Insulin-like growth factor-I has different effects on myogenin induction and cell cycle progression in human alveolar and embryonal rhabdomyosarcoma cells

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Abstract. Alveolar rhabdomyosarcoma (RMS) has a much poorer outcome than embryonal RMS. In this study, we found that IGF-I affected the induction of myogenin and cell cycle progression in alveolar RMS cells, but not in embryonal RMS cells. IGF-I enhanced the induction of myogenin protein in alveolar RMS SJ-Rh30 and KP-RMS-MS cells as it did in myoblast C2C12 cells, but not in embryonal RMS RD or KP-RMS-KH cells. IGF-I induction of myogenin protein was blocked by anti-IGF-IR monoclonal antibody αIR-3 and the mTOR-specific inhibitor rapamycin. In Rh30mTOR-rr cells, which stably express a rapamycin-resistant mutant mTOR, rapamycin did not inhibit IGF-I induction of myogenin protein. These data suggest that IGF-I induces myogenin in alveolar RMS cells through the IGF-IR/mTOR pathway. In C2C12 cells, IGF-I induces myogenin protein followed by cell cycle arrest leading to myogenic differentiation. IGF-I promoted G1-S cell cycle progression without any signs of terminal differentiation in alveolar RMS cells. On the other hand, IGF-I promoted neither cell cycle arrest nor G1-S cell cycle progression in embryonal RMS cells. In alveolar RMS SJ-Rh30 cells, 4E-BP1, one of two effectors downstream of mTOR, was continuously hyperphosphorylated by IGF-I, whereas in embryonal RMS RD cells, 4E-BP1 was only transiently hyperphosphorylated. These findings suggest that the different effects of IGF-I on myogenin induction and cell cycle progression in alveolar and embryonal RMS cells are due to a difference of phosphorylation status of 4E-BP1. These different responses to IGF-I help to explain immunohistochemical and clinical behavioral differences between alveolar and embryonal RMS.

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Abbreviations: IGF, insulin-like growth factor; IGF-IR, IGF-I receptor; mTOR, mammalian target of rapamycin; PI3K, phosphoinositide 3-kinase; p38MAPK, mitogen-activated protein kinase; RMS, rhabdomyosarcoma

Key words: rhabdomyosarcoma, IGF-I, 4E-BP1, myogenin, cell cycle progression

Introduction

Rhabdomyosarcoma (RMS) is the most common soft tissue tumor of childhood and adolescents (1). Although the survival rate has significantly improved as a result of the use of intensive therapy combined with surgery, radiation and chemotherapy, alveolar RMS, one of the two major subtypes of RMS, still has a poor prognosis compared with the other subtype, embryonal RMS (2). Approximately 60% of alveolar RMS cases are characterized by the recurrent translocation t (2;13)(q35;q14), which juxtaposes the 5'DNA binding domain-encoding sequence of the *PAX3* gene with the 3' sequence of the *FKHR* gene, to generate the chimeric gene *PAX3-FKHR* (3,4). Several studies have reported that the presence of *PAX3-FKHR* was an adverse prognostic factor in alveolar RMS (5,6). cDNA microarrays have revealed that PAX3-FKHR-overexpressing NIH3T3 cells overexpress *myogenin* and *igf2*, suggesting that *myogenin* and *igf2* are activated downstream of PAX3-FKHR (7).

Myogenin belongs to a group of myogenic regulatory proteins, the MyoD family (MyoD, myogenin, myf-5, and MRF-4) (8-12) and acts as a transcriptional activator of genes that encode skeletal muscle-specific proteins containing the myosin heavy chain (11). Myogenin knockout mice have been shown to have severe muscle deformity at birth (13,14). In cultured mouse myoblast C2C12 cells, myogenin protein and myogenic differentiation are induced by serum withdrawal (15-17). Insulin-like growth factors (IGFs) I and II appear to stimulate myogenesis in cultured myogenic cells, suggesting that they are required for normal skeletal muscle development during embryogenesis (15). IGFs appear to stimulate myogenesis by regulating myogenin protein induction (15-17). Almost all RMS samples show some degree of immunostaining with myogenin antibodies while non-RMS pediatric tumors are consistently negative (18-21). In addition, almost all alveolar

RMS samples show strong positive staining for myogenin, whereas embryonal RMS samples show weak and patchy staining for myogenin, and a large proportion of tumor cells are negative for myogenin (18-21).

