The Pennsylvania State University

The Graduate School

College of Medicine

MECHANISMS OF IGF-1 REGULATION OF S AND G2/M CELL CYCLE PHASES IN OLIGODENDROCYTE PROGENITOR (OP) CELLS

A Dissertation in

Molecular Medicine

by

Jungsoo Min

© 2009 Jungsoo Min

Submitted in Partial Fulfillment
of the Requirements
for the Degree of

Doctor of Philosophy

December 2009

The dissertation of Jungsoo Min was reviewed and approved* by the following:

Teresa L. Wood
Adjunct Professor of Neural and Behavioral Sciences
Thesis Advisor
Co-Chair of Committee

Craig Meyers
Professor of Microbiology and Immunology
Co-Chair of Committee

Jong K. Yun
Assistant Professor of Pharmacology

James M. Hammond
Distinguished Professor of Medicine, Emeritus

Steven W. Levison
Adjunct Professor of Neural and Behavioral Sciences

Charles H. Lang
Distinguished Professor of Cellular and Molecular Physiology, and Surgery
Co-Chair of Intercollege Graduate Degree Program in Molecular Medicine

*Signatures are on file in the Graduate School

ABSTRACT

Oligodendrocytes are macroglial cells in the central nervous system (CNS), which myelinate and provide electric insulation for the axons of nerve cells. Oligodendrocyte progenitor (OP) cells generated in the developing neural tube differentiate into oligodendrocytes capable of producing myelin. OP cells undergo extensive proliferation before they become post-mitotic, mature oligodendrocytes. Intrinsic and extrinsic factors influence OP cells to decide whether to continue cell cycle progression or exit the cell cycle and terminally differentiate. Growth factors are extrinsic signals involved in developmental regulation of OPs. Some growth factors act independently and other growth factors act coordinately to exert maximal effects. Previously, our laboratory reported that insulin like growth factor-1 (IGF-1), which itself is a weak mitogen in oligodendrocyte lineage cells, synergizes with fibroblast growth factor-2 (FGF-2) for G1 cell cycle progression and S phase entry, in part, by inducing cyclin D1 and enhancing activity of cdk2.

Here, we report that the combination of IGF-1 with FGF-2 accelerates S phase and promotes S phase progression. Particularly, IGF-1/FGF-2 enhances mRNA and protein expression of cyclin A and cdk2, the predominant regulators of S phase. Further, the growth factor combination increases effective complex formation in IGF-1/FGF-2 resulting in enhanced cdk2 activity. We further studied the signaling mechanism by which IGF-1 and FGF-2 synergize to exert their coordinated effects on cell cycle progression, specifically in S phase. Using specific inhibitors for MAP kinase and PI3 kinase, we determined that both pathways are necessary for expression of S phase proteins and mRNA and for phosphorylation of retinoblastoma protein (pRb), a target of cdk2. However, the effects of each pathway on S phase targets were distinct.

We also demonstrate that IGF-1 plays a critical role in G2/M progression. OP cells exposed to FGF-2 alone fail to progress through G2/M. In contrast, the combination of IGF-1 with FGF-2 promotes progression through G2/M. Protein expression of cyclin B and cdk1 increases and cyclin B/cdk1 complex formation increases with concomitant increase in cdk1 kinase activity in the combination growth factor treatment. Nuclear

localization of cyclin B and cdk1, one parameter for active cdk1, was enhanced in IGF-1/FGF-2 treated cells as well as the relative amount of cdc25C, an activator of cdk1. Using mechanism to arrest cells in S phase or with cells treated with FGF-2 alone into S phase, we demonstrated that IGF-1 by itself promotes G2/M progression. Overall, we have determined that IGF-1 plays an important role in cell cycle progression of OP cells, and furthermore, it is an essential factor for G2/M progression.

TABLE OF CONTENTS

LIST OF FIGURES viii

LIST OF ABBREVIATIONS x

ACKNOWLEDGEMENTS xiii

Chapter 1 Introduction 1

1.1 Oligodendrogenesis
1.1.1 The origin of oligodendrocyte progenitor cells 2
1.1.2 Oligodendrocyte progenitor specification and regulatory signals 5
1.1.3 Proliferation and migration of oligodendrocyte progenitor cells 7
1.1.4 Oligodendrocyte lineage progression and differentiation 9
1.1.5 Oligodendrocyte precursors in adult CNS and remyelination 14
1.2 Oligodendrocyte development and growth factors 16
1.2.1 Fibroblast growth factor 17
1.2.2 Insulin like growth factor 24
1.3 Cell cycle regulation 29
1.3.1 Overview of cell cycle 29
1.3.2 Retinoblastoma protein and cell cycle regulation 34
1.3.3 S phase progression and major regulators 35
1.3.4 G2/M transition and major regulators 39
1.4 Cell cycle studies in oligodendrocyte progenitor cells 45
1.5 Contribution of IGF-1 in cell cycle 47
1.6 FGF-2 and IGF-1 signaling pathway 50
1.6.1 Signaling pathway mediated by FGF-2 in oligodendrocyte progenitor cells 50
1.6.2 Signaling pathway mediated by IGF-1 in oligodendrocyte progenitor cells 55
1.7 Statement of thesis 60

Chapter 2 Materials and methods 62

2.1 Material 62
2.2 Primary oligodendrocyte progenitor cell culture 62
2.3 Growth factor treatment 63
2.4 Thymidine double block 64
2.5 Propidium iodide labeling for flow cytometry 64
2.6 Western blot analysis and immunoprecipitation 64
2.7 Kinase assay 65

2.8 Nuclear fractionation 66
2.9 Immunohistochemistry 66
2.10 RNA isolation and Real time Polymerase Chain Reaction (PCR) 67
2.11 Statistical analyses 67

Chapter 3 IGF-1 coordinates with FGF-2 to promote S phase progression of cell cycle in OP cells 69

3.1 Introduction 69
3.2 Results 71
3.2.1 IGF-1 accelerates S phase entry of OP cells in coordination with FGF-2 71
3.2.2 IGF-1 enhances protein expression of cyclin A and cdk2 in the presence of FGF-2 75
3.2.3 IGF-1 promotes complex formation of cyclin A and cdk2 78
3.2.4 Cdk2 kinase activity enhances in combination of IGF-1 and FGF-2 81
3.2.5 mRNA expression of cycin E and cyclin A is regulated by both PI3K pathway and ERK pathway 84
3.2.6 Protein expression of cyclin E and cyclin A is downregulated by MAPK pathway and PI3K pathway inhibition 87
3.2.7 Initial G1 events regulate expression of S phase cyclin/cdks, but maximal phosphorylation of pRb requires an additional stimulus 93
3.2.8 Effects of blocking IGF-1R signaling 96
3.3 Discussion 100

Chapter 4 IGF-1 promotes G2/M transition in OP cell cycle 104

4.1 Introduction 104
4.2 Results 107
4.2.1 FGF-2 treated OP cells fail to progress to G2/M, but IGF-1/FGF-2 promotes G2/M phase progression 107
4.2.2 Cyclin B and cdk1 expression are upregulated in IGF1/FGF-2 treated OP cells 111
4.2.3 Enhanced complex formation between cyclin B and cdk1 in IGF-1/FGF-2 conditions leads to higher cdk1 activity 114
4.2.4 IGF-1/FGF-2 increases nuclear localization of cyclin B, cdk1 and cdc25c 117
4.2.5 IGF-1 alone promotes G2/M phase progression 120
4.2.6 IGF-1 increases phosphorylated histone 3, a mitotic marker 123
4.2.7 IGF-1R blocking antibody increased cdk1 activity and cyclin B1 expression 126
4.2.8 Blocking of both MAPK and PI3K downregulate protein expression of cyclin B, cdk1 and Wee1, but had no effect on cdk1 inhibition 130

4.3 Discussion 133

Chapter 5 Final discussion 138

5.1 Summary of results 138
5.2 Summary Model of the combination of growth factors on cell cycle progression in OP cells 143
5.3 Cross talk between two different signaling pathways and cellular responses 146
5.4 The role of IGF-1 on proliferation in OP cells 149
5.5 Implication for a mitotic role of IGF-1 in Multiple Sclerosis 151
5.6 Conclusions 153

Bibliography 154

LIST OF FIGURES

Figure **1.1**: Lineage progression of oligodendrocytes with cell surface antigens........ 12

Figure **1.2**: The expression pattern of FGFR at oligodendrocyte lineage progression.. 22

Figure **1.3**: Regulation of cell cycle progression**.** 32

Figure **1.4**: Subcellular localization of G2/M proteins and regulation**.** 43

Figure **1.5**: The signaling pathway of FGFR**.** 53

Figure **1.6**: The signaling pathway of IGFR**.** 58

Figure **3.1**: Flow cytometry analysis of OP cells... 73

Figure **3.2**: Protein expression levels of cyclin A and cdk2 in OP cells...................... 76

Figure **3.3**: Effective complex formation of cyclin A with cdk2................................ 79

Figure **3.4**: Cdk2 kinase assay and cyclin A associated kinase activity 82

Figure **3.5**: Real time PCR analysis of cyclin E and cyclin A mRNA levels in the presence of specific inhibitors for the MAP kinase and PI3 kinase pathway**.**...... 85

Figure **3.6**: Protein expression levels of cyclin E and cyclin A, and phosphorylation of retinoblastoma protein (pRb) in the presence of a MAP kinase pathway inhibitor.. 89

Figure **3.7**: Protein expression levels of cyclin E and cyclin A, and phosphorylation of retinoblastoma protein (pRb) in the presence of a PI3 kinase pathway inhibitor.. 91

Figure **3.8**: Protein expression levels of cyclin A and cdk2, and phosphorylation of retinoblastoma protein (pRb) after 4 h pretreatment with full growth media**.**.. 94

Figure **3.9**: Cyclin A associated kinase assay, protein expression of cyclin A and cdk2, and activation of signaling proteins in the presence of IGF-1R blocking antibody 97

Figure **4.1**: Flow cytometry analysis of OP cells**.**.. 109

Figure **4.2**: Protein expression levels of cyclin B and cdk1 in OP cells**.** 112

Figure **4.3**: Effective complex formation of cyclin B with cdk1 and cyclin B associated kinase assay 115

Figure **4.4**: Subcellular localization of cyclin B and cdk1. 118

Figure **4.5**: Cell distribution after double thymidine block. 121

Figure **4.6**: Detection of phosphorylated histone 3. 124

Figure **4.7**: Cdk1 kinase assay and protein expression of cdk1, cyclin B with A12 antibodies. 127

Figure **4.8**: Protein expression level of cyclin B and cdk1, and Wee1 in the presence of signaling pathway inhibitors. 131

Figure **5.1**: Overall cell cycle regulation in OP cells 141

LIST OF ABBREVIATIONS

APC – anaphase promoting complex

bHLH –basic helix loop helix

BMP – bone morphogenic protein

CDC – cell division cycle

CDK – cyclin-dependent kinase

CDKI – cdk inhibitor

CIP/KIP – cdk interacting protein/ kinase interacting protein

CNPase – 2’3’-cyclic nucleotide 3’-phosphatase

CNS – central nervous system

CNTF – cilliary neurotrophic factor

CRS - cytoplasm retention signal

E1A – early region 1A

E2 – 17 β estradiol

EAE – experimental autoimmune encephalomyelitis

EGF – epidermal growth factor

ERK – extracellular signal regulated kinase

FGF –fibroblast growth factor

FGFR – FGF receptor

FRS – FGF receptor substrate

GalC - galactosylceramidase

GFP – green fluorescent protein

GM-CSF – granulocyte macrophage colony stimulating factor

Grb – growth factor receptor bound protein

HDAC – histone deacetylase

HSPG – heparin sulfacte proteoglycan

IGF – insulin like growth factor

IGFR – IGF receptor

IGFBP – IGF binding protein

IR – insulin receptor

IRS – insulin receptor substrate

LGE – lateral ganglionic eminence

MAG – myelin associated glycoprotein

MAPK – mitogen activated protein kinase

MBP – myelin basic protein

MCM - minichromosome maintenance

MGE - medial ganglionic eminence

MOG – myelin oligodendrocyte glycoprotein

MPF – mitosis promoting factor

MS – multiple sclerosis

mTOR – mammalian target of rapamycin

Myt1 - myelin transcription factor 1

O-2A – oligodendrocyte- type-2 astrocyte

OL - oligodendrocyte

OP – oligodendrocyte progenitor

ORC – origin recognition complex

PCNA – proliferating cell nuclear antigen

PDGF – platelet derived growth factor

PDGFR – PDGF receptor

PH – pleckstrin homology

PKA – protein kinase A

PLC – phospholipase C

PLP – proteolipid protein

POA – proligodendroblast antigen

PTB – phosphoprotein binding

RA – retinoic acid

pRb – retinoblastoma protein

SCF – stme cell factor

SEM – standard error of mean

SH – Shc homology

Shh – sonic hedge hog

Sos – son of sevenless

Sox - SRY-box containing

SVZ – subventricular zone

TGF – transforming growth factor

TH – thyroid hormone

TNF – tumor necrosis factor

ACKNOWLEDGEMENTS

My graduate school years have been a great challenge in both my academic and personal life. I have learnt that being a scientist requires tremendous patience and hard work, as well as a thoughtful mind. Living in a different country during my graduate studies has also provided a good opportunity to grow as an individual.

First, I want to thank my thesis adviser, Dr. Teresa Wood. She was the director of the Molecular Medicine program at Penn State when I applied and was the person that interviewed me on the phone. I think my long journey with her might begin from that moment. Since I have joined her lab, she has always inspired me and showed me great enthusiasm toward research. She was patient and considerate and understood the difficulties that I faced as an international student. I really appreciate her support, patience and guidance through my graduate studies. To me, Dr. Wood is an example of a passionate scientist.

Next, I want to thank all my committee members, Dr. Levison, Dr. Meyers, Dr. Yun and Dr. Hammond. They have critically evaluated and given me good comments on my work and directed me onto the right path. They encouraged and lead me to become a thoughtful scientist.

During my time in the graduate school, I have met great people in the lab and at school. Particularly, I am thankful to past and present members of the Wood's lab. They are good colleagues as well as good friends. They made the lab an enjoyable place to work. In addition, I could not have enjoyed my graduate school without friends through whom I have learnt a lot besides research.

Lastly, I want to give special thanks to my family. They have always support me even though they were never physically with me. Especially, my dad, he wanted to see me graduate, and I am sure he has been watching me up there. My mom, who understands and knows me more than myself, encouraged and made me see straight and go through tough moments. I extend a great thank to my sisters and brother for their unconditional love and encouragement.

Chapter 1

Introduction

1.1 Oligodendrogenesis

Oligodendrocytes, which produce myelin, are one type of macroglia cells in the central nervous system (CNS). The myelin sheath is a modified plasma membrane that wraps axons and promotes rapid conduction of electrical impulses (Bunge 1968). A single oligodendrocyte can myelinate up to 60 nerve axons (Butt and Ransom 1989) while a Schwann cell, the myelinating cell of the peripheral nervous system, myelinates one axon. Myelination provides normal nerve functioning in the mature CNS. Thus, the loss of an oligodendrocyte can cause neurological dysfunctions over a broad area (Waxman 1992).

The different neural cell types are generated at different developmental stages such that neurons arise first, followed by astrocytes and oligodendrocytes (Altman and Bayer 1984; Miller 2002). CNS myelnation occurs postnatally in most vertebrates. Due to its critical role for normal neuronal functions, the correct number of oligodendrocytes needs to be generated to match the number of axons to be myelinated. To ensure a sufficient number of oligodendrocytes for proper myelination,

a well-organized process regulates the generation and maturation of oligodendrocytes. The initial step in this process is the induction of oligodendrocyte progenitor (OP) cells, which arise from neuroepithelial cells in restricted regions in the CNS. OP cells proliferate and migrate to their final destination throughout the CNS. As they reach sufficient numbers and make contact with axons, OP cells differentiate to mature oligodendrocytes capable of myelination. These events result from coordination of intrinsic signals and extrinsic signals from the environment. This section will review the molecular mechanisms by which OP cells are generated and finally differentiate to myelin-producing mature oligodendrocytes.

1.1.1 The origin of oligodendrocyte progenitor cells

It has been a long debate to define the origin of neural precursor cells that give rise to OP cells. Oligodendrocyte precursor cells arise in the epithelium of the neural tube (Warf, Fok-Seang et al. 1991). Several lines of studies have suggested that the precursor cells originate in the restricted sites of the ventral neural tube. However, recent studies reported that precursor cells are produced also in the dorsal region (Richardson, Kessaris et al. 2006).

In early studies in the optic nerve and spinal cord where oligodendrocytes are enriched, investigators reported that oligodendrocyte precursor cells arise from restricted regions of the CNS. A study with the chick optic nerve demonstrated that oligodendrocyte precursors initially appear in a discrete region at the ventral midline of third ventricle and migrate into the chiasma and finally are distributed uniformly (Ono, Yasui et al. 1997). In addition, multiple groups have found oligodendrocyte precursors in the ventral ventricular zone in the spinal cord and observed that the

dorsal spinal cord is populated by oligodendrocyte precursors migrating from the ventral side (Warf, Fok-Seang et al. 1991; Ono, Bansal et al. 1995; Pringle, Guthrie et al. 1998). The ventral ventricular source of oligodendrocyte precursors in the spinal cord is a common feature of vertebrate development from *Xenopus* (Maier and Miller 1997) to chick, mouse, and rat (Warf, Fok-Seang et al. 1991; Pringle and Richardson 1993; Ono, Bansal et al. 1995). However, oligodendrocyte precursors derived from the dorsal region in the spinal cord also were reported (Cameron-Curry and Le Douarin 1995). Taken together, it is generally believed that 85% of oligodendrocyte precursor cells are derived from ventral regions and dorsal derived oligodendrocyte precursor cells comprise the remaining 15% of the total precursor population in the spinal cord.

The spinal cord is not the only region in which ventrally derived oligodendrocytes are found. Similarities in basic structure between the spinal cord and metencephalon lead to investigation of oligodendrocyte development in the metencephalon. These studies demonstrated a ventral-to-dorsal development of oligodendrocyte precursors (Ono, Fujisawa et al. 1997). However, Spassky and his colleagues reported a diverse source of oligodendrocyte precursor cells rather than only a ventral source, using two different markers (Spassky, Goujet-Zalc et al. 1998). Interestingly, another group observed that oligodendrocyte precursors are derived independently in lateral and dorsal regions, and an explant culture study suggested that intrinsic factors from the dorsal region could induce oligodendrogenesis (Davies and Miller 2001).

Oligodendrocyte development in the telencephalon also shows a diverse pattern. Oligodendrocyte precursors that populate the telencephalon arise in the medial ganglionic eminence (MGE) (He, Ingraham et al. 2001; Tekki-Kessaris,

Woodruff et al. 2001) and lateral ganglionic eminence (LGE) (He, Ingraham et al. 2001), followed by migration to the developing forebrain (He, Ingraham et al. 2001; Tekki-Kessaris, Woodruff et al. 2001). In another study, it was concluded that all oligodendrocyte precursors in the chick originate from the ventral telencephalon (anterior entopeduncular area) (Olivier, Cobos et al. 2001). Additionally, there is another view of oligodendrogenesis in cortex. Using retroviral lineage tracing, it was revealed that oligodendrocyte precursors arise postnatally from the subventricular zone (SVZ) (LeVine and Goldman 1988; Levison and Goldman 1997).

Recent studies finally have provided conclusive findings that resolve the issues on the origin of oligodendrocyte precursor cells. Ivanova et al provided evidence that there are two different origins of oligodendrocyte precursors in the cerebral cortex. The “First wave” of oligodendrocyte precursors originates from the MGE in SVZ and populate cortical gray matter while the “second wave” of oligodendrocyte precursors is generated postnatally from cortical SVZ (Ivanova, Nakahira et al. 2003). This study showed the temporal development of oligodendrocyte precursors and linked the two different views of oligodendrocyte precursor origin, ventral regions and SVZ. Subsequently, other studies claimed that there are other origins of oligodendrocyte precursor cells in the spinal cord and telencephalon (Cai, Qi et al. 2005; Fogarty, Richardson et al. 2005; Vallstedt, Klos et al. 2005). The previous idea in early studies supporting a ventral source of oligodendrocyte precursor cells is supplemented by the recent finding that dorsally and locally derived oligodendrocyte precursor cells appear in later developmental periods, which suggests that oligodendrogenesis is regulated in both a spatial and temporal manner. This view is well summarized in a paper published in 2006 as

follows: in the spinal cord, ventral origin oligodendrocyte precursors arise first and dorsal region derived oligodendrocyte precursors follow (Richardson, Kessaris et al. 2006). In the telencephalon (cortex), the first oligodendrocyte precursors are produced from the MGE and the second wave are generated from the LGE, followed by cortex derived precursors after birth (Richardson, Kessaris et al. 2006). The next section will discuss how oligodendrocyte development is temporally and spatially regulated.

1.1.2 Oligodendrocyte progenitor specification and regulatory signals

Oligodendrocyte development in the spinal cord and telencephalon shows a similar pattern as described above. Moreover, induction of early oligodendrocyte precursors in ventral regions in both areas suggest that restricted emergence of oligodendrocyte precursors reflects local influences. Several lines of evidence show that notochord explants are sufficient to induce oligodendrocyte precursors in dorsal spinal cord (Trousse, Giess et al. 1995; Pringle, Yu et al. 1996). The notochord is located ventral to the neural tube (van Straaten, Hekking et al. 1989) and produces a signaling molecule, sonic hedgehog (Shh) (Echelard, Epstein et al. 1993). Shh is the vertebrate homologue of the *Drosophila* pattern forming gene *hedgehog* (Roelink, Augsburger et al. 1994). Shh is secreted from the notochord and floor plate (ventral regions) and gradually diffuses to dorsal regions. The relationship between Shh and induction of oligodendrocyte precursors has been extensively studied in vivo and in vitro in a broad range of species (Pringle, Yu et al. 1996; Maier and Miller 1997; Orentas, Hayes et al. 1999; Nery, Wichterle et al. 2001; Loulier, Ruat et al. 2006). However, oligodendrocyte development occurs in Shh knock out mice (Nery, Wichterle et al. 2001) implying that there is a Shh independent pathway for

oligodendrogenesis specification. It is likely that other hedgehog family members can substitute for the absence of Shh since inhibition of all hedgehog family members blocks oligodendrocyte development (Tekki-Kessaris, Woodruff et al. 2001).

In vitro studies revealed that negative regulators of oligodendrocyte development also exist. Exposure of bone morphogenetic proteins (BMPs), a member of transforming growth factor (TGF) β family, to either oligodendrocyte-type-2 astrocyte (O-2A) progenitor cells (Mabie, Mehler et al. 1997) or perinatal progenitor cells (Mehler, Mabie et al. 2000) induces astrocyte lineage specification and suppresses oligodendrocyte commitment. In addition, explant culture experiments suggested that inhibitory factors other than BMP from dorsal spinal cords negatively control oligodendrocyte development (Wada, Kagawa et al. 2000).

Transcriptional regulation controls oligodendrocyte specification in the developing CNS at another level. Transcription factors involved in oligodendrocyte lineage specification include basic helix-loop-helix (bHLH) family members Olig1 and Olig2, SRY-box containing (Sox) family members, Sox 8, Sox 9, Sox 10, and Sox 17, homeobox transcription factors Nkk2.2 and Nkk 6 and the paired box gene 7 (Nicolay, Doucette et al. 2007).

Olig1 and Olig2 are the earliest known markers in oligodendrocyte specification (Zhou, Choi et al. 2001). Olig 1 is important for specification in certain areas in the brain (Lu, Sun et al. 2002; Zhou and Anderson 2002), and overexpression of Olig 1 increases OP cells (Lu, Yuk et al. 2000; Alberta, Park et al. 2001). Olig 2 is expressed throughout oligodendrocyte development and is required for oligodendrocotye and motor neuron specification in the spinal cord (Lu, Sun et al. 2002) and for cell maturation (Fu, Qi et al. 2002). Expression of Olig2 is induced by

Shh in motor neuron precursor domain (pMN) in the spinal cord, and Olig2 itself regulates platelet derive growth factor receptor PDGFR- α expression. Nkk2.2 is induced by Shh in a concentration dependent manner (Ericson, Rashbass et al. 1997). Expression of Nkk2.2 spreads dorsally and overlaps with expression of Olig2 in the spinal cord, which allows precursor cells to develop into oligodendrocyte progenitors in the spinal cord (Zhou, Choi et al. 2001). Nkk6 transcription factor expression also is under the control of Shh and in turn regulates expression of Olig2 (Cai, Qi et al. 2005; Vallstedt, Klos et al. 2005). Sox9 plays a role to switch lineage specification from neurogenesis to gliogenesis (Nicolay, Doucette et al. 2007). Sox9 ablation causes the initial reduction of OP cells although it has no effect on other transcription factors such as Olig2 and Nkk2.2, suggesting compensation by other Sox family members (Stolt, Lommes et al. 2003). Finally, one zinc finger DNA binding protein, myelin transcription factor 1 (Myt1) is also expressed in OP cells and promotes oligodendrocyte maturation (Armstrong, Kim et al. 1995).

1.1.3 Proliferation and migration of oligodendrocyte progenitor cells

Once OP cells are specified in the ventricular zone or SVZ, the progenitor cells undergo proliferation. Although OP cells start to proliferate in these areas, more extensive proliferation begins after cells migrate out to their final destination (Miller, Payne et al. 1997). Many types of growth factors are involved in the regulation of OP cell proliferation, which will be reviewed in section 1.2. The balance between intrinsic cues and extrinsic cues has been of considerable interest in regards to the fine control of OP cell division. Studies with O-2A cells demonstrated that the number of cell divisions is limited even in the presence of the mitogen, platelet derive growth factor

(PDGF) and suggested that an intrinsic clock controls the number of cell divisions required for final differentiation (Temple and Raff 1986; Raff, Lillien et al. 1988). However a paper published in 2001 suggested an alternative idea about intrinsic regulation of cell proliferation, reporting that progenitor cell proliferation depended on the environmental factor, PDGF, rather than on an intrinsic factor (van Heyningen, Calver et al. 2001). Since Raff et al used progenitor cells from 7 day old optic nerve and van Heyningen et al used progenitor cells from E17 spinal cord, the different stages of progenitor cells may respond to PDGF with different affinity. Moreover, Temple et al used type 1 astrocyte conditioned media as a PDGF source but the concentration was not determined whereas PDGF was overexpressed in van Heyningen's study. Thus, it may be that PDGF availability plays an important role as well. Taken together, both intrinsic and extrinsic cues seem to regulate differentiation timing in accord, and cell division numbers depend on the progenitor cell developmental stage. Mature oligodendrocytes are widely distributed and oligodendrocyte precursors arise predominantly in restricted foci in the CNS. Thus, oligodendrocyte precursor cells migrate widely to populate the remaining areas in the CNS. Transplantation of optic bulb segments is sufficient to induce myelination in shiverer mice (Lachapelle, Gumpel et al. 1983), which are autosomal recessive mutant mice and unable to produce compact myelin (Chernoff 1981). In addition, transplantation of early progenitor cells into shiverer mice also showed that OP cells migrate a long distance, differentiate into mature oligodendrocytes and finally produce myelin sheaths (Chernoff 1981; Warrington, Barbarese et al. 1992; Warrington, Barbarese et al. 1993). Taken together, the capacity of OP cells to migrate is critical for a wide range of normal myelination in the vertebrate CNS.

The molecular mechanisms mediating OP migration are not yet fully understood. Axons, adhesion molecules and extracellular matrix receptor, and integrin receptors have been proposed as substrates for promoting OP migration (Miller 2002). Specific directional signals (chemoattractants and chemorepellents) from local environments likely mediate the migration of OPs. One of the important mitogens in oligodendrocyte lineage cells, PDGF is considered a candidate for chemoattractants. PDGF promotes the migration of O-2A progenitor cells toward the area where it is present, which is independent of its growth stimulating function (Armstrong, Harvath et al. 1990). In the optic nerve, netrin 1 and Sema3a act as chemorepellents to direct NG2 positive glial precursors away from the chiasma (Sugimoto, Taniguchi et al. 2001). Data from another study in the spinal cord supported the conclusion that netrin 1 is a chemorepellent and even antagonizes the chemoattractant role of PDGF (Tsai, Tessier-Lavigne et al. 2003). However, analysis in other CNS regions revealed differences from the optic nerve. Migration of progenitor cells from the SVZ was visualized using green fluorescence protein (GFP). These cells were observed to migrate in a unidirectional fashion in the SVZ, but once progenitor cells moved into white matter, cortex and striatum, the cells migrated radially and tangentially (Kakita and Goldman 1999). These results suggest that each region in the CNS utilizes different mechanisms or guiding cue to direct OP cell migration.

1.1.4 Oligodendrocyte lineage progression and differentiation

OP cells progress to mature oligodendrocytes, undergoing 4 distinct stages, which express specific cell surface markers and show differential responses to growth

factors. Figure 1.1 illustrates lineage progression of oligodendroglia along with important cell surface markers and regulation by growth factors.

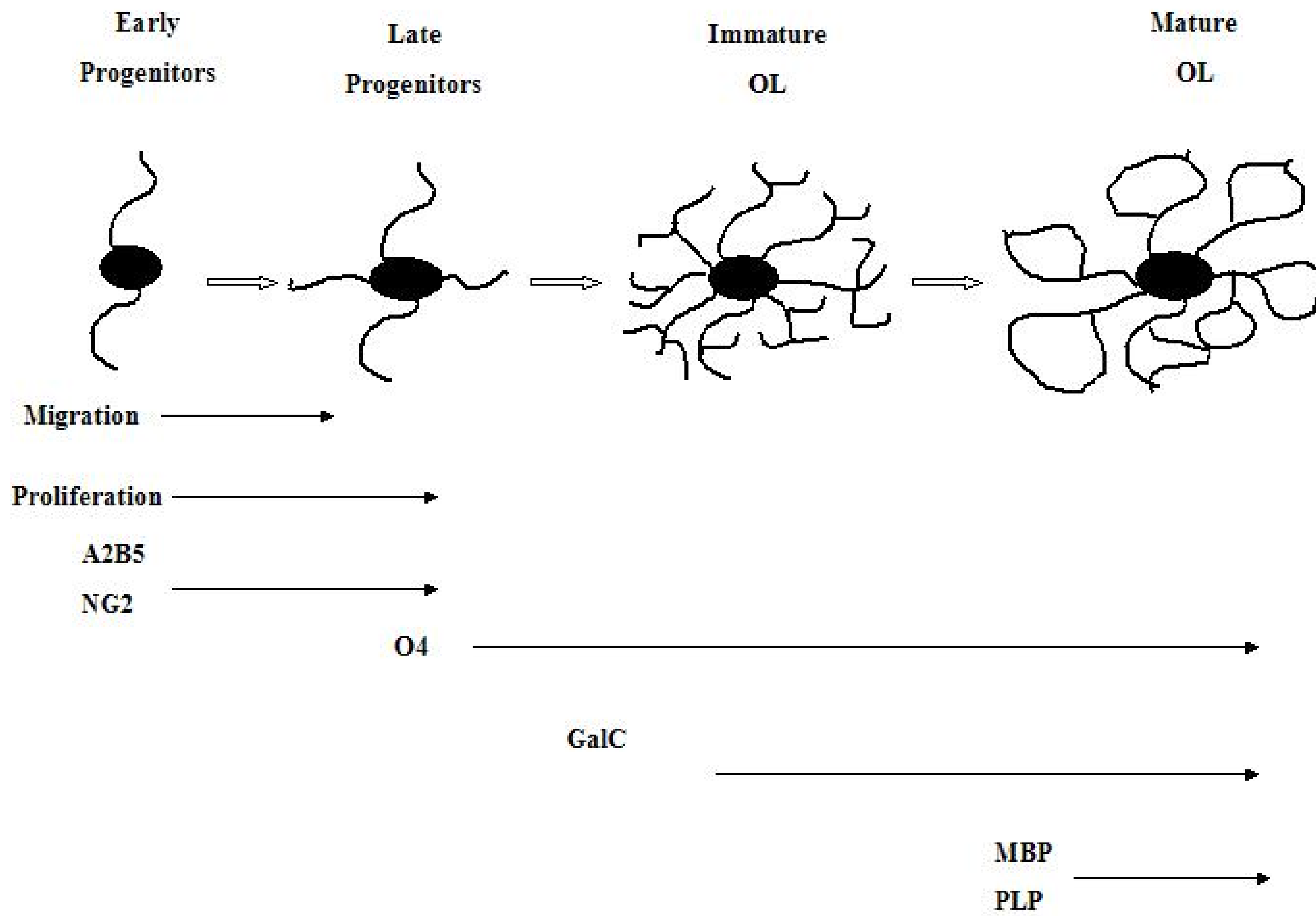
Early OP cells are characterized by a distinct bipolar morphology, expression of PDGFR-α (Hart, Richardson et al. 1989), a cell surface glycolipid recognized by A2B5 monoclonal antibody (Raff, Abney et al. 1984), a glycolipid, GD3 recognized by the monoclonal antibodies R24 and LB1 (Hardy and Reynolds 1991) and a chondroitin sulfate proteoglycan, NG2 (Nishiyama, Lin et al. 1996). Initially, early OP cells were termed O-2A cells since they can generate both oligodendrocytes and astrocytes in vitro, depending on environmental cues (Raff, Miller et al. 1983). However, an in vivo study failed to generate astrocytes from O-2A cells (Espinosa de los Monteros, Zhang et al. 1993), thus, the term O-2A has been replaced by OP cells.

Differentiation of early OP cells to late OP cells is initiated by mitogen withdrawal and exposure to differentiating factors. Late OP cells show a simple multipolar morphology and express the pro-oligodendroblast antigen (POA), a cell surface antigen recognized by the monoclonal antibodies O4 and A007 (Bansal, Stefansson et al. 1992). Late OP cells are still proliferative, but no longer migratory (Bansal, Stefansson et al. 1992).

As late OP cells further differentiate into immature oligodendrocytes, they extend more processes and express specific glycolipids recognized by monoclonal antibodies R-mAb (Ranscht monoclonal antibody) and O1, the latter recognizes the major myelin glycolipid galactocerebroside (GalC) (Sommer and Schachner 1981; Bansal and Pfeiffer 1992). Cells at this stage no longer respond to mitogens and have exited the cell cycle.

Immature oligodendrocytes terminally differentiate to mature oligodendrocytes, characterized by the expression of major myelin proteins and minor myelin proteins. Major myelin proteins include myelin basic protein (MBP) and proteolipid proteins (PLP). Minor myelin proteins include myelin-associated glycoprotein (MAG) and myelin/oligodendrocyte glycoprotein (MOG). Studies presented in this dissertation are focused on early and late OP cells and the regulation of their proliferation.

Figure 1.1 Lineage progression of oligodendrocytes with cell surface antigens.


Early
Progenitors
Late
Progenitors
Immature
OL
Mature
OL
Migration
Proliferation
A2B5
NG2
O4
GalC
MBP
PLP

1.1.5 Oligodendrocyte precursors in adult CNS and remyelination

Developmental studies of the CNS have revealed that there are stem/progenitor cells, including OP cells, present in the adult CNS that are able to differentiate to more lineage restricted cells. Early research with adult rat optic nerve showed the presence of OP cells in adults (Raff, Miller et al. 1983; Ffrench-Constant and Raff 1986) and suggested their ability to self-renew (Ffrench-Constant and Raff 1986). However, it was postulated that adult OP cells are distinct from perinatal OP cells and show a different morphology and antigenic phenotype (Wolswijk and Noble 1989; Wren, Wolswijk et al. 1992). Data from two studies were contradictory with respect to the capacity of adult OP cells to participate in remyelination. Wolswijk and colleagues suggested that adult OP cells have a longer cell cycle time and slower migratory capacity compared to perinatal OP cells, implying adult OP cells are unable to replace a sufficient number of oligodendrocytes (Wolswijk and Noble 1989). In contrast, Wren and colleagues claimed that adult OP cells are maintained for a longer time and go through symmetric divisions while prenatal OP cells disappear after birth and show asymmetric division (Wren, Wolswijk et al. 1992). The presence of adult OP cells was also confirmed in vivo. Results from analyzing mice at three different postnatal days suggested that NG2+/O4- cells differentiated to NG2+/O4+ and further GalC+ oligodendrocytes (Reynolds and Hardy 1997). Interestingly, the authors observed that a subpopulation of NG2+/O4+ remained in later postnatal stages. These results indicate that OP cells may reside in the adult brain for long periods and repopulate oligodendrocytes when needed.

Multiple sclerosis (MS) is an autoimmune demyelinating disease accompanied by loss of oligodendrocytes as well as myelin. In MS, oligodendrocytes

do not completely recover, particularly after repeated attacks. Many reasons have been proposed for lack of differentiation and remyelination of some MS plaques. One proposal is that there are insufficient OP cells to mature to myelin forming cells. However, as discussed above, several lines of evidence indicate that OP cells or earlier precursor cells exist in the adult CNS. The evidence for resident OP cells also came from normal human brain and MS patient specimens (Prineas, Barnard et al. 1993; Ozawa, Suchanek et al. 1994; Prabhakar, D'Souza et al. 1995; Scolding, Rayner et al. 1995). Cells with OP cell morphology from cerebral cortex (Prabhakar, D'Souza et al. 1995) and temporal lobe (Scolding, Rayner et al. 1995) were shown to be responsive to mitogens. Tissues from long surviving MS patients have more plaques with remyelination although active demyelination occurs in these lesions (Prineas, Barnard et al. 1993). Remyelination clearly occurs in acute and early chronic MS patients (Ozawa, Suchanek et al. 1994) and a large number of immature oligodendrocytes are present (Prineas, Kwon et al. 1989). However, Scolding et al reported that the number of PDGFR-α positive OP cells appeared insufficient in acute and chronic patients (Scolding, Franklin et al. 1998), indicating that the number of OP cells in the demyelinating lesions is critical.

It has been of interest to a number of investigators to understand the origin of new oligodendrocytes in the adult CNS. Considerable evidence supports the hypothesis that new oligodendrocytes arise from OP cells (Wood and Bunge 1991; Blakemore and Keirstead 1999). Moreover, proliferating cells in rat spinal cord are NG2+ OP cells (Horner, Power et al. 2000). Also, distinct OP cell markers can be found in remyelinating regions, such as PDGFR-α (Redwine and Armstrong 1998; Sim, Zhao et al. 2002), NG2+ (Di Bello, Dawson et al. 1999; Levine and Reynolds

1999) and Myt1 (Sim, Zhao et al. 2002). Studies with a LacZ-expressing retrovirus or [^{3}H]-thymidine labeling for proliferating cells revealed that cells in optice nerve or the adult CNS generate remyelinating oligodendrocytes after induced demyelination (Carroll and Jennings 1994; Gensert and Goldman 1997). Finally rat subependymal tissue transplants are able to remyelinate host animals (Zhang, Ge et al. 1999). Taken together, the data support the hypothesis that OP cells in the adult CNS are recruited to demyelinating lesions and participate in remyelination.

A recent study adds more complexity on the characteristics of remyelinating OP cells. Gensert et al selected BrdU positive cells and separated them into subtypes: 1) O4+ cells able to differentiate to GalC, and 2) vimentin+ cells able to differentiate to glial fibrillary acidic protein (GFAP), 3) A2B5+ cells and 4) NG2+ cells (Gensert and Goldman 2001). A heterogeneous population of OP cells may give different responses to environmental cues as reported in Mason et al (Mason and Goldman 2002). Consistent with the idea of OP cell heterogeneity, OP cells in chronic lesions do not proliferate (Wolswijk 1998) while MOG+ cells proliferate in active demyelinating lesions (Schonrock, Kuhlmann et al. 1998). In addition, NG2+ OP cells fail to renew in spinal cord after induced demyelination (Keirstead, Levine et al. 1998). Thus, it appears that OP cells are generated from diverse regions and developmental stages, which provides heterogeneous subpopulations and different responsivity to environmental signals. Thus, understating the identity of OP cells will be important to find successful cures for demyelinating diseases.

1.2 Oligodendrocyte development and growth factors

1.2.1 Fibroblast Growth factor

The Fibroblast growth factor (FGF) family has roles in diverse cellular processes such as proliferation, migration, and differentiation. A total of 22 FGF family members have been identified so far in human and mice: each family member is encoded by a single gene (Reuss and von Bohlen und Halbach 2003). Targeted deletion of FGF genes provides a good idea about specific and compensatory functions of FGFs (Eswarakumar, Lax et al. 2005). FGFs elicit their cellular response by binding to a receptor tyrosine kinase family, FGF receptor (FGFR). 4 FGFR (FGFR 1-4) are identified so far, but each of them has splice isoforms, which provides for diverse cellular effects (Reuss and von Bohlen und Halbach 2003). The FGFR is composed of an extracellular ligand-binding domain, a single transmembrane domain and a cytoplasmic tyrosine kinase domain (Eswarakumar, Lax et al. 2005). Half of the FGF family members and three FGFRs are expressed in the brain (Bansal and Pfeiffer 1997; Reuss and von Bohlen und Halbach 2003).

