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# Insulin-like growth factor-I induces the phosphorylation and nuclear exclusion of forkhead transcription factors in human neuroblastoma cells

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**Akt-mediated phosphorylation of forkhead transcription factors is linked to growth factor-stimulated cell survival. We investigated whether the survival activity of insulin-like growth factor-I (IGF-I) in SH-SY5Y human neuroblastoma (NBL) cells is associated with phosphorylation and/or localization changes in forkhead proteins. IGF-I induced phosphorylation of Erks (p42/p44), FKHR (FOXO1a) (Ser 253), FKHRL1 (FOXO3a) (Ser 256), and Akt (Ser 473). PI3-K inhibitor, LY294002, reduced IGF-I-stimulated phosphorylation of FKHR, FKHRL1, and Akt, but did not affect Erk phosphorylation. Using a GFP-FKHR construct, FKHR imported into the nucleus during growth factor withdrawal-induced apoptosis. In addition, IGF-I rescue from serum withdrawal-induced apoptosis is associated with a rapid export of GFP-FKHR into the cytoplasm. Leptomycin B, an inhibitor of Crm1-mediated nuclear export, decreased the level of FKHRL1 phosphorylation in the presence of IGF-I in vector and FKHR overexpressing cells, but had no effect on the phosphorylation status of FKHR. In addition, leptomycin B prevented IGF-I stimulated nuclear export of GFP-FKHR. These studies show IGF-I phosphorylation of FKHR and FKHRL1 via a PI3-K-dependent pathway in NBL cells.**

*Keywords:* FKHR; FKHRL1; forkhead; IGF-I; IGF-IR; neuroblastoma.

## Introduction

Neuroblastoma (NBL) is a devastating childhood cancer with poor prognosis for diagnosis over the age of 12 months.[1,2] Although NBL accounts for 10% of all childhood solid tumors, relatively little is known about the molecular events leading to NBL development, progression, and metastasis.[1] Furthermore, current treatment protocols are inadequate, with metastatic disease largely incurable. Therefore, any knowledge gained concerning tumorigenic properties of NBL or ways to induce apoptosis, leading to increased treatment efficacy, will be potentially clinically beneficial. One area that has been highly investigated is the role of growth factors in NBL development and progression. The most studied growth factor in NBL is nerve growth factor (NGF) which has a negative effect on NBL cell survival and growth.[3,4] In contrast, brain derived growth factor (BDNF) protects NBL from drug-induced apoptosis *in vitro*[5,6] and is associated with poor prognosis in NBL patients.[7]

We have recently shown that insulin-like growth factor-I (IGF-I) signaling through its receptor (type I IGF receptor, IGF-IR) is altered and IGF-IR expression levels are increased in tumorigenic NBL cells. Our laboratory has had a long-standing interest in the IGF-I/IGF-IR system, and we have shown that IGF-I and IGF-IR play important roles in NBL signaling, motility and survival, all of which occur through specific signaling mechanisms.[8–11] Ligand binding of IGF-I by IGF-IR mediates receptor autophosphorylation, which, in turn, enhances binding and tyrosine phosphorylation of downstream adaptor proteins.[12–14] These adaptor proteins recruit further signaling proteins activating signaling cascades such as the phosphatidylinositol 3-kinase (PI3-K) and the mitogen-activated protein kinase (MAPK) pathways. The MAPK pathway is primarily involved in cellular differentiation and growth,[15,16] whereas the PI3-K pathway is essential in mitogenesis, glycogen synthesis, and motility.[17–19] Interestingly, both the PI3-K and MAPK pathways are important in protecting cells from apoptosis.[20] Multiple downstream effectors of the Akt/MAPK pathways are implicated in cell survival and apoptosis. The best characterized of these is the serine/threonine protein kinase Akt.[21,22] The first report of an anti-apoptotic effect of Akt was in cerebellar neurons in which IGF-I treatment promoted both Akt phosphorylation and cell survival.[23] A variety of studies show that Akt is activated by PI3-K-dependent phosphorylation upon growth factor exposure leading to cell survival.[21,24–27] In addition, the use of wild type and/or dominant

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negative mutant Akt constructs provide further evidence that Akt activation leads to protection from apoptosis[22,28] (for review see.[29]).

Several reports show that activated Akt translocates to the nucleus upon growth factor exposure.[25,30] These data suggest an association with nuclear proteins such as transcription factors to directly regulate gene transcription.[30] Studies in *C. elegans* show that the forkhead transcription factor DAF-16 interacts with Akt in the nucleus.[31] Currently, there are three human homologues of DAF-16 located at chromosomal breakpoints in human tumors: FKHR (FOXO1a),[32,33] FKHRL1 (FOXO3a),[34,35] and AFX (FOXO4).[36] These human forkhead homologs contain three sites that are phosphorylated by Akt *in vitro* and appear to be phosphorylated by Akt *in vivo* in growth factor-stimulated cells.[37,38,38–45] Consistent with a role for forkhead proteins in PI3-K/Akt signaling, inhibitors of PI3-K block the growth factor-stimulated phosphorylation of FKHR and FKHRL1, as well as phosphorylation and activation of Akt.[38,41,44] In addition, it appears that Akt-mediated phosphorylation of these forkhead proteins causes translocation from the nucleus to the cytoplasm by a Crm1 dependent mechanism.[43] Forkhead protein translocation may block apoptosis by preventing transcription of genes responsible for cell death.[37,37,40,46,47] Together, these studies support a model in which PI3-K/Akt-dependent phosphorylation of forkhead proteins helps mediate growth factor-stimulated cellular survival.