On the other hand, in numerous carcinomas as well as malignant sarcomas including RMS, IGFs also act as growth/survival factors (22). RMS cells produce IGF-II and express IGF-I receptor (IGF-IR), suggesting that an autocrine pathway plays a role in regulating the growth of these cells (23,24). Furthermore, down-regulation of the IGF/IGF-IR pathway, through blocking ligand binding to the IGF-IR, was found to suppress the proliferation of RMS cells both *in vitro* and *in vivo* (25). Suppression of the IGF signaling pathway by IGF-IR antisense mRNA also resulted in the inhibition of RMS cell growth (26). We previously reported that mTOR, one of the molecular targets downstream of IGF-IR, plays a major role in the proliferation and survival of RMS cells and that an mTOR-specific inhibitor, rapamycin, blocks the proliferation and induces apoptosis of tumor cells (27,28). It remains unclear why alveolar RMS cells aggressively proliferate and easily invade surrounding tissue and why distant metastasis occurs without terminal differentiation in spite of its overexpression of IGF-IR, IGF-II and myogenin.

In this study, we investigated the responses of alveolar RMS cells and embryonal RMS cells to serum-starvation and IGF-I stimulation and which signaling pathways downstream of IGF-IR are responsible for the responses.

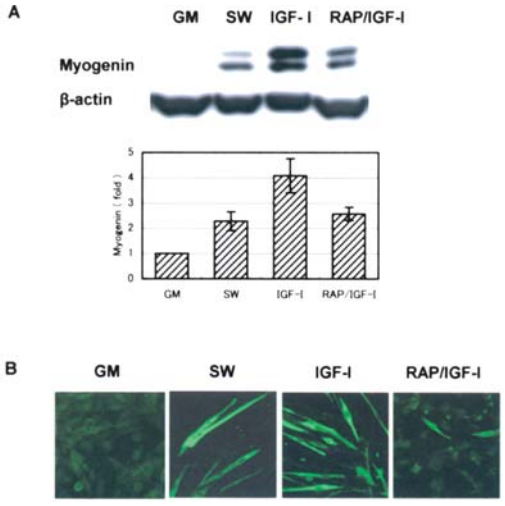


Figure 1. mTOR mediates both myogenin expression and myogenic differentiation induced by serum withdrawal and IGF-I in C2C12 murine myoblast cells. C2C12 cells were serum-fasted and then stimulated with IGF-I (50 ng/ml) in the absence or presence of rapamycin (100 ng/ml). (A) After 48 h, whole lysates were subjected to Western blot analysis to determine myogenin expression. The levels of myogenin protein were normalized against ß-actin (mean ± SE, n=3). (B) After 72 h, the cells on cover slips were fixed and stained with anti-myosin heavy chain. GM, growth medium (10% FBS medium); SW, serum withdrawal; IGF-I, cells were stimulated with IGF-I; RAP/IGF-I, cells were stimulated with IGF-I in the presence of rapamycin.

Materials and methods

Cell culture and reagents. Six cell lines were used in this study: mouse myoblast C2C12 cells (American Type Culture Collection, Manassas, VA), human alveolar RMS cell lines SJ-Rh30 (Rh30) and KP-RMS-MS (MS), both of which express the *PAX3-FKHR* chimeric gene, human embryonal RMS cell lines RD and KP-RMS-KH(KH), neither of which express the *PAX3-FKHR* chimeric gene, and Rh30mTOR-rr cells, which stably express a rapamycin-resistant mutant mTOR gene as well as the *PAX3-FKHR* gene (28). The cells were maintained in DMEM containing 10% fetal bovine serum (FBS) at 37˚C in a 5% CO_2 incubator. The mouse monoclonal antibody to myogenin was purchased from IMGENEX (San Diego, CA). The rabbit polyclonal antibodies to Ser 473 phospho-specific Akt, total Akt, and 4EB-P1 were purchased from Cell Signaling Technology (Beverly, MA). The rabbit polyclonal antibody to p70s6k was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The mouse monoclonal antibody to ß-actin, IGF-I, and rapamycin were obtained from Sigma Chemical Co. (St. Louis, MO). IGF-IR blocking antibody (αIR3) was purchased from Oncogene Science (Cambridge, MA). Horseradish peroxidase-conjugated anti-mouse IgG and antirabbit IgG were obtained from Amersham (Arlington, IL). Rapamycin was added 15 min prior to stimulation with IGF-I and αIR3, and SB203580 (Calbiochem) was added 1 h prior to stimulation.

Western blot analysis. Cells were plated into 2 ml of medium at a density of $5x10^5$ cells/35-mm well in six-well plates, incubated overnight at 37˚C and 5% CO_2, serum-starved, incubated with IGF-I (50 ng/ml) in the absence or presence of either rapamycin (100 mg/ml) or αIR3 (0.5 μg/ml) and lysed as described previously (29). Whole cell lysates were separated by SDS-polyacrylamide gel electrophoresis and transferred to an Immobilon-P membrane (Millipore Corp., Bedford, MA). The membrane was blocked for 1 h in phosphate-buffered saline-Tween 20 (PBS-T) with 5% nonfat dry milk, incubated with the primary antibodies, washed in PBS-T, and incubated with the appropriate secondary antibodies. Antibody binding was detected by using the enhanced ECL detection system (Amersham).