The role for FGFs in oligodendrocyte lineage cells has been well established (illustrated in Fig. 1.2). Early studies identified FGF receptor mRNA expression patterns during oligodendrocyte lineage progression (Bansal, Kumar et al. 1996) as illustrated in Figure 1.2. FGFR1 expression gradually increased with lineage progression from early progenitor cells to mature oligodendrocytes. FGFR2 is expressed in terminally differentiated mature oligodendrocytes, while FGFR3 expression peaks in late progenitor cells (Bansal 2002). The mRNA expression pattern of FGFRs during OP cell maturation led to the proposal of their temporal function during differentiation. FGFR1 stimulates proliferation and migration of progenitor cells. In turn, FGFR3 promotes further proliferation and inhibition of differentiation of

progenitors to oligodendrocytes (Bansal 2002). However, this proposal recently confronted challenges. Oh and colleagues reported that FGFR3 null mice showed reduced and delayed myelination, but no alterations in survival or proliferation of oligodendroglia, emphasizing the role of FGFR3 on regulation of differentiation timing (Oh, Denninger et al. 2003). In vitro study using receptor blocking antibodies demonstrated that FGFR1 is required for proliferation and FGFR3 inhibits terminal differentiation (Fortin, Rom et al. 2005). Targeted FGFR1 downregulation with siRNA inhibits proliferation and reverses inhibition of OP cell differentiation (Zhou, Flint et al. 2006). Taken together, the current view is that FGFR1 and FGFR3 function in OP cell proliferation and differentiation, respectively.

FGF is a potent mitogen for oligodendrocyte lineage cells. Chemically defined medium for oligodendrocyte cultures contains FGF along with insulin (at superphysiological levels) and transferrin (Saneto and de Vellis 1985). Moreover, data from several studies demonstrated that FGF is a potent mitogen for oligodendrocytes (Besnard, Perraud et al. 1989; McKinnon, Matsui et al. 1990). Either FGF-1 or FGF-2 stimulated proliferation of newborn rat oligodendrocyte and decreased activity of mature oligodendrocyte markers, 2’3’-cyclic nucleotide 3’-phosphodiesterase (CNP) and myelin positive oligodendrocytes, suggesting inhibition of oligodendrocytes maturation (Besnard, Perraud et al. 1989). Another study demonstrated that the mitogenic activity of FGF results in part from its ability to increase the level of the PDGF-α receptor in OP cells and inhibit their differentiation into oligodendrocytes (McKinnon, Matsui et al. 1990).

Migratory behavior is also a hallmark of progenitor cells to expand density of cells during development. Ganglioside GD3 positive oligodendrocyte progenitor cells

need growth factors, such as FGF-2 for migration (Decker, Avellana-Adalid et al. 2000). FGF-2 stimulates migration of OP cells, which is not affected by PDGF neutralizing antibodies (Milner, Anderson et al. 1997). Further, another group implicated Ca^{2++} and actin or tubulin polymerization in the migratory effect of FGF-2 in OP cells (Simpson and Armstrong 1999).

FGF maintains the progenitor stage of the oligodendrocyte lineage by blocking differentiation of progenitor cells to myelin producing oligodendrocytes. Addition of FGF-2 into mixed culture of oligodendrocytes and OP cells caused an increase in OP cell number and a decrease in GalC+ mature oligodendrocytes and MBP mRNA (Grinspan, Stern et al. 1993) suggesting FGF dedifferentiated oligodendrocytes to OP cells and stimulated their proliferation. Moreover, introduction of FGF-2 into differentiated oligodendrocytes resulted in loss of myelination, accumulation of PLP and MBP within the cell body of oligodendrocytes, disruption of myelin production, but no delay in differentiation (Goddard, Berry et al. 2001). The general conclusion from these studies is that FGF increases the progenitor cell population by blocking oligodendrocyte maturation.

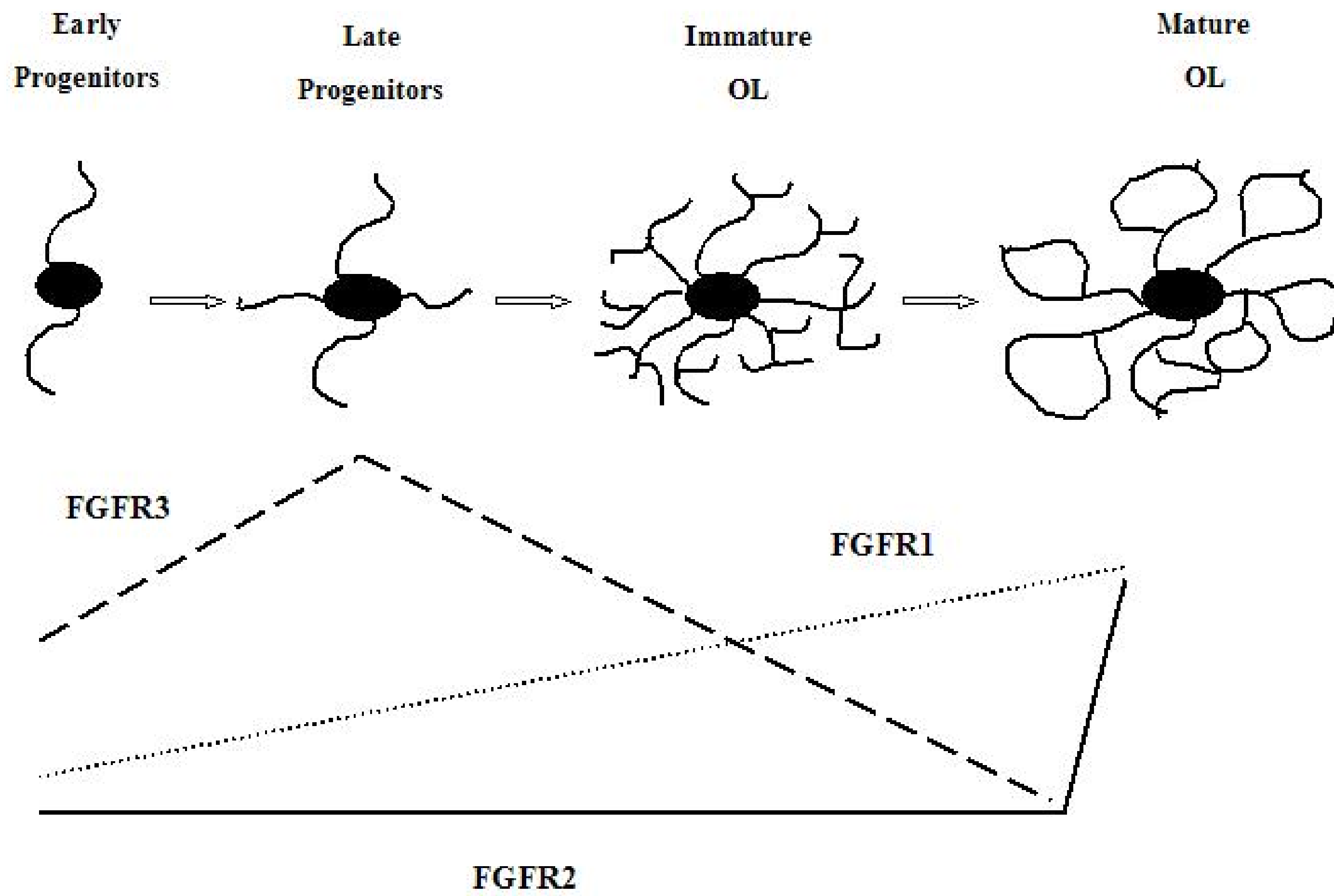
In vivo studies using specific gene ablation clearly demonstrated functions of FGF-2 in oligodendrocyte development as well as neurogenesis. Two different groups reported that FGF-2 knockout mice were viable, but showed cerebral cortex defects, deficits in neurogenesis, and cytoarchitecture abnormalities (Dono, Texido et al. 1998; Ortega, Ittmann et al. 1998), implying roles of FGF-2 in defining neuronal cell fate, and in migration and differentiation of progenitor cells (Dono, Texido et al. 1998). FGF-2 deletion increased the number of oligodendrocytes, without an effect on progenitor cell density, proliferation, and survival (Murtie, Zhou et al. 2005). The

same group investigated the effect of FGF receptor deletion, using a retrovirus expressing dominant negative FGFRs with GFP (Zhou, Flint et al. 2006). Similar to FGF-2 null mice, a higher portion of oligodendrocytes was reported and FGF-2 mediated OP proliferation was inhibited with decreased FGFR levels (Zhou, Flint et al. 2006). However, transient exposure of FGF-2 in oligodendrocyte/neuronal co-cultures enhanced BrdU uptake, increased CNPase activity and MBP expression and promoted differentiation, maturation and myelination of oligodendrocytes (Magy, Keita et al. 2003). The conflicting findings of Magy and colleagues may reflect a temporal expression of FGFRs in different developmental stages that regulate different cellular responses in oligodendrocyte lineage cells.

The action of FGF-2 on remyelination is also well studied. FGF-2 knockout mice were demyelinated by murine hepatitis virus strain A59 (MHV-A59) or cuprizone, both of which lead to remyelination after the demyelinating stimulus (Armstrong, Le et al. 2002). Results of these studies showed oligodendrocyte repopulation even though proliferation was unaltered (Armstrong, Le et al. 2002). Further in MHV-A59 induced demyelinated glial cultures, addition of FGF-2 stimulated progenitor proliferation while treatment with neutralizing antibodies resulted in increased differentiation of OP cells into oligodendrocytes (Armstrong, Le et al. 2002). Another group reported that in MHV-A59 induced demyelination lesions, FGF-2 mRNA peaked when remyelination was initiated. Oligodendrocyte lineage cells and astrocytes express multiple FGF receptors, suggesting paracrine and autocrine effects of FGF-2 on remyelination (Messersmith, Murtie et al. 2000). Taken together, FGF-2 expression has to increase for proliferation of OP cells at the time of remyelination, but levels of FGF-2 should be downregulated for differentiation to

mature oligodendrocytes, suggesting the importance of temporal regulation of FGF-2.

Figure 1.2 The expression pattern of FGFR during oligodendrocyte lineage progression. (Modified from Bansal, R 2002)


Early
Progenitors
Late
Progenitors
Immature
OL
Mature
OL
FGFR3
FGFR1
FGFR2

1.2.2 Insulin Like Growth Factor

Insulin like growth factor (IGF) is closely related to insulin as its name suggests. The IGF system proteins consist of IGF-1, IGF-2, the IGF type 1 receptor (IGF1R), the IGF2 receptor (IGF2R) which is also the high affinity mannose 6 phosphate receptor, and the six high affinity IGF binding proteins (IGFBPs) (Ye and D'Ercole 2006). IGF-1 and IGF-2 are 70 amino acid and 67 amino acid polypeptides encoded by single genes, respectively (Schmid 1995). IGF-1 and IGF-2 bind with high affinity (nanomolar range) to the IGF1R; insulin has only weak affinity (~100x less than the IGF ligands) for the IGF-1R but stimulates the IGF-1R at micromolar concentrations used in most neural cell culture systems (Bondy and Cheng 2004). IGF-1 synthesized in the liver is the primary source of circulating IGF-1. IGF-1 is also locally synthesized in many tissues including the brain. IGFBPs regulate IGF availability and function by preventing proteolysis and modulating IGF binding to receptors both positively and negatively (Bondy and Cheng 2004). However, IGFBP isoforms also have IGF independent functions. The IGF-1R has 60% sequence identity to the insulin receptor (IR) in structure. The IGF-1R is heterotetrameric, composed of two α subunits and two β subunits (Blakesley, Scrimgeour et al. 1996). The α subunits are the extracellular domain of the receptor and bind to ligands while the β subunits contain the transmembrane and tyrosine kinase domains. Many regulatory proteins associate with the β subunit after receptor activation such as insulin receptor substrate (IRS) proteins. IGF-1 is implicated in cell proliferation, differentiation, cell lineage specification and survival in neural cells (Ye and D'Ercole 2006). The function of IGF-1 is well studied: in this section, the diverse roles of IGF-1 will be discussed particularly in oligodendrocyte lineage cells.

Expression of IGF-1 and IGF-1R in the brain and especially in oligodendrocyte lineage cells has been well documented in rodents. Spatial and temporal mRNA expression of the IGF system has been reported in the central nervous system. IGF-1R mRNA increases at embryonic day 15 and 20 while the insulin receptor appears at embryonic day 20 and the day of birth (Baron-Van Evercooren, Olichon-Berthe et al. 1991). IGF-1 mRNA is widely expressed in the brain while the pattern of insulinis restricted in certain areas such as olfactory regions and hippocampus, where IGF-1 is co-expressed (Baron-Van Evercooren, Olichon-Berthe et al. 1991). IGF-1 is expressed predominantly in neurons throughout many brain areas while IGF-2 expression is limited to mesenchymal cells and cells of neural crest origin (D'Ercole, Ye et al. 1996). IGF-1R mRNA is ubiquitously expressed and IGFBP expression shows regional and developmental patterns, concomitantly with IGF expression (D'Ercole, Ye et al. 1996). In vitro studies support a role for IGF-1 in proliferation of neural progenitors and in survival of neurons and oligodendrocytes (Wilkins, Chandran et al. 2001). In vivo, transgenic mice overexpressing IGF-1 showed brain overgrowth due to hypermyelination (Carson, Behringer et al. 1993; Ye, Carson et al. 1995). In contrast, ectopic expression of IGFBP-1 (D'Ercole, Ye et al. 1996), which inhibits IGF signaling, or knockout of IGF-1 (Beck, Powell-Braxton et al. 1995) decreases brain size and result in hypomyelination. It has been reported that OP cells express IGF-1 mRNA (Shinar and McMorris 1995) as well as differentiating oligodendrocytes, but the latter at a lower level (Shinar and McMorris 1995). Taken together with the potential for axonal IGF-1 release, these data suggest autocrine and paracrine action of IGF-1 in regulating oligodendrocyte development.

IGF-1 stimulates proliferation or differentiation of other cell types. Using ^{125}I labeled IGF-1, IGF-1 was shown to bind to the cell surface in oligodendrocyte cultures. Early studies suggested that IGF-1 and IGF1R interaction leads to proliferation as well as differentiation of oligodendrocytes (McMorris, Smith et al. 1986; McMorris and Dubois-Dalcq 1988; Masters, Werner et al. 1991). In another study, it was suggested that IGF-1 acts predominantly as a proliferating factor (Satoh and Kim 1994). Studies with brain aggregates also supported a role for IGF-1 both in proliferation and myelination (Mozell and McMorris 1991). More recently, data from our laboratory demonstrated that IGF-1 by itself is a poor mitogen for OP cells: however, it cooperates with FGF-2 and PDGF to promote proliferation of these cells (Jiang, Frederick et al. 2001; Frederick and Wood 2004).

Another function of IGF-1 is in inducing survival of cells. Oligodendrocyte conditioned media or co-cultures with neurons revealed that IGF-1 promotes survival of both neurons and oligodendrocytes (Wilkins, Chandran et al. 2001; Ness and Wood 2002). IGF-1 prevents tumor necrosis factor (TNF)-α induced apoptosis in mouse glial cultures (Ye and D'Ercole 1999). Previous data from our laboratory have shown that IGF-1 prevents glutamate-mediated excitotoxicity of late OP cells ((Ness and Wood 2002; Ness, Scaduto et al. 2004) and prevents loss of oligodendroglia in white matter following hypoxia-ischemia in the immature brain (Wood, Loladze et al. 2007). The ability of IGF-1 to protect late OPs from excitotoxicity is unlike NT-3 which only provides short term protection of the cells or CNTF which fails to protect OPs from excitotoxic death (Ness and Wood 2002). The survival effect of IGF-1 as well as its cooperative effect on OP cell proliferation is mediated through activation of PI3K pathway and sustained AKT activation through IGF-1R trafficking and recycling

(Ness and Wood 2002; Romanelli, LeBeau et al. 2007; Romanelli, Mahajan et al. 2009).

In vivo studies with transgenic animals have clearly shown that IGF-1 plays a role in the CNS. IGF-1 overexpression results in increased brain size and increased myelination, including myelin content, MBP and PLP mRNA, increased numbers of myelinated axons and myelin sheath thickness (Carson, Behringer et al. 1993; Ye, Carson et al. 1995). In contrast, IGFBP-1 expressing mice reversed this effect by inhibiting IGF-1 actions (Ye, Carson et al. 1995). Studies on IGF-1 knockout mice were controversial in concluding that IGF-1 effects on myelination were direct effects on oligodendroglia and myelination or indirect effects as a result of reduced brain size and defects in neurons. One study reported reduced brain weight, thinner white matter structure in the brain and spinal cord, and decreased myelinated axons in transgenic mice compared to control mice (Beck, Powell-Braxton et al. 1995). However, a later study showed that the IGF-1 KO mice had no visible myelinopathy, no change in myelin index (myelin composition, myelin concentration, MBP, PLP, MAG and CNP), and comparable oligodendrocyte numbers proportional to the smaller animal size and brain weight (Cheng, Joncas et al. 1998). Interestingly, this paper described that oligodendrocytes and myelin depletion was concomitant with projection neuron pattern, pointing out that myelination is regulated by neural factors other than IGF-1 (Cheng, Joncas et al. 1998). The discrepancy between the two observations resulted from the way the myelin index was analyzed since the latter group calculated the myelination index proportionally. By investigating two different aged IGF-1 mutant mice, another group reported that the developing brains of IGF-1 knockout mice showed decreased myelination but no change in adult white matter (Ye, Li et al.

2002). Moreover IGF-2 mRNA was elevated in the IGF-1 null brains, implying that IGF-2 might compensate for loss of IGF-1 in adult mice (Ye, Li et al. 2002). The question of whether IGF signaling exerts direct effects on oligodendrocyte development was resolved by more recent studies using Cre-lox deletion of the IGF-1R in OP cells and more mature cells in the developing white matter (Zeger, Popken et al. 2007). These studies demonstrated that deletion of IGF-1R specifically in the oligodendrocyte lineage resulted in decreased numbers of progenitors as well as mature cells and myelin in the corpus callosum.

IGF-1 and the IGF-1R also are important for remyelination following a demyelination insult. Treatment with cuprizone, which is a demyelination inducing agent and leads to subsequent remyelination stimulates expression of IGF-1 mRNA and protein in astrocytes as well as in immature oligodendrocytes, and myelin gene expression, suggesting that IGF signaling is necessary for remyelination (Komoly, Hudson et al. 1992). These results were supported by other groups who reported that only IGF-1 mRNA increased unlike other growth factors, PDGF, NT3, FGF, jagged and notch following cuprizone (Mason, Jones et al. 2000) and lysolecithin demyelination (Hinks and Franklin 1999). IGF-1 overexpressing mice treated with cuprizone demonstrated less apoptosis and more rapid recovery (Mason, Jones et al. 2000). However, the therapeutic capacity of IGF-1 for demyelinating disorders is still controversial. IGF-1 injection into chronic relapsing experimental autoimmune encephalitis (EAE), an animal model for MS, reduced inflammation, demyelination and demyelinating lesions (Li, Quigley et al. 1998). However, another group reported no significant effect on remyelination after IGF-1 injection (Cannella, Pitt et al. 2000). They observed that TGF-β2 and TGF-β3 both decreased in EAE animals and

suggested that insufficiency of these factors may abolish IGF-1 function (Cannella, Pitt et al. 2000). Introduction of IGF-1 with IGF-1 expression adenoviral vector failed to increase remyelination even though IGF-1 mRNA was elevated in lysolecithin induced demyelination (O'Leary, Hinks et al. 2002). Taken together, IGF-1 may be a good candidate to treat demyelinating diseases based on in vitro and in vivo studies, however, coordination with other factors in the microenvironment is needed for better outcomes.

1.3 Cell cycle regulation

1.3.1 overview of cell cycle

The cell cycle or cell division cycle is a series of cellular events that a cell undergoes to duplicate the genetic material and divide into two daughter cells. Progression through the cell cycle is tightly regulated and well orchestrated to maintain genetic identity of organisms through several levels of control, which will be discussed below. A cell integrates extracellular and intracellular cues and depending on these signals, is induced to progress through the cell cycle. For example, mitogenic cues such as growth factors stimulate cells to initiate another round of cell division, while antiproliferative signals such as TGF-β cause cells to withdraw from the cell cycle and become quiescent or to differentiate (Massague and Polyak 1995).

The cell cycle is divided into 4 distinct phases, G1 (gap1), S (synthesis), G2 (gap2) and M (mitosis) (illustrated in Fig 1.3). In the G1 phase, cells respond to extracellular signals to determine whether to enter or continue another round of the cell cycle or exit to quiescent G0. The S phase is the time when DNA replication and

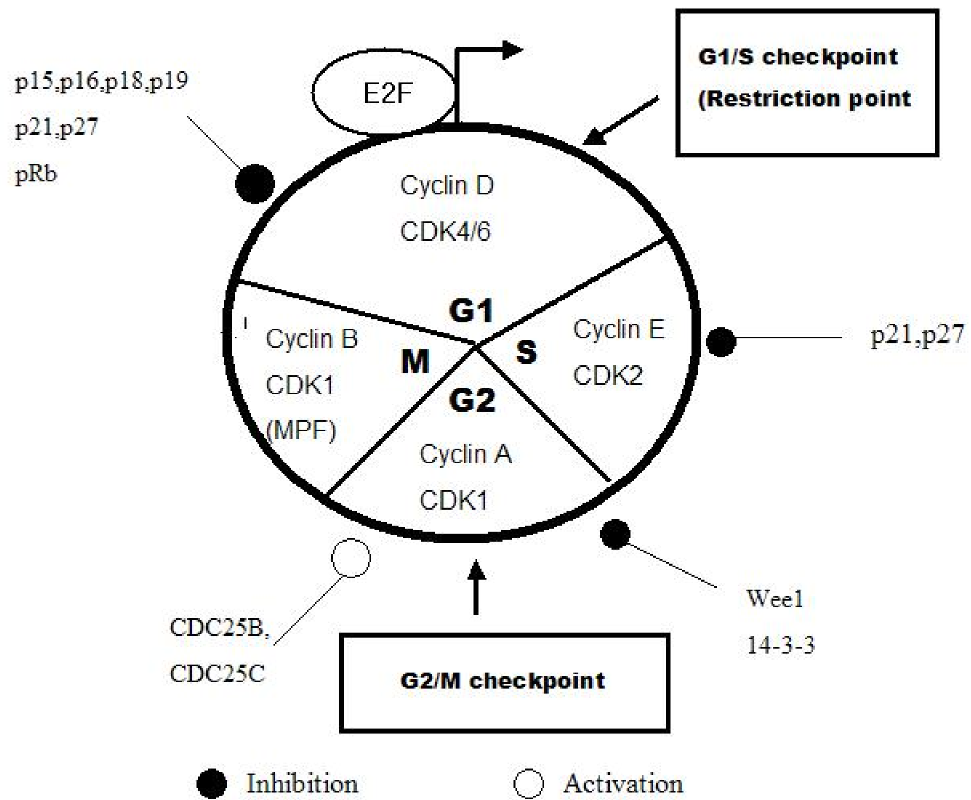
chromosome duplication is completed. In the G2 phase, cells determine whether all DNA, chromosome, and other components are accurately duplicated. Mitosis involves chromosome condensation, nuclear envelope breakdown, assembly of the mitotic spindle, centrosome separation and cytokinesis, sequentially and finally a single cell is divided into two new cells. Two transition points exist to ensure proper cell cycle progression, one in G1 phase and another in G2 phase (Elledge 1996). Sufficient nutrients and growth signals are required for a cell to progress through the G1 phase transition point. It was originally proposed that once a cell passes the transition point in G1 phase, also known as the restriction point (Pardee 1974), a cell commits to finish the rest of cell cycle without any further growth stimulation. The G2 transition point is the point where cells examine whether DNA damage has occurred or DNA synthesis has been completed prior to mitosis.

The most critical step for proper cell division is the transition from one phase to the next phase, which is regulated by cell cycle machinery. The cell cycle machinery mainly consists of cyclins, the regulatory subunits, and cyclin-dependent-kinases (cdks), the catalytic subunits that together form a holoenzyme for effective action ((Morgan 1997). Particular cyclins and cdks form complexes that have roles in different cell cycle phases, e.g. cyclin D/cdk4 or cyclin D/cdk6 in early G1 phase, cyclin E/cdk2 in late G1 phase, cyclin A/cdk2 in S phase and cyclin B/cdk1 in G2/M phase (Morgan 1997). Cyclins, the regulatory subunits, are expressed and degraded in a cell cycle phase specific manner. However, cdks, the catalytic subunits, are present consistently throughout the cell cycle. The cdks are threonine/serine kinases, which are the driving force of cell cycle progression: cdk activity is tightly controlled in a phase-specific manner. There are several mechanisms to govern cdk activity such as

expression and degradation of partner cyclins, holoenzyme formation, negative control by cdk inhibitors (cdki), and phosphorylation in the T loop of cdks for activation, and phosphorylation in the ATP binding site of cdks for inactivation ((Morgan 1997; Pines 1999).

In general, mitogens induce exit from G0 and passage through the G1-S transition. In turn, S phase entry allows cells to complete the remainder of the cell cycle. Therefore, in section 1.3.2 I will briefly review the mechanism by which cells enter S phase, following mitogen stimulation. Also, regulation of S phase progression and G2/M transition will be discussed in sections 1.3.3 and 1.3.4. This overview will include cyclins, cdks, cdk regulators as well as the molecular events to activate cdks in the individual cell cycle phases.

Figure 1.3 Regulation of cell cycle progression. (Modified from Tessema, M 2003)


p15,p16,p18,p19
p21,p27
pRb
E2F
G1/S checkpoint
(Restriction point
Cyclin D
CDK4/6
G1
Cyclin B
CDK1
(MPF)
M
S
Cyclin E
CDK2
p21,p27
G2
Cyclin A
CDK1
Wee1
14-3-3
CDC25B,
CDC25C
G2/M checkpoint
Inhibition
Activation

1.3.2 Retinoblastoma protein and cell cycle regulation

Retinoblastoma protein pRb is a member of what are known as pocket proteins first identified as tumor suppressors (van den Heuvel and Dyson 2008). pRb negatively regulates cellular proliferation, and mutated forms of the gene are found in many human tumors (Stevens and La Thangue 2003)). pRb plays an important role in the G1 phase to S phase transition, partially through interaction with transcription factor, E2F family members (Dyson 1998). Hypophosphorylated pRb binds to promoters on E2F genes (Chellappan, Hiebert et al. 1991) thereby blocking transcriptional activation of genes related to cell cycle progression (van den Heuvel and Dyson 2008). When pRb is phosphorylated by kinases such as cdks, E2Fs are released and initiate transcription of E2F responsive genes. pRb also recruits histone deacetylases (HDACs) and inhibits transcriptional activity of E2Fs (Harbour and Dean 2000). It is not clear yet whether pRb inhibits E2F activity by direct binding on promoter sites or indirectly by association with HDACs.

Inactivation of pRb by phosphorylation is promoted through cdk activity. Cyclin D induction upon mitogen stimulation and complex formation with cdk4/6 phosphorylates pRb (Mittnacht 1998). Sequentially, E2F induces transcription of cyclin E, and the cyclin E/cdk2 complex further phosphorylates pRb, resulting in S phase entry and DNA replication (Ohtani, DeGregori et al. 1995; Lundberg and Weinberg 1998).

E2F transcription factors are heterodimers consisting of an E2F subunit and a DP (DRTF1 protein) subunit. Six E2F gene family members and two DP gene family members have been identified in mammalian cells so far (van den Heuvel and Dyson 2008). E2F 1-3, interacting with pRb, are expressed in G1 and S phase and act as activators of E2F responsive genes (Stevens and La Thangue 2003). Combinatorial knockout of E2F 1-3 in mice results in a significant delay in S phase entry and abolishes cellular proliferation (Wu, Timmers et al. 2001). However, E2F 4-6 are expressed constitutively and are associated with p130 and p107, which are other members of the pocket protein family that also act as repressors of E2F dependent transcription (Stevens and La Thangue 2003). E2Fs are involved in transcription of

many genes responsible for cell cycle progression and apoptosis such as cell cycle regulators (cyclin E, cyclin A, cyclin D1, Cdc2, and Cdc25A), DNA synthesis enzymes (dihydrofolate reductase, DNA polymerase α, and thymidine kinase), DNA replication related proteins (Cdc6, ORC1, and minichromosome maintenance (MCM) proteins), and apoptosis associated proteins (apoptosis protease-activating factor 1(Agaf1), p73 and ARF) (Dyson 1998).

The pRb-E2F pathway is a complex mechanism to regulate G1/S transition and S phase entry. Since regulation of pRb activity and sequentially E2F activity is mainly controlled in G1 phase and specifically the passage through the restriction point, the status of pRb is a good marker to distinguish whether cells have entered S phase or are arrested at the G1 phase.

1.3.3 S phase progression and major regulators

Once cells enter S phase, DNA replication is initiated. DNA replication mechanisms are well understood in yeast. First the origin recognition complex 1 (ORC1) binds to DNA (Woo and Poon 2003) and in G1 phase, additional prereplication complex, which is composed of Cdc6, CDP1 and MCMs, associates with ORC1-DNA (Bell and Dutta 2002). DBF4-Cdc7 and cyclin-cdk complexes phosphorylate the prereplication complexes, which allow association with cdc45 and further activate the origin. Once prereplication complexes are activated, the replication origin is unwound and the single-stranded DNA binding protein RPA and DNA polymerase α are recruited for DNA replication (Woo and Poon 2003).

As described above, cyclin/cdk complexes play an essential role in cell cycle progression. Cdk2 binds to cyclin E and A to regulate S phase entry and progression. While expression of cyclin E is maximal at late G1, cyclin A starts to accumulate in early S phase. As discussed above, the cyclin E/cdk2 complex is necessary for passage through the restriction point by phosphorylation of pRb (Ohtani, DeGregori et al. 1995; Lundberg and Weinberg 1998) and for initiation of DNA replication by activation of prereplication complexes (Woo and Poon 2003). Cyclin A/cdk2 maintains phosphorylated pRb for S phase progression (Knudsen, Buckmaster et al.

1998) and regulates DNA replication (reviewed in (Yam, Fung et al. 2002). A critical role for cyclin A in S phase progression has been reported in numerous studies; overexpression of cyclin A promotes S phase entry (Resnitzky, Hengst et al. 1995; Rosenberg, Zindy et al. 1995) while downregulation of cyclin A by anti-sense DNA constructs or antibodies delays S phase progression (Girard, Strausfeld et al. 1991; Pagano, Pepperkok et al. 1992; Zindy, Lamas et al. 1992). Furthermore, several lines of evidence have implicated cyclin A in DNA replication (Fotedar, Cannella et al. 1996). Cyclin A is localized at DNA replication foci similar to proliferating cell nuclear antigen (PCNA) (Cardoso, Leonhardt et al. 1993; Sobczak-Thepot, Harper et al. 1993). Studies with parvovirus demonstrated that cyclin A dependent kinase activity, not cyclin E dependent kinase activity, is required for DNA polymerase δ-dependent elongation machinery (Bashir, Horlein et al. 2000). Since cyclin A predominantly functions in DNA replication and S phase progression as described above, the review below is restricted to discussion of cyclin A and cdk2.

Cyclin A was first described in mitosis of developing marine invertebrates, specifically clam oocytes (Swenson, Farrell et al. 1986). Human cyclin A was found as a virus associated gene in tumor cells. The cyclin A gene was initially identified in a Hepatitis B virus integration site (Wang, Chenivesse et al. 1990). In the same year, another group identified human cyclin A, using PCR based on sequences from Drosophila and *Xenopus* cyclin A and found that cyclin A mRNA and protein expression oscillates in a cell cycle dependent manner (Pines and Hunter 1990). Interestingly, these investigators found that cyclin A was identical to protein p60, initially reported as adenovirus early region 1A (E1A) associated protein (Harlow, Whyte et al. 1986). These two studies implicated cyclin A in DNA replication.

Cyclin levels are regulated to fine-tune cell cycle progression. In 1990, Pines's group reported that cyclin A mRNA and protein expression levels changed during the cell cycle and accumulate during S phase (Pines and Hunter 1990). Henglein and his colleagues reported that the cyclin A promoter was repressed during G1 phase and activated at S phase entry (Henglein, Chenivesse et al. 1994). Transcription of cyclin A is controlled by the E2F family, particularly by E2F3

(Humbert, Verona et al. 2000) as discussed in section 1.3.2. Interestingly, the cyclin A/cdk2 complex phosphorylates and inhibits the E2F-1/DP-1 transcription factor, suggesting negative feedback regulation (Xu, Sheppard et al. 1994). This negative feedback loop is postulated to inhibit DNA rereplication since cyclin A is a critical component for DNA replication. Cyclin A levels also are regulated by degradation. The N-terminal region of cyclin A, known as the destruction box (D-box), is a targeted site for cyclin A degradation (Fung and Poon 2005). Destruction of cyclin A is mediated by E3 ubiquinase in the anaphase promoting complex (APC)/cyclosome (reviewed in (Castro, Bernis et al. 2005), which was first identified as cyclin B destruction complex (King, Peters et al. 1995; Sudakin, Ganoth et al. 1995). Activated by association with cdc20, APC degrades cyclin A at the prometaphase to metaphase transition to induce mitosis (den Elzen and Pines 2001; Geley, Kramer et al. 2001; Castro, Bernis et al. 2005). Considering its expression and destruction time, cyclin A levels start to accumulate at S phase entry and then peak and are degraded just before mitosis.

As briefly described above, cyclin A functions through binding to cdks. Cyclin A normally binds to cdk2 in late G1 through S phase and to cdk1 in late S phase and early G2/M. Purified clam cyclin A was shown to activate cdc2 in *Xenopus* oocyte extracts (Roy, Swenson et al. 1991). In addition, studies with cultured human fibroblasts showed cdk2 association with cyclin A and activity (Rosenblatt, Gu et al. 1992). Furthermore, Pagano and colleagues clearly demonstrated dual aspects of cyclin A, suggesting that cyclin A is required at two points in the cell cycle (Pagano, Pepperkok et al. 1992). They injected anti-cyclin A antibodies at two different times, during G1 phase and S phase and found inhibition of DNA synthesis in S phase and entry into mitosis in early G2/M, respectively (Pagano, Pepperkok et al. 1992). In this report, the authors suggested that cyclin A would form a complex with cdk2 and cdk1 for the two distinct activities.

Spatial expression of cyclin A revealed that two different cyclin A proteins exist with distinct roles. A second cyclin A (cyclin A1) was reported 10 years after first cyclin A (cyclin A2) was reported, cyclin A1 was considered the embryonic form

and cyclin A2 as the somatic form (Yam, Fung et al. 2002). Cyclin A1 was demonstrated in mouse germ cells from testes and oocytes (Sweeney, Murphy et al. 1996) and in human testis (Yang, Morosetti et al. 1997). Cyclin A1 complexes with cdk2 and has kinase activity similar to that reported for cyclin A2. A knockout study in mice showed only defective spermatogenesis, but no other defects with loss of cyclin A1 (Liu, Matzuk et al. 1998). In contrast, targeted deletion of cyclin A2 in mice results in embryonic lethality, suggesting cyclin A2 is essential for embryo development and that no other cyclins can compensate for its function (Murphy, Stinnakre et al. 1997). However, the ability of cyclin A2 to compensate for cyclin A1 was not investigated in these studies (Murphy, Stinnakre et al. 1997).

It is well documented that cdk2 exerts its kinase activity throughout late G1 and S phase. In yeast, only one type of cdk is present and controls the cell cycle, cdc2 in the fission yeast, *Schizosaccharomyces pombe* and cdc28 in the budding yeast, *Schizosaccharomyces cerevisiae* (Morgan 1997). In mammalian cells, different cdks govern specific cell cycle phases. Cdk2 with cyclin E promotes late G1 and S phase entry, and with cyclin A promotes S phase progression. Human cdk2 was first identified as cdc2-like p33 protein, which associates with cyclin A, (Pines and Hunter 1990). Later, Tsai and his colleagues confirmed it as a new cyclin dependent kinase, cdk2, showing that $p33^{cdk2}$ is immunologically and physically distinct from $p34^{cdc2}$ (Tsai, Harlow et al. 1991). Another group subsequently reported that cdk2 was the only cell cycle kinase to associate with cyclin E and this complex peaked during G1 phase, suggesting a possible role as a G1 phase regulator (Koff, Giordano et al. 1992). The function of cdk2 also was reported: activity of cdk2 associated with cyclin A increased in late G1 and early S phase and decreased at mitosis (Rosenblatt, Gu et al. 1992). An elegant study in 2002 by Coverley and colleges delineated distinct roles for cyclin E-cdk2 and cyclin A-cdk2. This paper demonstrated that cyclin E induces assembly of the replication complex while cyclin A activates DNA synthesis and prevents new replication complex formation (Coverley, Laman et al. 2002). Binding with cyclin partners is a way to regulate cdk2 activity. However, three different phosphorylation sites also were identified on cdk2, threonine 14, tyrosine 15 and

threonine 160 (Gu, Rosenblatt et al. 1992). Point mutations on each site revealed that phosphorylation on T14 and Y15 inhibit cdk2 kinase while phosphorylation on T160 enhances cdk2 activity. Interestingly, cdk phosphorylation peaks during S and G2 phases (Gu, Rosenblatt et al. 1992), indicating that regulation of cdk2 activity is complex rather than dependent simply on phosphorylation. Another level of regulation is present for cdk2 activity. The Cip/Kip (cdk interacting protein/kinase inhibitory protein) family, one of two families comprising cdk inhibitors (cdkis), negatively regulates cdk2 activity. p21, one member of the CIP/KIP family, forms a complex with cyclin A/cdk2 (Dotto 2000). Antiproliferation growth factor, TGF-β 1, inhibits cyclin A-cdk2 activity, by increasing association of p21 with cyclin A-cdk2 complex (Gong, Ammanamanchi et al. 2003). The inhibitory effect of p27, another CIP/KIP family member, in cell cycle progression is well documented, including in the oligodendrocyte cell cycle (Durand, Fero et al. 1998; Casaccia-Bonnefil, Hardy et al. 1999). Moreover, Blain et al defined two distinct roles of p27: cyclin A-cdk2 as an inhibitory target of p27 and cyclin D-cdk4 as a p27 reservoir (Blain, Montalvo et al. 1997). It has long been believed that cdk2 is indispensable for S phase progression. However, recent studies demonstrated that cdk2 knockout mice are viable showing only a minor delay at S phase, indicating redundant functions between cdks (Berthet, Aleem et al. 2003; Ortega, Prieto et al. 2003). However both female and male cdk2 knockout mice are sterile (Berthet, Aleem et al. 2003; Ortega, Prieto et al. 2003).

Overall, it is clear that DNA replication is a major event controlled by cdk2 in complex with either cyclin E or A at S phase. Orchestrated action of each participator of S phase progression at different levels is critical for accurate DNA replication and inhibition of rereplication.

1.3.4 G2/M transition and major regulators

Once cells complete DNA replication and chromosome duplication, they go through another transition or checkpoint at G2 phase before mitosis and transit into mitosis. G2/M transition is governed by mitosis promoting factor (MPF), which consists of cdk1 and cyclin B (Lindqvist, Rodriguez-Bravo et al. 2009). Cdk1/cyclin

A complex was also considered a MPF, but recent work published in 2007 demonstrated that cdk1/cyclin B is a MPF, but not cdk1/cyclin A. These studies showed that down-regulation of cyclin A caused arrest at G2, rather than at entry into mitosis (Fung, Ma et al. 2007). Three subtypes of cyclin B have been reported so far, cyclin B1, cyclin B2, and cyclin B3. All of the cyclin B subtypes bind to cdk1, but their cellular localization is different. Cyclin B1 is associated with microtubules and translocated into the nucleus in late G2 phase. In contrast, cyclin B2 is associated with intracellular membranes in the Golgi apparatus in most of the cell cycle (Jackman 1995; Jackman, Firth et al. 1995). Specific knockout of either cyclin B1 or cyclin B2 demonstrated that cyclin B1 is essential for embryo development while cyclin B2 has an important role in meiosis (Brandeis, Rosewell et al. 1998) even though cyclin B1 is sufficiently expressed during spermatogenesis (Chapman and Wolgemuth 1993). Expression of cyclin B3 shows restricted expression in germ line cells and in adult testis (Nguyen, Manova et al. 2002).

Diverse activity of cdk1 has been well documented. As a maestro, activated cdk1 phosphorylates many substrates related to serial events in mitosis such as nuclear envelop breakdown, chromatin condensation, chromosome separation, spindle assembly and Golgi fragmentation (Nigg 2001). One group identified about 200 cdk1 substrates, using a proteomic library in the budding yeast (Ubersax, Woodbury et al. 2003), including proteins related to cdk1 regulation, DNA replication, mitosis, spindle assembly, actin polarization and so on. Moreover, a cdk1 knockout study demonstrated that cdk1 is the only essential kinase to drive the cell cycle in mammalian cells, showing cdk1 is able to bind to all the cyclins, phosphorylate pRb and turn on genes regulated by E2F transcription factor families (Santamaria, Barriere et al. 2007).

