Age-dependent FOXO regulation of p27<sup>Kip1</sup> expression via a conserved binding motif in rat muscle precursor cells

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Lees SJ, Childs TE, Booth FW. Age-dependent FOXO regulation of p27<sup>Kip1</sup> expression via a conserved binding motif in rat muscle precursor cells. Am J Physiol Cell Physiol 295: C1238–C1246, 2008; doi:10.1152/ajpcell.00349.2008.—Previously, we have demonstrated that forkhead box O3a (FOXO3a) overexpression increased p27<sup>Kip1</sup> promoter activity and protein expression, whereas it decreased proliferation in muscle precursor cells (MPCs). The objectives of the present study were to 1) locate and identify FOXO regulatory elements in the rat p27<sup>Kip1</sup> promoter using deletion analysis of a promoter/reporter construct and 2) determine if age-related differences exist in FOXO-induced p27<sup>Kip1</sup> expression. The full-length (−4.0/kb) rat p27<sup>Kip1</sup> promoter construct revealed that both FOXO1 and FOXO3a induced an increase in transcriptional activity. Interestingly, MPCs isolated from old animals exhibited an increased FOXO3a-induced p27<sup>Kip1</sup> promoter activity compared with MPCs isolated from young animals. Deletion of a 253-bp portion of the 5′-untranslated region (UTR) resulted in a significant decrease in FOXO-induced p27<sup>Kip1</sup> promoter expression. Site-specific mutation of a daf-16 family protein-binding element (DBE) within this 253-bp portion of the 5′-UTR also demonstrated a decrease in FOXO-induced p27<sup>Kip1</sup> promoter expression. These data suggest that a putative FOXO regulatory element located in the 5′-UTR of the rat p27<sup>Kip1</sup> gene plays a role in the age-dependent differences in FOXO3a-dependent p27<sup>Kip1</sup> promoter expression. These findings have implications for developing treatment strategies aimed at increasing the proliferation of MPCs and regenerative capacity of aged skeletal muscle.

MATERIALS AND METHODS

Animals. All procedures were approved by the Institutional Animal Care and Use Committee of the University of Missouri (Columbia, MO). Fischer-344 × Brown Norway F<sub>1</sub> hybrid male rats (3 and 32 mo old) were obtained from the National Institute on Aging. Animals were housed at 21°C on a 12:12-h light-dark cycle and allowed free access to food and water. At the time of death, animals were given an intraperitoneal injection of ketamine (80 mg/kg), xylazine (10 mg/kg), and acepromazine (4 mg/kg), and muscles were then excised.

MPC isolation and culture. MPC isolation was modified from Allen et al. (2) as previously described (24, 25). Briefly, cells isolated from the gastrocnemius and plantaris muscles by pronase digestion were preplated for 24 h on tissue culture-treated 150-mm plates. After the 24-h preplate, cells were seeded onto Matrigel (BD Biosciences, San Jose, CA)-coated 150-mm plates (0.1 mg/ml Matrigel for 60 min at 37°C) and cultured for 3 days in growth media (GM; 20% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 40 μg/ml gentamicin) and 35°C (HERAcell, Thermo Scientific). After 3 days, cells reached muscle atrophy (5). A mutant FOXO that was constitutively active induced MAFbx expression and caused atrophy in myotubes in culture (34). Moreover, RNA inhibitor knock down of FOXO3a in vivo decreased MAFbx expression (34). Interestingly, recent findings have revealed increased FOXO3a mRNA (32) in sarcopenia, which is defined as an age-associated loss of skeletal muscle mass and strength.

Muscle precursor cells (MPCs) are required for normal regenerative (35) and hypertrophic responses in skeletal muscle (1). However, sarcopenia has been linked to impaired skeletal muscle regeneration (6) and hypertrophy (3) as well as impaired MPC function (4, 12, 27). Previous work from our laboratory has demonstrated elevated nuclear FOXO1 and p27<sup>Kip1</sup> protein levels in MPCs isolated from sarcopenic animals (27). p27<sup>Kip1</sup> is a key cell cycle inhibitor that has been shown to be regulated by FOXO (14, 29, 31). Furthermore, we have demonstrated that adenosine-activated/FOXO3a overexpression increased p27<sup>Kip1</sup> promoter activity and protein expression in MPCs (31). This FOXO3a-mediated increase in p27<sup>Kip1</sup> expression was associated with a decrease in 5-bromo-2′-deoxyuridine incorporation and cell number (31).

