extent of homology between vertebrate and invertebrate species suggests RET may function similarly in terms of downstream signaling patterns across the organisms (Abrescia et al., 2005).
MODES of RECEPTOR ACTIVATION

Signaling pathways begin with a ligand binding to a receptor. While there are many receptors on the surface of a cell, the receptor will only bind to a specific ligand or set of ligands. The signaling molecule, which can be a functional group, growth factor, neurotransmitter, antigen or a hormone behaves as a ligand to change the properties of the receptor. Most signaling molecules are water-soluble and cannot bypass the cell membrane without binding to a cell surface receptor.

After a ligand binds to a cell surface receptor, other processes, such as internalization, degradation or recycling can determine which signaling pathway is activated or deactivated as well as the duration of the signal (Ullrich and Schlessinger, 1990). Ligand binding is necessary to stabilize the receptor regardless of whether the receptor is monomeric or oligomeric in its inactive state, but activation may also occur in the absence of the ligand. Most commonly, growth factor binding activates the receptor by inducing dimerization (Ullrich and Schlessinger, 1990).

RET is the signaling receptor for the glial-cell derived neurotrophic factor (GDNF) family of soluble, covalent, dimeric ligands (Trupp et al., 1996; Treanor et al., 1996; Durbec et al., 1996). GDNF was initially identified as a trophic factor for dopaminergic neurons (Lin et al., 1993) and later found involved in survival of sensory and motor neurons of the enteric nervous system (Baloh et al., 2000). The four proteins, GDNF, Neurturin (NTN), Persephin (PSP) and Artemin (ART) have been renamed GDNF family ligands (GFLs) (Baloh et al., 2000). This family of proteins form a subdivision of the transforming growth factor β (TGF-β) superfamily, being the only members of that family to signal via a RTK (Mason, Ivor, 2000). NTN, PSP and ART share similar characteristic to that of GDNF. However, GFLs do not bind to the extracellular domain of RET directly. They bind indirectly by first forming a complex with one of the four glycosyl-phosphatidylinositol (GPI) anchored GFRα co-receptors (GFRα1–4). The ligand binds to the co-receptor based on high affinity to form the following complexes: GDNF → GFRα1; NTN →
Although each GFL ligand exhibits a preference for a specific GFRα co-receptor, different complexes may be formed dependent on the receptor and co-receptor localization. Other functional complexes formed based on low affinity: GDNF → GFRα2; NTN → GFRα1; ART → GFRα1 may also activate RET (Trupp et al., 1996; Mason, Ivor, 2000).

The receptor's signal transduction is dependent on the assistance of lipid rafts on the plasma membrane. The complex formation between the ligand, co-receptor and receptor is mediated by the scaffolding properties of lipid rafts on the cell membrane. Jing et al., 1996 described the RET receptor complex formation with respect to the lipid rafts in two different ways. First, the dimeric GFL ligand ligates the two monomers of the GFRα co-receptor, which are commonly located in the lipid raft. Alternatively, the GFRα co-receptor is cleaved from the plasma membrane to produce a soluble form that associates with the dimeric GFL ligand. The complex then recruits RET to the lipid raft and promotes its homodimerization, which in turn, leads to activation. Dimerization of RET can stimulate autophosphorylation of specific tyrosine residues on the intracellular catalytic domain.

The activation mechanism by which ligand-induced dimerization leads to kinase activation differs for the particular RTK. The activation loop of the kinase domain in RTKs adopts a conformational change that mediates phosphotransferase catalytic activity. Protein kinases catalyze the transfer of the gamma phosphate group from adenosine triphosphate to a tyrosine residue on a substrate protein. Protein phosphatases transfer the phosphate group from a phosphoprotein to a water molecule. Such enzymes are termed phosphotransferases and catalyze opposing reactions to mediate kinase domain activity. Protein phosphorylation and dephosphorylation are a signaling motif that defines RTK activation. The crystal structures of the activated kinase domains of
RTKs are very similar because all kinase domains include an N and C-terminal lobe. The crystal structures of the inactivated kinase domains differ across RTKs, suggesting they may regulate in different ways (Huse and Kuriyan, 2002). In unstimulated conditions, the catalytic domains of most kinases are monomeric and auto-inhibited in cis by the C-lobe activation loop that keeps the kinase pocket closed. In stimulated conditions, inhibition is relived and the receptor is activated by the binding of specific ligands that stimulate the receptor’s protein tyrosine kinase activity (Ullrich and Schlessinger, 1990).

The RET intracellular catalytic tyrosine kinase domain is predicted to adopt the classical fold conformation involving the N-terminal lobe and C-terminal lobe connected by the linker region (Knowles et al., 2006). Instead of a cis conformation, RET adopts a trans conformation that blocks the substrate binding site of each monomer within the inactive dimer (Knowles et al., 2006). Ligand growth factor stimulation brings the two intracellular parts of the RET homodimer in proximity of one another, such that, the auto-inhibitory constraints are relieved, thereby favoring the formation of an active dimer that allows for catalytic activity of the kinase domain. The catalytic activity of the kinase domain that leads to different signaling pathways is described by the phosphorylation of specific tyrosine residues. Trans-autophosphorylation can be divided into two parts each with specific catalytic properties. The first wave of phosphorylation serves to enhance the catalytic activity of the kinase once the receptor binds to its ligand. The second wave creates the phosphotyrosine binding sites that recruit cytoplasmic signaling molecules that include binding domains. The resulting phosphotyrosines serve as docking sites for other protein-protein interactions involved in the RTK-mediated signaling pathway.
RET in DISEASE and DEVELOPMENT

RET plays an essential role in development of the nervous and enteric systems, and in signaling during development of the mammalian kidney. The neural crest cells originate in the dorsal most part of the neural tube and migrate extensively to generate differentiated cell types including subpopulations of the enteric, sensory and sympathetic nervous systems (Pachnis et al., 1993). The different substitutions and rearrangements in the ret gene underlie various human diseases including four different human cancers: papillary thyroid carcinoma (PTC), familial medullary thyroid carcinoma (MTC) and multiple endocrine neoplasia types 2A and 2B (MEN 2A and MEN 2B) (Grieco et al., 1990; Donis-Keller, Helen, et al. 1993; Mulligan et al., 1993; Hofstra et al., 1994). A chromosomal rearrangement of RET leads to oncogenic properties of the protein present in human papillary thyroid carcinomas (PTC) (Grieco et al., 1990).

MTC is a cancer syndrome that affects tissues derived from the neural ectoderm, specifically glands of the endocrine system. MTCs are rare tumors of the neural crest that arise from calcitonin secretion by a subset of cells in the thyroid glands named parafollicular C cells. MTC is classified into three types: familial MTC, MEN 2A and MEN 2B, and are characterized by the types of tumors and the age of onset of the disease (Donis-Keller, Helen, et al. 1993; Santoro et al., 1995). Mutations to RET account for a quarter of inherited MTCs that are either sporadic or familial. Sporadic MTC arises from somatic mutations of the RET protein and generally presents as a tumor confined to one thyroid lobe. Familial MTC results from germ-line mutations of the RET protein and often present as bilateral tumors (Donis-Keller, Helen, et al. 1993; Santoro et al., 1995).

MEN2 is a hereditary cancer defined by defects to major glands of the endocrine system including the thyroid, parathyroid and adrenal glands. MEN 2A usually occurs during early adulthood
and is characterized by MTC and tumors of the adrenal gland and neuroendocrine tissues named phaeochromocytoma (Donis-Keller, Helen, et al. 1993; Mulligan et al., 1993). The MEN 2A gene has been mapped to the DNA sequence on chromosome 10q11.2 that also encompasses the RET protein (Gardner et al., 1993). Germ-line missense mutations of RET have been identified in the majority of different MEN 2A families (Mulligan et al., 1993). The RET mutations associated with MEN 2A are the cysteine residues 609, 611, 618, 620, 630 and 634. The majority of mutations identified affect the same conserved cysteine residue 634 located on RET’s extracellular domain suggesting RET as a target for the MEN 2A gene (Mulligan et al., 1993). In the absence of the ligand, MEN 2A mutations leave an unpaired cysteine residue leading to the constitutive dimerization of the RET monomers by intramolecular disulfide bonds, followed by constitutive activation of the kinase domain (Santoro et al., 1995).

MEN 2B usually occurs in infancy or early childhood and is characterized by MTC and pheochromocytoma, but also by mucosal neuromas and ganglioneuromas, which are tumors composed of ganglion cells and nerve fibers of the gastrointestinal tract. MEN 2B has also been associated with mutations of the RET proto-oncogene by a substitution of a threonine for a methionine in the tyrosine kinase catalytic domain of the protein (Hofstra et al., 1994). The mutation that accounts for most of MEN 2B is mapped to the residue 918 in the catalytic domain of RET. This mutation induces conformation change of the kinase domain, resulting in activation of RET protein without dimerization (Iwashita et al., 1996). The different point mutations in MEN 2A, MEN 2B and FMTC that activate RET and disrupt development of the peripheral nervous system are described as gain-of-function mutations.

The enteric nervous system (ENS) derives from neural crest cells, which migrate and differentiate in the mesenchyme of the developing gut and subsequently become the gastrointestinal tract.
Hirschsprung disease (HSCR) is a congenital, genetic disorder of the neural crest development characterized by the absence of enteric parasympathetic neurons in the lower end of the gut. While the RET mutations that underline the cancer syndromes are described as gain-of-function mutations, HSCR is described by the inactivation of RET therefore are considered loss-of-function germ-line mutations (Schuchardt et al., 1994; Romeo et al., 1994). The disease is described as a blockage of the large intestine due to poor muscle movement caused by lack of nerves in the bowel and thus, results in an enlargement of the bowel and abdomen areas. Different types of mutation to the RET protein result in the HSCR phenotype. Missense and frameshift mutations found in the tyrosine kinase domain of RET show a decrease of kinase activity that is consistent with the HSCR phenotype (Romeo et al., 1994). Mutations to the extracellular domain inhibit RET’s cell surface expression caused by incorrect folding of the protein (Iwashita et al., 1996). Consequently, RET-mediated signaling pathways are impaired. Thus, RET signals are essential for the normal development of the enteric nervous system.

Besides RET’s role in the peripheral and enteric nervous system, it also plays a role in developing mammalian kidney. As described by Pietilä et al., 2013, the development of the mammalian kidney progresses through three stages, of which two are transient and the last remains as the functional kidney. An important step in kidney development is the formation of the Wolffian duct which can be defined as a simple epithelial tube from a group of cells that undergo mesenchyme-to-epithelial transition in the mesoderm. A subset of cells adjacent to the group that give rise to the Wolffian duct, gives rise to the non-functional primitive kidney, the pronephros. The pronephros eventually degenerates and then the next kidney, the mesonephros begins to form. The mesonephros consists of tubules that are connected peripherally to the Wolffian duct. The mesonephros is also a transient structure because it later regresses to become part of the epididymis in males and the metanephros remains as the predominant kidney structure present in
adult mammals. The metanephros appears as a tubular outgrowth of the Wolffian duct, known as the ureteric bud (UB), that invaginated the metanephric mesenchyme in a process known as UB induction. While in the metanephric mesenchyme, the UB folds into a T-shaped structure with two tips and a stalk and each subsequent tip continues to grow in a process termed branching morphogenesis. Branching morphogenesis is led by interactive signaling between the UB epithelium and metanephric mesenchyme to become the collecting system of the kidney. Formation of the kidney is completed before birth in humans, but continues after birth in mice.

