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Regulation of insulin-like growth factor–dependent myoblast differentiation by Foxo forkhead transcription factors

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Insulin-like growth factors promote myoblast differentiation through phosphoinositol 3-kinase and Akt signaling. Akt substrates required for myogenic differentiation are unknown. Forkhead transcription factors of the forkhead box gene, group O (Foxo) subfamily are phosphorylated in an insulin-responsive manner by phosphatidylinositol 3-kinase–dependent kinases. Phosphorylation leads to nuclear exclusion and inactivation. We show that a constitutively active Foxo1 mutant inhibits differentiation of C_2C_{12} cells and prevents myotube differentiation induced by constitutively active Akt. In contrast, a transcriptionally inactive mutant Foxo1 partially rescues inhibition of C_2C_{12} differentiation mediated by wortmannin, but not by rapamycin, and is able to induce aggregation-independent myogenic conversion of teratocarcinoma cells. Inhibition of Foxo expression by siRNA resulted in more efficient differentiation, associated with increased myosin expression. These observations indicate that Foxo proteins are key effectors of Akt-dependent myogenesis.

Introduction

Muscle development is a multi-step process that begins with the determination of myogenic precursors from mesodermal stem cells and concludes with differentiation of committed myoblasts (McKinsey et al., 2001). This program depends on myogenic effectors of the MyoD family (Weintraub et al., 1991) and their cooperation with myocyte-specific enhancer-binding factors, such as MEF2 (McKinsey et al., 2001). These proteins activate expression of genes required for muscle differentiation through heterodimer formation with other ubiquitous bHLH proteins and by binding to E boxes, cis-acting elements found in the promoter regions of muscle-restricted genes (Olson and Klein, 1994).

Most peptide growth factors stimulate myoblast proliferation and prevent differentiation (Coolican et al., 1997). In contrast, insulin-like growth factors (IGFs) promote myoblast differentiation in vitro (Coolican et al., 1997; Tureckova et al., 2001). In cultured myoblasts, autocrine production of IGF2 is associated with differentiation triggered by serum withdrawal (Rosenthal et al., 1991; Stewart and Rotwein, 1996; Lawlor and Rotwein, 2000a). Inactivation of IGF signaling by targeted mutagenesis of the gene-encoding IGF1 receptor leads to muscle hypoplasia (Liu et al., 1993), whereas IGF1 overexpression in muscle results in enlarged myofibers (Coleman et al., 1995), suggesting that IGFs participate in myogenesis in vivo.

IGF signaling activates the phosphatidylinositol 3-kinase (PI 3-kinase) and the MAPK pathways (Kim and Accili, 2002). Although several reports indicate a requirement for PI 3-kinase activity in the activation of myogenic program (Jiang et al., 1998; Kaliman et al., 1998), the MAPK pathway does not appear to contribute to IGF-dependent myogenesis (Coolican et al., 1997; Lawlor et al., 2000; Tureckova et al., 2001; Conejo et al., 2002). Among the effectors of PI 3-kinase, the serine/threonine kinase Akt has been shown to induce transcription of muscle-specific genes (Jiang et al., 1999), thus resulting in myoblast differentiation (Lawlor et al., 2000; Lawlor and Rotwein, 2000b). However, Akt substrates required for completion of the myogenic process are unknown. Some evidence indicates that $p70^{s6k1}$ participates in myogenesis (Cuenda and Cohen, 1999), whereas the role

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Abbreviations used in this paper: Foxo, forkhead box gene, group O; IGF, insulin-like growth factor; MyHC, myosin heavy chain; PI 3-kinase, phosphatidylinositol 3-kinase; RD, rhabdomyosarcoma-derived.

of mTOR is unclear. Analyses with the mTOR inhibitor rapamycin have yielded conflicting results, with some suggesting that rapamycin inhibits (Coolican et al., 1997; Cuenda and Cohen, 1999; Conejo et al., 2002) and others suggesting that rapamycin promotes myoblast differentiation (Erbay and Chen, 2001). Notably, other authors have reported no effect of rapamycin in this process (Canicio et al., 1998).

Forkhead transcription factors of the forkhead box gene, group O (Foxo) subfamily (Kaestner et al., 2000) are phosphorylated in an insulin-responsive manner by Akt and Sgk (Brunet et al., 1999, 2001; Nakae et al., 2000). Phosphorylation leads to Foxo inhibition through nuclear exclusion. Foxo proteins mediate several transcriptional effects of insulin and IGFs, including those on hepatic glucose production (Nakae et al., 2001b, 2002), pancreatic β-cell proliferation (Kitamura et al., 2002), and adipocyte differentiation (Nakae et al., 2003). To study the role of Foxo proteins in myoblast differentiation, we examined the effects of constitutively active and dominant-negative mutant Foxo1 on in vitro differentiation of cultured myoblasts, teratocarcinoma cells, and rhabdomyosarcoma cells. To examine pathways impinging on Foxo1 regulation of myogenesis, we also studied the ability of Foxo1 mutants to regulate myoblast differentiation in the presence of pharmacological inhibitors of the PI 3-kinase and mTOR pathways, as well as constitutively active Akt. Our data identify Foxo proteins as key effectors of Akt-dependent myogenesis.

Results and discussion

Expression and phosphorylation of Foxo isoforms during differentiation

C_2C_{12} cells undergo growth arrest and induction of the myogenic program after incubation in serum-free medium.