The purpose of the present study was twofold: 1) to locate and identify FOXO regulatory elements on the p27<sup>Kip1</sup> promoter using deletion analysis of a promoter/reporter construct and 2) to determine if age-related differences exist in FOXO-induced p27<sup>Kip1</sup> expression at the identified regulatory element.

EMERGING EVIDENCE suggests that forkhead box O (FOXO) transcription factors are at the nexus of aging, metabolism, and cell fate/function (9). The primary regulation of FOXO family proteins is downstream of insulin and IGF signaling. Akt-mediated phosphorylation of FOXO results in nuclear exclusion and inhibition of transcriptional activity (7, 8, 23, 30, 33). However, despite extensive study into the multitude of functional roles, the details of the regulatory regions for targets of FOXO are still mostly elusive.

FOXO proteins play a key role in multiple conditions of skeletal muscle wasting. Several models of skeletal muscle atrophy cause upregulation of the transcripts of the muscle-specific ubiquitin ligases muscle ring finger-1 (MuRF-1) and muscle atrophy F-box (MAFBx/atrogen-1) (5). Followup experiments revealed that overexpression of MAFbx induced myotube atrophy in culture, whereas mice lacking either the MuRF-1 or MAFbx genes were resistant to denervation-induced

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~80% confluence. Cells were then passaged one time and seeded onto appropriate Matrigel tissue culture plates. Greater than 95% desmin- and MyoD-positive cells are obtained using this isolation protocol (data not shown). As media depth is an important concern for 5% O2 culture conditions (36), 1.5 ml of GM were used for experiments carried out in six-well culture plates (25,000 cells/well). For experiments where MEC differentiation was induced, cells were washed once with PBS, and the media were replaced with differentiation media (DM), which consisted of 2% horse serum in DMEM.

DNA constructs, transfection, and promoter activity. The rat muscle creatine kinase (CK-M) promoter construct (−1500/+24 bp) was cloned from genomic DNA isolated from Fischer-344 × Brown Norway F1 hybrid skeletal muscle by PCR using the following primers: forward 5′-CGACGGCGTTGCGAGGTAGGATGAGAAGCCATGC-3′ and reverse 5′-TCCCCGGGGAGATCTTTCGTTGGAGGAGGGATGGA-3′. The cloned promoter was then ligated into the pGL3-Basic firefly luciferase reporter vector (Promega, Madison, WI). The cloned CK-M promoter sequence was verified by DNA sequencing using an Applied Biosystems 3730 DNA Analyzer and Applied Biosystems Prism BigDye Terminator cycle sequencing chemistry (Applied Biosystems, Foster City, CA). Expression plasmids for FOXO1 and its triple mutant, in which the inhibitory Akt threonine and serine phospho-residues were mutated by substitution with alanine residues (FOXO1 A3), were a kind gift from Dr. Terry Unterman (in pALTER-MAX, Promega). Expression plasmids for FOXO3a and its triple mutant, in which the inhibitory Akt threonine and serine phospho-residues were mutated by substitution with alanine residues (FOXO3a A3), were a kind gift from Dr. Paul Coffer (in pECE). The full-length rat p27Kip1 promoter (−4362/+0.421 kb) was cloned using the primers previously described (24). The full-length sequence is shown in supplemental Fig. 1S along with the human sequence. 1 Deletion constructs for the p27Kip1 promoter construct were created from the full-length (−4362/+0.421 kb) p27Kip1 construct using the primers shown in Table 1. The deletion of ~253 bp from the 5′-untranslated region (UTR) was achieved by restriction endonuclease digestion with HindIII (Table 1). The NF-κB cis-reporter construct contains five repeats of the transcription recognition sequence (5′-GGGACTTCCGCG-3′) linked to a basic promoter element (TATA box) and the firefly luciferase gene (Stratagene, La Jolla, CA).