The levels of myogenin protein were quantified by NIH Image Software 1.55 (NIH, Bethesda, MD) and were normalized against the levels of ß-actin protein. Values are the mean ± SE of results from three separate experiments.

Cell cycle analysis. Cells ($3x10^6$) were seeded in 100-mm dishes, incubated overnight, serum-fasted for 24 h, incubated with IGF-I (50 ng/ml) for 48 h, harvested by trypsinization, washed once in PBS, resuspended in 50 mg/ml propidium iodide solution containing 200 μg/ml boiled Rnase, incubated at room temperature for 30 min in the dark, and analysed by flow cytometry using a Becton Dickinson FACScan. Cell cycle distribution was determined with the ModiFit software (Verity Software House Inc., Topshame, ME).

Detection of myosin heavy chain by immunofluorescence. Cells on cover slips were fixed with absolute methanol, washed and incubated with the mouse monoclonal anti-myosin heavy chain antibody (15 μg/ml; Sigma) for 1 h, rinsed with PBS, incubated with fluorescein isothiocyanate-conjugated anti-mouse IgG (1:80;Santa Cruz) for 1 h, and examined under an inverted fluorescence microscope.

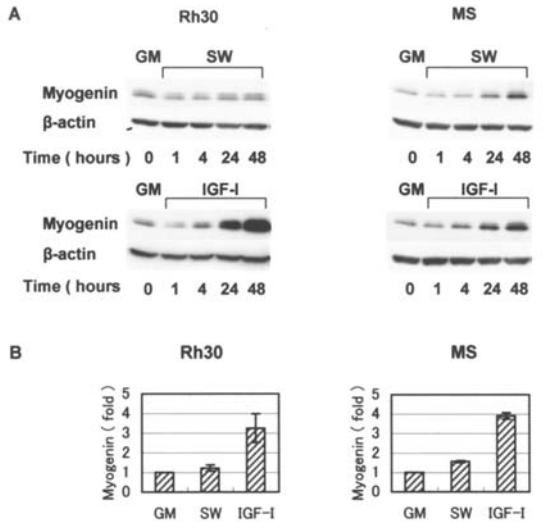


Figure 2. IGF-I, but not serum withdrawal induces myogenin in human alveolar rhabdomyosarcoma, Rh30 and KP-RMS-MS cells. (A) Time-course of myogenin expression with either serum withdrawal or IGF-I stimulation in Rh30 and KP-RMS-MS. Cells were serum-fasted and then stimulated with IGF-I (50 ng/ml) for the times indicated. (B) The levels of myogenin protein were normalized against ß-actin (mean ± SE, n=3). GM, growth medium (10% FBS medium); SW, serum withdrawal; IGF-I, cells were stimulated with IGF-I.