There are three Foxo isoforms in mice: Foxo1, -3 and -4 (Kaestner et al., 2000). *Foxo4* and *Foxo1* are the most abundant transcripts in skeletal muscle (Kitamura et al., 2002). Using Western analysis, we measured expression of the three proteins in myoblasts and myotubes. The relative abundance of all three isoforms declined in myotubes compared with myoblasts, but the difference was not statistically significant (Fig. 1 A). Next, we used phospho-specific antibodies to measure phosphate content of the main phosphorylation site (S253 in Foxo1, S193 in Foxo4). In myotubes, phosphate content increased by 38 and 40% for Foxo1 and Foxo4, respectively. To measure Foxo3 phosphorylation, we used an electrophoretic gel shift assay. We detected an 85% increase in the amount of low mobility (phosphorylated) protein in myotubes (Fig. 1 B). These data indicate that differentiation is associated with increased Foxo phosphorylation. From these observations, it can be inferred that myogenic differentiation requires Foxo1 inhibition, similar to what has been observed in adipocytes (Nakae et al., 2003) and thymocytes (Leenders et al., 2000).

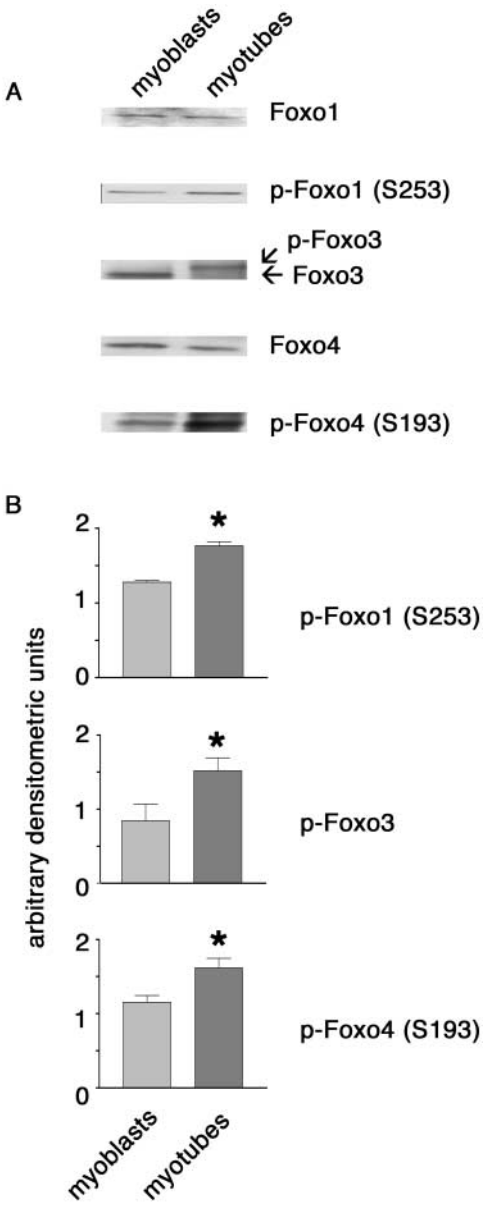

Figure 1. **Foxo isoform expression and phosphorylation levels in C_2C_{12} myoblasts and myotubes.** (A) Extracts of C_2C_{12} myoblasts and myotubes were immunoblotted with the antisera indicated on the right side. (B) Levels of phosphorylated Foxo1 and Foxo4 were assessed by immunoblotting with phospho-specific antisera (S253 for Foxo1, S193 for Foxo4), whereas electrophoretic gel mobility shift was used as a surrogate measure of Foxo3 phosphorylation. The phosphorylated protein migrates more slowly on SDS-PAGE. Mean ± SEM of densitometric scanning values was calculated from three independent experiments. Immunoblotting with anti-cyclophilin antiserum was used as a control for gel loading (not depicted). Asterisk indicates $P < 0.01$.

Foxo1 mutants modulate myoblast differentiation

Because Akt mediates IGF-dependent C_2C_{12} differentiation (Lawlor and Rotwein, 2000b; Tureckova et al., 2001) and Foxo isoforms are Akt substrates (Brunet et al., 1999), we investigated whether they mediate myoblast differentiation. We used adenovirus-mediated gene transfer to express constitutively active (ADA) and dominant-negative (Δ256) Foxo1 mutants in myoblasts. The constitutively active mutant cannot be phosphorylated and fails to translocate in response to insulin, whereas the dominant-negative mutant lacks the transactivation domain (Nakae et al., 2000). The Δ256-Foxo1 or ADA-Foxo1 adenoviruses were expressed at high levels after transduction (Fig. 2 A, lane 2 and lane 3). After 96 h in differentiation medium, cells transduced with

