Novel Fibroblast Growth Factor Receptor Signalling

Pathways Regulating Neuronal Differentiation

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Thesis submitted for the degree of

**Master of Philosophy**

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12 September 2013
To my parents, my wife and daughter, who have given me love, support, and encouragement to finish this study
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Abstract

The fibroblast growth factors (FGFs) consist of a family of growth factors that regulate diverse (or pleiotropic) responses, such as cell survival, proliferation, differentiation, and migration in both development and adulthood. The FGFs exert their biological activities through the binding and activation of four structurally related FGF receptors (FGFR1-4). The activation of FGFRs leads to FGFR tyrosine phosphorylation and the recruitment of SH2/PTB-domain signalling proteins, which are essential for initiating the downstream signalling pathways. Although receptor tyrosine phosphorylation is known to be important for cellular responses mediated by FGFRs, the precise mechanisms by which the FGFRs regulate pleiotropic biological responses remain unclear. In addition to receptor tyrosine phosphorylation, previous work in the Cell Growth and Differentiation Laboratory has shown that the FGFR cytoplasmic domain is also phosphorylated on a specific serine residue, Serine 779 (S779), which recruits and binds the 14-3-3 family of phosphoserine/threonine-binding adaptor/scaffold proteins. However the precise role of the receptor phosphoserine signalling event in regulating downstream pathways and biological events has remained unclear.

Using PC12 pheochromocytoma cells and primary mouse bone marrow stromal cells (BMSCs) as models for growth factor-regulated neuronal differentiation, our study aimed to understand the biological roles of S779 in the cytoplasmic domain of the FGFRs in regulating neuronal differentiation mediated by FGFs. We have shown that S779 in the cytoplasmic domain of FGFR2 is phosphorylated in response to ligand stimulation in PC12 cells. Phosphorylation of S779 is required for inducing the
maximal activation of the Ras/MAP kinase pathway but not for other FGFR phosphotyrosine pathways. Furthermore, we have shown that the maximal Ras/MAPK signalling promoted by S779 signalling is critical for PC12 cell neuronal differentiation in response to FGF9. We have further elucidated the functions of S779 of endogenous FGFR2 in BMSCs neuronal differentiation mediated by FGF2. Our results show that both Ras/MAP kinase and novel PKCs (nPKCs) signalling are important for the BMSCs neuronal differentiation. Further siRNA-mediated knockdown suggest the PKCε is required for BMSCs neuronal differentiation regulated by FGF2. Further studies from our laboratory have shown that in BMSCs FGF2 stimulation results in PKCε mediated S779 phosphorylation. Our results show that the increased phosphorylation of S779 is critical for the maximal Ras/MAP kinase signalling and neuronal differentiation mediated by FGF2 in BMSCs.

Collectively, we show the S779 phosphorylation mediated by PKCε is critical for regulating the intracellular signalling events necessary for maximal Ras/MAP kinase signalling and neuronal differentiation mediated by FGFs. Our findings indicate that in addition to FGFR tyrosine phosphorylation, the phosphorylation of S779, a conserved serine residue in cytoplasmic domains of FGFR2, can quantitatively and selectively regulate Ras/MAP kinase signalling to mediate neuronal differentiation.
Author Declaration

This work contains no material that has been accepted for the award of any other degree or diploma in any university or other tertiary institution to Yang Kong, and to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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Yang Kong
Publications arising from this study

Peer-reviewed journal article:


*equal 2nd author.

The reference number of this publication in the thesis is [242].

Conference presentation:

Acknowledgements

I sincerely appreciate my supervisor Dr Mark Guthridge for bring me into the fascinating cell signalling field as well as the guidance and encouragement. I am grateful that I could study in the Division of Human Immunology, and acknowledge the generosity and expertise of the fantastic team of scientists at the Centre for Cancer Biology, SA Pathology. Thank Dr Ana Lonic for establishing the foundation of my project and her support. I especially thank Dr Jason Powell and Dr Daniel Thomas for their friendship as well as their support and encouragement. Thank Emma Barry and Nhan Truong for being the most supportive colleagues. Special thanks go to Dr Yeesim Khew-Goodall for the encouragement and support. I thank Dr Gafar Sarvestani for his expert technical assistance in confocal microscopy, Dr Stan Gronthos and Dr Quenten Schwarz for their helpful advices.

Appreciation also goes to Ms Cathy Lagnado and Ms Anna Nitshke for their encouragement and kindness.

I would finally like to acknowledge with much appreciation my wife and daughter, who are always my sunshine, and my parents for their lifelong encouragement and support.
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<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>AD</td>
<td>Alzheimer's disease</td>
</tr>
<tr>
<td>Akt</td>
<td>v-akt murine thymoma viral oncogene homologue</td>
</tr>
<tr>
<td>ATP</td>
<td>adenine triphosphate</td>
</tr>
<tr>
<td>BAD</td>
<td>Bcl-2-associated death promoter</td>
</tr>
<tr>
<td>BAX</td>
<td>Pro-apoptotic Bcl2-associated X protein</td>
</tr>
<tr>
<td>βc</td>
<td>β chain</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>B-cell lymphoma 2</td>
</tr>
<tr>
<td>Bcl-xl</td>
<td>B-cell lymphoma-extra large</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>BM</td>
<td>Bone marrow</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenic protein</td>
</tr>
<tr>
<td>BMSCs</td>
<td>Bone marrow stromal cells</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>BRAF</td>
<td>v-raf murine sarcoma viral oncogene homologue B1</td>
</tr>
<tr>
<td>BrdUrd</td>
<td>5-bromo-2-deoxyuridine</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dsDNA</td>
<td>Double-stranded deoxyribonucleic acid</td>
</tr>
<tr>
<td>ECE</td>
<td>Endothelin-converting enzyme</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EGFP</td>
<td>Enhanced GFP</td>
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<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
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<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>EMT</td>
<td>Epithelial to Mesenchymal Transition</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>FGFR</td>
<td>Fibroblast growth factor receptor</td>
</tr>
<tr>
<td>Gab1</td>
<td>GRB2-associated-binding protein 1</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase activating proteins</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanosine diphosphate</td>
</tr>
<tr>
<td>GEF</td>
<td>Guanine nucleotide exchange factor</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte-macrophage colony stimulating factor</td>
</tr>
<tr>
<td>Grb2</td>
<td>Growth factor receptor-bound protein 2</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione S-transferase</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>HGF</td>
<td>Hepatocyte growth factor acetate</td>
</tr>
<tr>
<td>HSPG</td>
<td>Geparin or heparin sulfate proteoglycans</td>
</tr>
<tr>
<td>IC50</td>
<td>Half maximal inhibitory concentration</td>
</tr>
<tr>
<td>IEG</td>
<td>Immediate early gene</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IL-3</td>
<td>Interleukin-3</td>
</tr>
<tr>
<td>IPs</td>
<td>Immunoprecipitations</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinases</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MEK</td>
<td>Mitogen-activated protein kinase kinase</td>
</tr>
<tr>
<td>MKs</td>
<td>MAPK-activated protein kinases</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>MNCs</td>
<td>Mononuclear cells</td>
</tr>
<tr>
<td>MSC</td>
<td>Mesenchymal stem cell</td>
</tr>
<tr>
<td>MSK1</td>
<td>Mitogen- and stress-activated protein kinase-1</td>
</tr>
<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
</tr>
<tr>
<td>NGFR</td>
<td>Nerve growth factor receptor</td>
</tr>
<tr>
<td>NS</td>
<td>Not significant</td>
</tr>
<tr>
<td>pAb</td>
<td>Polyclonal antibody</td>
</tr>
<tr>
<td>PC12 cell</td>
<td>Pheochromocytoma cell</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
</tr>
<tr>
<td>PH</td>
<td>Pleckstrin homology domain</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PKD1</td>
<td>Phosphoinositide-dependent kinase 1</td>
</tr>
<tr>
<td>PLCγ</td>
<td>Phospholipase C γ</td>
</tr>
<tr>
<td>PI</td>
<td>Phosphatidylinositol</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PIP</td>
<td>Phosphatidylinositol phosphates</td>
</tr>
<tr>
<td>PTB</td>
<td>Phosphotyrosine-binding</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homolog</td>
</tr>
<tr>
<td>RACK1</td>
<td>Receptors for activated C-kinase 1</td>
</tr>
<tr>
<td>RAF kinase</td>
<td>Rapidly Accelerated Fibrosarcoma kinase</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor tyrosine kinase</td>
</tr>
<tr>
<td>RSK</td>
<td>Ribosomal S6 kinase</td>
</tr>
<tr>
<td>SCLC</td>
<td>Small cell lung cancer</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SH2</td>
<td>Src homology 2 domain</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
</tr>
<tr>
<td>----------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>TKIs</td>
<td>Tyrosine kinase inhibitors</td>
</tr>
<tr>
<td>Tm</td>
<td>Melting temperature</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming growth factor β</td>
</tr>
<tr>
<td>TM domain</td>
<td>Transmembrane domain</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumour necrosis factor α</td>
</tr>
<tr>
<td>wt</td>
<td>Wild-type</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>XIAP</td>
<td>X-linked inhibitor of apoptosis protein</td>
</tr>
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Chapter 1 Literature review: the Fibroblast Growth Factor Receptors and their regulation of cellular responses
1.1 Biological functions of FGFs and FGFRs

Cells within multicellular organisms utilize a complex, dynamic and highly integrated signalling network to orchestrate intracellular and cell-cell communications. Growth factors are proteins secreted by cells, which have evolved to achieve long-distance and long-term communications between cells. Cytokines and growth factors exert their signal transduction functions by binding and activating cell surface receptors. Growth factor and cytokine receptors consist of an extracellular ligand-binding domain, a trans-membrane domain and an intracellular domain that transduces signals within the cell. Through a series of biochemical events, growth factor binding to specific cell surface receptors results in the activation of intracellular signalling cascades that ultimately regulate targets in the cytoplasm and nucleus to mediate diverse cell behaviors, such as proliferation, survival and differentiation.

1.1.1 Introduction of FGFs and their receptors

The fibroblast growth factors (FGFs) are a large family of growth factors that play important roles in diverse biological processes such as embryonic development, tissue repair, angiogenesis and cancer [1]. The FGFs exert their functions through the binding of four structurally related FGF receptors (FGFRs), including FGFR1, FGFR2, FGFR3 and FGFR4, in a heparin-dependent manner [2].

FGF and FGFR genes have been identified in all metazoa but not in bacteria or yeast, highlighting their importance in mediating intercellular processes spanning an array of species. The FGFs were initially identified for their mitogenic activities toward murine fibroblasts in the mid-1970s. Since then, FGFs have been further shown to regulate diverse biological responses, such as cell survival, differentiation and
migration. FGFs and FGFRs are involved in repair processes in a wide range of mammalian tissues [3].

The work presented in this thesis is primarily focused on the ligands FGF1 and FGF2 together with their receptors FGFR1 and FGFR2. The FGFRs and their cognate ligands are potent regulators of cell survival, proliferation and differentiation within neuronal tissues. The overall aim of the present studies is to understand the molecular mechanisms by which the FGFRs regulate neurogenesis using a number of in vitro model systems.

1.1.2 The functions of FGF and their receptors in central nervous system

The FGFs play various roles within the central nervous system (CNS), such as stimulating neurogenesis, enhancing axonal branching, and promoting neuron survival [4, 5]. The FGF1 and FGF2 are extensively expressed in the CNS during both development and adulthood [6-9]. In CNS, while FGF1 is primarily expressed in different type of neurons, FGF2 is expressed in both neuronal and non-neuronal cells, such as glia cells [10, 11]. Importantly, the expression of FGF1 and FGF2 has been shown to be elevated within brain after multiple types of injuries, stroke and neurodegenerative disorders in the mouse models, indicating that the FGFs may have an important role in brain injuries recovery and neural regeneration [12-17]. The FGFR1 and FGFR2 also have distinct expression patterns. FGFR1 is predominantly localized on neurons, while FGFR2 is expressed primarily on glial cells [10, 11].

FGF1 and FGF2 are important mitogens for different type of neuronal precursors and can induce neuronal cells proliferation both in vivo and in vitro. For example, FGF2
has demonstrated potent mitogenic activity toward rat embryonic dopamine precursors *in vitro* [18]. Furthermore, FGF2 can stimulate the proliferation of primary gamma-aminobutyric acid (GABA) neurons derived from embryonic rat cerebral cortex. Interestingly, FGF2 can also facilitate GABA neurons to take up and store GABA [19]. Furthermore, administration of FGF2 *in vivo* has been shown to promote neurogenesis in both the hippocampus and the subventricular zone [20, 21], while other studies have shown that the injection of FGF2 into the cerebellum can increase granule neuron numbers to promote cerebellar neurogenesis [22].

Gene knockout studies in mice show that FGF signalling plays a critical role in neural tissues development and proper function maintenance [4, 23]. The *in vivo* biological functions of FGFs and FGFRs in CNS have been widely investigated by using gene deletion in knockout animal experiments. FGF1 knockout mice are viable and do not have any significant phenotype, indicating that the function of FGF1 might be compensated through the redundancy of FGFs [24]. FGF2 deficient mice have demonstrated impaired cerebral cortex development, caused by a significant reduction in neuronal density in most layers of the motor cortex [25, 26]. In terms of FGFRs, FGFR1 deficient mice die of severe growth retardation prior to or during gastrulation [27]. FGFR1 knockout mouse embryos have further demonstrated the impaired neural tube formation and limb bud development [28]. FGFR2 gene disruption results in an early embryonic lethality, which suggest that FGFR2 is required for early post-implantation embryogenesis [29]. These studies using knockout mice indicate that FGF1 and FGF2 together with their receptors play an essential role in diverse cellular processes in CNS both during development and adulthood [4, 23].
FGF2 can promote the survival of multiple types of neuron from the CNS. For example, the dorsal root ganglion (DRG) sensory neurons isolated at the later stages of development require FGF2 for survival and proliferation in vitro [30]. In addition, the immunosuppression of FGF2 could significantly reduce the number of DRG sensory neurons, suggesting that FG2 might be also required for (DRG) sensory neurons survival in vivo [30]. FGF2 has also been shown to promote rat neocortical neurons survival and the neurite growth in vitro [31]. Furthermore, FGF2 can also significantly promote the survival and differentiation of dentate gyrus neurons from neonatal rats [31]. Similarly, the survival of fetal neurons derived from the rat cerebral cortex, septum, striatum, midbrain, thalamus, and colliculus can be enhanced by FGF2 [32]. In other studies, FGF2 has been shown to have neurotrophic activity for adult rat hippocampal neurons and enhance their viability at least 3-fold in vitro without affecting the morphology [33]. Moreover, the continuous application of FGF-2 inhibites neuronal cell death induced by serum-deprivation in organotypic hippocampal culture in vitro [34]. Together, these studies demonstrate that both FGF1 and FGF2 can act as neurotrophic factors promoting the survival and differentiation of neurons both in vitro and in vivo.

1.2 FGF receptor activation and signal transduction

The FGFRs are a group of important members of the receptor tyrosine kinases (RTKs) family, which possess intrinsic tyrosine kinase activities [35]. FGFRs are composed of an extracellular ligand binding domain, a single trans-membrane domain and a cytoplasmic domain containing the protein tyrosine kinase activities (Figure
Activation of FGFR requires both FGF and heparin or heparin sulfate proteoglycans (HSPG) [36]. After the FGF and heparin bind to FGFRs, FGFRs undergo dimerization, which results in the activation of receptor tyrosine kinase activities and the phosphorylation of multiple tyrosine residues within the cytoplasmic domain [37]. These phosphotyrosine residues provide binding sites for signalling proteins containing Src homology 2 domain (SH2) or phospho-tyrosine-binding domain (PTB) domains [1]. The binding of SH2 proteins or PTB proteins to phosphotyrosine docking sites physically couples activated FGFRs to downstream signalling pathways, including the Ras/MAP kinase, phosphatidyl inositol 3-OH kinase (PI3 kinase), phospholipase C γ (PLCγ) and the signal transducer and activator of transcription (STAT) pathways (1,2).
Figure 1-1 Schematic representation of FGFR1/2 structure

Shown are extracellular immunoglobulin-like (Ig) domains, the transmembrane domain, the intracellular domain including the juxtamembrane domain, the split tyrosine kinase domain, and the C-terminal lobe. Conserved cytoplasmic tyrosine autophosphorylation sites are indicated. Tyrosine 653 (Y653) and tyrosine 654 (Y654) highlighted with red color are crucial for FGFRs intrinsic protein tyrosine kinase activities.
Seven tyrosine residues in FGFR1, including Y463, Y583, Y585, Y653, Y654, Y730 and Y766, have been reported to be phosphorylated after activation of FGFR1 [38]. Two of tyrosine residues, Y653 and Y654, within the activation loop of the tyrosine kinase domain, are essential for the activation of tyrosine kinase activity therefore important for all biological responses of FGFRs [38]. The remaining five tyrosine residues, including Y463, Y583, Y585, Y730 and Y766, are proposed to provide docking sites for the binding of SH2- and PTB-domain proteins [39, 40]. So far it has only been proved that Y766 and Y463 are able to physically associate with signal proteins [40, 41]. For example, phosphorylation of Y766 in the C-terminal tail of FGFR1 has been shown to be essential for PLCγ binding and the generation of inositol phosphates [42]. Mutation of Y766 resulted in a defect in PLCγ activation and phosphatidylinositol (PI) biphosphate hydrolysis [40]. In addition, phosphorylation of Y463 has been reported to recruit Crk adaptor protein and play a role in the proliferation of porcine aortic endothelial cells [41]. Although the remaining tyrosine residues have been shown to be phosphorylated, the specific binding partners or biological responses have not yet been identified.

The scaffold protein, FGFR substrate 2 (FRS2), is important for FGFRs signalling [43]. Two isoforms, FRS2α and FRS2β, are constitutively associated with the juxtamembrane domains of FGFR1 and FGFR2 [44]. After the activation of FGFRs, multiple tyrosine residues within FRS2 are phosphorylated and provide docking sites for signal proteins that include Growth factor receptor-bound protein 2 (Grb2), GRB2-associated-binding protein 1 (Gab1) and SH2 domain-containing protein tyrosine phosphatase-2 (Shp2) to form signalling complexes, which are essential for mediating the Ras/MAP kinase and PI3 kinase pathways [44, 45] (Figure 1-2).
Importantly, gene knockout studies have shown that the deletion of FRS2α gene blocks FGF induced signalling and therefore causes severe impairment of mouse development and embryonal lethality at E7–7.5 [44], demonstrating important roles of this molecule in FGFRs mediated biological responses.

A number of pathologies that arise from mutations in the FGFRs have been identified. For example, several skeletal disorders are caused by specific point mutations of the FGFRs [1]. The most common form of dwarfism is caused by a gain-of-function mutation in the trans-membrane domain of FGFR3 [1]. Over-expression of FGFRs has been found in different types of cancer, such as breast cancer [46], prostate cancer [47, 48], glioblastoma brain tumours [49] and several haematological malignancies [50-54].
Figure 1-2 FGFR activation and signal transduction.

FGFR requires both FGF ligands and heparin for activation. The activation of FGFR involves receptor dimerization/oligomerization, the activation of intrinsic receptor tyrosine kinase activity and the phosphorylation of specific tyrosine residues in the cytoplasmic domain of the receptor. The activated FGFR induces three major signalling pathways: Ras/MAP kinase, PI3 kinase, and PLCγ signalling pathways.
1.2.1 Ras/MAPK pathway

FGFs are potent regulators of the Ras/MAPK pathway [36, 55, 56]. The Ras/MAPK pathway plays a central role in regulating proliferation, differentiation, migration and survival mediated by growth factors including FGFs [57]. It is becoming increasingly apparent that the amplitude, duration and temporal distribution of Ras/MAPK signalling are important in determining cellular outcomes, such as proliferation, differentiation and survival [58]. The Ras small GTPases are signalling proteins downstream of growth factor receptors that bind and hydrolyze nucleotide guanosine triphosphate (GTP). Activation of Ras by growth factor receptors leads to recruitment of Raf kinases to cytoplasmic membrane, which in turn triggers the activation of the Raf kinases. The Raf kinases constitute a signalling module with MEK (mitogen-activated protein kinase kinase) and ERK (extracellular-signal-regulated kinase).