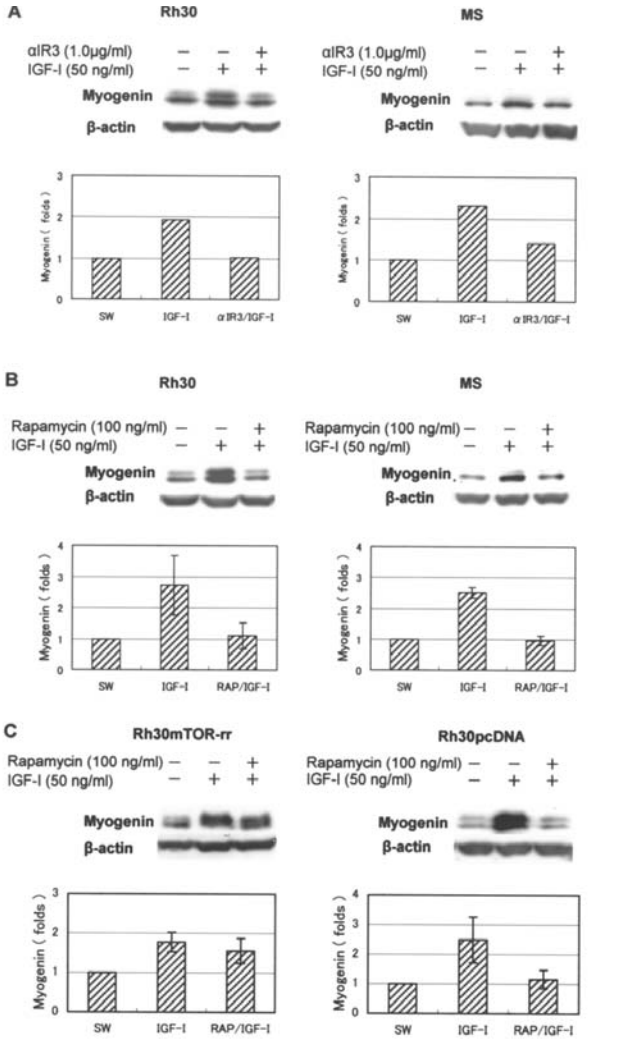


Figure 3. IGF-I induction of myogenin in alveolar RMS Rh30 and KP-RMS-MS cells is mediated mainly through the IGF-IR/mTOR pathway. Serum-fasted Rh30 and KP-RMS-MS (MS) cells were preincubated with α-IR3 (0.5 μg/ml) (A) or rapamycin (100 ng/ml) (B). Cells were then incubated with IGF-I (50 ng/ml) for 48 h. Whole lysates were subjected to Western blot analysis to determine myogenin expression. ß-actin was used as an internal control. (C) Serum-fasted Rh30mTOR-rr and Rh30pcDNA cells were preincubated with rapamycin (100 ng/ml). Cells were then incubated with IGF-I (50 ng/ml) for 24 h. Whole lysates were subjected to Western blot analysis to determine myogenin expression. ß-actin was used as an internal control.

Results

IGF-I enhances both myogenin expression and myogenic differentiation induced by serum withdrawal through the mTOR pathway in C2C12 cells. In mouse C2C12 myoblasts after 48 h of serum withdrawal, the level of myogenin (Fig. 1A, upper panel, SW) was 2.28 (±0.37)-fold greater than the level produced in the presence of 10% FBS (GM). The level of myogenin after 48 h of serum withdrawal in the presence of IGF-I (Fig. 1A, upper panel, IGF-I) was 4.08 (±0.67)-fold greater than the levels produced in the presence of 10% FBS (GM). In other words, after 48 h of incubation in the presence of IGF-I (50 ng/ml), the level of myogenin protein was 1.79 (±0.67)-fold higher than the level induced by serum withdrawal. Similar results were reported previously (15-17). IGF-I induction of myogenin protein was decreased by rapamycin (100 ng/ml) to the level induced by serum withdrawal (Fig. 1A, upper panel, RAP/IGF-I).

In C2C12 cells, a serum-free condition for 72 h induced morphological differentiation and myosin heavy chain expression. IGF-I (50 ng/ml) enhanced both morphological differentiation and myosin heavy chain expression. Rapamycin (100 ng/ml) inhibited myogenic differentiation both by serum withdrawal and IGF-I (Fig. 1B).

IGF-I induces myogenin protein in human alveolar RMS SJ-Rh30 and KP-RMS-MS cells. In the human alveolar RMS cell lines SJ-Rh30 (Rh30) and KP-RMS-MS (MS), both of which have the chimeric gene *PAX3-FKHR*, serum withdrawal for 48 h had a little effect on myogenin induction: the levels of myogenin after 48 h of serum withdrawal (Fig. 2A, upper panels, right lanes, SW) were 1.22 (±0.16)-fold and 1.56 (±0.05)-fold, respectively, greater than the level produced in the presence of 10% FBS (Fig. 2A, upper panels, left lanes, GM).

On the other hand, IGF-I (50 ng/ml) induced a remarkable increase of myogenin protein; the levels of myogenin in Rh30 cells and MS cells after 48 h of serum withdrawal in the presence of IGF-I (Fig. 2A, lower panels, right lanes, IGF-I) were 3.25 (±0.72) and 3.91 (±0.16)-fold, respectively, greater than the levels produced in the presence of 10% FBS (Fig. 2A, lower panels, left lanes, GM).

αIR-3 inhibits IGF-I-induced myogenin protein expression. αIR-3 (1.0 μg/ml) inhibited myogenin protein expression in alveolar RMS Rh30 and MS cells induced by IGF-I (50 ng/ml) to the level of the expression obtained in serum-starved condition (Fig. 3A, upper panels, middle lanes and lower panels, αIR3/IGF-I), confirming that IGF-IR is involved in myogenin protein induction by IGF-I in these cells.