Gene-targeting studies revealed the GDNF/GFRα1/RET signaling complex is physiologically essential in different stages of kidney development. In mice, the proliferating Wolffian duct is filled with cells that express RET and GFRα1. RET-GFRα1-expressing cells undergo cellular rearrangement prior to migrating to what will become the UB (Pachnis et al., 1993; Cacalano et al., 1998). GFRα1-GDNF-expressing cells are located in the mesenchyme of the urinary system adjacent to the Wolffian duct (Hellmich et al., 1996; Cacalano et al., 1998). The UB outgrowth from the Wolffian duct is induced by GDNF signals from the mesenchyme and GDNF is proposed to control the growth of the UB tips during branching morphogenesis (Vega et al., 1996). The proliferating UB has the greatest amount of RET-GFRα1 expression while the UB stalk harbors the least RET-GFRα1 expression (Schuchardt et al., 1994; Cacalano et al., 1998). During branching morphogenesis, the UB fold is led by the high concentration of RET-expressing cells at the distal UB tips (Pachnis et al., 1993). At this stage in development, the expression of GFRα1 in the UB is much greater than that of the mesenchyme (Keefe et al., 2013). The expression of RET-GFRα1 becomes weaker in the UB stalk, the ureter bud epithelium and the Wolffian duct as the common nephron duct (CND) forms (Hoshi et al., 2012).
Mutations in RET are associated with congenital anomalies of the kidney and urinary tract (CAKUT) and these mutations have been revealed in three different *in vivo* experimental approaches in mice. In the first approach, aberrant expression of RET caused multi-cystic dysplastic kidneys where the renal cortex is replaced by cysts during fetal development leading to a non-functional kidney (Srinivas et al., 1999). In the second approach, nonfunctional RET and the absence of GFRα1 caused downregulation of Erk in the Ras-Erk/MAPK pathway which resulted in unilateral or bilateral renal agenesis, or kidney hypoplasia due to failure of the ureteric bud to evaginate from the Wolffian duct and branch properly (Jain et al., 2004). In the last approach, both isoforms of RET, promoted branching morphogenesis to an extent, but mutations in the tyrosine residues Y1062, Y1015 and Y981, resulted in renal defects for RET 9 and not for RET 51 (Jain et al., 2006). The absence of Y1062 in kidney development disrupted the Ras-Erk/MAPK and PI3K/Akt pathways. The absence of Y1062 and Grb2 in RET 9 resulted in bilateral renal agenesis, a phenotype similar to non-functional RET mutations in mice. However, the absence of Y1062 in RET 51 did not affect kidney development. The absence of the Src and Grb2 adaptors on Y981 in RET 9 resulted in unilateral or bilateral kidney agenesis. Conversely, the absence of Src on Y981 in RET 51 resulted in normal kidneys. Moreover, loss of Y1015 signaling in RET prevents association with the adaptor phospholipase C gamma (PLC-γ). Lack of PLC-γ recruitment resulted in renal dysplasia described by multiplexed kidneys, enlarged ureters and failure of gonads to properly develop in male and females (Jain et al., 2006). RET 51 provides redundancy and promotes normal kidney formation via Y1096 when there are mutations to Y981 or Y1062 (de Graaff et al., 2001; Jain et al., 2006).
THE SIGNALING PATHWAYS ACTIVATED by RET

RTK signaling is described as an interconnected network because of the many phosphorylation sites present in the intracellular domain of RTKs and the multiple signaling proteins that may be recruited at those individual phosphorylation sites. The signaling molecules may be recruited to the phosphotyrosines in the receptor via receptor-proximal interactions such as Src homology 2 (SH2) and phosphotyrosine binding (PTB) binding domains (Schlessinger and Lemmon, 2003). There are approximately 100 SH2 domains that have been identified in the human genome and these domains mediate phosphorylation dependent protein-protein interactions. The signaling molecules are adaptor proteins that contain these binding domains with a conserved binding-pocket that specifically attaches to the autophosphorylated tyrosine residues present on the receptor. The formation of a signaling complex occurs when a signaling protein containing an SH2 or PTB domain binds directly or indirectly to a specific autophosphorylation site on the receptor.

Cells contain many proteins with SH2 and PTB domains that bind to phosphorylated tyrosine residues including adaptor proteins, docking proteins, transcription factors and enzymes. Adaptor proteins, such as growth factor receptor-bound protein 2 (Grb2), function as linkers that allow two signaling proteins to bind together to form a signaling complex. Docking proteins, such as fibroblast growth factor receptor substrate 2 (FRS2), insulin receptor substrate-1 (IRS1) and Grb2 associated binding proteins (GAB) provide receptors with additional tyrosine phosphorylation sites and these interactions that are spatially and temporally removed function as binding sites for other signaling proteins. (Schlessinger and Lemmon, 2003). Transcription factors may also bind to the activated receptor and subsequently become translocated to the nucleus such as members of the signal transducers and activators of transcription, (STAT) family. Enzymes such as (PLC-γ), a lipid phosphoinositide 3-kinase (PI3K) and a protein tyrosine phosphatase (SHP2) may also become activated by binding to an activated receptor (Schlessinger and Lemmon 2010).
RET regulates proliferation, differentiation, migration, survival, protein synthesis and other cellular processes as a result of downstream signaling pathways, including Ras-Erk/MAPK, PLC-γ, PKC, PI3K/Akt, JAK/STAT, RAC1 and JNK (figure 2). The alternative splicing of the RET protein adds complexity to the mechanism by which RET signals. The Shc proteins bind to both of the RET isoforms. However, RET 9 will only associate with the SH2 binding domain whereas RET 51 will only associate with the PTB binding domain of Shc adaptor proteins (Lorenzo et al., 1997), suggesting differences in downstream signaling and regulation of cellular interactions.

Previous studies showed that 14 out of 18 tyrosine residues present in the intracellular cytoplasmic region of RET can become autophosphorylated (Liu et al., 1996). The tyrosine residues Y905, Y981, Y1015, Y1062, and Y1096 are important for further signaling propagation because each of these sites allow for multiple adaptor proteins including Src (Y981), Grb7/10 (Y905), PLC-γ (Y1015), Shc/IRS1 or IRS2/DOK1,2,4,5 or DOK6 (Y1062) and Grb2/GAB1 or GAB2 (Y1096) to associate with activated RET (Mason, Ivor, 2000). Amongst these, Y1015 and Y1062 have been shown to be important for ligand-dependent and ligand-independent activation of RET (Takahashi, 2001). Some of these adaptor proteins bind to the activated RET within the lipid raft, while others prefer to bind to RET outside of the lipid raft. These binding sites have either redundant or unique roles that are dependent on the tissue and cell type.

Apart from tyrosine autophosphorylation, other autophosphorylation sites contribute to RET activation. The serine residue, S696, is associated with protein kinase A (PKA) (Fukuda et al., 2002). Mutations of this residue affect the ability of RET to activate the small GTPase Rac1 and to induce formation of a flattened extension of a cell termed lamellipodia, that is important for cell motility during cell migration (Fukuda et al., 2002). Alternatively, the S696 residue leads to the
activation of the JNK pathway through binding of the PKA adaptor protein. In addition, activation of PKA by forskolin, a chemical found in *Plectranthus barbatus* (Coleus forskohlii), was found to block the recruitment of SHP2 to RET and negatively affect the ligand growth factor stimulation (Perrinjaquet et al., 2010). Moreover, mutations to S687 promote SHP2 binding to the receptor and cancel the negative effects caused by forskolin.

The first tyrosine residue after the serine residue on the receptor that may become phosphorylated is Y687. The tyrosine residue, Y687, is mapped to the junxtamembrane region of the RET intracellular domain (Perrinjaquet et al., 2010). Upon growth factor stimulation, tyrosine-protein phosphatase non-receptor type 11 (PTPN11), which is the gene that in humans encodes the SHP2 protein, binds to Y687, but is not linked to the transduction of a signaling pathway directly associated with RET. The following tyrosine residue that can become phosphorylated is Y786. Phosphorylation of Y786 leads to the recruitment of the SHP2 and activation of the PI3K/Akt pathway. The PI3K/Akt pathway may be activated upon the recruitment of SHP2 to Y786 on the activated receptor, but also with cooperative binding of Y1062. Both of the tyrosine residues Y786 and Y1062 are necessary for stable recruitment of SHP2 to the receptor to activate the PI3K/Akt pathway (Perrinjaquet et al., 2010). SHP2 may promote survival and neurite outgrowth in primary neurons through the binding of phosphotyrosine Y786 (Perrinjaquet et al., 2010).

There are two tyrosine residues, Y900 and Y905, that map to the activation loop of the RET tyrosine kinase domain and undergo autophosphorylation (Knowles et al., 2006). The growth factor receptor-bound protein 7 (Grb7) and protein growth factor receptor-bound protein (Grb10) bind to the phosphotyrosine Y905 to induce signaling of the Ras-Erk/MAPK pathway. Following Y905 is Y981 which can also become phosphorylated. The tyrosine residue, Y981, binds to the
Src cytoplasmic tyrosine kinase and leads to the activation of the PI3K/Akt pathway (Encinas et al., 2004). Most recently Y981 was found to associate with Rap1GAP for RET binding and suppress growth factor induced stimulation of Erk and neurite outgrowth (Jiao et al., 2011). Finally, the recruitment and phosphorylation of SHC adaptors to phosphotyrosine Y1015 leads to the binding of PLC-γ and activation of the protein kinase C (PKC) (Borrello et al., 1996).

The most well-characterized site on all of the RET protein's isoforms is the tyrosine residue Y1062. Upon ligand growth factor stimulation, two different protein complexes assemble on the phosphorylated Y1062 via the Shc1 transforming protein 1 (Asai et al., 1996; Lorenzo et al., 1997). One of the signaling complexes leads to the activation of the PI3K/Akt pathway through the recruitment of the adaptor proteins Grb2 and Gab1 or Gab2 (Besset et al., 2000). The second signaling complex formation leads to the activation of the Ras-Erk/MAPK pathway through the recruitment and phosphorylation of adaptor proteins Grb2 and/or Gab1 or Gab2 (Besset et al., 2000). Alternatively, FRS2 associates with Y1062 in competition with Shc1 and leads to the activation of the Ras-Erk/MAPK pathway through the recruitment of the adaptor proteins Grb2 and sons of sevenless (SOS) (Kurokawa et al., 2001). The JNK pathway is activated upon binding of docking protein 1 (Dok1) and non-catalytic region of tyrosine kinase adaptor protein (Nck) to Y1062 (Murakami et al., 2002). The adaptor proteins IRS1, IRS2 and docking proteins Dok2, Dok4, Dok5 and Dok6 are also associated with Y1062 (Melillo et al., 2001; Grimm et al., 2001). Tyrosine residue Y1062 is important for RET function and has been shown to induce severe non-functional RET mutations in mice (Jijiwa et al., 2004). Growth retardation of the enteric nervous system was observed when the tyrosine residue was replaced with phenyalanine (Jijiwa et al., 2004). Mice with this mutation either lacked enteric neurons that line the inside of the intestinal tract or developed enteric neurons with reduced ganglion cells, but the formation of a small kidney was achieved. Thus proving the signing via Y1062 to be important for embryogenesis of the
enteric nervous system and development or growth of the kidney (Jijiwa et al., 2004). Further, the signaling complex formed via Shc1 contributes to the survival of RET in neuroblastoma cells. The survival properties of SK-N-MC cells were demonstrated in the competitive interaction of Sch/ENIGNMA and FRS2 to bind to Y1062 (Lundgren et al., 2006). Thus suggesting cell survival properties of RET are specific to cell type and occur in response to the competitive binding of different Src adaptor to the tyrosine residue (Lundgren et al., 2006).