1.2.1.1 Ras regulation

The Ras family contains three key members that are critical for regulating cellular responses: K-Ras, H-Ras, and N-Ras [59]. Activating mutations in Ras isoforms have been reported in more than 30% cancers [60]. RTKs including FGFRs are potent regulators of Ras family members [61]. The Ras small GTPases cycle between active (GTP bound) and inactive (GDP bound) conformational states. The association with GTP induces Ras conformational change into the "active" state, which is facilitated by guanine nucleotide exchange factors (GEFs), while the binding with nucleotide guanosine diphosphate (GDP) induces conformational change into the "inactive" state, which is accelerated by GTPase activating proteins (GAPs) [62, 63]. The balance between GEF and GAP activity determines the activation status of Ras, thereby regulating the Ras activity.
The most well-described GEF for Ras is the Son of Sevenless (Sos), and the most widely studied GAP for Ras is p120 GAP. The mechanisms regulating Ras activation mediated by RTKs have been extensively studied [64]. The growth factor receptor-bound protein 2 (Grb2) is the key adaptor protein in Ras activation, which forms a signalling complex with Sos [65]. When the Grb2/Sos complex is translocated to the cell membrane, Sos can activate Ras GTPase activity [65]. The SH2 domain of Grb2 can directly associate with phospho-tyrosine docking sites in RTK cytoplasmic domain [65], or indirectly associates with other scaffold proteins [43]. For example, the pheochromocytoma (PC12) cell line has been widely studied in examining the growth factor regulations and intracellular signalling pathways. PC12 cells differentiate into neuronal phenotype characterized by the up-regulation of neural-specific genes and the extension of neurite-like outgrowths in response to FGF or nerve growth factor (NGF). Importantly, PC12 cells demonstrate pleiotropic responses to growth factor stimulation. For example, while PC12 cells differentiate in response to FGFs or NGF, they undergo proliferation in response to epidermal growth factor (EGF) (the more detailed description of PC12 cells will be introduced in Chapter 2) [66]. Thus, the PC12 cell system has been used to explore the mechanism by which growth factors induce specific downstream signalling and cellular responses. Ras is a key regulator for PC12 cells neuronal differentiation mediated by FGF or NGF. For example, FGF or NGF stimulation induces the increased Ras activity [67], while microinjection of anti-Ras antibodies [68] and the expression of the dominant-negative H-Ras mutant [69] blocks cellular responses mediated by NGF and FGF. These results suggest that Ras activation is necessary for NGF and FGF to stimulate PC12 differentiation. Furthermore, the activation of Ras alone is sufficient
to mediate PC12 differentiation. For instance, both transfection of Ras gene [70] and microinjection of H-Ras protein could induce PC12 cell neuronal differentiation independent of NGF or FGF [71]. These studies suggest that Ras is important for signal transduction mediated by growth factor receptors.

### 1.2.1.2 Raf kinase regulation

Raf kinases are a group of serine/threonine kinases widely expressed in the eukaryotes and play a central role in intracellular signalling. Raf kinase family consists of three key members: Raf-1 (C-Raf), B-Raf and A-Raf. The activation of Raf kinases involves a series of signalling events, including translocalization, phosphorylation, proteins interaction and conformation change [72-74].

Raf-1 is crucial for mediating growth factor responses in diverse cellular settings [72-74]. For example, the antisense Raf-1 RNA or the dominant-negative Raf-1 mutant can block the cell proliferation mediated by growth factors or 12-O-Tetradecanoylphorbol-13-acetate (TPA) [72-74]. In addition, Raf-1 is required in the proliferation and anchorage-independent colony formation of Ras-transformed cells [75]. These findings suggest that Raf-1 can function as the signalling transducer to transmit proliferative signals.

Ras activates the Raf kinases through recruiting Raf to the plasma membrane. It has been shown that GTP-loaded Ras can physically interact with Raf-1 through an N-terminal regulatory domain of Raf-1 [76, 77]. In addition, activated Ras is able to specifically associated with the Ras binding domain (RBD) of Raf-1 in vitro [78]. Activated Ras also forms a complex containing Raf-1 and MEK from rat brain lysates
These results support the prevailing paradigm that Ras is a direct effector of Raf-1 [59, 63]. Although GTP-loaded Ras (active) can recruit Raf-1 to the plasma membrane, the recruitment alone is not sufficient for the full activation of Raf-1. In addition to cell membrane recruitment, the phosphorylation of Raf-1 on a number of key residues, including S338, Y341, T491, and S494, are required for Raf-1 kinase activation and signalling [79, 80]. The laboratory of Rapp has identified a model of Raf-1 activation in the PC12 cells [81]. In an unstimulated cell, the kinase activity of Raf-1 is constrained by two mechanisms: 1) the inter-molecular interaction between the N-terminal regulatory domain and the C-terminal catalytic domains; 2) the intra-molecular interaction between the 14-3-3 phospho-serine binding proteins and two phosphorylation sites, Ser259 and Ser621 [81]. After the growth factors stimulation, activated Ras displaces the 14-3-3 proteins from the Raf-1, which releases the inhibitory effects of the 14-3-3 protein and results in a full activation of Raf-1 [82].

B-Raf has been identified as a human oncprotein because B-Raf has been shown mutated in about 7% of total cancers and 70% of malignant melanomas [89]. B-Raf is regarded as a major MEK activator rather than C-Raf or A-Raf [83, 84]. The activation of B-Raf also requires signalling events including membrane recruitment, scaffold protein interaction and phosphorylation [85]. Translocalization to the plasma membrane driven by Ras is critical for the B-Raf activation. The studies based on the PC12 cells have shown that multiple growth factors, including EGF, NGF and FGF, induce rapid B-Raf phosphorylation, which indicates the increased activity of B-Raf [86]. There are multiple phosphorylation sites in the B-Raf, including Ser364, Ser428, Thr439, Ser445, Thr598 Ser601 and Ser728 [86, 87]. The phosphorylation of
conserved residues Thr598 and Ser601 localized in the kinase domain is essential for the B-Raf activation [87].

1.2.1.3 MEK regulation
MEKs consist of MEK 1 and MEK2 isoforms, which are a group of rare dual-specificity serine/tyrosine protein kinases [88]. The phosphorylation of MEK1/MEK2 on Ser217 and Ser221 residues respectively by Raf kinases leads to full activation of their kinase activities [89]. MEK1/MEK2 represent the key components of the MAP kinase pathway. The dominant–negative and constitutively active forms of MEK1 have been developed to elucidate the relationship between the activation of MAP kinase pathway and specific cellular responses. For example, expression of a dominant–negative MEK1 can block both growth factor mediated PC12 cell neuronal differentiation [90]. Furthermore, constitutively active MEK1 is also able to induce NIH 3T3 cell transformation [90]. Conversely, expression of a dominant–negative MEK1 can block PC12 cell neuronal differentiation mediated by NGF and NIH 3T3 cell proliferation [92].

1.2.1.4 ERK regulation
ERK1/ERK2 are highly conserved and widely expressed protein kinases, which are involved in many fundamental cellular responses including cell proliferation, survival, differentiation, apoptosis, migration and metabolism [57, 91]. The activation of ERK1/ERK2 has been observed in multiple cell systems in response to diverse growth factors including the FGFs, highlighting their important roles in regulating cell proliferation, survival [92-95] and cell cycle [96-98].
The full activation of ERK1/ERK2 requires the phosphorylation of residues Thr202/Tyr204 and Thr185/Tyr187 respectively by MEK1/MEK2 [99, 100]. The expression of dominant-negative MEK1 or the treatment of cells with a specific MEK1/2-inhibitor, PD98059, resulted in the inhibition of multiple cellular responses mediated by ERK, such as the transformed NIH 3T3 cells growth and colony formation, and NGF mediated PC 12 cells differentiation, suggesting that ERK1/ERK2 are the regulatory targets of MEK1/MEK2 [90, 101].

The spatial distribution of ERK1/ERK2 is also important for cellular responses mediated by ERKs. In the absence of growth factor stimulation, ERK1/2 remains in the cytoplasm. However, following growth factor stimulation, ERK1/2 phosphorylation and activation result in its translocation to the nucleus where it phosphorylates transcription factors such as AP1 to promote genes expression [102-105]. The ERK1/2 translocation into nucleus is rapid, reversible, and even more importantly, coupled to the signal amplitude [104, 105]. For example, in PC12 cells, the sustained ERKs activation leads to a rapid accumulation of activated ERKs in the nucleus, while the transient ERK activation fails to promote nuclear translocation of ERK [106].

Activated ERK1/ERK2 can phosphorylate a large number of substrates in both cytoplasm and nuclear, including kinase substrates, such as RSKs, MSKs, and MNKs, and nuclear substrates, such as Elk-1, MEF2, c-Fos, c-Myc, and STAT3 [102]. The ERKs exert their regulatory functions partially through the MAPK-activated protein kinases (MKs), which are a group of conserved serine/threonine kinases on the downstream of ERKs. The prototypes of this family of protein kinases are the 90 kDa
ribosomal S6 kinase (RSK) kinases, which are able to translocate into nucleus and share overlapping substrates with ERKs [107].

Diverse growth factors are able to promote pleiotropic biological responses at least in part through the regulation of the Ras/MAP kinase pathway. Thus, a long-standing question in growth factor biology has been how the Ras/MAP kinase pathway can specify pleiotropic cellular responses, such as proliferation, survival or differentiation. Blenis and Murphy propose a model that immediate early genes (IEG) and the expression of IEG-encoded protein products function as a sensor to interpret the amplitude of ERK signals [58]. The majority of the IEG-encoded proteins are transcription factors or components regulating gene transcription. ERKs/RSKs are able to phosphorylate IEG-encoded proteins and enhance their stabilities. For example, c-Fos is phosphorylated by sustained ERK activation, which stabilizes its structure. Enhanced stability of c-Fos results in increased target genes expression and eventually change cell behaviors [108].

1.2.2 The phosphoinositide 3 kinase pathway

The phosphoinositide 3 kinases (PI3 kinases) are a group of lipid kinases that are able to phosphorylate the D-3 position hydroxyl group in the inositol ring of phosphoinositides (PI). The activated PI3Ks transform the membrane located lipid phosphatidylinositol-4,5-bisphosphate (PIP2) into phosphatidylinositol-3,4,5-trisphosphate (PIP3) in response to growth factors, cytokines and hormones [109]. As the major products of PI3Ks, the PIP3 functions as lipid second messenger to recruit multiple cytoplasmic substrates to the membrane and sequentially activate those molecules after growth factor stimulation [110]. The PI3Ks are key regulators for
diverse cellular responses, including proliferation, growth, migration and survival [111]. There are three classes of PI3K in the PI3K family, including the class I, II and III. Of the three classes of PI3K, the class I isoforms are the major isoforms regulated by growth factors receptors for PIP3 lipid synthesis [110], which will be discussed with more details below.

1.2.2.1 The regulation of PI3 kinase signal

The class I PI3 kinases are heterodimers consisting of a p110 catalytic subunit and a p85 regulatory subunit. The p85 regulatory subunits possess two SH2 domains that bind specific phosphorylated tyrosine residues on the growth factor receptors or associated scaffold proteins [111]. After growth factor stimulation, PI3 kinases are recruited to phospho-tyrosine residues within the growth factor receptors, which not only brings PI3K into a close proximity of its PI substrates at the plasma membrane [112], but also triggers an increase in the PI3 kinase activity by releasing the inhibition of regulatory subunits [113]. The p110 catalytic subunit contains a Ras-binding domain, which also allows p110 catalytic subunits to interact with activated Ras and therefore provides a mechanism for further activation of PI3 kinase [114, 115].

Activated PI3 kinases phosphorylate PIP (4,5) P2 to generate PI (3,4,5) P3, which serves as the docking sites for the signalling proteins containing pleckstrin homology (PH) domains. The PH domain demonstrates a high affinity to bind PIP3 within the plasma membrane. Therefore, a range of signalling proteins containing PH domains are recruited to the plasma membrane following the generation of PIP3 by PI3 kinases in response to growth factors [81]. For example, the PH domains of protein kinases
such as Akt [116] and phosphoinositide-dependent kinase 1 (PDK1) [117] bind to PIP3 in the plasma membrane resulting in kinase recruitment and activation of downstream signalling. Ultimately, these events are critical in coordinating the key signalling networks to mediate multiple cellular responses including cell growth, actin polymerization, cell migration and cell survival. [110, 118].

Lipid phosphatases, which dephosphorylate PI (3,4,5) P3 into PI (4,5) P2, are able to antagonize PI3 kinase signalling. The Src-homology 2 containing phosphatases (SHIPs) and phosphatase and tensin homolog (PTEN) are two lipid phosphatases that can down-regulate and/or terminate PI3 kinase signalling. The SHIPs, including SHIP1 and SHIP2, convert PI (3,4,5) P3 into PI (3,4) P2 by dephosphorylating the 5 position of the inositol ring, while the PTEN converts PI (3,4,5) P3 into PI (4,5) P2 through hydrolyzing the phosphate group in D-3 position of inositol [119]. For example, SHIP2 is a potent negative regulator of the insulin signalling pathway through antagonizing the PI3 kinase signalling. The loss of SHIP2 significantly increases insulin sensitivity [120]. In a quite different manner, the lipid phosphatase PTEN attracts substantial interests because that loss of functions of PTEN has been observed to promote deregulated PI3K signalling and tumorigenesis in a wide range of human cancers [121, 122].

1.2.2.2 PI3 kinase pathway regulating cell survival

The PI3 kinase pathway has been widely shown to be important for growth factor mediated cell survival [109, 110, 123]. For example, the essential role of PI3 kinase in regulating cell survival has been firstly reported in PC12 cell survival mediated by NGF or PDGF under serum-deprived conditions [124]. The prominent role of the PI3
kinase in cell survival has been further shown in two rat fibroblast cell models including the NIH3T3 and the BALB 3T3 cell [125]. Since then, the important role of PI3 kinase in FGF mediated cell survival has been further confirmed in a panel of cell lines including Swiss 3T3, NIH 3T3, HEK293, COS-1 and SH-SY5Y cells [45].

The protein serine/threonine kinase Akt is the key mediator for PI3 kinase regulated cell survival [126-128]. Akt (also known as PKB), is a group of serine/threonine kinases consisting of three isoforms: Akt1, Akt2, and Akt3 [129, 130]. Akt1 is ubiquitously expressed at a high level in all tissues [130, 131], while Akt2 is specifically highly expressed in insulin-sensitive tissues such as the liver, skeletal muscle and adipose tissue [132, 133], with a very high expression in brain [134]. PI (3,4,5) P3, the major product of the PI3 kinase, translocates Akt to the membrane through PH domain binding to phospho-lipid [135]. The full activation of Akt requires further phosphorylation, and the dual phosphorylation of threonine 308 and serine 473 increases Akt activity significantly [136, 137]. PKD1 and Rictor-mTOR complex have been shown their essential roles in regulating Akt phosphorylation. For example, the Thre308 is phosphorylated by PDK1 [117, 138], while the Ser473 is phosphorylated by Rictor-mTOR [139].

Akt exerts its anti-apoptotic effects through the phosphorylation and inhibition of the pro-apoptotic protein Bad [140, 141]. Bad belongs to the Bcl-2 family, which consists of both pro-apoptotic and anti-apoptotic members. The phosphorylation of Bad by Akt results in the binding of the 14-3-3 proteins, which sequesters Bad to the cytoplasm thereby neutralizing its pro-apoptotic activity [140, 141]. In the absence of growth factor stimulation and PI3 kinase signalling, Bad remains unphosphorylated
and is free to translocate to the mitochondria, where it engages with Bcl-xL to induce apoptosis [140, 141]. Thus, the PI3 kinase pathway is able to directly regulate key members of Bcl-2 family to control cell survival [142-144].

1.2.3 The Phospholipase Cγ pathway

Phospholipase Cγ (PLCγ) belongs to the phospholipase C (PLC) family, which contains more than 10 isoforms. They are divided into 4 subfamilies, including β, γ, δ and ε in mammals [145]. The PLC proteins hydrolyze phosphatidylinositol 4,5-bisphosphate (PIP2) into inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG), which are important secondary messengers following the activation of diverse growth factor surface receptors such as FGFRs [146, 147]. IP3 stimulates the release of Ca\(^{2+}\) from intracellular stores [148]. Ca\(^{2+}\) binds to calmodulin and activates a family of Ca\(^{2+}\)/calmodulin-dependent protein kinases. For example, the production of both DAG and Ca\(^{2+}\) are known to activate the protein kinase C (PKC) family of protein kinases (described in more detail below), which are able to regulate diverse cellular responses [149].

PLCγ contains a catalytic domain and a regulatory domain. The regulatory domain consists of both PH domains and SH2 domains, which facilitate the recruitment of PLCγ to cell membrane and phospho-tyrosine residues of activated receptor tyrosine kinases respectively [149]. Receptor tyrosine kinases further phosphorylate PLCγ on tyrosine residues, including Y771, Y783 and Y1254, which are crucial for full activation of PLCγ [150, 151]. For example, Y766 in the C-terminal tail of FGFR1 is critical for PLCγ binding and activation, which is required for the generation of inositol phosphates by PLCγ [42].
1.2.4 Protein kinase C pathway

The PKC family plays an important role in regulating cell proliferation, differentiation, apoptosis and angiogenesis [152]. FGFs have been shown to be the key regulators for multiple isoforms of protein kinase C (PKC) in various cell types [153-155], and particularly in neuronal cells [156, 157]. The PKC family is a group of serine/threonine kinases consisting of 11 isoforms. Based on the requirement of second messengers for activation, they can be divided into three subfamilies: conventional Protein Kinase C (cPKCs), novel Protein kinase C (nPKCs) and atypical Protein kinase C (aPKCs). The cPKCs include the isoforms α, βI, βII, and γ. The activation of cPKC requires Ca$^{2+}$, DAG, and a phospholipid such as phosphatidylserine. The nPKCs, including the δ, ε, η, and θ isoforms, require DAG and phosphatidyserine but not Ca$^{2+}$ for their activation. Lastly, aPKCs, which include protein kinase Mζ and ι/λ isoforms, require neither Ca$^{2+}$ nor DAG but phosphatidyserine for activation [152]. The full activation of PKCs activity requires three mechanisms, including cofactor binding, phosphorylation and interaction with scaffold proteins [158]. DAG recruits PKCs from cytosol to membrane and also allosterically activates the kinases [159]. Both highly ordered phosphorylation by phosphoinositide-dependent kinase 1 (PKD1) and auto-phosphorylation further enhance the kinase activity [160]. Scaffold proteins, such as receptors for activated C-kinase 1 (RACK1), also play a crucial role in regulating PKCs activities through bridging PKCs to substrates, regulators and specific compartments [161].
1.2.4.1 The biological functions of PKCs in CNS

PKCα, PKCε, PKCδ and PKCγ, are highly expressed in neuronal cells of brain and spinal cord tissues [162, 163]. Indeed, PKCs have been shown to be a potential therapeutic target for treating neurodegenerative diseases, such as Alzheimer's disease, stroke and traumatic brain injury [163-165]. For example, in the mouse models of stroke, different isoforms have been shown to play opposite roles in cerebral ischemic and reperfusion injuries [165]. During cerebral ischemic and reperfusion injuries, PKCδ and PKCγ play a detrimental role through increasing apoptosis, necrosis and inflammation [165]. In contrast, PKCε exerts neuro-protective effects via the induction of ischemic tolerance, such as promoting neurons survival and synaptogenesis [163, 165, 166]. Consistent with its neuro-protective functions in stroke, in the mouse model of Alzheimer's disease, over-expression of PKCε in neurons selectively increases endothelin-converting enzyme (ECE) activity, which sequentially improves amyloid β protein degradation and relieves neuropathology symptoms of Alzheimer's disease [167, 168]. In a similar manner, increased PKCε activity by specific activators efficiently prevents synaptic loss, amyloid β protein elevation, and cognitive deficits in Alzheimer's disease transgenic mice [169]. Thus, the isoform-specific PKC activators are proposed to be particularly useful for treating neurodegenerative diseases [164].

The neurite outgrowth in neuronal cell lines as well as primary mouse neurons in response to growth factor stimulation has been thought to be mediated at least in part through the activation of PKCs [170]. For example, the neuronal differentiation PC12 cells in response to NGF has been enhanced significantly by TPA, which is a potent PKCs activator [171]. Furthermore, the over-expression of PKCε isoform also can
enhance PC12 cells neurite outgrowth and Ras/MAP kinase signalling mediated by NGF [172, 173]. In additional, co-treatment PC12 cells with EGF (which does not normally induce PC12 cells neuronal differentiation by itself) and TPA has induced robust neuronal differentiation [174]. Furthermore, in the PC12 cell neuronal differentiation mediated by NGF, PKCδ has been shown to function as the positive feedback loop to maintain the sustained activation of Ras/MAP kinase pathway [174]. In another example, PKCe has been shown to be involved in neuronal differentiation of primary mouse neuroblastoma cells [175-177]. While a number of studies have highlighted the roles of individual PKC isoforms in PC12 cells neuronal differentiation mediated by NGF, there are very limited studies conducted to investigate the role of PKC family in FGFs mediated PC12 cell differentiation.