The QwikChange Lightning Site-Directed Mutagenesis kit (Stratagene) was used for site-directed mutagenesis of the daf-16 family protein-binding element (DBE) site in the 5′-UTR (+348 to +355 bp) of the p27Kip1 gene from 5′-TTGGTTTAT-3′ to 5′-TTGGTTTAT-3′ using the following primers: forward 5′-CAAGGGGCGTTGCC-3′ and reverse 5′-TTGGTTTATGGTTTC-3′.

Transient transfections were carried out immediately after the cells had been seeded in antibiotic-free GM using Fugene 6 (Roche Applied Science, Indianapolis, IN) following the manufacturer’s instructions. The phRL-null Renilla luciferase reporter vector (Promega) was cotransfected in each experiment and used as an internal control promoter to normalize for transfection efficiency. For FOXO overexpression experiments, 0.1 μg/well of expression vector containing either FOXO1 or FOXO1A3 in pALTER-MAX or FOXO3a or FOXO3a A3 in pECE were used. A total of 0.7 μg of DNA were used for both firefly and Renilla luciferase reporter constructs at a firefly-to-Renilla ratio of 20:1. Cells were lysed using passive lysis buffer (Promega) and stored at −80°C. Firefly and Renilla luminescence were measured using the Dual-Luciferase Reporter Assay System (Promega) on a Veritas microplate luminometer (Turner Biosystems, Sunnyvale, CA).

Western blot analysis. After 2 days in GM, cells were lysed with RIPA buffer containing 1.04 mM 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride, 800 mM aprotinin, 20 μM leupeptin, 40 μM phenylmethylsulfonyl fluoride, 15 μM pepstatin A, 14 μM E-64, and phosphatase inhibitor cocktail 1 (P2850, Sigma-Aldrich, St. Louis, MO; a proprietary mix of cantharidin, bromotetramisole, and microcystin LR used at 1:100 dilution). Cell lysates were then frozen and stored at −80°C. Samples were thawed and then centrifuged at 12,000 g (4°C) for 15 min, the supernatant was collected, the protein concentration was determined using the DC protein assay, and samples were diluted to equal concentrations (0.4 mg/ml) in SDS reducing buffer. Equal amounts of protein were loaded and separated by SDS-PAGE and transferred to nitrocellulose membranes (Osmonics). To ensure equal loading, nitrocellulose membranes were stained with Ponceau S (Sigma-Aldrich), which allows for both the qualitative visualization and quantitation of the amount or protein in a given lane (22). Phospho-Akt(Serine473) antibody was purchased from Cell Signaling Technology (Beverley, MA). Horseradish peroxidase-conjugated secondary IgG antibody was purchased from Pierce Biotechnology (Rockford, IL). Immuno-plexes were visualized using SuperSignal West Dura Extended Duration Substrate (Pierce Biotechnology). Signal bands were scanned using a Kodak Image Station 4000R Digital Imaging System (Eastman Kodak, Rochester, NY) and quantified using Kodak molecular imaging software (version 4.0).

