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Insulin-Like Growth Factor I Regulates G2/M Progression Through Mammalian Target of Rapamycin Signaling in Oligodendrocyte Progenitors

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Abstract

Extrinsic factors including growth factors influence decisions of oligodendrocyte progenitor cells (OPCs) to continue cell cycle progression or exit the cell cycle and terminally differentiate into oligodendrocytes capable of producing myelin. Multiple studies have elucidated how the G1/S transition is regulated in OPCs; however, little is known about how S phase progression and the G2/M transition are regulated in these cells. Herein, we report that insulin-like growth factor (IGF)-I coordinates with FGF-2 to promote S phase progression but regulates G2/M progression independently. During S phase, IGF-I/FGF-2 enhances protein expression of cyclin A and cdk2, and further increases effective complex formation resulting in enhanced cdk2 activity. Surprisingly, however, OPCs exposed to FGF-2 in the absence of IGF-I fail to traverse through G2/M. Consistent with this observation, OPCs exposed to IGF-I, but not FGF-2, increase cell number over 48 h. IGF-I enhances cdk1 kinase activity during G2/M by promoting nuclear localization of cyclin B/cdk1 as well as of Cdc25C, an activator of cdk1. IGF-I also induces phosphorylation of histone 3 indicating traverse of cells through mitosis. Finally, we demonstrate that IGF-I-mediated G2/M regulation requires mammalian target of rapamycin activity. These data support an important function for IGF-I in G2/M progression in OPCs.

Keywords

oligodendrocyte; IGF-I; cell cycle; mTOR

INTRODUCTION

Oligodendrocyte progenitor cells (OPCs) arise in the subventricular zone in the brain and migrate to populate white matter and gray matter. Once OPCs reach their final destination, they proliferate and finally differentiate to mature oligodendrocytes capable of myelinating axons. Loss of oligodendrocytes is a distinctive feature of demyelinating diseases such as

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Multiple Sclerosis and in central nervous system trauma such as following hypoxia-ischemia to the immature brain or spinal cord injury. Generation of new oligodendrocytes and remyelination often fails despite the presence of OPCs around or within the lesions including in Multiple Sclerosis (Patrikios et al., 2006) and after prenatal hypoxia-ischemia (Billiards et al., 2008; Segovia et al., 2008). Thus, understanding how proliferation of OPCs is regulated in normal development is important to determine how to overcome blocks to proliferation and differentiation in pathological conditions. Understanding normal cell cycle regulation in glial progenitors also will provide insight into the mechanisms of cell cycle dysregulation in gliomas and oligodendrogliomas.

To date, there have been numerous studies elucidating how OPC cell cycle time is regulated by PDGF (Calver et al., 1998; van Heyningen et al., 2001; Wolswijk et al., 1991) and how G1 and S phases of the cell cycle are regulated in OPCs (Bansal et al., 2005; Belachew et al., 2002; Calver et al., 1998; Casaccia-Bonnel et al., 1997; Chew et al., 2005; Chittajallu et al., 2002; Ghiani and Gallo, 2001; Ghiani et al., 1999a, b; Tikoo et al., 1998; van Heyningen et al., 2001; Wolswijk et al., 1991). Our prior studies demonstrated that insulin-like growth factor-I (IGF-I) enhances FGF-2- or PDGF-induced DNA synthesis in OPCs and coordinates with FGF-2 in regulating the G1/S transition through distinct molecular actions on G1 phase regulators (Frederick et al., 2007; Frederick and Wood, 2004; Jiang et al., 2001). Consistent with these *in vitro* studies, disruption of IGF-I or of the IGF-IR in oligodendroglia *in vivo* reduces OPC proliferation and the number of mature oligodendrocytes (Beck et al., 1995; Mason et al., 2003; Ye et al., 2002; Zeger et al., 2007).

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 (Sherr, 1996). However, it is now clear that the G2/M transition represents another critical cell cycle restriction point, where cells respond to extracellular cues. Moreover, as it is thought that OPCs receive signals to differentiate when in G0/G1 phases (Tang et al., 1998), successful progression through G2/M is necessary for cell cycle exit and subsequent differentiation. Several studies have provided support for IGF-I as a mediator of S and G2/M progression in multiple cell types. Lack of IGF-I causes G2/M arrest in fibroblasts and myeloma cells (Sell et al., 1994; Stromberg et al., 2006). Analysis of uterine epithelial proliferation in *igf-1* null mice further supports a specific role for IGF-I in G2/M regulation (Adesanya et al., 1999). Herein, we tested the hypothesis that IGF-I regulates progression into or through G2/M in OPCs. We demonstrate that IGF-I and FGF-2 coordinately regulate S phase progression in OPCs, consistent with our previous data showing coordinate regulation of G1 and the G1/S transition by these factors. In contrast, IGF-I, but not FGF-2, is essential for further progression of OPCs through G2/M.