1.2.4.2 PKCs signalling regulating Ras/MAP kinase pathway

PKCs are the major mediators for the Ras/MAP kinase pathway. Non-specific PKCs activator TPA can induce ERKs phosphorylation in both quiescent chick cells and human monocyte-like cells [178, 179]. Indeed, further studies have shown that the TPA-induced activation of PKCs can regulate Ras/MAP kinase pathway via directly phosphorylating Raf kinases [180, 181]. For example, conventional PKCs have been shown to activate Raf-1 through direct phosphorylation in NIH3T3 cells treated with TPA [183]. Prototype of cPKCs, PKCa, can activate Raf-1 through direct phosphorylation in NIH3 cells treated with TPA [183]. nPKCs play an important role in regulating Ras/MAP kinase signalling in the multiple cellular systems. For instance, over-expression of constitutively active PKCδ activates ERK in COS1 and NIH 3T3 cells independent of growth factor stimulation, suggesting that PKCδ is involved in Ras/MAP kinase signalling.
regulation [181]. VEGF mediates primary endothelial cells proliferation and DNA synthesis through activation of MAP kinase pathway. During this process, the activation of MAP kinase pathway requires PKCs in a Ras-independent manner [184]. PKCε functions as a Raf activator in growth factor-mediated Ras/MAP kinase signalling in both NIH 3T3 cells and COS cells [185]. Over-expression of PKCε leads to the elevated Raf-1 kinase activity as well as cell transformation in R6 fibroblasts cells, suggesting PKCε exerts its oncogenic effects through enhancing activation of the Raf-1 kinase [186, 187].

FGF2 can prevent the apoptosis of small cell lung cancer (SCLC) mediated by etoposide [188]. In the anti-apoptotic effects of FGF2, PKCε plays a crucial role in activating Ras/MAP kinase signalling through the formation of a signalling complex containing PKCε, B-Raf and S6K2, and sequentially up-regulates anti-apoptotic proteins, such as X-linked inhibitor of apoptosis protein (XIAP) and Bcl-XL [189].

1.3 Signal transduction mediated by serine residues in growth factor receptors

Although the importance of growth factor receptor tyrosine phosphorylation has been widely reported, the regulation of receptor serine phosphorylation and its biological roles remain less clear. While the phosphorylation of serine/threonine residues in signalling proteins is regarded as a major mechanism for allosterically regulating protein functions (e.g. activating enzyme activities), recent discovery of phosphoserine/threonine binding domain proteins has changed this view. The phosphoserine/threonine binding proteins are able to act as adaptors or scaffold proteins through their ability to coordinately regulate protein-protein interactions. The
multiple phosphoserine/threonine binding proteins/domains have been discovered and include 14-3-3 proteins, WW domain proteins, forkhead-associated domain proteins, WD40 repeats and leucine-rich region proteins [190]. Our previous studies have identified 14-3-3 protein binding sites in the cytoplasmic domain of growth factors receptors, including FGFR2 and the βc subunit of the GM-CSF and IL-3 receptors [190-194]. Some of the key features and biological functions of 14-3-3 family phosphoserine/threonine binding proteins have been described as below.

### 1.3.1 The roles of 14-3-3 proteins in signalling transduction

The 14-3-3 proteins function as scaffold or adaptor proteins through their ability to bind phosphoserine/threonine residues within a sequence-specific context [195, 196]. The 14-3-3 proteins were initially identified as a group of the highly abundant proteins from brain. Later, different isoforms have been identified in a wide range of tissues. Now they are established as a group of cross-species highly conserved 30 KDa acidic proteins expressed in all eukaryotic cells [197]. There are seven known mammalian 14-3-3 isoforms: β,ε,δ,γ,η,τ and ζ [198]. Several studies have shown that the 14-3-3 proteins interact with a broad array of intracellular proteins involved in the regulation of functions such as cell survival, metabolism, proliferation and differentiation [45, 196]. So far, the list of 14-3-3 substrates has grown to over 260, and the number is growing at a daily-based rate [199]. The binding of 14-3-3 proteins to the phosphoserine and phosphothreonine residues in their “client” molecules can regulate intracellular signalling via a number of different mechanisms, such as changing enzymatic activities of substrates, translocating substrates, and facilitating the formation of protein complex [199].
Although not exclusive, the binding sites of 14-3-3 proteins mostly contain phosphorylated serine or threonine motif [200]. The consensus motif for 14-3-3 binding was originally defined as RSXpSXP (mode I) or RXY/FXpSXP (mode II), where pS is phosphorylated serine [201]. However, more recently, 14-3-3 binding motifs that distinct from mode I or mode II have been reported [202]. For example, our studies have shown that 14-3-3 zeta binds HSRpSLP in the βc subunit of GM-CSF and IL-3 receptor [192-194]. One of important features of 14-3-3 proteins is their ability to form stable homo or hetero dimmers in vivo spontaneously [203]. X-ray crystallography indicates that the 14-3-3 proteins form stable dimers via their N-terminal in which a central groove binds the phosphoserine motif [196, 203] (Figure 1-3). Each 14-3-3 monomer consists of a bundle of nine antiparallel helices, which form a highly conserved, concave-shape inner surface and a variable outer surface [204]. In addition, the dimmers of different isoforms seem to have a very similar tertiary structure, which is particularly useful for their adaptor or scaffold proteins functions to facilitate proteins assembly [205].
Figure 1-3 Crystal structure of 14-3-3 dimer associated with phosphoserine peptides

Monomers of 14-3-3 proteins are able to self-assemble into stable homo or heterodimers in vivo. Each monomer of the dimer is associated with phosphoserine peptide containing the 14-3-3 binding motif as shown in the picture (Structure Reference: PDB: 2B05). A negatively charged channel is formed between the two monomers as the consequence of dimerization, which also mediates the association between 14-3-3 proteins and their substrates.
1.3.2 The phosphorylated serine residues of growth factor receptor coupling 14-3-3 proteins to mediate multiple cellular responses

In addition to tyrosine phosphorylation, growth factor receptors can also be phosphorylated on serine and threonine residues to provide docking sites for the 14-3-3 proteins family of phosphoserine/threonine-binding proteins. It has been shown that 14-3-3 proteins bind with several cell surface receptors through phosphorylated serine residues located in the receptors, including IL-9 receptor α, insulin-like growth factor 1 receptor (IGF-1R), the epidermal growth factor (EGF) receptor and the transforming growth factor β receptor (TGF β) after the growth factor stimulation [206-210].

Previous studies from our laboratory have identified a unique serine/tyrosine bidentate motif, which is a conserved amino acid sequence consisting of a phosphotyrosine docking site adjacent to a phosphoserine docking site [211]. Specifically, the original bidentate motif identified in the βc subunit of the GM-CSF and IL-3 receptor is composed of a binding site for the SH2 domain of Shc (Tyrosine 577) adjacent to a 14-3-3 binding site (Serine 585) [211]. These early studies have identified putative bidentate motives in diverse growth factor receptors composed of: 1) a specific sequence surrounding key tyrosine residue to form the binding site for SH2 domain or PTB domain; 2) a specific sequence surrounding key serine or threonine residue to form the 14-3-3 proteins binding site [191] (Table 1-1). The serine/tyrosine bidentate motif conserved in both FGFR1 and FGFR2 is composed of 14-3-3 proteins binding site Serine 779 (S779) and PLCγ binding site Tyrosine 766 (Y766) (Figure 1-5).
Importantly, from Lonic et al [191].

Table 1-1 Multiple cell surface receptors contain putative conserved bidentate tyrosine/serine motif.

<table>
<thead>
<tr>
<th>Name</th>
<th>Conserved motif</th>
<th>Accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>GM-CSF/IL-3/IL-5 receptor βc subunit</td>
<td>YSVPVIPNPQY</td>
<td>NP_000386</td>
</tr>
<tr>
<td>FGFR1(Flg)</td>
<td>NEQYLDSPLTD</td>
<td>P13162</td>
</tr>
<tr>
<td>erbB4</td>
<td>NEQYLDSPLTD</td>
<td>NP_005220</td>
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The database search had been performed to identify putative bidentate tyrosine/serine motif in a range of growth factor and cytokine receptors. The generic feature of those motifs is that every motif consists of both a putative conserved PTB or SH2 binding site and a conserved putative 14-3-3 binding site. Therefore, we proposed that the tyrosine/serine bidentate motif can serve as a generic mechanism in regulating pleiotropic cellular responses in a more broad range of systems. The table is taken from Lonic et al [191].

Importantly, we have shown that the Ser585 within the serine/tyrosine bidentate motif of βc subunit GM-CSF receptors is phosphorylated and binds the 14-3-3 proteins to regulate PI3 kinase signalling and hemopoietic cell survival [192-194]. Recently, we have shown a specific serine residue S779 within the serine/tyrosine bidentate motif of FGFR2 has important biological functions in regulating multiple cellular responses mediated by FGF2 in Ba/F3 hemopoietic cell line and BALB/c 3T3 fibroblasts [191]. The stimulation of FGF2 causes increased S779 phosphorylation, which provides the
docking site for 14-3-3 proteins. The recruitment of 14-3-3 leads to full activation of Ras/MAP kinase and PI3 kinase pathways, eventually to mediate cell survival and proliferation. These results have provided the first evidence that a specific serine residue within FGFR2, S779, regulates intracellular signalling and specific cellular responses (Figure 1-4).
The Ba/F3 and BALB/c 3T3 cells stably expressing wt-FGFR2 and FGFR2-S779G mutant receptor have been used to investigate biological function of the S779 in regulating cellular responses. The stimulation of FGF2 causes the increased S779 phosphorylation, which provides the docking site for 14-3-3 proteins. The recruitment of 14-3-3 proteins leads to the full activation of Ras/MAP kinase and PI3 kinase pathways, which eventually mediates cell proliferation and survival. Model is based on the work of Lonic et al [211].
Figure 1-5 Schematic representation of FGFR1/2 and the serine/tyrosine motif in the FGFR.
Shown are extracellular immunoglobulin-like (Ig) domains, the transmembrane domain, intracellular domain including the juxtamembrane domain, the split tyrosine kinase domain, and the C-terminal lobe. The serine/tyrosine bidentate motifs conserved in both FGFR1 and FGFR2 consist of S779 that is 14-3-3 proteins binding site and Y766 that is PLCγ binding site. Conserved tyrosine autophosphorylation sites are indicated, and the putative 14-3-3 binding site surrounding S779 is underlined.
1.4 Aims and hypothesis

The FGFRs regulate pleiotropic cellular responses through initiating multiple downstream intracellular signalling cascades, which include the Ras/MAPK, PI3 kinase, PLCγ, and STAT pathways [212, 213]. Among the major signalling pathways mediated by FGFRs, the Ras/MAP kinase module is particularly important for its roles in regulating diverse cellular responses. The abilities of the FGFs to regulate pleiotropic cellular responses through Ras/MAP kinase signalling pathway raises a very important question: how do growth factors regulate specific biological outcomes via a limited repertoire of seemingly overlapping and redundant signalling pathways?

A number of models have been proposed to address this question. One hypothesis is that cell surface receptors regulate signalling pathways in quantitatively different manners (“strength of signalling”) to mediate different biological responses. Probably the most widely cited prototypic example of this “specificity versus redundancy” conundrum is the ability of EGF to promote the proliferation of PC12 cells, while growth factors such as FGF and NGF regulate differentiation [214]. What has remained an unresolved and open question is that while FGF and EGF are able to promote two distinct and entirely different cellular responses in PC12 cells, they appear to regulate similar and overlapping intracellular signalling pathways. For example, both FGF and EGF promote receptor tyrosine phosphorylation of their respective cognate receptors and activate signalling via the Ras/MAPK, PI3 kinase, PLCγ and STAT pathways. Despite significant work, no specific tyrosine residue in the FGFR has been functionally linked to PC12 differentiation [40, 215-217].
Similarly, no specific tyrosine residue in the EGFR has been functionally linked to proliferation [218-220].

Thus, how are FGF and EGF able to promote different biological responses when both ligands regulate a limited repertoire of overlapping and redundant signalling pathways? One explanation proposed by Chris Marshall in 1995 is the “transient versus sustained” model of intracellular signalling whereby sustained activation of the Ras/MAPK pathway by FGF or NGF promotes PC12 differentiation, while transient activation of the Ras/MAPK pathway by EGF promotes PC12 proliferation [106]. In other words, growth factor receptors may regulate pleiotropic biological responses by signalling in an analogue manner where “signal strength” determines specific cellular responses.

Another possibility is that growth factor receptors utilize phospho-serine mediated mechanisms for defining specific signalling events and cellular responses. For example, our previous studies have shown that the Serine 585 (S585) of βc subunit of the GM-CSF and IL-3 receptor is phosphorylated and binds the 14-3-3 proteins, which regulates PI3 kinase signalling to mediate hemopoietic cell survival [192-194]. We have further shown that S779 of FGFR2 is phosphorylated in response to FGF2 and binds the 14-3-3 proteins, which leads to the full activation of both Ras/MAP kinase and PI3 kinase pathways, and sequentially mediates cell proliferation and survival in Ba/F3 hemopoietic cell line and BALB/c 3T3 fibroblasts [191]. Whether S779, which is conserved between FGFR1 and FGFR2, is important for regulating specific intracellular signalling pathways or biological responses such as differentiation has remained unclear.
Therefore, in current studies, we aimed to examine the potential roles of S779 signalling in mediating the neuronal differentiation of PC12 cells and primary mouse bone marrow stromal cells (BMSCs) in response to the FGFs stimulation. Our hypothesis was that S779 of FGFR2 plays a central role in mediating neuronal differentiation. In particular, these studies have examined the possibility that the dynamics and amplitude of the Ras/MAP kinase pathway is regulated by S779 signalling, which in turn regulates neuronal differentiation.

Specifically, these studies aimed to:

1) Examine the roles of S779 in regulating FGFR-proximal signalling events;
2) Examine and identify the signalling pathways regulated by the S779 in the neuronal differentiation mediated by FGFRs;
3) Identify the protein kinases responsible for S779 phosphorylation.

The studies performed as part of this thesis employed two cell models for neuronal differentiation. Firstly, the PC12 pheochromocytoma cell line, which has been widely used to examine neuronal differentiation [215, 221-224], will be used to examine wt-FGFR2 and FGFR-2S779G mutant to promote the intracellular signalling and differentiation. Secondly, a model system has been developed as part of the current studies in which BMSCs differentiate into neuronal phenotype \textit{in vitro} with FGF2 treatment [225-228].
Chapter 2 The phosphorylation of Serine 779 in FGFR2 regulates ERK signalling to promote PC 12 cell neurite outgrowth
2.1 Abstract

The Fibroblast Growth Factors (FGFs) and their receptors (FGFRs) play important roles in regulating pleiotropic biological processes in diverse tissues. However, it is not clear how the FGFRs utilize limited signalling pathways to mediate pleiotropic cellular responses. Previous studies from our laboratory have identified a new phosphotyrosine-independent mechanism by which the FGFRs can mediate specific cellular responses. These studies have shown that phosphorylation of Serine 779 (S779) in the cytoplasmic domain of FGFR2 provides a docking site for the 14-3-3 proteins and couples the activated receptor to downstream signalling pathways and biological responses. However, these previous studies did not define the role of S779 signalling in mediating specific cellular responses such as neuronal differentiation.

Using the PC12 pheochromocytoma cell line that differentiates in response to FGF and extends neurite-like extensions, the specific roles of S779 signalling have been examined. PC12 cells expressing FGFR2-S779G mutant have a significant reduction in FGF9-mediated differentiation when compared with cells expressing wt-FGFR2, demonstrating that S779 signalling is required for PC12 cells neuronal differentiation mediated by FGF9. The stimulation of FGF9 triggers S779 phosphorylation in PC12 cells but S779 signalling is not required for either FGFR or FRS2 tyrosine phosphorylation. PC12 cells expressing the FGFR2-S779G mutant are defective in their ability to induce Ras-GTP loading and ERK phosphorylation in response to FGF9 when compared with PC12 cells expressing wt-FGFR2. Specifically, PC12 cells expressing the FGFR2-S779G mutant exhibit the shorter Ras activation kinetics as well as the reduced magnitude of ERK phosphorylation. Interestingly, MEK
phosphorylation is not defective in PC12 cells expressing the FGFR2-S779G mutant when compared to PC12 cells expressing wt-FGFR2, suggesting that phospho-S779 regulates ERK signalling independently of MEK phosphorylation. These results identify a novel mode of ERK activation in which the phosphorylation of S779 of FGFR2 is required for the sustained Ras activation and the maximal ERK phosphorylation. These findings are significant in that they demonstrate that growth factor receptors utilize phosphoserine residues to couple to specific downstream pathways and biological responses.
2.2 Introduction

The FGFs are a family of pleiotropic growth factor mediating diverse cellular responses such as cell survival, proliferation, differentiation, and migration [3, 36, 229]. The FGFs exert such pleiotropic activities through the binding and activation of structurally related FGF receptors (FGFR1-4), which initiate downstream intracellular signalling cascades [212, 213]. The activation of FGFRs involves receptor dimerization/oligomerization, the activation of intrinsic receptor tyrosine kinase and the phosphorylation of specific tyrosine residues in the cytoplasmic domains of the receptors [55]. The phosphorylated tyrosine residues in the cytoplasmic domains of the FGFRs provide docking sites for signalling proteins containing SH2 or PTB domain and allow an activated receptor to physically couple to downstream signalling pathways. The major signalling pathways regulated by FGFRs include Ras/MAP kinase, phosphatidylinositol 3-OH kinase (PI3 kinase), phospholipase C γ (PLCγ) and signal transducer and activator of transcription (STAT) pathways [55]. Additionally, the molecular scaffold protein FRS2, which is constitutively associated with juxtamembrane domains of FGFR1 and FGFR2, is tyrosine phosphorylated following receptor activation and recruits signalling proteins regulating PI3 kinase and Ras/MAP kinase signalling [45].

It has been proposed that FGFR phosphotyrosine signalling pathways initiate specific intracellular signalling events that lead to distinct biological responses, but the underlining mechanisms are not fully understood. One proposed mechanism is that individual receptor tyrosine residues may be able to couple to specific signalling pathways and thereby regulate specific biological responses [39]. There are some
examples showing certain receptor tyrosine residues are linked to specific cellular responses [230, 231]. However, this widely accepted paradigm is difficult to explain the biological specificities exerted by FGFRs. For example, the point mutation and domain deletion studies have shown that tyrosine residues are dispensable for cell differentiation and mitogenesis mediated by FGFRs [40, 215-217]. It seems that receptor tyrosine phosphorylation is important for maintaining “generic” signals [232, 233], and other non-tyrosine mechanisms could contribute to the determination of cellular specificity. These studies raise the possibility that FGFRs may use alternative mechanisms to regulate diverse biological responses.