Finally, the tyrosine residue, Y1096, is specific to only one of the RET isoforms, RET 51. The phosphotyrosine Y1096 binds to Grb2, which leads to recruitment of Gab1 or Gab2 to activate the PI3K/Akt pathway (Liu et al., 1996). Alternatively, Y1096 may recruit Celasitas B-lineage Lymphoma (CBL) and was shown to be required for ubiquitination and degradation of RET 51 (Calco et al., 2013).

TYROSINE KINASE INHIBITORS

The kinase domain, specifically the binding sites and the mechanism by which they regulate downstream signaling represents targets for drug discovery. Small-molecule tyrosine kinase inhibitors (TKIs) focus on the ATP-binding sites found on the intracellular kinase domain of RTKs and inhibit signaling by blocking phosphotransferase activity (Noble et al., 2004). While there is no RET-specific inhibitor present, there are multikinase inhibitors that have proven to work against RET. For instance, Vandetanib (ZD6474) proved to have potent inhibitory effects against the kinase domain of RET although it was originally developed as an epidermal growth factor receptor (EGFR) inhibitor. The inhibitor suppressed the secretion of calcitonin in 80 percent of the patients with sporadic or familial MTC and the remaining 20 percent were partial responses induced by the inhibitor (Carlomagno et al., 2003). Vandetanib binds to RET whether it is in its phosphorylated or unphosphorylated state with equal affinity, explaining its inhibitory effects.
against other mutated residues. While some residues have demonstrated resistance towards van-
detanib, it is to the date the most successful anti-RET inhibitor and was approved for treatment of
locally and advanced MTC tumors (Wells et al., 2012). Chow et al., 2007 described Sunitinib
(SU011248) as a multikinase inhibitor that had successfully transitioned from its molecular design
to an approved target-therapy as it effectively inhibited advanced RET-mediated renal carcinoma
during phase II clinical trials.

Other inhibitors have also been identified with anti-RET activity, but have had varying results
including Cabozantinib (XL-184) (Zhang et al., 2010) Sorafenib (BAY 43-9006) (Carlomagno et
al., 2006) and Motesanib (AMG 706) (Sherman et al., 2007). Other drugs have proven less effec­
tive as a RET inhibitor although described to work against RET-mediated malignancies. For ex­
ample, Imatinib (ST1 571), another multikinase inhibitor was described to block RET-mediated
MTC tumor growth. In vitro, phosphorylation of the Y1062 was blocked in the presence of Ima­
tinib. RET expression was affected with increased exposure to Imatinib, which lead to a decrease
in cell growth and thus blocked tumor proliferation (de Groot et al., 2006). The concentration of
Imatinib used to achieve results in vitro were considered far too elevated to attempt in target-thera­
py of MTC. In agreement, clinical trials of this drug proved to be ineffective, as the patients test­
ed either had stable diseases, reduced doses or stopped treatment because of toxic effects (de
Groot et al., 2006).

RET DEPENDENT TRANSCRIPTION CANDIDATES
Sprouty1 (Spry1)
Sprouty (Spry) proteins represent a class of ligand-inducible antagonists of the RTK-dependent
signaling pathways. Members of the Spry family are involved in anti-proliferation activity, inhibi­
tion of migration, favoring of differentiation, and promotion of survival. In Drosophila, Spry
inhibits FGFR and EGFR signaling during trachea and eye development (Hacohen et al., 1998). While it is known that Spry proteins suppress Ras-Erk signaling via RTKs, the exact mechanism by which Spry proteins function biochemically is not entirely understood (Mason et al., 2006). Specifically, because the method in which Spry proteins interact with components of RTK signaling pathways to induce signaling is unclear. While Spry proteins have been described in the etiology of several RTK-mediated diseases and disorders, the present study will focus only on the Spryl isoform. Spryl has been described as a negative regulator of GDNF/RET-mediated kidney induction and as a candidate tumor-suppressor gene in MTC, respectively (Basson et al., 2005; Macia et al., 2012).

Spry was first identified in *D. melanogaster* while genetically screening for regulators of tracheal and eye development (Hacohen et al., 1998). Four mammalian Spry homologs were identified based on similarity with the Drosophila Spry sequence. The Spry family includes four family members, Spryl-4, and there are three conserved sequences found across all four Spry proteins, including a tyrosine kinase-binding (TKB) binding motif, a serine-rich motif (SRM) and a cysteine-rich domain (CRD) (Hacohen et al., 1998). The mammalian Spry proteins are similar to the Drosophila Spry in the cysteine rich C-terminus, but differ in the N-terminus. The N-terminus of Spry represent a conserved sequence that promotes its ability to inhibit signaling (Hanufusa et al., 2002; Mason et al., 2006). The Spry genes encode relatively small proteins, approximately 63 Kda in Drosophila and 32-34 Kda in mammals (Kim et al., 2004). Tyrosine phosphorylation of Spry proteins is specific to the isoform, the growth factor and cell type, suggesting that Spry proteins are functionally different (Mason et al., 2006). The expression pattern of mammalian Spry proteins was described in embryonic development as well as in adult tissue, and near fibroblast growth factor (FGF) signaling complexes associated with mesenchyme-to-epithelial transition (Chambers and Mason, 2000).
The role of the Spryl has been described in the development of different organs, in particular, kidney development and RET-mediated ureteric branching (Gross et al., 2003; Basson 2005). In mice, Spryl is expressed in the developing metanephric mesenchyme that later becomes the nephron and in the branching UB that later becomes the collecting system in the kidney (Gross et al., 2003). In addition to RET and GFRα1, several other genes such as Wnt11 are co-expressed specifically in the UB tips (Kispert et al., 1996). Spryl behaves as a negative regulator of ureteric branching in kidney development via GDNF/RET/Wnt11 in the Ras-Erk/MAPK pathway (Basson et al., 2005). Basson et al. showed that mice with non-functional Spryl have multiplexed dysplastic kidneys with enlarged ureters and numerous UBs branching at random site along the WD (Basson et al., 2005). This phenotype results from failure of GDNF/RET signaling in the proliferating WD that leads to UB induction. An explanation for the phenotype is that increased RET signaling in the absence of Spryl leads to hyper activation of the Ras-Erk/MAPK pathway (Basson et al., 2005).

The opposite phenotype is observed in non-functional RET mutations. Mice with non-functional RET fail to develop kidneys during birth. Further, mutated Y1062 in which UB induction is blocked by decreased Ras-Erk activity also results in renal agenesis (Basson et al., 2005). An explanation for the phenotype observed is that in stimulated conditions, the Ras-Erk pathway is activated, followed by translocation of phosphorylated ERK to the nucleus, where it is activates transcription of Spry (Mason et al., 2006). Simultaneously, Spry is translocated to the plasma membrane where it is tyrosine phosphorylated by Src kinase activity. In this negative feedback mechanism, Spry suppresses the Ras-Erk pathway by inhibiting Ras activity (Gross et al., 2003). Loss-of-function mutations of Spryl and RET respectively, are opposite examples of defects to the kidneys and urinary tract that are caused from misregulation of the Ras-Erk pathway (Basson et al., 2005). The non-functional Spryl and RET phenotypes can be rescued (Rozen et al., 2009),
mice that lacked Y1062 activity and Spryl demonstrated morphological corrections for the renal defects seen in the individual non-functional phenotypes. Since Y1062 is a multi-adaptor docking site that can lead to PI3K/Akt and Ras-Erk/MAPK signaling, the morphological correction suggests UB branching may be achieved through RET-mediated signals other than those associated with Y1062.

A common outcome of aberrant expression of RTKs aside from development defects is malignancy. Given the Spry family's behavior as negative regulators has prompted studies to address the role of these proteins in cancer. The expression of Spryl has been shown to be down regulated in breast cancer, prostate cancer, and cardiovascular diseases with minimal explanation as for the mechanism by which Spryl functions. Recently, Macia et al. have proposed Spryl as a candidate tumor suppression gene in MTC in a mouse model (Macia et al., 2012). The expression of Spryl was traced to the thyroid glands from the embryonic state well into adulthood. Non-functional Spryl leads to calcitonin-producing C-cell hyperplasia, which is the primary reason for MTC. However, non-functional Spryl did not lead to MTC, but rather C-cell hyperplasia alone (Macia et al., 2012). Additionally, Spryl reduced proliferation of oncogenic RET-expressing cells by inducing cellular senescence. Macia et al., suggested that Spry-mediated senescence could explain the tumor suppression, but the mechanism by which expression of Spryl induces senescence is not clear.

Protein Tyrosine Phosphatase Non-receptor Type 11 (PTPN11) (SHP2)

The phosphatase SHP2 is a downstream signaling component of RET whether by GDNF stimulation of the wild type receptor or by RET mutant forms (Kurokawa et al., 2001). However, the biochemical function of SHP2 and the signaling components surrounding its activity are not entirely understood. Although the mechanism is not entirely understood, it is believed SHP2 is re-
lated to Spryl and a transcription factor downstream of RET, ETV4, while the transcriptional regulation of the associated signaling partners remain unclear.

The Src homology 2 domain-containing phosphatases (SHPs) are a sub-family of non-receptor protein tyrosine phosphatases that are conserved between vertebrates and invertebrates. There are two mammalian members SHP1 (encoded by PTPN6) and SHP2 (encoded by PTPN11) and both have conserved sequences including two SH2 domains, a protein tyrosine phosphatases (PTP) domain, a C-terminal tail with tyrosyl phosphorylations sites and a proline rich motif (Neel et al., 2003). The PTK phosphorylation and PTP dephosphorylation represent a signaling motif of RTKs. Under normal conditions, SHP2 has low catalytic activity because the SH2 domain and PTP domain keep it auto-inhibited. The auto-inhibition is released when the SH2 domain binds to a phosphotyrosine on the activated receptor or scaffolding proteins. SHP2 is ubiquitously expressed and functions as a signaling component of RTKs by promoting activation rather than down-regulation of signaling pathways. Mutations in PTPN11 results in Noonan, LEOPARDS syndrome and leukemia. Non-functional SHP2 mutations results in early embryonic defects in mice. Neel et al., 2003 has shown SHP2 is important for the development of the central nervous system and neural crest cells.

Recent studies suggest that SHP2 promotes the activation of Ras in the Erk/MAPK pathway. PTPs are thought to play opposing roles of PTKs in signal transaction and assumed to be negative regulators. While phosphatase activity is needed for SHP2 to promote activation of the pathway, the exact dephosphorylation targets are not clear. In a proposed method, SHP2 competes with Spry1 and FRS2 adaptor proteins for binding to the Grb2-SOS signaling complex on Y1062 to promote Erk/MAPK activation (Hanafusa et al., 2004). Another study suggests SHP2 promotes the Ras-Erk/MAPK signaling pathway by inactivating phosphorylation sites on Spryl
It has also been concluded that SHP2 acts downstream of GDNF/RET-mediated signaling in branching morphogenesis during kidney development.

Incoranato et al., studied the interactions between mutant RET and the two phosphatases, SHP1 and SHP2, in the rat phenochromocytoma cell line, PC12 cells (Incoranato et al., 2004). The RET mutations studied included cysteine residue 634 and methionine residue 918 causing MEN 2A and MEN 2B, respectively. It was concluded the complexes formed by SHP2 and the mutant RET were found outside the lipid rafts (Incoranato et al., 2004). Hereby concluding the SHP2-mediated signaling occurs outside the raft compartment.