MATERIALS AND METHODS

Materials

Cell culture medium was purchased from Gibco-BRL (Long Island, NY) or Mediatech (Manassas, VA). Fetal bovine serum (FBS) was purchased from Gibco-BRL (Long Island, NY). Recombinant human IGF-I and FGF-2 were purchased from Cell Signaling Technology (Danvers, MA) and R&D Systems (Minneapolis, MN), respectively. Cell culture

media supplements (biotin, transferrin, progesterone, putrescine, and selenite) were purchased from Sigma (St. Louis, MO). Standard laboratory reagents were purchased from Fisher Scientific (Pittsburgh, PA) or VWR (West Chester, PA). Antibodies to cyclin A (H-432), cdk2 (M2, D-12), cdk1 (C-19, PSTAIRE, 17), cdc25c (F-5), cyclin B (H-433, GNS1) as well as normal mouse IgG, normal rabbit IgG, and Protein A plus agarose were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies to pHistone 3, cyclin A (E23), and β -actin were purchased from Upstate-Millipore (Billerica, MA), Chemicon-Millipore (Billerica, MA), and Sigma (St. Louis, MO), respectively. Horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Jackson Laboratories (West Grove, PA). [γ -32P]ATP and enhanced chemiluminescence (ECL) detection system were purchased from PerkinElmer (Waltham, MA).

Primary OPC Culture

OPCs were prepared from newborn Sprague Dawley rats 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, 2 mM L-glutamine, 100 μ g/mL penicillin, 100 μ g/mL streptomycin, and 0.6% glucose] and plated in T75 flasks at a density of $2 \times 10^5/\text{cm}^2$. Mixed glial cell cultures were grown for 11 days, and OPCs were purified as previously described (McCarthy and de Vellis, 1980). Mixed glial cell cultures were shaken for 1.5 h at 260 rpm to remove microglia and remaining cells were shaken overnight for 18 h to detach from astrocytes. Purified OPCs were seeded onto poly-D-lysine coated T75 flasks at a density of $2 \times 10^4/\text{cm}^2$ 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 μ g/mL insulin, 20 nM progesterone, 100 μ M putrescine, 5 ng/mL selenium, 50 μ g/mL apotransferrin, 100 μ g/mL penicillin, 100 μ g/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. OPCs were amplified for 2 days and split once for another amplification before experiments were performed.

Thymidine Double Block

OPCs were plated and treated with N2S media for 16 h in 2 mM thymidine. After washing in $1 \times$ PBS twice, cells were released to N2S media for 8–10 h, followed by a second pulse of thymidine for another 16 h. After the double round of thymidine exposure, cells were treated with IGF-I, FGF-2, IGF-I/FGF-2, or no growth factors for specified times, depending on the experiment.

Propidium Iodide Labeling for Flow Cytometry Analysis

Cells were collected in 0.05% 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 min and then stained with 50 mM of propidium iodide (PI). PI stained cells were analyzed by flow cytometry with Becton Dickinson FACS scan. Cell-Quest software (Becton Dickinson, Franklin Lakes, NJ) and ModFitTM (Verity Software House, Topsham, ME) were used for acquisition and analysis, respectively.

Western Blot Analysis and Immunoprecipitation

Total cell lysates from OPCs were washed in ice-cold PBS and isolated in sodium dodecyl sulfate (SDS) buffer (62.5 mM Tris-HCl, 2% SDS, 10% glycol, 50 mM DTT, 1/100 protease inhibitor cocktail, 1 mM Na_3VO_4 , and 1 mM NaF). Lysates were briefly sonicated and then subjected to a protein assay (BioRad, Hercules, CA). A total of 15–30 μg of cell lysates were boiled at 100°C for 5 min and resolved on 7%, 10%, or 4%–12% mini gels by SDS polyacrylamide gel electrophoresis (SDS-PAGE). Separated proteins were electrotransferred to nitrocellulose membranes and blocked in 5% milk in TBS-1% Tween buffer for 1 h at room temperature. Membranes were incubated with primary antibodies overnight at 4°C (1:500, pHistone 3; 1:5000, β -actin; 1:250 for all other antibodies). The following day, membranes were washed 3 $\times$ 5 min in TBS-1% Tween and incubated with secondary antibodies, HRP-conjugated goat anti-mouse or goat anti-rabbit antibodies (1:5000) for 1 h at room temperature. The detection of HRP-conjugated secondary antibodies was performed with 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 in ice-cold 1 $\times$ 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 Na_3VO_4 , 50 mM NaF, 1 mM PMSF, 2.5 mM Napyrophosphate, and 1/100 protease inhibitor cocktail) for 45 min on ice. After brief sonication, protein concentration was determined using a Bio-Rad assay kit. A total of 500 μg of cell lysates were precleared 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. A total of 20 μL of protein A agarose was added to the cell lysates and incubated for 1 h at 4°C. Beads were washed 4 $\times$ in NP-40 buffer and 1 $\times$ in PBS. SDS buffer was added, and samples were boiled at 100°C for 5 min. Eluted proteins were resolved on 10% SDS-PAGE gels and subjected to western blot analysis.

Kinase Assays

Kinase assays were performed as previously described with slight modifications (Ghiani and Gallo, 2001). A total of 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 h at 4°C. Beads were washed 3 $\times$ in NP-40 buffer and 2 $\times$ 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 min at 30°C. 2 $\times$ SDS buffer was added to stop the reaction, and samples were heated for 5 min 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.