The PC12 pheochromocytoma cell line has provided an important model system for dissecting the molecular mechanisms by which growth factor receptors control intracellular signalling pathways leading to differentiation and neurite outgrowth [215, 221-224]. Treatment with NGF or FGF promotes the differentiation of PC12 cell in which they exit the cell cycle, extend neurite-like projections and express a range of neuronal-specific genes and cell surface markers such as neuroalfilament light chain (NF-L) and β-tublin (tuj1) [234-236]. In contrast, EGF stimulation promotes PC12 cell proliferation. However, despite the differential activities of FGF and EGF, both growth factors are able to promote signalling via the Ras/MAP kinase pathway. Thus, one longstanding question has been, how do FGF and EGF promote similar intracellular signals to mediate distinct cellular responses? One explanation proposed by Chris Marshall is the “transient versus sustained” model in which sustained activation of the Ras/MAP kinase pathway mediated by FGF (or NGF) promotes PC12 differentiation, while transient activation of the Ras/MAP kinase pathway by EGF leads to PC12 proliferation [106]. Such a model is supported by
previous studies showing that over-expression of the EGFR promotes more sustained ERK activation and leads to cell differentiation following EGF stimulation [237]. In contrast, PC12 cells that show a substantially reduced expression of NGF receptors demonstrate transient activation of ERK and proliferation rather than neuronal differentiation [238]. These studies highlight the mechanism that the kinetics of signalling pathways can be precisely regulated to control different specific biological outcomes (Figure 2-1).
Figure 2-1 The duration of ERK activation decides cell fates

EGF stimulation induces transient ERK activation and leads to proliferation, while NGF or FGF treatment induces sustained ERK activation and leads to differentiation. The figure is adapted from C.J. Marshall, Cell, 80: 179 (1995) [106]. The western blots are adapted from the S. Kao et al, JBC, 276:21 (2001) [239].
We have previously shown that S779 in the cytoplasmic domain of FGFR2 is phosphorylated in response to ligands and binds the 14-3-3 family proteins in Ba/F3 cells and BALB/c 3T3 fibroblasts [191]. The recruitment of the 14-3-3 proteins to S779 in the cytoplasmic domain of FGFR2 is essential for the full activation of the PI3 kinase and Ras/MAP kinase pathways, which sequentially regulate cell survival and proliferation in both Ba/F3 cells and BALB/c 3T3 fibroblasts [191]. To study the biological roles of S779 of FGFR2 in regulating PC12 cells neuronal differentiation, we generated wt-FGFR2 and FGFR2-S779G mutant constructs (Figure 2-2), and established stable PC12 cell lines expressing those specific constructs. PC12 cells do not express endogenous FGFR2 but do express endogenous FGFR1, FGFR3 [240]. To distinguish the transfected FGFR2 from endogenous FGFR1 and FGFR3 in our studies, we stimulated transfected PC12 cell lines with FGF9, which has higher affinity for FGFR2 [241] and therefore allows specific downstream responses of wt-FGFR2 and FGFR2-S779G to be assessed. In addition, specific phospho-S779 antibodies developed as part of previous studies were employed to investigate the regulation of S779 phosphorylation in response to growth factor stimulation. These unique tools allow us to examine the potential roles of S779 signalling in promoting the PC12 cells neuronal differentiation mediated by FGF9.
Figure 2-2 wt-FGFR2 and FGFR2-S779G mutant constructs

Schematic representation of FGFR2 and the identification of a putative 14-3-3 binding site at S779 located in the Serine/Tyrosine bidentate motif. The FGFR2 consists of extracellular immunoglobulin-like (Ig) domains, the transmembrane domain (TM), intracellular domain. In the FGFR2-S779G mutant, the S779 residue was substituted with glycine.
We found that PC12 cells expressing FGFR2-S779G mutant exhibited a reduced neuronal differentiation capacity compared with PC12 cells expressing wt-FGFR2 in response to FGF9, suggesting that S779 in FGFR2 plays a critical role in regulating PC12 cell neuronal differentiation mediated by FGF9. Regarding the FGFR proximal signalling events, we showed that the stimulation of FGF9 induced S779 phosphorylation in PC12 cells expressing wt-FGFR2. However, the loss of S779 signalling in the FGFR2-S779G mutant did not affect the tyrosine phosphorylation of FGFR or FRS2 in response to FGF9. To further determine the roles of S779 in regulating intracellular signalling, we compared the major signalling pathways mediated by FGFR2 between PC12 cells expressing wt-FGFR2 and PC12 cells expressing FGFR2-S779G mutant. We showed that only Ras/MAP kinase pathway was defective in PC12 cells expressing FGFR2-S779G mutant. Specifically, PC12 cells expressing the FGFR2-S779G mutant were defective in both Ras activation and ERK phosphorylation compared with PC12 cells expressing the wt-FGFR2. While the FGFR2-S779G mutant was still able to promote ERK phosphorylation with sustained kinetics, the overall magnitude of ERK phosphorylation was significantly reduced. Furthermore, the induction of Ras activity in PC12 cells expressing FGFR2-S779G was significantly more transient compared with PC12 cells expressing wt-FGFR2. These results identify a novel mode of ERK activation in which S779 signalling is required for the full activation of both Ras and ERK in PC12 cells.
2.3 Methodology

2.3.1 Antibodies

Anti-phosphotyrosine 4G10 (Millipore cat. no. 05-321); anti-phospho-S779 phospho-specific antibody were generated from laboratory according to the published method [191]; anti-FGFR2 C17 (Santa Cruz); anti-FRS2 G20 (Santa Cruz); anti-Ras 18/Ras (BD Transduction Laboratories); anti-phospho-Akt (Thr308) (244F9) Rabbit mAb (Cell Signalling Technology); anti-phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (E10) Mouse mAb (Cell Signalling Technology); anti-phospho-MEK1/2 (S217/221) (41G9) Rabbit mAb (Cell Signalling Technology); anti-phospho-PLCγ (Tyr783) (Biosource); anti-actin antibodies C4 (Chemicon); anti-14-3-3 protein pan antibody (Cell Signalling Technology).

2.3.2 Buffers

NP40 buffer: 10 mM Tris [pH 7.4], 137 mM NaCl, 10% glycerol, 1% Nonidet.

NP40 cell lysis buffer: NP40 buffer supplemented with 10 mM β-glycerolphosphate, 1 mM phenylmethylsulfonyl fluoride, 10 mM NaF, 10 mM Na orthovanadate, 4.5 U/ml aprotinin(Sigma), 1 mg/ml leupeptin (Sigma).

MT-PBS: To prepare 50ml MT-PBS, add 0.5ml Triton X-100, 0.5ml 500mM EDTA, 0.5ml aprotinin, 0.5ml 100mM PMSF, 50ul 1mg/ml leupeptin into 50ml PBS.

2.3.3 FGFR constructs, stable PC12 cell lines establishment and cell culture

Dulbecco’s modified Eagle’s medium (DMEM), Selective antibiotic G418, penicillin/streptomycin were all from Invitrogen. Fetal Bovine Serum (FBS), Horse serum (HS) were from Gibco.
Dr. Ana Lonic from our laboratory has generated constructs of wt-FGFR2 and FGFR2-S779G mutant (Figure 2-2) and the method has been published [191]. The PC12 cell lines were transfected with individual constructs by Lipofectamine according to protocol. The transfected cell lines were selected in 0.5mg/ml G418 for 5 weeks to generate stable pools. Cells were cultured in DMEM containing 10% FBS and 5% HS. The final concentration of G418 was 100µg/ml. The final concentration of penicillin was 100 U/ml of and the final concentration of streptomycin was 100 µg/ml.

2.3.4 Differentiation assay

Recombinant human fibroblast growth factor 9 (FGF9) from R&D Systems and heparin from Sigma were used.

Plates were pre-coated with collagen-I for 2h and then washed 3x with PBS. PC12 cells stably expressing FGFR2 constructs were plated at 8x10³ cells per well (24-well plates) in growth factor deprived DMEM containing 0.3% HS in the presence of 0.5 ng/ml heparin and in the presence or absence of 8 ng/ml FGF9. Growth factor and heparin were added daily. Differentiation was quantified by phase-contrast microscopy on day 4 with at least 20 fields of view/well from triplicate wells and each review containing more than 30 cells. As established by other studies [215, 221-224], the cells exhibiting neurites more than 2 times the length of cell body were scored as having undergone differentiation.

2.3.5 Recombinant GST-protein purification

Luria-Bertani (LB) broth, LB-agar, ampicillin from Sigma and IPTG from Difao laboratories were used. The BL21 strain E.coli transformed with plasmid expressing
the fusion GST-14-3-3 protein was used to inoculate 10 ml LB medium containing 100ug/ml ampicillin and cultured overnight. On the following day, the culture was expanded to 3 L size and grow until OD_{600}=0.7. The expression of recombinant proteins was induced by 0.1mM IPTG for 2.5-3 h. Cells were harvested by centrifugation at 4°C for 10 min at 4200 rpm. Supernatant was discarded and pellets were frozen at -20°C. Pellets were thawed in 30ml MT-PBS buffer. The pellets were re-suspended with MT-PBS buffer and sonicated on ice until 90% of cells were disrupted. 1 ml glutathione sepharose beads were washed with MT-PBS 3 times then added into cell lysate. Rotated mixture at RT for 2 h or 4°C for overnight. Glutathione sepharose beads were washed with PBS 3 times and kept in 4°C. The purified GST or GST-14-3-3 or GST-RBD (Ras binding domain) were subjected to SDS-PAGE and then stained with Coomassie blue for purity examination. The titrations of BSA were run along the purified proteins on the SDS-PAGE to estimate purified protein’s concentrations.

2.3.6 Immunoprecipitation/Pulldowns/Western-blotting

PC12 cells (2x10^7) were starved in growth factor deprived DMEM containing 0.3% HS for 20 h. Cells were then stimulate with 8 ng/ml FGF9 and 0.5 ng/ml heparin for the various time. Cells were harvested by centrifugation and then were lysed in 1 ml NP-40 lysis buffer.

For FGFR2 or FRS2 immunoprecipitation, cleared lysates were subjected to immunoprecipitation with 1 µg anti-FGFR2 C17 or anti-FRS2 antibody antibodies for 2hr at 4°C on a rotating wheel. 60 ul protein A sepharose beads were incubated with each cell lysate for 2 h at 4°C on a rotating wheel. Sepharose beads were washed with
NP40 buffer 3 times. Immunoprecipitates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then immunoblot analysis with 4G10, anti-phospho-S779FGFR2, anti-FGFR2 C17, anti-FRS2 antibodies, anti-14-3-3 antibodies. For 14-3-3 pulldown FGFR2, cleared lysates were incubated with GST-Sepharose (control) or GST-14-3-3-Sepharose for overnight at 4°C on a rotating wheel. Beads were washed with NP40 buffer 3 times. Pulldowns were subjected to SDS-PAGE and then immunoblot analysis with anti-FGFR2 C17 antibodies. For Ras pulldown, cleared lysates were incubated with GST-Sepharose (control) or GST-RBD-Sepharose for 2 h at 4°C on a rotating wheel. Beads were washed with NP40 buffer 3 times. Pulldowns were subjected to SDS-PAGE and then immunoblot analysis with anti-Ras antibodies. Cleared cell lysates were subjected to SDS-PAGE and then immunoblot analysis with anti-phospho-ERK1/2, anti-phospho-MEK1/2 antibodies. The signals were developed by ECL Western Blotting System (GE) and visualized on the X-ray films (Fuji film).
2.4 Results

2.4.1 S779 in FGFR2 is required for growth factor regulated PC12 cell neuronal differentiation

Our previous published studies have shown that S779 in the FGFR2 initiates a novel signalling pathway to promote cell survival and proliferation in Baf/3 hemopoietic cells and BALB/c 3T3 fibroblasts [191]. However, the role of S779 of FGFR2 in mediating cellular responses such as neuronal differentiation has not been examined in these previous studies. Now we employed the PC12 cell line to examine the role of S779 in regulating cell neuronal differentiation.

To investigate whether S779 in FGFR2 is functionally linked to PC12 cell neuronal differentiation, we compared the ability of FGF9 to promote neuronal differentiation in PC12 cells expressing either wt-FGFR2 or the FGFR2-S779G mutant. The PC12 cells that stably express wt-FGFR2 or FGFR2-S779G had been treated with FGF9 (8 ng/ml) or NGF (50ng/ml) for 4 days. Following FGF or NGF treatment, the PC12 cells were observed to undergo morphology change in which multiple neurite-like extensions protruded from the cell body.

We found that while PC12 cells expressing wt-FGFR2 showed a high proportion of neuronal differentiation in response to FGF9, differentiated cells expressing FGFR2-S779G mutant were significantly reduced (Figure 2-3 A). Quantification of differentiated cells indicated that PC12 cells expressing FGFR2-S779G mutant had a significant defect in FGF9-mediated differentiation when compared with cells expressing wt-FGFR2 (Figure 2-3 B) with an observed 3 fold reduction in neurite outgrowth (p<0.05). Stimulation of both PC12 cells expressing wt-FGFR2 and
FGFR2-S779G mutant with NGF resulted in robust differentiation (94% and 91% respectively), indicating that there was not intrinsic defect in the ability of the PC12 cells expressing FGFR2-S779G to differentiate (Figure 2-3 B). These results demonstrate that S779 is required for PC12 cell neuronal differentiation mediated by FGF9. The above results have been published in JBC, 2013 288(21): 14874-85, Figure 2 B, C. Lonic A et al [242].
Figure 2-3 S779 in FGFR2 is crucial for PC12 cell neuronal differentiation

The PC12 cells stably expressing wt-FGFR2 and FGFR2-S779G mutant were seeded in DMEM/0.3% horse serum (HS). (A) Phase microscopy of PC12 cells expressing wt-FGFR2 or FGFR2-S779G following no stimulation (untreated) or FGF9 stimulation (8ng/ml) or NGF (50 ng/ml) for 4 days. (B) Quantification of PC12 cell differentiation was determined by neurite extension under the phase microscope. The cells were counted blind and triplicate wells were set up for each counting. Error bars represent standard deviations and results are typical of 3 independent experiments (* p<0.05).
2.4.2 S779 in FGFR2 is required for growth factor regulated maximal activation of ERK in PC12 cells

A number of studies have shown that Ras/MAP kinase pathway is the key regulator of cell differentiation in diverse systems [58, 243, 244], and in particular, Ras/MAP kinase pathway is important for regulating PC12 cell differentiation in response to FGF and NGF [105]. We therefore decided to examine whether the lack of differentiation in PC12 cells expressing FGFR2-S779G mutant was due to the impairment of Ras/MAP kinase pathway.

As shown in (Figure 2-4 A), FGF9 treatment promoted ERK1/2 phosphorylation, which was detectable within 5 min and remained elevated at 120 min in PC12 cells expressing either wt-FGFR2 or FGFR2-S779G mutant. However, the signal strength of ERK phosphorylation was significantly reduced in the PC12 cells expressing FGFR2-S779G mutant compared with PC12 cells expressing wt-FGFR2 at each time point (Figure 2-4 B). Thus, while the amplitude of ERK1/2 phosphorylation was significantly reduced, the sustained signalling kinetics did not appear to be affected in PC12 cells expressing FGFR2-S779G mutant with elevated ERK1/2 phosphorylation clearly detectable up to 2h following FGF9 stimulation, reflecting the important biological functions of S779 in regulating maximal activation of ERK. In addition, while major downstream signalling events regulated by FGFR, such as PI3 kinase/Akt signalling pathway, phosphorylation of MSK1 and ATF2, have been systematically investigated by others in our laboratory [242], only Ras/MAP kinase pathway has been shown to be significantly defective in PC12 cells expressing FGFR2-S779G compared with PC12 cells expressing wt-FGFR2, suggesting the Ras/MAP kinase pathway is a major target of S779 signalling. We conclude that S779 is essential for
maximal ERK phosphorylation mediated by FGF9 in PC12 cells. The above results have been published in JBC, 2013 288(21): 14874-85, Figure 3 B, E. Lonic A et al [242].
Figure 2-4 S779 in FGFR2 is important for maximal activation of ERK.

PC12 cells expressing wt-FGFR2 or FGFR2-S779G were starved in DMEM/0.3% HS for 20 h and then stimulated with FGF9 (8 ng/ml) for the indicated times. (A) Cells were lysed and cell lysates were subjected to immunoblot analysis with anti-phospho-ERK and anti-actin pAb. (B) The quantification of phospho-ERK signals following FGF9 stimulation of PC12 cells expressing either wt-FGFR2 or FGFR2-S779G from 3 independent experiments. Error bars represent standard deviations and results are typical of 3 independent experiments (* p<0.05).
2.4.3 Phosphorylation of S779 in FGFR2 is regulated by FGF9

To examine the molecular mechanisms by which S779 might promote ERK signalling, we firstly examined the receptor-proximal signalling events regulated by FGFR2. Previous studies showed that in Ba/F3 cells, FGF2 induced S779 phosphorylation of FGFR2, which provided a binding site for 14-3-3 proteins [191]. We therefore decided to determine whether S779 in FGFR2 was phosphorylated in response to FGF9 in PC12 cells. Using anti-phospho-S779 specific antibodies previously developed in the lab, we showed that while S779 phosphorylation was induced by FGF9 stimulation in PC12 cells expressing wt-FGFR2, no such signals were detected in cells expressing the FGFR2-S779G mutant (Figure 2-5 A). On the other hand, no overall defect in receptor tyrosine phosphorylation was observed for the FGFR2-S779G mutant compared with wt-FGFR2 (Figure 2-5 B). These results demonstrate that while FGF9 stimulation of PC12 cells induces S779 phosphorylation, this signalling event is not required for FGFR2 tyrosine phosphorylation. The above results have been published in JBC, 2013 288(21): 14874-85, Figure 4 A. Lonic A et al [242].
Figure 2-5 Phosphorylation of S779 in FGFR2 is regulated by FGF9

PC12 cells expressing wt-FGFR2 or FGFR2-S779G mutant were starved in DMEM/0.3% HS for 20 h and then stimulated with FGF9 (8 ng/ml) for the indicated times. The cells were lysed and FGFR2 were immunoprecipitated by anti-FGFR2 pAb. The immunoprecipitations were then subjected to immunoblot analysis with (A) Anti-phospho-S779 and anti-FGFR2 pAb or (B) 4G10 anti-phosphotyrosine (P-Y) mAb and anti-FGFR2 pAb. Results are typical of 3 independent experiments.
2.4.4 S779 is not required for ligand induced FGFR2 tyrosine phosphorylation or FRS2 tyrosine phosphorylation

To further investigate the mechanism by which S779 regulates ERK phosphorylation, we examined the role of S779 in promoting FGFR2 and FRS2 tyrosine phosphorylation. Tyrosine phosphorylation of the cytoplasmic tail of FGFR2 as well as the associated scaffold protein, FRS2, has been shown to be important for initiating intracellular signalling including Ras/MAP kinase pathway. Both cytoplasmic tail of FGFR2 and FRS2 are the key substrates of FGFR tyrosine kinase.

Consistent with the data shown in Figure 2-5 B, there was no detectable difference in tyrosine phosphorylation of wt-FGFR2 and FGFR2-S779G following FGF9 stimulation (Figure 2-6 A). Similarly, there was no detectable defect in FRS2 tyrosine phosphorylation following FGF9 stimulation in PC12 cells expressing FGFR2-S779G mutant compared with PC12 cells expressing wt-FGFR2 (Figure 2-6 B). Collectively, these results show that S779 does not affect the overall FGFR2 and FGRS2 tyrosine phosphorylation. The above results have been published in JBC, 2013 288(21): 14874-85, Figure 4 B. Lonic A et al [242].
Figure 2-6 S779 is not required for ligand induced receptor tyrosine phosphorylation or FRS2 tyrosine phosphorylation

PC12 cells stably expressing wt-FGFR2 or FGFR2-S779G were starved for 20 h in DMEM 0.3% HS and then stimulated with FGF9 (8 ng/ml) for the indicated time. (A) wt-FGFR2 or FGFR2-S779G were immunoprecipitated by anti-FGFR2 pAb from cell lysates and then subjected to immunoblot analysis with 4G10 anti-phosphotyrosine (P-Y) mAb and anti-FGFR2 pAb. (B) The scaffold protein FRS2 was immunoprecipitated by anti-FRS2 pAbs from cell lysates and then subjected to immunoblot analysis with 4G10 anti-phosphotyrosine (P-Y) mAb and anti-FRS2 pAb. Results are typical of 2 independent experiments.
2.4.5 Recombinant GST 14-3-3 proteins unspecifically pull down FGFR2

We identified S779 in FGFR2 as the putative 14-3-3 binding site (775Q-Y-S-P-S-Y-P781, where S represents the phospho-S779). Importantly, S779 is conserved across mammalian species in both FGFR2 and FGFR1 [191]. We have previously shown that S779 in cytoplasmic domains of FGFR2 is phosphorylated in response to ligand and binds the 14-3-3 family proteins in Ba/F3 cells and BALB/c 3T3 fibroblasts [191]. The recruitment of the 14-3-3 proteins to FGFR2 is essential for the full activation of the PI3 kinase and Ras/MAP kinase pathways, which sequentially regulate cell survival and proliferation in both Ba/F3 cells and BALB/c fibroblasts. The association of 14-3-3 with FGFR2 via phospho-S779 is also supported by computational modeling of a FGFR1 peptide containing phospho-S779 into the crystal structure of 14-3-3ζ. It have been shown that the peptide exhibited numerous interactions within the 14-3-3 binding cleft (computational model established by our collaboration and published in JBC, 2013 288(21): 14874-85, Lonic A et al [242]). These interactions fit with the known critical binding motif for phosphoserine containing peptides including the binding of c-Raf to 14-3-3 [245]. We have previously shown in PC12 cells the S779 in cytoplasmic domains of FGFR2 is also phosphorylated in response to FGF9, which might provide putative binding site for 14-3-3 proteins. To investigate the roles of 14-3-3 in S799 mediated Ras/MAP kinase signalling, we examined the ability of 14-3-3 to interact with FGFR2 via phospho-S779 in PC12 cells after FGF9 stimulation.