In mice, non-functional SHP2 produces a phenotype similar to that of non-functional RET mutations in which there is reduced UB branching and reduced kidney size. In situ hybridization on sections of the kidney showed down regulation of RET target proteins including Wnt11 and transcription factor ETV4 (Willecke et al., 2011). Studies have shown ETV4 signals are essential for RET signaling, but are down regulated in nonfunctional SHP2 mice which may occur via the PI3K/Akt pathway (Besset et al., 2000). The development of the kidneys was then observed in an organ culture. The reduced UB branching phenotype on the explant remained the same even after GDNF stimulation. In agreement with other studies, ERK/MAPK signaling was reduced in the UB bud tips of the mutated explants (Willecke et al., 2011). Lack of SHP2 in the UB prevents mice with non-functional Spryl from developing numerous UB induction sites. In summary, SHP2 may act downstream of Spryl in the GDNF/RET signaling pathway (Willecke et al., 2011).
ETS Translocation Factor Variant 4 (ETV4)

Several genes described to be important during kidney development are downstream regulators of the GDNF/GFRα1/RET signaling pathway. Recently, Lu et al., described the role of overlapping ETV4 and ETV5 in the developing mammalian kidney, specifically in the UB tips during branching morphogenesis (Lu et al., 2009). ETV4 and ETV5 are co-expressed during kidney formation in a similar pattern as RET in the Wolffian duct, UB outgrowth and UB tips. In mice, nonfunctional Etv4 and non-functional ETV5, together, result in phenotypes similar to the non-functional RET phenotypes. Additionally, those phenotype resemble the nonfunctional SHP2 phenotypes (Willecke et al., 2011). That is, the absence of both Etv4 alleles and a single Etv5 allele displayed renal agenesis whereas mice with a variation of the mutation displayed different degrees of kidney reduction and UB branching (Lu et al., 2009). Further analysis demonstrated the PI3K/Akt pathway important for ETV4 and ETV5 expression in UB tips whereas the Ras-Erk/MAPK and PLC pathways did not affect the expression of these genes.

There are 28 human ETS genes and their homologues are found across vertebrates and invertebrates. The polyomavirus enhancer activator 3 (PEA3) subfamily of ETS transcription factors consists of three members ETV1 (Ews), ETV4 (Pea3) and ETV5 (Erm). The defining characteristic of the encoded proteins is the ETS domain that has three conserved α helices, and four antiparallel β-sheets. The ETS domain DNA binding motif is approximately 85 amino acids long and binds to DNA sequences with a 5'-GGA(A/T)-3' (Sharrocks et al., 2001). There is speculation about the way in which the ETS transcription factors regulate gene expression of specific genes as they all potentially bind to the same DNA target. Possible reasons include tissue or cell-type specificity, the sequence surrounding the 5'-GGA(A/T)-3' sequence or the ability of the transcription factors that bind in close proximity to the ETS proteins (Oh et al., 2012). Expression of ETV1, ETV4 and ETV5 has been traced in the development of numerous organs from
the embryonic stages well into adulthood. The expression of two closely related genes, ETV4 and ETV5, has been seen in the branching morphogenesis of organs including the lung, mammary gland, salivary gland and the UB of the developing kidney (Chotteau-Lelievre, et al., 2003). The co-expression of these two genes has been demonstrated to be essential in neuronal, spermatagonial and limb development (Oh et al., 2012). ETV4 was identified as a GDNF-RET target in motor neurons during limb development in the chicken (Hasse et al., 2002). It was shown that GDNF induces expression of ETV4, but only in a restricted subset of cervical motor neurons showing the spatial restriction of ETV4 response (Hasse et al., 2002).

While many proteins associated with the RET signaling pathway have been identified and discussed in the context of developmental biology, the transcriptional changes induced by the wild type receptor, particularly with respect to these signaling proteins in neuroblastoma cells, is less clear. The molecular mechanism(s) that connects the mutations or mutated sites on the receptor to the disease phenotypes are not entirely understood, in part, because the transcriptional changes of the putative signaling proteins involved in the proposed mechanistic pathways remain elusive. Analyzing the expression levels of each gene using real-time quantitative PCR in response to the activation of RET, will aid in elucidating RET-mediated mechanisms that contribute to these diseases, and understanding the role of RET on downstream signaling and cellular function. In the present study, the gene expression of Spryl, SHP2 and ETV4 in response to the activated wild type receptor RET is presented.
MATERIALS and METHODS

Primer Design
All primers were designed based on published genomic sequences obtained from NCBI Genebank or published papers using Primer-Blast. The primers are listed as three candidate genes followed by two housekeeping genes.

Gene name: (Spryl) Sprouty homolog 1
NCBI accession number: NM_001258038
forward primer, 5-ACATGGCACAGGCTCGTCTCGT-3
reverse primer, 5-GTCTTTGGTGCTGTCCGAGGAG-3
Amplicon size (bp) 194

Gene name: (Shp2) Tyrosine-protein phosphatase non-receptor type 11
NCBI accession number: NM_002834
forward primer 5-CGGGACAGGGACGTTCATTG-3
reverse primer: 5-GCTTGTGTCTGGACCATCCC-3
Amplicon size (bp) 135

The primers for Etv4 were designed according to (Pellecchia et al., 2012).

Gene name: (Etv4) ETS translocation variant 4
NCBI accession number: NM_001986.2
forward primer 5-GCTCGCTGTAAGCTAGGT-3
reverse primer 5-TCTCTTTGTGATCCTGTTGGTGT-3
Amplicon size (bp) 112

The housekeeping genes GAPDH and β-Actin were designed according to (Sikand et al., 2012).

Gene name: (GAPDH) Glyceraldehyde 3-phosphate dehydrogenase
NCBI accession number:
forward primer 5-ACCCACTCCTCCACCTTTGAC-3
reverse primer 5-TGTTGCTGTAGCCAAATTCGTT-3
Amplicon size (bp) 100

Gene name: (Actb) β-Actin
NCBI accession number:
forward primer 5-GCCGGGACCTGACTGACTAC-3
reverse primer 5-TTCTCCTTAATGTCACGCACGAT-3
Amplicon size (bp) 100

Cell Culture

Human neuroblastoma cell line SK-N-SH [American Type Culture Collection (ATCC #HTB-11)] and were cultured in EMEM supplemented with 10% FBS according to manufacturer’s recommendations. All cells were maintained at an atmosphere of 37°C with 2% relative humidity and 5% CO₂. Cells were passed from T-125 flasks to 6 well plates at a concentration of 1e⁶ cells/well and grown overnight. The plates were then aspirated, rinsed with PBS, and starved in serum free medium at 2 hours. Following the serum starvation, the cells were treated with serum free medium containing the GDNF growth factor (Promega, cat. No. #G2781). The GDNF treatment was diluted to final concentrations of 1ng/mL or 0.1ng/mL in serum free media for subsequent experiments. Cells were collected at 0 hours, 2 hours, 4 hours, 6 hours, 8 hours and 12 hours, and harvested for RNA extraction at the end of each time interval tested. RNA was extracted using a Trizol-based method. Total RNA was resuspended in sterile DEPC treated water and quantitated by OD₂₆₀. The final concentration of the RNA was diluted prior to being stored at -80°C.
Extracted total RNA was subjected to reverse transcription prior to polymerase chain reaction in the two-step protocol. The reverse transcriptase reaction was performed using oligo(dT) primers obtained from Invitrogen Life Technologies. In brief, a 20 μL reaction was set up using 10 μL of sterile DEPC treated H₂O, 1 μL of oligo(dT) primers, 1 μL of 2.5mM deoxynucleotide triphosphate dNTP mix, 1 μL of total RNA. The reactions were dispensed in 8-strip PCR tubes and sealed with domed caps and amplification was carried out using Applied Biosystems Veriti Thermal Cycler using the following conditions: 60°C for 3 minutes followed by 4°C for 1 minute. The reaction was paused and the remaining reagents were added to the reaction, 4 μL of Invitrogen 5X Buffer, 2 μL of Invitrogen DTT, 1 μL of Invitrogen RNAase OUTand 1 μL of Invitrogen SuperScript II Reverse Transcriptase and cDNA synthesis continued under the following conditions: 42°C 30 minutes, 70°C 5 minutes. The sequence specific lyophilized primers obtained from Invitrogen were diluted in autoclaved H₂O to make 100μM stock solutions, which were then diluted in autoclaved H₂O to make 10μM working solutions. The reverse transcriptase reaction was performed according to the conditions described in the previous section. Unlike the primer oligo(dT) reaction, the sequence specific reaction only requires a reverse 3’ primer for cDNA synthesis.

Standard Polymerase Chain Reaction (PCR)

The PCR reactions were prepped using reagents obtained from Invitrogen and Sigma-Aldrich. In brief, a 25 μL reaction was set up containing: 15 μL nucleated free PCR grade autoclaved H₂O, 1 μL of 2.5mM deoxynucleotide triphosphate dNTP mix, 1 μL of 10 μM sequence specific forward and reverse primers obtained from Invitrogen, 5 μL Sigma 10X PCR Buffer, 1.5 μL of 1.5mM Sigma MgCl₂, 1 μL of cDNA template and 0.5 μL of 5 U/μL Sigma Taq Polymerase. The reactions were dispensed in 8-strip PCR tubes and sealed with domed caps and amplification was carried out using Applied Biosystems Veriti Thermal Cycler using the following conditions: 1 cycle of
95°C for 5 minutes, followed by 40 cycles of 95°C for 30 seconds, 58°C for 30 seconds, and 72°C for 1 minute, followed by 1 cycle of 72°C for 7 minutes. The experiments included ‘no reverse transcriptase’ and ‘no template’ controls. The PCR conditions were then altered to 1 cycle of 95°C for 2 minutes, followed by 35 cycles of 95°C for 15 seconds, 55°C for 15 seconds, and 68°C for 45 seconds, followed by 1 cycle of 68°C for 5 minutes. The annealing temperature was altered to the melting temperature of each gene, respectively.

**Gel Electrophoresis**

1% and 1.5% agarose gels were used for the gel electrophoreses reactions. The gels were prepared with 1g or 1.5g agarose into 100mL of the respective buffer used in the experiment either 1X TAE or 1X TBE which were diluted from 50X TAE or 10X TBE stock solutions, respectively. The components of the buffers included: (TAE) Tris Base, Glacial Acetic Acid, Na_2_ EDTA, pH ~8.5; (TBE) Tris Base, Boric Acid and 0.5M EDTA, pH ~8.0. The running buffer used in the reactions were the buffer used to make the gel and stained with ethidium bromide (EtBr). 10 μL of the PCR product were diluted with 3 μL of DNA Loading Dye, 10 μL of the mixture were loaded in each respective well on the gel. The gel ran for approximately 45 minutes at 100 volts. Results were analyzed using the Launch TS Software on the UVP Ultraviolet Gel Imaging System.

**Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR)**

Two different approaches were employed for qRT-PCR, SYBR and TaqMan assays. The real-time PCR reactions were prepped using Brilliant SYBR Green Master Mix including ROX passive reference dye obtained from Applied Biosystems. In brief, a 25 μL reaction was set up containing 12.5 μL of 2X SYBR Green PCR Master Mix, 1.5 μL of 10 μM of each of the sequence specific forward and reverse primers, 1 μL of 1 in 5 diluted template cDNA synthesized from
RNA extracted from SK-N-SH treated neuroblastoma cells, and 9.5 μL of nucleated free PCR grade H2O. The reactions were dispensed in qPCR 8-strip flat-capped tubes and amplification was carried out using Applied Biosystems StepOne Plus Real-Time PCR System using the following conditions: 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The experiment included a 'no reverse transcriptase' control. Otherwise, the real-time qPCR reactions were set up using Taqman RNA-to-Ct 1-Step Kit obtained from Applied Biosystems with 900nM primers (forward and reverse) and 250nM probe. In brief, a 10 μL reaction was set up containing 5 μL of TaqMan RT-PCR 2X, 3 μL of sterile DEPC treated H2O, 0.5 μL of 20X Taqman Gene Expression Assay for each respective gene being tested, 0.5 μL of RT Taqman Enzyme diluted to 20X and 1 μL of 1 in 10 diluted 100ng/mL RNA template. The reactions were dispensed into 96-well optical plates and sealed with Applied Biosystems sealing tape and amplification was carried out using Applied Biosystems StepOne Plus Real-Time PCR System using the following conditions: 48°C for 15 minutes, 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Three technical replicates were performed per RNA sample along with a 'no template' control. Specificity and amplification were confirmed by running the samples on a 1% agarose gel. Gene expression was quantified by using the relative standard curve method and ΔΔCt Livak method. Expression of Spry1, Shp2 and Etv4 were normalized to either β-Actin or GAPDH expression, relative to treatment. The treatments included, serum-free (untreated) control, 4-hour serum treatment and 4-hour 100 ng/mL GDNF treatment. Ten-fold serial dilutions of 100ng/mL RNA were used to construct a standard curve, including, (1X, 0.1X and 0.01X). The mean and ± standard deviation of the candidate genes normalized to the housekeeping, endogenous controls were calculated from four independent experiments.

The TaqMan Gene Expression Assays used are listed below:
Gene Name: sprouty homolog 1, antagonist of FGF signaling (Spryl)
NCBI Location: Chromosome Chr.4: 124317956 - 124324910
NCBI Accession no.: NM_001258038.1 Amplicon Length: 103
Dye Label and Assay Concentration: FAM-MGB / 20X

Gene Name: protein tyrosine phosphatase, non-receptor type 11 (Ptpn11)
NCBI Location: Chromosome Chr.12: 112856536 - 112947717
NCBI Accession no.: NM_002834.3 Amplicon Length: 78
Dye Label and Assay Concentration: FAM-MGB / 20X

Gene Name: ets variant 4 (Etv4)
NCBI Location: Chromosome Chr.17: 41605211 - 41623762
NCBI Accession no.: NM_001079675 Amplicon Length: 70
Dye Label and Assay Concentration: FAM-MGB / 20X

Gene Name: Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) Endogenous Control
(probe, non-primer limited)
NCBI Accession no.: NM_002046.3 Amplicon Length: 122
Dye Label and Assay Concentration: FAM-MGB / 20X

Gene Symbol: β-actin (Actb) Endogenous Control
NCBI Location: Chromosome Chr.7: 5566779 - 5570232
NCBI Accession no.: NM_001101.3 Amplicon Length: 63
Dye Label and Assay Concentration: FAM-MGB / 20X
RESULTS

PRIMER DESIGN of CANDIDATE GENES

In order to evaluate expression changes of specific genes in response to RET activation, RT-PCR and real-time PCR were performed. Primer design is an essential application component for reverse transcription polymerase chain reaction (RT-PCR) in order to yield successful amplification of a target sequence. The reaction requires two PCR primers that are specific for the sequence amplified. When measuring gene expression it is also important for the primers to differentiate between cDNA and genomic DNA amplification. The forward and reverse primers designed for Spry1 span a single exon in the mRNA sequence (figure no. 3). The forward Shp2 primer was designed to span an exon junction while the reverse primer lies within a single exon to reduce the risk of false positives from amplifying genomic DNA (figure no. 3). Theses primers will anneal to cDNA from spliced mRNAs, but not to genomic DNA. Likewise, the forward primer designed for Etv4 spans an exon junction, while the reverse primer spans a different exon (Pellecchia et al., 2012). The primers for target and reference genes were designed using Primer-Blast to have similar properties so that they could be tested simultaneously in subsequent reactions. The primers for β-actin and GAPDH, were designed according to the primer sequences used for real-time QPCR by (Sikand et al., 2012) (figure no. 4). Both forward and reverse β-actin primers were designed within a single exon. The forward and reverse GAPDH primers flank a region that contains an intron towards the end of the mRNA sequence. The GAPDH primers were designed to bind to different exons to promote amplification of cDNA such that genomic DNA contamination is avoided. The PCR product amplified from the region of cDNA excluding introns, will be smaller in size compared to a region containing introns. Thus, amplification of contaminating genomic DNA should be eliminated.
PRIMER CHARACTERIZATION of CANDIDATE GENES

Extracted total RNA (work by a different graduate student from the lab) was subjected to reverse transcription followed by polymerase chain reaction in order to determine the specificity of the primers designed. The synthesis of DNA from an RNA template, via reverse transcription, produces complementary DNA, termed cDNA. The reaction requires an RNA template and a short primer complementary to the 3' end of the RNA sequence to direct the synthesis of the first strand of cDNA. The cDNA template is then used for the polymerase chain reaction to exponentially amplify the target DNA sequence. The PCR product is then evaluated by gel electrophoresis. In this method, an electrical field is applied such that the PCR product travels through the gel, from negative end to the positive end, at a speed that is inversely proportional to the size of the molecule. Thus, smaller fragments travel quicker than larger fragments.

The reverse transcription reaction was originally performed with oligo(dT) primers to synthesize cDNA from the total RNA extracted from SK-N-SH 1ng/mL GDNF-treated neuroblastoma cells. While some genomic DNA contamination is possible, the purity of the total RNA is critical in order to capitalize on the sensitivity of the technique. The purity of the starting total RNA template was $1.64 A_{260/280}$. Oligo(dT) primers are nonspecific primers that offer flexibility of the cDNA so that the cDNA, in a two-step reaction, may be used as template for subsequent polymerase chain reactions with gene-specific primers, for genes in the present study. Oligo(dT) primers initiate reverse transcription at the 3' polyA tail of the transcript, but do not ensure that the 5' end of long mRNAs is converted to cDNA. In that case, a different priming method, random primers could be used to increase the probability that the 5' mRNAs convert to cDNA, resulting in shorter cDNA fragments across the mRNA sequence. Transcriptase activity depends in part, on the purity and integrity of the starting material, but also by secondary structures, which
can obstruct the reverse transcriptase activity and lead to incomplete cDNA synthesis. The temperature of the reaction is equally as important for proper cDNA synthesis.

The primers for GAPDH and β-actin were subjected to reverse transcription with oligo(dT) primers followed by polymerase chain reaction as shown in (figure no. 5). The DNA ladder in the first lane represents a marker of known DNA fragment sizes ranging from 0.1 base pairs (bp) to 10 kilo bases (kb). In the initial experiment, none of the experimental samples, including the 'no reverse transcriptase' and 'no template' controls, showed any amplification (figure no.5). Therefore, the use of oligo(dT) primers for reverse transcription proved to be ineffective as they did not lead to the amplification of Gapdh and β-actin. The lack of PCR product can be explained by poor quality of PCR template, primer design or the reagents used in the reaction.

Sequence-specific primers can provide high specificity but the cDNA synthesized can only be used to study the particular gene for which the primers were designed, unlike oligo(dT) primer dependent reverse transcription which can be used to study many possible genes. Gene-specific primers were used in subsequent reactions to improve data analysis. In figure no. 6, lane 2, GAPDH amplification was observed. Unfortunately, amplification was also seen in the 'no reverse transcriptase' and 'no template' control (lanes 3 and 4, respectively), suggesting that the amplification was not mRNA specific. However, when amplifying for β-actin, a much more specific signal was obtained. While the intensity of the band is high and no distinct, lane 6 represents amplification of β-actin. The negative control reactions represented in lanes 7 to 9, suggest there is no genomic DNA amplification or genomic DNA contamination in the sample. To confirm the amplification of β-actin was the correct size, 100 bp, subsequent experiments used 1.5% agarose gels. A higher gel percentage has smaller pores, which, in turn, allow for more clear measurements of small fragments.
The specificity of the β-actin primers was confirmed by single distinct bands present in lanes 2 and 5 (figure no.7). This was accomplished by diluting the RNA template used in the reverse transcription reaction to 1 in 10 diluted 100ng/mL and 1 in 5 diluted 100ng/mL. Second, decreasing the Taq polymerase volume from 1 μL in a 25 μL reaction to 0.5 μL. Lastly, the final primer concentration was set to 0.6 μM per 25 μL reaction volume. The Taq polymerase and final primer concentration modifications were maintained in subsequent experiments. The specificity of Etv4 and Spryl primers were confirmed by distinct bands present in lanes 2 and 4, respectively (figure no. 8). The inclusion of β-actin in this experiment served as a positive control.

Amplification of the genes were present in the ‘+ reverse transcriptase’ controls, but not in the ‘no reverse transcriptase’ controls. The two separate experiments show the intensity of the band is dependent, in part, on the concentration of RNA template used in the reverse transcription reaction.

β-actin were subjected to reverse transcription with oligo(dT) primers in order to determine if the modifications made to the reaction components would yield cDNA synthesis using oligo(dT) primers. β-actin was chosen as it maintained positive results in the experiments thus far. Figure no.9 shows amplification of β-actin with sequence-specific primers and oligo(dT) primers in lanes 2 and 6, respectively. Reverse transcription reactions were tested using oligo(dT) primers followed by polymerase chain reaction with other sequence specific primers, but the results were not reproducible (data not shown).

The specificity of Shp2 was confirmed by the band present in lane 8 (figure no.10). In this experiment β-actin and GAPDH served as controls. The inclusion of Etv4 in this experiment owed to the PCR product smear present in lane 4 in figure no.8. Smearing can be caused by too much starting template evident in the (left) undiluted RNA experiment, which was corrected by
the (right) 1 in 5 diluted 100 ng/mL RNA template (figure no. 8). Also, because suboptimal primer concentrations can affect band quality, the final concentration of the primers in this experiment were set to 0.4 μM. The number of cycles during the polymerase chain reaction were also reduced from 40 to 35. The agarose gel was stained with EtBr overnight and visualized the following morning, but there was no amplification of Etv4 seen in lane 4 (figure no. 10).

Subsequent experiments were performed to confirm the specificity of the primers for the appropriate sequences. The experiments continued to use the following final concentrations: 0.6 μM forward and reverse primer, 1 U/25 μL Taq polymerase, 0.125 mM dNTPs, 1.5 mM MgCl2+, included 35 cycles during polymerase chain reaction. The reverse transcription reactions was performed with sequence-specific reverse primers and 1 in 5 diluted 100 ng/mL RNA templates, all of which proved successful in preceding experiments. Such conditions were used in combination with increasing annealing temperature to avoid primer dimers or unspecific DNA binding. However, distinct amplification of the genes were achieved in inconsistent patterns (figure no. 11), regardless of the optimization method and thermal cycler conditions used.

**TRANSCRIPTIONAL PROFILING of CANDIDATE GENES**

Because of the difficulties in obtaining reproducible results using standard RT-PCR subsequent experiments used real-time PCR. Real-time quantitative polymerase chain reaction, also referred to as real-time PCR, or qPCR, provides a method for determining the amount of a target sequence present within a sample. The reaction is characterized by the point at which amplification of the target sequence achieves a significant level of fluorescence as the reaction progresses, thus, in real time, rather than by end-point analysis as in standard PCR. Irrespective of the real-time PCR method used, the data is read as the number of PCR cycles necessary to achieve amplification that is detected by fluorescence signals. The amplification curve can be divided into four
parts, the baseline, the exponential phase, the linear phase and the plateau phase. The baseline refers to the earlier cycles where there is no significant change in fluorescence signal. The threshold is set above the baseline in the linear phase, but sufficiently low to be within the exponential phase of the amplification curve. The accumulation of fluorescence becomes significantly greater compared to the baseline during the exponential phase. The relative measure of the concentration of the template in the PCR reaction is read as the intersection between the amplification curve and the threshold value. This is termed cycle threshold (Ct) value.