Nuclear Fractionation

Cells were harvested in PBS and pellets were collected by centrifugation at 12,000 rpm for 10 min. 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

Na₃VO₄) and incubated on ice for 5 min. Lysates were centrifuged at 14,000 rpm for 3 min 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 Na₃VO₄) and gently rotated for 30 min at 4°C. Lysates were centrifuged at 14,000 rpm for 20 min at 4°C, and supernatants were collected as nuclear extracts. Cytoplasmic and nuclear extracts were stored at –80°C before western blot analysis.

Immunohistochemistry

OPCs were seeded onto poly-L-ornithine coated coverslips at 3×10^2 cells/cm². Cells were washed 2× in ice-cold PBS and fixed with 4% paraformaldehyde for 15 min at room temperature followed by 3× washes in PBS. Cells were permeabilized in 100% methanol for 10 min 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 h at room temperature. Primary antibodies for A2B5 (1:3) and pHistone 3 (1:300), or cyclin B (1:100), were diluted in Triton/PBS and incubated overnight at 4°C. Residual antibodies were removed by 3× washes in PBS, and secondary antibodies (goat-anti-mouse- IgM-FITC and goat-anti-rabbit-Alexa 546) were added and incubated for 1–2 h at room temperature. After washing 3× in PBS, coverslips were mounted with Biomedica mounting solution (Foster City, CA).

AMNIS Imaging Flow Cytometry

OPCs were fixed with 1% paraformaldehyde overnight. Cells were stained with anti-cyclin B, anti-Cdc25C, or anti-Wee1 followed by secondary AF488. Before sample acquisition by the Amnis Imagestream 100, Draq5 was added to each sample to stain nuclei. Samples were analyzed using the Amnis IDEAS 3.0 analysis software. Briefly, single cells were identified by plotting intensity of the nuclear stain versus the aspect ratio intensity of the nuclear stain (measures the roundness of an image). Cells in focus were selected by using the gradient root mean square of the nuclear stain, followed by selecting cells positive for the transcription factor (AF488-positive events). Finally, similarity score was plotted to determine if the AF488 stain also occupied the same region as the nuclear stain. A similarity score between 1.5 and 2.0 and above was determined to be positive for nuclear translocation, which was verified by visual inspection of the images to determine the boundary for nuclear translocation.

RESULTS

IGF-I and FGF-2 Regulation of S and G2/M Progression

To examine cell cycle progression after S phase entry in cycling OPCs, we initially used flow cytometry to quantify the percentage of OPCs specifically in S and G2/M phases following growth arrest and stimulation with FGF-2 and/or IGF-I. Our previous study showed that the percentage of OPCs in S phase peaks at 16 h after serum starvation and treatment with growth factors (Frederick et al., 2007; Frederick and Wood, 2004). Therefore, we examined OPC cell cycle distribution profiles by flow cytometry analysis after permeabilization and staining with PI from 16 to 24 h following growth arrest and

stimulation with FGF-2, IGF-I or a combination of the two growth factors. PI is a fluorescent dye which intercalates into DNA, thus, the DNA content in a cell correlates with fluorescent intensity providing a profile of cells in different phases of the cell cycle. FGF-2 treated OPCs successfully entered S phase 16 h after growth factor stimulation as expected and gradually accumulated in S phase with a peak accumulation at 22 h (Fig. 1A,B). In IGF-I/FGF-2 treated OPCs, the peak of cells in S phase was significantly greater at 16 h than in FGF-2 conditions, consistent with our previous results (Frederick and Wood, 2004). Also in contrast to FGF-2-treated cells, the number of OPCs in S phase started to decrease as cells progressed to G2/M as early as 18 h in IGF-I/FGF-2 conditions (Fig. 1A,B). The increased number of cells in G2/M in IGF-I/FGF-2 corresponded to a reduced number of cells in S phase (Fig. 1B). FGF-2 alone failed to promote traverse of OPCs into G2/M, demonstrating only 11% of cells in G2/M phases at the highest point, significantly less than in either control cells or in cells treated with IGF-I (Fig. 1B). IGF-I had minimal effects on S phase entry of OPCs; both control (no growth factor) and IGF-I-treated cells were found predominantly in G1 although some cells accumulated in G2/M indicating a slow traverse of the cells seen in S phase at T0 (10%) after growth arrest (Fig. 1A,B). Interestingly, the proportion of cells in either G2/M or G1 was less in FGF-2 treated OPCs than in control cells supporting the conclusion that these cells remained in S phase.

To confirm that OPCs exposed to IGF-I/FGF-2, but not FGF-2, completed the cell cycle, we determined total cell number in each condition after 24 and 48 h. Cells were treated with FGF-2 and/or IGF-I or no growth factors for 24 and 48 h after serum starvation, and total number of cells was counted by V-cell coulter counter. FGF-2 treated OPCs increased total cell number by 50% at 24 h, but no increase was observed at 48 h (Fig. 1C) suggesting that the increase at 24 h was due to cells completing mitosis initiated before growth arrest. However, the combination of FGF-2/IGF-I increased cell number by 90% and 130% at 24 and 48 h, respectively (Fig. 1C). As expected from our previous data showing that IGF-I by itself does not induce significant S phase entry in OPCs, IGF-I alone failed to increase cell number similarly to no growth factor conditions. IGF-I/FGF-2 was the only condition where cell number increased by 40% between 24 and 48 h and 150% between 0 and 48 h (Fig. 1C, bottom panel). These data further support the hypothesis that FGF-2 alone is insufficient to allow cells to complete mitosis, whereas IGF-I is indispensable for G2/M transition of OPCs in IGF-I/FGF-2 conditions.