<table>
<thead>
<tr>
<th>Construct Size, kb</th>
<th>Primer(s) Abbreviation</th>
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<tbody>
<tr>
<td>−3.941 to +0.421</td>
<td>Forward: 5′-CTTCAAGCCTCTGATGGGGGCTCTAATTGCTGCT-3′</td>
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<tr>
<td>−3.539 to +0.421</td>
<td>Reverse: 5′-CAGCTTGAGTTCATTCCCTCCTCCTCCTCCTC-3′</td>
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<tr>
<td>−3.041 to +0.421</td>
<td>Forward: 5′-CTTCAAGCCTCTGATGGGGGCTCTAATTGCTGCT-3′</td>
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<tr>
<td>−2.521 to +0.421</td>
<td>Forward: 5′-GGCAAGCCTCTGATGGGGGCTCTAATTGCTGCT-3′</td>
</tr>
<tr>
<td>−2.088 to +0.421</td>
<td>Forward: 5′-CTTCAAGCCTCTGATGGGGGCTCTAATTGCTGCT-3′</td>
</tr>
<tr>
<td>−1.573 to +0.421</td>
<td>Forward: 5′-GAAGCCAGCTCTGATGGGGGCTCTAATTGCTGCT-3′</td>
</tr>
<tr>
<td>−1.074 to +0.421</td>
<td>Forward: 5′-CTTCAAGCCTCTGATGGGGGCTCTAATTGCTGCT-3′</td>
</tr>
<tr>
<td>−0.543 to +0.421</td>
<td>Forward: 5′-CTTCAAGCCTCTGATGGGGGCTCTAATTGCTGCT-3′</td>
</tr>
<tr>
<td>−0.400 to +0.421</td>
<td>Forward: 5′-GGCAAGCCTCTGATGGGGGCTCTAATTGCTGCT-3′</td>
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<tr>
<td>−0.303 to +0.421</td>
<td>Forward: 5′-GAAGCCAGCTCTGATGGGGGCTCTAATTGCTGCT-3′</td>
</tr>
<tr>
<td>−0.200 to +0.421</td>
<td>Forward: 5′-GGCAAGCCTCTGATGGGGGCTCTAATTGCTGCT-3′</td>
</tr>
<tr>
<td>−0.543 to +0.168</td>
<td>HindIII</td>
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Underlined sequences show the BglII restriction endonuclease recognition site. *HindIII was used to digest 253 bp from the 3′-end of the −0.5/+0.4 p27Kip1 promoter.
Since ectopic expression of p27Kip1 has been demonstrated and increased as an indicator of MPC differentiation, CK-M promoter activity was measured and increased 4.0/+/−0.4 p27Kip1 promoter construct and determined the effects of both FOXO1 and FOXO3a and their triple mutant counterparts (FOXO1 A3 and FOXO3a A3), which are not responsive to Akt-induced nuclear export/inhibition. Overexpression of FOXO1 and FOXO3a in rat MPCs resulted in −1.5- and −6-fold increases, respectively, in rat −4.0/+0.4 p27Kip1 promoter activity (Fig. 1). Moreover, the FOXO-induced p27Kip1 response was further increased for both FOXO1 A3 and FOXO3a A3, resulting in −2- and 14-fold increases in rat −4.0/+0.4 p27Kip1 promoter activity, respectively, compared with the empty vector. These data establish that 1) our −4.0/+0.4 rat p27Kip1 promoter construct is expressed in proliferating rat MPCs and is responsive to both FOXO1 and FOXO3a and 2) the triple mutants FOXO1 A3 and FOXO3a A3 enhance this response compared with the wild type.

p27Kip1 is a key cell cycle regulator that induces cell cycle arrest. The temporal regulation of p27Kip1 expression can dramatically affect the function of MPCs in skeletal muscle repair/regeneration. For example, during regeneration, MPCs must first proliferate and then differentiate. In the proliferation stage, increased p27Kip1 expression would impair MPC proliferation and subsequent repair/regeneration. However, for MPCs to effectively become new myonuclei, cell cycle arrest must occur to allow for terminal differentiation. Therefore, upregulation of p27Kip1 at the differentiation stage would induce cell cycle arrest, which might help MPCs differentiate. By changing MPCs to low-serum media conditions (DM), MPCs upregulate p27Kip1 expression, induce cell cycle arrest, and subsequently differentiate into myotubes. These data provide further proof that the rat p27Kip1 promoter is expressed in rat MPCs as demonstrated by −3 and −10-fold increases after exposure to DM for 24 and 48 h, respectively (Fig. 2A). As an indicator of MPC differentiation, CK-M promoter activity was measured and increased ∼4.5-fold between 24 and 48 h in DM (Fig. 2B). Since ectopic expression of p27Kip1 has been demonstrated to promote myoblast differentiation (40), the effect of FOXO3a overexpression during MPC differentiation was determined. As previously found (Fig. 1), FOXO3a increased p27Kip1 promoter activity in GM (0 h). After 24 h of exposure to DM, p27Kip1 expression increased in MPCs, and this effect was further increased when FOXO3a was overexpressed (Fig. 3A). CK-M promoter activity was not different between FOXO3a and the empty vector after 24 h of exposure to DM; however, there was a modest FOXO3a-induced increase in CK-M after 48 h of exposure to DM (Fig. 3B). Taken together, FOXO3a caused similar fold inductions in the −4.0/+0.4 p27Kip1 promoter construct in both GM and DM; therefore, we chose to focus experiments on the ability of FOXO3a-induced p27Kip1 expression in proliferating MPCs.