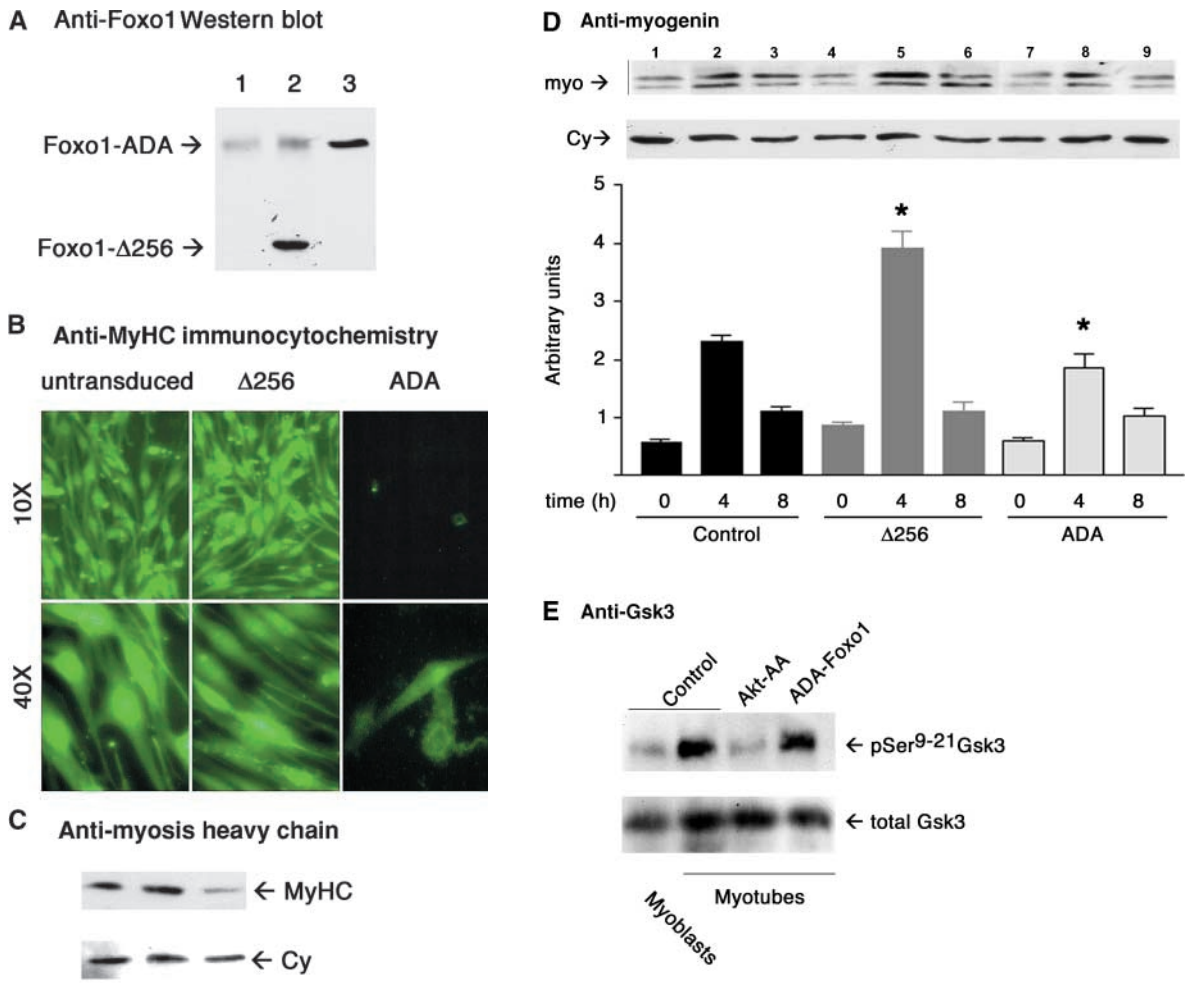

Figure 2. **Expression of early and late differentiation markers in C_2C_{12} transduced with Foxo1 mutants.** (A) Expression of Foxo1 mutants following adenovirus-mediated gene transfer. Extracts from control cells (lane 1) or from cells transduced with D256-Foxo1 (lane 2) or ADA-Foxo1 (lane 3) were analyzed by immunoblotting with anti-Foxo1 antiserum. (B) Detection of myosin expression by immunocytochemistry. (C) Immunoblot analysis of MyHC expression in cells expressing ADA-Foxo1 or Δ256-Foxo1. A representative blot is shown at the top, and a graph summarizing several experiments is shown on the bottom. The same filter was stripped and reprobed with anti-cyclophilin antiserum (Cy) to normalize protein content (bottom). Asterisk indicates $P < 0.01$. (D) Myogenin expression in cells expressing mutant Foxo1. Extracts were obtained from untransduced cells (lanes 1–3), cells expressing Δ256-Foxo1 (lanes 4–6), and ADA-Foxo1 (lanes 7–9) at the indicated time points and analyzed by SDS-PAGE, followed by immunoblot with anti-myogenin (top) or anti-cyclophilin antiserum (bottom). Data from three separate experiments are summarized in the bar graphs at the bottom. Scanning densitometry of the autoradiograms was used to measure MyHC and myogenin expression. Data are plotted as mean ± SEM. An asterisk indicates $P < 0.01$. (E) Phosphorylation of Gsk3 in cells transduced with ADA-Foxo1. Lysates were analyzed by Western blotting with a phosphoSer$^{9-21}$-specific Gsk3 antiserum (top) and a total Gsk3 antiserum (bottom).

the ADA mutant failed to convert to myotubes, whereas cells transduced with the Δ256 mutant were morphologically indistinguishable from control cells (Fig. 2 B). Accordingly, the ADA mutant prevented expression of myosin heavy chain (MyHC), a marker of terminally differentiated myotubes. Conversely, transduction with Δ256 caused a 30% increase in MyHC expression (Fig. 2, B and C).