Among several ways of regulating cdk activity, the expression levels of the partner cyclin is fundamental. Both mRNA and protein levels of cyclin B increase during G2 phase, and cyclin B protein is abruptly destroyed at mitosis (Pines and Hunter 1989). Transcription of cyclin B1 is regulated by several transcription factors, such as B-Myb, NF-Y and p300, which are activated by cyclin A/cdk2

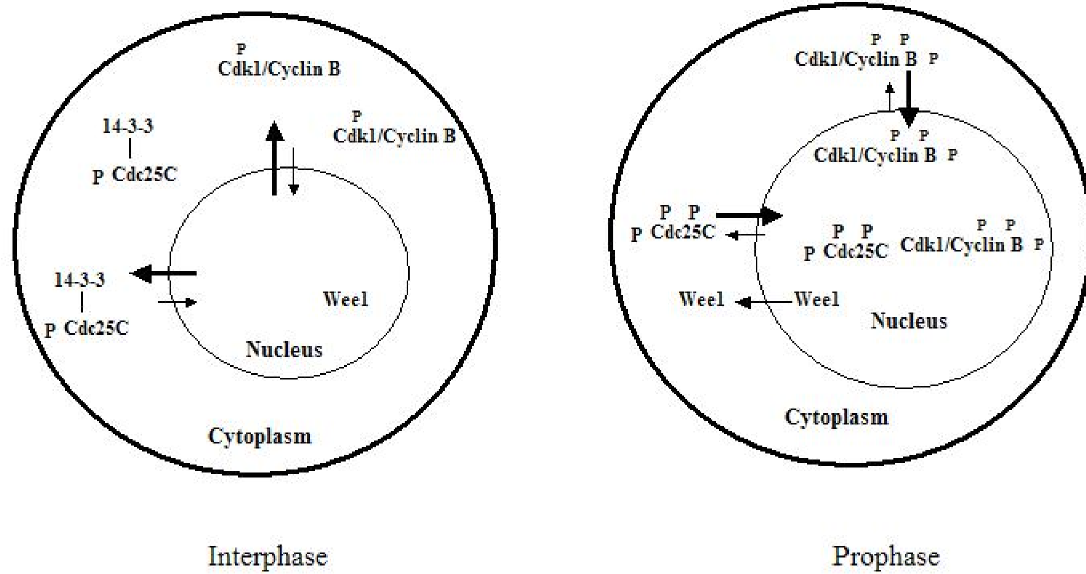
phosphorylation (Fung and Poon 2005). Cyclin B is destroyed in two different ways, depending on the cell cycle phase. An early study with purified clam cyclin A showed that exogenous cyclin A prevents degradation of both endogenous cyclin A and B (Roy, Swenson et al. 1991). Repression of E2F transcription factor or inhibition of cyclin A associated kinase activity in mammalian cells trigger cyclin B1 destruction, confirming previous results (Lukas, Sorensen et al. 1999). Degradation of cyclin B mediated by APC/C in metaphase is an important event to trigger mitosis.

Different subcellular localization of proteins provides another level of regulation of certain proteins (illustrated in Fig. 1.4). Cyclin B accumulates in the cytoplasm during interphase in association with cdk1 (Pines and Hunter 1991). In contrast, cyclin B translocates into the nucleus in mitosis. Several lines of study have elucidated the mechanisms by which cyclin B shuttles between the cytoplasm and nucleus. Yang et al reported that CRM1, a member of the importin β family (Fornerod, Ohno et al. 1997; Stade, Ford et al. 1997), is responsible for cyclin B cytoplasmic localization. They used leptomycin B, which interferes with cyclin B and CRM1 interaction and further showed that phosphorylations on the cytoplasm retention signal (CRS) on cyclin B inhibit interactions between cyclin B and CRM1 and cause nuclear localization of cyclin B in *Xenopus* oocytes. Live cell imaging, using fusion proteins between human cyclin B and GFP, confirmed the results from *Xenopus* (Hagting, Jackman et al. 1999).

Phosphorylation of specific sites on cdk1 contributes to cdk1 activity. Phosphorylation on threonine 14 (T14) and tyrosine (Y15) maintains inactive cdk1 while phosphorylation on threonine 161 (T161) is necessary for cdk1 activity (Morgan 1997). Phosphorylation on T14 and Y15 is regulated by a balance between the kinases, Wee1 and Myt1, and the phosphatase, cdc25. The wee1 kinase exclusively phosphorylates Y15 (McGowan and Russell 1993) and is present in the nucleus (Heald, McLoughlin et al. 1993). The Myt1 kinase has dual activity to phosphorylate both T14 and Y15 and it is membrane associated (Mueller, Coleman et al. 1995). The phosphatase cdc25 was identified in yeast as a mitosis inducer (Russell and Nurse 1986). A line of evidence reported that cdc25 from Drosophila (Gautier,

Solomon et al. 1991) and human cdc25 homologue to yeast (Strausfeld, Labbe et al. 1991) dephosphorylated and activated cdc2 in *Xenopus* oocytes. Three isoforms of cdc25 have been reported: cdc25A, cdc25B and cdc25C. Cdc25A was initially known for its role in G1/S transition (Jinno, Suto et al. 1994; Blomberg and Hoffmann 1999), but later it was shown to have a role in the G2/M transition (Molinari, Mercurio et al. 2000; Zhao, Watkins et al. 2002). Cdc25B and cdc25C activate cyclin B/cdk1 at the centrosome during G2/M transition (Gabrielli, De Souza et al. 1996) and in the nucleus at mitosis (Gabrielli, Clark et al. 1997), respectively. Their expression pattern is slightly different. While cdc25C expression is consistent through the cell cycle, cdc25A and cdc25B are expressed in a cell cycle specific manner (Boutros, Lobjois et al. 2007). In fact, cdc25 phosphatase activity is regulated by several mechanisms, including cyclin B/cdk1 regulation of the autophosphorylation loop (Hoffmann, Clarke et al. 1993), polo-like kinase (PLK) (Kumagai and Dunphy 1996; Roshak, Capper et al. 2000) and cellular localization (Toyoshima-Morimoto, Taniguchi et al. 2002; Myer, Bahassi el et al. 2005). Cdc25C binding to 14-3-3 protein family, following phosphorylation by checkpoint kinase, chk and ctak1, leads to cytoplasmic localization and finally inactivation (Hermeking and Benzinger 2006). The kinase responsible for T161 on cdk1 is cdk activating kinase (Cak) (Morgan 1997).

Figure 1.4 Subcellular localization of G2/M proteins and regulation. (Modified from Takizawa, CG 2000)



Interphase Prophase

1.4 Cell cycle studies in OP cells

Multicellular organisms integrate intrinsic and extrinsic signals to process essential cellular events to coordinate proper development. Both proliferation and differentiation are important for normal development. Numerous studies have investigated processes that regulate when progenitor cells cease proliferation and undergo differentiation. Results of these studies have led to the suggestion that there are two different mechanisms to regulate the final decision whether to proliferate or to exit the cell cycle and differentiate in OPs: 1) a counting mechanism, and 2) an effecter mechanism (Barres, Lazar et al. 1994; Gao, Durand et al. 1997). The counting mechanism means that cells intrinsically count the number of divisions or the time lapse while the effecter mechanism posits that extracellular cues regulate division number (Temple and Raff 1986; Barres, Lazar et al. 1994). However, a study using different growth temperatures (33˚C vs 37˚C) demonstrated that cells count the period of time prior to differentiation (Gao, Durand et al. 1997). Although cells possess intrinsic cues, extrinsic cues (effecter) also appear necessary for regulating proliferation and decision of differentiation. For example, once a mitogen such as PDGF is withdrawn from OP cells in cell culture, the cells differentiate no matter how many cell divisions they have completed. In addition, withdrawal from cell cycle also does not lead to differentiation automatically, but requires other signals such as thyroid hormone (TH) or retinoic acid (RA) (Durand and Raff 2000) for final differentiation. Taken together, it appears that both mechanisms can contribute to the transition from cell proliferation to differentiation in OP cells. Components of the effecter mechanism are mitogen or growth factors, which are discussed in section 1.2 and will no longer be reviewed here. Since proliferation is defined as progression through the cell cycle, cell cycle regulatory molecules function to regulate cell cycle exit or continuous cycling. Hence, in this section I will review how cell cycle regulatory molecules control the link between proliferation and differentiation.

Several studies have shown that cdk2, critical for the G1/S transition, plays an important role in cell cycle exit. Decreased cdk2/cyclin E complex causes G1 arrest and further reduces cdk2 activity resulting in withdrawal from the cell cycle in

primary OP cell culture (Ghiani and Gallo 2001). Cdk2 activity is high in dividing OP cells and downregulated in adult brain (Ghiani and Gallo 2001). Expression of dominant negative cdk2 significantly decreases proliferation due to reduced cdk2 activity, but the growth arrest caused by dominant negative cdk2 fails to induce differentiation (Belachew, Aguirre et al. 2002).

Cdkis inhibit cell cycle progression and arrest cells at specific cell cycle phases. Numerous researchers have focused on the relationship between cdkis and differentiation. Accumulation of p27 is observed in differentiated OP cells (Casaccia-Bonnefil, Tikoo et al. 1997; Durand, Gao et al. 1997), and OP cells in p27 knockout mice rarely undergo differentiation (Casaccia-Bonnefil, Tikoo et al. 1997). Ectopic expression of p27 inhibits cell division even in the presence of mitogen and abolishes cdk2 activity (Tikoo, Osterhout et al. 1998; Tang, Beesley et al. 1999) although these cells fail to express the late progenitor stage antigen O4 or mature oligodendrocyte markers including GalC and MBP (Tikoo, Osterhout et al. 1998; Tang, Beesley et al. 1999), suggesting that cell cycle arrest is not sufficient for differentiation. Taken together, growth arrest results from accumulation of p27 (Friessen, Miskimins et al. 1997; Ghiani, Eisen et al. 1999) without a change in expression of cell cycle regulatory molecules (Friessen, Miskimins et al. 1997). In contrast to p27, loss of p21 does not arrest OP cells, although p21 is required for normal differentiation (Zezula, Casaccia-Bonnefil et al. 2001). From the results described above, it can be concluded that cell cycle exit alone does not result in differentiation and that other extrinsic factors are needed for OL differentiation.

p53 is a tumor suppressor gene and sequence specific transcription factor that regulates expression of many genes related to cell cycle arrest and apoptosis (Billon, Terrinoni et al. 2004). Initial studies of localization of p53 in OP cells, neurons and PC12 cells suggested a role in differentiation of oligodendrocyte lineage cells (Eizenberg, Faber-Elman et al. 1996). Expression of a dominant-negative p53 inhibits differentiation, sequestering p53 exclusively in the cytoplasm (Eizenberg, Faber-Elman et al. 1996). Furthermore, expression of a dominant-negative p53 blocks TH or RA induced differentiation (Tokumoto, Tang et al. 2001). When dominant-negative

p53 was expressed with TH or RA, differentiation was blocked, although the affected molecules are slightly different (Tokumoto, Tang et al. 2001). However, expression of dominant-negative p53 does not affect differentiation related to PDGF withdrawal (Tokumoto, Tang et al. 2001). These results indicate that p53 is closely related to the TH or RA pathways inducing differentiation. A recent paper agreed with the previous role of p53, supporting a role in TH induced OP cell differentiation (Billon, Terrinoni et al. 2004). Moreover, p73, a member of the p53 family, controls both TH induced differentiation and differentiation induced by PDGF withdrawal in OP cells (Billon, Terrinoni et al. 2004).

1.5 Contribution of IGF-1 in cell cycle

Growth factors mediate multiple cellular processes such as proliferation, cellular growth, differentiation, survival and migration. One of the best studied actions of growth factors is in the control of proliferation and cell number, by regulating cell cycle progression. However, the final effect of each growth factor results from the coordination of the intracellular environment within the cell and the extracellular environment where the cell is located. Therefore, different cellular responses result from different tissue environments or cellular maturation stages. As depicted in section 1.3.2, IGF-1 participates in multiple cellular events in oligodendrocyte lineage cells. Since the goal of this dissertation is to investigate the role of IGF-1 on cell cycle regulation, this section will review literature that address how IGF-1 contributes to proliferation and cell cycle progression.

Several studies have reported that IGF-1 acts as a mitogen in the brain. IGF-1 overexpressing transgenic mice demonstrate an increase in brain size (Carson, Behringer et al. 1993; Ye, Xing et al. 1996) and weight due in part to increased DNA content and increased number of cells (Ye, Xing et al. 1996). Another in vivo study also concluded that IGF-1 increases cell number and decreases apoptosis (Popken, Hodge et al. 2004)), suggesting that IGF-1 may increase cell number by controlling both proliferation and survival. In contrast, IGF-1 knockout mice were characterized by a smaller brain size, reduced brain volume and number of cells in certain areas

(Beck, Powell-Braxton et al. 1995; Ye, Li et al. 2002). In vitro studies also support a role for IGF-1 in proliferation in neuroepithelial cells (Drago, Murphy et al. 1991), OP cells (McMorris and Dubois-Dalcq 1988; Masters, Werner et al. 1991; (Bhat, Hauser et al. 1992), tyrosine-hydroxylase positive neurons (DiCicco-Bloom and Black 1988) and cortical precursors (Mairet-Coello, Tury et al. 2009).

Several studies have provided information on the mechanisms for IGF-1 proliferation. Studies in fibroblasts suggested that IGF-1 acts primarily as a progression factor to stimulate G1 progression in the presence of PDGF, a competence factor (Stiles, Capone et al. 1979) Wharton, Van Wyk et al. 1981; Russell, Van Wyk et al. 1984). IGF-1 promotes progression to the G1/S transition and DNA synthesis including inducing the cyclin regulatory proteins in mammary epithelial cells, (Stull, Richert et al. 2002). In cortical precursors, both in vivo and in vitro, IGF-1 induces G1 cyclins and downregulates p27 and p57 resulting in increased proliferation (Mairet-Coello, Tury et al. 2009). Finally, studies on neuroepithelial cells from IGF-1 overexpressing transgenic mice provided evidence that IGF-1 decreases total cell cycle duration and G1 phase length and increases mitotic index and cell cycle reentry in ventricular zone neural precursors at embryonic day 15 but does not affect the duration of G2/M and neurogenesis (Hodge, D'Ercole et al. 2004). Taken together, these results suggest that IGF-1 regulates G1 progression with or without other growth factors.

Nevertheless, several groups claim that IGF-1 functions at later cell cycle phases. In 1994, Sell and colleagues suggested that IGF-1 regulates not only early cell cycle but also later cell cycle events. They demonstrated that all cell cycle phases are lengthened in fibroblasts lacking the IGF-1R (R- cells) (Sell, Dumenil et al. 1994). Another group reported that mGrb10 α, a protein that interacts with the IGF-1R and inhibits growth, does not affect S phase entry, but delays cells in S and G2 phases (Morrione, Valentinis et al. 1997). Studies in uterine epithelial cells in IGF-1 null mice agreed with the previous results, showing G2 arrest without an effect on G1 and S phase progression (Adesanya, Zhou et al. 1999). Recently, Stromberg and colleagues demonstrated that blocking IGF-1R resulted in G2/M arrest in multiple

myeloma cells, by suppressing cdk1 activity (Stromberg, Ekman et al. 2006). These studies suggest not only a novel role of IGF-1 in S and G2/M phases, but also support the hypothesis that extracellular signals can regulate cell cycle progression after cells pass the G1 restriction point, challenging the traditional view of cell cycle progression.

The cell cycle is regulated by coordination of multiple events induced by positive regulators or negative regulators. In order to control cell cycle progression, IGF-1 must act on at least one of these molecules. Based on variety of studies, it appears that IGF-1 directly regulates expression of positive regulators, including the G1 cyclins, cyclin D and cyclin E (Lai, Sarcevic et al. 2001; Wilker, Lu et al. 2005; Kashima, Shiozawa et al. 2009; Ren, Zhong et al. 2009), as well as cyclin A (Wilker, Lu et al. 2005; Loladze, Stull et al. 2006), and cyclin B (Loladze, Stull et al. 2006). In one study, data also supported a role for IGF-1 in decreasing negative cell cycle regulators (Lai, Sarcevic et al. 2001). IGF-1 enhances complex formation of cyclins and cdks (Lai, Sarcevic et al. 2001; Wilker, Lu et al. 2005) and controls cellular localization of regulators (Agudo, Ayuso et al. 2008). Thus, the final molecular targets of IGF-1 are cell type specific and varied, depending on cellular environment.

Multiple growth factors are present in vivo and work coordinately to regulate cell proliferation. Studies from several laboratories including our own have investigated how the combination of growth factors affects cell cycle progression. Our data suggest that IGF-1 by itself, is a weak mitogen in OP cells, but enhances S phase entry in OP cells when in combination with well-known mitogens such as PDGF and FGF-2 (Jiang, Frederick et al. 2001). Two additional reports investigated the molecular mechanisms by which IGF-1 regulates OP cell cycle in coordination with FGF-2 (Frederick and Wood 2004; Frederick, Min et al. 2007). Our laboratory is not the only group to suggest combinational effects of growth factors that include IGF-1. Activation of IGF-1 depends on transactivation of epidermal growth factor (EGF) for proliferation of hepatocytes (Hallak, Moehren et al. 2002). In contrast, IGF-1 is required for EGF mediated cycle progression in mammary epithelial cells (Stull, Richert et al. 2002). IGF-1 and EGF also synergize to stimulate proliferation of

human esophageal epithelial cells (Qureshi, Tchorzewski et al. 1997). IGF-1 combined with estrogen (Lai, Sarcevic et al. 2001) or 17 β estradiol (E2) also enhances G1/S progression in breast cancer cell lines (Hamelers, van Schaik et al. 2002).

Taken together, it can be concluded that whereas the ability of IGF-1 to regulate the cell cycle is well established its mechanisms of action are diverse. In certain circumstances, it acts as a competence factor, which gives a cell the ability to respond to a progression factor, which by itself is rarely a mitogen (Winston, Dong et al. 1996). However, IGF-1 also has been reported to be a progression factor. The traditional view of cell cycle regulation is that a cell responds to extracellular signals only at the G1 phase before the restriction point and then finishes the cell cycle without any further cues (Sherr 1996). The cell cycle is controlled at several levels and each level is finely regulated by multiple factors to prevent incorrect division, which means that fine-tuning is preferred for accuracy. Therefore, it is possible that each event is governed by the coordination of immediate signals from the environment and intrinsic signals received during the G1 phase.

1.6. FGF-2 and IGF-1 signaling pathway

1.6.1. Signaling pathway mediated by FGF-2 in OP cells

FGF-2 elicits its biological effects by transducing signals through tyrosine kinase receptors, FGFRs. As discussed previously, the FGFR family consists of 4 different types, R1-R4, encoded by 4 separate genes. FGFRs share a common structural similarity. Each receptor contains, 1) extracellular domains composed of 2 or 3 cystein-flanked immunoglobulin like domains, an acid box, a heparin binding region and a cell adhesion molecule recognition domain, 2) a transmembrane domain, and 3) intracellular domains composed of a tyrosine kinase domain and a cytoplasmic terminus with several tyrosine residues (Ornitz 2001; Jackson, Nurcombe et al. 2006). The FGFR system contains other unique molecules, heparin or heparin sulfate proteoglycan (HSPG), which interact with FGF and increase affinity and half-life of

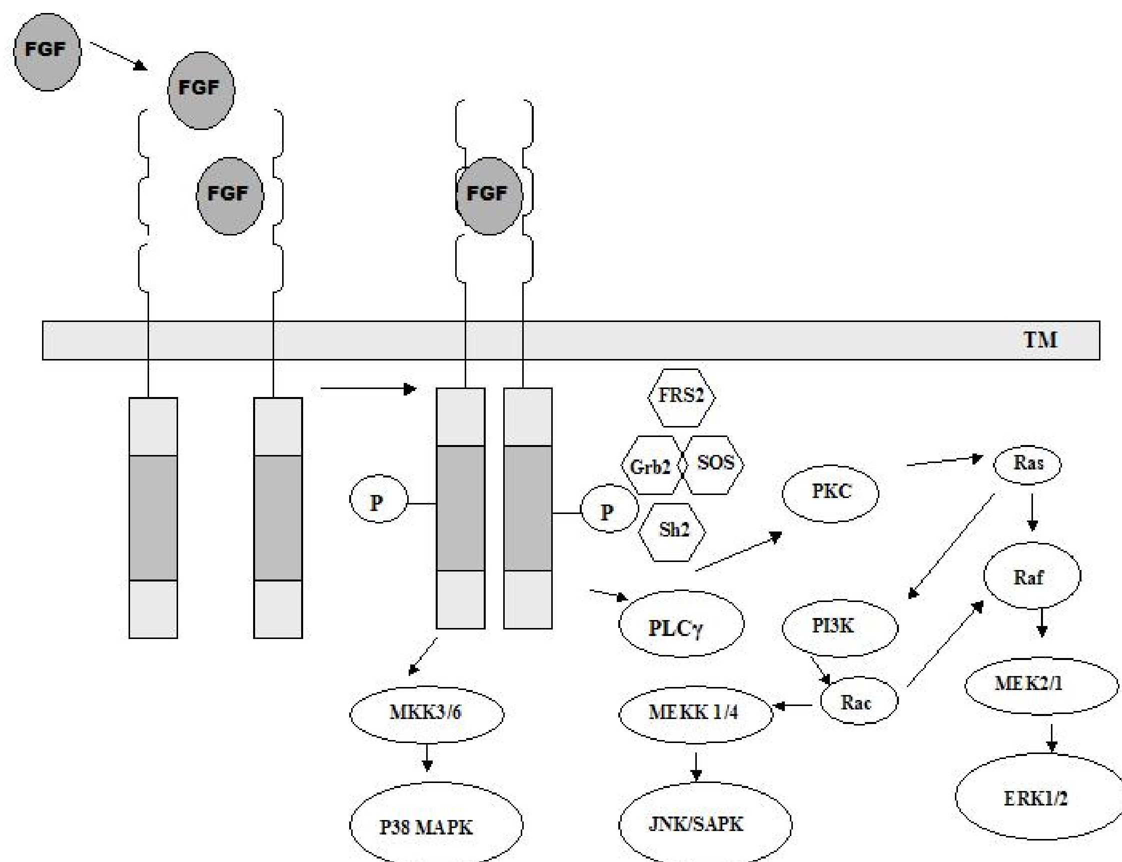
the FGF/FGFR complexes (Ornitz 2001). Upon binding of dimerized FGFs, a specific FGFR is dimerized and activated, initiating down-stream signaling cascades that are conserved in many tyrosine kinase receptor families (figure 1.5). Phosphorylated tyrosine kinase domains interact with proteins that contain Shc homology 2 (SH2) domains or phosphoprotein binding (PTB) domains (Thisse and Thisse 2005)). For example, phospholipase C γ (PLC γ) interacts with FGFRs through SH2 domains while FGF receptor substrate 2 (FRS2) binds through PTB domain (Thisse and Thisse 2005)). One of the common downstream signaling pathways activated by FGFs is the Ras oncogene pathway, which leads to proliferation in many cell types. Autophosphorylated FGFR induces tyrosine phosphorylation on FRS2, which recruits an adaptor protein, growth factor receptor bound protein 2 (Grb2). Grb2 forms a complex with a guanine nucleotide exchange factor, son of sevenless protein (Sos) that recruits Ras to the plasma membrane and activates the Ras oncogene, by exchanging GDP for GTP. Sequentially activated Ras leads to serial activation of downstream molecules, including Raf/MEK/ MAPK (ERK) signaling cascades. Other FGF-mediated signaling pathways such as PLCγ and PI3K are activated in a cell type specific manner (Cotton, O'Bryan et al. 2008).

The role of FGF-2 in oligodendrocyte lineage cells has been well established as discussed in section 1.2.1. Expression of three receptors, R1-R3 have been reported in a developmental stage specific manner (Bansal 2002). In vitro studies revealed that FGFR1 mRNA expression is low in OP cells (Bansal 2002) and upregulated in oligodendrocytes (Bansal 1996a, cohen 2000). Exposure of OP cells to FGF-2 or FGF-9 upregulates FGFR1 mRNA (Bansal, Kumar et al. 1996; Cohen and Chandross 2000) and ciliary neurotrophic factor (CNTF) in oligodendrocytes (Jiang, Frederick et al. 2001). However, FGFR2 expression in vitro is opposite to that of FGFR1 or FGFR2. FGFR2 mRNA is down regulated in the presence of FGF2 or FGF9 (Bansal, Kumar et al. 1996; Cohen and Chandross 2000; Yim, Hammer et al. 2001) and, in addition, FGFR2 mRNA is expressed upon terminal differentiation in oligodendrocytes (Bansal 2002). FGFR2 protein is also detected in oligodendrocytes in adult spinal cord (Messersmith, Murtie et al. 2000; Yim, Hammer et al. 2001),

cerebellum and corpus callosum (Cohen and Chandross 2000) in vivo. FGFR3 mRNA is maximally expressed in OP cells and downregulated in oligodendrocytes in vitro (Bansal, Kumar et al. 1996). One in vivo study reported the presence of FGFR3 protein in PDGFR-α positive cells (Messersmith, Murtie et al. 2000).

Signaling cascades by FGF-2 in oligodendrocytes has been studied mainly focusing on proliferation as an endpoint. When OP cells are exposed to FGF-2, both FGFR1 and FGFR2 are autophosphorylated (Yim, Hammer et al. 2001). This results in phosphorylation of downstream effectors, including MAPK, ERK2, ERK4 (Bhat and Zhang 1996; Cohen and Chandross 2000; Frederick, Min et al. 2007) and p70S6 kinase (Pende, Fisher et al. 1997). Phosphorylation of p44/42 (ERK 1/2), p38 MAPK and p70S6 kinase are blocked when FGF-2-mediated proliferation is inhibited by PKA via elevated cAMP (Baron, Metz et al. 2000). Inhibition of FGFRs with a specific chemical inhibitor blocks MAPK activation (Baron, Metz et al. 2000; Bansal, Lakhina et al. 2003). In other studies FGF-2 was shown to phosphorylate MARKS, which are downstream substrates of PKC (Baron, Metz et al. 2000). It is generally agreed that FGF-2 does not activate the PI3K pathway in OP cells and that PI3K is not involved in the FGF-2 mitogenic response (Baron, Metz et al. 2000; Frederick, Min et al. 2007). Overall, FGF-2 appears to activate the Ras signaling pathway for its mitogenic effects at least in OP cells.

Figure 1.5 The signaling pathway of FGFR (Modified from Jackson 2006)


FGF
FGF
FGF
FGF
TM
FRS2
Grb2
SOS
Sh2
P
P
PKC
Ras
Raf
PLCγ
PI3K
Rac
MEK2/1
MKK3/6
MEKK 1/4
ERK1/2
P38 MAPK
JNK/SAPK

1.6.2. Signaling pathways mediated by IGF-1 in OP cells

The IGF system consisting of IGF ligands, several IGF signaling receptors and IGFBPs as mentioned in section 1.2.2 play a critical role in development and adult biological processes. Two different categories of receptors that bind IGF ligands are expressed in mammalian cells, but the only IGF type 1 receptor (IGF-1R) will be reviewed here since the IGF-2R, also known as the cation-independent mannose-6-phosphate receptor, has no known signaling from binding to IGF-2 but instead removes IGF-2 from the extracellular environment and targets it for lysosomal degradation (LeRoith and Roberts 2003). The IGF-1R is a tyrosine kinase receptor, which means that it shares common downstream effectors with other tyrosine kinase receptors such as the insulin receptor. IGF-1R is a heterotetramer structurally related to the insulin receptor consisting of two extracellular α subunits and two membrane spanning β subunits (Denley, Cosgrove et al. 2005). Consistent with their localization, the α subunits contain ligand binding while the β subunits transduce intracellular signals via the cytoplasmic tyrosine kinase domain. Once IGF ligand binds to the IGF-1R, receptors undergo a conformational change in the β subunit, which causes trans-autophosphorylation of the cytoplasmic tyrosine kinase domain (LeRoith and Roberts 2003). Additional multiple phosphorylations occur in series on the tyrosine kinase domain. Among them, phosphorylation on tyrosine 950 particularly serves as a docking site for the insulin receptor substrate (IRS) and Shc adaptor proteins (LeRoith and Roberts 2003). Phosphorylation on tyrosine residues on IRS and Shc recruits other effecter proteins and results in diverse cellular responses (figure 1.6). Alternative interactions of IGF-1R with two different docking proteins, IRS and Shc, provide further complexity of signaling cascades by IGF-1 since these two proteins recruit different downstream signaling molecules.

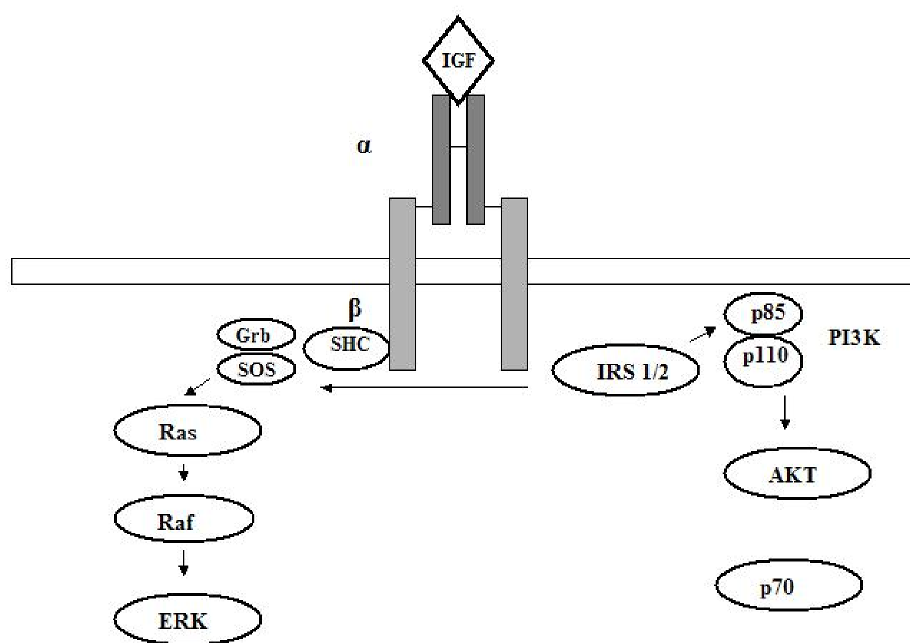
The IRS proteins function as docking proteins, with no catalytic activity. A total of six members of the IRS family have been reported to date. IRS-1 and IRS-2 are widely expressed in many tissues, including brain, muscle, heart, adipocyte, kidney, ovary and mammary gland (Chan and Lee 2008). Only rodent IRS-3 has been reported while expression of IRS-4 is limited to brain and thymus (Chan and Lee

2008). IRS-5 and IRS-6 have less similarity to other IRS members (Chan and Lee 2008). Most studies have focused on IRS-1 and IRS-2 since they are the most ubiquitously expressed. Henceforth, I will refer to IRS to mean either IRS-1 or IRS-2 unless otherwise specified. IRS is composed of an N-terminally located pleckstrin homology (PH) domain, PTB protein and multiple tyrosine and serine/tyrosine phosphorylation sites in the C terminal domain (Oldham and Hafen 2003) with which other signaling molecules interact (LeRoith and Roberts 2003). Therefore, IRS contributes unique specificities, by binding with diverse signaling molecules such as PI3K, Grb2, SHP2 (phosphotyrosine phosphatase), and the Src-like kinases Fyn and Lyn. In general, IRS binds to the p85 regulatory subunit of PI3K and sequentially activates the p110 catalytic subunit of PI3K and AKT/PKB. As just mentioned, IGF-1 triggers diverse signaling cascades owing to its various downstream effecter molecules, including the PI3K pathway and the ERK pathway that regulate biological processes by turning on several transcription factors. Another docking protein, Shc, also mediates IGF signaling in some cell types. As discussed above for FGF-2 signaling, Shc recruits the Grb2/Sos complex and activates the Ras/Raf/MEK/ERK pathway. Since IRS and Shc bind to the same site on IGF-1R, they can compete for this binding site (LeRoith and Roberts 2003). Generally, the effect of growth factors is determined by the interaction of the specific extracellular ligands with cognate receptors. However, a number of downstream signaling molecules also modulate the action of growth factors as seen for the IGF-1 signaling pathway. In fact, differential expression of downsteam targets in a cell type specific manner can lead to different signaling cascades and cellular responses.

An important role for IGF-1 in oligodendrocyte lineage cells has been well established as described in section 1.2.2. IGF-1R mRNA is expressed in OP cells (Masters, Werner et al. 1991) and rat brain in vivo (Garcia-Segura, Rodriguez et al. 1997). Furthermore, Cre-lox deletion of the IGF-1R in either OP cells or immature oligodendrocytes in mice reduces the number of oligodendrocytes and amount of myelin in the corpus callosum (Zeger, Popken et al. 2007). However the signaling pathways by which IGF-1 exerts cellular processes in oligodendroglia has not been

elucidated until recently. IGF-1 increases survival of oligodendrocytes in a PI3K pathway dependent manner (Vemuri and McMorris 1996; Ness and Wood 2002; Zaka, Rafi et al. 2005) although activation of the ERK pathway has also been reported in OP cells but only with IGF-1 concentrations higher than the receptor Kd or in mixed glial cultures (Zaka, Rafi et al. 2005; Pang, Zheng et al. 2007). Our recent studies demonstrated that IGF-1 at physiological concentrations stimulates the PI3K/AKT pathway but not the ERK pathway in OP cells (Frederick, Min et al. 2007). A recent paper reported that IGF-1 induces signaling pathways that regulate protein synthesis, including AKT, mTOR (mammalian target of rapamycin), S6 kinase and 4E-BP1 (Bibollet-Bahena and Almazan 2009). Signaling pathways and downstream targets induced by IGF-1R activation have just begun to be defined, and many new studies will be needed to elucidate the specific targets of IGF signaling in oligodendrocyte lineage cells.

Figure 1.6 The known signaling pathways of IGF-1R



IGF
α
β
Grb
SHC
SOS
Ras
Raf
ERK
IRS 1/2
p85
p110
PI3K
AKT
p70

1.7 Statement of thesis

IGF-1 is a well-characterized growth factor that mediates various biological processes in diverse cell types including in oligodendrocyte lineage cells. Relevant to oligodendrocytes, early studies have shown that IGF-1 regulates proliferation and survival of OP cells (McMorris and Dubois-Dalcq 1988; Masters, Werner et al. 1991; Bhat, Hauser et al. 1992; McMorris and McKinnon 1996). In vivo studies in which IGF-1 is overexpressed reported an increase in brain size, oligodendrocyte numbers and myelin content (Carson, Behringer et al. 1993; Goddard, Berry et al. 2001). Conversely, deletion of IGF-1 in mice results in decreased numbers of oligodendrocytes and myelin content (Beck, Powell-Braxton et al. 1995; Ye, Li et al. 2002). However, the mechanisms by which IGF-1 exerts mitogenic effects in oligodendroglia has not been well documented.

IGF-1 acts through binding to the IGF-1R, which is also activated by micromolar levels of insulin present in vitro in media for primary OP cell cultures (Saneto and de Vellis 1985). Specific deletion of the IGF-1R in oligodendroglia results in decreased OP cells as well as mature oligodendrocytes and myelin (Zeger, Popken et al. 2007). These results led us to speculate a mitogenic role for IGF-1 in OP cells. Recently our laboratory has provided evidence that IGF-1 promotes cell cycle entry in coordination with PDGF-AA and/or FGF-2 (Jiang, Frederick et al. 2001) although IGF-1 alone is a weak mitogen for oligodendrocyte lineage cells. Moreover, IGF-1 and FGF-2 promote S phase entry (Frederick and Wood 2004), by different mechanisms during G1 progression (Frederick, Min et al. 2007).

Previous studies from our laboratory, interestingly, showed that OP cells treated with FGF-2 alone fail to progress through G2/M phases even though they enter S phase. Additionally, several studies have highlighted a specific role for IGF-1 in S phase and G2/M transition in other cell types (Sell, Dumenil et al. 1994; Morrione, Valentinis et al. 1997; Adesanya, Zhou et al. 1999; Stromberg, Ekman et al. 2006). Taken together, we hypothesized that IGF-1, not FGF-2, regulates S and G2/M progression in OP cells.

Based on studies from our own and other laboratories as described in this chapter, the goal of this dissertation is to examine the specific role of IGF-1 in S and G2/M phase progression in OP cells. Thus, we hypothesize that *IGF-1 is an important cell cycle progression factor in S and G2/M phase in OP cells*. This hypothesis is tested in the following chapters:

- *In chapter three*, we examine the effect of IGF-1 on S phase progression in the presence of FGF-2 and determine the signaling pathways through which these two growth factors regulate S phase progression.
- *In chapter four*, we examine the role of IGF-1 on G2/M phase progression and define the molecular mechanism for its actions in G2/M of OP cells.

Chapter 2

Materials and methods

2.1 Materials

Cell culture medium was purchased from Gibco-BRL (Long Island, NY) or Mediatech, Inc. (Manassas, VA). Fetal bovine serum (FBS) was purchased from Gibco-BRL (Long Island, NY). Recombinant human IGF-1 was purchased from Cell Signaling Technology, Inc. (Danvers, MA). Recombinant human FGF-2 was purchased from R&D System (Minneapolis, MN). Cell culture media supplements (biotin, transferrin, progestrone, putrescine and selenite) were purchased from Sigma (St. Louis, MO). Standard laboratory reagents were purchased form Fisher Scientific (Pittsburgh, PA) or VWR (West Chester, PA). Antibodies to cyclin E, cyclin A, cdk2, cdk1, cdc25c, wee1, lamin B, lamin A/C, normal mouse IgG, normal rabbit IgG, Protein A plus agarose were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies to Rb, p21, p27 were purchased form BD PharMingen (San Diego, CA). Antibody to pHistone 3 was purchased from Upstate-Milllipore (Billerica, MA). Antibodies to P-p44/42 MAPK (Thr202/Tyr204), p44/42 MAPK, P-AKT (Ser473), AKT were from Cell Signaling Technology, Inc. (Danvers, MA). Antibodies to β-actin were purchased from Sigma (St. Louis, MO). Horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Jackson Laboratories (West Grove, PA). [γ-^{32}P]ATP and Enhanced Chemiluminescence (ECL) detection system were purchased form PerkinElmer (Waltham, MA). LY294002 was purchased from BioMol (Plymouth Meeting, PA). U0126 was purchased from Upstate-Millipore (Billerica, MA). IMC-A12 antibody and control IgG were from ImClone Systems (New York, NY). Trizol reagent and RT PCR reagents were purchased from Invitrogen (Carlsbad, CA).

2.2 Primary oligodendrocyte progenitor cell culture

Oligodendrocyte progenitor (OP) cells were prepared from newborn Sprague Dawley rat as previously described (Levison and McCarthy 1991). In brief, forebrain cortices were removed from postnatal day 0-2 rat pups and dissected. Tissues were enzymatically digested with DNase I and 2.5% trypsin and then mechanically dissociated. Cells were resuspended in MEM-C medium [minimal essential media (MEM) supplemented with 10% FBS, 2mM L-glutamine, 100 μ/ml penicillin, 100 μg/ml streptomycin and 0.6% glucose] and plated in T75 flasks at a density of $2X10^5/cm^2$. Mixed glial cell cultures were grown for 11 days and then OP cells were purified as previously described (McCarthy and de Vellis 1980). Mixed glial cell cultures were shaken for 1.5 hours at 260 rpm to remove microglia and remaining cells were shaken overnight for 18 hours to detach from astrocytes. Purified OP cells were seeded onto poly-d-lysine coated T75 flasks at a density of $2X10^4/cm^2$ in N2S media. N2S consisted of 66% of N2B2 media (DMEM/F-12 supplemented with 0.66 mg/ml BSA, 10 ng/ml d-biotin, 5 μg/ml insulin, 20 nM progesterone, 100 μM putrescine, 5 ng/ml selenium, 50 μg/ml apo-transferrin, 100 μ/ml penicillin, 100 μg/ml streptomycin, and 0.5 % FBS), 34% of B104 conditioned medium (N2B2 preconditioned by B104 neuroblastoma cell line), 5 ng/ml FGF-2 and 0.5% FBS. OP cells were amplified for 2 days and split once for another amplification before experiments were performed.

2.3 Growth factor treatment

OP cells were seeded on 60 mm dishes for western blot analysis or 100 mm dishes for flow cytometry analysis and RNA isolation or 150 mm dishes for nuclear fractionation, immunoprecipitation, and kinase assays at a density of $2.5\text{-}3X10^4/cm^2$. OP cells were starved in N1A-S media for 10-15 hours to arrest cells at G_0 phase 20-24 hours after seeding. N1A-S media contains all the supplements used for N2B2 media except with a reduced concentration of insulin, 5 ng/ml, which is sufficient to activate the insulin receptor but not the IGF-1R (Rechler and Nissley 1985). Cells were treated with N1A in the presence of IGF-1 (10 ng/ml), and/or FGF-2 (10 ng/ml), or without growth factors (control) for indicated times, depending on experiments. N1A media is identical to N1A-S with deletion of insulin. For experiments in Chapter 3 using IGF-1 blocking antibody,

IMC-A12, cells were serum starved as described above and then treated with N2S for the first 4 hours, pre-incubated with IMC-A12 antibody or control IgG for 30 minutes in N1A media and then treated with FGF-2 or IGF-1/FGF-2 with antibody or control IgG for an additional 12 hours.

2.4 Thymidine double block

OP cells were plated as described in section 2.3 and then treated with N2S media for 16 hours in 2 μM thymidine. After washing in 1X PBS twice, cells were released to N2S media for 8-10 hours, followed by a second pulse of thymidine for another 16 hours. After the double round of thymidine exposure, cells were treated with IGF-1, FGF-2, IGF-1/FGF-2 or no growth factors for specified times, depending on the experiment.

2.5 Propidium Iodide labeling for flow cytometry analysis

Cells were collected in 0.05 % of trypsin-EDTA, fixed in 70% ethanol and then stored at –20°C until analysis. Cells were incubated with RNase 1 (Sigma, St. Louis, MO) for 15 minutes and then stained with 50 mM of propidium iodide (PI). PI stained cells were analyzed by flow cytometry with Becton Dickinson FACS scan. CellQuest™ software (Becton Dickinson, Franklin Lakes, NJ) and ModFit™ (Verity Software House, Inc., Topsham, ME) were used for acquisition and analysis, respectively.