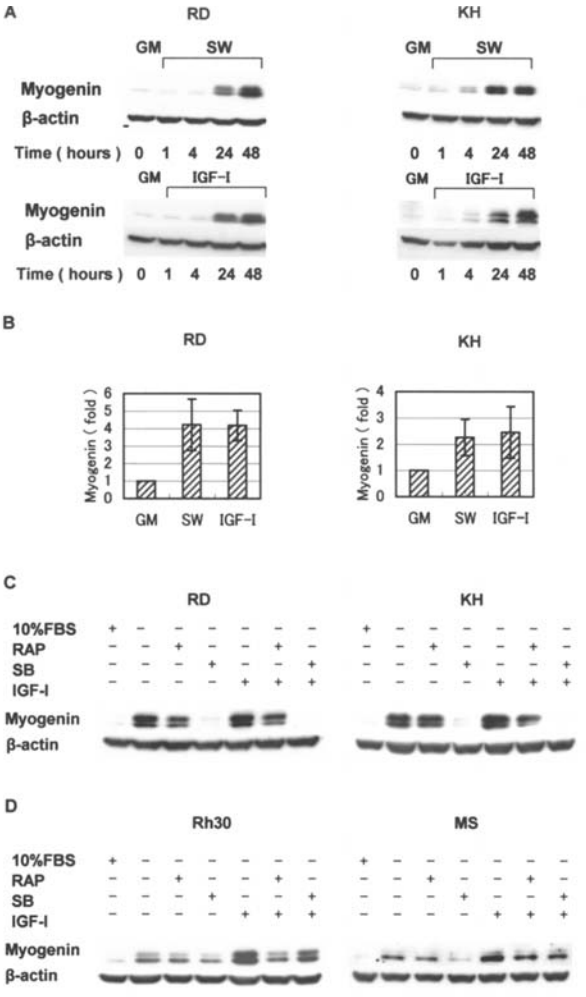


Figure 4. Serum withdrawal, but not IGF-I induces myogenin in embryonal RMS, RD and KP-RMS-KH cells. Serum withdrawal induces myogenin protein mainly through the p38MAPK pathway in embryonal RMS cells, but not in alveolar RMS cells. (A) Time-course of myogenin expression with either serum withdrawal or IGF-I stimulation in RD and KP-RMS-KH (KH). Cells were serum-fasted and then stimulated with IGF-I (50 ng/ml) for the times indicated. (B) The levels of myogenin protein were normalized against ß-actin (mean ± SE, n=3). GM, growth medium (10% FBS medium); SW, serum withdrawal; IGF-I, cells were stimulated with IGF-I. Serum-fasted RD and KP-RMS-KH (KH) cells (C) and Rh30 and KP-RMS-MS (MS) (d) were preincubated with rapamycin (100 ng/ml) or SB203580 (10 μM). Cells were then incubated with IGF-I (50 ng/ml) for 48 h. Whole lysates were subjected to Western blot analysis to determine myogenin expression. ß-actin was used as an internal control.

Rapamycin inhibits IGF-I-induced myogenin protein expression in alveolar RMS Rh30 and MS cells. In Rh30 and MS cells, IGF-I (50 ng/ml) induced a remarkable increase of myogenin protein up to 48 h (Fig. 2B, upper panels, middle lines) and demonstrated a maximum increase of 2.72 (±0.95) and 2.51 (±0.18)-fold at 48 h, respectively, compared with the values of myogenin protein induced by serum withdrawal (Fig. 3B, lower panels, IGF-I).

After 48 h in the presence of 100 ng/ml rapamycin, induction of myogenin protein in Rh30 and MS cells by IGF-I decreased to the levels induced by serum withdrawal (Fig. 3B).

Rapamycin does not inhibit IGF-I-induced myogenin protein expression in Rh30mTOR-rr cells resistant to rapamycin. To confirm that mTOR affects IGF-I induction of myogenin

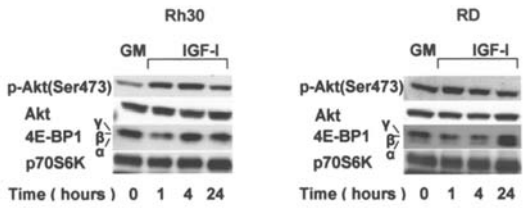


Figure 5. IGF-I phosphorylates Akt, 4E-BP1 and p70s6k in alveolar RMS Rh30 cells and embryonal RMS RD cells. Serum-starved cells were stimulated with IGF-I (50 ng/ml) for the times indicated. Phosphorylated Akt was detected with a phospho-specific Akt antibody. Blots were stripped and reprobed with antibody that recognizes Akt regardless of its phosphorylation state. Phosphorylation of p70s6k and 4E-BP1 was evaluated with a gel mobility shift. Three 4E-BP1 bands represent various isoforms with α being basally phosphorylated and γ being hyperphosphorylated.