Myogenin is an early differentiation marker. In myoblasts, its expression was induced between 4 and 8 h of serum withdrawal. The transient decrease in myogenin expression during differentiation has been observed by others (Langley et al., 2002), and does not appear to interfere with the cells' ability to undergo complete differentiation into myotubes (Fig. 2 B). Cells transduced with Δ256 showed a 70% increase of myogenin levels compared with untransduced cells. In contrast, ADA-transduced cells showed a 20% decrease (Fig. 2 D). These data are consistent with the observation that the Foxo1 gain-of-function mutant impairs differentiation, whereas the dominant-negative Foxo1 increases the efficiency of differentiation.

The effect of the ADA mutant could not be accounted for by nonspecific inhibition of Akt function because phosphorylation of Gsk3, another Akt substrate, was unaffected in cells expressing the ADA-Foxo1 mutant (Fig. 2 E).

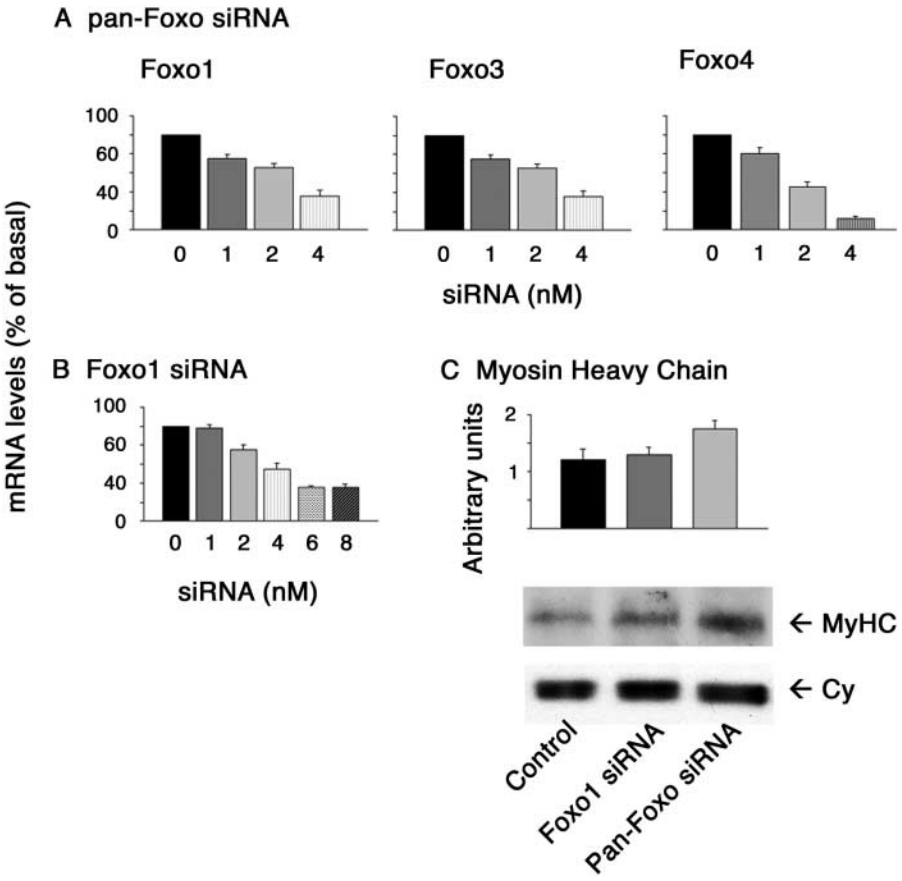

Figure 3. **siRNA-mediated inhibition of Foxo expression.** Expression of mRNA encoding the three Foxo isoforms in cells transfected with increasing concentrations of pan-Foxo (A) or Foxo1-specific siRNA (B). Results are expressed as a percentage of control. (C) Immunoblot analysis of MyHC expression in cells transfected with pan-Foxo (light gray), Foxo1-specific siRNA (dark gray), or control siRNA (black, top). The same filter was stripped and reprobed with anti-cyclophilin antiserum (Cy) to normalize protein content (bottom).

We obtained additional evidence that Foxo inhibition is required for differentiation using siRNA to decrease Foxo mRNA levels. We designed two siRNAs, one directed against a Foxo1-specific sequence, and one against a pan-Foxo–specific sequence. Transfection of the pan-Foxo siRNA resulted in 70–90% decreases in mRNA levels encoding all three isoforms (Fig. 3 A), and was accompanied by a 30% increase in MyHC expression (Fig. 3 C). In contrast, transfection of the Foxo1-specific siRNA inhibited expression of Foxo1 (Fig. 3 B), but not of Foxo3 and -4, and induced a lesser increase in MyHC expression (Fig. 3 C).

Δ256 Foxo1 partly rescues wortmannin inhibition of myoblast differentiation

Inhibition of PI 3-kinase prevents myoblast differentiation (Jiang et al., 1998; Tureckova et al., 2001). Because Foxo proteins are regulated by PI 3 kinase–dependent and –independent pathways (Brunet et al., 2001; Nakae et al., 2001a), we evaluated the ability of Δ256 to rescue inhibition of myogenesis by wortmannin. Treatment with wortmannin inhibited differentiation and prevented MyHC expression. Transduction with Δ256 restored MyHC expression to ~50% of control levels and was associated with partial restoration of the myotube phenotype (Fig. 4 A). These data indicate that Foxo1 mediates some of the effects of PI 3-kinase on differentiation. However, the failure of Δ256 to completely reverse the effect of wortmannin is consistent with the presence of Foxo-independent mechanisms of myogenesis downstream of PI 3-kinase.