Based upon the above reports, the current study examines the effect of IGF-I signal transduction on the forkhead transcription factors (FKHR and FKHRL1) in human SH-SY5Y NBL cells exposed to serum withdrawal and mannitol-induced apoptotic stimuli. IGF-I induces the phosphorylation of FKHRL1 and FKHR via a PI3-K-dependent pathway. FKHR is predominantly localized to the nucleus upon growth factor withdrawal, while IGF-I exposure results in FKHR export from the nucleus to the cytoplasm. Exposure of FKHR overexpressing cells to leptomycin B, a specific inhibitor of nuclear export, reveals a decrease in the phosphorylation status of FKHRL1, while no changes were seen in phospho-FKHR levels. In addition, leptomycin B blocks IGF-I mediated nuclear export of FKHR. Collectively, these data indicate that IGF-I stimulates the phosphorylation and nuclear export of forkhead transcription factors in SH-SY5Y human NBL cells.

## Materials and methods

### Cell culture

All cell culture reagents were purchased from GIBCO BRL (Gaithersburg, MD) unless otherwise stated. SH-SY5Y human NBL cells were cultured in DMEM with 10% calf serum (CS) (Hyclone Lab, Logan, UT) at 37°C with 10% CO2 in a humidified incubator. Human recombinant IGF-I was a generous gift from Cephalon, Inc. (West Chester, PA). All other chemicals and reagents were purchased from Sigma Chemical Company (St. Louis, MO).

For indicated experiments, cells were transfected with 8 $\mu$g of pCMV-Tag-2C-FKHR, pEGFP-C3-FKHR, pCMV-Tag-2C, or pEGFP-C3 vectors using Lipofectamine 2000 reagent following the manufacturer's protocol. The pCMV-Tag-2C-FKHR and pEGFP-C3-FKHR vectors were generous gifts from David Powell (Baylor College of Medicine, Houston, Texas). The pCMV-Tag-2C vector was purchased from Stratagene Cloning Systems (La Jolla, CA), while the pEGFP-C3 vector was purchased from Clonetech Laboratories, Inc. (Palo Alto, CA). Transfected cells were selected in 250 $\mu$g/mL geneticin for 1 month prior to beginning experiments.

### Western blot analysis

Cells were rinsed with ice-cold HBSS and solubilized in lysis buffer (50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 $\mu$g/ml aprotinin, 5 $\mu$g/ml leupeptin, and 1 mM sodium orthovanadate). Cell lysates were briefly sonicated, centrifuged for 10 min at 4°C, and boiled for 5 min in 2X sample buffer (20 mM Tris-HCl, pH 8.0, 2 mM EDTA, 2% SDS, 20 mM DTT, 0.1% bromophenol blue, 20% glycerol) prior to loading. 50 $\mu$g protein cell lysates were loaded and run on 7.5 or 10% SDS-polyacrylamide gels followed by transfer to nitrocellulose membranes (Amersham Pharmacia Biotech, Piscataway, NJ). Membranes were blocked overnight in 5% nonfat milk in TBS-T (20 mM Tris, 0.16 M NaCl, and 0.10% Tween-20, pH 7.4) at 4°C. Membranes were incubated in primary antibodies (Akt and Erk antibodies 1:2,000; pFKHRL1 1:500; and FKHR 1:1,000) in 5% nonfat milk in TBS-T for 7–14 h at 4°C, followed by a 1 h incubation with secondary anti-rabbit HRP antibody (1:2000) at room temperature in 5% nonfat milk in TBS-T. An enhanced chemiluminescent detection system was utilized following the manufacturer's protocol followed by exposure to film (Hyperfilm-ECL, Amersham Pharmacia Biotech). For phospho-FKHR immunoblot analysis, SH-SY5Y cells were directly lysed in 300 $\mu$l of 2X sample buffer, briefly sonicated, and centrifuged for 10 min at 4°C. 40 $\mu$l aliquots were boiled for 5 min and loaded on SDS-polyacrylamide gels. For phosphatase treatments, protein phosphatase I purchased from Calbiochem (San Diego, CA) was added directly to the cells and incubated for 30–60 min at 30°C prior to collection of cell lysates. In some experiments, the nitrocellulose membranes were stripped by incubation with stripping

solution (2% SDS, 0.1 M dithiothreitol, and 0.1 M Tris, pH 6.8). All blots were stained with Ponso-S to verify equal loading of all wells. All experiments were repeated at least 3 times and typical representative results are shown.

### Antibodies

Antibodies against FKHR, phospho-FKHR (serine 256), phospho-p42/p44 MAP kinase, and phospho-Akt (serine 473) were purchased from Cell Signaling Technology (Beverly, MA), while antibodies against FKHRL1 and phospho-FKHRL1 (serine 253) were purchased from Upstate Biotechnology (Lake Placid, NY). Horseradish peroxidase-conjugated polyclonal goat anti-rabbit antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and ECL western blot reagents were purchased from Amersham Pharmacia Biotech (Piscataway, NJ).