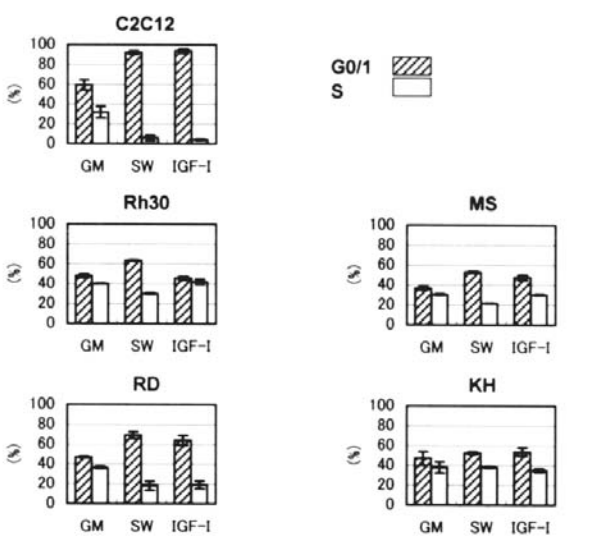
protein, we used Rh30mTOR-rr cells that were stably expressing a rapamycin-resistant mutant mTOR. IGF-I (50 ng/ml) induced a remarkable increase of myogenin protein in Rh30mTOR-rr cells as it did in parent Rh30 cells, and rapamycin (100 ng/ml) did not decrease the induction; the levels of myogenin protein induced by IGF-I in the absence and presence of rapamycin were 1.48 (±0.05) and 1.20 (±0.12)-fold greater, respectively, than the values of myogenin protein induced by serum withdrawal (Fig. 3C, left panels). On the other hand, IGF-I induction of myogenin protein in Rh30p-cDNA cells that were transfected with an empty vector alone was decreased by rapamycin to the level induced by serum withdrawal (Fig. 3C, right panels).

Serum withdrawal induces myogenin protein, whereas IGF-I does not enhance the induction in embryonal RMS RD and KP-RMS-KH cells. On the other hand, in embryonal RMS RD and KP-RMS-KH (KH) cells, which do not have the chimeric gene *PAX3-FKHR*, serum withdrawal induced myogenin protein. After 48 h in a serum-free condition, myogenin protein levels in embryonal RMS RD and KH cells were 4.22 (±1.47) and 2.26 (±0.69)-fold greater, respectively, than the levels produced in the presence of 10% FBS (GM) [Fig. 4A (upper panels) and B (SW and GM)]. However, IGF-I (50 ng/ml) did not cause an additional induction of myogenin protein [Fig. 4A (lower panels) and B (IGF-I)].

In embryonal RMS RD and KH cells, SB203580 inhibits serum withdrawal-induced and IGF-I-induced myogenin protein. The p38MAPK pathway was previously shown to play a role in myogenesis in non-tumor mouse myoblast cells (30-33). To examine whether this pathway was involved in IGF-I induction of myogenin protein, we used a p38MAPK inhibitor, SB203580. SB203580 (10 μM) completely inhibited myogenin protein induction by both serum withdrawal and IGF-I (50 ng/ml) in embryonal RMS RD and KH cells (Fig. 4C). On the other hand, SB203580 (10 μM) did not appear to inhibit induction of myogenin protein by IGF-I in human alveolar RMS Rh30 and MS cells (Fig. 4D).

IGF-I phosphorylates 4E-BP1 continuously in alveolar RMS Rh30 cells, but transiently in embryonal RMS RD cells. Akt, 4E-BP1 and p70s6k have been identified as effectors on the IGF-IR/PI3K/mTOR signaling pathway (34-37). In both

A



B

Cell Line	G0/1 (%)			S (%)		
	GM	SW	IGF-I	GM	SW	IGF-I
C2C12	59.2±5.0	92.0±1.6	93.6±2.0	31.8±5.7	6.11±3.0	4.3±0.9
Rh30	48.1±2.0	63.3±0.9	45.6±1.7	40.1±0.5	30.3±0.7	42.1±2.7
MS	36.4±1.6	53.0±0.9	47.5±2.7	30.6±1.0	21.4±0.3	30.3±0.8
RD	46.8±1.3	69.2±3.5	63.9±5.1	36.4±1.0	18.2±4.5	19.1±3.7
KH	47.0±7.0	52.5±1.3	53.5±4.1	38.0±5.6	37.9±0.8	35.1±1.9