ADA Foxo1 prevents differentiation induced by Myr-Akt

Akt stimulates myoblast differentiation (Coolican et al., 1997; Lawlor and Rotwein, 2000b). Transduction with a constitutively active Myr-Akt promoted differentiation and increased MyHC expression twofold. However, cotransduction of Myr-Akt and ADA-Foxo1 prevented differentiation induced by Myr-Akt and decreased MyHC expression to the same levels seen in cells transduced with ADA-Foxo1 alone (Fig. 4 B). These data suggest that Akt-induced myogenesis is mediated by Foxo.

Δ256-Foxo1 fails to reverse rapamycin inhibition of differentiation

Among the PI 3-kinase effectors, some authors have shown that $p70^{s6k}$ is required for differentiation (Coolican et al., 1997; Cuenda and Cohen, 1999; Conejo et al., 2002). Inhibition of mTOR by rapamycin resulted in an approximate 70% decrease of MyHC levels and blocked myogenic differentiation. The inhibition of MyHC expression by rapamycin was unaffected by Δ256-Foxo1 (Fig. 4 C; $P < 0.001$), suggesting that the mTOR pathway does not act through Foxo to promote differentiation.

Δ256-Foxo1 induces differentiation of teratocarcinoma, but not of rhabdomyosarcoma cells

P19 teratocarcinoma cells are able to undergo myogenic conversion when cultured with DMSO. When P19 cells were transduced with the Δ256-Foxo1, differentiation oc-

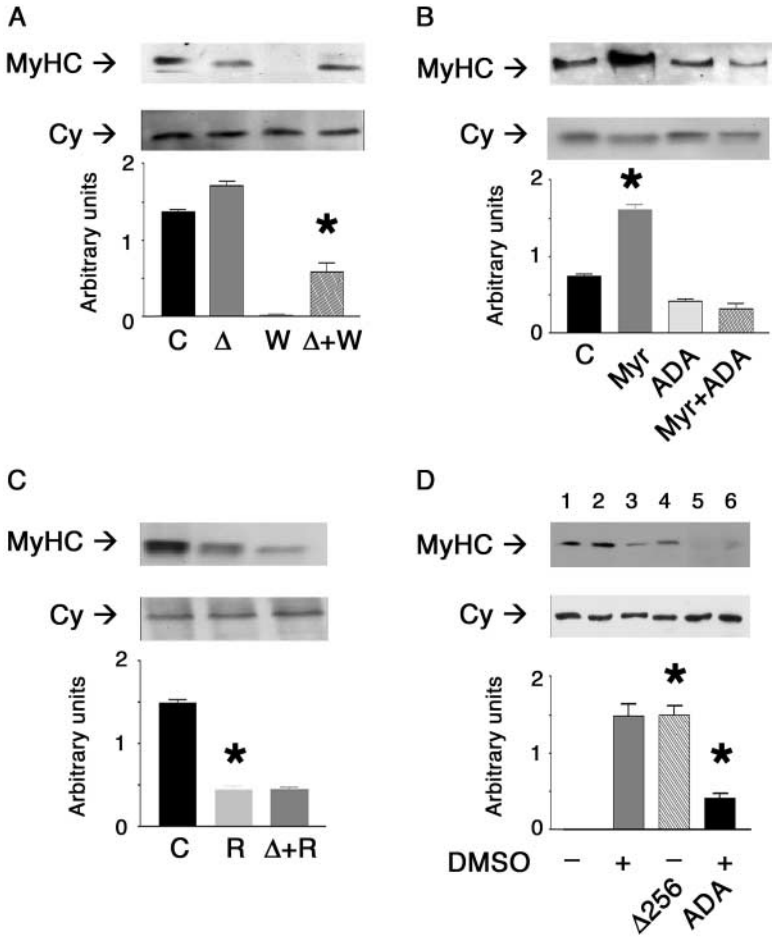

Figure 4. **Signaling pathways involved in Foxo1 regulation of myoblast differentiation.** (A) Δ256-Foxo1 partly rescues wortmannin inhibition of myoblast differentiation. C, control cells; W, wortmannin-treated cells; Δ, cells expressing Δ256-Foxo1; Δ + W, Δ 256-expressing cells that have been treated with wortmannin. Extracts were obtained 96 h after the induction of differentiation. The bar graph summarizes the results of three experiments. (B) ADA-Foxo1 overrides the effect of Myr-Akt to stimulate myoblast differentiation. Extracts from control cells (C) and cells transduced with Myr-Akt (Myr), ADA-Foxo1 (ADA), or both (Myr + ADA) were prepared 96 h after the induction of differentiation. Equal amounts of protein were subjected to SDS-PAGE and were immunoblotted with anti-MyHC (top) or anti-cyclophilin antiserum (bottom). (C) Δ256-Foxo1 fails to override the inhibitory effect of rapamycin on C_2C_{12} differentiation. Immunoblot analyses with anti-MyHC (top) and anti-cyclophilin (bottom) antisera were performed on extracts obtained from control (C), rapamycin-treated (R), and Δ256-expressing cells (Δ + R) as indicated above. (D) Δ256-Foxo1 induces myogenic conversion of P19 teratocarcinoma cells independently of DMSO treatment. P19 cells were differentiated in the presence (lane 1 and lane 4) or absence (lane 2 and lane 3) of DMSO and were transduced with either Δ256-Foxo1 (lane 2) or ADA-Foxo1 (lane 4). Lane 5 and lane 6 show control RD cells and RD transduced with Δ256-Foxo1. The bar graphs represent mean ± SEM densitometric values from three or four experiments for each condition. One asterisk indicates $P < 0.01$ in A–C, and $P < 0.005$ in D. Cyclophilin was used to normalize gel loading.

curred in the absence of DMSO, and MyHC expression increased to levels similar to DMSO-treated cells (Fig. 4 D, lanes 1–4 and bar graph). In contrast, transduction with the ADA mutant inhibited DMSO-induced differentiation and reduced MyHC expression by ~70%. Thus, a loss-of-function Foxo1 mutation enhances myoblast differentiation of P19 cells, whereas a gain-of-function mutation impairs it.