To identify potential FOXO regulatory elements in the rat −4.0/+0.4 p27Kip1 promoter, we carried out a deletion analysis by sequentially removing ∼500 bp from the 5′-end of the promoter (Fig. 4A). There was a modest decrease in FOXO3a-induced p27Kip1 promoter activity after the deletion of the 500 bp between −3.5 and −3.0 kb. Nonetheless, the ap-

**Fig. 1.** Forkhead box O (FOXO)-mediated p27Kip1 promoter activity in growth media. A: muscle precursor cells (MPCs) were transiently transfected with the full-length rat −4.0/+0.4 p27Kip1 promoter/reporter construct and cotransfected with either wild-type FOXO1, mutant FOXO1 lacking the three Akt inhibitory phosphorylation sites (A3), or the corresponding empty vector (EV). B: MPCs were cotransfected with either wild-type FOXO3a, FOXO3a A3, or the corresponding EV. Data are presented as group means ± SE; n = 4 for FOXO1 and 6 for FOXO3a. *Significantly different from EV; #significantly different from FOXO1 or FOXO3a.
proximately sixfold FOXO3a-induced increase in the p27kip1 promoter was maintained even after the promoter was shortened to -0.5/-0.4 kb. Since the smallest promoter fragment from this deletion analysis retained the majority of the FOXO3a-induced response observed in the full-length -4.0/+0.4 construct (>5-fold increase over the empty vector), the sequence of the -0.5/+0.4 construct was analyzed. Three interesting putative regulatory elements were identified in the 5’-UTR. We next pursued the putative regulatory elements identified in the 253 bp of the 5’-UTR of the p27kip1 gene.

Luciferase signal, likely due to the fact that this portion of the promoter contained the TATA box (data not shown). Since the -0.5/+0.4 p27kip1 promoter construct retained the vast majority of the FOXO3a-induced response observed in the full-length -4.0/+0.4 construct (>5-fold increase over the empty vector), the sequence of the -0.5/+0.4 construct was analyzed. Three interesting putative regulatory elements were identified in the 5’-UTR. We next pursued the putative regulatory elements identified in the 253 bp of the 5’-UTR of the p27kip1 gene.

Fig. 3. FOXO3a potentiates p27kip1 and CK-M promoter activity during MPC differentiation. MPCs were transiently transfected with either wild-type FOXO3a or EV and cotransfected with the rat full-length p27kip1 promoter/reporter construct (A) or the rat CK-M promoter/reporter construct (B). MPCs were allowed to differentiate for either 24 or 48 h before they were lysed. Proliferating MPCs had undetectable CK-M promoter activities. Data are presented as group means ± SE; n = 4. *Significantly different from 24 h; #significantly different from 0 h.
Two of the putative regulatory elements in the 5'-UTR of the p27Kip1 promoter demonstrated homology to a conserved NF-κB binding motif (Table 2). Although the existence of regulatory elements in the 5'-UTR is less common than upstream of the transcription start site, NF-κB has previously been identified to regulate gene transcription in the 5'-UTR region of Bcl10 (38). Four of the ten nucleotides (1, 2, 3, 4, and 10) in the NF-κB binding motif are highly conserved, with the most frequent sequence being 5'-GGGACTTTCC-3' (39). The first putative NF-κB regulatory element in the rat p27Kip1 promoter (p27Kip1-1, +188/+197 bp) is 5'-GGGACGTCCC-3' and the second (p27Kip1-2, +321/+330 bp) is 5'-GGGGCGTTTC-3' (Table 2). In a pilot study to determine whether to pursue the mutation of the two putative NF-κB sites, we used a NF-κB cis-reporter construct. As expected, cells treated with TNF-α caused an increase in NF-κB cis-reporter activity (Fig. 5). However, surprisingly, TNF-α did not increase p27Kip1 promoter activity that contained the aforementioned two NF-κB sequences. FOXO3a, without TNF-α, increased NF-κB cis-reporter activity. Interestingly, an additive effect of FOXO3a overexpression and TNF-α treatment on NF-κB cis-reporter activity was evident, indicating that FOXO3a-induced NF-κB activation may act in a parallel pathway to TNF-α. Even though FOXO3a did increase NF-κB cis-reporter activity, TNF-α, which induced NF-κB cis-reporter activity, did not enhance −0.5/+0.4 p27Kip1 promoter activity. Therefore, we concluded that FOXO3a does not act via NF-κB signaling to increase p27Kip1 expression, and we then selected the third regulatory region for a mutational experiment.