2.6 Western blot analysis and immunoprecipitation

Total cell lysates from OP cells were washed in ice-cold PBS and isolated in SDS buffer (62.5 mM Tris-HCl, 2% SDS, 10% glycol, 50 mM DTT, 1/100 protease inhibitor cocktail, 1 mM Na3VO4 and 1 mM NaF). Lysates were briefly sonicated and then subjected to a protein assay (BioRad, Hercules, CA). 15-30 μg of cell lysates were boiled at 100°C for 5 minutes and resolved on 7%, 10% or 4-12% mini gels by SDS polyacrylamide gel electrophoresis (SDS-PAGE). Separated proteins were elctrotransferred to nitrocellulose membranes and blocked in 5% milk in TBS-1% Tween buffer for 1 hour at room temperature. Membranes were incubated with primary antibodies overnight at 4°C (1:500 for pHistone 3, P-p44/42, p44/42, P-AKT, AKT;

1:5000 for β actin; 1:250 for all other antibodies). The following day, membranes were washed 3X for 5 minutes in TBS- 1% Tween and incubated with secondary antibodies, HRP-conjugated goat anti-mouse or goat anti-rabbit antibodies (1:5000) for 1 hour at room temperature. The detection of HRP-conjugated secondary antibodies was performed with enhanced chemiluminescence (ECL; Perkin Elmer, Boston, MA) using the Ultra-LUM imaging device (Claremont, CA). Protein expression levels were quantified using NIH Image 1.62 software.

Protein immunoprecipitations were performed as previously described with slight modifications (Ghiani and Gallo, 2001). Cells were harvested by scraping cells in ice-cold 1X PBS. Cell pellets were lysed in NP-40 buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 1 mM EDTA, 1 mM NaVO3, 50 mM NaF, 1 mM PMSF, 2.5 mM Napyrophosphate, and 1/100 protease inhibitor cocktail) for 45 minutes on ice. After brief sonication, protein concentration was measured using Bio-Rad kit. 500 µg of cell lysates were pre-cleared by incubating with protein A agarose for 30 min at 4°C. Supernatants were collected and incubated with 2 µg of primary antibodies overnight at 4°C. 20 µl of protein A agarose was added to the cell lysates and incubated for 1 hour at 4°C. The beads were washed 4X in NP-40 buffer and 1X in PBS. SDS buffer was added and samples were boiled at 100°C for 5 minutes. Eluted proteins were resolved on 10% SDS-PAGE gels and subjected to western blot analysis.

2.7 Kinase assay

Kinase assays were performed as previously described with slight modifications (Ghianhi and Gallo, 2001). Cells were harvested by scraping in ice-cold PBS. Cell pellets were lysed in NP-40 buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 1 mM EDTA, 1 mM NaVO3, 50 mM NaF, 1 mM PMSF, 2.5 mM Na-pyrophosphate, and 1/100 protease inhibitor cocktail) for 45 minutes on ice. After brief sonication, protein concentration was measured using Bio-Rad kit. 500 µg of cell lysates were incubated with 2 µg of primary antibodies (cyclin A, cdk2, or cdk1) overnight at 4°C. 20 µl of protein A agarose was added to the cell lysates and the samples were incubated for 1 hour at 4°C. The beads were washed 3X in NP-40 buffer and 2X in kinase assay buffer (50

mM HEPES pH 7.4, 50 mM $MgCl_2$, 1 mM DTT). Kinase assay reactions were performed in kinase assay reaction buffer containing 2 μg Histone H1, 20 μM ATP, and 2 μCi[γ-^{32}P]-ATP for 30 minutes at 30°C. 2X SDS buffer was added to stop the reaction and samples were heated for 5 minutes at 100°C. Proteins were eluted by centrifugation and resolved on 10% or 12% mini SDS-PAGE gels. The histone H1 band was visualized and quantified by PhosphorImager analysis.

2.8 Nuclear fractionation

Cells were harvest in PBS and pellets were collected by centrifugation at 12,000 rpm for 10 minutes. Cell pellets were resuspended in ice-cold lysis buffer (50 mM KCl, 25 mM HEPES, 1.25% NP-40, 1 mM PMSF, 0.1 mM DTT, 1:100 protease inhibitor cocktail, and 1 mM NaVO3) and incubated on ice for 5 minutes. Lysates were centrifuged at 14,000 rpm for 3 minutes at 4°C and supernatants were collected as cytoplasmic extracts. Lysates were washed in lysis buffer without NP-40 and resuspended in ice-cold extraction buffer (500 mM KCl, 25 mM HEPES, 10% glycerol, 1 mM PMSF, 0.1 mM DTT, 1:100 protease inhibitor cocktail, and 1 mM NaVO3) and gently rotated for 30 minutes at 4°C. Lysates were centrifuged at 14,000 rpm for 20 minutes at 4°C and supernatants were collected as nuclear extracts. Both extracts were stored at -80°C prior to western blot analysis.

2.9 Immunohistochemistry

OP cells were seeded onto poly-L-ornithine coated coverslips at $3X10^4$ cells/cm^2. Cells were washed twice in ice-cold PBS and fixed with 4% paraformaldehyde for 15 minutes at room temperature followed by washing 3 times in PBS. Cells were permeablized in 100% methanol for 10 minutes at -20°C for intracellular protein staining followed by two washes in PBS. Cells were incubated with blocking buffer (5% goat serum in 0.3% Triton X-100 in PBS) for 1 hour at room temperature. Primary antibodies for A2B5 (1:3) and pHistone 3 (1:300), cyclin B (1:100), cdc25c (1:100) or Wee1 (1:100) were diluted in Triton/PBS and incubated overnight at 4°C. Residual antibodies were removed by 3X washes in PBS, and secondary antibodies (goat-anti-mouse- IgM-FITC

and goat-anti-rabbit-Alexa 546) were added and incubated for 1-2 hours at room temperature. After washing 3X in PBS, coverslips were mounted with Biomeda mounting solution (Foster city, CA).

2.10 RNA isolation and Real time Polymerase Chain Reaction (PCR)

OP cells were harvested in ice-cold PBS followed by washing twice in PBS. Cells were pelleted by centrifugation at 12,000 rpm for 10 minutes at 4°C. Pellets were stored at -80°C prior to RNA isolation.

Frozen cell pellets were thawed briefly and resuspended in 1 ml of Trizol (Invitrogen, Carlsbad, CA). Total RNA was isolated, following manufacture's instructions. The RNA concentration was measured using a NanoDrop spectrophotometer (NanaDrop Technologies, Inc.; Wilmington, DE). 1 μg of total RNA was treated with DNase 1 (Invitrogen) and cDNA was synthesized by Superscript 3 reverse transcriptase following manufacturer's protocol (Invitrogen). cDNA concentration was measured using NanoDrop spectrophotometer.

All reactions contained 1X QuantiTect SYBR Green PCR Master Mix (Qiagen, Valencia, CA), 1X QunatiTect Primer Mix (Qiagen, Valencia, CA) for cyclin A and cyclin E and 150 ng of cDNA for quantitative polymerase chain reaction (Q-PCR). All reactions were performed on the Applied Biosystmes 7900HT Fast Real-time PCR system with associated Sequence Detection System Software Version 2.2.2. (SDS2.2.2, Foster City, CA). The thermal reaction profiles for the PCR reactions were set as follows: 50°C for 2 minutes, 9°C for 10 minutes, 55 cycles of 95°C for 15 seconds and 58°C for 1 minute. Amplification levels were normalized to β actin expression level for each reaction.

2.11 Statistical analyses

Statistical analyses were performed using StatView statistical analysis software. For all experiments, one-way ANOVA was used to assess the statistical significance between treatment groups. All experiments were performed using triplicate samples and analyzed for statistical differences. Most experiments were performed at least three times

unless otherwise stated. Statistical comparisons were performed across three experiments where possible with exceptions noted.

Chapter 3

IGF-1 coordinates with FGF-2 to promote S phase progression of cell cycle in OP cells

3.1 Introduction

Oligodendrocyte progenitor (OP) cells arise in the subventricular zone in the brain and widely migrate to populate white matter and gray matter. Once OP cells reach their final destination, they proliferate and finally differentiate to mature oligodendrocytes capable of myelinating nerve axons. The distinctive feature of demyelinating diseases such as Multiple Sclerosis is loss of oligodendrocytes as well as myelin in the pathological lesions. Moreover, remyelination ultimately fails in many lesions despite the presence of OP cells around the lesions and periods of remyelination. Thus, it is important to understand how proliferation of OP cells is regulated in normal development since OP cells possess the capability to proliferate and undergo maturation into myelin forming cells.

Extracellular signals such as growth factors and cytokines regulate multiple cellular processes, including proliferation, differentiation, survival, and migration. Fibroblast growth factor-2 (FGF-2) is one of the identified mitogens for OP cells, promoting proliferation (Besnard, Perraud et al. 1989; McKinnon, Matsui et al. 1990) and migration (Decker, Avellana-Adalid et al. 2000). FGF-2 also is reported to modulate PDGF mitogenic actions by enhancing expression of its receptor (McKinnon, Matsui et al. 1990). The effect of FGF-2 on promoting proliferation of OP cells is well documented. FGF-2 also inhibits differentiation of OP cells into oligodendrocytes (Grinspan, Stern et al. 1993; Goddard, Berry et al. 2001), and has been reported to dedifferentiate terminally differentiated oligodendrocytes (Bansal and Pfeiffer 1997).

Insulin like growth factor-1 (IGF-1) is involved in various cellular processes in oligodendrocyte lineage cells, which is suggested by the expression of IGF-1 (Shinar and McMorris 1995), IGF-1R (McMorris and Dubois-Dalcq 1988; Masters, Werner et al. 1991) and IGFBPs (Mewar and McMorris 1997) in oligodendroglia. Transgenic studies

highlight the essential role of IGF-1 in oligodendrocyte lineage cells. IGF-1 overexpression in vivo significantly increases brain weight, oligodendrocyte number, and myelin sheath thickness (Carson, Behringer et al. 1993). IGF-1 administered into cerebrospinal fluid increases oligodendrocytes, CNPase (2'3'-cyclic nucleotide 3'-phosphodiesterase), and myelin proteins (Goddard, Berry et al. 2001). In contrast, disruption of IGF-1 or of the IGF-1R in oligodendroglia reduces OP cell proliferation as well as the number of mature oligodendrocytes and myelin thickness (Beck, Powell-Braxton et al. 1995; Ye, Li et al. 2002; Mason, Xuan et al. 2003; Zeger, Popken et al. 2007).

The mechanism for the mitogenic effect of IGF-1 has not been studied extensively. Our laboratory has shown previously that IGF-1 has a mitogenic effect on OP cells when in combination with FGF-2 or PDGF (Jiang, Frederick et al. 2001; Frederick and Wood 2004). In our studies, IGF-1 alone showed little effect on S phase entry (Frederick and Wood 2004) whereas another study reported that IGF-1 stimulates DNA synthesis (Cui and Almazan 2007). Studies from our own laboratory demonstrated that the combination of IGF-1/FGF-2 showed the most powerful effect on DNA synthesis versus other combinations such as FGF-2/PDGF and PDGF/IGF-1 (Jiang, Frederick et al. 2001).

The cell cycle is tightly regulated from eukaryotes to multicellular organisms, including in mammalian cells. In general, the cell cycle consists of four different phases, G1, S, G2 and M. Transition from one cell cycle phase to the next cell cycle phase is controlled by cyclin-dependent kinases (cdks) (Morgan 1997). As a serine kinase, cdks exert their control by phosphorylating numerous substrates, most of which are cell cycle related proteins. Because of their critical role in cell cycle regulation, activation of cdks is also tightly controlled by availability of partner cyclins, formation of complexes with cyclins, inhibitory or activating phosphorylations, and cdk inhibitors.

Cells integrate extracellular signals and intracellular signals before they decide whether to proceed through another round of the cell cycle or to exit from the cell cycle and differentiate. Historically, it was thought that once a cell passes the G1 restriction point, it will complete the rest of cell cycle without any further extracellular signals

(reviewed (Sherr 1996). However, it was recently suggested that there might be another cell cycle restriction point where cells respond to extracellular cues although these signals are not as potent as the initial signals received during G1. In fact, several investigators have suggested that IGF-1 might have a specific role in S and G2/M phases (Sell, Dumenil et al. 1994; Morrione, Valentinis et al. 1997; Adesanya, Zhou et al. 1999). A previous finding in our laboratory supports this hypothesis. OP cells exposed FGF-2 in the absence of IGF-1 to linger in S phase, without traversing into G2/M (Frederick and Wood 2004). In contrast, OP cells cultured in the presence of IGF-1/FGF-2 progress normally to G2/M (Frederick and Wood 2004). Taken together, these results suggest that whereas FGF-2 is critical for S phase entry, IGF-1 has a distinct role in promoting S phase progression.

In this chapter, we examine how IGF-1 promotes S phase progression in coordination with FGF-2. We investigate the underlying molecular mechanisms for S phase progression, including protein expression of cyclin A and cdk2, holoenzyme formation and finally kinase activity. Further, we explore signaling pathways regulating these cell cycle targets to determine whether IGF-1 and FGF-2 act via similar or distinct pathways.

3.2 Results

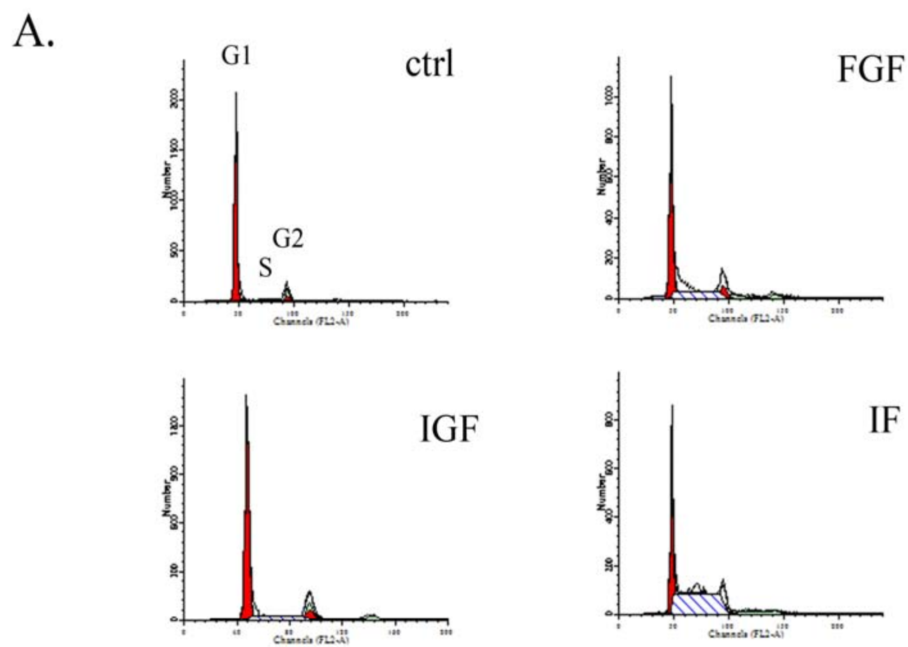
3.2.1 IGF-1 accelerates S phase entry of OP cells in coordination with FGF-2

In order to investigate how IGF-1 and FGF-2 regulate cell cycle progression of OP cells, we examined how cells were distributed in different cell cycle phases over time following growth arrest and growth factor stimulation as described in the Methods. Previous studies reported that the binding affinity (Kd) of IGF-1 to IGF-1R is 0.2 nM (Pandini, Frasca et al. 2002); in these studies, we used 1.3 nM (10 ng/ml) of IGF-1, which is standard concentration for maximally stimulating the IGF-IR at physiologically relevant concentrations without cross-activating the insulin receptor. Rusnati et al reported that the Kd of the FGFR for FGF-2 is 20 pM (Rusnati, Urbinati et al. 1993), and 10 ng/ml (606 pM) of FGF-2 showed maximal effect on DNA synthesis in OP cells

(Wolswijk and Noble 1992). We used 10 ng/ml of FGF-2 for our studies. We utilized propidium iodide to stain DNA, which intercalates into cellular DNA, and then analyzed stained cells using flow cytometry. In the histograms shown in Figure 3.1A, the left peaks and right peaks indicate cells in G1 phase and G2 phase, respectively, for cells treated with no growth factors (ctrl) or with IGF-1 and/or FGF-2. The area between the two peaks represents cells in S phase. At T0, immediately after 15 h serum starvation, 64% and 26% of cells reside in G1 and S phase, respectively (Fig. 3.1 B, C). In the no growth factor control group, cells in G1 phase continued to increase while cells in S phase declined over the next 16 h (Fig. 3.1 B, C). FGF-2 treated cells entered G1 phase (Fig. 3.1 B) and started to appear in S phase at 8 h increasing to 28% of cells in S phase at 12 h through 16 h (Fig. 3.1 A, C). In contrast, IGF-1 treated cells were present in G1 phase (Fig. 3.1 B), but failed to enter S phase (Fig. 3.1 A, C) similar to the no growth factor treated control cells. IGF-1/FGF-2 accelerated S phase entry to 30% at 12 h and to 60% at 16 h, which is more than 2 fold higher than FGF-2 treated cells (Fig. 3.1 A, C). These results agree with the previous work in our laboratory (Frederick and Wood 2004), and suggest that IGF-1 enhances S phase entry of OP cells in the presence of FGF-2.

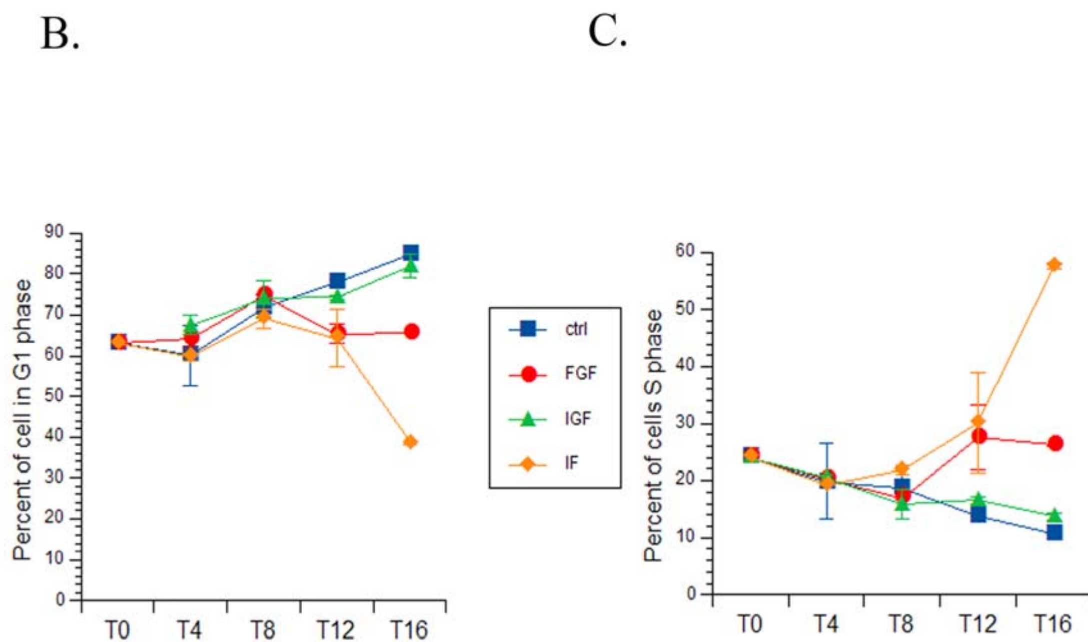
Figure 3.1. Flow cytometry analysis of OP cells. Growth arrested OP cells were treated with IGF-1, FGF-2, IGF-1/FGF-2 (10 ng/ml each) or no growth factor for 4, 8,12 and 16 hours. Propidium iodide was used to stain DNA. (A) Histogram representative of 16 hours treatment. (B, C) Percentage of cells in G1 phase (B) and S phase (C) was quantified using the DNA staining analysis software ModFit$^{TM.}$ Data reflect mean and SEM from a single experiment. Two additional experiments showed similar results.

A.



B.

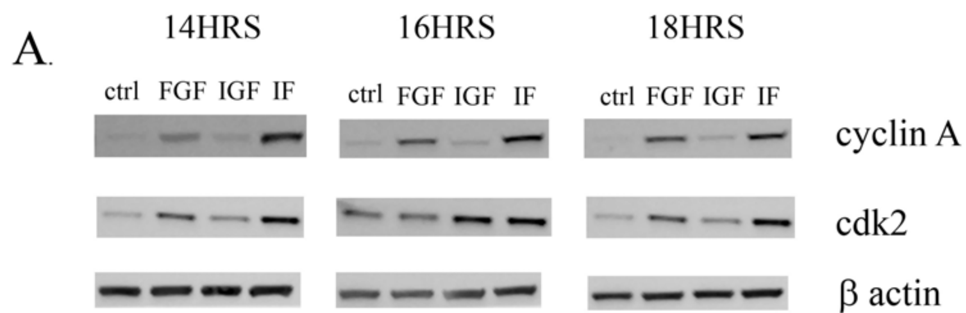
C.

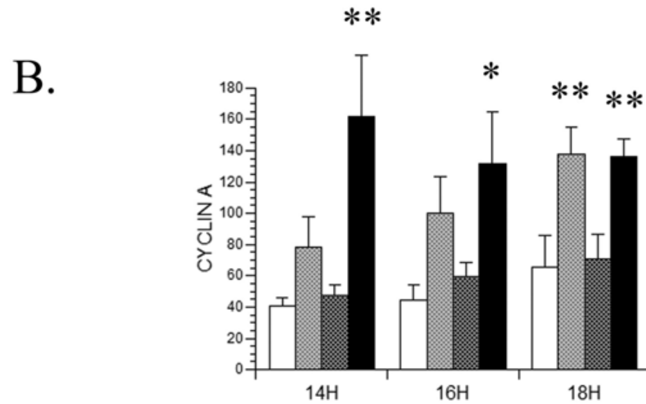


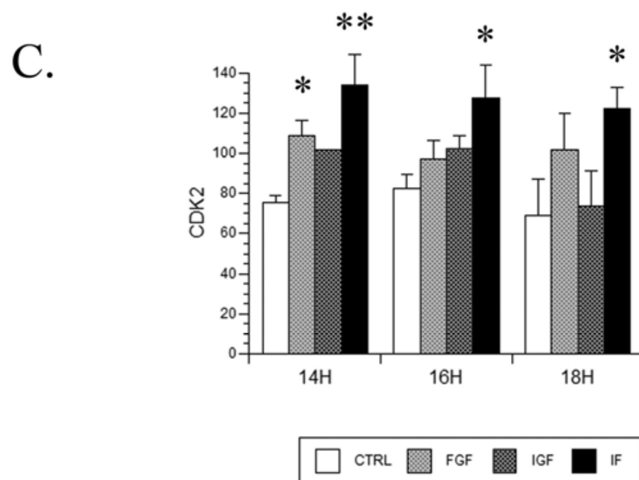
3.2.2 IGF-1 enhances protein expression of cyclin A and cdk2 in the presence of FGF-2

In order to understand the molecular mechanisms underlying cell cycle progression observed by flow cytometry analysis, we investigated protein expression of cell cycle regulators, which control S phase entry. The cyclin dependent kinase, cdk2, governs cell cycle progression from late G1 initially in coordination with cyclin E. In early S phase, cyclin A predominantly regulates cell cycle progression in complex with cdk2. Therefore we examined expression of cyclin A and cdk2 in OP cells during S phase in response to IGF-1 and/or FGF-2. Three time points following arrest and addition of growth factors were chosen for analysis based on the prior flow cytometry study. These time points, 14 h, 16 h and 18 h represent the beginning of S phase, peak of S phase and exit of S phase, respectively. FGF-2 induced cyclin A expression through S phase (Fig 3.2 A and B, ** $p<0.01$ vs. control, 18h). In contrast, IGF-1 had no effect on cyclin A expression compared to no growth factor treatment (control, Fig 3.2 A and B). The combination of IGF-1 and FGF-2 significantly increased cyclin A expression at all time points (Fig 3.2 A and B, ** $p<0.01$ vs control, 14h; * $p<0.05$ vs control, 16h; ** $p<0.01$ vs control, 18h). Cyclin A levels in IGF-1/FGF-2 treated OP cells were significantly higher than in FGF-2 treated cells at both 14 h and 16 h. The combined effect of IGF-1/FGF-2 also induced cdk2 expression. Cdk2 was highly expressed in all growth factor treated groups (Fig 3.2 A and B) at 14 h and 16 h although the combination of IGF-1 and FGF-2 maintained higher levels of cdk2 overall (Fig 3.2 A and B, ** $p<0.01$ vs control at 14 h, $p<0.05$ at 16 h and 18 h).

Figure 3.2. Protein expression levels of cyclin A and cdk2 in OP cells. (A, B, C) OP cells were growth arrested for 12 h and treated with IGF-1, FGF-2, IGF-1/FGF-2 (10 ng/ml each) and no growth factor for 14, 16, and 18 h. (A) Total cell lysates (10-30 µg) harvested at each indicated time point were processed for SDS-PAGE and western immunoblot. (B, C) Western blot results were quantified by densitometric analysis. Levels of cyclin A (B) and cdk2 (C) were normalized to β actin. Statistical analyses were performed on data from three independent experiments. Values represent the mean ± SEM for each condition. (B, C) *** $p<0.001$ vs control, ** $p<0.01$ vs control and * $p<0.05$ vs control.


A.
14HRS
16HRS
18HRS
ctrl FGF IGF IF
ctrl FGF IGF IF
ctrl FGF IGF IF
cyclin A
cdk2
β actin


B.
CYCLIN A
14H
16H
18H

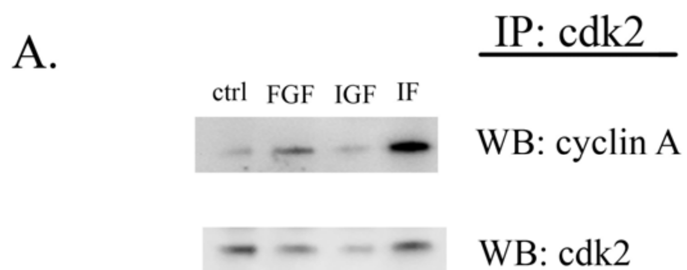

C.
CDK2
14H
16H
18H
CTRL
FGF
IGF
IF

3.2.3 IGF-1 promotes complex formation of cyclin A and cdk2

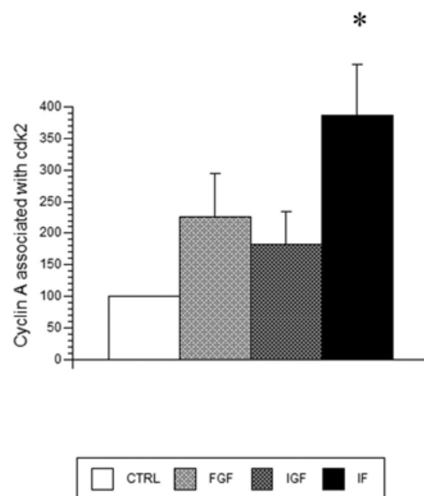
Cdk2 binds to cyclin A to form a functional holoenzyme that then has kinase activity. It is complex formation and ultimately activity of cdk2 that are most relevant to determining S phase progression. Thus we investigated how growth factors regulate these events in OP cells. Since more than 30% of FGF-2 treated cells and IGF-1/FGF-2 treated cells enter S phase between 14 h and 18 h after growth arrest and factor treatment, we chose 16 h to analyze complex formation. Cells were treated as for the previous study with different growth factors or with no growth factors for 16 h and then isolated protein was used for immunoprecipitation of cdk2. Cyclin A bound to cdk2 was elucidated by western blot analysis (Fig. 3.3A). Cyclin A associated with cdk2 was increased by 2.8 fold in FGF-2 treated OP cells as determined by expression of the data as a ratio of cyclin A/cdk2. Interestingly, IGF-1 induced cyclin A/cdk2 association by 2.2 fold compared to no growth factor control cells. However, the combination of IGF-1/FGF-2 increased cyclin A and cdk2 association by 4.4 fold versus control cells (*$p<0.05$). Taken together, the data suggest that the two growth factors are additive for cyclin A/cdk2 complex formation.

Figure 3.3. Effective complex formation of cyclin A with cdk2. (A, B, C) Growth arrested OP cells were treated with IGF-1, FGF-2, IGF-1/FGF-2 (10 ng/ml each) or no growth factor for 16 h. 500 μg of total cell lysates were immunoprecipitated with cdk2 antibodies as described in chapter 2. (A) Immune complexes were processed for western immunoblot analysis. (B) Graph represents levels of cyclin A associated with cdk2 (*$p<0.05$ vs control). Statistical analyses were perfomed on data from three independent experiments. Values represent the mean ± SEM for each condition.

A.



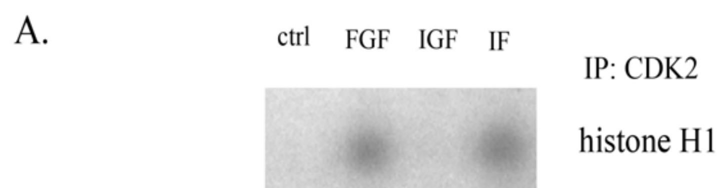
B.

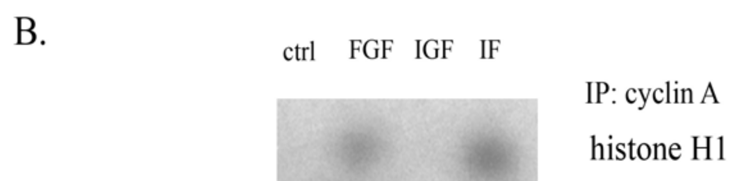


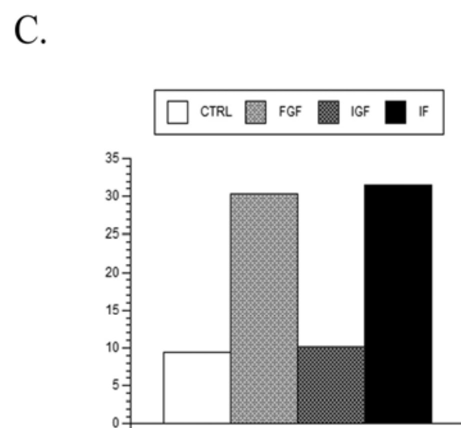
3.2.4 Combination of IGF-1 and FGF-2 enhances Cdk2 kinase activity

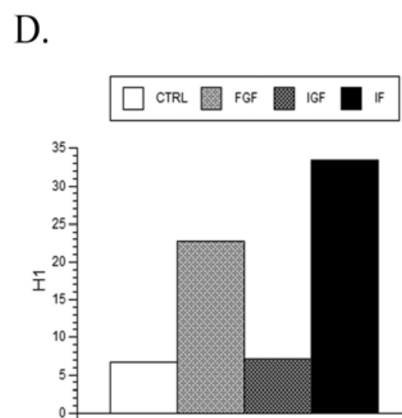
Complex formation between cyclins and cdks does not always correspond to effective kinase activity because cdk inhibitors such as p27 also bind to cdk2 and inhibit cdk2 activity. Therefore it is necessary to investigate kinase activity separately from complex formation. Kinase assays were performed, using histone H1 as a substrate following cdk2 immunoprecipitation. OP cells treated with FGF-2 showed enhanced cdk2 kinase activity by 3.3 fold (Fig. 3.4 A, C) compared to no growth factor control cells. IGF-1 treated cells failed to activate cdk2 although cyclin A and cdk2 formed a complex (Fig. 3. 4 A, B). IGF-1/FGF-2 combined treatment enhanced cdk2 activity by 3.4 fold, but this activity was not much greater than in FGF-2 treated cells (Fig. 3.4 A,C). Since cdk2 binds cyclin E (late G1 phase) as well as cyclin A (S phase), the cdk2 activity we observed could be from both cyclin E associated- and cyclin A associated- cdk2. Thus, we performed kinase assays with cyclin A immunoprecipitates to determine cyclin A associated kinase activity, specifically. FGF-2 treated OP cells showed 3.8 fold increased kinase activity (Fig. 3.4 B, D), similar to the previous cdk2 kinase assay results. Additionally, the IGF-1/FGF-2 combination increased kinase activity by 5.5 fold (Fig. 3.4. B, D). These results suggest that complex formation of cyclin A/cdk2 corresponds to kinase activity in both FGF-2 and IGF-1/FGF-2 treated OP cells but not in IGF-1 treated OP cells.

Figure 3.4. Cdk2 kinase assay and cyclin A associated kinase activity. (A-D) Growth arrested OP cells were treated with IGF-1, FGF-2, IGF-1/FGF-2 (10 ng/ml each) or no growth factor for 16 h. (A, C) Cdk2 activity was determined using histone H1 protein as a substrate. (B, D) Cyclin A associated kinase activity was determined using histone H1 as a substrate. (C, D) Autoradiographs were subjected to densitometric analysis. Experiments were repeated three times with similar results.


A.
ctrl
FGF
IGF
IF
IP: CDK2
histone H1


B.
ctrl
FGF
IGF
IF
IP: cyclin A
histone H1


C.
CTRL
FGF
IGF
IF
35
30
25
20
15
10
5
0

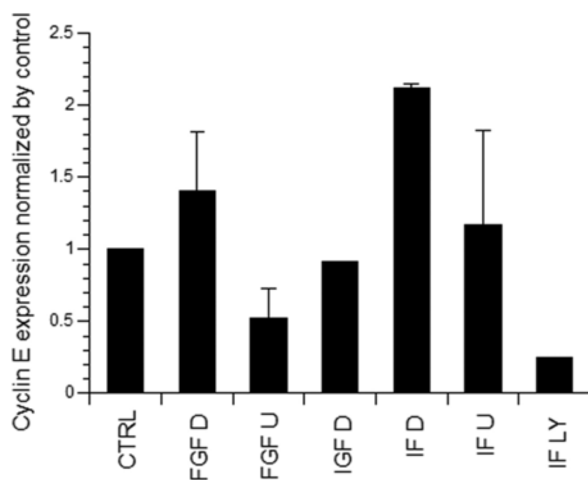

D.
CTRL
FGF
IGF
IF
H1
35
30
25
20
15
10
5
0

3.2.5 mRNA expression of cyclin E and cyclin A is regulated by both the PI3K pathway and ERK pathway.

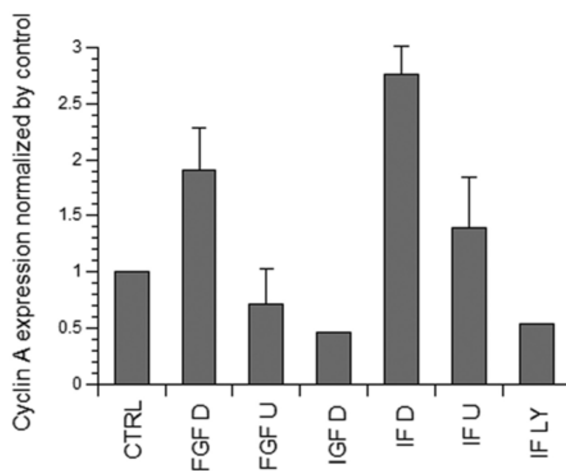
Since cyclin E (Frederick and Wood 2004) and cyclin A protein (Fig. 3.2 A, B) were highest in IGF-1/FGF-2 conditions (Fig. 3.2 A, B), we were interested to determine whether mRNA expression of cyclin E and cyclin A were also regulated by FGF-2 and/or IGF-1. Moreover, a previous study in our laboratory showed that FGF-2 induced cyclin D1 mRNA through activation of the MAPK pathway whereas IGF-1, which predominantly activates the PI3K pathway in OP cells, had no effect on cyclin D1 mRNA expression (Frederick, Min et al. 2007). Therefore, we investigated which pathways control cyclin E and cyclin A mRNA induction by IGF-1 and FGF-2. We used the specific MEK1/2 inhibitor, U0126 (10 nM) or PI3K inhibitor, LY294002 (30 nM), to inhibit MAPK and PI3K signaling pathways, respectively. Previous studies in OP cells showed that 1 μM U0126 (Horiuchi, Itoh et al. 2006) and 5 μM LY294002 (Cui, Zheng et al. 2005) caused apoptosis. The concentrations we used here did not show cytotoxicity. Since protein expression levels of cyclin E and cyclin A peaked at 16 h followed serum starvation, we chose 12 h to analyze mRNA expression. FGF-2 induced cyclin E mRNA by 1.4 fold compared to no growth factor treated cells, and blocking the MAPK pathway abolished this induction to below basal levels (ctrl-Fig. 3.5 A). IGF-1 had no effect on cyclin E mRNA expression. However, when IGF-1 was combined with FGF-2, cyclin E mRNA expression was increased by 2.2 fold compared to levels in control cells and was greater than in FGF-2 treated cells (Fig. 3.5 A). The MEK inhibitor significantly reduced IGF-1/FGF-2 mRNA expression. Surprisingly, cyclin E mRNA expression also was dramatically reduced with PI3K pathway inhibition. Cyclin A mRNA expression showed a similar pattern of growth factor regulation as cyclin E mRNA expression (Fig. 3.5 B).

Figure 3.5. Real time PCR analysis of cyclin E and cyclin A mRNA levels in the presence of specific inhibitors for the MAP kinase and PI3 kinase pathway. (A, B) Growth arrested OP cells were treated with IGF-1, FGF-2, IGF-1/FGF-2 (10 ng/ml each) or no growth factor in the presence of DMSO, U0126 or LY294002 for 12 h (see the text for details). Total RNA was isolated and real time PCR was performed using specific cyclin E, cyclin A and β actin primers. C_T values were calculated as described in Methods in Chapter 2. mRNA levels were normalized to β actin. Data are presented from two independent experiments. (n=2 replicate cultures in each experiment)

A.



B.



3.2.6 Protein expression of cyclin E and cyclin A is downregulated by MAPK pathway and PI3K pathway inhibition.

A previous study showed that the ERK pathway and PI3K pathway both are required for mRNA expression of cyclin E and cyclin A. In general, the ERK and PI3K pathways regulate transcription and protein translation of several genes, including cyclin D1 (Frederick, Min et al. 2007). Thus, we examined protein expression of cyclin E and cyclin A in the presence of the MEK pathway inhibitor, U0126 and the PI3K inhibitor, LY2940. OP cells were serum starved for 8-12 h and then treated with FGF-2, IGF-1 or both growth factors with or without inhibitors for 16 h.

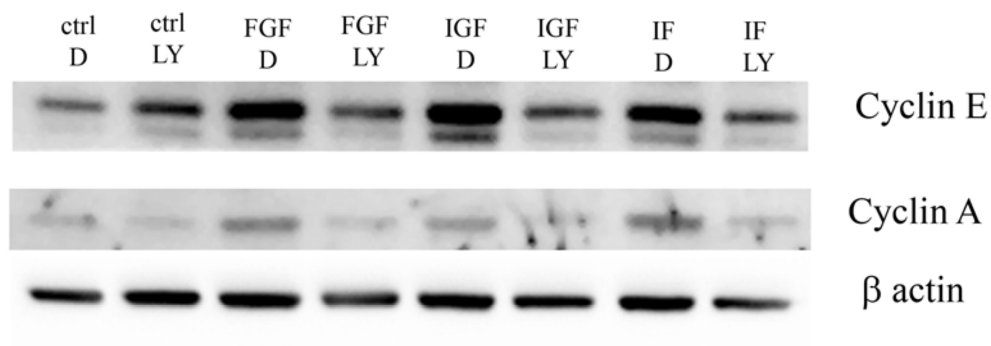
First, we determined whether the ERK pathway regulates expression of late G1 and S phase cyclin proteins in OP cells. Cells were treated as previously described in section 3.6. U0126 treatment decreased cyclin E expression overall (Fig. 3.6 A, B). Interestingly, blocking the ERK pathway down-regulated cyclin E expression up to 30% of levels in control cells. Furthermore, U0126 treatment significantly downregulated cyclin E protein expression in FGF-2 and FGF-2/IGF-1 (Fig. 3.6 A, B, * $p<0.05$ vs DMSO control). Expression of cyclin A was also downregulated by inhibition of the ERK pathway, (Fig. 3.6 A, C), but the effect was more moderate, and statistically insignificant. Since the cyclins are a critical regulatory subunit of the kinase holoenzyme, we investigated the phosphorylation status of pRb, one of the targets of cyclin E/A-associated kinase. Both FGF-2 and FGF-2/IGF-1 treated cells had a significant amount of pRb hyperphosphorylation although FGF-2/IGF-1 induced 1.5 fold more hyperphosphorylated pRb than that seen in FGF-2 treated cells (Fig. 3.6 D, E, ***$p<0.001$ vs DMSO control). The no growth factor treated control cells and IGF-1 treated cells had low levels of hyperphosphorylated pRb, and treatment with U0126 slightly inhibited pRb hyperphosphorylation (Fig. 3.6 D, E). Cells treated with FGF-2 alone in the presence of U0126 had nearly 50% reduction in hyperphosphorylated pRb (Fig. 3.6 D, E). Blocking the ERK pathway similarly inhibited phosphorylation of pRb in FGF-2/IGF-1 treated cells (Fig. 3.6 D, E).