Quantification may either be absolute or relative depending on the experimental goals. In the present study, relative quantification was used to compare changes in gene expression, to determine the fold-difference in expression. This method requires normalization of fluorescence signals for the target being tested against an endogenous control. An endogenous control is a reference gene that should be expressed at similar levels in all samples tested. Housekeeping genes can be used as endogenous controls as they are involved in basic cellular functions and assumed to be constitutively expressed regardless of treatment.

In a two-step reaction (figure no.13), cDNA was first synthesized with sequence-specific primers and then subjected to real-time PCR using the SYBR Green method. SYBR green binds to the double stranded DNA by intercalating between adjacent base pairs. This method quantifies cDNA, reverse transcribed from RNA, by measuring the fluorescence signal emitted by the SYBR green dye at the end of each cycle bound to double stranded DNA (Wittwer et al., 1997). While quantification of β-actin and Spryl was obtained, Spryl did not yield expected results. The Ct value for the '+ reverse transcriptase' reaction did not register above background and amplification of Spryl was not detected. Instead, amplification was detected in the 'no reverse transcriptase' reaction probably owing to recognition of nonspecific double stranded DNA. SYBR Green
experiments were continued with different annealing temperature, primer concentrations and cDNA dilutions (data not shown), but proved to be unsuccessful.

To improve gene expression data analysis, a different real-time PCR method was employed using the TaqMan method. TaqMan real-time PCR uses specific primers to quantify cDNA, where 5'-3' exonuclease activity of Taq DNA polymerase degrades 5' fluorophore group of the probe and the fluorescence emitted is measured at the end of each cycle during exponential stages of the PCR reaction (Livak et al., 1995). The probe contains a fluorescent FAM dye, termed a fluorophore, on the 5' end and a minor groove binder (MGB) quencher molecule on the 3' end. The proximity of the quencher molecule to the fluorophore keeps it from fluorescing due to fluorescent resonance energy transfer (FRET) (Livak et al., 1995). In the presence of the template, the probe bind downstream of the forward primer and during the extension phase of PCR is removed by the 5' nuclease activity of Taq polymerase. When the probe is removed, the fluorophore and quencher are separated allowing the fluorescence signal to be detected as the quencher no longer inhibits its activity. This continues at each cycle during the reaction and the accumulation of signal emitted from the fluorophore is proportional to the amount of template present in the sample.

The previously designed sequence-specific primers were not used for the Taqman real-time PCR reactions. Instead, predesigned primer and probe gene expression assays were selected. The real-time PCR reaction was performed as a one-step reaction in which the reverse transcription and polymerase chain reaction were carried out consecutively in a single reaction tube. The real-time PCR reactions were performed using technical replicates where the template used derived from the same SK-N-SH RNA extraction. Three replicates were used to control for validity of the method. The bar height indicates the average expression and ± standard deviations of the genes in response to the experimental conditions: untreated (serum-free), 4hr serum treatment and 4hr
GDNF treatment, respectively (figure no.14). The data is presented as the average Ct value which denotes the cycle at which fluorescence signal, indicative of the template present, was first significantly greater compared to the baseline.

GAPDH and β-actin represent the endogenous controls in the present study. While the average Ct values are consistent for GAPDH across the treatments (22.95, 22.48, 22.10), they are not for β-actin (30.55, 27.15, 30.21). In the target genes, the average Ct for serum is lower and the average Ct for GDNF is higher relative to the untreated (serum-free) control. While the resultant averages are graphed similarly for Spryl and Etv4, they are different for Shp2. The gene expression of the housekeeping genes were compared by calculating the Δ in Ct average. The value is expressed as the average Ct of the housekeeping gene subtracted from the average Ct of the target gene. There is great disparity in the Δ in Ct average compared between the two housekeeping genes (figure no.14). When compared to β-actin, Etv4 and Shp2 appear to have negative values and Spryl has positive values. When compared to GAPDH, the Δ in Ct average results in positive values for all of the genes.

Relative quantification requires a standard curve to compare the expression levels of a gene in a treated sample versus an untreated sample. The untreated (serum-free) control was a reference sample used as basis for relative quantification results. The template used to construct the three-point standard curve was RNA extracted from SK-N-SH untreated (serum-free) neuroblastoma cells. The cDNA reverse transcribed from RNA is expected to express both the target gene and the housekeeping gene. Standard curves were plotted to known concentrations and then created by performing real-time PCR on serial dilutions of the template. The highest concentration was undiluted 100ng/mL RNA plotted as (1X) on the standard curve. When the initial concentration of the template is high (1X), the Ct is reached at an earlier amplification cycle. The second con-
centration was 1 in 10 diluted 100ng/mL RNA plotted as (0.1X) on the standard curve. The most diluted template to construct the standard curve was 1 in 100 diluted 100ng/mL plotted as (0.01X) on the curve. The Ct of the most diluted template is reached at a later amplification cycle. A linear regression of the Ct values derived from the template dilutions are plotted against the log of the initial copy number. The regression line is used to determine the concentration of the unknown samples from their Ct values relative to the standard. Figures no. 15-18 and 20-23 represent the standard curves for each gene plotted as grey boxes, respectively.

The results show aberrant fold differences when normalized to β-actin (figure no.19). The reference gene should have stable expression levels in all samples and should not change with treatment. The serum treatment and GDNF treatment were tested using 1 in 10 diluted 100ng/mL RNA (0.1X) to ensure the expression level of the template fell within the limits of the standard curve, such that the Ct value is between the highest and lowest dilutions on the standard curve. The unsuitability of β-actin as an endogenous control is reflected in the efficiency of the assays (table no.1). The efficiency of the assay is determined by the rate at which the PCR product is detected and that is measured as a percentage. In table no.1, the efficiency for β-actin is well above the accepted 110%, suggesting possible pipetting error in serial dilutions or co-amplification of nonspecific product and in the second experiment, the efficiency is below 90% indicating sub-optimal reaction conditions. The efficiency of an assay is generated from the standard curve. Because the replicates were stochastic for all treatment conditions, the normalization to β-actin resulted in incorrect relative quantification of gene expression.

A second efficiency measurement is the $R^2$ value. This value indicates how well the value of $Y$ (Ct) can be used to accurately predict the value of $X$ (quantity) in a standard curve. The $R^2$ value when close to 1 assumes there is a 2-fold difference between each Ct value on the standard curve.
While the normalization to β-actin was not considered, Shp2 showed a distinct difference in the in response to GDNF treatment (figure no.17). The earlier Ct values suggest a possible 2-fold upregulation of Shp2 in response to the activated RET receptor when compared to the untreated (serum-free) control. While these results are interesting they were not reproducible in subsequent experiments.

The results show minimal fold differences when normalized to GAPDH (figure no.24). The expression of GAPDH in response to the three experimental conditions was uniform and the variation between replicates was minimal (figure no. 20). In table no.2, the efficiency of GAPDH is well above 110% in the earlier experiment, but within the acceptable range in latter experiments. Because the replicates for each treatment were much less stochastic compared to β-actin, relative quantification of gene expression normalized to GAPDH was made possible.

DISCUSSION

In order to evaluate the transcriptional changes that might occur in genes associated with RET dependent signaling, we studied the gene expression of RET-dependent signaling proteins in the human SK-N-SH neuroblastoma cell line using real-time PCR. Initially, sequence-specific primers were designed using Primer-Blast to amplify the genes Spry1, Shp2 and Etv4. Different reverse transcription priming methods including oligo(dT) primers, random primers and sequence-specific primers resulted in inconsistent amplification results (figure no. 5-12). While amplification of the target genes was achieved with sequence-specific primers, the results were not reproducible, despite continued optimization efforts. To improve data analysis real-time PCR was performed using the SYBR Green method, but also proved unsuccessful (figure no.12). To establish whether the signaling proteins under study were either induced or repressed in response to the activation of RET, TaqMan gene expression primer and probe assays were selected. The real-
time PCR data was analyzed to show the relative changes in gene expression levels using the Livak
\( \Delta \Delta Ct \) method based on the \( 2^{-\Delta \Delta Ct} \) equation (Livak and Schmittgen, 2001). Relative quantifica­
tion relates the expression of a gene in a given sample to that of another sample such as an un­
treated control. The correction of experimental variability in a real-time PCR experiment is
achieved by normalization to an endogenous control, more specifically, a housekeeping gene.
Housekeeping genes are involved in basic cellular functions and assumed to be constantly ex­
pressed at the same level regardless of treatment; however, the expression of \( \beta \)-actin did not ex­
hibit those properties (figure no.15 and table no.1). Variation in the expression of a housekeeping
gene can potentially mask the changes in expression of the target gene(s). Further, recent studies
have shown expression of the commonly used housekeeping genes including 18S RNA, GAPDH,
and \( \beta \)-actin to vary under diverse conditions (Dheda et al., 2005). The unsuitably of \( \beta \)-actin as an
endogenous control in the real-time PCR experiments resulted in normalizing gene expression
levels to GAPDH.

The gene expression normalized to Gapdh showed discrete fold differences, less than two-fold, in
response to the activated RET receptor when treated with GDNF for 4 hours (figure no 24). The
biological significance of a given-fold change is likely to depend on the gene and on the experi­
mental context. A two-fold differential is a relatively small change, but considered functionally
significant in the development of the mammalian kidneys. The changes in gene expression lead
to functionally significant changes in the protein that cause profound developmental effects in the
kidney. The expression of Etv4 is upregulated by a 2.9-fold in response to GDNF-RET signaling
during kidney development and is essential for ureteric bud branching morphogenesis (Lu et al.,
2009). While the expression levels in that experiment were accessed via Affymetrix microarray
analysis, minimal fold difference of 0.24 and 0.61 were observed in neuroblastoma cells using
real-time PCR (Table no.2). Although direct comparison between real-time PCR reactions can-
not be made, an average fold change of 0.42 is a small numeric value, indication of a weak response to 4-hour GDNF stimulation.

The expression of Spryl is upregulated by a 3.3-fold in response to GDNF-RET signaling in the ureteric bud (Lu et al., 2009). However, those changes were not observed in neuroblastoma cells. When normalized to β-actin the fold difference ranged from 0.15 to 1.50 (figure no. 19) and when normalized to GAPDH the fold difference ranged from 0.11 to 1.52 (figure no. 24). The fold difference is so small that functionally, it may be considered unimportant. One explanation for the minimal change in gene expression is the use of the human SK-N-SH neuroblastoma cell line, as there are genes that are only expressed transiently or only in certain tissues. In addition, the SK-N-SH cells are a differentiated cell line and the developmental activity of the genes of interest, Spryl, Shp2 and Etv4, were described early stages of kidney development. While neuronal and kidney systems derived from neural crest cells and differentiate similarly, neuronal systems may behave differently from kidney systems.