Table 2. NF-κB binding motifs

<table>
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<th>Sequence</th>
<th>Underlined sequences show common nucleotides with a highly conserved NF-κB binding motif (39)</th>
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<tr>
<td>Highly conserved NF-κB motif*</td>
<td>5'-GGGACGTCCC-3'</td>
</tr>
<tr>
<td>pNF-κB-luc</td>
<td>5'-GGGACGTCCC-3'</td>
</tr>
<tr>
<td>p27Kip1-1</td>
<td>5'-GGGACGTCCC-3'</td>
</tr>
<tr>
<td>p27Kip1-2</td>
<td>5'-GGGGCGTTTC-3'</td>
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Fig. 4. A and B: sequential 500-bp deletions from the 5'-end of the full-length p27Kip1 promoter (A) and 100-bp deletions from the 5'-end of the −0.5/+0.4-kb p27Kip1 promoter construct (B). MPCs were transiently cotransfected with either the full-length rat p27Kip1 promoter/reporter construct (−4.0/+0.4 kb) or one of the deletion constructs shown in the schematic representation and either wild-type FOXO3a or the corresponding EV. Data are represented as the fold increase in reporter activity induced by FOXO3a compared with EV. Data are presented as group means ± SE; n = 4. *Significantly different from the −4.0/+0.4-kb promoter (in A) or −0.5/+0.4-kb promoter (in B).
The third putative regulatory element in the 5′-UTR of the p27Kip1 gene is a DBE (DBE: +348/+355 bp, 5′-TTGT TTAT-3′). Similar to NF-κB regulation in the 5′-UTR of Bcl10, a DBE has been previously reported in the 5′-UTR of the mouse atrogin-1 gene (34). The DBE sequence has been shown to bind four members of the FOXO protein family, including FOXO1 and FOXO3a (17). To test whether potential regulatory sites within the 5′-UTR are responsive to FOXO3a, a 253-bp region of the 5′-UTR (that contained putative NF-κB sites and the DBE site) was deleted from the −0.5/+0.4-kb construct, resulting in a construct we designated as −0.5/+0.2. Since the second purpose of our study was to determine if age-related differences exist in FOXO-induced p27Kip1, MPCs isolated from old rats were also tested. Interestingly, MPCs isolated from old animals demonstrated a greater upregulation of p27Kip1 compared with MPCs isolated from young animals (Fig. 6). Moreover, the 5′-UTR 253-bp deletion resulted in ~25% and ~34% decreases in the FOXO3a-induced p27Kip1 response in MPCs isolated from young and old rats, respectively (Fig. 6).

Previous reports have demonstrated that site-directed mutagenesis of four nucleotides within the DBE core sequence prevented FOXO1 A3 binding (16). Therefore, we mutated the DBE in the −0.5/+0.4 rat p27Kip1 promoter construct (DBE-mut: +348/+355 bp, 5′-TCCCCTAT-3′), as with the sites previously mutated for pyruvate dehydrogenase kinase 4 (PDK 4) (16). Mutation of the DBE caused a decrease in the FOXO3a-induced p27Kip1 promoter activity (Fig. 6); importantly, the age differential was removed by the greater reduction in the MPCs isolated from 32-mo-old animals. It is important to note that DBE-mut resulted in a ~50% decrease in p27Kip1 promoter activity in MPCs isolated from old animals, which reduced promoter activity to the same level observed for DBE-mut in MPCs isolated from young animals (Fig. 6). These findings imply that FOXO3a caused an increased response in MPCs isolated from old animals compared with young animals, and most of the FOXO3a signaling in the 253-bp region deleted from the 5′-UTR in the −0.5/+0.2 rat p27Kip1 promoter construct acts via the DBE.