We firstly examined whether the 14-3-3 proteins were able to directly interact with FGFR2 through phospho-S779 in the FGF9 treated PC12 cells by performing the recombinant GST 14-3-3ζ protein pulldown assay. The GST-Sepharose resin (control)
or GST 14-3-3ζ-Sepharose was used to pulldown FGFR2 from PC12 cells expressing wt-FGFR2 or FGFR2-S779G mutant after FGF9 treatment. The expectation was to observe regulated association between GST 14-3-3ζ proteins and FGFR2 in PC12 cells expressing wt-FGFR2, and no association in PC12 cells FGFR2-S779G mutant. Interestingly, although the recombinant GST 14-3-3ζ was able to significantly precipitate FGFR2 compared with GST control, there was no difference between treated and untreated cells, neither for cells expressing wt-FGFR2 or FGFR2-S779G mutant (Figure 2-7). Therefore, the ability of 14-3-3ζ to precipitate FGFR2 did not appear to be regulated by FGF9 stimulation.

The GST 14-3-3ζ protein pulldown experiments did not address whether endogenous 14-3-3 proteins were able to associate with FGFR2 via S779 in response to ligands. We therefore examined the ability of FGFR2 to co-immunoprecipitate with endogenous 14-3-3 proteins in FGF9 treated PC12 cells by co-immunoprecipitation experiments. We immunoprecipitated FGFR2 from PC12 cells expressing wt-FGFR2 or FGFR2-S779G mutant after FGF9 stimulation. We then used the specific 14-3-3 antibodies to examine whether FGFR2 could associate with endogenous 14-3-3 proteins. The expectation was to observe wt-FGFR2 coimmunoprecipitation with endogenous 14-3-3 in response to FGF9 stimulation, and no such detectable interaction for the FGFR2-S779G mutants with endogenous 14-3-3. However, although 14-3-3 proteins were identified in cell lysates, no detectable 14-3-3 proteins were observed in either PC12 cells expressing wt-FGFR2 or PC12 cells expressing FGFR2-S779G mutant (data are not shown). Whether endogenous 14-3-3 can interact with phospho-S779 in FGFR2 after FGF9 stimulation in PC12 cells remains unknown.
In conclusion, the precise role of the 14-3-3 proteins interaction with phosphorylated S779 for regulating downstream signalling pathways and specific cellular responses remains unclear.

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<tr>
<td>14-3-3 pulldown</td>
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**Figure 2-7 Recombinant GST 14-3-3 proteins pull down FGFR2 unspecifically**

PC12 cells stably expressing wt-FGFR2 or FGFR2-S779G were starved for 20 h in DMEM 0.3% HS and then stimulated with FGF9 (8 ng/ml) for the indicated time. The cells were lysed and cell lysates were then incubated with Glutathione Sepharose coupled with GST alone (control) or Glutathione Sepharose coupled with GST 14-3-3ζ. The resins and cell lysates were then subjected to immunoblot analysis with anti-FGFR2 Abs. Data are representative of three independent experiments.
2.4.6 S779 is required for the sustained Ras activation

Previous results showed that mutation of S779 reduced the amplitude of ERK phosphorylation, which suggest that Ras/MAP kinase pathway is the main regulated target of S779. Ras, MEK and ERK constitute a signalling cassette in which Ras has been widely shown to be an upstream regulator of ERK. We firstly compared the activation of Ras between the PC12 cells expressing either wt-FGFR2 or FGFR2-S779G mutant. We employed an approach that took advantage of the Ras binding domain (RBD) of c-Raf to selectively bind GTP-loaded Ras and examined the ability of a recombinant fusion protein GST-RBD to precipitate (pull-down) activated Ras from the lysates of FGF9-stimulated PC12 cells expressing either wt-FGFR2 or FGFR2-S779G.

PC12 cells expressing either wt-FGFR2 or FGFR2-S779G both showed the Ras activation within 2 minutes as evidenced by a significantly increased detectable Ras in the GST-RBD pulldowns (Figure 2-8 A, B, C, D). No Ras was detected in the GST controls. While some degree of variability was observed between the four repeat experiments depicted in Figure 2-8, the quantification of the Western blots demonstrated that while PC12 cells expressing the wt-FGFR2 or the FGFR2-S779G mutant activated Ras to similar maximal levels at 5 minutes, Ras activation was significantly impaired at later time-points (Figure 2-8 E). Overall, the kinetics of Ras activation was significantly more transient in FGFR2-S779G mutant cells compared with wt-FGFR2 cells. These results indicate that S779 in FGFR2 is required for sustained Ras activation in PC12 cells treated with FG9. The above results have been published in JBC, 2013 288(21): 14874-85, Figure 4 E, F. Lonic A et al [242].
Figure 2-8 S779 in FGFR is required for sustained Ras activation in PC12 cells

PC12 cells stably expressing wt-FGFR2 or FGFR2-S779G had been starved for 20 h in DMEM 0.3% HS and then stimulated with FGF9 (8 ng/ml) for the indicated time. Cells were lysed and the lysates were then subjected to pulldowns by using either GST-Sepharose (control) or GST-RBD-Sepharose (which binds GTP-bound active Ras but not GDP-bound inactive Ras). (A, B, C, D) Pulldowns were then immunoblotted with anti-Ras pAb. Increased Ras precipitation by GST-RBD following FGF9 stimulation indicates the presence of increased levels of GTP-loaded active Ras within cells. (E) Quantitation of 4 independent Ras pulldown experiments was performed by laser densitometry. The signal of GTP-loaded Ras was normalized with signal of GTP-loaded Ras of at 15-minute time point. (* p<0.05). Data are representative of four independent experiments and error bars represent SEM from four independent experiments.
2.4.7 

*S779 is not required for the MEK phosphorylation*

We then investigated whether S779 could regulate ERK phosphorylation through regulating MEK1/2 kinases signalling, which are another key upstream components of the Ras/MAP kinase module. The MEK1/2 kinases are dual-specificity serine/tyrosine protein kinases and activate ERK1/2 by phosphorylating on both threonine and tyrosine residues within the activation loop of ERK1/2 kinase [99, 100]. We therefore investigated the abilities of S779 in regulating MEK activation by examining the phosphorylation status of MEK1/2.

Consistent with the data in Figure 2-4, ERK phosphorylation in S779G mutant cells was significantly reduced at each time point compared with cells expressing wt-FGFR. However, equivalent levels of MEK phosphorylation were observed in PC12 cells expressing wt-FGFR2 and FGFR2-S779G mutant (Figure 2-9). These results suggest that despite the important role of S779 in promoting maximal ERK phosphorylation, it was not essential for regulating MEK phosphorylation. These results were somewhat surprising as Ras, MEK and ERK are known to constitute a signalling module and a defect in Ras and ERK, as occurs with PC12 cells expressing the FGFR2-S779G mutant, would also be expected to have a defect in MEK signalling. These findings are discussed in more detail in the Discussion but raise the possibility that S779 promotes maximal phosphorylation of ERK independently of upstream MEK signalling.
Figure 2-9 S779 is not required for MEK phosphorylation

PC12 cells stably expressing wt-FGFR2 or FGFR2-S779G had been starved for 20 h in DMEM 0.3% HS and then stimulated with FGF9 (8 ng/ml) for the indicated time. (A, B and C) The cells were lysed and cell lysates were then subjected to immunoblot analysis with anti-phospho-MEK and anti-actin antibodies. (D) The signal of phospho-MEK from three independent experiments was quantified by laser densitometry. Data are representative of three independent experiments and error bars represent SEM from three independent experiments.
2.5 Discussion

Understanding the mechanism by which a growth factor regulates pleiotropic cellular activities is a central question in cell biology. Although it is known that growth factors exert their biological activities through the binding of cell surface receptors and the activation of multiple intracellular signalling cascades, how specificity in both signalling and biological responses is achieved is unclear. While the ability of growth factor receptor tyrosine phosphorylation to control diverse cellular responses represents a central paradigm in cell biology, the functional roles of receptor serine phosphorylation are less well understood.

Our previously published studies using Ba/F3 and BALB/c cell lines have shown that a key serine residue in the FGFR2 cytoplasmic tail, S779, is phosphorylated in response to growth factor stimulation and binds the 14-3-3 proteins [191]. In current studies, we now show that S779 in FGFR2 is critical for mediating specific signalling pathways and biological responses in PC12 cells. Using PC12 cells expressing the wt-FGFR2 and the FGFR2-S779G mutant, we have shown that S779 signalling is required for maximal activation of Ras/MAP kinase pathway and PC12 cell neuronal differentiation. We show that while both the wt-FGFR2 and the FGFR2-S779G mutant are able to induce Ras activation and ERK1/2 phosphorylation, the kinetics of Ras activation is shorter while the magnitude of ERK phosphorylation is reduced in cells expressing the FGFR2-S779G mutant. Importantly, S779 is phosphorylated after FGF9 stimulation in PC12 cells but S779 signalling is not required for either FGFR or FRS2 tyrosine phosphorylation.
We have shown that the mutation of S779 FGFR2 significantly can impair the PC12 cell differentiation ability in response to FGF9 (Figure 2-3), suggesting that S779 in the FGFR2 is crucial for PC12 neuronal differentiation mediated by FGF9. In order to examine the molecular mechanism by which S779 regulates PC12 cell neuronal differentiation, we have compared the downstream signalling pathways initiated by FGFR2 between PC12 cells expressing wt-FGFR2 and PC12 cells expressing FGFR2-S779G mutant. Among the major signalling pathways regulated by FGFR2, Ras/MAP kinase pathway is impaired in the PC12 cells expressing FGFR2-S779G mutant as evidenced by reduced ERK phosphorylation compared with PC12 cells expressing the wt-FGFR2 (Figure 2-4). It is particularly interesting that other major signalling pathways mediated by FGFR2, including the PI3 kinase signalling pathway, phosphorylation of MSK1 and ATF2, are not altered in the PC12 cells expressing FGFR2 S779G mutant (experiments performed by others in our laboratory and published in JBC, 2013 288(21): 14874-85, Lonie A et al [242]). Our results suggest that Ras/MAP kinase pathway is the major target of the S779 and the reduction of Ras/MAP kinase signalling leads to the impaired PC12 cell differentiation. It is important to point out that the overall signal amplitude of ERK phosphorylation is reduced in PC12 expressing FGFR2-S779G mutant, but the kinetics of ERK phosphorylation is similar between PC12 cell lines expressing wt-FGFR2 and FGFR2-S779G mutant. It seems that S779 is able to regulate the strength instead of kinetics of Ras/MAP kinase pathway to determine the specific cellular responses.

This observation is different with the “transient versus sustained” model proposed by Chris Marshall based on PC12 cells studies, which suggests that duration but not
strength of signal events is sufficient to determine specific cellular responses [106] (also refer to Figure 2-1). The duration or strength of signals has been proposed to explain how independent and distinct cellular responses can arise from the activation of common (or redundant) downstream pathways [91]. We show that it is the strength but not the duration of ERK signalling that determines PC12 cell differentiation. Many other studies have shown that quantitative differences in ERK signalling lead to qualitative differences in cellular responses such as in fibroblasts [108, 246, 247] and T cells [248, 249]. In particular, over-expressed EGFRs in PC12 cells promote an elevated and prolonged activation of ERK with treatment of EGF, which leads to PC12 cell neuronal differentiation [237]. The varied magnitude of ERK signalling is important for the cell cycle control. For example, moderate levels of ERK activity promote cell cycle progression, while high activation of ERK causes cell cycle arrest [250, 251]. The elevated ERK signalling is particularly required for cancer cell proliferation and survival, such as melanomas and colon carcinoma cells [252, 253]. Our results suggest it is the signal strength rather than the duration of ERK signalling that determines whether PC12 cells undergo neuronal differentiation.

Since our results indicate that Ras/MAP kinase signalling is affected in PC12 cells expressing S779G mutant FGFR2, we have questioned whether the key FGFR2 proximal signalling events such as S779 phosphorylation, FGFR2 tyrosine phosphorylation and FRS2 tyrosine phosphorylation are altered in PC12 cells expressing FGFR2-S779G mutant. Our results show that S779 in FGFR2 is phosphorylated in response to FGF9 stimulation in wt-FGFR2 cells (Figure 2-5). Phosphorylation of S779 in FGFR2 raises two possibilities: 1) phosphorylation of S779 could be a prerequisite for the induction of FGFR2 intrinsic tyrosine kinase.
activity following growth factor stimulation; 2) phosphorylation of S779 could provide docking sites for downstream signal molecules such as 14-3-3. To examine the first possibility, we have compared the tyrosine phosphorylation of both FGFR2 and FRS2 in PC12 cells expressing either the wt-FGFR2 or the FGFR2-S779G mutant. Our results show no overall defect in the tyrosine phosphorylation of either FGFR2 or FRS2 in PC12 cells expressing the FGFR2-S779G mutant (Figure 2-6). These results suggest that the inability of the FGFR2-S779G mutant to promote PC12 cell differentiation is not simply due to a global defect in phospho-tyrosine signalling pathways.

We have further examined whether phosphorylated S779 can recruit downstream signal molecules to FGFR2 to activate specific signalling pathways. Therefore, we have examined whether 14-3-3 proteins can also associate with phospho-S779 of FGFR2 in response to ligands in PC12 cells. We have investigated both the ability of GST-14-3-3 proteins to pull down FGFR2 and the ability of FGFR2 to coimmunoprecipitate with endogenous 14-3-3 proteins in FGF9 treated PC12 cells. Despite of several attempts, we are unable to show the direct physical association between 14-3-3 and FGFR2 through phospho-S779 in PC12 cells. In the GST-14-3-3 protein pulldown assay, although GST 14-3-3 protein can interact specifically with FGFR2 compared to the GST control, there was no difference between treated and untreated cells, neither for cells expressing wt-FGFR2 or FGFR2-S779G mutant (Figure 2-7). Several possibilities may explain the non-specific binding between GST-14-3-3ζ proteins and FGFR2. Firstly, in addition to S779, FGFR2 may contain other serine residues forming extra 14-3-3 protein binding sites that potentially interact with GST-14-3-3ζ proteins. Diminishing those serine residues by point-
mutation study, the interaction of 14-3-3 and S779 can be accurately examined. Secondly, the 14-3-3 proteins have been shown to interact with more than 200 proteins. Therefore, GST-14-3-3ζ may indirectly associate FGFR2 through other signalling proteins independently of S779.

We also examined whether FGFR could associate with endogenous 14-3-3 through phospho-S779 using a co-immunoprecipitation approach. Although the 14-3-3 proteins were detected in PC12 cell lysates, we could not show wt-FGFR2 co-immunoprecipitation with endogenous 14-3-3 in response to FGF9 stimulation (data not shown). However, the current studies have only examined the 14-3-3ζ isoform. Thus, we cannot exclude the possibility of other 14-3-3 isoforms might be involved in FGF signalling. While the precise role of 14-3-3 interaction with phospho-S779 in regulating downstream signalling pathways and specific cellular responses remains unclear, the findings presented here suggest a mechanism in which S779 signalling selectively regulates Ras and ERK to promote neuronal differentiation in PC12 cells.

Ras, MEK and ERK constitute a signalling cassette in which Ras has been widely shown to be an upstream regulator of ERK [57]. In fact, results presented in this Chapter clearly demonstrate that S779 signalling is required for sustained Ras activation (Figure 2-8). In contrast, while S779 in FGFR2 is important for the full activation of both Ras (Figure 2-8) and ERK (Figure 2-4), S779 signalling is not required for phosphorylation of MEK (Figure 2-9). It is not clear how S779 regulates full ERK phosphorylation and Ras activation independently of MEK phosphorylation. Although the Ras/Raf/MEK/ERK module has been well established in diverse cellular systems, our results suggest that S779 of cytoplasmic tail of FGFR2 activates ERK
without the requirement for MEK. Such a non-canonical mechanism for the MEK-independent activation of ERK has also been described by others [254-256]. For example, Grammer et al have shown that while the early ERK activation relied on MEK activity, the prolonged activation of ERK mediated by PDGF in Swiss 3T fibroblast cells was dependent on conventional PKCs signalling and PI3 kinase pathways instead of MEK [255]. Jiang et al have shown that in the B-RAF inhibitor-resistant melanoma cells, the highly activated ERK led to cell survival and proliferation in which ERK signalling was largely mediated by the PI3K/Akt pathway but not by MEK [256]. Similarly, in T47D breast cancer cells, ERK activation mediated by EGF has been shown to be MEK-independent but rather relies on PI3 kinase signalling [254].

Ras/MAP kinase pathway is the evolutionarily conserved signalling module and regulates cell differentiation, proliferation and migration in response to diverse growth factors and cytokines [91]. Considering its centre role in signal transduction, intensive research has been focused on the regulation of Ras/MAP kinase pathway and some basic understandings have been established [57, 91, 257]. However, the detailed mechanism is yet fully characterized and the complexity of Ras/MAP kinase pathway regulation has been reflected on multiple levels. For example, small GTPases Ras and Rap1 both function as upstream modulator but regulate activation of Ras/MAP kinase pathway in a different manner. While both NGF and EGF induce the transient activation of Ras, the ability of NGF to induce the prolonged activation of Rap1 leads to sustained activation ERK [258, 259]. Other factors including the expression of scaffolding such as RSK proteins and 14-3-3 proteins [257, 260], crosstalk between C-Raf and B-Raf [261, 262], crosstalk between Raf kinases and
PKCs [263], and the interplay between kinases and phosphatases, may also play important roles in kinetics of Ras/MAP kinase activation, which will be investigated in future studies.

Table 2-1 The putative conserved tyrosine/serine motifs in FGFR2 and FGFR1

We have performed database searches to identify putative tyrosine/serine bidentate motifs in FGFR2 from different species and FGFR isoforms. (A) The bidentate motifs have been found in the FGFR2 for cross species. (B) The putative 14-3-3 binding site at S779 in is conserved in both FGFR1 and FGFR2 but not in FGFR3 and FGFR4.
Our laboratory has discovered a novel tyrosine/serine putative “bidentate motif” consisting of a conserved putative PTB or SH2 binding site (phosphotyrosine) that is followed by a conserved putative 14-3-3 binding site (phosphoserine). The prototypic example of a bidentate motif has been identified in the GM-CSF receptor, which is composed of a Shc binding site at Y577 and a 14-3-3 binding site at S585 [211]. Similar putative bidentate motifs have been identified in a range of other growth factor receptors including the FGFRs [191], raising the possibility that the bidentate motifs might serve as a novel generic signalling device for the control pleiotropic cellular responses. In the FGFR2, the bidentate motif consists of the PLCγ binding site at Y766 and the 14-3-3 binding site at S779 and are conserved from zebra fish to humans (Table 2-1 A). The 14-3-3 binding site at S779, which is the key structural and functional component of putative bidentate motifs, is conserved in cytoplasmic tails of both FGFR2 and FGFR1 but not in FGFR3 and FGFR4 (Table 2-1 B). In this regard, it is interesting that FGFR4 is unable to regulate cell proliferation of Ba/F3 cells [264], and FGFR3 is significantly less potent than FGFR1 in promoting PC12 cell differentiation [217].

The studies presented in this Chapter address fundamental questions regarding how growth factors such as the FGFs promote pleiotropic biological responses such as proliferation, survival and differentiation. It is arguable that the current concepts of how growth factor pleiotropy is regulated have not ventured beyond the “sustained versus transient” model proposed by Marshall in 1995 or other models based on receptor tyrosine phosphorylation. The current studies identify a new mechanistic basis in which specific serine phosphorylation sites in the cytoplasmic tails of growth
factor receptors are important in defining distinct cellular responses and therefore provide an important conceptual advance over current paradigms used to explain pleiotropy. Our findings demonstrate that growth factor receptors can differentially utilize phosphoserine or phosphotyrosine pathways to provoke different modes of Ras/MAP kinase signalling, which may impart specificity in signalling.
Chapter 3 The regulation of neuronal differentiation by S779 of FGFR2 in primary mouse bone marrow stromal cells is regulated by PKCε and ERK signalling
3.1 Abstract

The FGFs have long been known to regulate cellular responses in neuronal cells including differentiation. While it has been assumed that such biological activities of the FGFs are regulated by phospho-tyrosine signalling, functionally linking individual tyrosine residues in the cytoplasmic tails of FGFRs to specific cellular responses such as differentiation has been proved difficult. Studies presented in Chapter 2 have demonstrated a critical role for S779 of FGFR2 in promoting the neuronal differentiation of the PC12 cell line. In this Chapter, a model system has been established in which FGF2 can induce the neuronal differentiation of primary bone marrow stromal cells (BMSCs) in vitro. The neuronal differentiated BMSCs promoted by FGF2 are characterized by the appearance of neurite extensions and the expression of the classical neuronal markers such as neurofilament (NF) and β-Tubulin III. This model system provides an in vitro approach for the analysis of the pathways and mechanisms by which FGF2 promotes neuronal differentiation. Using both pharmacological inhibitors and selective siRNA knockdown approaches, we have shown that Ras/MAP kinase and protein kinase Cε (PKCε) signalling are crucial for FGF2-mediated BMSC neuronal differentiation.