Further, transcription of these genes may not be dependent on RET activation alone, but rather in combination with other ligands. It has been established that the SK-N-SH neuroblastoma cell lines expresses RET, but different cell types express characteristic sets of transcriptional regulators. Neuroblastoma cells have demonstrated to require TGF-β for GDNF expression in the RET signaling pathway as the GDNF family of proteins form a subdivision of the transforming growth factor β (TGF-β) superfamily (Mason, Ivor, 2000). The co-expression, of a ligand other than GDNF may be necessary for gene expression of these signaling proteins. And because different cell types often respond in different ways to the same extracellular signal.
The Ct value in real-time PCR is a dependent variable affected by treatment and concentration of template. The Ct values become less efficient at high dilutions because they are more stochastic in the presence of less template. While the template may have not presented an issue, it seems likely that the time-course of GDNF stimulation would elicit a more significant change in gene expression. Perhaps the Spry1, Shp2 and Etv4 genes are not expressed transiently or rapidly in response to RET-GDNF signaling, but rather by late responses to longer stimulation with GDNF. More time points, other than the 4-hour GDNF stimulation, are needed to elicit elevated gene expression responses. Longer GDNF stimulation, such as 6-hour, 8-hour, 12-hour treatment may promote transcription of the genes. Alternatively, in stimulated conditions, the RET receptor exists in a phosphorylated state, but the magnitude of the signal is not sufficient to transduce the signal to the nucleus so that target proteins, including transcription factors, may be activated to change patterns in gene expression.

The lack of biological replicates could have also contributed to the minimal changes in gene expression observed. The use of technical replicates provided a limitation in this study as the data represents a single group of RNA that was extracted from SK-N-SH neuroblastoma cells, therefore the biological material analyzed is the same. The findings from these experiments only represent that specific RNA extraction and measure the reproducibility of the results. To draw meaningful conclusions, independent biological replicates are needed. Biological samples in which different RNA extractions from different SK-N-SH neuroblastoma cell cultures were stimulated with GDNF and then subjected to real-time PCR for relative gene expression analysis. The findings from such experiments would show similar patterns of gene expression and how those expression patterns are differ between untreated (serum-free), 4-hour serum and 4-hour GDNF treatments.
CONCLUSION

Because it has been proposed Etv4 may act downstream of Shp2 (Besset et al., 2000) and Shp2 may act downstream of Spryl (Willecke et al., 2011) in the GDNF/RET signaling pathway, it is not surprising that the fold differences observed, for the genes tested, were similar in response to GDNF and serum stimulation (figure no.24). Expression of the genes increase with serum stimulation and decreases in response to GDNF. These findings, show quantitatively minimal effect on Spryl, Shp2 and Etv4 expression in response to 4-hour GDNF treatment in the human SK-N-SH neuroblastoma cell line by real-time PCR. Our data, therefore, suggests that the fold difference is too discrete to alter the physiological state of the cell or for the cell to have to compensate for the change. The magnitude of the GDNF-RET signal, by 4-hour GDNF stimulation, is not transduced to the nucleus to activate transcription of GDNF-RET dependent proteins. Finally, transcription of these signaling proteins may not depend on RET activation alone. Future studies include analyzing transcriptional changes of these signaling proteins in response to RET activation with longer GDNF treatment times. Second, analyze the transcriptional changes of the signaling proteins in response to RET activation with co-expression of different ligands including GDNF and TGF-β. And third, the inclusion of biological replicates as opposed to technical replicates. All of which should elicit a significant change in expression and thereby, contribute to understanding the role of RET in these RET-dependent signaling protein. In summary, the present findings contribute to elucidating the role of RET on downstream signaling and in cellular function.
Figure No. 1 Schematic representation of RET receptor tyrosine kinase

The extracellular region of RET consists of four phylogenetically conserved cadherin-like domains (CLD) that are each 110 residues long, including a calcium binding site between CLD2 and CLD3, followed by a 120-residue cysteine rich domain (CRD) that is connected to the transmembrane domain (TM). The intracellular region of RET begins with a 50-residue juxtamembrane (JM) followed by a catalytic tyrosine kinase domain that is separated by a 14-residue linker region and ends with the 100-residue carboxyl terminal tail that results in two functional RET isoforms as a result of alternative splicing, RET 9 (short) and RET 51 (long).
Figure No. 2 Schematic of Signaling Pathway Activity by the RET Receptor
The RET receptor is represented as the green structure at the raft compartment of the plasma membrane. The assembly of the ligand, co-receptor and RET complex leads to dimerization. Dimerization allows each monomer to auto-phosphorylate the tyrosine residues in orange on the opposing monomer. Auto-phosphorylation activates the kinase activity of the receptor thereby signaling activation of other components downstream. The black arrows represent the putative pathways involving adaptor proteins, docking proteins, transcription factors and enzymes (left to right): growth factor receptor bound protein 2 (GRB2), Grb2 associated binding type1 (GAB1), (AKT), lipid phosphoinositide 3-kinase (PI3K), phospholipase C gamma (PLC-γ), protein kinase C (PKC), protein tyrosine phosphatase, non-receptor type 11(PTPN11), (GRB2), sons of sevenless (SOS), extracellular signal-regulated kinase (Erk) and activation of mitogen-activated protein kinase (MAPK), janus kinase and signal transducer and activation of transcription (JAK/STAT). The candidate genes are represented in grey at their presumed location based on gene knockout studies. The signal is transduced to the nucleus and transcription of growth-factor-responsive genes is activated. The possible cellular outcomes are highlighted at the bottom of the figure.
Figure No. 3 Standard RT-PCR Primer Design for Candidate Genes (listed top to bottom)

**SPRY1** NM_001258038.1 - The forward and reverse primers both span an exon. The PCR product length is 194 base pairs. The forward primer parameters: 20 nucleotides, 55% GC content, and the melting temperature 62°C. The reverse primer parameters: 21 nucleotides 61% GC content, and the melting temperature 61°C.

**PTPN11 (SHP2)** NM_002834.3 - The primer was designed so that half of the forward primer hybridizes to the 3' end of one exon and the 5' end of the adjacent exon. The reverse primer spans an exon. The PCR product length is 135 base pairs. The forward primer parameters: 60% GC content, and the melting temperature 61°C. The reverse primer parameters: 60% GC content, and the melting temperature 60°C.

**ETV4** NM_001986.2 - The forward primer spans an exon junction while the reverse spans an exon. The forward primer parameters: 18 nucleotides, 61% GC content, and the melting temperature 59°C. The reverse primer parameters: 20 nucleotides, 55% GC content, and the melting temperature 55°C.
Figure No. 4 Standard RT-PCR Primer Design for Housekeeping Genes (listed top to bottom)
ACTB NM_001101.3 - The forward and reverse primers both span an exon. The PCR product length is 100 base pairs. The forward primer parameters: 20 nucleotides, 65% GC content, and the melting temperature 61°C. The reverse primer parameters: 23 nucleotides, 43% GC content, and the melting temperature 60°C.

GAPDH NM_002046.3 - The forward and reverse primers flank an intron. The PCR product length is 100 base pairs. The forward primer parameters: 21 nucleotides, 57% GC content, and the melting temperature 61°C. The reverse primer parameters: 22 nucleotides, 40% GC content, and the melting temperature 59°C.
Figure No. 5 Gel Electrophoresis: Agarose gel of RT-PCR product
Reverse transcriptase was performed using undiluted 100ng/mL of RNA template and primer oligo(dT) followed by polymerase chain reaction with primers specific for GAPDH and β-actin. The gel conditions are: 1% agarose gel in 1X TBE ran at 100 volts for 45 minutes.
(1) DNA ladder
(2) GAPDH + reverse transcriptase
(3) GAPDH ‘no reverse transcriptase’ control
(4) GAPDH ‘no template’ control
(5) GAPDH + reverse transcriptase without Taq polymerase
(6) β-actin + reverse transcriptase
(7) β-actin ‘no reverse transcriptase’ control
(8) β-actin ‘no template’ control
(9) β-actin + reverse transcriptase without Taq polymerase
Reverse transcriptase was performed using undiluted 100ng/mL of RNA template using sequence-specific reverse primer followed by polymerase chain reaction with primers specific for Gapdh and β-actin. The gel conditions are: 1% agarose gel in 1X TBE ran at 100 volts for 45 minutes.

1. DNA ladder
2. GAPDH + reverse transcriptase
3. GAPDH 'no reverse transcriptase' control
4. GAPDH 'no template' control
5. GAPDH + reverse transcriptase without Taq polymerase
6. β-actin + reverse transcriptase
7. β-actin 'no reverse transcriptase' control
8. β-actin 'no template' control
9. β-actin + reverse transcriptase without Taq polymerase
Figure No. 7 Gel Electrophoresis: Agarose gel of RT-PCR product
Reverse transcriptase was performed using (left) 1 in 10 diluted 100ng/mL RNA template and (right) 1 in 5 diluted 100ng/mL RNA template with the β-actin reverse primer followed by polymerase chain reaction with primers specific for β-actin. The gel conditions are: 1.5% agarose gel in 1X TBE ran at 100 volts for 45 minutes.
(1) DNA ladder
1 in 10 diluted 1μg/μL of RNA template.
(2) β-actin + reverse transcriptase
(3) β-actin ‘no reverse transcriptase’ control
(4) β-actin ‘no template’ control
1 in 5 diluted 1μg/μL of RNA template.
(5) β-actin + reverse transcriptase
(6) β-actin ‘no reverse transcriptase’ control
(7) β-actin ‘no template’ control
Reverse transcriptase was performed with (left) undiluted 100 ng/mL RNA template and (right) 1 in 10 diluted 100 ng/mL RNA template using sequence-specific reverse primers followed by polymerase chain reaction with primers specific for Etv4, Spry1 and β-actin. The gel conditions are: 1% agarose gel in 1X TBE ran at 100 volts for 45 minutes.

1. DNA ladder
2. Etv4 + reverse transcriptase
3. Etv4 'no reverse transcriptase' control
4. Spry1 + reverse transcriptase
5. Spry1 'no reverse transcriptase' control
6. β-actin + reverse transcriptase
7. β-actin 'no reverse transcriptase' control
Figure No. 9 Gel Electrophoresis: Agarose gel of RT-PCR product
Reverse transcriptase was performed using 1 in 5 diluted 100ng/mL of RNA template and (left) sequence-specific reverse primer or (right) primer oligo(dT) followed by polymerase chain reaction with primers specific for β-actin. The gel conditions are: 1.5% agarose gel in 1X TBE ran at 100 volts for 45 minutes.
(1) DNA ladder
(2) β-actin + reverse transcriptase
(3) β-actin ‘no reverse transcriptase’ control
(4) β-actin ‘no template’ control
(5) β-actin + reverse transcriptase without Taq polymerase Primer oligo(dT)
(6) β-actin + reverse transcriptase
(7) β-actin ‘no reverse transcriptase’ control
(8) β-actin ‘no template’ control
(9) β-actin + reverse transcriptase without Taq polymerase
Figure No. 10 Gel Electrophoresis: Agarose gel of RT-PCR product
Reverse transcriptase was performed with 1 in 5 diluted 100ng/mL RNA template using sequence-specific reverse primers followed by polymerase chain reaction with primers specific for β-actin, Etv4, GAPDH and Shp2. The gel conditions are: 1% agarose gel in 1X TBE ran at 100 volts for 45 minutes.

1) DNA ladder
2) β-actin + reverse transcriptase
3) β-actin 'no reverse transcriptase' control
4) Etv4 + reverse transcriptase
5) Etv4 'no transcriptase' control
6) GAPDH + reverse transcriptase
7) GAPDH 'no reverse transcriptase' control
8) Shp2 + reverse transcriptase
9) Shp2 'no transcriptase' control
Reverse transcriptase was performed with 1 in 5 diluted 100ng/mL of RNA template using sequence specific reverse primers followed by polymerase chain reaction with primers specific for β-actin, Spry1, GAPDH, and Shp2. The gel conditions are: 1% agarose gel in 1X TBE ran at 100 volts for 45 minutes.