Next, we investigated how the PI3K pathway controls cyclin protein expression. Cells were treated as previously described in section 3.6. PI3K pathway inhibitor

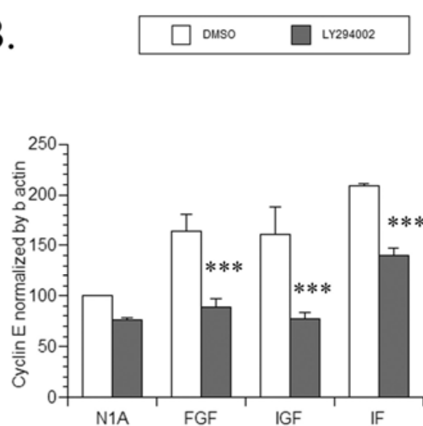
significantly downregulated cyclin E protein expression in all growth factor treatment groups (Fig. 3.7 A, B *** $p<0.001$ vs DMSO control group). The PI3K inhibitor treatment also significantly decreased expression of cyclin A protein in all growth factor treated groups, showing nearly equivalent levels to the no growth factor control in FGF-2 and IGF-1 (Fig. 3.7 A, C ** $p<0.01$ vs DMSO control). However, cyclin A levels were maintained despite PI3K inhibitor treatment (Fig. 3.7 A, C # $p<0.05$ vs FGF-LY and IGF-LY) in FGF-2/IGF-1. Hyperphosphorylation of pRb was also determined in the presence of the PI3K inhibitor. LY294007 treatment partially abolished phosphorylation of pRb in all groups to 50%, 60% and 62% of DMSO control conditions in FGF-2, IGF-1 and FGF-2/IGF-1, respectively (Fig. 3.7 D, E).

Figure 3.6. Protein expression levels of cyclin E and cyclin A, and phosphorylation of retinoblastoma protein (pRb) in the presence of a MAP kinase pathway inhibitor. (A-E) Growth arrested OP cells were treated with IGF-1, FGF-2, IGF-1/FGF-2 (10 ng/ml each) or no growth factor in the presence of DMSO or U1026 for 12 hours. (A, D) Total cell lysates (10-30 μg) harvested at 12 h were processed for SDS-PAGE and western immunoblotting. (B, C) Levels of cyclin E (B) and cyclin A (C) were normalized to levels of β actin. (E) Quantification of pRb phosphorylation is shown as a ratio of ppRb/pRb and expressed as percentage of control. Statistical anlyses were performed on data from three independent experiments. * $p<0.05$ vs DMSO control, , ***$p<0.001$ vs DMSO control.Values represent the mean ± SEM for each condition from triplicate samples in one representative experiment.

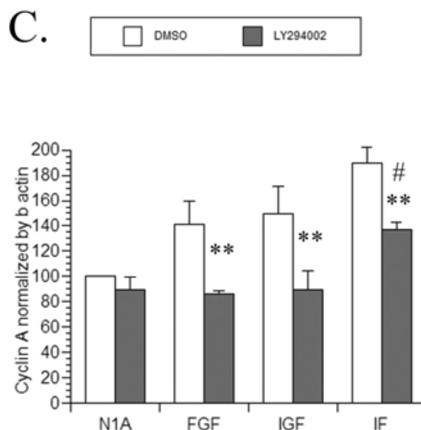
A.



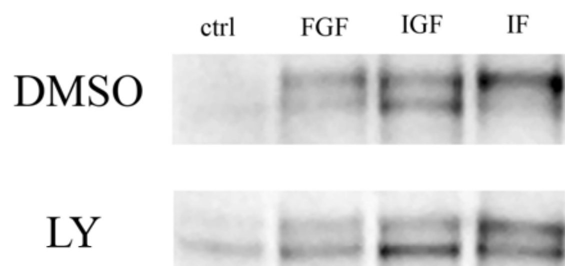
B.



C.



D.



E.

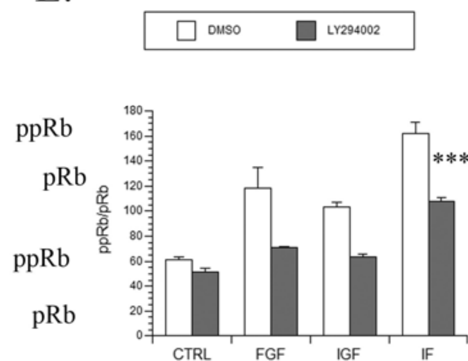
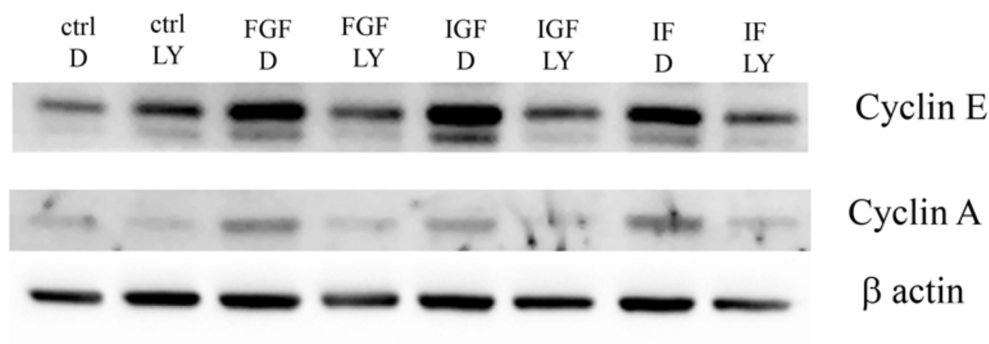
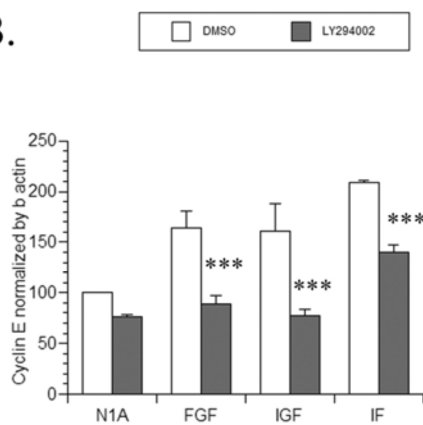


Figure 3.7. Protein expression levels of cyclin E and cyclin A, and phosphorylation of retinoblastoma protein (pRb) in the presence of a PI3 kinase pathway inhibitor. (A-E) Growth arrested OP cells were treated with IGF-1, FGF-2, IGF-1/FGF-2 (10 ng/ml each) or no growth factors in the presence of DMSO or LY294002 for 12 h. (A, D) Total cell lysates (10-30 μg) harvested at 12 h were processed for SDS-PAGE and western immunoblotting. (B, C) Levels of cyclin E (B) and cyclin A (C) were normalized to levels of β actin and expressed as percentage of control. (E) Quantification of pRb phosphorylation is shown as a ratio of ppRb/pRb. Statistical analyses were performed on data from three independent experiments. (E) ** $p<0.01$ vs DMSO control, *** $p<0.001$ vs DMSO control. Values represent the mean ± SEM for each condition from triplicate samples within one experiment.

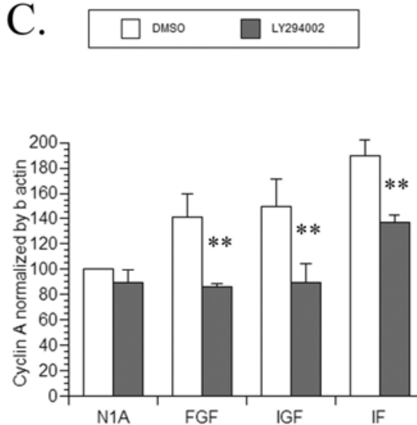
A.



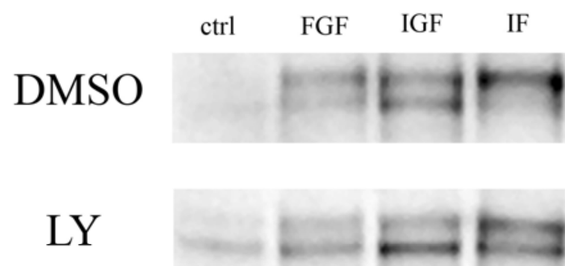
B.



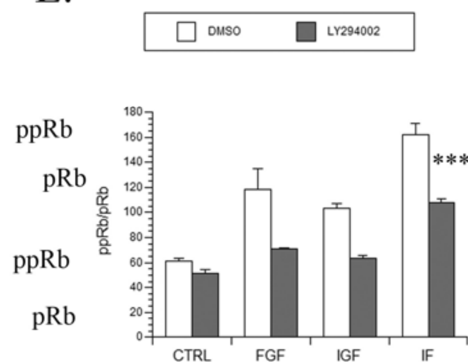
C.



D.



E.

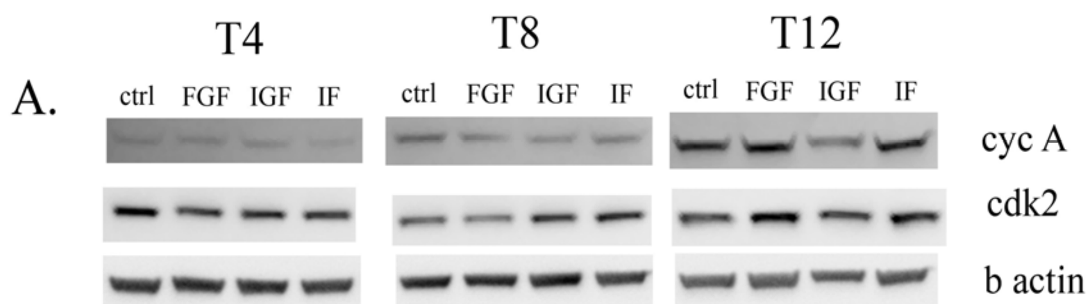


3.2.7 Initial G1 events regulate expression of S phase cyclin/cdks, but maximal phosphorylation of pRb requires an additional stimulus

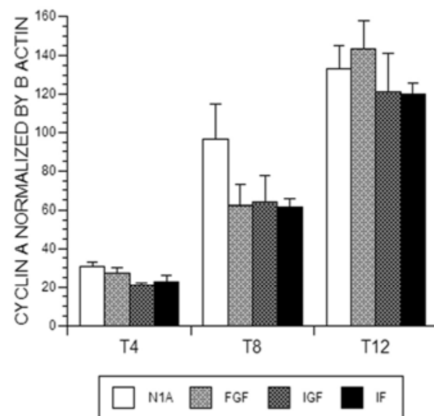
It has been postulated that once cells pass the G1 restriction point, they are committed to complete the cell cycle without any further mitogen stimulation (Pardee 1974). Induction of cyclin D early in G1 is an initiator of the G1 events leading to traverse past the G1 restriction point (Planas-Silva and Weinberg 1997). A previous study in our laboratory showed that cyclin D expression peaks in cycling OP cells at 4 h (Frederick and Wood 2004). Therefore, we were interested in testing whether exposure of OP cells to mitogen stimulation for 4 h is sufficient to induce progression through the cell cycle. Thus, we designed an experiment in which cells were growth arrested by serum and growth factor starvation for 12 h then treated with full growth media for 4 h, and finally switched to media containing FGF-2 and/or IGF-1 or no growth factors for indicated times. The levels of cyclin A and cdk2 as well as hyperphosphorylated pRb were used as indicators of S phase progression. The expression levels of cyclin A and cdk2 protein were unchanged in the different growth factor treatments (Fig. 3.8 A, B and C) at all time points. However, hyperphosphorylation of pRb was significantly increased in FGF-2 and FGF-2/IGF-1 with maximal induction seen in the combination condition (Fig. 3.8 D and E, $^{**}p<0.01$ vs control). These results suggest that the G1 complex activities and S phase entry are enhanced by growth factors subsequent to maximal cyclin D1 expression in early G1.

Figure 3.8. Protein expression levels of cyclin A and cdk2, and phosphorylation of retinoblastoma protein (pRb) after 4 h pretreatment with full growth media. (A-E) Growth arrested OP cells were treated with full growth media for 4 h and then switched to media containing IGF-1, FGF-2, IGF-1/FGF-2 (10 ng/ml each) or no growth factor. (A, D) Total cell lysates (10-30 μg) harvested at the indicated time points were processed for SDS-PAGE and western immunoblotting. (B, C) Levels of cyclin A (B) and cdk2 (C) were normalized to the levels of β actin. (E) Quantification of pRb phosphorylation is shown as a ratio of ppRb/pRb. Statistical analyses were performed on data from three independent experiments. Values represent the mean ± SEM for each condition. (E) ** $p<0.01$.

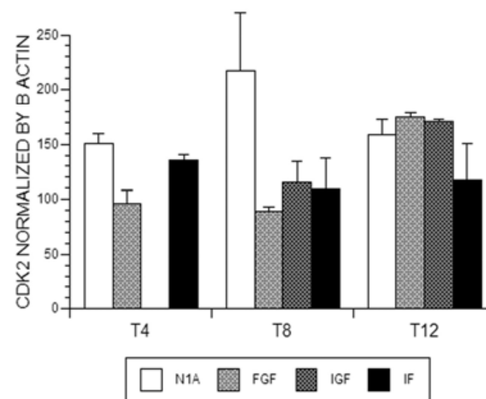
A.



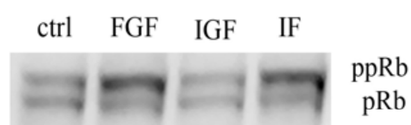
B.



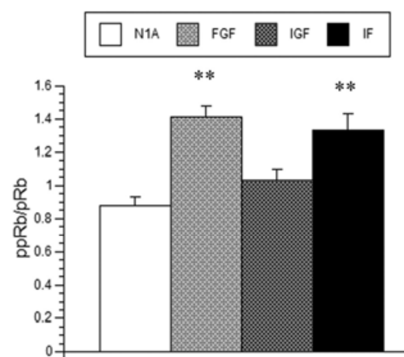
C.



D.



E.

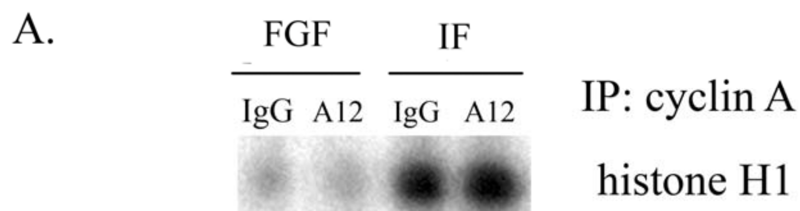


3.2.8 Effects of blocking IGF-1R signaling

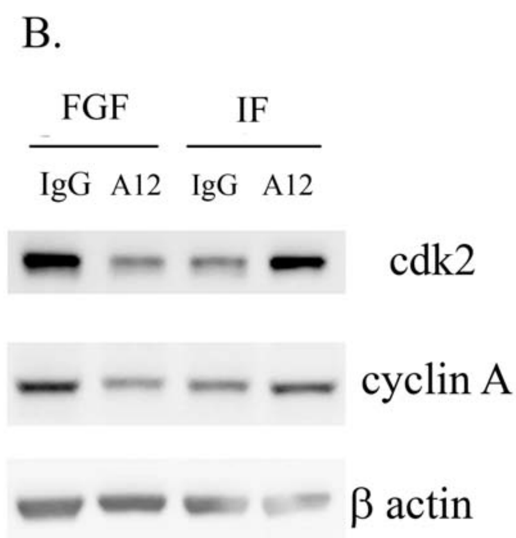
Several studies have reported that oligodendrocytes themselves secrete trophic factors, including IGF-1 (reviewed in (Du and Dreyfus 2002). A study of signaling pathways after a short exposure to FGF-2 alone showed little stimulation of the PI3K pathway in OP cells (Frederick, Min et al. 2007). However, most studies performed in this dissertation were analyzed after longer treatment times. Therefore we cannot rule out the possibility that OP cells in our culture system secrete autocrine IGF-1 that enhances FGF-2 cell cycle progression. Recently IGF-1R blocking antibodies, which specifically inhibit human and rodent IGF-1R have been developed for use in cancer treatment and also for research purposes (Burtrum, Zhu et al. 2003; Yeh, Litz et al. 2008). The previous study showed that the first 4 h of G1 is critical to induce expression of cyclin A and cdk2. To determine the specific effect of IGF-1R signaling in S phase progression, we repeated the previous experiment in the presence of IGF-1R blocking antibodies (IMC-A12) or control IgG antibodies. Serum starved OP cells were cultured in full growth media for 4 h and then switched to FGF-2 or IGF-1/FGF-2 containing media for 12 h, which corresponds to the peak of S phase. In order to determine cdk2 activity, we performed cyclin A associated kinase assays. Surprisingly, OP cells treated with IGF-1/FGF-2 in the presence of IGF-1R blocking antibody showed high kinase activity similar to control IgG treated cells (Fig. 3.9 A). These results led us to examine expression of underlying regulatory molecules, cyclin A and cdk2. Expression levels of these two molecules were unchanged by the IGF-1R blocking antibody (Fig. 3.9 B, C) In addition, we investigated molecules involved in signaling pathways activated by FGF-2 and IGF-1 to verify that the presence of the blocking antibody inhibited IGF-1 signaling. The IGF-1R antibody decreased phosphorylation of AKT, a downstream signaling target of PI3K activated predominantly by IGF-1, but similarly decreased total AKT (Fig. 3.9 D, E). Phosphorylated p44/42 (ERK 1/2) was unaffected as expected since this pathway is not activated by IGF-1 treatment in OP cells (Fig. 3.9 D, E).

Figure 3.9. Cyclin A associated kinase assay, protein expression of cyclin A and cdk2, and activation of signaling proteins in the presence of IGF-1R blocking antibody. (A, B) Growth arrested OP cells were treated with full growth media for 4 h and then replenished with media containing FGF-2 and IGF-1/FGF-2 (10 ng/ml each) in the presence of control IgG or IGF-1R blocking antibodies (IMC-A12) for 12 h. (A) Cyclin A associated kinase assays were performed with histone H1 as a substrate. (B-E) Total cell lysates (30 µg) before incubation with cdk2 antibodies were processed for SDS-PAGE and western immunoblotting. Statistical analyses were performed on data from three independent experiments. Values represent the mean ± SEM for each condition.

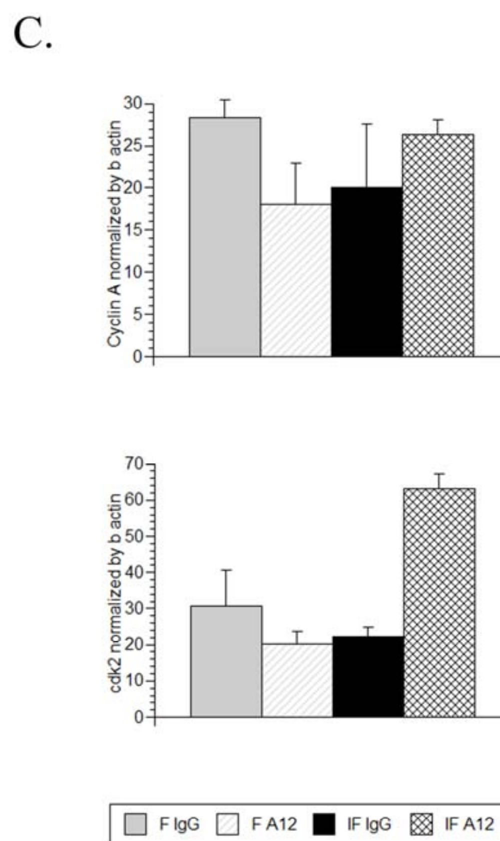
A.



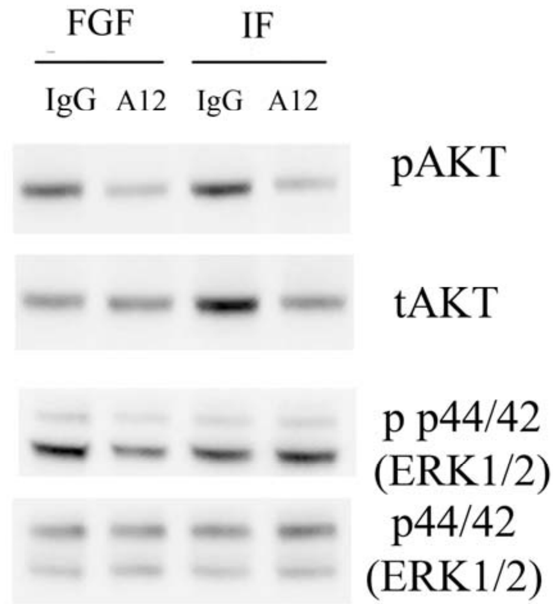
B.



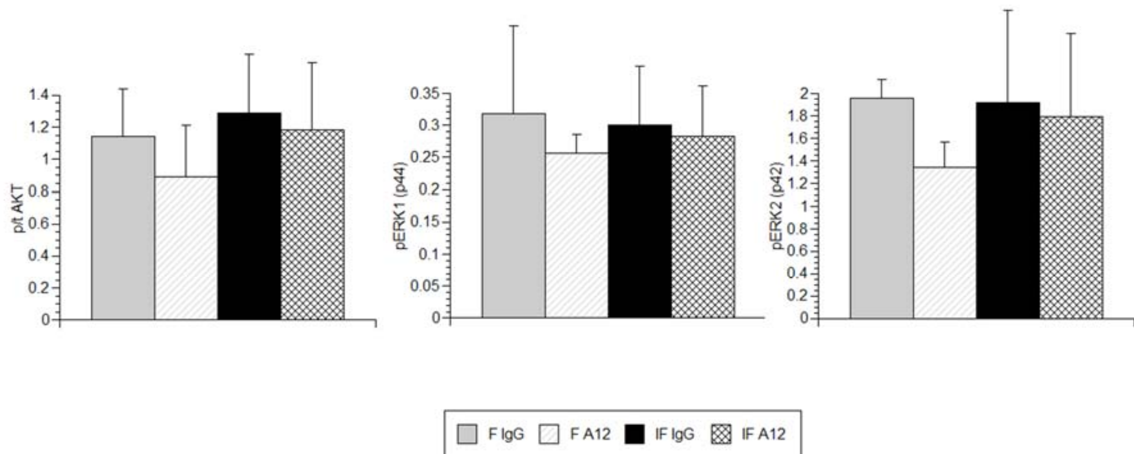
C.



D.



E.



3.3 Discussion

Growth factor stimulation promotes cell cycle progression of proliferating cells or can induce quiescent cells to enter the cell cycle. In some cell types, one growth factor is sufficient to induce a mitogenic signal while other cell types may need combined signals from several growth factors. Previous studies from our laboratory demonstrated that a combination of IGF-1 and FGF-2 in OP cells increased DNA synthesis to a greater extent than either FGF-2/PDGF or PDGF/IGF-1 (Jiang, Frederick et al. 2001). Furthermore, IGF-1 and FGF-2 promoted S phase entry, acting differentially on molecular targets (Frederick and Wood 2004). Here, we present data showing that IGF-1/FGF-2 coordinate to enhance S phase progression in OP cells.

The mitogenic effect of FGF-2 in OP cells has been well documented based on measurement of the number of cells (Saneto and de Vellis 1985; Besnard, Perraud et al. 1989). Consistent with these data, the studies presented here show that OP cells exposed to FGF-2 accumulate in S phase by 12 h (figure 3.1). Similarly, IGF-1/FGF-2 treated OP cells enter S phase at 12 h (figure 3.1). However, almost two fold more cells enter S phase in IGF-1/FGF-2 compared to FGF-2 alone, which agrees with our previous results where we also demonstrated increased cyclin D and E expression and subsequently enhanced cdk4 and cdk2 activity (Frederick and Wood 2004). Consistent with our previous findings, IGF-1 alone does not induce S phase entry. The studies here were designed to test the hypothesis that IGF-1/FGF-2 promotes S phase progression as well as S phase entry.

Expression of specific cyclins is used commonly to determine how cells are actively progressing through the specific cell cycle phases. Protein levels of cyclin A, the predominant cdk2 partner in S phase, accumulate during S phase (Pines and Hunter 1990). We showed that cyclin A protein starts to accumulate earlier and is maintained at a high level over time in OP cells treated with IGF-1/FGF-2. In FGF-2 alone conditions, cyclin A slowly accumulates but the expression level is less than observed in the IGF-1/FGF-2 combination. Cyclin A expression is positively controlled by the transcription factor, E2F, which is activated by release from hyperphosphorylated pRb (Dyson 1998).

Previous studies showed that IGF-1/FGF-2 enhances phosphorylation of pRb via increased cyclin E/cdk2 activity (Frederick and Wood 2004). In addition, we determined that almost 2 fold more cyclin mRNA is found in IGF-1/FGF-2 treated cells. Taken together, increased cyclin A expression in IGF-1/FGF-2 likely results from enhanced E2F transcriptional activity. However we cannot rule out the possibility that degradation of cyclin A, which is mediated by the APC, might be decreased by IGF-1/FGF-2. The relationship between APC and either IGF-1 or FGF-2 has not been identified although regulators of APC have been well established.

In addition to increased cyclin expression, holoenzyme formation between cyclins and cdks is important to regulate cdk activity. Co-immunoprecipitation experiments clearly showed that association of cyclin A/cdk2 is enhanced in IGF-1/FGF-2 compared to FGF-2. This result, in turn, correlated with enhanced cdk2 kinase activity in IGF-1/FGF-2. Intriguingly, FGF-2 alone showed potent cdk2 activity although cyclin A/cdk2 complex formation is less than in IGF-1/FGF-2 treated cells. Cdk2 exerts activity via binding to either cyclin E or cyclin A, therefore, we could not distinguish which cyclins were bound to cdk2 in our experiments since both cyclins are present in the cultures. Co-immunoprecipitation with cdk2 and immunodetection of cyclin A or cyclin E in immune complex will reveal the proportional association of the two cyclins with cdk2. Another interesting observation was that cyclin A/cdk2 complexes also are increased in OP cells treated with IGF-1 when compared to control cells. It is possible that IGF-1 increases association of cyclin A and cdk2. However, a cdk2 kinase assay did not show any activity in IGF-1 treated cells suggesting that the complex formed is inactive. In a previous study, our laboratory observed that a significant amount of cyclin E/cdk2 was detected in IGF-1 treated OP cells during G1, but more p27 cdk inhibitor was found in the complex of cyclin E/cdk2 since less p27 was bound to cdk4/cyclin D complex in early G1 phase (Frederick and Wood 2004). We predict that there are increased levels of p27 similarly associated with the cyclin A/cdk2 complex in IGF-1 treated OP cells, although this needs to be confirmed. It is of note that cyclin A associated kinase activity is greatly enhanced in IGF-1/FGF-2 treated OP cells versus FGF-2 alone cells unlike cdk2 kinase activity. Cyclin A is able to associate with cdk2 in S phase and cdk1 in G2 (Pagano, Pepperkok et

al. 1992; Morgan 1997). We observed that cyclin A associated kinase activity could be either cdk2 activity or cdk1 activity. Increased cyclin A/cdk1 complex formation and subsequent kinase activity may be responsible for the difference in S to G2/M transition in the two treatment groups (see Chapter 4).

Our signaling studies showed complicated results unlike the previous study in which we showed that FGF-2 induces cyclin D1 mRNA expression via the ERK pathway and IGF-1 stabilizes cyclin D1 protein via the PI3K pathway in OP cells (Frederick, Min et al. 2007). However, mRNA expression of cyclin E and cyclin A during late G1 phase is regulated by both the ERK and PI3K pathways. It was reported in a previous study that IGF-1 transiently activates the ERK pathway in OP cells (Cui, Zheng et al. 2005), and this may account for downregulation of cyclin E and A mRNA when the PI3K pathway is inhibited. The signaling studies in our laboratory showed that IGF-1 does not activate the ERK pathway (Frederick, Min et al. 2007), but use of high concentrations of IGF-1 (100 ng/ml) may result in the activation of the ERK pathway in OP cells (Cui, Zheng et al. 2005). We observed that the ERK pathway is activated in IGF-1 treated cells 16 h after growth factor stimulation (data not shown), suggesting possible indirect activation of the ERK pathway. Since we did not examine the activation status of both signaling molecules for these experiments, we cannot currently specify what mechanisms cause further downregulation of cyclin E and A mRNA expression in the presence of PI3K inhibitors. Protein expression of cyclin E and cyclin A show a similar pattern to the mRNA regulation. Inhibition of either signaling pathway downregulated cyclin E and cyclin A proteins and further abolished phosphorylation of pRb.

Induction of cyclin D initiates cell cycle progression and then sequential events allow the cells to overcome the G1 restriction point by hyperphosphorylating pRb (sherr 1996). We determined that the initial 4 h of cell cycle induction in G1, when cyclin D1 is maximal (Frederick and Wood 2004), results in expression of cyclin A and cdk2. However, our results suggest that phosphorylation of pRb is regulated by events after early G1. The lack of correlation between cyclin A protein profiles and phosphorylation status of pRb may result from a relatively low threshold of phosphorylated pRb for cyclin A transcription. In addition, it is accepted that phosphorylation of pRb is mediated by at

least two kinases, cyclin D/cdk4,6 and cyclin E/cdk2 (Zarkowska and Mittnacht 1997; Lundberg and Weinberg 1998) and additionally, cyclin A is able to phosphorylate pRb (Tsukada, Tanaka et al. 2004). Taken together, it is plausible that increased cyclin protein levels do not correspond to an increase in associated kinase activity.

In order to investigate the role of IGF-1 in S phase progression, we attempted to block the IGF-1 signaling pathway using a specific IGF-1R blocking antibody, IMC-A12. The A12 monoclonal antibody was designed to block human IGF-1R with high affinity and also recognizes the rodent IGF-1R (Burtrum, Zhu et al. 2003; Rowzee, Ludwig et al. 2009). Our experiments with the A12 antibody gave complicated results. Blocking IGF-1R does not change cyclin A associated kinase activity in FGF-2 and slightly enhances in IGF-1/FGF-2 treated OP cells. Levels of phosphorylated AKT decrease after IGF-1 treatment in the presence of the blocking Ab suggesting that the Ab treatment is effective in blocking IGF-1 signaling in OP cells. Levels of phosphorylated ERK are unchanged by the IGF-1R Ab in OP cells, which is consistent with our data showing that IGF-1 activates the PI3K pathway, but not the MAPK pathway in OP cells. Another intriguing observation was that pAKT is seen in FGF-2 treated OP cells, since our previous studies showed only a minor and brief (5 min) elevation of p-AKT in OP cells treated with FGF-2. Since cells were treated with FGF-2 for 12 h, FGF-2 activation of the PI3K pathway is likely indirect possibly due to autocrine secretion of IGF-1 (Shinar and McMorris 1995; Du and Dreyfus 2002).

The present study demonstrates that IGF-1/FGF-2 coordinately activate S phase progression, by enhancing cyclin A associated kinase activity via increased complex formation of cyclin A and cdk2. The signaling pathways underlying these effects need to be further delineated.

Chapter 4

IGF-1 promotes G2/M transition in OP cell cycle

4.1 Introduction

Cell cycle regulation past S phase is not as well studied as the earlier phases, in part, because it was proposed that once growth factors stimulate cells to overcome the restriction point, cells finish the remainder of the cell cycle without any further growth stimulation. However, data from several papers suggest that IGF-1 might have a specific role in S and G2/M phase progression, in which lack of IGF-1 causes G2/M arrest in fibroblast cells and myeloma cells (Sell, Dumenil et al. 1994; Stromberg, Ekman et al. 2006). In addition, our previous studies showed that FGF-2, a potent mitogen in OP cells, fails to promote OP cell progression through G2/M while the combination of FGF-2 with IGF-1 results in normal progression of the cells through G2/M. Therefore, we hypothesized that IGF-1 may have an important role in progression into G2/M in OP cells.

As the transition from one cell cycle phase to the next is tightly regulated, progression through the G2/M transition requires extra caution since inappropriate entry into mitosis leads to genetic catastrophes. Entry into mitosis is governed by two main regulators, cyclin B and cdk1. The importance of timely activation of cdk1 entails several levels of regulation. In addition to canonical regulation such as expression of cyclin B and complex formation of cyclin B and cdk1, other levels of control exist as well: nuclear localization of cyclin B/cdk1 complexes and activation of cdk1 by phosphorylation and dephosphorylation on inhibitory sites for temporal activation.

Subcellular localization of cyclin B is an essential regulatory mechanism to prevent premature mitosis. During interphase, the cyclin B/cdk1 complex is found predominantly in the cytoplasm (Pines and Hunter 1991), while in prophase, the complex rapidly translocates into the nucleus (Clute and Pines 1999; Hagting, Jackman et al.

1999). The effect of nuclear localization of cyclin B/cdk1 on cdk1 activation is still unclear. Early studies reported that cdk1 is activated before it translocates into the nucleus (Ookata, Hisanaga et al. 1993). Another study showed that removal of the nucleus does not affect cdk1 activity in the cytoplasm (Wasserman and Smith 1978). However, Ferrell proposed that nuclear import of cyclin B is a mechanism for switch-like control of cdk1. On the other hand, nuclear localization of cyclin B/cdk1 is required for mitotic events, which are triggered by phosphorylated substrates of cdk1, for example, nuclear envelope breakdown by phosphorylated lamins (Miake-Lye and Kirschner 1985).

Activation of cdk1 is regulated by multiple kinases and phosphatases for appropriate timing. Thr 14 and Tyr 15 on cdk1 are responsible for inactivation of cdk1 during interphase. The tyrosine kinase Wee1 and the dual-specificity kinase Myt1 phosphorylate these two sites on cdk1 in the nucleus and membranes of the endoplasmic reticulum and Golgi complex, respectively (Heald, McLoughlin et al. 1993; McGowan and Russell 1993). Cdc25 is a phosphatase, which dephosphorylates the cdk1 inhibitory phosphate resulting in cdk1 activation (Gautier, Solomon et al. 1991; Strausfeld, Labbe et al. 1991). Three isoforms of cdc25, cdc25A, cdc25B and cdc25C, have been reported to date, and their expression and activation are regulated in a cell cycle specific manner (reviewed in (Boutros, Lobjois et al. 2007). Cdc25C activation is also regulated by subcellular location (Dalal, Schweitzer et al. 1999; Kumagai and Dunphy 1999) as for the cyclin B/cdk1 complex. Cdc25C phosphorylated on Ser216 (human) binds to 14-3-3 protein and is sequestered in the cytoplasm during interphase (Kumagai, Yakowec et al. 1998).

Recently, several studies have revealed the mechanism by which signaling pathways regulate the G2/M transition. It has been well documented that Ras/Raf/MAPK pathway is involved in G1 progression (Kerkhoff and Rapp 1998; Takuwa and Takuwa 2001), however the underlying signaling pathways regulating G2/M have not been fully established. However, ectopic expression of MAPKK, a molecule upstream of ERK, caused a delay in G2 progression as well as G0/G1 (Wright, Munar et al. 1999). Similarly phosphorylated MAPKK increases in G0/G1 and G2/M phases (Morgan, Dolp et al. 2001). Using a specific ERK pathway inhibitor, two groups demonstrated that the ERK

pathway is required for early G2 progression, but not late G2 and timely entry into mitosis (Shinohara, Mikhailov et al. 2006; Matkovic, Lukinovic-Skudar et al. 2009). In contrast, the opposite result has also been reported. EGF activation of ERK in cells arrested in S phase causes G2/M arrest or delay due to an increase in p21 (Dangi, Chen et al. 2006). Downregulation of a specific phosphatase for ERK results in accumulation of active ERK, upregulation of p21 and finally G2/M arrest. The disagreement in these results suggests that a basal level of ERK is required for G2/M progression and an active ERK threshold plays an important role in G2/M progression.

The PI3K pathway also has a role in G2/M progression, but its role is more complicated than the ERK pathway. Shtivelman et al reported that AKT, a downstream target of PI3K, is activated at the entry of G2 and during progression to mitosis. Moreover, inhibition of the PI3K pathway causes G2 arrest and apoptosis. In addition, studies with constitutively active AKT reached the same conclusion that G2/M arrest and apoptosis by induced DNA damage was overridden by active AKT (Kandel, Skeen et al. 2002; Lee, Park et al. 2005; Faurschou, Gniadecki et al. 2008). The mechanisms underlying this effect of PI3K are various: inactivation of the chk1 check point kinase (Shtivelman, Sussman et al. 2002) increases mRNA and protein expression of cyclin B and cdk1 via the NF-Y transcription factor (Lee, Park et al. 2005) and reversal of cdk1 inhibition (Kandel, Skeen et al. 2002). However, the PI3K kinase pathway is generally activated to prevent apoptosis, including in OP cells (Ness and Wood 2002; Romanelli, Mahajan et al. 2009), and G2/M phase arrest is regulated at the G2/M checkpoint, depending on DNA damage. Therefore, it is still in debate whether activation of PI3K pathway is really required for G2/M progression in the normal cell cycle.

Several studies have reported that the ERK PI3K pathways regulate G2/M phase regulators. Wee kinase, which inhibits cdk1 activation, is phosphorylated and translocates to the cytoplasm by AKT stimulation and results in activation of cdk1 (Katayama, Fujita et al. 2005). However, another report demonstrated that activation of ERK suppressed Wee1 activation (Chen and Gardner 2004). The ERK pathway regulates nuclear translocation of cyclin B by phosphorylation on serine residues (Walsh, Margolis et al. 2003). Cdc25c regulation is dependent on either the ERK pathway (Wang, He et al.

2007) or the PI3K pathway (Dangi, Cha et al. 2003), suggesting that activation of cdc25c might be regulated by several pathways (Lindqvist, Rodriguez-Bravo et al. 2009).

In this chapter, we examine how G2/M phase progression is controlled by FGF-2 and IGF-1 individually or coordinately. Expression of cyclin B1 and cdk1, complex formation and subsequent activity is investigated as well as subcellular localization of cyclin B1 and cdk1. The effect of individual growth factors on G2/M progression is determined as well. Finally, we report initial studies of signaling pathways governing these cell cycle phases.

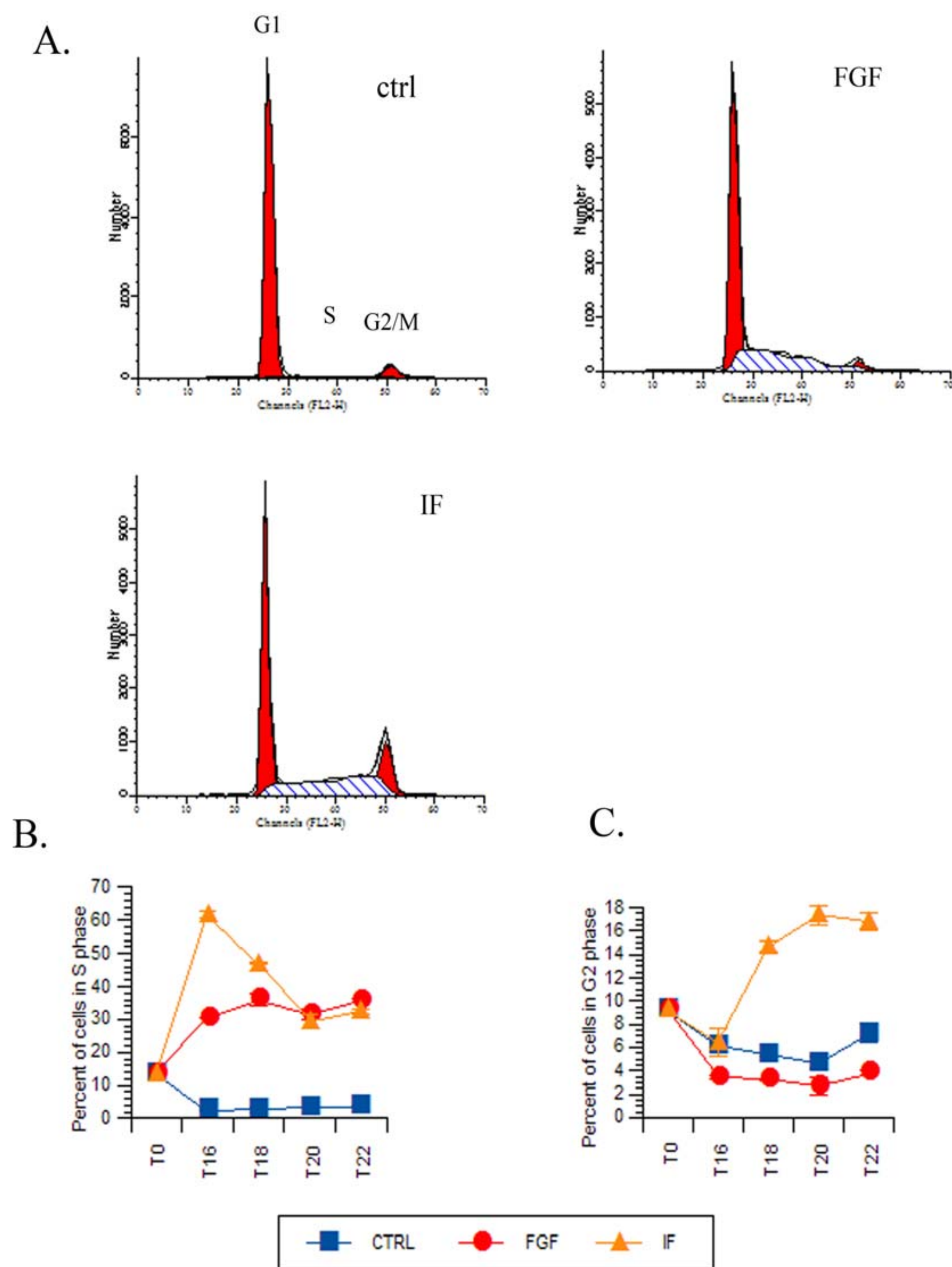
4.2 Results

4.2.1 FGF-2 treated OP cells fail to progress to G2/M, but IGF-1/FGF-2 promotes G2/M phase progression

To determine how FGF-2 and/or IGF-1 regulate cell cycle progression in G2/M phases, we were interested in quantifying the percentage of cells specifically in S and G2/M phases. Since G2/M phases are relatively shorter than either G1 or S phases, it was essential to find the best time point when most of the OP cells go through this transition phase. First, we examined OP cell distribution profile by flow cytometry analysis after staining with PI as performed in chapter 3. OP cells were growth arrested as for previous experiments and then stimulated with FGF-2 and or IGF-1 or no growth factors, harvested at the indicated time (every 2 hours). For these experiments, IGF-1 alone was excluded because the cell distribution pattern of IGF-1 was similar to the no growth factor control group. As presented in the histogram in Fig. 4.1, FGF-2 treated OP cells successfully entered S phase from 16 h after growth factor stimulation, and more than 30% of cells were maintained in S phase (Fig. 4.1 A, B). However, a different pattern was observed in IGF-1/FGF-2 treated OP cells. As for the FGF-2 group, OP cells successfully entered S phase, but the number of cells in S phase started to decreased as cells gradually progressed to G2/M as early at 18 h (Fig 4.1 A, B). The increased number of cells in G2/M in IGF-1/FGF-2 corresponded to a reduced number of cells in S phase (Fig 4.1 B, C). FGF-2 alone almost completely failed to promote traverse of OP cells into

G2/M, showing only 8% of cells in G2/M phases at the maximum time point (Fig. 4.1 C). When IGF-1 was added to FGF-2 treated cells, almost 2 fold more cells (16%) were detected in G2/M (Fig. 4.1 C). In addition, the peak of G2/M was delayed in FGF-2 alone cultures compared to IGF-1/FGF-2 (Fig. 4.1 C).