C

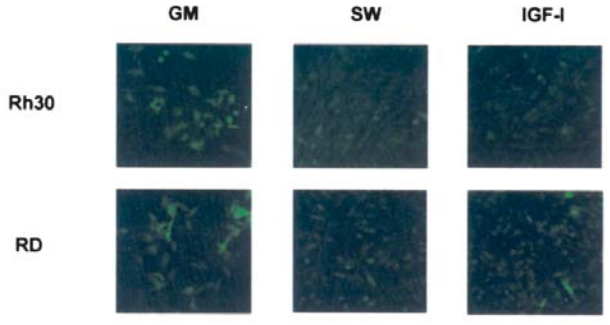


Figure 6. Myogenin induction does not induce myogenic differentiation in either alveolar or embryonal RMS cells. (A) Serum-starved C2C12, Rh30, KP-RMS-MS (MS), RD and KP-RMS-KH (KH) cells were incubated with or without IGF-I (50 ng/ml). After propidium iodide staining, the cell cycle distributions were determined (mean ± SE, n=3). (B) Effects of FBS and IGF-I on percent of cells of various cell types in different phases of the cell cycle. (C) Rh30 and RD cells were serum-fasted and then stimulated with IGI-I (50 ng/ml) in the absence or presence of rapamycin (100 ng/ml). After 72 h, the cells on cover slips were fixed and stained with anti-myosin heavy chain. GM, growth medium (10%FBS medium); SW, serum withdrawal; IGF-I, cells were stimulated with IGF-I; RAP/IGF-I, cells were stimulated with IGF-I in presence of rapamycin.

alveolar RMS Rh30 cells and embryonal RMS RD cells, Akt and p70s6k were phosphorylated continuously at least for 24 h after stimulation with 50 ng/ml of IGF-I (Fig. 5, left panel, Akt and p70s6k), although the phosphorylation of Akt and p70s6k peaked at 1-4 h after the stimulation (Fig. 5, right panel, Akt and p70s6k). However, 4E-BP1, the other effector downstream of mTOR was continuously phosphorylated up to 24 h by IGF-I in Rh30 cells, whereas in RD cells the phosphorylation by IGF-I stimulation was only transient showing the dephosphorylation at 24 h (Fig. 5, 4E-BP1). IGF-I maintained 4E-BP1 hyperphosphorylation as shown by the signal of the hyperphosphorylated form (γ) without any increase of the less-phosphorylaed form (ß, α) at least for 24 h after IGF-I stimulation in Rh30 cells (Fig. 5, left panel, 4E-BP1), whereas ß-4E-BP1 and α-4E-BP1 began to increase at 4 h after IGF-I stimulation in RD cells (Fig. 5, right panel, 4E-BP1).

IGF-I induces cell cycle arrest in C2C12 cells, but not in alveolar or embryonal RMS cells. Serum withdrawal as well as IGF-I induced cell cycle arrest and myogenic differentiation in C2C12 cells (Fig. 6A). FACS analysis demonstrated that the percent of C2C12 cells that were in the G0/G1 phase was 59.2% in 10% FBS medium, 92.0% 24 h after serum withdrawal, and 93.6% 24 h after IGF-I stimulation (Fig. 6B). On the other hand, serum withdrawal increased the G0/G1 population only modestly in alveolar RMS Rh30 and MS cells and in embryonal RMS RD and KH cells (Fig. 6A and B). Further, IGF-I did not induce G0/G1 cell cycle arrest in either alveolar or embryonal RMS cells, but it did induce G1-S cell cycle progression in alveolar RMS cells (Fig. 6A and B).

Neither Rh30 cells nor RD cells show myogenic terminal differentiation. In mouse myoblast C2C12 cells, myosin heavy chain expression was observed after 72 h of serum withdrawal condition and was enhanced by IGF-I (50 ng/ml). However, neither Rh30 cells nor RD cells expressed myosin heavy chain during either serum withdrawal or IGF-I (50 ng/ml) treatment up to 72 h (Fig. 6C).

Discussion

Because alveolar RMS has an extremely poor outcome, even with the newest multimodal therapy, it is necessary to find a biological characteristic that is specific to alveolar RMS in order to develop a new treatment. In alveolar RMS, the chimeric gene *PAX3-FKHR* has been widely accepted as an indicator of poor prognosis. Myogenin has been reported to be expressed in alveolar RMS at a much higher rate than in embryonal RMS in paraffin-embedded tissues (18-21). Myogenin is a member of the MyoD family, whose members regulate the myogenic differentiation program (8-12) IGFs are reported to stimulate myogenesis by inducing the expression of myogenin as one of the molecular mechanisms of skeletal muscle differentiation (15-17). IGFs (IGF-I and -II) strongly stimulate myogenesis in cultured myogenic cells and are required for normal skeletal muscle development during embryogenesis (15-17). IGFs regulate myogenic differentiation mainly at the level of myogenin gene transcription via phosphoinositide 3-kinase (PI3K) and mTOR (15). Although embryonal RMS RD cells have some defects in the IGF-IR/PI3K/mTOR pathway in which IGF-I induces the expression of myogenin protein (16), there is no evidence yet that the IGF-I/PI3K/mTOR pathway is involved in myogenin protein induction in alveolar RMS.