1. DNA ladder
2. β-actin + reverse transcriptase
3. β-actin 'no reverse transcriptase' control
4. Spry1 + reverse transcriptase
5. Spry1 'no transcriptase' control
6. GAPDH + reverse transcriptase
7. GAPDH 'no reverse transcriptase' control
8. Shp2 + reverse transcriptase
9. Shp2 'no transcriptase' control
**Figure No. 12** Real-Time PCR using SYBR Green

The real-time PCR reaction was performed as two-step reaction. The reverse transcriptase reaction was performed with gene-specific reverse primers for Spryl and β-actin using 1 in 5 diluted 100ng/mL RNA. The polymerase chain reaction with cDNA and SYBR green was carried out subsequently in a separate reaction tube. The amplification plot shows the fluorescence signal versus the cycle number. The normalized reported (Rn) is the ratio of the fluorescence emission of the reporter dye to the fluorescence emission of the passive dye. The baseline refers to the initial cycles of the reaction where there is minimal change fluorescence signal. The (ΔRn) is the baseline subtracted from the Rn. The cycles represent the cycles as the polymerase chain reaction progresses. Spryl is represented by the yellow line and β-actin is represented by the green line. The ‘+ reverse transcriptase’ control for Spryl resulted in a Ct value of 37.07, the ‘no reverse transcriptase’ 21.89, and the ‘no template’ control resulted in a Ct value of 34.02. The ‘+ reverse transcriptase’ control for β-actin resulted in a Ct value of 14.5, the ‘no reverse transcriptase’ control, 36.01 and the ‘no template’ control 26.87.
Figure No. 13 Average Ct value of RET dependent signaling proteins
Technical replicates were used for the Real-Time qPCR TaqMan reactions as opposed to biological replicates. Three replicates were used to control for validity of the method. The average and ± standard deviations of the replicates are presented as statistical validation for the replicates tested. The bar height indicates the average expression of each gene in response to the three experimental conditions: (serum-free) untreated, 4-hr serum treatment and 4-hr GDNF treatment.
**Figure No. 14** The Δ in Ct Average of RET dependent signaling proteins

The bar height represent the Δ in Ct Average that is calculated by subtracted the average Ct of the endogenous control form the average Ct of the target gene. The three bar per gene represent the experimental conditions, (serum-free) untreated, 4-hr serum treatment and 4-hr GDNF treatment, respectively. The top graph represents the Δ in Ct average for GAPDH. The bottom graph represent the Δ in Ct Average for β-actin.
### Table No. 1 Data Normalized to β-actin relative to treatment using Livak ΔΔCt Method

Relative quantification of gene expression measures the differences in expression levels of a target between samples. It is the change in expression of a target in a sample relative to the same target in a reference sample. The table represents the separate real-time qPCR experiments performed with TaqMan gene expression assays that were normalized to β-actin. The data is presented as the experimental conditions followed by the averages with ± standard deviations of the three technical replicates. The data is read as a fold-difference of expression levels. The amplification efficiency of the assays are listed on the right.

<table>
<thead>
<tr>
<th>Experimental Condition</th>
<th>Actin Average Ct</th>
<th>ACTβ Relative</th>
<th>ΔCt Relative</th>
<th>ΔΔCt Relative</th>
<th>Efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unstimulated Serum-Free</td>
<td>30.348 ± 0.026</td>
<td>1.000 ± 0.000</td>
<td>0.000 ± 0.000</td>
<td>0.000 ± 0.000</td>
<td>100% ± 0.00%</td>
</tr>
<tr>
<td>Serum Treatment (5%)</td>
<td>30.348 ± 0.026</td>
<td>1.000 ± 0.000</td>
<td>0.000 ± 0.000</td>
<td>0.000 ± 0.000</td>
<td>100% ± 0.00%</td>
</tr>
<tr>
<td>IgG Treatment (5%)</td>
<td>30.348 ± 0.026</td>
<td>1.000 ± 0.000</td>
<td>0.000 ± 0.000</td>
<td>0.000 ± 0.000</td>
<td>100% ± 0.00%</td>
</tr>
</tbody>
</table>
Figure No. 15 Standard Curve for β-actin
The amplification efficiency of real-time PCR assays were assessed by standard curves constructed with serial dilutions of the 100ng/mL RNA template extracted from SK-N-SH untreated (serum-free) neuroblastoma cells. The Ct versus the RNA concentration (log scale) are plotted to calculate slope and R² value. The experimental conditions were tested with 1 in 10 diluted (0.1X) 100ng/mL untreated RNA template. The replicates for the dilutions are plotted as the grey boxes labeled ‘not assigned’ in the legend.
The amplification efficiency of real-time PCR assays were assessed by standard curves constructed with serial dilutions of the 100ng/mL RNA template extracted from SK-N-SH untreated (serum-free) neuroblastoma cells. The Ct versus the RNA concentration (log scale) are plotted to calculate slope and R² value. The experimental conditions were tested with 1 in 10 diluted (0.1X) 100ng/mL untreated RNA template. The replicates for the dilutions are plotted as the grey boxes labeled ‘not assigned’ in the legend. The 0.01X values are not plotted in the curve.
The amplification efficiency of real-time PCR assays were assessed by standard curves constructed with serial dilutions of the 100ng/mL RNA template extracted from SK-N-SH untreated (serum-free) neuroblastoma cells. The Ct versus the RNA concentration (log scale) are plotted to calculate slope and $R^2$ value. The experimental conditions were tested with 1 in 10 diluted (0.1X) 100ng/mL untreated RNA template. The replicates for the dilutions are plotted as the grey boxes labeled 'not assigned' in the legend.

**Figure No. 17** Standard Curve for Shp2

The amplification efficiency of real-time PCR assays were assessed by standard curves constructed with serial dilutions of the 100ng/mL RNA template extracted from SK-N-SH untreated (serum-free) neuroblastoma cells. The Ct versus the RNA concentration (log scale) are plotted to calculate slope and $R^2$ value. The experimental conditions were tested with 1 in 10 diluted (0.1X) 100ng/mL untreated RNA template. The replicates for the dilutions are plotted as the grey boxes labeled 'not assigned' in the legend.

- **Slope**: -3.087
- **$R^2$**: 0.98
- **Efficiency Percent**: 110.83%
Figure No. 18 Standard Curve for Etv4
The amplification efficiency of real-time PCR assays were assessed by standard curves constructed with serial dilutions of the 100ng/mL RNA template extracted from SK-N-SH untreated (serum-free) neuroblastoma cells. The Ct versus the RNA concentration (log scale) are plotted to calculate slope and R² value. The experimental conditions were tested with 1 in 10 diluted (0.1X) 100ng/mL untreated RNA template. The replicates for the dilutions are plotted as the grey boxes labeled ‘not assigned’ in the legend.
Figure No. 19 Data Normalized to β-actin relative to treatment using Livak ΔΔCt Method

Real-time PCR analysis of the expression levels using predesigned TaqMan gene expression assays. Data was normalized to β-actin. The values represent fold changes relative to the untreated (serum-free) control and are given as the fold difference with ± standard deviations and p values. The values are presented as relative fold changes with upregulation >1 and downregulation <1.
Table No. 2 Data Normalized to GAPDH relative to treatment using Livak ΔΔCt Method

Relative quantification of gene expression measures the differences in expression levels of a target between samples. It is the change in expression of a target in a sample relative to the same target in a reference sample. The table represents the separate real-time PCR experiments performed with TaqMan gene expression assays that were normalized to GAPDH. The data is presented as the experimental conditions followed by the averages with ± standard deviations of the three technical replicates. The data is read as a fold-difference of expression levels. The amplification efficiency of the assays are listed on the right.

<table>
<thead>
<tr>
<th>Experimental Condition</th>
<th>Sample Average Ct</th>
<th>ΔΔCt Sample - GAPDH</th>
<th>ΔΔCt Treatment - GAPDH</th>
<th>ΔΔCt Treatment - ΔΔCt GAPDH</th>
<th>Efficiency %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated 48h</td>
<td>27.1628±0.10</td>
<td>27.1628±0.10</td>
<td>27.1628±0.10</td>
<td>27.1628±0.10</td>
<td>2.05%</td>
</tr>
<tr>
<td>Untreated 48h</td>
<td>27.1628±0.10</td>
<td>27.1628±0.10</td>
<td>27.1628±0.10</td>
<td>27.1628±0.10</td>
<td>2.05%</td>
</tr>
<tr>
<td>Untreated 48h</td>
<td>27.1628±0.10</td>
<td>27.1628±0.10</td>
<td>27.1628±0.10</td>
<td>27.1628±0.10</td>
<td>2.05%</td>
</tr>
<tr>
<td>Untreated 48h</td>
<td>27.1628±0.10</td>
<td>27.1628±0.10</td>
<td>27.1628±0.10</td>
<td>27.1628±0.10</td>
<td>2.05%</td>
</tr>
</tbody>
</table>
Figure No. 20  Standard Curve for GAPDH

The amplification efficiency of real-time PCR assays were assessed by standard curves constructed with serial dilutions of the 100ng/mL RNA template extracted from SK-N-SH untreated (serum-free) neuroblastoma cells. The Ct versus the RNA concentration (log scale) are plotted to calculate slope and $R^2$ value. The experimental conditions were tested with 1 in 10 diluted (0.1X) 100ng/mL untreated RNA template. The replicates for the dilutions are plotted as the grey boxes labeled 'not assigned' in the legend.
Figure No. 21 Standard Curve for Spryl
The amplification efficiency of real-time PCR assays were assessed by standard curves constructed with serial dilutions of the 100ng/mL RNA template extracted from SK-N-SH untreated (serum-free) neuroblastoma cells. The Ct versus the RNA concentration (log scale) are plotted to calculate slope and $R^2$ value. The experimental conditions were tested with 1 in 10 diluted (0.1X) 100ng/mL untreated RNA template. The replicates for the dilutions are plotted as the grey boxes labeled 'not assigned' in the legend.
The amplification efficiency of real-time PCR assays were assessed by standard curves constructed with serial dilutions of the 100ng/mL RNA template extracted from SK-N-SH untreated (serum-free) neuroblastoma cells. The Ct versus the RNA concentration (log scale) are plotted to calculate slope and R^2 value. The experimental conditions were tested with 1 in 10 diluted (0.1X) 100ng/mL untreated RNA template. The replicates for the dilutions are plotted as the grey boxes labeled 'not assigned' in the legend.
The amplification efficiency of real-time PCR assays were assessed by standard curves constructed with serial dilutions of the 100ng/mL RNA template extracted from SK-N-SH untreated (serum-free) neuroblastoma cells. The Ct versus the RNA concentration (log scale) are plotted to calculate slope and $R^2$ value. The experimental conditions were tested with 1 in 10 diluted (0.1X) 100ng/mL untreated RNA template. The replicates for the dilutions are plotted as the grey boxes labeled 'not assigned' in the legend.
Data Normalized to GAPDH relative to treatment using Livak ΔΔCt Method

Figure No. 24 Data Normalized to GAPDH relative to treatment using Livak ΔΔCt Method
Real-time PCR analysis of the expression levels using predesigned TaqMan gene expression assays. Data was normalized to GAPDH. The values represent fold changes relative to the untreated (serum-free) control and are given as the fold difference with ± standard deviations and p values. The values are presented as relative fold changes with upregulation >1 and downregulation <1.
Works Cited