Growth Factor Regulation of S and G2/M Cyclins and cdk

The previous experiments demonstrated that FGF-2 and IGF-I/FGF-2 have distinct effects on cell cycle progression after S phase entry. These results led us to investigate the molecular mechanisms underlying the cell cycle profiles revealed by flow cytometry analysis. In early S phase, cyclin A in complex with cdk2 regulates cell cycle progression, whereas cyclin B in complex with cdk1 regulates progression through G2 and entry into mitosis. Therefore, we examined expression of cyclin A and cdk2 during S phase and of cyclin B and cdk1 during G2/M in response to IGF-I and/or FGF-2. Three time points following cell cycle arrest and addition of growth factors were chosen for analysis based on the prior flow cytometry: 16 h to analyze the beginning of S phase, 18 h for the peak of S phase, and 20 h for entry into G2/M. Both FGF-2 and IGF-I/FGF-2 significantly induced

cyclin A expression in OPCs at 16 h and 18 h during S phase (Fig. 2A, $P < 0.001$ vs. control); however, the combination treatment showed a greater induction of cyclin A at 16 h (Fig. 2A, $P < 0.001$ vs. control). In contrast, IGF-I alone had no effect on cyclin A expression compared with no growth factor control (Fig. 2A). Expression of cdk2 was significantly induced only by the combination of IGF-I/FGF-2 (Fig. 2A, $P < 0.05$ vs. ctrl at 16 and 18 h).

Although FGF-2 treated OPCs increased cyclin A expression, cyclin B expression levels were unaffected at either 18 or 20 h in these cells (Fig. 2B). In contrast, the combination of IGF-I with FGF-2 significantly induced the expression of cyclin B at 18 and 20 h ($P < 0.01$ and $P < 0.001$ vs. control, respectively, Fig. 2B). Overall, cdk1 protein levels were unchanged across growth factor treatment groups at 18 h (Fig. 2B). Only IGF-I/FGF-2 treated OPCs showed a statistically significant increase in cdk1 protein levels at 20 h, a time corresponding to the peak of G2/M (Fig. 2B, $P < 0.05$ vs. control).

FGF-2 and IGF-I/FGF-2 Enhance cdk2 Activity During S Phase but Only IGF-I/FGF-2 Induces cdk1 Activity During G2 Progression

Protein expression of cyclins is a primary mechanism to control cell cycle progression. However, functional holoenzyme formation is an essential step for regulating cdk kinase activity and cell cycle phase transition. Thus, we investigated how growth factors regulate cyclin A/cdk2 and cyclin B/cdk1 complex formation and activity in OPCs. Cyclin A bound to cdk2 was analyzed following immunoprecipitation of cdk2 (Fig. 2C). Cdk2 associated with cyclin A was increased in both FGF-2 and IGF-I/FGF-2 treated OPCs although threefold more cyclin A was found complexed with cdk2 in IGF-I/FGF-2 treated OPCs (Fig. 2C). IGF-I treated and control OPCs had a similar low level of complex formation consistent with the low level of cyclin A expression at 16 h (Fig. 2C). Complex formation between cyclins and cdks does not always correspond to effective kinase activity because of additional regulatory events including the presence of cdk inhibitors. Therefore, we analyzed kinase activity using Histone H1 as a substrate following cdk or cyclin immunoprecipitation. FGF-2 enhanced cdk2 kinase activity compared with no growth factor control or IGF-I treated cells (Fig. 2C). IGF-I/FGF-2 enhanced cdk2 activity but only moderately over FGF-2 treated OPCs (Fig. 2C). These results suggest that complex formation of cyclin A/cdk2 corresponds to kinase activity in both FGF-2 and IGF-I/FGF-2 treated OPCs.

During G2 progression, complex formation of cyclin B and cdk1 is important for cdk1 activation and further G2/M progression. Cdk1 was associated with cyclin B in both FGF-2 and IGF-I/FGF-2 treated OPCs (Fig. 2D) even though both cyclin B and cdk1 protein levels in FGF-2 treated OPCs were lower than in IGF-I/FGF-2 treated cells (Fig. 2B,D). However, analysis of kinase activity revealed that cdk1 activity was detectable only in IGF-I/FGF-2 treated OPCs (Fig. 2D). These results suggest that cdk2 activity and progression through S phase is insufficient to allow cells to progress through G2/M and that additional regulation is required to promote progression through G2/M. G2/M progression and cdk1 activity were observed only in combination treated OPCs although cyclin B/cdk1 complex formed in FGF-2 treated OPCs.