Further studies from our laboratory have shown that siRNA-mediated knockdown of PKCε results in a decrease in S779 phosphorylation of the endogenous FGFR2 mediated by FGF2, which suggests that PKCε is required for S779 phosphorylation of FGFR2. In addition, S779 signalling is required for the maximal ERK phosphorylation and BMSCs neuronal differentiation. Collectively, we conclude that PKCε mediated S779 phosphorylation is essential for maximal Ras/MAP kinase signalling and BMSC neuronal differentiation mediated by FGF2.
3.2 Introduction

FGFs play multiple critical roles in CNS during both development and adulthood through inducing neurogenesis, enhancing axonal growth, promoting neural progenitors proliferation and differentiation [20, 265]. FGFs have been also shown to be important for brain injury recovery [266]. For example, multiple types of brain injuries induce increased expression of endogenous FGF2, which promotes survival, proliferation and differentiation of neural progenitors in the adult hippocampus [267, 268]. Intraventricular infusion of exogenous FGF2 or FGF2 gene further enhances the newborn neurons to form functional synapses and integrate into the existing brain circuitry [268, 269]. FGFs exert their neuro-protective functions through binding and activating FGFR1 and FGFR2, which are predominantly expressed in neurons and glial cells [10, 11]. Although the signalling pathways initiated by FGFRs have been well characterized in neuronal cells, how these signalling pathways are regulated to promote specific neuronal cellular responses is less clear.

Bone marrow stromal cells (BMSCs) have the potential to differentiate into a variety of cell types, including osteoblasts, chondrocytes, myocytes and adipocytes both in vitro and vivo [270, 271]. Located in the bone marrow with hematopoietic stem cells (HSCs), BMSCs are a group of heterogeneous adult stem/progenitor cells that are able to adhere to plastic surfaces in vitro culture. In response to FGF2, BMSCs can differentiate into a neuronal phenotype with extending neurites and expressing the neuronal markers [272, 273]. BMSCs have been shown to be an excellent source for cell and gene therapies for their multipotency/pluripotentcy, easy to isolate and easy to expand into a large number in cell culture [271, 274, 275]. Importantly, the transplantation of BMSCs into the injured brain or spinal cord have been shown
therapeutic benefits in multiple CNS disease models, including ischemic stroke, traumatic brain injury and traumatic spinal cord injury in different animals, and recently in human trials [228, 276-279].

In this study, we aimed to understand the molecular mechanisms determining BMSCs neuronal differentiation mediated by FGF2 using a BMSC-derived in vitro model system. In Chapter 2, we show that the FGF9 stimulation of PC12 cells can trigger the phosphorylation of S779 in the cytoplasmic tail of FGFR2, which is essential for the maximal activation of ERK phosphorylation leading to neuronal differentiation. Here, we sought to examine the potential roles of S779 signalling in regulating BMSC neuronal differentiation mediated by FGF2. Using both pharmacological inhibitions as well as selective siRNA knockdown approaches, we show that both Ras/MAP kinase and PKCε signalling are crucial for FGF2-mediated BMSC neuronal differentiation. Further studies from our laboratory have shown that FGF2 induces the PKCε mediated S779 phosphorylation of endogenous FGFR2 in BMSCs, which is critical for regulating maximal Ras/MAP kinase signalling and neuronal differentiation. Therefore, we conclude that PKCε mediated S779 phosphorylation is essential for maximal Ras/MAP kinase signalling and BMSCs neuronal differentiation mediated by FGF2, which is consistent with our findings in PC12 cells.

3.3 Methodology

3.3.1 Antibodies and secondary antibodies

Two neuronal biomarker antibodies were used to examine neuronal marker expression levels in differentiated BMSCs. Neurofilament 160 kDa (NF) rabbit monoclonal
antibody (clone NN18) was from Millipore (clone A60). β-Tubulin III (tuji) mouse monoclonal antibody was from Sigma (clone SDL.3D10). Alexa Fluor 594(red) goat anti-rabbit IgG and Alexa Fluor 488(green) goat anti-mouse IgG secondary antibodies were from Invitrogen. Hoechst from Invitrogen was for nuclei staining.

3.3.2 Collagen coated coverslip preparation

Collagen is the major structural component of extracellular matrices. It has also been used in the study of a range of cell lines and primary cell growth, differentiation, migration. BMSCs differentiation assay needs to be performed on the collagen coated coverslips. Collagen type I from rat tail was from Sigma (catalogue no. C3867-IVL). 25mmx25mm glass coverslips were placed into 6-well plates by one coverslip/well and then exposed under the UV in the hood for 2 h. The 4 mg/ml stock collagen solution was diluted into 0.05mg/ml solution by sterilized PBS. The 0.05mg/ml collagen solution was added into the 6 well plate containing coverslips to cover the coverslips completely. The coverslips were incubated in collagen solution for 2h at RT or overnight at 4°C on the shaker. The extra collagen solution was aspirated and coverslips were kept in the culture hood overnight to dry completely. The coverslips were washed with sterilized water or PBS before use.

3.3.3 BMSCs isolation and culture

BMSCs were isolated from 10-12 weeks old CD1 female scavenged mice. The Institute of Medical and Veterinary Science Animal Ethics Committee authorized the Executive Approval for using scavenged animal tissue. The scavenged tissue approval number was ST 04/10 and ST 20/10.
Mice were left in CO\textsubscript{2} box to lose consciousness and then killed by cervical dislocation. Mice bodies were spayed with 70% ethanol thoroughly. Limbs were cut at anklebone and then dissected from the bodies. Limbs were sprayed with 70% ethanol. Further dissection was operated in the hood. Muscle and tissues were removed from limbs thoroughly. The ends of the tibia and femur were removed. The bone marrow was flush out of the bones by using 10ml syringe containing culture medium. 25-G needles were for tibia marrow flushing and 19-G needles were for femur marrow flushing. The bones were flushed with medium twice or until bones look white and clear. The bone marrow was re-suspend thoroughly. The bone marrow derived cells isolated from one mouse were cultured in one T175cm\textsuperscript{2} flask containing 20 ml culture medium. A total of 7-10\times10\textsuperscript{6} cells were obtained from one mouse. The cells were incubate at 37 °C with 5% CO\textsubscript{2} in a humidified chamber without disturbing. It took 2 days for BMSCs to attach to the surface of flasks. The non-adherent cells were removed by changing the medium.

BMSCs were cultured in Low glucose DMEM supplemented with 20% FBS. The final concentration of penicillin was 100 U/ml of and the final concentration of streptomycin was 100\textmu g/ml. The adherent cells were split when cells were less than 80% confluent. Cells were pre-treated with 5 ml 1x typsin/0.01M EDTA (V: V=1:1) mixture for 10 min before passaging. Low glucose DMEM was from Invitrogen, penicillin/ streptomycin was from Invitrogen. FBS was from Gibco. Tyspin was from Invitrogen.
3.3.4 Differentiation assay and immunofluorescence staining

BMSCs were plated at 6x10^4 cells/well density on collagen coated coverslips. Cells were cultured overnight before stimulation. Cells were stimulated with 10ng/ml FGF2 (R&D Systems) for 4 days. Stimulation was stopped by adding 4% PFA and the differentiated BMSCs were analyzed by immunofluorescence staining with specific neuronal marker antibodies.

Cells were fixed in 4% PFA in PBS for 20 min at RT, washed and then permeabilized in 0.1% Triton-X100 in PBS for 10min. Cells were blocked in PBS containing 0.1% Triton-X100 and 10% normal goat serum (Millipore) for 1 h and then stained with 1:250 of anti-Neurofilament or anti-β-tubulin III anti-antibodies overnight at 4°C followed by 1:200 dilution of either a Alexa Fluor-594 or Alexa Fluor-488 goat anti-mouse secondary antibodies for 2 h with a Hoechst 33342 counterstain. Differentiation was quantified by confocal and phase-contrast microscopy with at least 20 fields-of-view containing more than 30 cells from duplicate slides.

BMSCs were scored as having undergone neuronal differentiation if: 1) NF expression was induced, 2) cells had extended at least two neurites; 3) and all neurites were at least three times the cell body in length.

3.3.5 Pharmacological assay

A total of 4 drugs with various concentrations were used in pharmacological assay. MEK inhibitor U0126 was from Cell Signalling Technology; FGFR inhibitor SU5402 was from Calbiochem; two commercial PKCs inhibitors were GF 109203 X (Tocris Bioscience) and Gö 6976 (Calbiochem). All inhibitors were dissolved in DMSO for
various concentrations. Cells were pre-treated with inhibitors for 1hr before adding FGF2. Equal volume of DMSO was added into control wells or plates. In all pharmacological assays, the DMSO final concentration was kept below 0.05% to prevent toxicity. Cells were re-feed with fresh medium containing both fresh inhibitors and FGF2 for every 2 days. Differentiation analysis was performed on the day 4.

3.3.6 PKCs specific siRNA sequence

siRNA was manufactured by Stealth RNAi™ siRNA Technology according to the cDNA sequences of specific protein kinase C isoforms (Table 3-1).

Table 3-1 siRNA sequence of specific PKCs

<table>
<thead>
<tr>
<th>PKCs isoform</th>
<th>NCBI reference code</th>
<th>siRNA sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>PKCα</td>
<td>NM_001105713</td>
<td>5’ UGA AUU UGU GGU CUU UCACCUCAU G</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3’ CAU GAG GUA AAA GAC CAC AAA UUC A</td>
</tr>
<tr>
<td>PKCε</td>
<td>NM_017171</td>
<td>5’ AAU AGA GUU GGG UUA GAU AAG GGU G</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3’ CAC CCU UAU CUA ACCCAA CUC UAU U</td>
</tr>
<tr>
<td>PKCδ</td>
<td>NM_133307</td>
<td>5’ AAU UGA AGG AGA UGC GCA GGA ACG G</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3’ CCG UUC CUG CGC CUG CGC AUG UCC UUC AAU U</td>
</tr>
</tbody>
</table>

3.3.7 siRNA transfection

The protocol titled “Transfecting Stealth™ RNAi or siRNA into Human Mesenchymal Stem Cells Using Lipofectamine™ RNAiMAX” from Invitrogen was chosen as our standard method. The first passage BMSCs were plated at 4x10⁴ cells/well density on collagen coated coverslips. Cells were cultured overnight
before transfection. Cells were transfected with 100nM specific isoform PKC siRNA or GC-control siRNA and 50nM BLOCK-iT™ Fluorescent Oligo by using Lipofectamine™ RNAiMAX. The final concentration of Lipofectamine™ RNAiMAX was 5 ul/ml. siRNA transfection was stopped by changing fresh culture medium. Cells were stimulated with 10ng/ml FGF2 at 48 h after siRNA transfection and then cultured for another 4 days. Stimulation was stopped by adding 4% PFA and the differentiated BMSCs were analyzed by immunofluorescence staining with specific neuronal marker antibodies.

### 3.3.8 siRNA knockdown

1x10^6 BMSCs were transfected with 100nM PKC siRNA targeting specific isoforms (Invitrogen) and 50nM BLOCK-iT™ Fluorescent Oligo by using Lipofectamine™ RNAiMAX for 6 h. Cells were cultured for 48 h to knockdown expression of PKCs isoforms. Total RNA was isolated from BMSCs according to the protocol of TRIzol® RNA Isolation Reagents (Invitrogen). Knockdown was confirmed by Quantitative RT-PCR on purified (Trizol) total RNA using PKC isoform-specific primers.

**Table 3-2 Forward and reverse primers specific PKCs**

<table>
<thead>
<tr>
<th>PKCs isoform</th>
<th>Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>PKCa</td>
<td>Forward</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
</tr>
<tr>
<td>PKCe</td>
<td>Forward</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
</tr>
<tr>
<td>PKCd</td>
<td>Forward</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
</tr>
</tbody>
</table>
Quantitative RT-PCR was performed with QuantiTect SYBR Green (Qiagen) using RotorGene (Corbett Research 2004) with 0.5µM of PKC isoform-specific forward and reverse primers. Primer sequences were listed in Table 3-2. Amplification cycling was performed as follows: 94°C for 15 minutes, followed by 38 cycles of 94°C for 28 seconds, 60°C for 28 seconds, and 72°C of 28 seconds. Fluorescence data were acquired at 510nm during each 72°C extension phase. Amplification of actin (ACTB) was used as an internal standard to normalize expression. Relative expression values were calculated from duplicate samples by cycle threshold value (C_T) extrapolation to standard curves of known concentration. Relative gene expressions were expressed in arbitrary units (AU) defined as ng of RNA from 2µg of total RNA relative to ACTB expression. The Mann-Whitney U test was used to establish the statistical significance of differences in gene expression between BMSCs treated with siRNA targeting specific PKC isoforms and control siRNA.
3.4 Results

3.4.1 FGF2 induces BMSCs differentiate into neuronal phenotype

FGF2 has been shown to induce BMSCs neuronal differentiation in vitro [272, 273]. We initially performed differentiation assays as originally described by Woodbury et al by using early-passage BMSCs treated with FGF2 [272]. Under these conditions, BMSCs treated with FGF2 underwent morphological changes in which neurite-like extensions were observed, while cells cultured in control medium without FGF2 did not exhibit any measurable morphological changes (Figure 3-1 A). To further examine whether the morphological changes was correlated with the expression of neuronal markers, we examined the expression of neurofilament (NF) and β-tubulin III using specific antibodies. Compared with the control cells, FGF2 treated cells not only showed morphological changes consistent with neuronal differentiation, but also increased the expression of NF (Figure 3-1 B) and β-tubulin III (Figure 3-1 C).
The BMSCs were seeded on the coverslips coated with collagen in low glucose DMEM/20% FBS. Cells were then non-stimulated or stimulated with FGF2 (10 ng/ml) for 4 days. The stimulation was stopped by incubating cells with 4% PFA. The fixed cells were then stained with specific neuronal marker antibodies. (A) The images were taken by phase contrast microscope. (B) The fixed cells were stained with anti-neuronfilament (NF) primary antibody followed by Alexa Fluor 594 secondary antibody. (C) The fixed cells were stained with anti-β-tublin III (tuji) primary antibody followed by Alexa Fluor 488 secondary antibody. The images were taken by either the phase contrast microscope or confocal microscope at 20X magnifications. Data are representative of three independent experiments.
Initial experiments demonstrated that the abilities of BMSCs to differentiate in response to FGF2 varied according to the passage number of the BMSCs. We therefore compared the neuronal differentiation capacities of early- and late-passage BMSCs. Passage 1 BMSCs demonstrated a robust neuronal differentiation capacity with 17% neurite-positive cells (Figure 3-2). The differentiation capacities of BMSCs were found to decrease with increasing passage number. Furthermore, we also observed some variabilities in differentiation capacity with later passage BMSCs with ranged from 3-12% (data are not shown). Therefore, all subsequent assays employed passage 1 BMSCs to ensure the consistent and robust differentiation capacity in response to FGF2.
Figure 3-2 First passage BMSCs demonstrated robust and consistent capacity of neuronal differentiation

The first passage BMSCs were seeded on the coated coverslips in LG DMEM/20% FBS. Cells were then non-stimulated or stimulated with FGF2 (10 ng/ml) for 4 days. The stimulation was stopped by incubating cells with 4% PFA. The fixed cells were stained with anti-NF antibody. Cells were stained with both Alexa Fluor 594 secondary antibody and Hoechst, and then analysed by confocal microscopy. The neuronal differentiation were quantified and indicated. Data are representative of six independent experiments. Error bars represent S.E.M from six independent experiments (* p<0.05).
3.4.2 BMSCs 7 days growth curve

We next examined the proliferation rate of BMSCs directly isolated from bone marrow. We named this batch of cells as 0 passage BMSCs and it took 48h for BMSCs to attach to the plastic surface. Stem cells lose their proliferation and differentiation potentials in the prolonged cell culture. Therefore, we aimed to expand enough cell numbers by cell culture without impacting the abilities of BMSCs neuronal differentiation in response to FGF2. BMSCs started to form colonies from the third day but the colonies were small and just detectable. More colonies were formed and sizes were becoming bigger following the culture. The cell shape also changed from round cell body shape into fibroblast like cell body shape (Figure 3-3 A). The results showed that BMSCs grew slowly in the first five days and then went into an exponential growth phase during following two days (Figure 3-3 B).
Cells isolated from bone marrow were seeded in 6-well Falcon plate at density of $1.6 \times 10^6$ cell/well. Cells were cultured for 48 h to allow BMSCs to attach to the plastic surface. Non-adhesive cells were washed away by changing medium. BMSCs were harvested on 2 day, 3 day, 4 day, 5 day, 6 day and 7 day and then performed cell counting. (A) The photos were taken by the phase contrast microscope before harvest. (B) The graph showed the 7 days log growth curve of BMSCs. Data are representative of two independent experiments. Error bars represent standard deviations.
3.4.3 Initial culture time impacts BMSCs neuronal differentiation capacity

Although the first passage BMSCs had demonstrated robust neuronal differentiation capacity, some degree of variability in neuronal differentiation capacity was observed. The neurite positive cells on the passage 1 BMSCs from different batches of BMSCs different experiments varied from 12% to 23% (Figure 3-2). In our initial procedure, a total of 1x10^7 cells containing both adhesive and non-adhesive cells were cultured in one T175cm^2 flask. It normally took 4-9 days for BMSCs that were adhesive cells to reach the 70%-80% confluence, which was called as the initial culture time. The initial culture time allowed the BMSCs to attach to the surfaces of cell culture dishes and to expand to the required cell amount. We thought that different initial culture time might cause various neuronal differentiation capacities. Therefore, we decided to examine the influence of initial culture time on the BMSCs neuronal differentiation capacity. BMSCs isolated from bone marrow were cultured for 2 days, 3 days, 4 days, 5 days and 6 days respectively, and then cells were harvested to perform differentiation assay (Figure 3-4 A). When the initial culture time was shorter than 5 days, the neurite positive cells were about 15%. Interestingly, the differentiation ability dropped to 7% when the initial culture time was 6 day (Figure 3-4 B). The 5 days initial culture time allowed BMSCs to reach the maximal cell amount without defecting neuronal differentiation potential. Therefore, we chose the BMSCs with 5 days initial culture time for the following experiments.
Cells isolated from bone marrow were cultured for 2 days and then non-adhesive cells were washed away by changing medium. Cells were cultured until 6 days and were harvested at 2, 3, 4, 5, 6 days respectively. BMSCs were then seeded on the coated coverslips in LG DMEM/20% FBS and stimulated by FGF2 (10 ng/ml) for 4 days. The stimulation was stopped by incubating cells with 4% PFA. (A) Cells were stained with anti-NF primary antibody and then stained with both Alexa Fluor 594 secondary antibody and Hoechst. The coverslips were analysed by confocal microscopy. (B) The neuronal differentiation were quantified and indicated. Data are representative of two independent experiments. Error bars represent standard deviations.
3.4.4 FGF treatment time course

We further examined whether the FGF2 treatment time could affect BMSCs neuronal differentiation capacity. We compared the neuronal differentiation capacities of BMSCs stimulated with FGF2 over different times. While morphological changes in FGF2-treated BMSCs were detected from 48 h, no increased NF expression level was observed (Figure 3-5 A). After 72 h, BMSCs showed more significant morphological changes together with an increased NF expression up to 7 days (Figure 3-5 A). The maximal neuronal differentiation was observed on day 5 (Figure 3-5 B, 18% neurites positive cells). Interestingly, the percentage of neurite positive cells started to drop on the sixth day and kept dropping until seventh day (Figure 3-5 B). The results suggest that FGF2 stimulation for 4 or 5 days can induce the maximal and most consistent neuronal differentiation of BMSCs. Therefore, the 4-day FGF2 treatment was used in all following experiments.