### Fluorescent microscopy

SH-SY5Y cells transfected with either pEGFP-C3-FKHR or pEGFP-C3 vector were grown on coverslips for several days, serum-starved for 5.5 h, followed by a 2 h treatment with 10 nM IGF-I. After treatment, cells were fixed with 4% paraformaldehyde at room temperature, washed twice with 1X phosphate-buffered saline (PBS), and counterstained with 1 $\mu$g/ml bisbenzamide in 1X PBS for 10 min. Coverslips were mounted onto slides with Biomedia Gel Mount (Electron Microscopy Sciences, Ft. Washington, PA) and allowed to dry overnight. Fluorescent microscopy analysis was performed using a Zeiss Axiophot microscope. For leptomycin B experiments, cells were serum starved, treated for 6 h with 20 nM leptomycin B, followed by a 30 or 60 min 10 nM IGF-I exposure.

### Flow cytometry

Hypodiploid (sub-$G_o$) apoptotic analyses of SH-SY5Y transfectants were performed in triplicate using propidium iodide as previously described.[8] Briefly, cells were treated with or without 300 mM mannitol and with or without IGF-I for 12 h and collected. Cells were fixed using 70% ice cold ethanol and stained with 18 $\mu$g/mL propidium iodide containing 40 $\mu$g/mL RNase A. Propidium iodide specifically intercalates into DNA, allowing total DNA content to be measured for each cell. The cells are then placed into each phase of the cell cycle based on DNA content. Apoptotic cells characteristically have DNA that is fragmented into 180–200 base-pair fragments. These fragments are then represented as a sub-Go peak on the cell cycle histogram using an Epics flow cytometry system (Coulter Cytometry, Hialeah, FL).

## Results

### IGF-I stimulates the phosphorylation of FKHRL1 in SH-SY5Y human NBL cells

Our laboratory is interested in the mechanisms involved in IGF-I mediated survival of human NBL cells. Although increasing evidence points to a role for the forkhead transcription factors in apoptosis, their function in NBL cells has not been reported. Therefore, we examined endogenous levels of FKHRL1 in SH-SY5Y human NBL cells, a well-characterized cell line in which IGF-I acts as a potent survival factor.[8,9,48] Initial experiments utilized Western blotting to examine the phosphorylation of FKHRL1, as well as Akt and p42/p44 MAP kinases (Erks). SH-SY5Y cells were serum starved overnight and then treated with or without 10 nM IGF-I in serum-free media for either 30 min or 2 h. IGF-I treatment leads to Akt phosphorylation at Ser 473, a site required for Akt activation[24] and p42/p44 Erk phosphorylation by 30 min which continues through 2 h (Figure 1). Thus, IGF-I induces a robust activation of both the PI3-K and MAP kinase pathways. In addition, IGF-I treatment produces a shift in electrophoretic mobility in the FKHRL1 lanes (Figure 1). Treatment of cells with protein phosphatase eliminates the upper band in the FKHRL1 lanes (data not shown), confirming that the mobility shift is caused by phosphorylation of the protein. This phosphorylation was further confirmed by Western blotting with a monoclonal antibody specific for FKHRL1 phosphorylated at Ser 253, a site phosphorylated by Akt[38,41–43] (Figure 1). Using densitometry, a 1.6 fold increase is observed in phospho-FKHRL1 protein levels treated with IGF-I at both 30 min and 2 h timepoints compared to lanes receiving no IGF-I.

### FKHRL1 and FKHR phosphorylation is dependent on PI3-K activity

Several studies suggest that the forkhead proteins are regulated by the PI3-K pathway.[37,38,42,43,49] Specifically, Akt, a key downstream effector of PI3-K, is thought to either phosphorylate FKHR and FKHRL1 directly,[37,38,40,43] or to promote their phosphorylation by other kinases.[37] To determine if PI3-K regulates these proteins in SH-SY5Y cells, we examined the effect of a PI3-K inhibitor, LY294002 (LY)[50] on FKHRL1 phosphorylation. In these experiments, serum-starved SH-SY5Y cells were treated with 0, 10, or 50 $\mu$M LY for 1 h prior to a 30 min treatment with 1 or 10 nM IGF-I. As shown in Figure 2A, treatment with 10 $\mu$M LY causes a significant reduction in the IGF-I stimulated phosphorylation

**Figure 1**. IGF-I Promotes Phosphorylation of Endogenous FKHRL1 in SH-SY5Y Neuroblastoma Cells. SH-SY5Y neuroblastoma cells were grown to approximately 75% confluency and serum starved overnight. Cells were treated with either 10 nM IGF-I or DMEM (0) for 30 min and 2 h. 50 $\mu$g total protein of cell lysate was loaded in each lane and separated on a 7.5% polyacrylamide gel prior to Western blotting for FKHRL1, phospho-Akt (Ser 473), phospho-Erk, or phospho-FKHRL1 (Ser 253). Arrows indicate the different electrophoretic mobilities of the phosphorylated (*asterisk*) and unphosphorylated forms of FKHRL1. All data are representative of 3 independent experiments.