IGF-I/FGF-2 Increases Nuclear Localization of Cyclin B, cdk1, and Cdc25C

The previous results support the hypothesis that the combination of IGF-I/FGF-2 regulates G2/M progression by stimulating cdk1 activation. Cdk1 activation is tightly regulated by several mechanisms, which prevent its premature activation, resulting in mitotic catastrophes such as endoreplication of DNA. In addition to canonical regulation of cyclin B/cdk1 activity including expression of cyclin B and complex formation of cyclin B and cdk1, cyclin B/cdk1 activity is regulated by nuclear localization and by phosphorylation and dephosphorylation of inhibitory sites on cdk1 (Lindqvist et al., 2009). Nuclear localization of the cyclin B/cdk1 complex is an essential mechanism of regulation; the complex shuttles in and out of the nucleus before mitosis (Takizawa and Morgan, 2000). Thus, during interphase, nuclear export is overridden, and cdk1 nuclear levels are low, while in prophase, the balance is reversed and nuclear import is prominent. Therefore, we investigated whether nuclear localization of cyclin B and cdk1 is differentially regulated by FGF-2 and/or IGF-I. At 20 h after growth factor stimulation, OPCs were collected and lysates processed for subcellular fractionation. More cdk1 and cyclin B were found in the nucleus in IGF-I/FGF-2 treated OPCs compared with all other treatment conditions including FGF-2 (Fig. 3A). In addition to cyclin B and cdk1, nuclear localization of the cdk1 activator, Cdc25C, was also enhanced in the growth factor combination (Fig. 3A). To confirm these results, nuclear translocation was determined using Amnis Imagestream 100 multispectral imaging flow cytometry. Cyclin B and Cdc25C were colocalized in OPCs with a nuclear marker (Draq). Colocalization was determined by the value of similarity of two channels and similarity above a threshold indicated by R2 (Fig. 3B). In IGF-I/FGF-2 treated OPCs, nearly 42% of single cells showed colocalization of cyclin B with the nuclear marker versus 32% in FGF-2 treated OPCs (Fig. 3C), consistent with the percentage increase seen in G2/M in the previous flow cytometry analysis of cell cycle phases. Interestingly, more OPCs in FGF-2 treated conditions showed nuclear cyclin B than in either IGF-I treated (22%) or control (14%) conditions even though the prior data demonstrated no cyclin B/cdk1 activity in FGF-2 treated cells (see Fig. 2D). These data suggest that the increased cdk1 activity in IGF-I/FGF-2 treated OPCs is in part regulated by increased nuclear localization of Cdc25C.

IGF-I Regulates Translocation of Cyclin B and Cdc25C

As discussed previously, cells normally progress rapidly through G2/M phases of the cell cycle. 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 (Fig. 1B). Another difficulty with the previous studies is in distinguishing specific IGF-I effects on G2/M because IGF-I treated OPCs do not progress into S phase without coordinate stimulation with FGF-2. Moreover, a greater number of cells progress into S phase with IGF-I/FGF-2 than with FGF-2 alone (see Frederick and Wood, 2004) making it difficult to accurately determine specific differences in G2/M progression between growth factor conditions. Therefore, we designed an experiment to identify specific effects of growth factors on G2/M by first normalizing the number of cells entering S phase using a double thymidine block, which arrests cells at the G1/S phase boundary. This also allowed us to culture cells in full media to allow maximal progression of all cells to S phase. OPCs were subjected to a double thymidine block and then switched to FGF-2, IGF-I, or no growth factor media (Fig. 4A). Almost 80% of cells were in S phase 4 h after release from

the double thymidine block (Fig. 4B), confirming cell arrest in early S phase. By 8 h following release from the double thymidine block, OPCs treated with IGF-I alone had exited S phase and were observed in both G2/M and G1 suggesting they were re-entering a new cell cycle. In contrast, a smaller proportion of FGF-2 treated cells were seen exiting S phase and re-entering G1. So, although the percentage of cells in G2/M at T8 is similar between IGF-1 and FGF-2 treated cells, more cells have traversed G2/M in the IGF-1 conditions. In subsequent experiments, we also determined that the combination of IGF-I and FGF-2 provided no benefit over IGF-I alone in progression of OPCs into G2/M (data not shown). To delineate a specific role of each growth factor on nuclear localization of cyclin B and Cdc25C, we performed Amnis flow cytometry analysis using the double thymidine block paradigm. IGF-I alone significantly enhanced cyclin B and Cdc25C translocation to the nucleus, with 78% and 70% colocalized cells, respectively (Fig. 4C,D). However, FGF-2 alone did not show any further increase in cyclin B translocation compared with control conditions with no growth factors (Fig. 4C), consistent with the decreased proportion of cells re-entering G1 in the previous experiment. Moreover, FGF-2 decreased nuclear translocation of Cdc25C (Fig. 4D).

IGF-I Alone Induces G2/M Transition and Enhances Phosphorylation of Histone 3

To further analyze the effect of IGF-I and FGF-2 on G2/M regulation, we examined the level of phosphorylated histone 3 (pHis3), a mitotic marker, in OPCs arrested at the G1/S boundary by double thymidine block and treated with IGF-I and/or FGF-2. The combination of IGF-I/FGF-2 accelerated G2/M phase progression, showing an earlier increase of pHis3 6 h after the double thymidine block (Fig. 5A). IGF-I alone showed a significant increase of pHis3 also at 9 h after the double thymidine block ($P < 0.05$ vs. control, Fig. 5A), whereas FGF-2 alone had no significant effect on pHis3 expression. Consistent with the previous results, pHis3 positive cells detected by immunofluorescence were more abundant in IGF-I alone conditions whereas FGF-2 treated OPCs showed no induction of pHis3 (Fig. 5B). The IGF-I/FGF-2 combination showed a slightly lower number of pHis3+ cells compared with IGF-I alone conditions likely due to more rapid progression through G2/M similar to the pHis3 western blot analyses (Fig. 5B).