Rhabdomyosarcoma-derived (RD) cells lack functional IGF signaling (Merlino and Helman, 1999). Most rhabdomyosarcomas carry chromosomal translocations in which the DNA-binding domain of Foxo1 is fused to the transactivation domain of Pax3 or Pax8 (Wang et al., 1998; Merlino and Helman, 1999). We tested whether Foxo1 inhibition would restore differentiation by transducing cells with Δ256-Foxo1 and by culturing them in differentiation medium. However, we failed to detect morphological changes or increases in MyHC expression (Fig. 3 D, lane 5 and lane 6). Thus, Foxo1 inhibition alone cannot rescue the transformed phenotype of RD cells.

Conclusions

Our data provide evidence for a key role of Foxo transcription factors as mediators of IGF-dependent myoblast differentiation. We provide two lines of evidence to support our conclusions. Foxo1 gain-of-function is associated with impaired myoblast differentiation, whereas loss-of-function is able to partly restore inhibition of differentiation by wortmannin. Furthermore, constitutively active Foxo1 inhibited, whereas dominant-negative mutant Foxo1 caused a slight, but significant increase in the expression of differentiation markers. Consistent with the notion that Foxo is regulated by Akt, we also found that the ability of constitutively active Akt to induce differentiation (Rommel et al., 2001) can be blocked by the phosphorylation-defective Foxo1 mutant. These data identify Foxo1 as a key component of the Akt pathway in differentiation. This pathway requires activation of the cell cycle inhibitor p21 (Lawlor and Rotwein, 2000b), a Foxo1 target in differentiating adipocytes (Nakae et al., 2003). Although we have not examined p21 expression in C_2C_{12}, it is likely to be an important Foxo target in myoblasts as well.

The inability of the dominant-negative Foxo1 to completely reverse the inhibition of differentiation caused by wortmannin suggests that multiple effectors of differentiation act downstream of PI 3-kinase. Similarly, the dominant-negative Foxo1 failed to reverse rapamycin inhibition of myoblast differentiation, indicating that Foxo1 phosphorylation by mTOR is not required for this process. We suggest that signals regulating myoblast differentiation diverge downstream of PI 3-kinase, with both Akt and mTOR playing a role in the process.

Foxo1 inhibition restored myogenic differentiation of teratocarcinoma-derived cells, but not of rhabdomyosarcoma-derived cells. RD cells express myogenic factors, such as

MyoD and myogenin, but these factors are transcriptionally inactive (Tapscott et al., 1993). It has also been shown that RDs are defective in their PI 3-kinase→Akt signaling (Xu et al., 2002), and that constitutively active forms of PI 3-kinase and Akt fail to restore differentiation. Now, we extend those data by showing that inhibition of Foxo1 function is equally unable to restore the differentiated phenotype, suggesting that the defect in RD cells is distal to Foxo1.

After the submission of this manuscript, it has been reported that Foxo1 promotes differentiation of primary myoblasts (Bois and Grosveld, 2003). These conclusions are at odds with our data, and appear to be due to substantial differences in the experimental system, such as the dissociation of Foxo1 phosphorylation from nuclear localization, the lack of Akt regulation of Foxo1, and the failure of dominant-negative Akt to affect differentiation.

In conclusion, we show that Foxo1 plays in important role in regulating skeletal muscle differentiation through the PI3K→Akt pathway. Further work will identify Foxo1 targets involved in this process.

Materials and methods

Reagents

We obtained C_2C_{12}, P19, and RD cells from the American Type Culture Collection, anti- Foxo1 antibodies from Santa Cruz Biotechnology, Inc., anti-HA antibody 12CA5 from Boehringer, anti-MyHC (clone MF 20) and anti-myogenin antibodies (clone F5D) from the Hybridoma Bank (University of Iowa, Iowa City, IA), and anti phospho-Gsk3 and anti-Gsk3 antisera from Upstate Biotechnology. C_2C_{12}, P19 (Wilton and Skerjanc, 1999), and RD cells (Merlino and Helman, 1999) were maintained and differentiated as described previously (Astolfi et al., 2001). Foxo1 and Akt adenoviral vectors have been described previously (Nakae et al., 2001b). Western blotting was performed according to standard procedures.

Design and transfection of siRNAs

We used the target finder and design tool (Ambion) to identify target siRNAs. The Foxo1-specific sequence was 5′-AAAAGTCCTTCAGATTGTCTG-3′. The pan-Foxo sequence was 5′-AAGGATAAGGGCGACAGACC-3′. SiRNA synthesis and transfection with siPORT™ reagents was performed according to the manufacturer's instructions (Ambion). Foxo expression was measured by real-time RT-PCR using a LightCycler PCR instrument (Roche) and LightCycler SYBR® Green I reaction mix (Roche). Each reaction was performed in triplicate under standard reaction conditions. β-Actin was used as a control of amplification efficiency.