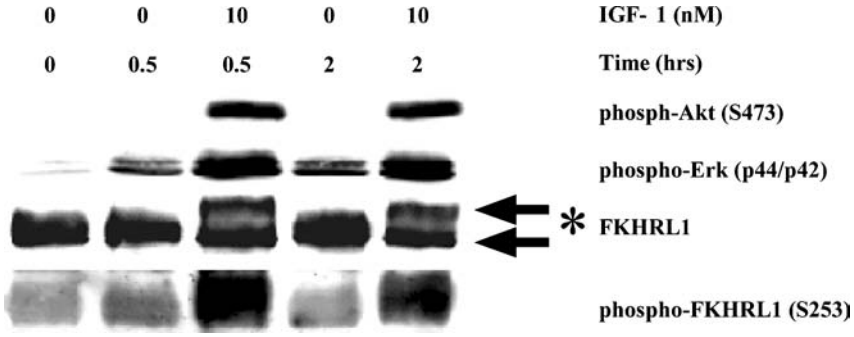
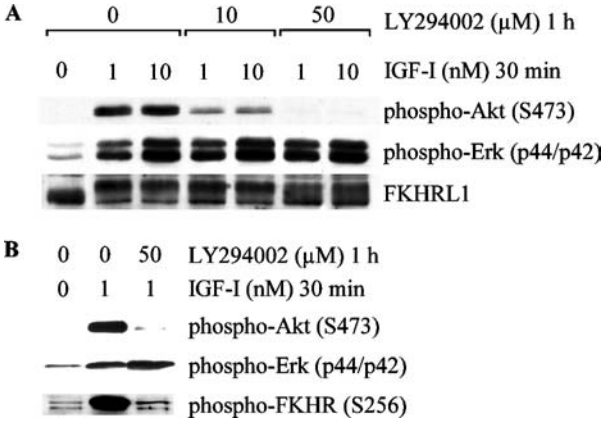



**Figure 2**. IGF-I Mediated Phosphorylation of FKHR and FKHRL1 is Dependent on PI3-K Activation. SH-SY5Y cells were grown to approximately 75% confluency and serum starved overnight. (A) Cells were treated with 0, 10, or 50 $\mu$M LY for 1 h followed by a 0, 1, or 10 nM IGF-I treatment for 30 min. Western blot analysis was performed as in Figure 1 for phospho-Akt (Ser 473), phospho-Erk, and phospho-FKHRL1. (B) Cells were treated with 0 or 50 $\mu$M LY for 1 h followed by a 0 or 1 nM IGF-I treatment for 30 min. Western blot analysis was performed as in Figure 1 for phospho-Akt (Ser 473), phospho-Erk, and phospho-FKHR (Ser 256).




of Akt. Treatment with 50 $\mu$M LY, however, eliminates the phosphorylation of Akt in IGF-I-stimulated cells. As expected, IGF-I stimulated phosphorylation of Erks is unaffected by LY, indicating that the MAP kinase pathway remains activated despite full inhibition of PI3-K. In addition, pretreatment with 50 mM LY significantly reduces the phosphorylation of FKHRL1 as evidenced by a shift from the phosphorylated to the unphosphorylated form (Figure 2A).

FKHR, another forkhead homolog, is also phosphorylated by Akt and is thought to play a role in growth factor-stimulated survival.[49] Similar to FKHRL1, IGF-I enhances the phosphorylation of FKHR on Ser 256, a site targeted by Akt (Figure 2B). Indeed, Akt phosphorylation (Ser 473) is enhanced in parallel with FKHR phosphorylation. Further, IGF-I enhancement of both FKHR and Akt phosphorylation is blocked by 50 $\mu$M LY. Specifically, phospho-FKHR levels of LY treated cells decreased by 8-fold compared to cells receiving IGF-I only upon densitometry analyses. As in Figure 2A, Erk phosphorylation is unaffected by LY. Together, these results show that IGF-I stimulates the PI3-K-dependent phosphorylation of Akt, FKHR, and FKHRL1 in human neuroblastoma cells. However, the fact that MAPK pathway may also play a role in the phosphorylation of FKHR and FKHRL1 can not be completely ruled out.

## Regulation of FKHR subcellular localization in SH-SY5Y cells

In several cell lines, phosphorylation of FKHR and FKHRL1 results in nuclear export, a process necessary and sufficient for inhibition of their transcriptional activity.[40,43,49,51] To examine the effects of IGF-I on the subcellular localization of forkhead proteins, cells were transfected with pEGFP-C3-FKHR, a vector that expresses FKHR fused to the C-terminus of a modified variant of the Aequorea victoria green fluorescent protein. Fluorescence microscopy revealed that EGFP-FKHR is localized equally between the nucleus and the cytoplasm in a population of asynchronous cells grown in 10% CS (Figures 3A and E). However, EGFP-FKHR is predominantly localized to the nucleus when cells undergo serum withdrawal for 5.5 h (Figures 3B and E) and 7.5 h (Figures 3C and E). Addition of 10 nM IGF-I to serum-starved cells for 2 h results in an increase in cytoplasmic localization of EGFP-FKHR and a decrease in its nuclear localization (Figures 3D and E) indicating that IGF-I causes a rapid nuclear export of FKHR. Similar

results are also observed after 1 h and 5 h treatments with 10 nM IGF-I (data not shown). In contrast, pEGFP-C3 vector transfected cells demonstrate diffuse (nuclear and cytoplasmic) expression for each individual treatment indicated above (data not shown).