IGF-I Promotes G2/M Progression via the Mammalian Target of Rapamycin Pathway

The serine-threonine kinase, mammalian target of rapamycin (mTOR), is a downstream target of the IGF-I/PI3K/Akt pathway and is a well known translation regulator acting through the raptor/mTORC1 protein complex. Several studies have shown that mTORC1 regulates cell cycle progression through protein translation, and recently two independent studies demonstrated mTORC1 regulates G2/M progression in other cell types (Gwinn et al., 2010; Ramirez-Valle et al., 2010). As our previous studies demonstrated IGF-I activation of PI3K/Akt pathway in OPCs (Frederick et al., 2007), we investigated whether IGF-I regulates G2/M progression in OPCs via activation of mTOR. OPCs were treated with the mTOR specific inhibitor, rapamycin, following 14 h in IGF-1/FGF-2 to allow cells to reach S phase. Initially, we determined OPC cell cycle progression by flow cytometry. OPCs treated with rapamycin were slow to progress through G2/M phases, and the percentage of cells in G2/M was reduced at 22 h compared with control OPCs (Fig. 6A). In the absence of rapamycin, OPCs in G2/M and G1 increased from 18 to 22 h concomitant with their exit from S phase

consistent with our previous results. In contrast, the OPCs treated with IGF-I/FGF-2 in the presence of rapamycin showed no increase in G2/M from 18 to 22 h and remained in S phase longer (Fig. 6A). Consistent with these results, Cdc25C was downregulated by rapamycin (Fig. 6B) although the change in cyclin B and pcdk1 was negligible (data not shown). The level of the mitotic marker pHis3 was also reduced by rapamycin confirming that mTOR activation is necessary for normal progression of OPCs through G2/M.

DISCUSSION

The cell cycle contains two highly regulated transitions, G1/S and G2/M. The G2/M transition is less considered and often overlooked in studies that focus on DNA synthesis as a measure of proliferation competence. Cell cycle studies in OPCs also have focused predominantly on G1/S regulation. Herein, we demonstrate that OPCs exposed to FGF-2 or IGF-I/FGF-2 accumulate in S phase by 12–14 h after growth arrest. However, almost twofold more cells enter S phase in IGF-I/FGF-2 conditions compared with FGF-2 alone. Consistent with our previous findings (Frederick et al., 2007; Frederick and Wood, 2004), IGF-I alone at physiological concentrations does not induce S phase entry. However, once cells enter S phase, IGF-I, but not FGF-2, stimulates OPCs to progress through G2/M.

S Phase Regulation in OPCs

This study demonstrates that IGF-I and FGF-2 coordinately activate S phase progression by enhancing cyclin A levels and associated kinase activity. Interestingly, cells in FGF-2 alone showed potent cdk2 activity although cyclin A/cdk2 complex formation was less than in IGF-I/FGF-2 treated cells. Cdk2 exerts activity via binding to either cyclin E or cyclin A. We could not distinguish which cyclins were bound to cdk2 in these assays as both cyclins are present in the cells. Another interesting observation was that cyclin A/cdk2 complexes also were increased in OPCs treated with IGF-I. It is possible that IGF-I increases association of cyclin A and cdk2. However, a cdk2 kinase assay did not show any activity in IGF-I treated cells suggesting that the complex formed is inactive. In a previous study, we observed that a significant amount of cyclin E/cdk2 was detected in IGF-I treated OPCs during G1, but more p27 cdk inhibitor was found in the complex of cyclin E/cdk2 (Frederick and Wood, 2004). Increased levels of p27 may similarly associate with the cyclin A/cdk2 complex in IGF-I treated OPCs. It is of note that cyclin A associated kinase activity is greatly enhanced in IGF-I/FGF-2 treated OPCs versus FGF-2 treated OPCs unlike cdk2 kinase activity. Cyclin A associated kinase activity could result from either cdk2 or cdk1 because cyclin A is able to associate with cdk2 in S phase and cdk1 in G2 (Morgan, 1997; Pagano et al., 1992). Therefore, we cannot rule out the possibility that cdk1 also participates in cyclin A associated kinase activity.

IGF-I Regulates G2/M Progression in OPCs

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 OPCs (Cui and Almazan, 2007; Frederick et al., 2007; Frederick and Wood, 2004; Ghiani and Gallo, 2001; Jiang et al., 2001). Herein, we present data showing that IGF-I has a distinct role in G2/M progression in OPCs.

OPCs exposed to FGF-2 in the absence of IGF-I failed to traverse through G2/M while IGF-I/FGF-2 treated OPCs normally progressed through G2/M. We demonstrated increased cyclin B expression as well as cdk1 kinase activity in IGF-I/FGF-2 conditions although complex formation of cyclin B/cdk1 was similar between FGF-2 and IGF-I/FGF-2 treated OPCs. These results indicate that complex formation does not always correspond to direct kinase activation and confirms that other factors are required for full cdk1 activation. Similar results were reported with cdk2 and cyclin A in human osteosarcoma cells after IGF-I stimulation (Zhang et al., 1999).