Figure 4.1. Flow cytometry analysis of OP cells. Growth arrested OP cells were treated with FGF-2, IGF-1/FGF-2 (10 ng/ml each) or no growth factor for 16, 18, 20, and 22 h. Propidium iodide was used to stain DNA. (A) Histogram representative of 20 h treatment. (B, C) Percentage of cells in S phase (B) and G2/M phases (C) were quantified using the DNA staining analysis software ModFit$^{TM.}$

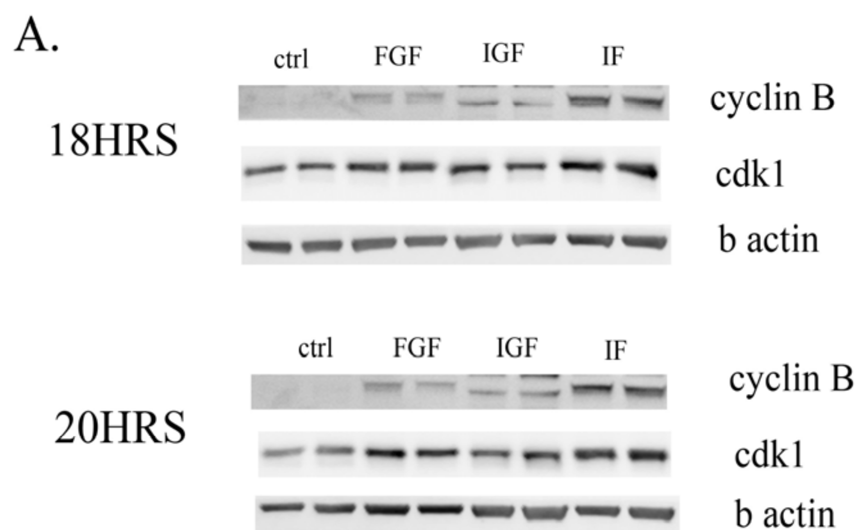

A.
G1
ctrl
S
G2/M
Number
Channels (FL2-W)
FGF
IF
B.
C.
Percent of cells in S phase
Percent of cells in G2 phase
T0
T16
T18
T20
T22
CTRL
FGF
IF

4.2.2 Cyclin B and cdk1 expression are upregulated in IGF1/FGF-2 treated OP cells

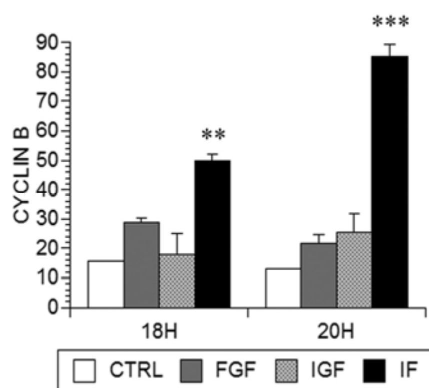
The previous experiments clearly showed that FGF-2 and IGF-1/FGF-2 had distinct effects on cell cycle progression after S phase entry. These results led us to investigate the molecular mechanisms underlying the cell cycle progression profiles revealed by flow cytometry analysis. We first examined protein expression of regulatory molecules responsible for the G2/M transition. The previous flow cytometry study indicated that the peak of G2/M was 20 h and 22 h in IGF-1/FGF-2 and FGF-2 alone conditions, respectively (Fig. 4.1 C). Therefore, we chose 18 h and 20 h for the analysis of protein expression of cyclin B and cdk1. FGF-2 induced expression of cyclin B1 more than IGF-1 or no growth factor control treated cells at 18 h, but this effect was modest and not statistically significant (Fig. 4.2 A, B). In contrast, the combination of IGF-1 with FGF-2 significantly induced the expression of cyclin B at 18 h and 20 h ($p<0.01$ and $p<0.001$ vs control, at 18h and 20h, respectively, Fig. 4.2 A, B). Interestingly, cyclin B levels were maintained in the combination treatment although other treatment groups failed to maintain levels of cyclin B protein (Fig. 4.2 A, B). The protein expression level of cdk1 showed a different pattern from that of cyclin B. Overall, cdk1 protein levels were not different across growth factor treatment groups at 18 h although all were moderately elevated versus control conditions (Fig. 4.2 A, C). Only IGF-1/FGF-2 treated OP cells showed a significant increase in cdk1 protein levels at 20 h ($p<0.05$ vs control, Fig. 4.2 A, C) corresponding to the peak of G2/M. Changes in protein expression of cdk1 was not dramatic overall although IGF-1/FGF-2 treated OP cells showed a slight increase as the cell cycle progressed, which agrees with the general model of cell cycle regulation. However, unexpectedly even cyclin B protein level was not dramatically changed as cells progressed towards G2/M in the FGF-2 treated cells, and, in fact, it was slightly downregulated. In contrast, IGF-1/FGF-2 treated OP cells showed a dramatic increase of cyclin B protein towards G2/M entry (Fig. 4.2).

Figure 4.2. Protein expression levels of cyclin B and cdk1 in OP cells. (A, B, C) OP cells were growth arrested for 12 hours and treated with IGF-1, FGF-2, IGF-1/FGF-2 (10 ng/ml each) and no growth factor for 18 and 20 h. (A) Total cell lysates (10-30 μg) harvested at each indicated time point were processed for SDS-PAGE and western immunoblotting. (B, C) Western blot results were quantified by densitometric analysis. Levels of cyclin B (B) and cdk1 (C) were normalized to β actin. Statistical analyses are shown for triplicate samples within one representative experiment. Experiments were repeated three times with consistent result but the fold change was diverse in each experiment so could not be used for pooled anayses. Values represent the mean ± SEM for each condition. (B, C) *** $p<0.001$ vs control, ** $p<0.01$ vs control and * $p<0.05$ vs control.

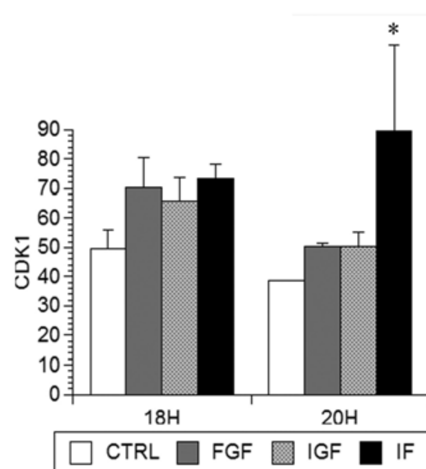
A.



B.



C.



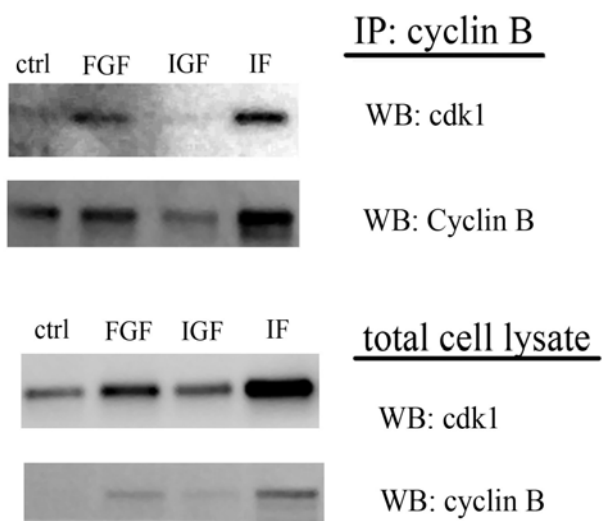
4.2.3 Enhanced complex formation between cyclin B and cdk1 in IGF-1/FGF-2 conditions leads to higher cdk1 activity

Protein expression of each regulatory molecule is a primary mechanism to control cell cycle progression. However, functional holoenzyme formation is an essential level of regulation of kinase activity. Complex formation of cyclin B and cdk1 is important for cdk1 activation and further G2/M phase progression. Thus, we investigated whether different growth factor treatments would change complex formation between cyclin B and cdk1. Flow cytometry analysis demonstrated that the number of cells in G2/M phase increased at 18 h in IGF-1/FGF-2 treated cells (Fig. 4.1 C). Thus, the 18 h time point after growth factor stimulation was chosen for immunoprecipitation analyses of complex association. As shown in Figure 4.3 A, more cdk1 was associated with cyclin B in IGF-1/FGF-2 treated OP cells. FGF-2 alone treatment also caused cdk1 association with cyclin B even though both cyclin B and cdk1 protein levels in FGF-2 treated OP cells were lower than in IGF-1/FGF-2 treated OP cells.

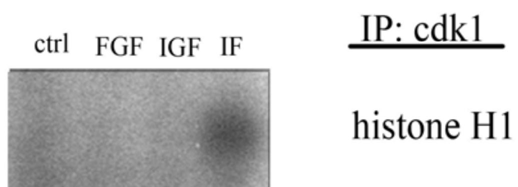
The purpose of forming a holoenzyme between cyclins and cdks is to induce cdk activity. Complex formation of cyclins and cdks does not necessarily correspond to effective cdk activity since there are several levels of regulation for cdk activation as described in chapter 1. Therefore, to assess cdk1 activity, a kinase assay was performed using radiolabeled ATP with histone H1 as a substrate. OP cells were treated for the immunoprecipitation analyses and cell lysates were used for kinase assays. Cdk1 activity was highest in IGF-1/FGF-2 treated OP cells and almost undetectable in the other growth factor groups (Fig. 4.3 B).

Figure 4.3. Effective complex formation of cyclin B with cdk1 and cyclin B associated kinase activity. (A) Growth arrested OP cells were treated with IGF-1, FGF-2, IGF-1/FGF-2 (10 ng/ml each) or no growth factor for 18 hours. 500 μg of total cell lysates were immunoprecipitated with cyclin B as described in chapter 2. Immune complexes were processed for western immunoblot analysis. Total cell lysates (30 μg) before reacting with cyclin B antibodies were processed for SDS-PAGE and western immunoblot analysis. (B) Cdk1 activity was determined using histone H1 protein as a substrate. Experiments were repeated three times with consistent result but the background levels were too high in each experiment so could not be used for pooled anayses.

A.



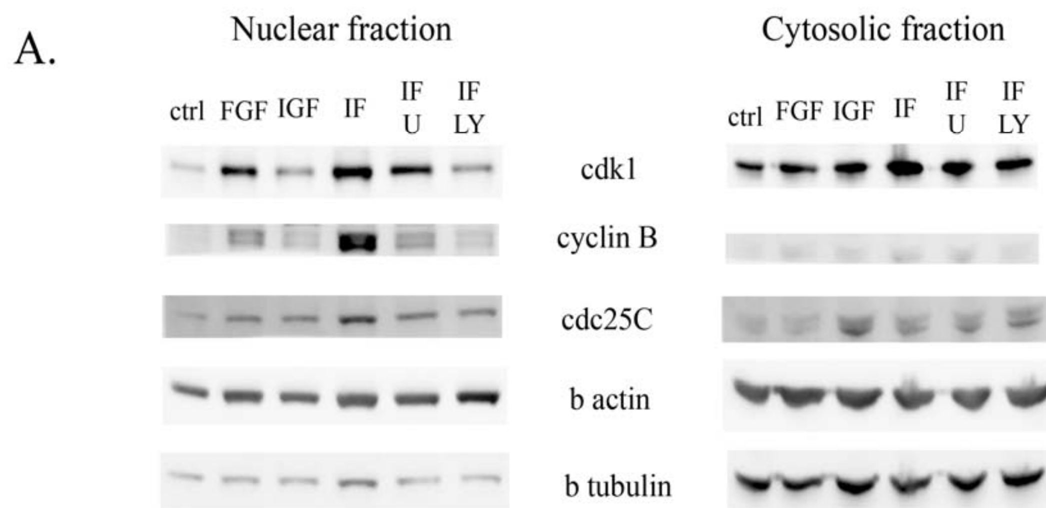
B.

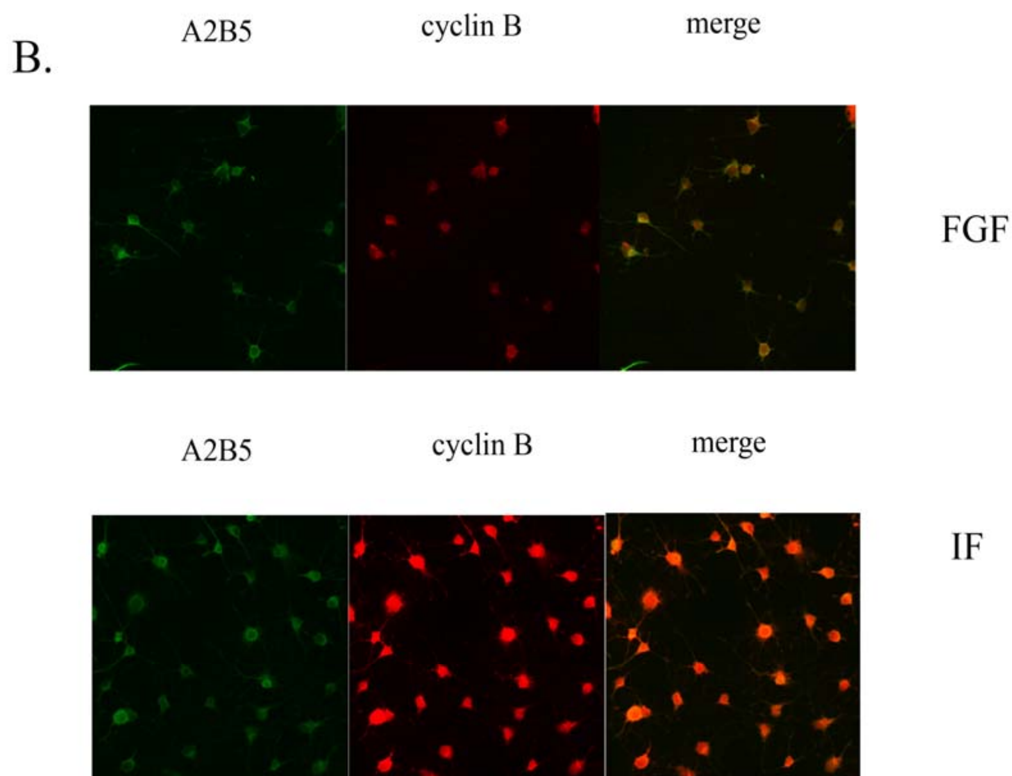


4.2.4 IGF-1/FGF-2 increases nuclear localization of cyclin B, cdk1 and cdc25C

To prevent premature activation of Cdk1, which may cause mitotic catastrophes such as endoreplication of DNA, activation of cdk1 is tightly regulated by several mechanisms as described in section 1.3.4. Previously, we observed that enhanced complex formation between cyclin B and cdk1 did not lead to concomitant activation of cdk1 in FGF-2 treated OP cells. The cyclin B/cdk1 complex is known to shuttle in and out of the nucleus prior to mitosis (reviewed in (Takizawa and Morgan 2000). Thus, during interphase, nuclear export is overridden and cdk1 levels are maintained at a low level, while in prophase, the balance is reversed and nuclear import is prominent. Therefore, we questioned whether nuclear localization of cyclin B and cdk1 is differentially regulated by FGF-2 and/or IGF-1. At 20 h after growth factor stimulation, OP cells were collected and lysates processed for subcellular fractionation. More cdk1 and cyclin B were translocated into the nucleus in the IGF-1/FGF-2 treated OP cells compared to IGF-1 alone and no growth factor control cells (Fig. 4.4 A). In FGF-2 treated OP cells, cdk1 and cyclin B were present in the nucleus, but at a much lower level than in the IGF-1/FGF-2 treated cells, particularly for cyclin B (Fig. 4.4 A). In addition to cyclin B and cdk1, nuclear localization of cdc25c, a cdk1 activator (refer section 1.3.4) was also enhanced in the growth factor combination. To confirm these results, we used immunohistochemistry to localize cyclin B in the nucleus versus cytoplasm in IGF-1/FGF-2 versus FGF-2 treated cells. A2B5 was used as a cell surface marker to detect early OP cells. Most cells were stained with A2B5, confirming that our cultures are enriched in early progenitor cells (Fig. 4.4 B). Consistent with the fractionation results, the intensity of nuclear staining of cyclin B was much higher in OP cells exposed to IGF-1/FGF-2 versus FGF-2.

Figure 4.4. Subcellular localization of cyclin B and cdk1. (A) Growth arrested OP cells were treated with IGF-1, FGF-2, IGF-1/FGF-2 (10 ng/ml each) or no growth factor for 20 h. Cells were processed for subcellular fractionation and analyzed by western immunoblotting. (B) OP cells grown on poly-d-ornithine coated coverslips were growth starved and treated with FGF-1 and IGF-1/FGF-2 for 20 h. Cells were processed for immunohistochemistry. A2B5 cell surface antigen was used for detection of OP cells with a FITC conjugated secondary antibody (green) and cyclin B was visualized using Alexa-conjugated secondary antibody (red). Images were taken by confocal microscopy.


A.
Nuclear fraction
Cytosolic fraction
ctrl
FGF
IGF
IF
IF U
IF LY
cdk1
cyclin B
cdc25C
b actin
b tubulin


B.
A2B5
cyclin B
merge
FGF
A2B5
cyclin B
merge
IF

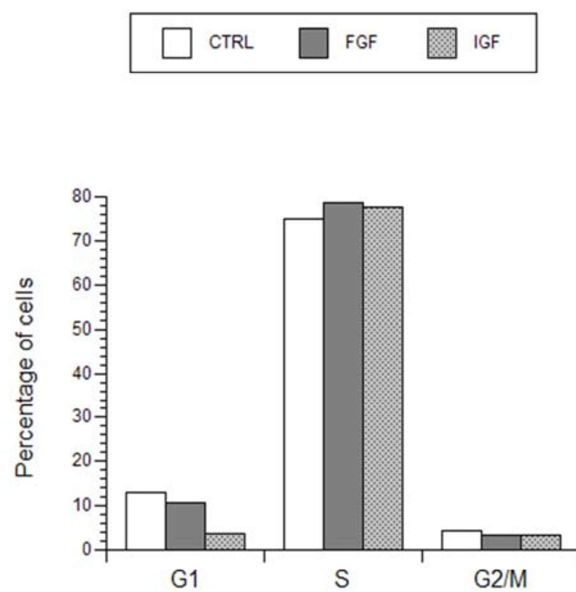
4.2.5 IGF-1 alone promotes G2/M phase progression

As mentioned previously, G2/M phases are only observed for a limited time period since cells progress rapidly through G2/M. For this reason, only a small percentage of cells were detected in G2/M phase by flow cytometry analysis even though the cells were synchronized by overnight serum and growth factor starvation. We detected, at most, 18% of cells in G2/M phase in the combined growth factor condition (Fig. 4.1 C). Another difficulty with the previous studies is that it is difficult to separate specific IGF-1 effects on G2/M since IGF-1 treated OP cells do not progress into S phase without coordinate stimulation with FGF-2. Moreover, a greater number of cells progress into S phase with IGF-1/FGF-2 than with FGF-2 alone. Therefore, we designed an experiment to identify specific effects of growth factors on G2/M using a double thymidine block, which arrests cells at late G1/S phase boundary. This allowed us to treat cells in full media to allow maximal progression of all cells to S phase. OP cells were treated with thymidine in normal growing media and then switched to FGF-2, IGF-1 or no growth factor media. 4 h after release from double thymidine block almost 80% of cells were in S phase (Fig. 4.5 A), confirming that the double thymidine block method was effective in arresting cells in early S phase. Interestingly, cells analyzed at 8 h after release form early S showed a significant difference from those analyzed at 4 h. Specifically, treatment with IGF-1 dramatically reduced the percentage of cells in S phase by 50% (Fig. 4.5 A, B). This reduction led to a 32% increase in G2/M phases and a 25% increase in G1 phase suggesting cells were traversing G2/M and re-entering G1. FGF-2 treated cells also showed a 42% decrease in S phase, a 30% increase in G2/M phases and an 18% increase in G1 phase, similar to IGF-1 treated cells but less pronounced. Overall, IGF-1 treated cells finished the cell cycle rapidly and re-entered G1 earlier than FGF-2 treated cell. Interestingly, no growth factor treated cells followed a similar pattern but with a more modest change in each phase. This suggests that some signals to promote G2/M were induced during G1 even though the second growth factor stimulation after the G1/S phase boundary had differential effects that were growth factor specific.

Figure 4.5. Cell distribution after double thymidine block. (A, B) OP cells were exposed to 2 μM of thymidine for 16 h, released to growth media for 10 h and exposed to thymidine for another 16 h. After the second thymidine pulse, cells were treated with IGF-1, FGF-2 or no growth factor for 4 h (A) or 8 h (B). The percentage of cells in each phase was quantified using the DNA staining analysis software ModFit™ .

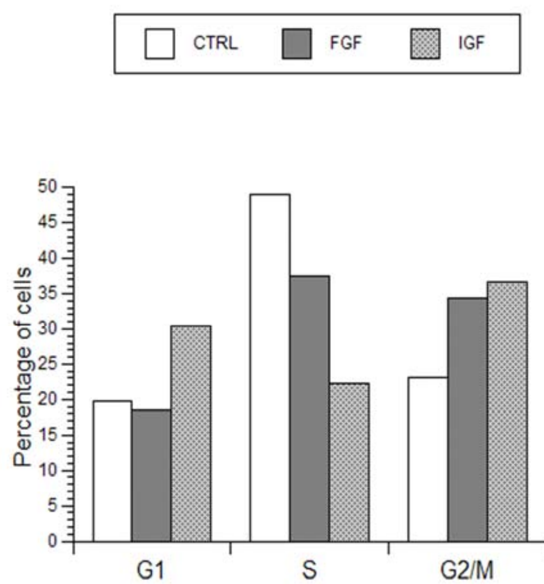
A.

T4



B.

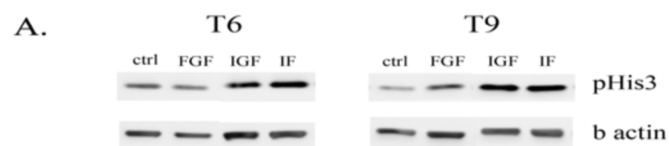
T8



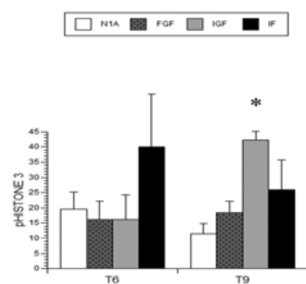
4.2.6 IGF-1 increases phosphorylated histone 3, a mitotic marker

To further analyze the effect of IGF-1 and FGF-2 on G2/M regulation, we examined the level of phosphorylated histone 3 (pHis3), a mitotic marker. Cells were arrested at the G1/S boundary by double thymidine block, treated with growth factors as above and cell lysates were used for western blot analysis. The combination of IGF-1/FGF-2 accelerated G2/M phase progression, showing an earlier increase of pHis3 6 h after the double thymidine block (Fig. 4.6 A, B). IGF-1 alone showed a significant increase of pHis3 also at 9 h after the double thymidine block ($p<0.05$ vs control, Fig. 4.6 A, B) while FGF-2 alone gradually increased pHis3 over time. We performed immunohistochemistry to confirm the results we observed from western blot analysis. Cells synchronized in S phase by double thymidine block were treated with IGF-1 and/or FGF-2 or no growth factors for 9 h and stained for: DAPI (4,6-diamidino-2 phenylindole) to detect the nucleus, A2B5 to detect early OP cells (FITC; green) and pHis3 (Alexa 546; red). Again, pHis3 positive cells were more abundant in IGF-1 alone treated cells while the IGF-1/FGF-2 combination showed a slightly lower number likely due to more rapid progression as seen in the pHis3 western blot analysis (Fig. 4.6 C, D). IGF-1 alone was more potent than FGF-2 in regulating G2/M progression.

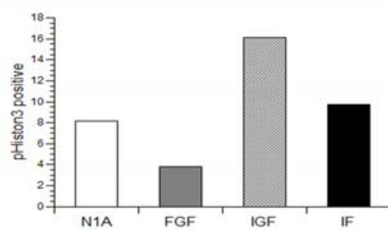
Figure 4.6. Detection of phosphorylated histone 3. (A-B) OP cells were exposed to 2 μM of thymidine for 16 h, released to growing media for 10 hours and exposed to another 16 h of thymidine. After the second thymidine pulse, cells were treated with IGF-1, FGF-2 or no growth factor for 6 or 9 h, harvested and processed for SDS-PAGE and western immunoblotting. Graph represents pooled data from 3 separate experiments. (C, D) OP cells grown on poly-d-ornithine coated coverslips were exposed to 2 μM of thymidine for 16 h, released to growth media for 10 h and exposed to thymidine for another 16 h. After the second thymidine pulse, cells were treated with IGF-1, FGF-2 or no growth factor for 9 h and processed for immunohistochemistry to detect DAPI, A2B5 (FITC; green) and pHis3 (Alexa 546; red). (C) DAPI and pHis3 positive cells were counted from (D). Values represent the mean ± SEM for each condition. (B) * $p<0.05$ vs control



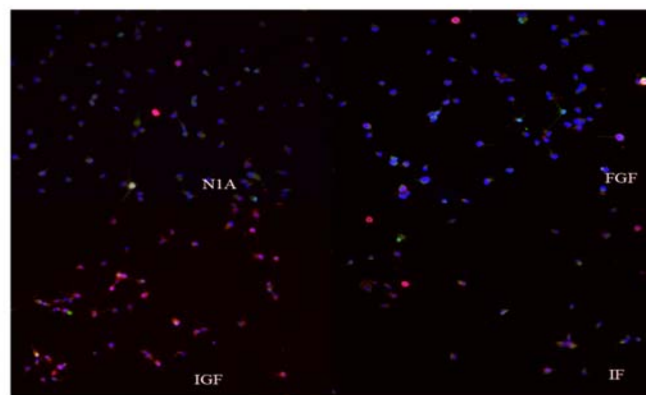
B.



C.



D.



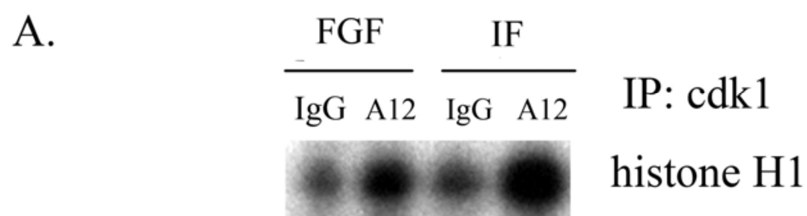
4.2.7 IGF-1R blocking antibody increased cdk1 activity and cyclin B1 expression

The previous studies clearly showed that IGF-1 independently regulates G2/M progression. These results led us to ask what are the IGF-1 signaling pathways that promote G2/M progression. Since the IGF-1R blocking antibody was available, we tested how OP cells would respond to IGF-1R antibodies during G2/M. Since OP cells are able to secrete autocrine IGF-1, we included FGF-2 and the IGF-1/FGF-2 treatment groups in this experiment. Serum starved OP cells were treated with IGF-1/FGF-2 for 14 h to induce more cells into S phase and then switched to FGF-2 and/or IGF-1 in the presence of IMC-A12 IGF-1R blocking antibody or control IgG antibody for 6 h for a 20 h total treatment time to correspond to the timing of G2/M phases by conventional cell cycle analysis.

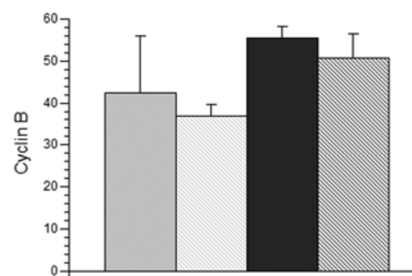
Surprisingly, more cdk1 activity was detected after treatment with the IGF-1R blocking antibody in both FGF-2 and IGF-1/FGF-2 conditions (Fig. 4.7.A). This unexpected result led us to examine protein expression under these experimental conditions. Expression of cdk1 was consistent, however cyclin B1 expression was increased in IGF-1/FGF-2 treated cells in the presence of the IGF-1R blocking antibody. Cyclin B expression did not change in FGF-2 treated cells with the blocking antibody (Fig.4.7 B). We also examined signaling pathway targets to determine whether IGF-1R signaling was inhibited by the blocking antibody. Downregulation of phosphorylated AKT indicated that IGF-1R signaling pathway was inhibited significantly in both IGF-1 and IGF-1/FGF-2 treated OP cells (Fig.4.7 C). In contrast, phosphorylated p44/42 (ERK 1/2) was not reduced with the IGF-1R blocking antibody treatment (Fig.4.7 C).

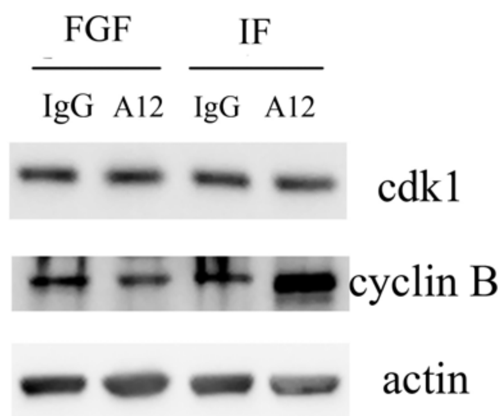
Figure 4.7. Cdk1 kinase assay and protein expression of cdk1, cyclin B in the presence of IGF-1R blocking or control antibodies. (A, B) Growth arrested OP cells were treated with IGF-1/FGF-2 for 14 h and switched to FGF-2 or IGF-1/FGF-2 for 6 h. Meanwhile, cells were pretreated with IgG and IMC-A12 (IGF-1R blocking antibody) for 30 minutes. (A) 500 μg of total cell lysates were immunoprecipitated with cyclin B as described in chapter 2. Immune complexes were processed for western immunoblot analysis (B) Total cell lysates (30 μg) were processed for SDS-PAGE and western immunoblot. Statistical analyses were performed on data from three independent experiments.

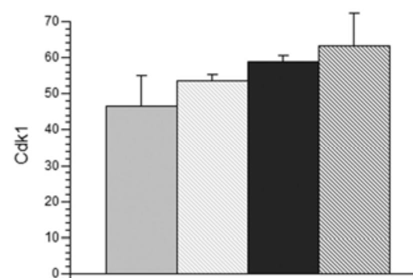
A.

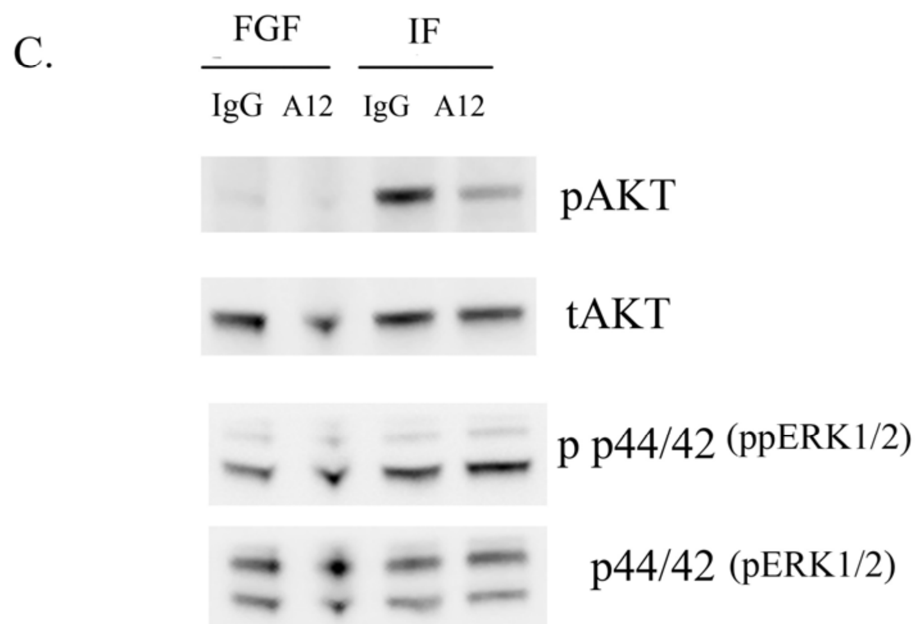


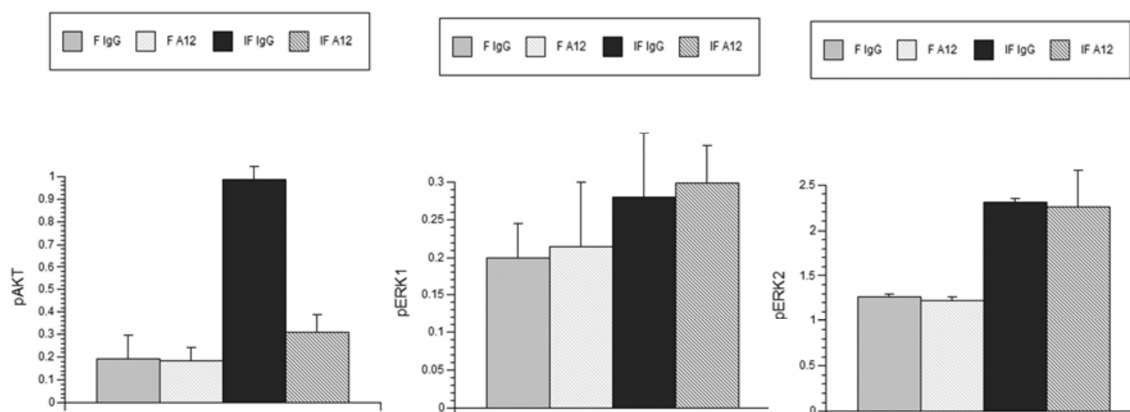
B.







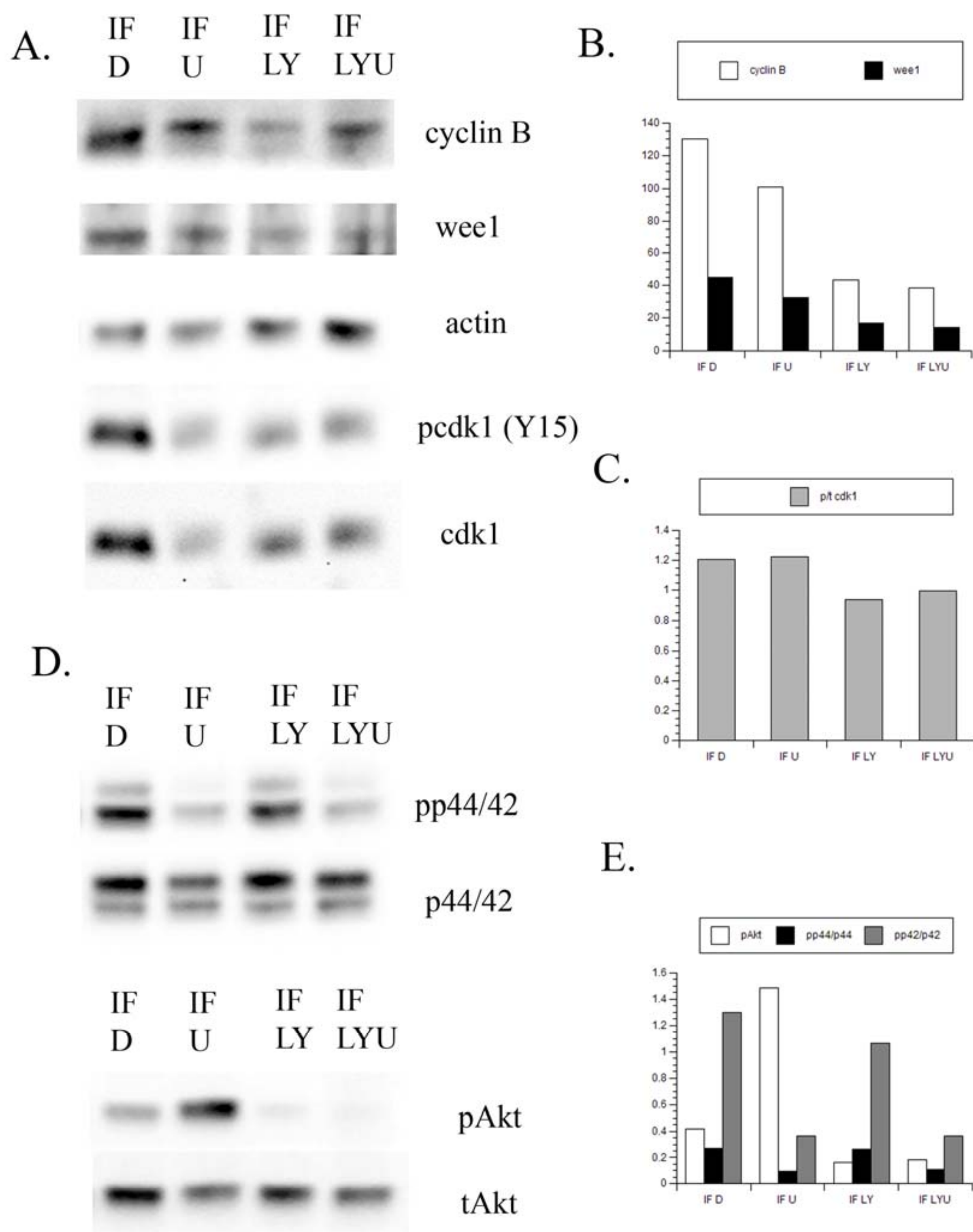

C.
FGF
IF
IgG A12 IgG A12
pAKT
tAKT
p p44/42 (ppERK1/2)
p44/42 (pERK1/2)


F IgG
F A12
IF IgG
IF A12
pAKT
pERK1
pERK2

4.2.8 Blocking of both MAPK and PI3K downregulate protein expression of cyclin B, cdk1 and Wee1, but had no effect on cdk1 inhibition.

In order to examine the role of the MAPK and PI3K pathway on G2/M progression, we used specific inhibitors, U0126 for the MAPK pathway and LY294002 for the PI3K pathway. Inhibition of both pathways from G1 phase resulted in downregulation of cyclin D1(Frederick, Min et al. 2007), cyclin E and A and hypophosphorylation of pRb (section 3.6), which suggested inhibition of S phase entry. Additionally, our goal was to determine effects of both pathways on G2/M progression. Therefore, we first allowed cells to enter S phase as one cohort, by treating them with IGF-1/FGF-2 for 14 h. We then exposed cells to the inhibitor for 6 h. Inhibition of the PI3K pathway decreased expression of cyclin B, cdk1 and Wee1 compared to blocking the MAPK pathway (Fig. 4.8 A, B). Surprisingly, blocking the PI3K pathway downregulated inhibitory phosphorylation of cdk1 while MEK pathway inhibition did not affect cdk1 phosphorylation (Fig 4.8 A, C). We observed interesting results, which showed compensatory activation of the other pathway when one pathway was inhibited. Blocking of the PI3K pathway activated ERK while inhibition of the MAPK pathway activated AKT (Fig. D, E).

Figure 4.8. Protein expression level of cyclin B and cdk1, and Wee1 in the presence of signaling pathway inhibitors. (A-E) Growth arrested OP cells were treated with IGF-1/FGF-2 for 14 h. Meanwhile, cells were pretreated with DMSO, U0126 and/or LY294002 for 30 minutes. Cells were treated for another 6 h with IGF-1/FGF-2 in the presence of inhibitors. Total cell lysates (10-30 μg) were processed for SDS-PAGE and western immunoblot. (B) Levels of cyclin B and Wee1 were normalized to β actin. (C) Phosphorylated cdk1 was divided by total cdk1. (E) Graphs showing P-Akt/Total Akt and P-p44/P-p42/total p44/p42.