In alveolar RMS Rh30 and MS cells, both of which had been confirmed to express *PAX3-FKHR*, IGF-I (50 ng/ml) induced a remarkable increase of myogenin protein up to 48 h (Fig. 2). This prompted us to examine the signaling pathway downstream of IGF-IR for myogenin protein induction in these alveolar RMS cells. PI3K, Akt and mTOR serve as downstream mediators of IGF action on muscle differentiation in C2C12

and L6 myoblasts (38-41). Our findings that both αIR-3 and rapamycin decreased myogenin protein induction by IGF-I (Fig. 3A and B) and that rapamycin did not inhibit the myogenin expression induced by IGF-I in Rh30mTOR-rr cells stably expressing a rapamycin-resistant mutant mTOR (Fig. 3C) indicate that IGF-I induces myogenin protein mainly through the IGF-IR/mTOR pathway and mTOR regulates the induction of myogenin in these alveolar RMS cells.

On the other hand, in embryonal RMS RD and KH cells, serum withdrawal appeared to induce an increase of myogenin protein up to 48 h after serum withdrawal, whereas IGF-I did not cause any additional induction (Fig. 4A and B). The p38MAPK pathway was previously shown to play a role in myogenesis in non-tumor mouse myoblast cells in a parallel but distinct route from the PI3K pathway (30-33). A p38MAPK inhibitor (SB203580) has been reported to inhibit the expression of myogenin in cardiac myoblast H9c2 cells (33). We found that a p38MAPK inhibitor (SB203580) completely inhibited myogenin protein induction by serum withdrawal in embryonal RMS RD and KH cells (Fig. 4C). These data indicate that serum withdrawal induces myogenin protein mainly through the p38MAPK pathway rather than through the IGF-IR/mTOR pathway in embryonal RMS cells.

Together, these results indicate that myogenin induction by serum withdrawal in alveolar RMS does not involve the p38MAPK pathway and that myogenin protein induction by IGF-I does not involve the IGF-IR/mTOR pathway in embryonal RMS cells. Because IGFs are naturally present in the human body, our results may also explain why alveolar RMS has a greater percentage of myogenin-positive tumor cells than embryonal RMS pathologically.

Because the levels of IGF-IR protein in alveolar and embryonal RMS cell lines used in this study were equal (data not shown), we further examined the phosphorylation status of effectors downstream of IGFI/mTOR signaling and found some differences between alveolar and embryonal RMS cells. Akt, 4E-BP1 and p70s6k have been identified as effectors of the IGF-IR/PI3K/mTOR signaling pathway (34-37). Some studies reported that p70S6K activity was not required for myogenesis and that myogenic differentiation was dependent on mTOR kinase function and the phosphorylation of 4E-BP1 by mTOR (42,43). The phosphorylation statuses of Akt and p70s6k were not different between Rh30 and RD cells. We found that 4E-BP1 was constitutively hyperphosphorylated by IGF-I in alveolar RMS Rh30 cells at least up to 24 h, whereas, in embryonal RMS RD cells, the phosphorylation of 4E-BP1 by IGF-I was transient; it was dephosphorylated by 24 h post stimulation. Here relatively small changes in 4E-BP1 phosphorylation caused an association of 4E-BP1 and eIF4E, resulting in an inhibition of myogenic differentiation in C2C12 cells (43). Thus, the different effects of IGF-I on myogenin induction in alveolar and embryonal RMS cells are not due to either the level of IGF-IR expression or the signaling status of Akt and p70s6k downstream of IGF-IR, but might be due to the difference of phosphorylation status of 4E-BP1 between alveolar and embryonal RMS cells.

In addition to myogenin expression, cell cycle arrest is also necessary for terminal muscle differentiation. In our study, both serum-withdrawal and IGF-I treatment induced complete cell cycle arrest in C2C12 cells. However, in alveolar RMS cells, serum-withdrawal did not induce cell cycle arrest, while IGF-I promoted cell cycle progression. Further, IGF-I did not cause any change of cell cycle phase distribution in embryonal RMS cells (Fig. 5).

In summary, our data suggest that IGF-I induces myogenin in both alveolar RMS cells and non-tumor myoblasts, but promotes G1-S cell cycle progression without terminal muscle differentiation only in alveolar RMS cells, and has little effect on myogenin induction or cell cycle progression in embryonal RMS cells. These different responses to IGF-I may help to explain the differences in myogenin staining between alveolar and embryonal RMS. Further, our findings suggest that a specific inhibitor of 4E-BP1 hyperphosphorylation might be a useful anti-tumor agent for alveolar RMS.

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