To understand the basis of the cdk1 activity in IGF-I/FGF-2 treated OPCs, we investigated subcellular localization of cyclin B and cdk1. Nuclear localization of cyclin B and cdk1 was enhanced in IGF-I/FGF-2 treated OPCs, confirming that cdk1 translocation to the nucleus is associated with enhanced cdk1 activity. 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, 1990), while in prophase, the complex rapidly translocates into the nucleus (Clute and Pines, 1999; Hagting et al., 1999). Nuclear import of cyclin B has been proposed as a mechanism for switch-like control of cdk1 (Ferrell, 1998).

Activation of cdk1 is regulated by multiple kinases and phosphatases for appropriate timing. Cdc25C is a phosphatase, which dephosphorylates the cdk1 inhibitory phosphate resulting in cdk1 activation (Gautier et al., 1991; Strausfeld et al., 1991). In contrast, Wee1 and Myt1 kinases phosphorylate cdk1 on inhibitory sites (T14 and Y15) (McGowan and Russell, 1993; Mueller et al., 1995). For full activation of cdk1, activated Cdc25C translocates into the nucleus while Wee1 moves to the cytoplasm (Takizawa and Morgan, 2000). Our studies clearly showed increased nuclear localization of Cdc25C in IGF-I/FGF-2 treated OPCs. However, we did not detect loss of Wee1 from the nucleus although inactive Wee1 was present in IGF-I/FGF-2 treated OPCs (data not shown).

Using a double thymidine block to study G2/M progression, we showed that IGF-I promotes and accelerates S phase cells to G2/M. OPCs treated with FGF-2 progressed slowly to G2/M. This result seems contradictory to the earlier results showing complete lack of G2/M progression in OPCs treated with FGF-2 alone from the start of G1. It is thus likely that the presence of serum or IGF type 1 receptor activation (by superphysiological levels of insulin) during the double thymidine block acts during late G1 or early S phase to regulate G2/M regulatory events before cell arrest. Histone 3 phosphorylation on serine 10 (pHis3) occurs during mitosis and is used as a mitotic marker (Prigent and Dimitrov, 2003; Shoemaker and Chalkley, 1978). IGF-I increased pHis3 to a greater extent and at an earlier time point versus FGF-2 after release of OPCs from a double thymidine block. These results indicate that FGF-2 has only a minor effect on G2/M by itself, but that it accelerates G2/M progression in the presence of IGF-I.

Taken together, this study showed a specific role for IGF-I in G2/M progression distinct from G1 progression in OPCs. The presence of IGF-I enhanced cyclin B and cdk1 expression and increased cdk1 kinase activity, positioning cyclin B, cdk1, and Cdc25C in the nucleus in OPCs. The ability of IGF-I to enhance G2/M progression in OPCs may also

extend to PDGF-stimulated proliferation as we found increased expression of cyclin B and p-His3 in OPCs treated with IGF-I/PDGF versus PDGF alone (data not shown). These findings are consistent with demonstrations that IGF-I promotes G2/M progression in several other cell types, including in uterine epithelial cells analyzed *in vivo* in the *igf1* null mice (Adesanya et al., 1999), although no prior analyses defined the mechanistic basis for this regulation as shown here. Moreover, as for most analyses of cell proliferation, studies on conditional deletion of the IGF-1R in oligodendroglia focused on reductions in DNA synthesis and cell number without specific analyses of G2/M regulation (Zeger et al., 2007).

The mTOR, a serine/threonine kinase, regulates cell growth and cell proliferation and is a downstream target of IGF signaling in many cell types. Recently, several lines of evidence showed that mTORC1 regulates the G2/M transition (Gwinn et al., 2010; Petersen and Nurse, 2007; Ramirez-Valle et al., 2010). Inhibition of mTOR with rapamycin treatment impeded progression through G2/M in IGF-I/FGF-2 treated OPCs. In addition, inhibition of mTOR decreased protein expression of Cdc25C, but cyclin B expression was minimally affected (data not shown). In parallel, inactive cdc2 was increased with rapamycin treatment (data not shown). As a result, p-His3 was also decreased in rapamycin treated OPCs. A recent study reported that phosphorylation of raptor, a component of mTORC1, is important for G2/M transition in other cell types after overexpression (Ramirez-Valle et al., 2010). We speculated whether raptor is phosphorylated in OPCs but we were unable to determine this due to the inability to detect endogenous levels of phosphorylated raptor using available antibodies. As raptor can be phosphorylated by active cdc2 (Gwinn et al., 2010), coordination between cdc2 and mTORC1 may control appropriate G2/M progression.

Taken together, these data support a unique role for IGF-I in G2/M progression in OPCs via its activation of PI3K/Akt/mTOR signaling. It is of interest that stimulation of this pathway also is critical for OPC differentiation suggesting coordinate regulation of G2/M and differentiation in these cells. This is intriguing because signals for differentiation are thought to occur during the G1 phase of the cell cycle. Considerable evidence supports the idea that cell cycle exit is necessary but not sufficient for OPC differentiation (Belachew et al., 2002; Tikoo et al., 1998). Thus, the requirements for differentiation must include completion of mitosis, absence of mitogens that promote G1/S such that the cells enter G1/G0, and, finally, the presence of differentiation signals. This sequence of events is also relevant to OPC differentiation and remyelination in Multiple Sclerosis lesions, where OPCs often fail to differentiate around chronic lesions. It will be of interest in future studies to determine whether failure to complete mitosis may contribute to the differentiation failure in such pathologies.