### Effects of FKHR overexpression on SH-SY5Y apoptosis

Mutation of all three putative Akt phosphorylation sites on FKHR or FKHRL1 results in a protein that cannot be phosphorylated by Akt, thereby promoting apoptosis.[37,40] Therefore, overexpression of wild type FKHR could alter the level of apoptosis in SH-SY5Y cells. To examine this possibility, cells were transfected with either FKHR or empty vector, and the degree of apoptosis was determined after serum withdrawal in the absence or presence of IGF-I. In these experiments, the level of apoptosis was determined by propidium iodide staining followed by flow cytometric analysis.[8]

Twelve h serum deprivation induces apoptosis in transfected SH-SY5Y cells (Figure 4A). This correlates with a low level of FKHR (Figure 1) or FKHRL1 (Figure 2) phosphorylation. Additionally, IGF-I prevents serum withdrawal-induced apoptosis in the transfected cells, which correlates with a high level of FKHR or FKHRL1 phosphorylation (Figures 1 and 2). Compared to the vector control, cells expressing EGFP-FKHR have a slightly lower degree of apoptosis. Also, there is no difference in the level of apoptosis in cells grown in 10% CS ($T_0$) (Figure 4A). Hyperosmotic media is a potent inducer of apoptosis in SH-SY5Y cells and IGF-I prevents apoptosis caused by this treatment.[8,48] As expected, a 12 h treatment with 300 mM mannitol produces higher levels of apoptosis than 12 h of serum withdrawal (Figure 4A). Furthermore, a lower degree of mannitol-induced apoptosis is observed in cells expressing EGFP-FKHR than control cells. FKHR expression has no effect on IGF-I protection or on the level of apoptosis in the presence of 10% CS ($T_0$). The above results suggest that FKHR overexpression has a mildly protective effect on the induction of apoptosis in SH-SY5Y cells. To determine whether this was due to an artifact of GFP or GFP-FKHR expression, apoptosis was examined in cells transfected with an alternative FKHR construct, FKHR expressed with an attached N-terminal FLAG peptide (pCMV-Tag2C-FKHR). Overexpression of this construct again produces a slight protective effect in serum withdrawal and hyperosmotic conditions (Figure 4B). Also, compared to control, FLAG-FKHR expression causes no difference in the level of apoptosis in the presence of 10% CS ($T_0$) or on protection from apoptosis by IGF-I. Similar results are also observed in both transfected cells lines at 24 and 48 h (data not shown).

### Phosphorylation of forkhead proteins

Akt translocates to the nucleus where it may associate with and phosphorylate nuclear proteins such as transcription factors to directly regulate gene transcription.[25,30] Genetic studies in *C. elegans* show that the forkhead transcription factor DAF-16 interacts with Akt in the nucleus.[31] However, it is unknown whether forkhead proteins are phosphorylated in the nucleus or in the cytoplasm. One mechanism by which cells can translocate from the nucleus into the cytoplasm involves the CRM1 receptor specific for nuclear localization sequences found in various proteins.[52] Nuclear export of certain proteins is blocked by the CRM1 inhibitor leptomycin B.[53,54] Interestingly, the localization of AFX, the third human forkhead homolog, is altered with leptomycin B exposure.[55] Therefore, to determine whether phosphorylation and/or localization of FKHR and FKHRL1 is affected by leptomycin B exposure in human neuroblastoma cells, EGFP-FKHR transfected SH-SY5Y cells were treated for 6 h with 20 nM leptomycin B prior to stimulation with 10 nM IGF-I. Leptomycin B exposure decreases the level of FKHRL1 phosphorylation in the presence of IGF-I in vector (1.5 fold) and FKHR overexpressing cells (2.5 fold) using densitometry analyses of Western blots (Figure 5A). However, leptomycin B exposure of IGF-I treated FKHR overexpressing cells resulted in no significant changes in phospho-FKHR levels compared to cells receiving no leptomycin B (Figure 5B). Lastly, leptomycin B prevents nuclear export of FKHR in cells exposed to IGF-I (Figure 5C and D). Taken together, these data suggest that nuclear export is not necessary for FKHR phosphorylation in human NBL cells. In contrast, nuclear export of the FKHRL1 may be involved in the phosphorylation of this protein.

## Discussion

Our laboratory is interested in IGF-I protection of neuronal cells from apoptosis. We have previously demonstrated that IGF-I prevents apoptosis of NBL cells,[8,48,56,57] Schwann cells,[58,59] and neurons.[60,61] Activation of the PI3-K/Akt pathway is one way IGF-I protects cells from undergoing apoptosis.[23,62,63] Recently, forkhead transcription factors have been identified as potential downstream effectors of the PI3-K/Akt pathway that may mediate the survival activity of many growth factors.[37,38,41] Therefore, the goal of this study was to determine whether forkhead transcription factors play a role in IGF-I mediated cell survival of human NBL cells. These studies focused on two of the most investigated forkhead homologs, FKHR and FKHRL1.

In SH-SY5Y human NBL cells, IGF-I activates both the PI3-K/Akt and the MAPK pathways as demonstrated by the phosphorylation of Akt and p42/p44 Erks

**Figure 3**. IGF-I Regulates the Cellular Localization of FKHR in SH-SY5Y Neuroblastoma Cells. Transfected SH-SY5Y cells were grown to approximately 75% confluency in 10% CS. Cells were then treated as follows: (A) maintained in 10% CS, (B) serum-starved for 5.5 h, (C) serum-starved for 7.5 h, (D) serum-starved for 5.5 h followed by 2 h with 10 nM IGF-I. After treatments, cells were fixed and analyzed by fluorescent microscopy. (E) Percentage of cells with either nuclear or cytoplasmic localization of EGFP-FKHR for each individual treatment. Results represent the mean percentage ± S.D. of three individual random counts of 200–300 cells per count.