Immunofluorescence

Cells were cultured on microscope slides, differentiated, and fixed in 4% PFA. After incubation with anti-MyHC IgG2b at 1:500, detection was performed using DAPC-conjugated secondary antibody (Nakae et al., 2003).

Statistical analysis

Statistical analysis was performed using *t* test for paired data.

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References

Astolfi, A., C. De Giovanni, L. Landuzzi, G. Nicoletti, C. Ricci, S. Croci, L. Scopece, P. Nanni, and P.L. Lollini. 2001. Identification of new genes related to the myogenic differentiation arrest of human rhabdomyosarcoma cells. *Gene.* 274:139–149.

Bois, P.R., and G.C. Grosveld. 2003. FKHR (FOXO1a) is required for myotube fusion of primary mouse myoblasts. *EMBO J.* 22:1147–1157.

Brunet, A., A. Bonni, M.J. Zigmond, M.Z. Lin, P. Juo, L.S. Hu, M.J. Anderson, K.C. Arden, J. Blenis, and M.E. Greenberg. 1999. Akt promotes cell survival by phosphorylating and inhibiting a forkhead transcription factor. *Cell.* 96:857–868.

Brunet, A., J. Park, H. Tran, L.S. Hu, B.A. Hemmings, and M.E. Greenberg. 2001. Protein kinase SGK mediates survival signals by phosphorylating the forkhead transcription factor FKHRL1 (FOXO3a). *Mol. Cell. Biol.* 21:952–965.

Canicio, J., E. Gallardo, I. Illa, X. Testar, M. Palacin, A. Zorzano, and P. Kaliman. 1998. p70 S6 kinase activation is not required for insulin-like growth factor-induced differentiation of rat, mouse, or human skeletal muscle cells. *Endocrinology.* 139:5042–5049.

Coleman, M.E., F. DeMayo, K.C. Yin, H.M. Lee, R. Geske, C. Montgomery, and R.J. Schwartz. 1995. Myogenic vector expression of insulin-like growth factor I stimulates muscle cell differentiation and myofiber hypertrophy in transgenic mice. *J. Biol. Chem.* 270:12109–12116.

Conejo, R., C. de Alvaro, M. Benito, A. Cuadrado, and M. Lorenzo. 2002. Insulin restores differentiation of Ras-transformed C2C12 myoblasts by inducing NF-kappaB through an AKT/P70S6K/p38-MAPK pathway. *Oncogene.* 21: 3739–3753.

Coolican, S.A., D.S. Samuel, D.Z. Ewton, F.J. McWade, and J.R. Florini. 1997. The mitogenic and myogenic actions of insulin-like growth factors utilize distinct signaling pathways. *J. Biol. Chem.* 272:6653–6662.

Cuenda, A., and P. Cohen. 1999. Stress-activated protein kinase-2/p38 and a rapamycin-sensitive pathway are required for C2C12 myogenesis. *J. Biol. Chem.* 274:4341–4346.

Erbay, E., and J. Chen. 2001. The mammalian target of rapamycin regulates C2C12 myogenesis via a kinase-independent mechanism. *J. Biol. Chem.* 276:36079–36082.

Jiang, B.H., J.Z. Zheng, and P.K. Vogt. 1998. An essential role of phosphatidylinositol 3-kinase in myogenic differentiation. *Proc. Natl. Acad. Sci. USA.* 95: 14179–14183.

Jiang, B.H., M. Aoki, J.Z. Zheng, J. Li, and P.K. Vogt. 1999. Myogenic signaling of phosphatidylinositol 3-kinase requires the serine-threonine kinase Akt/protein kinase B. *Proc. Natl. Acad. Sci. USA.* 96:2077–2081.

Kaestner, K.H., W. Knochel, and D.E. Martinez. 2000. Unified nomenclature for the winged helix/forkhead transcription factors. *Genes Dev.* 14:142–146.

Kaliman, P., J. Canicio, P.R. Shepherd, C.A. Beeton, X. Testar, M. Palacin, and A. Zorzano. 1998. Insulin-like growth factors require phosphatidylinositol 3-kinase to signal myogenesis: dominant negative p85 expression blocks differentiation of L6E9 muscle cells. *Mol. Endocrinol.* 12:66–77.

Kim, J.J., and D. Accili. 2002. Signalling through IGF-I and insulin receptors: where is the specificity? *Growth Horm. IGF Res.* 12:84–90.

Kitamura, T., J. Nakae, Y. Kitamura, Y. Kido, W.H. Biggs, III, C.V. Wright, M.F. White, K.C. Arden, and D. Accili. 2002. The forkhead transcription factor Foxo1 links insulin signaling to Pdx1 regulation of pancreatic beta cell growth. *J. Clin. Invest.* 110:1839–1847.

Langley, B., M. Thomas, A. Bishop, M. Sharma, S. Gilmour, and R. Kambadur. 2002. Myostatin inhibits myoblast differentiation by down-regulating MyoD expression. *J. Biol. Chem.* 277:49831–49840.

Lawlor, M.A., and P. Rotwein. 2000a. Coordinate control of muscle cell survival by distinct insulin-like growth factor activated signaling pathways. *J. Cell Biol.* 151:1131–1140.