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Fig. 1.

Profile of cell cycle progression in OPCs. Growth-arrested OPCs were treated with FGF-2, and/or IGF-I or no growth factors for indicated times. **A,B:** Cells were stained with PI and then analyzed by flow cytometry. **A:** Representative pictures 18 h after growth factor stimulation are shown. Left peak, right peak, and area between two peaks in each graph represent cells in G1 phase, G2 phase and S phase, respectively. **B:** The percentage of OPCs in each cell cycle phase was quantified using the DNA analyzing program ModFit. The data represent the mean $\pm$ SEM ($n = 3$). **C:** Total cell number was counted 24 and 48 h after growth factor treatments. Based on V-cell coulter, only viable cells were included in the analysis. Graphs show average of two experiments. Top graph shows number of cells at 24 or 48 h expressed as percentage of cells at T0. Bottom graph shows percentage change between indicated times with each treatment condition. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Fig. 2.

Protein expression, complex formation, and activity of S and G2/M cyclin/cdk regulators in growth factor treated OPCs. Serum starved OPCs were treated with growth factors or no growth factors for indicated times. **A, B:** Total cell lysates harvested at 16 and 18 h (A), and 18 and 20 h (B) were processed for SDS-PAGE and western immunoblot for S phase molecules (A), and G2/M phase molecules (B). Western blot results were quantified by densitometric analysis. Levels of cyclin A, cdk2, cyclin B, and cdk1 were normalized to β actin. Statistical analyses were performed on data from three independent experiments. Values represent the mean $\pm$ SEM ($n = 3$, *** $P < 0.001$ vs. control, ** $P < 0.01$ vs. control and * $P < 0.05$ vs. control). **C, D:** Effective complex formation and related kinase activity. A total of 500 μ g of total cell lysates were immunoprecipitated with cdk2 (C) or cyclin B (D) as described in Materials and methods section. Immune complexes were processed for western immunoblot analysis. Total cell lysates before reacting with cdk2 or cyclin B antibodies were processed for SDS-PAGE and western immunoblot analysis. Cyclin A

associated kinase activity (C, bottom panel) or cdk1 activity (D, bottom panel) was determined using histone H1 protein as a substrate following immunoprecipitation with cyclin A (C) or cdk1 (D) at 16 or 18 h after growth stimulation.

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Fig. 3. Nuclear localization of G2/M regulatory proteins. Growth stimulated OPCs were cultured for 18 h following serum starvation. **A:** Subcellular fractionation assay was performed and protein levels were analyzed by western immunoblot analysis. **B:** Confocal microscopy images of OPCs after processing for immunofluorescence to detect A2B5, an OPC cell surface antigen, using an FITC conjugated secondary antibody (green) and cyclin B using Alexa-conjugated secondary antibody (red). **C:** Population of OPCs with nuclear localized cyclin B was quantified using Amnis Imagestream system. Digital images and histogram

showing the similarity scores for serum stimulated OPCs at 18 h are shown as a positive control. Cyclin B in cells with similarity above 2 indicate translocation from the cytoplasm to the nucleus. **D**: Nuclear translocation of cyclin B was analyzed by Amnis after growth factor stimulation. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

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Fig. 4.

Nuclear translocation of cyclin B and Cdc25C after double thymidine block. **A:** OPCs were exposed to 2 mM of thymidine for 16 h, released to growth media for 10 h and exposed to thymidine for another 16 h. **B:** After the second thymidine pulse, cells were treated with IGF-I, FGF-2, or no growth factor for 4 or 8 h. The percentage of cells in each phase was quantified using the DNA staining analysis software ModFit™ (Data shown is representative of two independent experiments). **C, D:** Nuclear translocation of cyclin B (C) or Cdc25C (D) was analyzed by Amnis 7 h after release from double thymidine block.

Fig. 5.

Detection of mitotic marker, pHistone 3 after double thymidine block. **A:** OPCs were treated with IGF-I, FGF-2, or no growth factors for 6 or 9 h after release from double thymidine block, harvested, and processed for SDS-PAGE and western immunoblotting. **B:** OPCs grown on poly-D-ornithine coated coverslips were treated with IGF-I, FGF-2, or no growth factors for 9 h after release from double thymidine block, and processed for immunohistochemistry to detect DAPI, A2B5 (FITC; green), and pHis3 (Alexa 546; red). DAPI and pHis3 positive cells were quantified and plotted as shown. Values represent the mean $\pm$ SEM for each condition. * $P < 0.05$ versus control.

Fig. 6. mTOR inhibition and G2/M progression. Growth arrested OPCs were treated with IGF-I/FGF-2 for 14 h and then treated with fresh IGF-I/FGF-2 $\pm$ rapamycin for indicated time. **A:** Cells were stained with PI and then analyzed by flow cytometry. **B:** Total cell lysates harvested at 20 and 22 h were processed for SDS-PAGE and western immunoblot. Western blot results were quantified by densitometric analysis. Levels of Cdc25C, p-cdk1, and p-

histone 3 were normalized to β actin. Statistical analyses were performed on data from three independent experiments. Values represent the mean $\pm$ SEM ($n = 3$, $*P < 0.05$ vs. control).

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