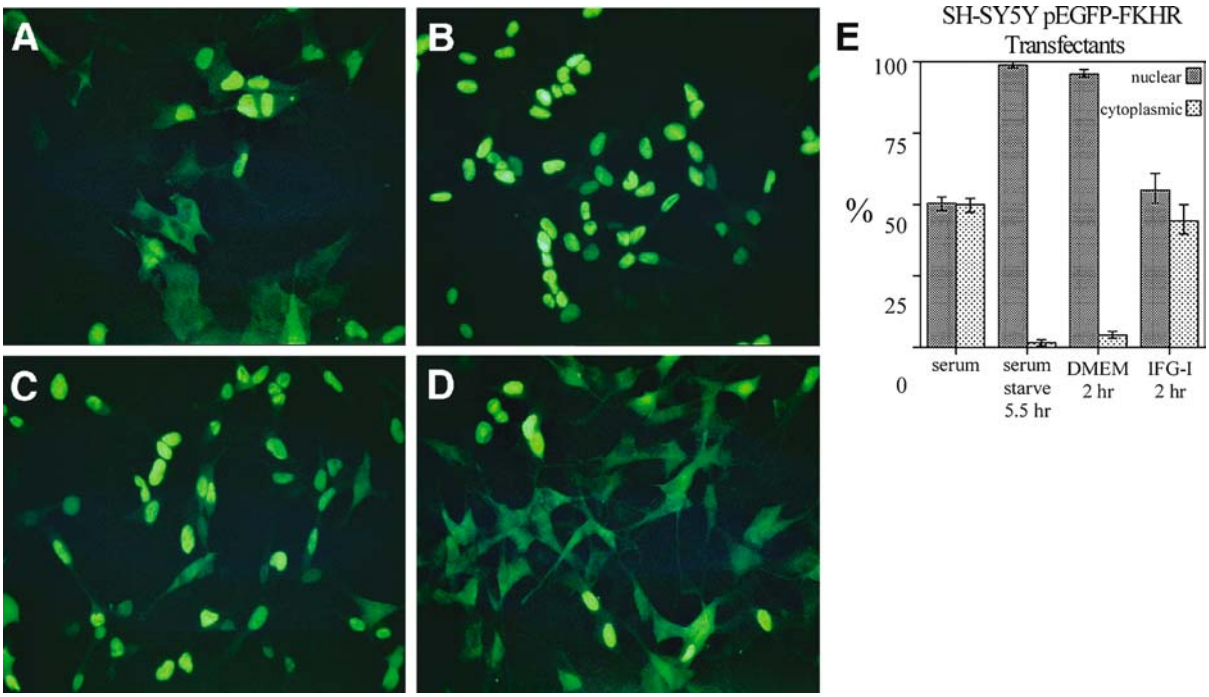



**Figure 5**.

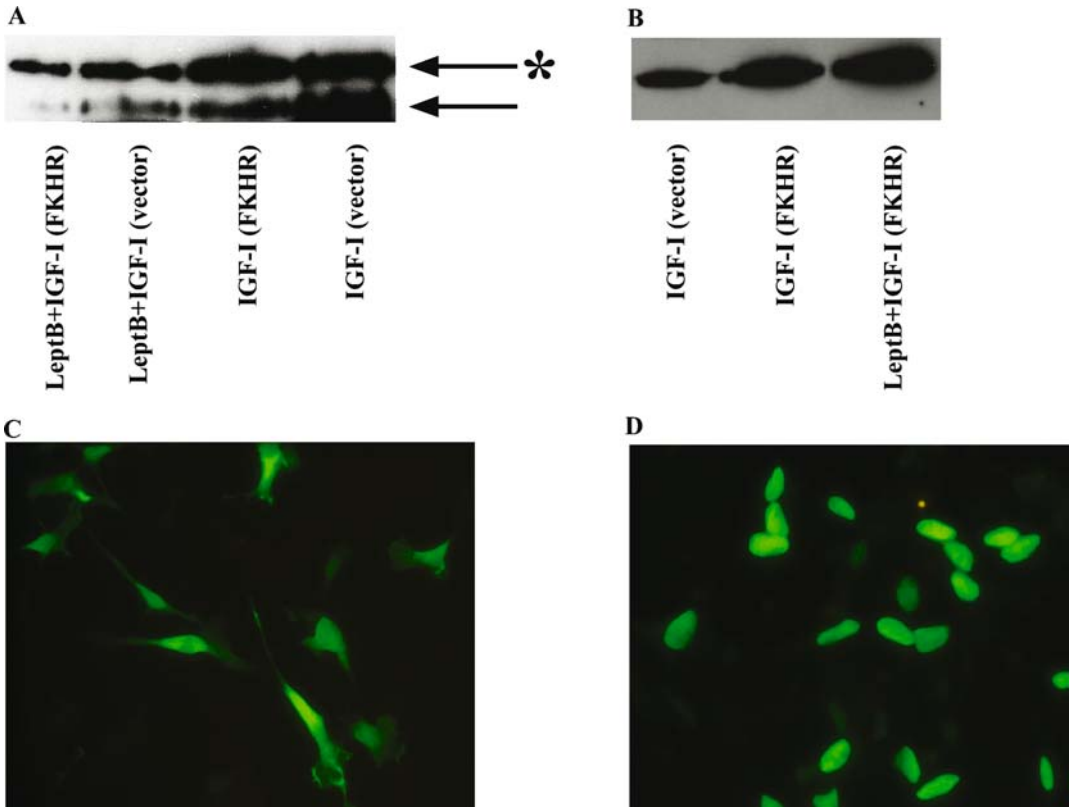
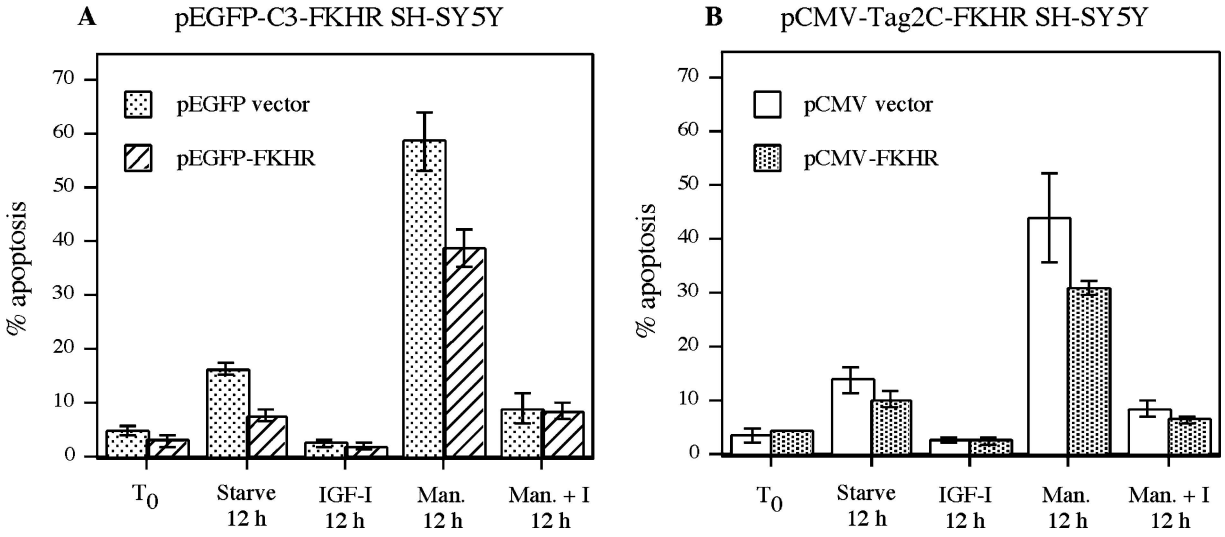


**Figure 4**. Effect of EGFP-FKHR and FLAG-FKHR Overexpression on SH-SY5Y Apoptosis and Protection by IGF-I. SH-SY5Y neuroblastoma cells were grown to approximately 75% confluency in 10% CS prior to individual treatments (in triplicate). Apoptosis was assayed using propidium iodide staining followed by flow cytometric analysis. Cells were deprived of serum for 12 h and then treated for 12 h with either 10 nM IGF-I, 300 mM mannitol, or 300 mM mannitol plus 10 nM IGF-I. Percent apoptosis was assessed as the total number of cells counted within a hypodiploid gate and is the mean ± SD ($n = 3$). $T_0$ represents cells grown in 10% CS and not serum deprived. (A) pEGFP-C3-FKHR and p-EGFP-C3 transfected cells. (B) pCMV-Tag 2C-FKHR and pCMV-Tag2C transfected cells.




respectively. These data are in agreement with our laboratory's previous findings in SH-SY5Y cells.