Since FOXO proteins have been demonstrated to be central to the biology of aging, metabolism, and cell fate (9), we next tested the separate effects of overexpression of FOXO1 and FOXO3a and their triple mutants in MPCs isolated from aged animals (32 mo old). We first examined FOXO1 (Fig. 7A). As demonstrated previously for the −4.0/+0.4 p27Kip1 construct, wild-type FOXO1 and mutant FOXO1 A3 overexpression in MPCs isolated from 3-mo-old animals resulted in ~1.5- and
FOXO3a-induced p27Kip1 promoter activity in MPCs isolated from both 3- and 32-mo-old sites directed mutagenesis of the DBE within the 5'-UTR decreased promoter activity (Fig. 7A). MPCs isolated from 32-mo-old animals exhibited similar ~1.5- and 2.5-fold increases in FOXO1 and FOXO1 A3-induced ~0.5/+0.4 p27Kip1 promoter activity, respectively, as 3-mo-old animals. DBE-mut did not decrease the FOXO1-induced effect for MPCs isolated from either 3- or 32-mo-old animals. However, DBE-mut did cause a decrease in the FOXO1 A3-induced response in MPCs isolated from both 3- and 32-mo-old animals. Next, FOXO3a was tested (Fig. 7B). When wild-type FOXO3a was examined, an aging effect was observed. FOXO3a caused ~50% greater p27Kip1 promoter activity in 32-mo-old MPCs compared with 3-mo-old MPCs (Fig. 7B). Interestingly, as mentioned above, DBE-mut abolished the age difference caused by FOXO3a. Furthermore, mutant FOXO3a A3 resulted in an ~21-fold increase in p27Kip1 promoter activity in MPCs isolated from 32-mo-old animals compared with the ~10-fold increase in 3-mo-old MPCs. Importantly, in MPCs isolated from 32-mo-old animals, DBE-mut reduced the FOXO3a-induced response by >50%, which remained slightly greater than DBE-mut in MPCs isolated from 3-mo-old animals.

DISCUSSION

Previous reports have demonstrated that FOXO proteins induce p27Kip1 expression (31); however, the mechanism of this induction was unknown. To our knowledge, the present study is the first to identify a conserved DBE FOXO binding motif in the 5'-UTR of the rat p27Kip1 gene. Moreover, we demonstrated that site-directed mutagenesis of the 5'-UTR DBE regulatory element reduced FOXO-induced p27Kip1 expression. As FOXO proteins are implicated to be at the nexus of aging and cell fate and function (9), another key finding of the present study is that MPCs isolated from aged animals exhibit a greater upregulation of p27Kip1 promoter activity in response to FOXO3a. Interestingly, mutation of the DBE site in the ~0.5 to +0.4 p27Kip1 promoter eliminated the age differential observed in MPCs isolated from young and old rats in FOXO3a-induced p27Kip1 expression.