A.
IF D
IF U
IF LY
IF LYU
cyclin B
wee1
actin
pcdk1 (Y15)
cdk1
B.
cyclin B
wee1
IF D
IF U
IF LY
IF LYU
C.
p/t cdk1
D.
pp44/42
p44/42
pAkt
tAkt
E.
pAkt
pp44/p44
pp42/p42

4.3 Discussion

The function of growth factors in regulating G2/M progression is largely unknown although cell cycle regulation by growth factors has been well-studied in G1 and S phase entry in many cell types, including OP cells (Jiang, Frederick et al. 2001; Frederick and Wood 2004; Cui and Almazan 2007). A growing body of evidence indicates inhibitory or stimulatory effects of growth factors in G2/M progression. Exposure to EGF or hepatocyte growth factor (HGF) during G2 arrested or delayed G2/M progression in HeLa cells (Dangi, Chen et al. 2006; Nam, Kim et al. 2008). In contrast, abrogation of IGF-1 signaling in myeloma cells or TGF-β signaling in bone marrow stromal cells similarly arrested G2/M progression (Stromberg, Ekman et al. 2006; Fujita, Epperly et al. 2008). Here, we present data showing that IGF-1 alone has a distinct role in G2/M progression in OP cells.

We demonstrated that OP cells exposed to only FGF-2 failed to transverse through G2/M while IGF-1/FGF-2 treated OP cells normally progressed through G2/M. To understand the underlying molecular mechanisms for this differential regulation of G2/M, we investigated protein expression of cyclin B and cdk1 and demonstrated increased cyclin B expression in IGF-1/FGF-2 treated OP cells compared to FGF-2 treated OP cells. However, complex formation of cyclin B/cdk1 did not clearly distinguish FGF-2 and IGF-1/FGF-2 effects. In contrast, cdk1 kinase activity was significantly enhanced in IGF-1/FGF-2 treated OP cells compared to all other groups. These results indicate that complex formation does not correspond to kinase activation. Similar results were reported with cdk2 and cyclin A, in which cdk2 complexed with cyclin A was activated after IGF-1 stimulation through activation of the ERK pathway in human osteosarcoma cells (Zhang, Lee et al. 1999). In addition, other mechanisms to active cdk1 have been well characterized during G2/M as described in chapter 1.

We demonstrated that nuclear localization of cyclin B and cdk1 were enhanced in IGF-1/FGF-2 treated OP cells, confirming that cdk1 translocation to the nucleus is associated with enhanced cdk1 activity. Nuclear localization of cyclin B is facilitated by CRM1, nuclear export protein (Yang, Bardes et al. 1998) and regulation of CRM1 is not

yet fully understood. CRM1 is also involved in nuclear localization of cyclin D1 via the PI3K-AKT-GSK-β pathway (Gladden and Diehl 2005), which is regulated by IGF-1 in OP cells (Frederick, Min et al. 2007). However it has not been determined whether the same pathway regulates cyclin B1 nuclear transport. Although its exact mechanism remaines unclear, phosphorylation on cyclin B is required to translocate into the nucleus. Cdc25C nuclear localization might contribute to enhanced cdk1 activity as well since cytoplasmic cdc25C is normally bound to 14-3-3 protein thus restricting its access to cdk1 (Kumagai, Yakowec et al. 1998; Kumagai and Dunphy 1999). However, it may not be appropriate to compare proportional changes in nuclear fraction versus cytosolic fraction since the G2/M phase is relatively short and only 20% of cells (at most) are in G2/M phase at any time, which means that more than 80% of cells will be suppressing cdk1 activity. ERK activation has been reported to control nuclear localization of cyclin B, by phosphorylating serine residues (Walsh, Margolis et al. 2003). Cyclin B is also partially phosphorylated by polo-like kinase (Takizawa and Morgan 2000). In addition to phosphorylation, subcellular localization of cdc25C is regulated by signaling pathways, however the main kinase responsible for this is not yet clear. One group reported that cdc25C localization is dependent upon the ERK/MAPK pathway in ovarian epithelial cancer cells (Wang, He et al. 2007) whereas another group showed that it is PI3K dependent in HeLa cells (Dangi, Cha et al. 2003) indicating that control of cdc25C localization might be cell type specific In OP cells, inhibition of both PI3K and ERK pathways downregulated nuclear localization of cyclin B and cdk1, but their specific roles in these processes need further investigation.

In order to understand the contribution of IGF-1 in G2/M progression, we synchronized cells after G1, using a double thymidine block method, which arrests cells at the G1/S boundary in early S phase. Using this method, we clearly showed that IGF-1 promotes and accelerates S phase cells to G2/M. OP cells treated with FGF-2 or no growth factor progressed slowly to G2/M. This result appears contradictory to the earlier results showing complete lack of G2/M progression in OP cells treated with FGF-2 alone from the start of G1. It is thus likely that the presence of serum or IGF-1R activation (by superphysiological levels of insulin) during the double thymidine block plays a role in

G2/M regulatory events prior to arrest in early S phase. It is also possible that FGF-2 may induce moderate progression through G2/M phase, but the effect is significantly less than that of IGF-1. We confirmed this result with another approach, analysis of histone 3 phosphorylation on serine 10 (pHis3). pHis3 is a well known mitotic marker since it is phosphorylated at Ser10 during mitosis (Shoemaker and Chalkley 1978; Prigent and Dimitrov 2003) We reported that IGF-1 increased pHis 3, compared to FGF-2 or no growth factor conditions in OP cells after release from double thymidine block. We observed that pHis3 was induced to a greater extent and at an earlier time point by IGF-1 versus FGF-2. These results indicate that FGF-2 has only a minor effect on G2/M by itself, but it accelerates G2/M progression in the presence of IGF-1.

To determine the molecular mechanisms for IGF-1 effects on G2/M progression, we used a specific IGF-1R blocking antibody, IMC-A12. Unexpectedly, blocking IGF-1R did not abolish cdk1 activity. In contrast, it enhanced cdk1 activity. Control cells treated with treated IGF-1/FGF-2 in the presence of the IgG control antibody showed slightly increased cdk1 activity compared to FGF-2 treatment, indicating an effect of IGF-1 on cdk1 activation. Inhibition of IGF-1R activated cdk1 more robustly in IGF-1/FGF-2 than in FGF-2 treated cells. There could be several possibilities to explain these results: one possibility is that IGF-1 actually inhibits cdk1 activity, another possibility is that IGF-1 may activate other receptors rather than IGF-1R, and the last possiblitiy is that blocking IGF-1R indirectly activates other signaling pathways and in turn, further activates cdk1 activity. The first possibility is unlikely since, taken together, the results presented in this chapter do not support this conclusion. In the second possibility, IGF-1 is able to bind to insulin receptor and hybrid receptors between IGF-1R and the insulin receptor (Rowzee, Lazzarino et al. 2008). However, the concentration of IGF-1 we used here is too low to activate insulin receptors. Signaling of IGF-1 through hybrid receptors still remains conceivable although information about expression of hybrid receptors in OP cells is limited and enhanced cdk1 activity does not seem likely since phosphorylation of AKT was decreased by antibody treatment. For the last possibility, in fact, there is evidence from other studies that show cross-talk between PI3K and ERK pathways in proliferation (Moelling, Schad et al. 2002; Cui and Almazan 2007), survival

(Levinthal and DeFranco 2004; Bradley, Ruan et al. 2008), chemotaxis (Campbell, Allen et al. 2004) and ER stress response (Dai, Chen et al. 2009). In most cases, activation of one pathway is required for transactivation of the other pathway. However, two papers published in 1999 showed that inhibition of AKT increased ERK activation (Rommel, Clarke et al. 1999; Zimmermann and Moelling 1999), and suggested that Raf is the intermediate for this crosstalk. Dai et al showed that constitutively active AKT inhibited ERK phosphorylation and a PI3K inhibitor promoted ERK activation after induction of ER stress. Taken together, it is thought that PI3K and ERK pathways cross-talk to increase cellular responses or to buffer adverse circumstances. IGF-1R activates AKT via the PI3K pathway and ERK via Grb2/Sos-Ras-Raf pathway, but IGF-1R predominantly activates the PI3K-AKT pathway in OP cells. Thus, it is plausible that OP cells compensatorily activate the ERK pathway when the PI3K-AKT pathway is blocked.

To further investigate signaling pathways regulating G2/M, we used specific inhibitors. Consistent with the IMC-A12 antibody experiments, we observed compensatory activation of PI3K and ERK pathways. We showed that the PI3K pathway regulates protein expression of cyclin B, cdk1 and Wee1. However, the PI3K inhibitor decreased inhibitory phosphorylation on cdk1 suggesting putative activation of cdk1. This result could be from low expression of Wee1 rather than an effect from the signaling pathway because AKT was reported to inhibit Wee1 activity (Katayama, Fujita et al. 2005) and activate cdc25c (Dangi, Chen et al. 2006) in other cell types. Compensatory activation of the ERK pathway by inhibition of the PI3K pathway may be responsible for decreased inhibitory phosphorylation on cdk, which was suggested by another group (Wang, He et al. 2007). As mentioned previously, it is possible that the molecular mechanism underlying G2/M progression is somewhat difficult to discern in the primary OP cells since only 20% of cells at most are progressing through G2/M. An experiment to arrest cells in G2/M such as nocodazole treatment would be required to accurately decipher the molecular mechanism by which G2/M is regulated in these cells. Even further, comparison of double thymidine block methods and nocodazole treatment will provide a better understanding of whether a growth factor such as IGF-1 is required for G2 or M.

The present study showed a specific role for IGF-1 in G2/M progression distinct from G1 progression in OP cells. Cyclin B and cdk1 expression was increased by IGF-1/FGF-2. In turn, cdk1 activity was enhanced, corresponding to increased nuclear localization of cyclin B, cdk1 and cdc25c. In addition, we found that the PI3K and ERK pathways were interwoven in regulating G2/M, and pathway cross-talk occurred when one pathway was blocked. It is of note that cyclin A/cdk1 plays a role in early G2, but its role was not investigated here. Therefore, this complex possibly contributes to the biochemical results we present in this chapter.

Chapter 5

Discussion

5.1 Summary of results

IGF-1 is a well-characterized survival and differentiation factor in oligodendrocyte lineage cells. In addition, in vivo and in vitro studies have shown that IGF-1 promotes proliferation in OP cells, particularly in combination with other mitogens. Previous studies in our laboratory have reported that IGF-1 combined with FGF-2 increased DNA synthesis and S phase entry. However, the role of IGF-1 in progression through later cell cycle phases after S phase entry has not been elucidated. The data presented in this thesis demonstrate that IGF-1 coordinates with FGF-2 for S phase progression and further, that IGF-1 has a distinct role in G2/M progression in OP cells.

S phase progression in the cell cycle is predominantly regulated by activated cdk2 kinase. Cdk2 is also implicated in S phase entry, through associated with cyclin E in late G1 phase, whereas during S phase it forms a complex with cyclin A and promotes S phase progression. Timely activation of cdk2 is controlled by several mechanisms, including expression of partner cyclins, association with cyclins and inhibitory proteins as well as activating or inhibiting phosphorylation events. In our study, we presented evidence that the IGF-1/FGF-2 combination increased cyclin A and cdk1 protein expression. FGF-2 alone was able to induce cyclin A and cdk2 protein, but less than IGF-1/FGF-2. Association of cyclin A and cdk2 was enhanced in IGF-1/FGF-2 compared to cells treated with FGF-2, IGF-1 or no growth factors. Finally, cdk2 activity also was enhanced in IGF-1/FGF-2 treated OP cells. Taken together, our data support the conclusion that the combination of IGF-1/FGF-2 in OP cells promotes S phase progression by increasing cyclin A and cdk2 protein levels and subsequent cdk2 activity.

In order to understand whether IGF-1 and FGF-2 target the same molecules for cell cycle progression, we utilized specific signaling pathway inhibitors. Inhibition of the ERK and PI3K pathways down-regulated mRNA expression of both cyclin E and cyclin A, but blocking the PI3K pathway had greater effects. Similarly, protein levels of cyclin E and cylin A were downregulated by both ERK and MAPK pathway inhibitors, resulting in diminished hyperphosphorylated pRb. It is likely that both pathways coordinate to regulate mRNA and protein expression of cyclin E and cyclin A in OP cells.

Cell cycle progression is a continuous event and molecular changes in the previous cell cycle phase affect not only the transition to the next cell cycle, but also the molecular events in the subsequent cell cycle. To separate the effect of growth factors in early G1 from their effect in later stages, we induced cell cycle entry of OP cells for 4 h in full media and then switched cells to defined growth factor conditions. The initial 4 h, which we previously demonstrated resulted in full induction of cyclin D1 (Frederick and Wood 2004), was sufficient for induction of cyclin A and cdk2 proteins. In contrast, it was insufficient for ppRb phosphorylation since only FGF-2 and IGF-1/FGF-2 stimulated cells demonstrated hyperphosphorylated pRb. To specifically block the IGF-1 signaling pathway, an IGF-1R blocking antibody, IMC-A12, was used 4 h after initial growth stimulation. The IMC-A12 antibody unexpectedly increased cdk2 activity and activated the ERK pathway.

To understand the molecular mechanism for G2/M progression by growth factors in OP cells, we investigated expression levels of cyclin B and cdk1 as well as cyclin B/cdk1 complex formation and kinase activity. Both proteins were increased in IGF-1/FGF-2 treated OP cells. FGF-2 also induced cyclin B, but at lower levels. Both FGF-2 and IGF-1/FGF-2 treated OP cells enhanced holoenzyme formation compared to IGF-1 alone or no growth factor conditions. However, only cdk1 kinase activity in IGF-1/FGF-2 treated OP cells was significant, suggesting that complex formation between cyclin B and cdk1 by itself is insufficient for cdk1 activation.

Cdk1 is activated by several mechanisms such as binding to cyclin B, nuclear localization and dephosphorylation of inhibitory residues on Thr14 and Tyr15. Since cyclin B/cdk1 complex formation did not correspond to cdk1 activity, we investigated

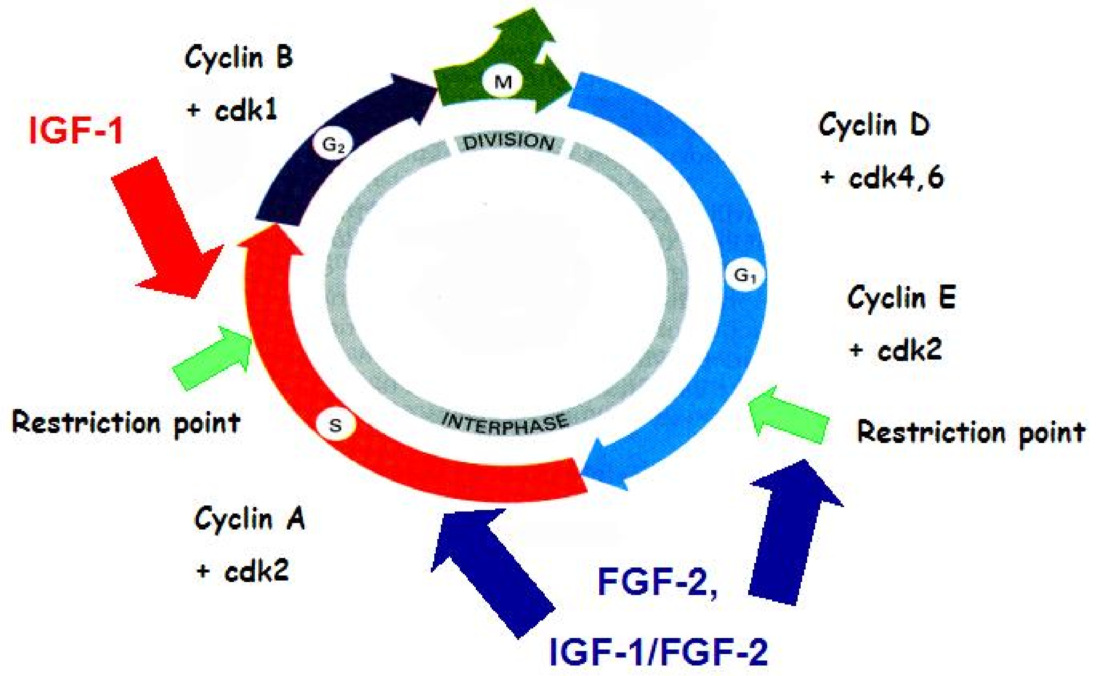
subcellular localization of cdk1, cyclin B and cdc25C. IGF-1/FGF-2 enhanced nuclear localization of all the proteins we analyzed via both PI3K and ERK pathways.

A double thymidine block experiment allowed us to analyze the specific role of IGF-1 in G2/M phases. Cell distribution assessed by flow cytometry analysis clearly showed that IGF-1 promoted and accelerated S and G2/M progression compared to FGF-2 alone. Furthermore, more pHis3, a mitotic marker, was induced in IGF-1 treated OP cells than in FGF-2 treated OP cells. We also observed that IGF-1/FGF-2 accelerated entry into mitosis identified by more rapid induction of pHis3.

Our attempt to investigate the signaling pathways by which each growth factor regulates progression led to interesting results. Use of IMC-A12 antibody enhanced cdk1 activity as seen in cdk2 activity in S phase, possibly via activated ERK. We found that blocking of one pathway with a specific inhibitor caused unexpected activation of the other pathway. We observed that the inhibitory kinase Wee1 was downregulated by the PI3K pathway inhibitor. Also, less P-cdk1 (inactive site) was also found in PI3K inhibited OP cultures. However, ERK pathway inhibitors did not alter expression of Wee1 and P-cdk1. Taken together, it can be concluded that blocking the PI3K pathway may contribute to cdk1 activity either directly or indirectly via activation of ERK. Further experiments will be necessary to define these possibilities in OP cells.

Altogether, this study provides interesting evidence that IGF-1, which is a weak mitogen by itself for S phase entry in OP cells, promotes S phase progression in cooperation with FGF-2 and further plays an essential role in G2/M progression. As illustrated in figure 5.1, FGF-2 and FGF-2/IGF-1 promote OP cells to overcome the restriction point in the late G1 phase and further promote S phase progression. IGF-1 alone induces cells to overcome another restriction point in late S or early G2 phase and to traverse into mitosis. The underlying mechanism for these effects can be attributed to regulating activation of cdk2 and cdk1. I will discuss the major contributions and implications of this dissertation in the following sections.

Figure 5.1 Overall cell cycle regulation in OP cells (modified from Molecular Biology of the Cell. III. 1995)


Cyclin B
+ cdk1
IGF-1
M
G2
DIVISION
Cyclin D
+ cdk4,6
G1
Cyclin E
+ cdk2
Restriction point
S
INTERPHASE
Restriction point
Cyclin A
+ cdk2
FGF-2,
IGF-1/FGF-2

5.2 Summary Model of the combination of growth factors on cell cycle progression in OP cells

The cell cycle in mammalian cells is regulated by integration of multiple signals from intrinsic and extrinsic cues. Growth factors are well known extrinsic signals that induce cell cycle progression. In vitro studies have revealed that a single growth factor is sufficient for cell cycle progression in some cell types, while multiple growth factors are required for proper proliferation of other cell types. In the latter, growth factors act sequentially or together to promote proliferation. However, the molecular mechanisms by which a combination of growth factors exert mitogenic activity have not been fully elucidated.

Growth factor combinations have been studied previously in retinal endothelial cells (Okazaki, Sakai et al. 1996; Kaven, Spraul et al. 2000; Castellon, Hamdi et al. 2002), articular chondrocytes (Okazaki, Sakai et al. 1996), murine hematopoietic cells (Neben, Donaldson et al. 1994; Wu, Nayar et al. 1994) and esophageal epithelial cells (Qureshi, Tchorzewski et al. 1997). In addition, an effort to enhance cellular processes by growth factor combinations has been studied in oligodendrocyte lineage cells. The combination of PDGF and FGF-2, both well-known mitogens in OP cells, promotes proliferation of OP cells to a greater extent than either factor alone (Bogler, Wren et al. 1990; Wolswijk and Noble 1992; McKinnon, Smith et al. 1993). PDGF and FGF-2 treatment of adult OP cells, which divide slowly, converted these cells to rapidly dividing cells more similar to perinatal OP cells (Wolswijk and Noble 1992). The addition of PDGF to OP cell cultures treated with only FGF-2 provided better mobility and maintained the OP cells as early progenitors (McKinnon, Smith et al. 1993).

Numerous investigators have attempted to understand how two growth factors coordinate to promote cellular responses. The effect of combined growth factor treatment on proliferation was first explored in fibroblast cell lines. 3T3 fibroblasts were originally shown to require a specific serum derived growth factor, somatomedin-C (now, known as IGF-1) for DNA synthesis in the presence of PDGF (Stiles, Capone et al. 1979; Russell, Van Wyk et al. 1984). A later study using PDGF and platelet poor plasma (PPP) reported that PDGF was able to induce cyclin D, E and A protein, but not sufficient to overcome

p27 inhibitory effects, resulting arrest in G1 phase. Addition of PPP into PDGF stimulated 3T3 cells further increased cyclin proteins, further reduced p27 and finally enhanced cdk2 activity, leading to S phase entry. In fact, PPP itself is a poor mitogen, which cannot induce cell cycle entry by itself. In this cell culture system, PDGF acts as “competent factor”, which primes cells to respond to a further extrinsic stimulus or “progression factor”, PPP. The authors thus proposed this model for how two growth factors coordinate to induce S phase entry.

Another study supported the competence/progression model, by showing that two signaling pathways are required for S phase entry. The authors demonstrated that transient activation of a receptor kinase, PDGF α receptor, in early G1 is insufficient for cells to pass through the restriction point without activation of a second signaling pathway (Jones and Kazlauskas 2001). In this study, investigators used discontinuous growth factor stimulations to identify the time frame for the second pulse as well as the types of growth factors required. Both PDGF and FGF-2 stimulate first and second signaling pathways for DNA synthesis, however, EGF and insulin are only required for activation of the second signaling pathway in the NIH3T3 cell line. Taken together, it was concluded that PDGF and FGF-2 act as both competence and progression factors while EGF and insulin act only as progression factors.

The competence and progression hypothesis fits well with many growth factor combination studies. However, this hypothesis appears inappropriate for other studies, including the previous study from our laboratory on OP cells. The IGF-1/FGF-2 combination synergistically increased DNA synthesis, while FGF-2/PDGF or IGF-1/PDGF showed additive effects on DNA synthesis (Jiang, Frederick et al. 2001). Since IGF-1 by itself is a poor mitogen for S phase entry in OP cells we postulate that IGF-1 may act as a progression factor along with a competence factor, FGF-2. However, the study of the molecular mechanisms underlying this synergism demonstrated that this postulation is only partially true. The combination of IGF-1 with FGF-2 increased sufficient levels of cyclin D, E, and cdk2 while FGF-2 alone induced protein expression (Frederick and Wood 2004). In addition, cdk2 kinase activity was enhanced in the combination group although FGF-2 stimulated cdk2 activity at a weak level (Frederick

and Wood 2004). However, we cannot rule out the possibility that IGF-1 might be present in FGF-2 alone OP cell cultures since OP cells secrete autocrine IGF-1 (Shinar and McMorris 1995; Du and Dreyfus 2002). In fact, we observed phosphorylated AKT from the FGF-2 alone treated group, which does not activate the AKT pathway to a great extent in oligodendrocyte lineage cells. Of particular interest is our observations here that IGF-1 might be critical for S phase and G2/M progression (Frederick and Wood 2004).

Most of the prior growth factor studies have focused on G1 phase progression and S phase entry since it was thought that once a cell progresses through the G1/S restriction point it will finish the cell cycle without further extrinsic signals. Moreover, some investigators suggested that addition of a growth factor during G2 disturbs the cell cycle and causes G2/M arrest (Dangi, Chen et al. 2006; Nam, Kim et al. 2008). In contrast, another paper emphasized that TGF- mediated timely degradation of cell cycle proteins leads to appropriate mitosis (Fujita, Epperly et al. 2008). However, several investigators suggested that IGF-1 might be necessary for S and G2/M phase progression in other cell types (Sell, Dumenil et al. 1994; Morrione, Valentinis et al. 1997; Adesanya, Zhou et al. 1999; Stromberg, Ekman et al. 2006). Altogether, the addition of growth factors during G2 may disturb the entire signaling pathway since it may activate unnecessary pathways, which in turn, counteracts the already activated pathways. However, this does not necessarily mean that growth factors do not have any role in G2/M phases since the cell cycle is regulated by coordination of multiple cellular events such as synthesis and degradation of proteins, activation and deactivation of kinases and subcellular localization of these critical proteins. Moreover, a G2/M delay mediated by lack of IGF-1 provides the possibility for the competence/progression model in later cell cycle progression. Originally, the term “competence” factor means a growth factor that makes a cell capable of initiating the cell cycle, while “progression” factor means a growth factor that promotes competent cell to progress into S phase (Mason, Jones et al. 2000).

Kamijo et al reported interesting data concerning a two growth factor combination in proliferation of hematopoietic cells. Stem cell factor (SCF) and granulocyte macrophage colony stimulating factor (GM-CSF) is a well-studied growth factor combination for hematopoietic cells (Kamijo, Koike et al. 2002; Lennartsson, Shivakrupa

et al. 2004). However, cell lines derived from different origins respond to these two growth factors differently. A megakaryoblastic cell line is growth arrested in G0/G1 phase in the presence of SCF alone while GM-CSF alone treated cells showed no G2/M progression. The difference from separate treatment of the two growth factors was that SCF induced only cyclin D1 and GM-CSF induced cyclin D3, cyclin E but not cyclin A and B. However, the combination of the two growth factors stimulated exponential proliferation via reciprocal interaction. In this case, it is possible that GM-CSF is a competence factor for S phase entry while SCF is a progression factor for G2/M progression. Nonetheless, there are still limited data to support this hypothesis.

Synergism of two growth factors results from multiple events since the cell cycle is regulated by diverse mechanisms. Each growth factor may target the same or different molecular events. Expression of cell cycle regulatory proteins, cdks and cyclins, is upregulated by growth factors. Growth factors also enhance kinase activity by suppressing expression of cdk inhibitors. Subcellular localization of regulatory molecules is also controlled by growth factor activation of signaling pathways. To identify whether two growth factors synergize to promote cell cycle progression, we need to examine how molecular events and signaling pathways are altered by addition of a growth factor.

We presented data that FGF-2 alone has a weak ability to induce OP cells to traverse G2/M phases, while IGF-1 induces OP cells to normally progress through G2/M. IGF-1 synergizes with 17-β-estradiol (E2) in MCF-7S cells (Hamelers, van Schaik et al. 2002) and with EGF in esophageal epithelia cells (Qureshi, Tchorzewski et al. 1997) and mammary epithelial cells (Stull, Richert et al. 2002)for cell cycle and S phase entry. However, a synergistic role for IGF-1 in S and G2/M progression has not yet been reported. Therefore, the studies presented here report novel findings supporting the hypothesis that IGF-1 modulates cell cycle regulation, particularly during S and G2/M phases.

5.3 Cross talk between two different signaling pathways and cellular responses

Binding of growth factors to their cognate receptors initiates signal transduction and leads to cellular responses. Since growth factors generally activate several signaling

pathways in a cell, proper activation of each signaling pathway is critical to induce specific biological events. IGF-1 activates both the PI3K-AKT pathway and transiently the Raf-MEK-ERK pathway in MCF-7 cells whereas prolonged activation of Raf cascades by IGF-1 inhibits proliferation, implying that duration of Raf pathway activation determines proliferation or growth arrest (Alblas, Slager-Davidov et al. 1998).

Growth factors are involved in many cellular events, and in some cases, their actions contradict each other. For instance, IGF-1 can regulate both proliferation and differentiation. The Ras/Raf/MEK/ERK pathway and PI3K/AKT pathways counteract in myoblast differentiation (Rommel, Clarke et al. 1999). In order to differentiate, only the PI3K pathway is required and for this effect, activated AKT phosphorylates Raf and inhibits further downstream cascades (Rommel, Clarke et al. 1999). Therefore, when AKT is blocked with a pharmacological inhibitor, ERK is activated due to reduced inhibition by AKT. We observed this same pattern of counteractivation of ERK and ATK. However, we have not determined whether the two pathways directly regulate each other in OP cells.

In many cases, different growth factors can activate the same signaling pathways and show similar or differnet cellular responses. PDGF and FGF-2 are well known mitogens for OP cells, and their signaling pathways are fully elucidated. Both PDGF and FGF-2 activate MAPK, p38 MEK and pp70S6K and exert mitogenic effects on OP cells (Baron, Metz et al. 2000). Addition of PKA, which plays a role in differentiation in OP cells, into FGF-2 treated OP cultures abolishes the FGF-2 mitogenic effect. In contrast, PKA has no effect on PDGF treated cultures (Baron, Metz et al. 2000). These results imply that other pathways may be involved in OP cell proliferation mediated by PDGF and FGF-2. Importance of the PI3K pathway in OP cell proliferation has also been reported (Ebner, Dunbar et al. 2000; McKinnon, Waldron et al. 2005; Cui and Almazan 2007). Proliferation of A2B5+ early OP cells requires activation of a wortmannin (a PI3K inhibitor)-sensitive PI3K, while O4+ OP cell proliferation can be either dependent on wortmannin-resistent PI3K or independent of PI3K (Ebner, Dunbar et al. 2000). Additionally, these investigators reported that only PDGF activates PI3K, not FGF-2 (Ebner, Dunbar et al. 2000), supporting the idea that PDGF and FGF-2 have different

mechanisms for OP cell proliferation. In other studies, IGF-1 activates the p38 signaling pathway to direct chondrogenic differentiation of mesenchymal stem cells or tansformation of human sarcoma and carcinoma cell lines. However, the p38 signaling pathway is implicated in differentiation and myelination in oligodendrocyte lineage cells (Jungsoo add ref). Another report demonstrated that IGF-1 alone has mitogenic effects via activated PI3K, ERK, and the Src-like tyrosine kinases, Fyn and Lyn (Cui and Almazan 2007). Results from Cui et al contradict our data that IGF-1 alone does not promote cell cycle entry and S phase entry. However, the investigators used 10 fold higher IGF-1 in their experiments, and it is possible that IGF-1 above a physiological relevant threshold may stimulate several signaling pathways and induce proliferation of OP cells via alternative signaling pathways. It is of interest to understand whether multiple growth factors independently or dependently induce cellular responses since generally cells in vivo are exposed to more than one growth factor. SCF and GM-CSF are both mitogenic for hematopoietic cells as discussed previously (Lennartsson, Shivakrupa et al. 2004). SCF receptor kinase, c-kit, binds to GM-CSF receptor and causes prolonged ERK activation via the PI3K pathway, resulting in synergism on cell proliferation. To elucidate growth factor synergism in OP cell proliferation, previously we reported that FGF-2 and IGF-1 activate distinct signaling pathways and act at different levels on proliferation targets (Frederick, Min et al. 2007).

Signaling studies implicated in the cell cycle have been focused on cell cycle entry and G1 phase progression. Recently, however, several investigators reported that signal transduction during G2/M is important for fine-tuning of cell cycle progression. The role of the ERK pathway in G2/M has been controversial. Observation that an upstream signaling molecule of ERK is activated in G0/G1 and G2/M phases suggests that activation of the ERK pathway is required for proper progression of G2/M in fibroblasts (Wright, Munar et al. 1999; Morgan, Dolp et al. 2001). However, several lines of evidence demonstrate that activation of ERK during G2 causes G2/M arrest (Dangi, Chen et al. 2006). In contrast, an ERK specific inhibitor arrests cells in G2/M (Shinohara, Mikhailov et al. 2006; Dumesic, Scholl et al. 2009; Matkovic, Lukinovic-Skudar et al. 2009). There are several possible reasons for this discrepancy. First, the requirement for

ERK activation might be cell type specific. Second, the thymidine used for S phase arrest may cause aberrant behavior of cells. Third, the amount of activated ERK is different in each experiment, and the threshold of ERK activation for G2/M progression may vary. Since many groups use the same cell lines and methods for synchronization, the third hypothesis is the most likely.

Activation of PI3K also is required for G2/M progression. Activated AKT is essential for G2/M progression, by inactivating a checkpoint kinase (Shtivelman, Sussman et al. 2002). Other investigators have shown that the PI3K pathway enhances cdk1 activity and, in turn, promotes G2/M (Kandel, Skeen et al. 2002; Stromberg, Ekman et al. 2006). However, another group claimed that both the PI3K and the ERK pathways are required for timely entry into mitosis. The PI3K pathway is necessary for cyclin B expression, activation of cdk1 and mitotic entry (Roberts, Shapiro et al. 2002). In contrast, inhibition of the ERK pathway interferes with mitotic entry and causes a delay in progression from metaphase to anaphase (Roberts, Shapiro et al. 2002). We observed that sustained ERK activation correlated with cdk1 kinase activity, but blocking the PI3K pathway decreased P-cdk1 (inactive cdk1). Therefore, G2/M phase regulation is more complex and not solely dependent on cdk1 activation. Orchestration of multiple regulators in a timely manner governs errorless progression into mitosis and further directs serial events, including chromosome condensation, nuclear envelope breakdown, assembly of the mitotic spindle, centrosome separation and cytokinesis.

5.4. The role of IGF-1 on proliferation in OP cells

The mitogenic effect of IGF-1 in OP cells has not been emphasized so far although the role of IGF-1 on maturation and survival of OP cells has been studied more extensively. The presence of micromolar levels of insulin in growth media for OP cells suggests that IGF-1 may have mitogenic effects since insulin at these levels activates the IGF-1R. Supporting this hypothesis, previous studies in our laboratory showed that the combination of IGF-1 with FGF-2 increased S phase entry and DNA synthesis in OP cells (Jiang, Frederick et al. 2001; Frederick and Wood 2004)

In vivo studies reported in 2007 delineated the role of the IGF-1R in oligodendrocyte development. Zeger and colleagues ablated IGF-1R, utilizing Cre/Lox deletion of IGF-1R in OP cells and oligodendrocytes (Zeger, Popken et al. 2007). Disruption of IGF-1R in OP cells resulted in decreased proliferation and number of NG2+ OP cells. As expected, olidogendrocytes with IGF-1R deletion showed reduced myelination. This study clearly showed that IGF signaling is involved in both proliferation (in OP cells) and myelination (in oligodendrocytes), strongly supporting in vitro work in our laboratory. The prior study was of particular importance not only because it demonstrated a direct effect of IGF signaling on the oligodendrocyte lineage but also because it demonstrated an essential role for IGF-1 in proliferation that was not compensated by the known OP cell mitogens, PDGF and FGF-2, which were present in vivo. Whether some compensation occurred in part by other growth factors should be further investigated. In addition, the mechanism by which different cellular responses are elicited by IGF-1 needs to be identified.

The putative role of IGF-1 on proliferation was previously explored in OP cells although the results were not entirely clear. IGF-1 alone increases the population of A2B5+ and O4+ adult OP cells although it had no effect on BrdU incorporation (Mason and Goldman 2002). However, when it is combined with other growth factors, FGF-2 or PDGF, IGF-1 showed increased proliferative effects (Mason and Goldman 2002). Interestingly, FGF-2/IGF-1 in this study enhanced differentiation of O4+ OP cells into O1+ oligodendrocytes, which contradicts results from our laboratory that FGF-2/IGF-1 enhances cell cycle progression as well as prior studies from other laboratories that FGF-2 inhibits oligodendrocyte differentiation (Goddard, Berry et al. 2001; Murtie, Zhou et al. 2005). The discrepancies in these studies may be due to adult versus neonatal OP cells. A recent study from the same group further support this hypothesis since they reported that adult OP cells preferentially differentiate to mature cells compared to neonatal OP cells (Lin, Mela et al. 2009). Taken together, it can be concluded that neonatal OP cells efficiently respond to the mitogenic effect of FGF-2/IGF-1 compared to adult OP cells.

As described above, IGF-1 has been implicated in both proliferation and maturation/myelination in OP cells. Two different effects by one growth factor possibly

results from a different threshold for cellular responses, which is related to receptor expression level or activation in OP cells. Expression level of IGF-1R in oligodendrocyte lineage cells has not been fully established. IGF-1R expression showed no difference in adult A2B5+ OP cells and O4+ OP cells, (Mason and Goldman 2002). However, our previous studies demonstrated maturation-stage differences in IGF-1 stimulation of the IGF-1R and AKT in OP cells (Ness and Wood 2002) supporting the idea of stage-dependent cellular responses. This information may explain why adult OP cells and neonatal OP cells behave differently in response to IGF-1.

5.5 Implication for a mitotic role of IGF-1 in Multiple Sclerosis

Multiple sclerosis (MS) is an autoimmune demyelinating disorder causing death of oligodendrocytes and loss of myelin. Although remyelination occurs, repeated remyelination and demyelination during the disease course leads to relapsing symptoms and finally a failure to remyelinate. Several reasons have been proposed for the limited remyelination in MS lesions, including robust demyelination versus slow remyelination, depletion of OP cells in lesions, recruitment failure of OP cells into lesions, or inhibited differentiation and death of repopulated oligodendrocytes from OP cells (Armstrong 2007). Several lines of evidence have shown that early oligodendrocyte precursor cells or OP cells are present in the adult CNS (Prineas, Kwon et al. 1989; Ozawa, Suchanek et al. 1994; Prabhakar, D'Souza et al. 1995; Scolding, Rayner et al. 1995; Blakemore and Keirstead 1999; Zhang, Ge et al. 1999). However, one report demonstrated that the number of PDGFR-α positive OP cells appeared insufficient in acute and chronic MS patients (Scolding, Franklin et al. 1998), which suggests that expansion of OP cells may improve the remyelinating process in MS plaques.

As described above, incomplete remyelination is attributable in part to depletion of OP cells in the lesions (Blakemore and Keirstead 1999). Impaired OP cell recruitment in demyelinating lesions decreases remyelination in an age dependent manner (Sim, Zhao et al. 2002). In addition, ablation of IGF-1R abolishes OP cell accumulation, proliferation and survival after demyelination, resulting in impaired reymelination (Mason, Xuan et al. 2003). In support of these findings, results from another study showed decreased

proliferation and increased apoptosis in IGF-1R null mice during normal development (Zeger, Popken et al. 2007). Taken together, sufficient numbers of OP cells is important for adequate remyelination, and IGF-1 plays an important role in maintaining OP cell numbers.

Most of the analysis for proliferation is based on [^{3}H] thymidine incorporation or BrdU incorporation to identify cells undergoing DNA synthesis in S phase. However, our results demonstrate that IGF-1 has a distinct role in S and G2/M progression, which is excluded when simply analyzing initial DNA synthesis. It may be critical for IGF-1 to be present to promote proliferation of OP cells if a growth factor, such as FGF-2, is present in the lesions. In addition, an in vitro culture study demonstrated that FGF-2 fails to enhance proliferation of human OP cells (Wilson, Onischke et al. 2003). Interestingly, examination of cerebrospinal fluid revealed that IGF-1 levels in control versus MS patients showed no difference (Poljakovic, Zurak et al. 2006) whereas FGF-2 increased in MS patients (Sarchielli, Di Filippo et al. 2008). Thus, it is possible that one reason for failure of resident OP cells to divide in MS lesions is lack of IGF-1.

Progenitor cells go through a number of cell divisions based on intrinsic and extrinsic cues and then differentiate to mature cell types. Failing demyelination may result from the fact that sufficient OP cells are not generated to differentiate into mature oligodendrocytes because of lack of extrinsic cues. Administration of IGF-1 may help to increase OP cells and promote further remyelination.

IGF-1 has been widely speculated as a potential therapeutic target for MS to enhance remyelination since numerous studies reported that IGF-1 promotes differentiation/maturation of OP cells in vitro (McMorris and Dubois-Dalcq 1988; Mozell and McMorris 1991; Barres, Lazar et al. 1994), and myelination in vivo (Carson, Behringer et al. 1993; Ye, Xing et al. 1996). However, administration of IGF-1 results in various outcomes in an EAE animal model. Some investigators reported enhanced clinical outcomes (Yao, West et al. 1995; Li, Quigley et al. 1998) whereas others showed no beneficial effect of IGF-1 in animal models (Cannella, Pitt et al. 2000; O'Leary, Hinks et al. 2002). The failure of some of these studies to show beneficial effects of IGF-1 may be due to experimental design. It is possible that systemic administration of IGF-1 does

not provide sufficient IGF-1 in the lesions in the CNS due to the blood brain barrier (Cannella, Pitt et al. 2000). Local expression of IGF-1 using adenoviral vectors also may cause immune responses and change the microenvironment (O'Leary, Hinks et al. 2002). Co-administration or co-expression of other proteins to enhance bioavailability of IGF-1 may help with continuous provision and effectivenss of IGF-1.

5.6. Conclusion

We present here that IGF-1 modulates cell cycle progression particularly in S and G2/M progression in OP cells. This finding is a novel finding in OP cells since IGF-1 by itself is a weak mitogen. IGF-1 coordinates with FGF-2 to enhance cdk2 kinase activity for progression through S phase and entry into G2/M. IGF-1 alone in G2/M enhances cdk1 kinase activity and entry into mitosis. The specific molecular events underlying these results need to be further investigated. Taken together, however, the present studies widen our understanding of how the cell cycle in OP cells is regulated by FGF-2 and IGF-1. Ultimately, the study of primary OP cell cultures in vitro is important to understand normal development of the oligodendrocyte lineage. Furthermore, understanding the exact mechanisms by which oligodendrocyte development is regulated in a spatial and temporal manner will help to develop better therapeutic targets, especially for demyelinating diseases such as MS.

Biblography

Adesanya, O. O., J. Zhou, et al. (1999). "Insulin-like growth factor 1 is required for G2 progression in the estradiol-induced mitotic cycle." Proc Natl Acad Sci U S A **96**(6): 3287-91.

Agudo, J., E. Ayuso, et al. (2008). "IGF-I mediates regeneration of endocrine pancreas by increasing beta cell replication through cell cycle protein modulation in mice." Diabetologia **51**(10): 1862-72.