[64–66] In addition, IGF-I induces the phosphorylation of the forkhead proteins FKHRL1 and FKHR. Similarly, IGF-I has been shown to promote the phosphorylation of FKHRL1 in human epithelial kidney cells[37] and PC12 cells[67] and FKHR in 293 cells.[38]

Western blotting with selective monoclonal antibodies reveals that IGF-I stimulates phosphorylation of FKHR on Ser 256 and FKHRL1 on Ser 253. These data suggest that IGF-I stimulates Akt-mediated phosphorylation of FKHRL1 and FKHR in SH-SY5Y cells.[37,42,44,67] Consistent with the possibility that Akt phosphorylates these sites, the phosphorylation of Akt, FKHRL1, and FKHR is reduced in parallel by LY294002, a known inhibitor of the PI3-K pathway. Such inhibition of growth factor-stimulated forkhead phosphorylation by LY294002 also occurs in primary human erythroid progenitor cells,[68] human leukemic cells,[44] and PC12 cells.[67] These data are in agreement with other studies demonstrating IGF-I mediated phosphorylation of forkhead proteins via Akt activation.[67,69,70] Taken together, these data suggest that IGF-I-stimulated phosphorylation of FKHR and FKHRL1 is dependent on activation of the PI3-K/Akt pathway.