The data presented from the pilot experiments designed to optimising the neuronal differentiation of BMSCs depicted the raw differentiation values. These experiments demonstrated the consistent and reproducible neuronal differentiation of BMSCs ranging from 16% to 20% (Figure 3-5). However, in all subsequent experiments including the pharmacological assay and siRNA-mediated knockdown, the neuronal differentiation of BMSCs was normalised with the control treatment.
Figure 3-5 FGF2 treatment time course

The first passage BMSCs were seeded on the coated coverslips in LG DMEM containing 20% FBS and stimulated by FGF2 (10 ng/ml) for the time indicated. The stimulation was stopped by incubating cells with 4% PFA. (A) The fixed cells were stained with anti-NF antibody, and then stained with both Alexa Fluor 594 secondary antibody and Hoechst. The coverslips were analysed by confocal microscopy. (B) The neuronal differentiation were quantified and indicated. Data are representative of two independent experiments. Error bars represent standard deviations.
3.4.5 Ras/MAP kinase and novel PKCs signalling are important for BMSCs neuronal differentiation mediated by FGF2

To investigate the molecular mechanisms regulating BMSCs neuronal differentiation mediated by FGF2, we examined the role of Ras/MAP kinase signalling in mediating BMSCs neuronal differentiation by using the small molecule specific MEK kinase inhibitor U0126.

MEK inhibition resulted in a significant reduction in BMSC neuronal differentiation, while the selective blockage of FGFR signalling using SU5402 essentially abolished FGF2-mediated differentiation (Figure 3-6). Although a small proportion of U0126 treated BMSCs were still able to express NF in their cell bodies, the average neurite length of those cells was much shorter than the neurite length of BMSCs treated with FGF2 alone. These results suggest that Ras/MAP kinase signalling is important for BMSCs neuronal differentiation mediated by FGF2. The above results have been published in JBC, 2013 288(21): 14874-85, Figure 7 B. Lonic A et al [242].
Chapter 3

Figure 3-6 Ras/MAP kinase signalling is the key signalling event in regulating BMSCs neuronal differentiation

The first passage BMSCs were seeded on the coated coverslips in LG DMEM/20% FBS. The cells were pre-incubated with DMSO or inhibitors indicated for 1 h then stimulated by FGF2 (10 ng/ml) for 4 days. The inhibitors included FGFR inhibitor SU 5402 (25 µM) and MEK inhibitor U0126 (20 µM). The stimulation was stopped by incubating cells with of 4% PFA. (A) The fixed cells were stained with anti-NF antibody. Cells were then stained with Alexa Fluor 594 secondary antibody and Hoechst. The coverslips were analysed by confocal microscopy. (B) The neuronal differentiation of inhibitors pre-treated BMSCs were quantified and indicated. Data are representative of three independent experiments. Error bars represent S.E.M from three independent experiments (* p<0.05).
We then examined the impact of PKC inhibition on BMSC differentiation. The pan-PKC inhibitor GF109203X showed a significant 80% reduction in BMSCs differentiation at 5µM concentration, while the cPKC selective inhibitor, Gö6976 failed to reduce differentiation (Figure 3-7), suggesting a role for nPKC (including δ, ε, η, and θ isoforms) rather than cPKCs (including α, βI, βII, and γ isoforms) in regulating BMSCs neuronal differentiation. The above results have been published in JBC, 2013 288(21): 14874-85, Figure 7 C. Lonic A et al [242].

In conclusion, these results based on the pharmacological assay suggest that both Ras/MAP kinase and nPKCs signal pathways are crucial for BMSCs neuronal differentiation mediated by FGF2.
Figure 3-7 nPKCs signal is also important in regulating BMSCs neuronal differentiation mediated by FGF2

The first passage BMSCs were seeded on the coated coverslips in LG DMEM/20% FBS. The cells were pre-incubated with DMSO or GF 109203X (5 µM) or Go 6976 (5 µM) for 1 h then stimulated by FGF2 (10 ng/ml) for 4 days. The stimulation was stopped by incubating cells with of 4% PFA. (A) The cells were fixed and stained with anti-NF antibody. Cells were then stained with Alexa Fluor 594 secondary antibody and Hoechst. The coverslips were analysed by confocal microscope. (B) The neuronal differentiation were quantified and indicated. Data are representative of two independent experiments. Error bars represent S.E.M from three independent experiments (* p<0.05).
3.4.6 PKCs siRNA knockdown confirmation

Previous pharmacological studies suggest that nPKCs but not cPKCs are important for BMSCs neuronal differentiation mediated by FGF2. We therefore decided to perform siRNA-mediated knockdown of the specific nPKC members, PKCε and PKCδ in BMSCs. Although our pharmacological studies suggest that cPKC isoforms are not involved in FGF2-mediated BMSC differentiation, we also performed siRNA knockdown of PKCα because PKCα is highly expressed in neuronal cells [280].

Firstly, we examined the efficiency of siRNA transfection protocol in BMSCs. Transfection of BMSCs with a FITC-labelled tracker oligonucleotide demonstrated that more than 90% cells were FITC-positive 6 h after the transfection (Figure 3-8). The transfection efficiency dropped slightly but still remained above 80% 48 h after the transfection.
The first passage BMSCs were seeded on the coated coverslips in LG DMEM/20% FBS. Transfected cells with both medium GC siRNA control and BLOCK-iT™ Fluorescent Oligo (green) by using Lipofectamine™ RNAiMAX according to the protocol (Invitrogen). The transfection was stopped after 6 h transfection by incubating cells with of 4% PFA or transfected cells were cultured for another 48 hours then fixed by 4% PFA. (A) The fixed cells were stained with Hoechst and analysed by confocal microscopy. (B) The siRNA transfection efficiency were quantified and indicated. Data are representative of two independent experiments. Error bars represent standard deviations from two independent experiments.
The mRNA levels of specific PKCs in BMSCs were examined by quantitative RT-PCR at 48 h after the siRNA transfection. Compared with the GC-control siRNAs, siRNA-mediated PKCs knockdown resulted in a significant decrease in mRNA expression of PKCs (Figure 3-9).

Figure 3-9 siRNA knockdown is confirmed by quantitative RT-PCR

The first passage BMSCs were seeded on the coated coverslips in LG DMEM/20% FBS. BMSCs cells were transfected with 100 nM GC control siRNA or siRNA targeting PKCα, PKCε or PKCδ. After 48h, cells were harvested and then total mRNA was isolated according to the Trizol protocol. The knockdown was confirmed by quantitative RT-PCR performed with 50nM PKC specific primers. Data are representative of two independent experiments. Error bars represent standard deviations.
3.4.7 PKCε is important for BMSCs neuronal differentiation mediated by FGF2

To identify specific PKC isoforms involved in the BMSCs neuronal differentiation mediated by FGF2, we examined the differentiation capacities of BMSCs in response to FGF2 after the siRNA-mediated knockdown of specific PKC isoforms. As shown in Figure 3-10, siRNA knockdown of the nPKC isoform, PKCε, but not the related nPKC members, PKCδ, or the cPKC isoform, PKCα, significantly reduced BMSC differentiation following the FGF2 stimulation. siRNA-mediated PKCε knockdown resulted in a 60% reduction in BMSCs neuronal differentiation compared with GC-control siRNA (Figure 3-10).

These results suggest that novel isoform PKCε, but not conventional isoform PKCα or other novel isoform PKCδ, is important for BMSCs neuronal differentiation mediated by FGF2. This is consistent with the inhibitor-based studies, which also suggested important roles of nPKCs subfamily but not cPKCs subfamily in BMSCs neuronal mediated by FGF2. The siRNA-mediated knockdown also confirmed PKCα, which belongs to cPKCs, is not important for BMSCs neuronal differentiation mediated by FGF2. The above results have been published in JBC, 2013 288(21): 14874-85, Figure 7 D. Lonic A et al [242].

Importantly, these findings are consistent with those in PC12 cells, showing that PKCε is the key PKC isoform involved in PC12 cells neuronal differentiation mediated by FGF9.
Figure 3-10 PKCε is important for BMSCs neuronal differentiation mediated by FGF2

The BMSCs were transfected with 100 nM siRNA targeting PKCα, PKCε or PKCδ isoforms or the medium GC siRNA control. Transfected cells were cultured for 48 h to knockdown the expression of PKCs and then stimulated with FGF2 for 4 days. The stimulation was stopped by incubating cells with 4% PFA. (A) The cells were fixed and stained with anti-NF antibody. Cells were then stained with Alexa Fluor 594 secondary antibody and Hoechst. The coverslips were analysed by confocal microscope. (B) The neuronal differentiation were quantified and indicated. Data are representative of three independent experiments. Error bars represent S.E.M from three independent experiments (* p<0.05).
3.5 Discussion

FGF2 has been shown to induce BMSCs neuronal differentiation in vitro [272, 281]. In addition, an elevated FGF2 expression has been observed in the injured CNS systems [20, 266-268, 282]. For example, the increased expression of both FGF2 peptide and FGF2 mRNA has been identified in multiple sites of brain after induced stroke in the animal models [267, 282]. The intracerebral transplantation of BMSCs improved function recovery in both stroke and traumatic brain injury mice models [283-285]. However, the relationship between the elevated FGF2 expression and BMSCs neuronal differentiation in vivo is unknown. Therefore, a better understanding of the molecular mechanisms of BMSCs neuronal differentiation mediated by FGF2 in vitro would provide invaluable information to enhance the application of BMSCs as the stem cell based therapy in treating CNS disorders.

In this study, we have examined the molecular mechanisms underpinning FGF2-mediated neuronal differentiation using a primary mouse BMSC model system. Our results show that the Ras/MAP kinase and PKC\(\varepsilon\) pathways are crucial for BMSCs neuronal differentiation mediated by FGF2. Importantly, our studies suggest that the phosphorylation of S779 by PKC\(\varepsilon\) is required for the maximal activation of Ras/MAP kinase signalling and neuronal differentiation of BMSCs in response to FGF2.

Several groups have utilized BMSCs neuronal differentiation in vitro to examine growth factor biology [286-289]. The culture conditions for mouse primary BMSCs, passage number used and the criteria established for neuronal quantification were optimized as part of the current studies to provide a robust model system for the analysis of FGF2 biology in neuronal differentiation. For example, other studies have
employed only morphological changes as evidence of differentiation [272, 281] possibly losing the ability to discriminate subtle changes in cellular responses to growth factors. In addition, our studies also suggest that NF is a suitable neuronal marker to score neuronal differentiated BMSCs.

Our results show that the first passage BMSCs has the robust and consistent neuronal differentiation capacity (Figure 3-2). The later passages of BMSCs show a much lower and significantly inconsistent differentiation capacity ranging from 3% to 12% in different batches of BMSCs (data are not shown). Our results further show that the prolonged initial culture time in the first passage cells can also reduce the BMSCs neuronal differentiation capacity (Figure 3-4). There might be two reasons for the lower neuronal differentiation from the latter passages BMSCs or the prolonged initial culture BMSCs. One reason could be that BMSCs gradually lost their neuronal differentiation capacities during the long-term culture. Another possible reason could be that the lower neuronal differentiation capacities are caused by the heterogeneity of BMSCs. BMSCs contain many different cell types by nature and those cells follow different growth rates. It is likely that only specific sub-populations of BMSCs are able to respond to FGF2 and that this specific sub-population is lost during extended culture in vitro.

Using the first passage BMSCs treated with FGF2 for 4 days, we have been able to examine the mechanisms by which FGF2 promotes neuronal differentiation (Figure 3-5). We used the unsorted BMSCs without performing further cell isolation. So far multiple protocols have been developed to try to isolate more homogenous BMSCs from bone marrow. These methods include the exposure of cultures to cytotoxic
materials [290, 291], low-density [292] and high-density culture [293], negative [294] and positive selection [295, 296]. However, cells isolated by these protocols have not been systematically analysed to prove that they are the same population cells. In addition, these protocols have been shown to impair BMSCs proliferation and differentiation [297]. Isolating BMSCs based on their capacity to attach to the plastic surface (pre-plating) is still the most accepted method in the BMSCs research field [297].

A total of four small molecule kinase inhibitors have been used to identify the key signalling pathways in BMSCs neuronal differentiation mediated by FGF2. SU5402 is a kinase inhibitor that selectively targets FGFR1 and FGFR2. SU5402 has been shown by others to block both FGFR tyrosine phosphorylation and ERK phosphorylation induced by FGF2 in NIH 3T3 cells [298]. In the current studies, BMSCs treated with SU5042 retained their fibroblast morphology and showed no evidence of neuronal differentiation (Figure 3-6). These results confirm that FGF2 signalling is crucial for BMSCs neuronal differentiation.

Ras/MAP kinase pathway has been shown to be important for both stem cell and neuronal cell differentiation. For example, the activation of ERK signalling mediated by FGFs drives mouse ES cell differentiation [299, 300]. Furthermore, sustained Ras/MAP kinase signalling is efficient to induce PC12 cells neuronal differentiation (see Chapter 2). U0126 has been widely used as a selective MEK1/2 inhibitor and has been shown to block ERK phosphorylation and neuronal differentiation mediated by NGF in PC12 cells [90]. While U0126 significantly reduced BMSCs neuronal differentiation mediated by FGF2, it did not totally abolish this response (Figure 3-6).
These results suggest that Ras/MAP kinase is important but might not be the only critical pathway in regulating BMSCs differentiation mediated by FGF2.

The PKC family of protein kinases have been shown to play multiple important roles in neuronal cells [170]. For example, the activation of PKCε enhances neurites outgrowth in several cell lines and primary neurons [170]. PKCε is also involved in the neuronal differentiation of neuroblastoma cells and the over-expression of PKCε is able to induce growth factor-independent neuroblastoma differentiation [175-177].

In the current studies, the pan-PKC inhibitor GF 109203X, was able to significantly inhibit BMSC neuronal differentiation. The cPKC inhibitor, Gö 6976, which selectively inhibits PKC α, β and γ, did not show significant inhibition of differentiation even at high concentrations (Figure 3-7). siRNA knockdown of the PKCε, but not PKCδ, or PKCα, showed a significant reduction in BMSCs neuronal differentiation compared with GC-control siRNA. These results suggest that PKCε is an important regulator of BMSC differentiation mediated by FGF2 (Figure 3-10).

Further studies performed by others in our laboratory show that in addition to promoting BMSCs neuronal differentiation, FGF2 stimulation also induced an increased S779 phosphorylation of endogenous FGFR2 in BMSCs. siRNA-mediated knockdown of PKCε, but not PKCδ, resulted in a decrease in growth factor-induced S779 phosphorylation of FGFR2 in BMSCs. Furthermore, the mutation of S779 resulted in the reduced ERK phosphorylation and the decreased cell differentiation, suggesting S779 signalling is critical for regulating the maximal Ras/MAP kinase signalling and neuronal differentiation in BMSCs (experiments performed by others in our laboratory and published in JBC, 2013 288(21): 14874-85, Lonic A et al [242]).
In a similar manner, we have shown that the pan PKC inhibitor GF109203X and siRNA knockdown of PKCε could significantly reduce S779 phosphorylation as well as neuronal differentiation in PC12 cells expressing wt-FGFR2. In addition, PKCε can phosphorylate a peptide encompassing S779 residue in vitro kinase assay. Thus, these results suggest that PKCε is responsible for S779 phosphorylation in both BMSCs and PC12 cells (experiments performed by others in our laboratory and published in JBC, 2013 288(21): 14874-85, Lonic A et al [242]).

The PKC family of kinases have been shown to regulate Ras/MAP kinase pathways through several different mechanisms. For example, PKCε can increase the kinase activity of both Raf kinases and ERK through direct phosphorylation [172, 173, 186]. Co-treatment PC12 cells with EGF and PMA induce sustained ERK and neuronal differentiation, while EGF stimulation of PC12 cells alone promotes transient ERK and cell proliferation [174]. Other studies have shown that the over-expression of PKCε enhanced PC12 cell differentiation and ERK phosphorylation [172, 173]. Increased PKCε expression induces by ethanol also promoted PC12 cell neurite outgrowth [301]. It has also been shown that PKCε induced differentiation of neuroblastoma cells through regulating Raf/MEK/ERK module [302, 303]. Combining computing models and siRNA–mediated knockdown, PKCδ has been observed to serve as the positive feedback modulator to regulate sustained Ras/MAP kinase pathway activation and PC 12 cell neuronal differentiation mediated by NGF [174]. Our studies identify a novel role PKCε in regulating maximal Ras/MAP kinase signalling through phosphorylation of S779 in FGFR2, which provides important mechanistic insights into the regulation of neuronal differentiation mediated by FGF2.
Collectively, our results suggest that PKCε-mediated phosphorylation of S779 in FGFR2 is critical for maximal Ras/MAP kinase signalling and neuronal differentiation in BMSCs. Consistent with the findings in Chapter 2, our results again demonstrate that in addition to the roles of phosphotyrosine residues in promoting intracellular signalling, the phosphorylation of S779 in FGFR2 can also initiate intracellular signalling to control specific cellular responses such as neuronal differentiation.

Our study may have important implications in developing the stem cell-based therapies for treating CNS disorders. The studies herein as well as those by other have shown that FGF2 can potently induce BMSCs neuronal differentiation [288, 289, 304]. Prior to transplantation, induction of BMSCs into neuronal phenotype cells significantly increases the engraftment efficiency and functional recovery benefits for treating stroke as well as brain trauma [285, 305]. Transplantation of post-mitotic cells with neuronal characteristics offers extra advantages such as less possibility for tumor genesis and neural overgrowth. Therefore, elucidation of the molecular mechanisms underlying BMSCs neuronal differentiation mediated by FGF2 might improve the manipulation BMSCs in treating CNS disorders. Our study highlights the critical roles of both Ras/MAP kinase and PKCs signalling pathways in regulating BMSCs neuronal differentiation, therefore it is reasonable to speculate that the enhancement of these signalling pathways might significantly enhance neuronal differentiation. Thus, synergetic administration of multiple growth factors or pharmaceutical activators that foster both Ras/MAP kinase and PKCs signalling would further promote BMSCs neuronal differentiation, and subsequently improve
therapeutic functions of BMSCs in treating stroke and other neuronal degenerative diseases.
Chapter 4 Discussion and conclusion
The underlying mechanisms by which growth factors exert their pleiotropic (diverse) biological responses are not fully understood. The FGFs are an important family of growth factors capable of mediating diverse cellular responses such as cell survival, proliferation, differentiation and migration [306]. In this study, we have examined the mechanisms by which the FGFRs regulate intracellular signalling and neuronal differentiation.

FGFR tyrosine phosphorylation is a critical event in the activation of downstream signalling cascades leading to specific cellular responses. However, in addition to FGFR tyrosine phosphorylation, we have also shown that FGFR2 is phosphorylated on specific serine residues [191]. Previous studies from the Cell Growth and Differentiation laboratory have shown that Serine 779 (S779) located in cytoplasmic domain of FGFR2 is important for downstream signalling pathways regulation and cell survival [191]. While these earlier studies identified S779 phosphorylation on FGFR2 as an important event in activating downstream signalling, the model systems examined were limited to Baf/3 hemopoietic cells and BALB/c 3T3 fibroblasts. We therefore have examined the potential role of S779 signalling in FGFR2 in neuronal differentiation model systems that are more relevant to FGFR biology and physiological functions. Specifically, the current studies aimed at examining the potential roles of S779 signalling in regulating the neuronal differentiation of both PC12 cells and BMSCs.

We show that the FGF stimulation of either PC12 cells or BMSCs triggers the phosphorylation of S779 in the cytoplasmic tail of FGFR2, which is essential for maximal ERK phosphorylation together with neurite outgrowth and differentiation.
We further demonstrate that the nPKC isoform, PKCe, is responsible for S779 phosphorylation, which leads to the full activation of Ras/MAP kinase signal pathway and neuronal differentiation. These findings demonstrate that, in addition to phosphotyrosine residues in the cytoplasmic tails of FGFR2, the phosphorylation of S779 can also initiate intracellular signalling to control specific cellular responses such as neuronal differentiation. Thus, the studies presented in this thesis which also contribute to a manuscript published by Lonic et. al. in *Journal of Biological Chemistry* establish a new mechanism by which the FGFRs are able to regulate intracellular signalling and cellular responses [242].

### 4.1. Key findings

Despite many studies that have examined the regulation of FGFR2 intracellular signalling, no specific tyrosine residue in the FGFR2 cytoplasmic domain has been functionally linked to neuronal differentiation. Thus, the studies described herein demonstrating that FGFR2 promotes neuronal differentiation of PC12 cells and BMSCs via serine phosphorylation represent an important conceptual advance and provide new mechanistic insights into growth factor receptor biology.