Armstrong, R. C. (2007). "Growth factor regulation of remyelination: behind the growing interest in endogenous cell repair of the CNS." Future Neurol **2**(6): 689-697.

Armstrong, R. C., L. Harvath, et al. (1990). "Type 1 astrocytes and oligodendrocyte-type 2 astrocyte glial progenitors migrate toward distinct molecules." J Neurosci Res **27**(3): 400-7.

Armstrong, R. C., J. G. Kim, et al. (1995). "Expression of myelin transcription factor I (MyTI), a "zinc-finger" DNA-binding protein, in developing oligodendrocytes." Glia **14**(4): 303-21.

Bansal, R. (2002). "Fibroblast growth factors and their receptors in oligodendrocyte development: implications for demyelination and remyelination." Dev Neurosci **24**(1): 35-46.

Bansal, R., M. Kumar, et al. (1996). "Regulation of FGF receptors in the oligodendrocyte lineage." Mol Cell Neurosci **7**(4): 263-75.

Bansal, R., V. Lakhina, et al. (2003). "Expression of FGF receptors 1, 2, 3 in the embryonic and postnatal mouse brain compared with Pdgfralpha, Olig2 and Plp/dm20: implications for oligodendrocyte development." Dev Neurosci **25**(2-4): 83-95.

Bansal, R. and S. E. Pfeiffer (1997). "FGF-2 converts mature oligodendrocytes to a novel phenotype." J Neurosci Res **50**(2): 215-28.

Baron-Van Evercooren, A., C. Olichon-Berthe, et al. (1991). "Expression of IGF-I and insulin receptor genes in the rat central nervous system: a developmental, regional, and cellular analysis." J Neurosci Res **28**(2): 244-53.

Baron, W., B. Metz, et al. (2000). "PDGF and FGF-2 signaling in oligodendrocyte progenitor cells: regulation of proliferation and differentiation by multiple intracellular signaling pathways." Mol Cell Neurosci **15**(3): 314-29.

Bell, S. P. and A. Dutta (2002). "DNA replication in eukaryotic cells." Annu Rev Biochem **71**: 333-74.

Besnard, F., F. Perraud, et al. (1989). "Effects of acidic and basic fibroblast growth factors on proliferation and maturation of cultured rat oligodendrocytes." Int J Dev Neurosci **7**(4): 401-9.

Bhat, N. R., K. F. Hauser, et al. (1992). "Cell proliferation and protooncogene induction in oligodendroglial progenitors." J Neurosci Res **32**(3): 340-9.

Bhat, N. R. and P. Zhang (1996). "Activation of mitogen-activated protein kinases in oligodendrocytes." J Neurochem **66**(5): 1986-94.

Bibollet-Bahena, O. and G. Almazan (2009). "IGF-1-stimulated protein synthesis in oligodendrocyte progenitors requires PI3K/mTOR/Akt and MEK/ERK pathways." J Neurochem **109**(5): 1440-51.

Blakemore, W. F. and H. S. Keirstead (1999). "The origin of remyelinating cells in the central nervous system." J Neuroimmunol **98**(1): 69-76.

Blakesley, V. A., A. Scrimgeour, et al. (1996). "Signaling via the insulin-like growth factor-I receptor: does it differ from insulin receptor signaling?" Cytokine Growth Factor Rev **7**(2): 153-9.

Bondy, C. A. and C. M. Cheng (2004). "Signaling by insulin-like growth factor 1 in brain." Eur J Pharmacol **490**(1-3): 25-31.

Boutros, R., V. Lobjois, et al. (2007). "CDC25 phosphatases in cancer cells: key players? Good targets?" Nat Rev Cancer **7**(7): 495-507.

Brandeis, M., I. Rosewell, et al. (1998). "Cyclin B2-null mice develop normally and are fertile whereas cyclin B1-null mice die in utero." Proc Natl Acad Sci U S A **95**(8): 4344-9.

Bunge, R. P. (1968). "Glial cells and the central myelin sheath." Physiol Rev **48**(1): 197-251.

Burtrum, D., Z. Zhu, et al. (2003). "A fully human monoclonal antibody to the insulin-like growth factor I receptor blocks ligand-dependent signaling and inhibits human tumor growth in vivo." Cancer Res **63**(24): 8912-21.

Butt, A. M. and B. R. Ransom (1989). "Visualization of oligodendrocytes and astrocytes in the intact rat optic nerve by intracellular injection of lucifer yellow and horseradish peroxidase." Glia **2**(6): 470-5.

Cameron-Curry, P. and N. M. Le Douarin (1995). "Oligodendrocyte precursors originate from both the dorsal and the ventral parts of the spinal cord." Neuron **15**(6): 1299-310.

Cannella, B., D. Pitt, et al. (2000). "Insulin-like growth factor-1 fails to enhance central nervous system myelin repair during autoimmune demyelination." Am J Pathol **157**(3): 933-43.

Carson, M. J., R. R. Behringer, et al. (1993). "Insulin-like growth factor I increases brain growth and central nervous system myelination in transgenic mice." Neuron **10**(4): 729-40.

Casaccia-Bonnefil, P., R. Tikoo, et al. (1997). "Oligodendrocyte precursor differentiation is perturbed in the absence of the cyclin-dependent kinase inhibitor p27Kip1." Genes Dev **11**(18): 2335-46.

Castro, A., C. Bernis, et al. (2005). "The anaphase-promoting complex: a key factor in the regulation of cell cycle." Oncogene **24**(3): 314-25.

Chan, B. T. and A. V. Lee (2008). "Insulin receptor substrates (IRSs) and breast tumorigenesis." J Mammary Gland Biol Neoplasia **13**(4): 415-22.

Chapman, D. L. and D. J. Wolgemuth (1993). "Isolation of the murine cyclin B2 cDNA and characterization of the lineage and temporal specificity of expression of the B1 and B2 cyclins during oogenesis, spermatogenesis and early embryogenesis." Development **118**(1): 229-40.

Chellappan, S. P., S. Hiebert, et al. (1991). "The E2F transcription factor is a cellular target for the RB protein." cell **65**(6): 1053-61.

Cheng, C. M., G. Joncas, et al. (1998). "Biochemical and morphometric analyses show that myelination in the insulin-like growth factor 1 null brain is proportionate to its neuronal composition." J Neurosci **18**(15): 5673-81.

Chernoff, G. F. (1981). "Shiverer: an autosomal recessive mutant mouse with myelin deficiency." J Hered **72**(2): 128.

Cohen, R. I. and K. J. Chandross (2000). "Fibroblast growth factor-9 modulates the expression of myelin related proteins and multiple fibroblast growth factor receptors in developing oligodendrocytes." J Neurosci Res **61**(3): 273-87.

Cotton, L. M., M. K. O'Bryan, et al. (2008). "Cellular signaling by fibroblast growth factors (FGFs) and their receptors (FGFRs) in male reproduction." Endocr Rev **29**(2): 193-216.

Coverley, D., H. Laman, et al. (2002). "Distinct roles for cyclins E and A during DNA replication complex assembly and activation." Nat Cell Biol **4**(7): 523-8.

Cui, Q. L. and G. Almazan (2007). "IGF-I-induced oligodendrocyte progenitor proliferation requires PI3K/Akt, MEK/ERK, and Src-like tyrosine kinases." J Neurochem **100**(6): 1480-93.

Cui, Q. L., W. H. Zheng, et al. (2005). "Inhibition of Src-like kinases reveals Akt-dependent and -independent pathways in insulin-like growth factor I-mediated oligodendrocyte progenitor survival." J Biol Chem **280**(10): 8918-28.

D'Ercole, A. J., P. Ye, et al. (1996). "The role of the insulin-like growth factors in the central nervous system." Mol Neurobiol **13**(3): 227-55.

Dai, R., R. Chen, et al. (2009). "Cross-talk between PI3K/Akt and MEK/ERK pathways mediates endoplasmic reticulum stress-induced cell cycle progression and cell death in human hepatocellular carcinoma cells." Int J Oncol **34**(6): 1749-57.

Dangi, S., H. Cha, et al. (2003). "Requirement for phosphatidylinositol-3 kinase activity during progression through S-phase and entry into mitosis." Cell Signal **15**(7): 667-75.

Davies, J. E. and R. H. Miller (2001). "Local sonic hedgehog signaling regulates oligodendrocyte precursor appearance in multiple ventricular zone domains in the chick metencephalon." Dev Biol **233**(2): 513-25.

Denley, A., L. J. Cosgrove, et al. (2005). "Molecular interactions of the IGF system." Cytokine Growth Factor Rev **16**(4-5): 421-39.

DiCicco-Bloom, E. and I. B. Black (1988). "Insulin growth factors regulate the mitotic cycle in cultured rat sympathetic neuroblasts." Proc Natl Acad Sci U S A **85**(11): 4066-70.

Dono, R., G. Texido, et al. (1998). "Impaired cerebral cortex development and blood pressure regulation in FGF-2-deficient mice." EMBO J **17**(15): 4213-25.

Dotto, G. P. (2000). "p21(WAF1/Cip1): more than a break to the cell cycle?" Biochim Biophys Acta **1471**(1): M43-56.

Du, Y. and C. F. Dreyfus (2002). "Oligodendrocytes as providers of growth factors." J Neurosci Res **68**(6): 647-54.

Durand, B. and M. Raff (2000). "A cell-intrinsic timer that operates during oligodendrocyte development." Bioessays **22**(1): 64-71.

Dyson, N. (1998). "The regulation of E2F by pRB-family proteins." Genes Dev **12**(15): 2245-62.

Eizenberg, O., A. Faber-Elman, et al. (1996). "p53 plays a regulatory role in differentiation and apoptosis of central nervous system-associated cells." Mol Cell Biol **16**(9): 5178-85.

Elledge, S. J. (1996). "Cell cycle checkpoints: preventing an identity crisis." Science **274**(5293): 1664-72.
Espinosa de los Monteros, A., M. Zhang, et al. (1993). "O2A progenitor cells transplanted into the neonatal rat brain develop into oligodendrocytes but not astrocytes." Proc Natl Acad Sci U S A **90**(1): 50-4.
Eswarakumar, V. P., I. Lax, et al. (2005). "Cellular signaling by fibroblast growth factor receptors." Cytokine Growth Factor Rev **16**(2): 139-49.
Ffrench-Constant, C. and M. C. Raff (1986). "Proliferating bipotential glial progenitor cells in adult rat optic nerve." Nature **319**(6053): 499-502.
Fotedar, A., D. Cannella, et al. (1996). "Role for cyclin A-dependent kinase in DNA replication in human S phase cell extracts." J Biol Chem **271**(49): 31627-37.
Frederick, T. J., J. Min, et al. (2007). "Synergistic induction of cyclin D1 in oligodendrocyte progenitor cells by IGF-I and FGF-2 requires differential stimulation of multiple signaling pathways." Glia **55**(10): 1011-22.
Frederick, T. J. and T. L. Wood (2004). "IGF-I and FGF-2 coordinately enhance cyclin D1 and cyclin E-cdk2 association and activity to promote G1 progression in oligodendrocyte progenitor cells." Mol Cell Neurosci **25**(3): 480-92.
Fung, T. K. and R. Y. Poon (2005). "A roller coaster ride with the mitotic cyclins." Semin Cell Dev Biol **16**(3): 335-42.
Gabrielli, B. G., C. P. De Souza, et al. (1996). "Cytoplasmic accumulation of cdc25B phosphatase in mitosis triggers centrosomal microtubule nucleation in HeLa cells." J Cell Sci **109 (Pt 5)**: 1081-93.
Gao, F. B., B. Durand, et al. (1997). "Oligodendrocyte precursor cells count time but not cell divisions before differentiation." Curr Biol **7**(2): 152-5.
Garcia-Segura, L. M., J. R. Rodriguez, et al. (1997). "Localization of the insulin-like growth factor I receptor in the cerebellum and hypothalamus of adult rats: an electron microscopic study." J Neurocytol **26**(7): 479-90.
Gensert, J. M. and J. E. Goldman (2001). "Heterogeneity of cycling glial progenitors in the adult mammalian cortex and white matter." J Neurobiol **48**(2): 75-86.
Gladden, A. B. and J. A. Diehl (2005). "Location, location, location: the role of cyclin D1 nuclear localization in cancer." J Cell Biochem **96**(5): 906-13.
Goddard, D. R., M. Berry, et al. (2001). "Fibroblast growth factor-2 inhibits myelin production by oligodendrocytes in vivo." Mol Cell Neurosci **18**(5): 557-69.
Grinspan, J. B., J. L. Stern, et al. (1993). "Trophic effects of basic fibroblast growth factor (bFGF) on differentiated oligodendroglia: a mechanism for regeneration of the oligodendroglial lineage." J Neurosci Res **36**(6): 672-80.
Hagting, A., M. Jackman, et al. (1999). "Translocation of cyclin B1 to the nucleus at prophase requires a phosphorylation-dependent nuclear import signal." Curr Biol **9**(13): 680-9.
Hallak, H., G. Moehren, et al. (2002). "Epidermal growth factor-induced activation of the insulin-like growth factor I receptor in rat hepatocytes." Hepatology **36**(6): 1509-18.
Hamelers, I. H., R. F. van Schaik, et al. (2002). "Synergistic proliferative action of insulin-like growth factor I and 17 beta-estradiol in MCF-7S breast tumor cells." Exp Cell Res **273**(1): 107-17.

Harbour, J. W. and D. C. Dean (2000). "The Rb/E2F pathway: expanding roles and emerging paradigms." Genes Dev **14**(19): 2393-409.

Hardy, R. and R. Reynolds (1991). "Proliferation and differentiation potential of rat forebrain oligodendroglial progenitors both in vitro and in vivo." Development **111**(4): 1061-80.

Harlow, E., P. Whyte, et al. (1986). "Association of adenovirus early-region 1A proteins with cellular polypeptides." Mol Cell Biol **6**(5): 1579-89.

Hart, I. K., W. D. Richardson, et al. (1989). "PDGF receptors on cells of the oligodendrocyte-type-2 astrocyte (O-2A) cell lineage." Development **105**(3): 595-603.

He, W., C. Ingraham, et al. (2001). "Multipotent stem cells from the mouse basal forebrain contribute GABAergic neurons and oligodendrocytes to the cerebral cortex during embryogenesis." J Neurosci **21**(22): 8854-62.

Henglein, B., X. Chenivesse, et al. (1994). "Structure and cell cycle-regulated transcription of the human cyclin A gene." Proc Natl Acad Sci U S A **91**(12): 5490-4.

Hermeking, H. and A. Benzinger (2006). "14-3-3 proteins in cell cycle regulation." Semin Cancer Biol **16**(3): 183-92.

Horiuchi, M., A. Itoh, et al. (2006). "MEK-ERK signaling is involved in interferon-gamma-induced death of oligodendroglial progenitor cells." J Biol Chem **281**(29): 20095-106.

Ivanova, A., E. Nakahira, et al. (2003). "Evidence for a second wave of oligodendrogenesis in the postnatal cerebral cortex of the mouse." J Neurosci Res **73**(5): 581-92.

Jiang, F., T. J. Frederick, et al. (2001). "IGF-I synergizes with FGF-2 to stimulate oligodendrocyte progenitor entry into the cell cycle." Dev Biol **232**(2): 414-23.

Jones, S. M. and A. Kazlauskas (2001). "Growth-factor-dependent mitogenesis requires two distinct phases of signalling." Nat Cell Biol **3**(2): 165-72.

Kakita, A. and J. E. Goldman (1999). "Patterns and dynamics of SVZ cell migration in the postnatal forebrain: monitoring living progenitors in slice preparations." Neuron **23**(3): 461-72.

Keirstead, H. S., J. M. Levine, et al. (1998). "Response of the oligodendrocyte progenitor cell population (defined by NG2 labelling) to demyelination of the adult spinal cord." Glia **22**(2): 161-70.

Koff, A., A. Giordano, et al. (1992). "Formation and activation of a cyclin E-cdk2 complex during the G1 phase of the human cell cycle." Science **257**(5077): 1689-94.

Lachapelle, F., M. Gumpel, et al. (1983). "Transplantation of CNS fragments into the brain of shiverer mutant mice: extensive myelination by implanted oligodendrocytes. I. Immunohistochemical studies." Dev Neurosci **6**(6): 325-34.

Lai, A., B. Sarcevic, et al. (2001). "Insulin/insulin-like growth factor-I and estrogen cooperate to stimulate cyclin E-Cdk2 activation and cell Cycle progression in MCF-7 breast cancer cells through differential regulation of cyclin E and p21(WAF1/Cip1)." J Biol Chem **276**(28): 25823-33.

Lee, S. R., J. H. Park, et al. (2005). "Akt-induced promotion of cell-cycle progression at G2/M phase involves upregulation of NF-Y binding activity in PC12 cells." J Cell Physiol **205**(2): 270-7.
LeRoith, D. and C. T. Roberts, Jr. (2003). "The insulin-like growth factor system and cancer." Cancer Lett **195**(2): 127-37.
Levison, S. W. and K. D. McCarthy (1991). "Characterization and partial purification of AIM: a plasma protein that induces rat cerebral type 2 astroglia from bipotential glial progenitors." J Neurochem **57**(3): 782-94.
Lin, G., A. Mela, et al. (2009). "Neonatal and adult O4(+) oligodendrocyte lineage cells display different growth factor responses and different gene expression patterns." J Neurosci Res.
Lindqvist, A., V. Rodriguez-Bravo, et al. (2009). "The decision to enter mitosis: feedback and redundancy in the mitotic entry network." J Cell Biol **185**(2): 193-202.
Liu, D., M. M. Matzuk, et al. (1998). "Cyclin A1 is required for meiosis in the male mouse." Nat Genet **20**(4): 377-80.
Magy, L., M. Keita, et al. (2003). "Transient exposure to FGF2 enhances myelination in embryonic brain cell cocultures." Exp Neurol **181**(1): 17-24.
Maier, C. E. and R. H. Miller (1997). "Notochord is essential for oligodendrocyte development in Xenopus spinal cord." J Neurosci Res **47**(4): 361-71.
Mason, J. L. and J. E. Goldman (2002). "A2B5+ and O4+ Cycling progenitors in the adult forebrain white matter respond differentially to PDGF-AA, FGF-2, and IGF-1." Mol Cell Neurosci **20**(1): 30-42.
Massague, J. and K. Polyak (1995). "Mammalian antiproliferative signals and their targets." Curr Opin Genet Dev **5**(1): 91-6.
Masters, B. A., H. Werner, et al. (1991). "Insulin-like growth factor I (IGF-I) receptors and IGF-I action in oligodendrocytes from rat brains." Regul Pept **33**(2): 117-31.
McCarthy, K. D. and J. de Vellis (1980). "Preparation of separate astroglial and oligodendroglial cell cultures from rat cerebral tissue." J Cell Biol **85**(3): 890-902.
McKinnon, R. D., T. Matsui, et al. (1990). "FGF modulates the PDGF-driven pathway of oligodendrocyte development." Neuron **5**(5): 603-14.
McKinnon, R. D., C. Smith, et al. (1993). "Distinct effects of bFGF and PDGF on oligodendrocyte progenitor cells." Glia **7**(3): 245-54.
McMorris, F. A. and M. Dubois-Dalcq (1988). "Insulin-like growth factor I promotes cell proliferation and oligodendroglial commitment in rat glial progenitor cells developing in vitro." J Neurosci Res **21**(2-4): 199-209.
McMorris, F. A. and R. D. McKinnon (1996). "Regulation of oligodendrocyte development and CNS myelination by growth factors: prospects for therapy of demyelinating disease." Brain Pathol **6**(3): 313-29.
Messersmith, D. J., J. C. Murtie, et al. (2000). "Fibroblast growth factor 2 (FGF2) and FGF receptor expression in an experimental demyelinating disease with extensive remyelination." J Neurosci Res **62**(2): 241-56.
Mewar, R. and F. A. McMorris (1997). "Expression of insulin-like growth factor-binding protein messenger RNAs in developing rat oligodendrocytes and astrocytes." J Neurosci Res **50**(5): 721-8.

Miake-Lye, R. and M. W. Kirschner (1985). "Induction of early mitotic events in a cell-free system." cell **41**(1): 165-75.

Miller, R. H. (2002). "Regulation of oligodendrocyte development in the vertebrate CNS." Prog Neurobiol **67**(6): 451-67.

Miller, R. H., J. Payne, et al. (1997). "Spinal cord oligodendrocytes develop from a limited number of migratory highly proliferative precursors." J Neurosci Res **50**(2): 157-68.

Mittnacht, S. (1998). "Control of pRB phosphorylation." Curr Opin Genet Dev **8**(1): 21-7.

Morgan, D. O. (1997). "Cyclin-dependent kinases: engines, clocks, and microprocessors." Annu Rev Cell Dev Biol **13**: 261-91.

Morrione, A., B. Valentinis, et al. (1997). "The role of mGrb10alpha in insulin-like growth factor I-mediated growth." J Biol Chem **272**(42): 26382-7.

Mozell, R. L. and F. A. McMorris (1991). "Insulin-like growth factor I stimulates oligodendrocyte development and myelination in rat brain aggregate cultures." J Neurosci Res **30**(2): 382-90.

Murphy, M., M. G. Stinnakre, et al. (1997). "Delayed early embryonic lethality following disruption of the murine cyclin A2 gene." Nat Genet **15**(1): 83-6.

Murtie, J. C., Y. X. Zhou, et al. (2005). "In vivo analysis of oligodendrocyte lineage development in postnatal FGF2 null mice." Glia **49**(4): 542-54.

Ness, J. K., R. C. Scaduto, Jr., et al. (2004). "IGF-I prevents glutamate-mediated bax translocation and cytochrome C release in O4+ oligodendrocyte progenitors." Glia **46**(2): 183-94.

Ness, J. K. and T. L. Wood (2002). "Insulin-like growth factor I, but not neurotrophin-3, sustains Akt activation and provides long-term protection of immature oligodendrocytes from glutamate-mediated apoptosis." Mol Cell Neurosci **20**(3): 476-88.

Nicolay, D. J., J. R. Doucette, et al. (2007). "Transcriptional control of oligodendrogenesis." Glia **55**(13): 1287-99.

Nigg, E. A. (2001). "Mitotic kinases as regulators of cell division and its checkpoints." Nat Rev Mol Cell Biol **2**(1): 21-32.

Okazaki, R., A. Sakai, et al. (1996). "Effects of transforming growth factor beta s and basic fibroblast growth factor on articular chondrocytes obtained from immobilised rabbit knees." Ann Rheum Dis **55**(3): 181-6.

Oldham, S. and E. Hafen (2003). "Insulin/IGF and target of rapamycin signaling: a TOR de force in growth control." Trends Cell Biol **13**(2): 79-85.

Olivier, C., I. Cobos, et al. (2001). "Monofocal origin of telencephalic oligodendrocytes in the anterior entopeduncular area of the chick embryo." Development **128**(10): 1757-69.

Ono, K., H. Fujisawa, et al. (1997). "Early development of the oligodendrocyte in the embryonic chick metencephalon." J Neurosci Res **48**(3): 212-25.

Ono, K., Y. Yasui, et al. (1997). "Focal ventricular origin and migration of oligodendrocyte precursors into the chick optic nerve." Neuron **19**(2): 283-92.

Ookata, K., S. Hisanaga, et al. (1993). "Association of p34cdc2/cyclin B complex with microtubules in starfish oocytes." J Cell Sci **105 (Pt 4)**: 873-81.

Ornitz, D. M. (2001). "Regulation of chondrocyte growth and differentiation by fibroblast growth factor receptor 3." Novartis Found Symp **232**: 63-76; discussion 76-80, 272-82.

Ozawa, K., G. Suchanek, et al. (1994). "Patterns of oligodendroglia pathology in multiple sclerosis." Brain **117 (Pt 6)**: 1311-22.

Pandini, G., F. Frasca, et al. (2002). "Insulin/insulin-like growth factor I hybrid receptors have different biological characteristics depending on the insulin receptor isoform involved." J Biol Chem **277**(42): 39684-95.

Pang, Y., B. Zheng, et al. (2007). "IGF-1 protects oligodendrocyte progenitors against TNFalpha-induced damage by activation of PI3K/Akt and interruption of the mitochondrial apoptotic pathway." Glia **55**(11): 1099-107.

Pardee, A. B. (1974). "A restriction point for control of normal animal cell proliferation." Proc Natl Acad Sci U S A **71**(4): 1286-90.

Pende, M., T. L. Fisher, et al. (1997). "Neurotransmitter- and growth factor-induced cAMP response element binding protein phosphorylation in glial cell progenitors: role of calcium ions, protein kinase C, and mitogen-activated protein kinase/ribosomal S6 kinase pathway." J Neurosci **17**(4): 1291-301.

Pines, J. and T. Hunter (1989). "Isolation of a human cyclin cDNA: evidence for cyclin mRNA and protein regulation in the cell cycle and for interaction with p34cdc2." cell **58**(5): 833-46.

Pines, J. and T. Hunter (1990). "Human cyclin A is adenovirus E1A-associated protein p60 and behaves differently from cyclin B." Nature **346**(6286): 760-3.

Pines, J. and T. Hunter (1991). "Human cyclins A and B1 are differentially located in the cell and undergo cell cycle-dependent nuclear transport." J Cell Biol **115**(1): 1-17.

Planas-Silva, M. D. and R. A. Weinberg (1997). "The restriction point and control of cell proliferation." Curr Opin Cell Biol **9**(6): 768-72.

Poljakovic, Z., N. Zurak, et al. (2006). "Growth hormone and insulin growth factor-I levels in plasma and cerebrospinal fluid of patients with multiple sclerosis." Clin Neurol Neurosurg **108**(3): 255-8.

Popken, G. J., R. D. Hodge, et al. (2004). "In vivo effects of insulin-like growth factor-I (IGF-I) on prenatal and early postnatal development of the central nervous system." Eur J Neurosci **19**(8): 2056-68.

Prabhakar, S., S. D'Souza, et al. (1995). "Phenotypic and cell cycle properties of human oligodendrocytes in vitro." Brain Res **672**(1-2): 159-69.

Prigent, C. and S. Dimitrov (2003). "Phosphorylation of serine 10 in histone H3, what for?" J Cell Sci **116**(Pt 18): 3677-85.

Prineas, J. W., R. O. Barnard, et al. (1993). "Multiple sclerosis: remyelination of nascent lesions." Ann Neurol **33**(2): 137-51.

Prineas, J. W., E. E. Kwon, et al. (1989). "Multiple sclerosis. Oligodendrocyte proliferation and differentiation in fresh lesions." Lab Invest **61**(5): 489-503.

Qureshi, F. G., M. T. Tchorzewski, et al. (1997). "EGF and IGF-I synergistically stimulate proliferation of human esophageal epithelial cells." J Surg Res **69**(2): 354-8.

Raff, M. C., E. R. Abney, et al. (1984). "Two glial cell lineages diverge prenatally in rat optic nerve." Dev Biol **106**(1): 53-60.

Raff, M. C., R. H. Miller, et al. (1983). "A glial progenitor cell that develops in vitro into an astrocyte or an oligodendrocyte depending on culture medium." Nature **303**(5916): 390-6.

Rechler, M. M. and S. P. Nissley (1985). "The nature and regulation of the receptors for insulin-like growth factors." Annu Rev Physiol **47**: 425-42.

Reuss, B. and O. von Bohlen und Halbach (2003). "Fibroblast growth factors and their receptors in the central nervous system." Cell Tissue Res **313**(2): 139-57.

Reynolds, R. and R. Hardy (1997). "Oligodendroglial progenitors labeled with the O4 antibody persist in the adult rat cerebral cortex in vivo." J Neurosci Res **47**(5): 455-70.

Richardson, W. D., N. Kessaris, et al. (2006). "Oligodendrocyte wars." Nat Rev Neurosci **7**(1): 11-8.

Roberts, E. C., P. S. Shapiro, et al. (2002). "Distinct cell cycle timing requirements for extracellular signal-regulated kinase and phosphoinositide 3-kinase signaling pathways in somatic cell mitosis." Mol Cell Biol **22**(20): 7226-41.

Rosenblatt, J., Y. Gu, et al. (1992). "Human cyclin-dependent kinase 2 is activated during the S and G2 phases of the cell cycle and associates with cyclin A." Proc Natl Acad Sci U S A **89**(7): 2824-8.

Rowzee, A. M., D. A. Lazzarino, et al. (2008). "IGF ligand and receptor regulation of mammary development." J Mammary Gland Biol Neoplasia **13**(4): 361-70.

Roy, L. M., K. I. Swenson, et al. (1991). "Activation of p34cdc2 kinase by cyclin A." J Cell Biol **113**(3): 507-14.

Rusnati, M., C. Urbinati, et al. (1993). "Internalization of basic fibroblast growth factor (bFGF) in cultured endothelial cells: role of the low affinity heparin-like bFGF receptors." J Cell Physiol **154**(1): 152-61.

Russell, P. and P. Nurse (1986). "cdc25+ functions as an inducer in the mitotic control of fission yeast." cell **45**(1): 145-53.

Saneto, R. P. and J. de Vellis (1985). "Characterization of cultured rat oligodendrocytes proliferating in a serum-free, chemically defined medium." Proc Natl Acad Sci U S A **82**(10): 3509-13.

Santamaria, D., C. Barriere, et al. (2007). "Cdk1 is sufficient to drive the mammalian cell cycle." Nature **448**(7155): 811-5.

Sarchielli, P., M. Di Filippo, et al. (2008). "Fibroblast growth factor-2 levels are elevated in the cerebrospinal fluid of multiple sclerosis patients." Neurosci Lett **435**(3): 223-8.

Satoh, J. and S. U. Kim (1994). "Proliferation and differentiation of fetal human oligodendrocytes in culture." J Neurosci Res **39**(3): 260-72.

Schmid, C. (1995). "Insulin-like growth factors." Cell Biol Int **19**(5): 445-57.

Schonrock, L. M., T. Kuhlmann, et al. (1998). "Identification of glial cell proliferation in early multiple sclerosis lesions." Neuropathol Appl Neurobiol **24**(4): 320-30.

Scolding, N., R. Franklin, et al. (1998). "Oligodendrocyte progenitors are present in the normal adult human CNS and in the lesions of multiple sclerosis." Brain **121 (Pt 12)**: 2221-8.

Scolding, N. J., P. J. Rayner, et al. (1995). "A proliferative adult human oligodendrocyte progenitor." Neuroreport **6**(3): 441-5.

Sell, C., G. Dumenil, et al. (1994). "Effect of a null mutation of the insulin-like growth factor I receptor gene on growth and transformation of mouse embryo fibroblasts." Mol Cell Biol **14**(6): 3604-12.
Sherr, C. J. (1996). "Cancer cell cycles." Science **274**(5293): 1672-7.
Shinar, Y. and F. A. McMorris (1995). "Developing oligodendroglia express mRNA for insulin-like growth factor-I, a regulator of oligodendrocyte development." J Neurosci Res **42**(4): 516-27.
Shoemaker, C. B. and R. Chalkley (1978). "An H3 histone-specific kinase isolated from bovine thymus chromatin." J Biol Chem **253**(16): 5802-7.
Shtivelman, E., J. Sussman, et al. (2002). "A role for PI 3-kinase and PKB activity in the G2/M phase of the cell cycle." Curr Biol **12**(11): 919-24.
Spassky, N., C. Goujet-Zalc, et al. (1998). "Multiple restricted origin of oligodendrocytes." J Neurosci **18**(20): 8331-43.
Stevens, C. and N. B. La Thangue (2003). "E2F and cell cycle control: a double-edged sword." Arch Biochem Biophys **412**(2): 157-69.
Stiles, C. D., G. T. Capone, et al. (1979). "Dual control of cell growth by somatomedins and platelet-derived growth factor." Proc Natl Acad Sci U S A **76**(3): 1279-83.
Stolt, C. C., P. Lommes, et al. (2003). "The Sox9 transcription factor determines glial fate choice in the developing spinal cord." Genes Dev **17**(13): 1677-89.
Strausfeld, U., J. C. Labbe, et al. (1991). "Dephosphorylation and activation of a p34cdc2/cyclin B complex in vitro by human CDC25 protein." Nature **351**(6323): 242-5.
Stromberg, T., S. Ekman, et al. (2006). "IGF-1 receptor tyrosine kinase inhibition by the cyclolignan PPP induces G2/M-phase accumulation and apoptosis in multiple myeloma cells." Blood **107**(2): 669-78.
Stull, M. A., M. M. Richert, et al. (2002). "Requirement for IGF-I in epidermal growth factor-mediated cell cycle progression of mammary epithelial cells." Endocrinology **143**(5): 1872-9.
Sugimoto, Y., M. Taniguchi, et al. (2001). "Guidance of glial precursor cell migration by secreted cues in the developing optic nerve." Development **128**(17): 3321-30.
Sweeney, C., M. Murphy, et al. (1996). "A distinct cyclin A is expressed in germ cells in the mouse." Development **122**(1): 53-64.
Swenson, K. I., K. M. Farrell, et al. (1986). "The clam embryo protein cyclin A induces entry into M phase and the resumption of meiosis in Xenopus oocytes." cell **47**(6): 861-70.
Takizawa, C. G. and D. O. Morgan (2000). "Control of mitosis by changes in the subcellular location of cyclin-B1-Cdk1 and Cdc25C." Curr Opin Cell Biol **12**(6): 658-65.
Thisse, B. and C. Thisse (2005). "Functions and regulations of fibroblast growth factor signaling during embryonic development." Dev Biol **287**(2): 390-402.
Tsai, H. H., M. Tessier-Lavigne, et al. (2003). "Netrin 1 mediates spinal cord oligodendrocyte precursor dispersal." Development **130**(10): 2095-105.
Ubersax, J. A., E. L. Woodbury, et al. (2003). "Targets of the cyclin-dependent kinase Cdk1." Nature **425**(6960): 859-64.

van den Heuvel, S. and N. J. Dyson (2008). "Conserved functions of the pRB and E2F families." Nat Rev Mol Cell Biol **9**(9): 713-24.
van Heyningen, P., A. R. Calver, et al. (2001). "Control of progenitor cell number by mitogen supply and demand." Curr Biol **11**(4): 232-41.
van Straaten, H. W., J. W. Hekking, et al. (1989). "Effect of the notochord on proliferation and differentiation in the neural tube of the chick embryo." Development **107**(4): 793-803.
Vemuri, G. S. and F. A. McMorris (1996). "Oligodendrocytes and their precursors require phosphatidylinositol 3-kinase signaling for survival." Development **122**(8): 2529-37.
Walsh, S., S. S. Margolis, et al. (2003). "Phosphorylation of the cyclin b1 cytoplasmic retention sequence by mitogen-activated protein kinase and Plx." Mol Cancer Res **1**(4): 280-9.
Wang, J., X. Chenivesse, et al. (1990). "Hepatitis B virus integration in a cyclin A gene in a hepatocellular carcinoma." Nature **343**(6258): 555-7.
Warf, B. C., J. Fok-Seang, et al. (1991). "Evidence for the ventral origin of oligodendrocyte precursors in the rat spinal cord." J Neurosci **11**(8): 2477-88.
Wasserman, W. J. and L. D. Smith (1978). "The cyclic behavior of a cytoplasmic factor controlling nuclear membrane breakdown." J Cell Biol **78**(1): R15-22.
Waxman, S. G. (1992). "Demyelination in spinal cord injury and multiple sclerosis: what can we do to enhance functional recovery?" J Neurotrauma **9 Suppl 1**: S105-17.
Wilker, E., J. Lu, et al. (2005). "Role of PI3K/Akt signaling in insulin-like growth factor-1 (IGF-1) skin tumor promotion." Mol Carcinog **44**(2): 137-45.
Wilson, H. C., C. Onischke, et al. (2003). "Human oligodendrocyte precursor cells in vitro: phenotypic analysis and differential response to growth factors." Glia **44**(2): 153-65.
Winston, J., F. Dong, et al. (1996). "Differential modulation of G1 cyclins and the Cdk inhibitor p27kip1 by platelet-derived growth factor and plasma factors in density-arrested fibroblasts." J Biol Chem **271**(19): 11253-60.
Wolswijk, G. (1998). "Chronic stage multiple sclerosis lesions contain a relatively quiescent population of oligodendrocyte precursor cells." J Neurosci **18**(2): 601-9.
Wolswijk, G. and M. Noble (1989). "Identification of an adult-specific glial progenitor cell." Development **105**(2): 387-400.
Wolswijk, G. and M. Noble (1992). "Cooperation between PDGF and FGF converts slowly dividing O-2Aadult progenitor cells to rapidly dividing cells with characteristics of O-2Aperinatal progenitor cells." J Cell Biol **118**(4): 889-900.
Wood, T. L., V. Loladze, et al. (2007). "Delayed IGF-1 administration rescues oligodendrocyte progenitors from glutamate-induced cell death and hypoxic-ischemic brain damage." Dev Neurosci **29**(4-5): 302-10.
Wren, D., G. Wolswijk, et al. (1992). "In vitro analysis of the origin and maintenance of O-2Aadult progenitor cells." J Cell Biol **116**(1): 167-76.
Wright, J. H., E. Munar, et al. (1999). "Mitogen-activated protein kinase kinase activity is required for the G(2)/M transition of the cell cycle in mammalian fibroblasts." Proc Natl Acad Sci U S A **96**(20): 11335-40.

Yam, C. H., T. K. Fung, et al. (2002). "Cyclin A in cell cycle control and cancer." Cell Mol Life Sci **59**(8): 1317-26.

Yang, J., E. S. Bardes, et al. (1998). "Control of cyclin B1 localization through regulated binding of the nuclear export factor CRM1." Genes Dev **12**(14): 2131-43.

Ye, P., J. Carson, et al. (1995). "In vivo actions of insulin-like growth factor-I (IGF-I) on brain myelination: studies of IGF-I and IGF binding protein-1 (IGFBP-1) transgenic mice." J Neurosci **15**(11): 7344-56.

Ye, P. and A. J. D'Ercole (1999). "Insulin-like growth factor I protects oligodendrocytes from tumor necrosis factor-alpha-induced injury." Endocrinology **140**(7): 3063-72.

Ye, P. and A. J. D'Ercole (2006). "Insulin-like growth factor actions during development of neural stem cells and progenitors in the central nervous system." J Neurosci Res **83**(1): 1-6.

Ye, P., L. Li, et al. (2002). "Myelination is altered in insulin-like growth factor-I null mutant mice." J Neurosci **22**(14): 6041-51.

Yeh, J., J. Litz, et al. (2008). "Selective inhibition of SCLC growth by the A12 anti-IGF-1R monoclonal antibody correlates with inhibition of Akt." Lung Cancer **60**(2): 166-74.

Yim, S. H., J. A. Hammer, et al. (2001). "Differences in signal transduction pathways by which platelet-derived and fibroblast growth factors activate extracellular signal-regulated kinase in differentiating oligodendrocytes." J Neurochem **76**(6): 1925-34.

Zaka, M., M. A. Rafi, et al. (2005). "Insulin-like growth factor-1 provides protection against psychosine-induced apoptosis in cultured mouse oligodendrocyte progenitor cells using primarily the PI3K/Akt pathway." Mol Cell Neurosci **30**(3): 398-407.

Zeger, M., G. Popken, et al. (2007). "Insulin-like growth factor type 1 receptor signaling in the cells of oligodendrocyte lineage is required for normal in vivo oligodendrocyte development and myelination." Glia **55**(4): 400-11.

Zhang, S. C., B. Ge, et al. (1999). "Adult brain retains the potential to generate oligodendroglial progenitors with extensive myelination capacity." Proc Natl Acad Sci U S A **96**(7): 4089-94.

VITA

Jungsoo Min

Education

August 2003-Presnet	The Pennsylvania State University College of Medicine, Graduate Research Assistant, Ph. D Candidate in Molecular Medicine
February 2001	Yeungnam University, Kyungsan, South Korea, Bachelor of Science in Pharmacy

Positions

2002-2003	Research volunteer, Yeungnan University, Kyungsan, South Korea

Publication and selected abstracts

Min, J. and Wood, T.L. IGF-1 promotes S phase progression in coordination with FGF-2 in oligodendrocyte progenitor cells. (in preparation)

Min, J and Wood, T.L. IGF-1 plays an important role in regulation of G2/M transition in oligodendrocyte progenitor cells. (in preparation)

Frederick TJ, Min J, Altieri SC, Mitchell NE and Wood TL. Synergistic induction of cyclin D1 in oligodendrocyte progenitor cells by IGF-1 and FGF-2 requires differential stimulation of multiple signaling pathway (2007). Glia. 55(10):1011-22

Min, J. and Wood, T.L. (2008) IGF-1 coordinates with FGF-2 to enhance S and G2/M phase progression in oligodendrocyte progenitor cells. American Society for Neurochemisry Meeting, *J. Neurochem.* 104 (Suppl.1) Abstr. PSM08-14, p.47

Min, J. and Wood, T.L. (2007) IGF-1 enhances S and G2/M progression in coordination with FGF-2 in oligodendrocyte progenitor cells. International Society for Neurochemistry/American Society for Neurochemistry Meeting, *J. Neurochem.* 102 (Suppl.1) Abstr. P.406

Wood, T.L., Romanelli, R.J., Ness, J.K., Frederick, T.J., Altieri, S. and Min, J. (2006) IGF-1 mediated signaling pathways and downstream targets in oligodendrocyte progenitors. American Society for Neurochemistry Meeting. *J. Neurochem.*96 (S.1) Abstr. #CO2-2, p.8.

Award

Young Investigator Enhancement Award. ASN 39th Annual Meeting (2008)