Lawlor, M.A., and P. Rotwein. 2000b. Insulin-like growth factor-mediated muscle cell survival: central roles for Akt and cyclin-dependent kinase inhibitor p21. *Mol. Cell. Biol.* 20:8983–8995.

Lawlor, M.A., X. Feng, D.R. Everding, K. Sieger, C.E. Stewart, and P. Rotwein. 2000. Dual control of muscle cell survival by distinct growth factor-regulated signaling pathways. *Mol. Cell. Biol.* 20:3256–3265.

Leenders, H., S. Whiffield, C. Benoist, and D. Mathis. 2000. Role of the forkhead transcription family member, FKHR, in thymocyte differentiation. *Eur. J. Immunol.* 30:2980–2990.

Liu, J.P., J. Baker, A.S. Perkins, E.J. Robertson, and A. Efstratiadis. 1993. Mice carrying null mutations of the genes encoding insulin-like growth factor I (Igf-1) and type 1 IGF receptor (Igf1r). *Cell.* 75:59–72.

McKinsey, T.A., C.L. Zhang, and E.N. Olson. 2001. Control of muscle development by dueling HATs and HDACs. *Curr. Opin. Genet. Dev.* 11:497–504.

Merlino, G., and L.J. Helman. 1999. Rhabdomyosarcoma–working out the pathways. *Oncogene.* 18:5340–5348.

Nakae, J., V. Barr, and D. Accili. 2000. Differential regulation of gene expression by insulin and IGF-1 receptors correlates with phosphorylation of a single

amino acid residue in the forkhead transcription factor FKHR. *EMBO J.* 19: 989–996.

Nakae, J., T. Kitamura, W. Ogawa, M. Kasuga, and D. Accili. 2001a. Insulin regulation of gene expression through the forkhead transcription factor Foxo1 (Fkhr) requires kinases distinct from Akt. *Biochemistry.* 40:11768–11776.

Nakae, J., T. Kitamura, D.L. Silver, and D. Accili. 2001b. The forkhead transcription factor Foxo1 (Fkhr) confers insulin sensitivity onto glucose-6-phosphatase expression. *J. Clin. Invest.* 108:1359–1367.

Nakae, J., W.H. Biggs, T. Kitamura, W.K. Cavenee, C.V. Wright, K.C. Arden, and D. Accili. 2002. Regulation of insulin action and pancreatic beta-cell function by mutated alleles of the gene encoding forkhead transcription factor Foxo1. *Nat. Genet.* 32:245–253.

Nakae, J., T. Kitamura, Y. Kitamura, W.H. Biggs, K.C. Arden, and D. Accili. 2003. The forkhead transcription factor foxo1 regulates adipocyte differentiation. *Dev. Cell.* 4:119–129.

Olson, E.N., and W.H. Klein. 1994. bHLH factors in muscle development: dead lines and commitments, what to leave in and what to leave out. *Genes Dev.* 8:1–8.

Rommel, C., S.C. Bodine, B.A. Clarke, R. Rossman, L. Nunez, T.N. Stitt, G.D. Yancopoulos, and D.J. Glass. 2001. Mediation of IGF-1-induced skeletal myotube hypertrophy by PI(3)K/Akt/mTOR and PI(3)K/Akt/GSK3 pathways. *Nat. Cell Biol.* 3:1009–1013.

Rosenthal, S.M., A. Brunetti, E.J. Brown, P.W. Mamula, and I.D. Goldfine. 1991. Regulation of insulin-like growth factor (IGF) I receptor expression during muscle cell differentiation. Potential autocrine role of IGF-II. *J. Clin. Invest.* 87:1212–1219.

Stewart, C.E., and P. Rotwein. 1996. Insulin-like growth factor-II is an autocrine survival factor for differentiating myoblasts. *J. Biol. Chem.* 271:11330–11338.

Tapscott, S.J., M.J. Thayer, and H. Weintraub. 1993. Deficiency in rhabdomyosarcomas of a factor required for MyoD activity and myogenesis. *Science.* 259:1450–1453.

Tureckova, J., E.M. Wilson, J.L. Cappalonga, and P. Rotwein. 2001. Insulin-like growth factor-mediated muscle differentiation: collaboration between phosphatidylinositol 3-kinase-Akt-signaling pathways and myogenin. *J. Biol. Chem.* 276:39264–39270.

Wang, W., P. Kumar, W. Wang, J. Epstein, L. Helman, J.V. Moore, and S. Kumar. 1998. Insulin-like growth factor II and PAX3-FKHR cooperate in the oncogenesis of rhabdomyosarcoma. *Cancer Res.* 58:4426–4433.

Weintraub, H., V.J. Dwarki, I. Verma, R. Davis, S. Hollenberg, L. Snider, A. Lassar, and S.J. Tapscott. 1991. Muscle-specific transcriptional activation by MyoD. *Genes Dev.* 5:1377–1386.

Wilton, S., and I. Skerjanc. 1999. Factors in serum regulate muscle development in P19 cells. *In Vitro Cell. Dev. Biol. Anim.* 35:175–177.

Xu, Q., L. Yu, L. Liu, C.F. Cheung, X. Li, S.P. Yee, X.J. Yang, and Z. Wu. 2002. p38 Mitogen-activated protein kinase-, calcium-calmodulin-dependent protein kinase-, and calcineurin-mediated signaling pathways transcriptionally regulate myogenin expression. *Mol. Biol. Cell.* 13:1940–1952.