Specifically, while FGF9 promotes neuronal differentiation of PC12 cells expressing the wt-FGFR2, PC12 cells expressing the FGFR2-S779G mutant were defective in their ability to differentiate in response to FGF9, confirming that S779 is critical for initiating intracellular signals that promote the differentiation of PC12 cells (*Figure 2-3*). The studies presented in this thesis have also shown that S779 in the cytoplasmic tail of FGFR2 was subjected to phosphorylation in response to FGF9 stimulation in PC12 cells (*Figure 2-5*). More detailed analyses of the Ras/MAP kinase pathway...
revealed that PC12 cells expressing the wt-FGFR2 treated with FGF9 showed the sustained ERK1/2 phosphorylation, while cells expressing FGFR2-S779G mutant with FGF9 stimulation showed a decreased ERK1/2 phosphorylation (Figure 2-4). Importantly, while the amplitude of ERK1/2 phosphorylation was significantly reduced in cells expressing the FGFR2-S779G mutant, the signalling kinetics did not appear to be affected with an elevated ERK1/2 phosphorylation clearly detectable up to 2h following FGF9 stimulation (Figure 2-4). The reduced amplitude of ERK phosphorylation observed in cells expressing the FGFR2-S779G mutant was not due to an overall defect in FGFR2 or FRS2 tyrosine phosphorylation (Figure 2-6). Furthermore, while Ras activation was reduced in PC12 cells expressing the FGFR2-S779G mutant (Figure 2-8), paradoxically, there was no defect in MEK phosphorylation (Figure 2-9). On the other hand, no defect was observed in the ability of the FGFR2-S779G mutant to induce the PI3K/Akt signalling or the phosphorylation of MSK1 and ATF2 (experiments performed by others in our laboratory and published in JBC, 2013 288(21): 14874-85, Lonic A et al [242]). Collectively, these results indicate that the phosphorylation of S779 is selectively required for the full activation of Ras/MAP kinase signalling and PC12 cells neuronal differentiation.

In BMSCs, treatment of MEK inhibitor U0126 resulted in the reduced neuronal differentiation, suggesting Ras/MAP kinase signalling is important for neuronal differentiation mediated by FGF2 (Figure 3-6). The pan-PKC inhibitor GF109203X showed an 80% reduction in BMSCs differentiation, while the cPKCs selective inhibitor, Gö6976 failed to reduce BMSCs neuronal differentiation even at a high concentration, suggesting that the nPKCs are involved in the BMSCs neuronal
differentiation (Figure 3-7). Selective siRNA-mediated knockdown of PKCε, but not PKCδ or PKCα, significantly reduced the ability of FGF2 to induce BMSC neuronal differentiation (Figure 3-10). Furthermore, further studies in our laboratory have also identified PKCε as a key kinase responsible for the phosphorylation of S779. siRNA-mediated knockdown of PKCε has resulted in a decrease in growth factor-induced S779 phosphorylation of the endogenous FGFR2 [242]. In addition, PKCε was shown to directly phosphorylate S779 in FGFR2 in vitro [242]. Mutation of S779 has resulted in the reduced ERK phosphorylation as well as the decreased BMSCs neuronal differentiation [242]. Collectively, our results show that PKCε mediated phosphorylation of S779 in FGFR2 is critical for regulating the intracellular signalling events necessary for maximal Ras/MAP kinase signalling and BMSCs neuronal differentiation.

4.2. Innovations and challenges

Cells in metazoans are exposed to numerous extracellular stimuli, which include growth factors and cytokines over the course of their lifespan [307]. All cells are equipped with specific sensors, i.e. cell surface receptors, which allow them to respond to external stimuli in different manners that may include proliferation, differentiation, migration and survival. When activated by growth factors, a specific cell surface receptor is able to regulate multiple cellular activities (pleiotropy). Growth factor receptors propagate stimuli from the plasma membrane into the intracellular environment by initiating signalling cascades. However, in many cases, the signals generated by distinct and diverse receptors appear highly redundant and often utilize common or overlapping signalling pathways. How such seemingly generic signal events are coordinated to generate specific cellular outcomes is a
fascinating and not yet fully understood question. While it is known that growth factors exert their biological activities through the binding of cell surface receptors and the activation of multiple intracellular signalling cascades, how specificity in both signalling and biological responses can be achieved is unclear.

One way to address this fundamentally important question is to carefully analyse proximal signalling events of the cytoplasmic domain of receptors after the receptor activation. Protein phosphorylation is one of the most important post-translation events enabling cells to rapidly and reversibly transduce signals throughout the cell. Among protein phosphorylation, the serine/threonine and tyrosine phosphorylation account for more than 90% of all phospho-proteins. The phosphorylation events occurring on the cytokine and growth factor receptors have important functions during the transmembrane signalling transduction. For example, the RTK family is a group of important growth factor receptors. After activation, tyrosine phosphorylation occurs within seconds of ligand stimulation and provides docking sites for signalling proteins containing phospho-tyrosine binding domains such as SH2 and PTB [39]. The recruitment of SH2- and PTB-domain proteins to the cytoplasmic tail of growth factor receptors is essential for the propagation of signals within cells [149]. While tyrosine phosphorylation pathways may be essential in providing generic signals [232, 308], other non-tyrosine mechanisms may regulate specific cellular responses.

4.3.1. Serine residues in growth factor receptors serve as the novel signalling devices to regulate pleiotropic cellular responses

Although less well studied than receptor tyrosine phosphorylation, serine phosphorylation has been observed in multiple cell surface receptors and is proposed
to play important roles in regulating cellular functions. For example, previous work in our laboratory has shown that Serine 585 (S585) phosphorylation in GM-CSF receptor common β subunit is crucial for cell survival mediated by growth factors in both hemopoietic cell lines and primary AML patient samples [192, 194, 238]. Others have shown that Serine 777 phosphorylation in FGFR1 may be important for promoting the translocation of exogenous FGF1 to the cytosol and nucleus in the NIH 3T3 cells [309, 310]. Interestingly, FGFR4, which is a less potent FGFR compared with FGFR1 or FGFR2, has been shown a prominent serine phosphorylation after the FGF treatment in L6 myoblast and NIH3T3 fibroblast cell lines, although the biological function of serine phosphorylation in FGFR4 is unclear [311]. Importantly, recent studies from our laboratory have shown that the S779 phosphorylation in FGFR2 mediates survival and proliferation in Ba/F3 hemopoietic cell line and BALB/c 3T3 fibroblasts [191]. The studies presented in this thesis and those published by Lonic et al have further shown that phosphorylation of S779 in the FGFR2 is crucial for PC12 cells and primary BMSCs neuronal differentiation mediated by FGFs [242].

In addition to FGFRs, serine phosphorylation has also been found in other growth factor receptors. For example, Serine 696 (S696) of the RET receptor tyrosine kinase has been identified as a putative substrate of protein kinase A (PKA) and the phosphorylation of S696 is critical for the lamellipodia formation mediated by glial cell line-derived neurotrophic factor (GDNF) [312]. Subsequent studies have shown that RET Ser697-Ala mutant mice are defective in neural crest cell migration in the developing gut, leading to aganglionosis in the distal colon [313]. Serine residues in the C-terminal of insulin-like growth factor I receptor (IGF-IR), which also belongs to
receptor tyrosine kinase family, could play important roles in mediating specific cellular responses. For example, the four serine residues at 1280-1283 of the IGF-IR are important for its transforming activity in both mouse embryo derived fibroblasts and the rat glioblastoma C6 cell line [314]. Furthermore, others have shown that S1280-1283 are crucial for regulating cell survival in response to IGF1 by enhancing the phosphorylation of BAD in a murine hemopoietic cell line [315]. The TGFβ family is another group of essential growth factors whose receptors employ serine phosphorylation to mediate their biological functions during the signalling transduction. TGFβ mediates its biological activity by binding and oligomerizing cognate TGFβ type I and II receptors, which belong to the receptor serine/threonine kinases family. The type II receptors phosphorylate serine/threonine residues in the type I receptors and then activate type I receptors. The activated type I receptors further transduce the signal by phosphorylating the Smads [316]. Together with our work, these studies demonstrate that the phosphorylation of serine residues in growth factor and cytokine receptors are also important in transmembrane signalling transduction and regulating specific biological responses.

4.3.2. The phosphorylation of S779 can selectively and quantitatively control Ras/MAP kinase signalling to promote specific cellular responses

The Ras/MAP kinase module has been extensively studied both genetically and pharmacologically in diverse cellular systems. The strength and duration of signalling have been shown to regulate specific biological responses. For example, the sustained activation of ERK mediated by FGFs or NGF promotes PC12 differentiation, while the transient activation of ERK mediated by EGF promotes PC12 proliferation [221]. We now show that while the FGFR2-S779G mutant was able to induce Ras activation
and ERK phosphorylation, the kinetics of Ras activation was significantly more transient while the magnitude of ERK phosphorylation was reduced when compared with cells expressing wt-FGFR2. Furthermore, S779 signalling was specifically involved in Ras/MAP kinase signalling as there was no defect in PI3K activation or the phosphorylation of Akt and MSK1 in PC12 cells expressing the FGFR2-S779G mutant [242]. However, while the mutation of S779 did not affect FGFR2 tyrosine phosphorylation, blockage of FGFR2 tyrosine phosphorylation with the kinase inhibitor, SU5402, blocked the differentiation of both PC12 and BMSCs, indicating that the ability of S779 to promote neuronal differentiation requires FGFR tyrosine kinase activity [242]. Therefore, our results suggest that the phosphorylation of S779 selectively and quantitatively controls Ras/MAP kinase signalling to promote neuronal differentiation mediated by FGFs.

4.3.3. PKCε mediates the maximal activation of Ras/MAP kinase signalling through S779 phosphorylation

Work presented in this thesis as well as those published by Lonic et al have identified a novel role of PKCε in promoting Ras/MAP kinase signalling and neuronal differentiation via its ability to phosphorylate S779 of FGFR2 in both PC12 cells and primary BMSCs [242]. Previous studies using Predikin analysis, which is a predictive algorithm that can be used to identify substrates for known kinases, identified the PKC family of kinases as being involved in S779 phosphorylation [191]. PKC isoforms have been identified to play an important role in regulating the Ras/MAPK pathway through different mechanisms. For example, PKCε functions as a Raf activator in growth factor-mediated Ras/MAP kinase signalling in both NIH 3T3 cells and COS cells [185]. Over-expression of PKCε leads to the elevated Raf-1 kinase
activity as well as cell transformation in R6 fibroblasts cells, suggesting that PKCε exerts its oncogenic effects through enhancing activation of the Raf-1 kinase [186, 187]. In the anti-apoptotic effects of FGF2, PKCε plays a crucial role in activating Ras/MAP kinase signalling through the formation of a signalling complex containing PKCε, B-Raf and S6K2, and sequentially up-regulates anti-apoptotic proteins, such as X-linked inhibitor of apoptosis protein (XIAP) and Bcl-XL [183, 317].

However, while these studies clearly show that PKC exerts the regulatory functions through downstream targets within the Ras/MAP kinase pathway, the findings presented in this thesis as well as those published by Lonic et. al suggest a novel upstream receptor-proximal role of PKCε in regulating Ras/MAP kinase pathway through phosphorylation of S779. We show that PKCε directly phosphorylates S779 in vitro. In addition, PKC activation (PMA) or PKCε over-expression increases S779 phosphorylation [242]. Furthermore, PKC inhibition by GF109203X or PKCε siRNA-mediated knockdown blocks S779 phosphorylation [242]. Importantly, the S779 phosphorylation mediated by PKCε is crucial for the maximal activation of Ras/MAP kinase signalling and neuronal differentiation of PC12 (Chapter 2). Over-expression of PKCε has been shown to enhance PC12 differentiation in response to NGF and FGF [318], while our previous studies have identified conserved putative phosphoserine 14-3-3 binding sites in the C-terminal tails of members of the NGF and FGF receptor families [191], raising the possibility that PKC may phosphorylate other growth factor receptors to promote Ras/MAP kinase signalling.

PKCε signalling has been suggested to be a therapeutic target for enhancing neural regeneration after CNS injuries [162]. PKCε has also been proposed to play a key role
in neurogenesis through the regulation of Ras/MAPK signalling [319, 320]. For example, the pharmacological activation of PKC has been shown the therapeutic effects in a rat model of ischaemic stroke [321]. Thus, PKCε may play an important role in neural regeneration, not only through its ability to regulate canonical downstream Ras/MAP kinase targets, but also through its ability to phosphorylate growth factor receptors such as FGFR and promote neuronal survival and differentiation. Collectively, our findings suggest that PKCε mediates the maximal activation of Ras/MAP kinase signalling through S779 phosphorylation.

4.3. Shortcomings and further research

While the current study has demonstrated that S779 in FGFR2 is able to activate Ras/MAP kinase pathway and is important for neuronal differentiation in cell lines and primary cells, the relevant signalling phenomena and mechanism have not been investigated in vivo. Future studies will be important to determine whether S779 in FGFR2 can regulate Ras/MAP kinase and neuronal differentiation within in vivo physiological settings.

Furthermore, the detailed mechanisms by which phospho-S779 couples to downstream pathways are still unclear. We could not determine whether the 14-3-3 proteins are also able to physically associate with phospho-S779 in response to ligands in PC12 cells. Pulldown experiments using recombinant GST-14-3-3 failed to selectively precipitate FGFR2. The 14-3-3 proteins have been shown to interact with over 200 proteins in diverse cell types [199]. Application of more suitable lysis buffer may resolve the non-specific binding of GST-14-3-3 to both the wt-FGFR2 and the FGFR2-S779G mutant. In co-immunoprecipitation experiments, the association
between endogenous 14-3-3 and FGFR2 proteins could not be detected. However, given that we have only tested endogenous 14-3-3ζ, we cannot exclude the possibility of other 14-3-3 isoforms being involved. Therefore, more 14-3-3 isoforms could be examined in future studies.

While our results show that PKCε is responsible for phosphorylating S779 of FGFR2, the mechanism by which PKCε is recruited to FGFR2 remains unclear. PKCε contains the C2 phosphotyrosine binding domain, which might facilitate its recruitment to phosphotyrosine docking sites either on FGFR or associated scaffold proteins to mediate S779 phosphorylation [322]. Furthermore, the activation PKCε requires DAG but not calcium [159]. The FGFR tyrosine phosphorylation actives PLCγ and lead to the DAG production [42], which is crucial for PKCε activation and S779 phosphorylation. Future work examining how phosphoserine and phosphotyrosine signalling events intersect at the level of FGFRs will be important to have a better understanding of how multiple pathways are integrated to allow specificity in signalling and cellular responses.

4.4. **Significance**

The conclusions and knowledge drawn from presented studies address fundamental questions regarding growth factor function and pleiotropy, and therefore have the potential to impact on diverse fields of biology, including normal physiological processes, such as CNS development and maintenance, as well as neuronal pathological diseases, such as stroke, brain injury, and Parkinson disease.
The growth factor and cytokine receptors exert their diverse cellular functions through activating a limited repertoire of overlapping pathways [323]. The duration or strength of signals generated downstream of cell surface receptors has been proposed to explain how independent and distinct cellular responses can arise from the activation of common (or redundant) downstream pathways. Through either negative or positive feedback loops, the Raf-1-MEK-ERK module is wired in such a way that it can generate signalling outputs across a range of amplitudes and kinetics depending on the specific signalling inputs from upstream receptors [324]. In addition to the prototypic example of sustained-versus-transient ERK activation regulating distinct and independent outcomes in PC12 cells [221], many other examples in which quantitative differences in ERK signalling lead to qualitative differences in cellular responses have been reported [325, 326]. Thus, growth factor receptors can provide instructive signals that determine whether downstream signalling is sustained or transient thereby dictating cellular outcomes. The ability to provide such instructive signals would imply that at least some growth factor receptors may have unique intrinsic features that allow them to generate specific signals and biological responses.

The individual receptor tyrosine residues have been proposed to couple to specific signalling pathways and thereby regulate specific biological responses [39]. Although there are clear instances such as c-Kit and Met receptors where individual growth factor receptor tyrosine residues have been functionally linked to specific cellular responses [230, 231], the overall picture emerging is significant redundancy in receptor tyrosine phosphorylation [39, 232]. In fact, redundancy in receptor tyrosine phosphorylation has been observed for a range of growth factor and cytokine receptors, including the FGFRs and it has been difficult to functionally link individual
receptor tyrosine residues are difficult to be functionally linked to the regulation of specific cellular responses [308, 327]. Our findings now demonstrate that in addition to tyrosine phosphorylation, growth factor receptor serine phosphorylation can also provide specificity in signalling to control pleiotropic biological responses.

FGFs play multiple roles in CNS development and adulthood maintain. These roles include the promotion of neurogenesis, the enhancement of axon growth, and the improvement of differentiation. Although generic signalling pathways initiated by FGFRs have been identified, the detailed molecular mechanisms of those pathways in regulating neuronal cell differentiation remain unclear. Our results show that PKCe-mediated phosphorylation of S779 in FGFR2 is critical for regulating the intracellular signalling events necessary for maximal Ras/MAP kinase signalling and neuronal differentiation. Considering that the enhancement of neuronal differentiation is one of the major tasks in treating CNS disorders, our study will significantly contribute to the development of therapeutic strategies for treating these diseases. For instance, administration of small molecule activators for Ras/MAP kinase and PKCs would mimic and enhance FGF functions in improving neuronal differentiation with extra advantages, such as improved penetration and less immune rejection responses. Our studies will also assist the implication of stem cell based therapies to treat neuronal degenerative diseases, such as strokes, Parkinson disease and brain trauma.

4.5. Conclusion

The findings presented in this thesis demonstrate that in addition to FGFR tyrosine phosphorylation, the phosphorylation of S779, a conserved serine residue in cytoplasmic domains of FGFR2, can quantitatively and selectively control Ras/MAP
kinase signalling to promote PC12 cells and BMSCs neuronal differentiation (Figure 4-1).

The FGFRs have been shown to regulate diverse (or pleiotropic) cellular responses, such as cell survival, proliferation, differentiation, and migration, in both development and adulthood. The activation of FGFRs leads to FGFR tyrosine phosphorylation and the recruitment of SH2/PTB-domain signalling proteins, which is essential to initiate the downstream signalling pathways. Although receptor tyrosine phosphorylation is known to be important for cellular responses mediated by FGFRs, we have previously shown that S779 in cytoplasmic domains of FGFR2 is also phosphorylated in response to ligand and binds the 14-3-3 family of phosphoserine/threonine-binding adaptor/scaffold proteins. The recruitment of the 14-3-3 proteins is essential for the full activation of the PI3 kinase and Ras/MAP kinase pathways, which sequentially regulates cell survival and proliferation in both Ba/F3 cells and BALB/c 3T3 fibroblasts [191].

Using PC12 cells and primary mouse BMSCs as models for growth factor-regulated neuronal differentiation, we show that S779 in the cytoplasmic domains of FGFR2 is required for cell neuronal differentiation. Results indicate that S779 is subjected to phosphorylation in response to FGFs stimulation. We further show phospho-S779 signalling is selective required for the maximal activation of Ras and ERK but not for other FGFR phosphotyrosine pathways, which is critical for cell neuronal differentiation mediated by FGFs. Further studies from our laboratory show that PKCε regulates the maximal Ras/MAP kinase signalling through directly S779 phosphorylation. Collectively, we are able to demonstrate that the PKCε mediated
S779 phosphorylation of FGFRs regulates maximal Ras/MAP kinase signalling to mediate neuronal differentiation [242].

Our findings indicate that the growth factor receptor serine phosphorylation can also regulate specific signalling pathways and biological responses. Therefore, we propose that serine residues in growth factor receptors may serve as the novel signalling devices to regulate pleiotropic cellular responses. Our studies have provided evidences to establish a new mechanistic basis to interpret how different biological outcomes are determined, which may provide an important conceptual advance over current paradigms used to explain pleiotropy.
Figure 4-1 Phosphorylation S779 mediated by PKCɛ mediates neuronal differentiation by regulating Ras/MAP kinase signalling

The diagram has been used to summarise the key findings of current studies. Using PC12 cell lines and primary mouse BMSCs as models, we show that S779 is subjected to phosphorylation with stimulation of FGFs. The phosphorylation of S779 is selectively required for maximal activation of Ras/MAP kinase pathways, which is crucial for the neuronal differentiation mediated by FGFs. We further show PKCɛ is responsible for phosphorylation of S779 in response to FGFs. Collectively, we demonstrate that phosphorylation of S779 mediated by PKCɛ selectively and quantitatively regulates Ras/MAP kinase pathway to mediate neuronal differentiatio
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Appendix: Copy of publications
Signal Transduction:
Phosphorylation of Serine 779 in Fibroblast Growth Factor Receptor 1 and 2 by Protein Kinase C\textsuperscript{\textregistered} Regulates Ras/Mitogen-activated Protein Kinase Signaling and Neuronal Differentiation

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doi: 10.1074/jbc.M112.421669 originally published online April 5, 2013

Access the most updated version of this article at doi: 10.1074/jbc.M112.421669

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