Unphosphorylated forkhead transcription factors are found primarily in the nucleus where they can bind to several DNA domains.[37,71,72] However, several studies show that Akt-dependent phosphorylation of forkhead corresponds with their export from the nucleus.[37,43,47] Mutation of Akt phosphorylation sites on FKHRL1 and FKHR prevents phosphorylation, causing accumulation of these proteins in the nucleus.[37,47] Similarly, epidermal growth factor stimulates both the phosphorylation and nuclear export of FKHR.[73] Using a GFP fusion protein, we now show that IGF-I stimulates the export of FKHR from the nucleus into the cytoplasm in human NBL cells. FKHR is expressed in both the nucleus and cytoplasm in NBL cells maintained in 10% CS, while serum withdrawal results in accumulation of FKHR in the nucleus. In addition, our laboratory has recently shown IGF-I mediated phosphorylation and nuclear exclusion of FKHR in rat embryonic dorsal root ganglia neurons.[74]

Next, we wanted to determine if overexpression of FKHR has any effect on levels of apoptosis in cells exposed

**Figure 5**. Effect of Leptomycin B Exposure on Phosphorylation and/or Localization of FKHR and FKHRL1. pEGFP-C3-FKHR transfected SH-SY5Y cells (FKHR) or the empty vector (vector) were grown on 10 cm tissue culture plates or glass coverslips in 10% CS for several days in media containing 10% calf serum. Cells were serum starved, treated for 6 h with 20 nM leptomycin B, followed by a 30 min 10 nM IGF-I exposure. (A) Western blot analysis for FKHRL1. Arrows represent the phosphorylated (*) and unphosphorylated bands. (B) Western blot analysis for pFKHR. (C) Immunofluorescence of pEGFP-C3-FKHR cells exposed to IGF-I only. (D) Immunofluorescence of pEGFP-C3-FKHR cells exposed to leptomycin B and IGF-I.

to hyperosmotic treatment. Overexpression of wild type FKHR has no effect on the survival activity of IGF-I, as shown previously by Brunet *et al*.[37] Wild type FKHR appears to provide weak protection from serum withdrawal or mannitol-induced apoptosis. Whether or not this protective effect is significant, it contrasts with the ability of wild type FKHR to induce apoptosis in 293T cells.[40] In addition, other investigators report that only overexpression of the forkhead phosphorylation mutants leads to apoptosis.[37,40] Therefore, it is possible that wild type FKHR does not normally play a major role in the apoptosis of SH-SY5Y cells. Rather, its physiological role in these cells may be in mediating growth factor-stimulated transcription.

One mechanism of nuclear export of proteins involves the CRM1 receptor. Biggs and coworkers[43] utilized leptomycin B to show that CRM1 mediates this export of forkhead proteins from the nucleus. Therefore, we wanted to investigate the effects of IGF-I on the cellular localization of FKHR utilizing leptomycin B in human NBL cells. Leptomycin B prevents IGF-I-stimulated FKHR nuclear export, indicating a role for CRM1 in this process. In addition, leptomycin B treatment had no effect on the phosphorylation of FKHR in transfected cells. These data agree with the observations that activated Akt is transported to the nucleus where it can interact and phosphorylate forkhead proteins.[30,38,75] In fact, Akt associates with forkhead proteins in the nucleus in *C. elegans*.[31] However, leptomycin B decreases the level of FKHRL1 phosphorylation in FKHR transfected cells exposed to IGF-I. These data suggest that the phosphorylation of the forkhead homologs in human NBL cells may involve different mechanisms.

In conclusion, our data show that IGF-I exposure phosphorylates two forkhead homologs, FKHR and FKHRL1, in human NBL cells via a PI3-K/Akt pathway. In addition, IGF-I exposure causes accumulation of FKHR in the cytoplasm, while serum withdrawal causes accumulation of FKHR in the nucleus. Overexpression of wild type FKHR does not lead to increased apoptosis in cells exposed to hyperosmotic treatment when compared with vector transfected cells. Lastly, leptomycin B does not affect phosphorylation of FKHR and FKHRL1 while also blocking nuclear export of cells exposed to IGF-I.

Functional apoptotic studies suggest that forkhead homologs promote cellular apoptosis by inducing genes responsible for cell death such as the Fas ligand.[37,40] However, mannitol may not induce Fas ligand, therefore explaining the apoptosis data in the current study. We are currently investigating other types of apoptotic stimuli in our FKHR transfected cells including traditional chemotherapeutic drugs and radiation treatment. Other apoptotic inducers, potentially acting through a Fas mediated pathway, may indicate a more definitive role for the forkhead proteins in the apoptosis of human NBL cells.

## Conclusion

IGF-I induced phosphorylation of Erks (p42/p44), FKHR (Ser 253), FKHRL1 (Ser 256), and Akt (Ser 473). PI3-K inhibitor, LY294002, reduced IGF-I-stimulated phosphorylation of FKHR, FKHRL1, and Akt, but did not affect Erk phosphorylation. Using a GFP-FKHR construct, FKHR imported into the nucleus during growth factor withdrawal-induced apoptosis. In addition, IGF-I rescue from serum withdrawal-induced apoptosis is associated with a rapid export of GFP-FKHR into the cytoplasm. An inhibitor of CRM1-mediated nuclear export, leptomycin B, decreased the phosphorylated levels of FKHRL1, while having no effect on the phosphorylation status of FKHR. Leptomycin B also prevented IGF-I stimulated nuclear export of GFP-FKHR. These studies show IGF-I phosphorylation of FKHR and FKHRL1 via a PI3-K-dependent pathway in NBL cells.

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