The DBE was first identified to have high-affinity binding to FOXO1, FOXO3a, and FOXO4 (17). One copy of the DBE binding sequence has been previously identified upstream of the Sod-3 gene (17). MnSOD is the product of Sod-3 and has been shown to be responsive to FOXO (19). Later, PDK4 gene expression has been demonstrated that site-directed mutagenesis of the 5'-UTR decreased FOXO1 A3-induced p27Kip1 promoter activity in MPCs isolated from both 3- and 32-mo-old rats. MPCs from 3- and 32-mo-old rats were transiently cotransfected with either DBE-wt or DBE-mut and with one of three plasmids containing either wild-type FOXO1, FOXO1 A3, or the corresponding EV. The DBE was first identified to have high-affinity binding to FOXO1, FOXO3a, and FOXO4 (17). One copy of the DBE binding sequence has been identified upstream of the Sod-3 gene (17). MnSOD is the product of Sod-3 and has been shown to be responsive to FOXO (19). Later, PDK4 gene expression has been demonstrated to be regulated by direct binding of FOXO1 to a DBE in the PDK4 promoter (16). Mutation of the DBE from 5'-TTGTTCAT-3' to 5'-TCCCTTAC-3' (DBE-mut) caused an elimination of FOXO1-induced luciferase activity and binding of FOXO1 to DBE-mut oligonucleotides using gel mobility shift assay (EMSA) (16). Although transcription factor binding sites are not as common in the 5'-UTR as upstream of the transcription start site, a DBE site has been previously identified in the atrogin-1 5'-UTR (34). Constituently active FOXO3a has been previously shown to act on the atrogin-1 promoter and cause atrophy in muscle fibers. Sandri et al. (34) reported two DBE sites in their atrogin-1 promoter construct. One partially overlapped with the TATA box (~94), and the other was just past the transcription start site in the 5'-UTR (~2). Mutation of these sites decreased FOXO3a-induced atrogin-1 promoter activity and FOXO binding using EMSA (34).
In the present study, we identified a DBE in the 5′-UTR of the p27kip1 gene and demonstrated that site-directed mutagenesis of this DBE resulted in significant decreases in FOXO-dependent p27kip1 transcriptional activity. Although the DBE-mut construct still retained ~1.5- and ~3-fold increases in p27kip1 promoter activity for FOXO1 and FOXO3a, respectively, this is likely due to that fact that p27kip1 is a key cell cycle regulator, and there are likely many other coregulators that are involved in the upregulation of p27kip1. Although we did not perform EMSA to measure FOXO/DNA binding in our p27kip1 construct, we used a previously described mutation of the core sequence of the DBE that has been verified using EMSA to dramatically reduce FOXO binding (16). Interestingly, upon inspection of the upstream region of the human p27kip1 gene, a putative DBE was observed (supplemental Fig. 1S). Although it does not align in the same region as the DBE in the rat, this may give some insight into the conservation across species. Collectively, it seems that the FOXO-induced regulation of gene expression via the DBE suggests a conserved regulatory mechanism in a group of target genes. To our knowledge, we are the first to identify this relationship for FOXO and p27kip1.

We investigated the possibility that FOXO can act via NF-κB, as previously reported (20, 26). Although FOXO3a did cause a modest increase in the NF-κB cis-reporter construct (Fig. 5), TNF-α, a major regulator of canonical (classical) NF-κB activation, did not influence p27kip1 transcriptional activity. Moreover, while deletion of 253 bp in the 5′-UTR that contains both putative NF-κB sites and DBE decreased FOXO3a-induced p27kip1 promoter expression, it is important to note that the site-directed mutagenesis of the DBE in the 5′-UTR accounted for all of this response (Fig. 6). Therefore, we conclude that FOXO3a signaling in the 253-bp region deleted from the 5′-UTR in the −0.5/+0.2 rat p27kip1 promoter construct acts via the DBE and not via NF-κB signaling.

The FOXO subfamily of proteins is a major target of Akt signaling, which transmits environmental stimuli into upregulation of target gene expression (18). Insulin and IGF-I are two growth factors that have been widely demonstrated to link Akt/FOXO signaling to increased organellar lifespan (9, 18). In terms of MPC function, previous work from our laboratory has demonstrated that IGF-I directly administered to the atrophied gastrocnemius muscle from old rats facilitated an improved regrowth of muscle mass and increased MPC proliferation following reembalulation (11). We next found that IGF-I increased phosphorylation of Akt at Ser256 in MPCs, inactivating FOXO1, and thereby downregulating p27kip1 promoter activity (28). Moreover, MPCs isolated from old rats exhibited increased cytoplasmic MnSOD and nuclear FOXO1 and p27kip1 protein compared with MPCs isolated from young rats (27). To our knowledge, it is not currently known whether aged MPCs have elevated endogenous nuclear FOXO3a protein levels; however, MPCs isolated from regenerating aged skeletal muscle have increased p27kip1 protein levels compared with MPCs isolated from young regenerating muscle (10). Findings from our present study revealed that FOXO3a overexpression causes a higher upregulation of p27kip1 transcriptional activation in MPCs isolated from aged rats compared with young rats. However, when wild-type DBE and DBE-mut promoter activities were compared, without exogenous overexpression of FOXO3a, there was no effect of the mutation, and no age effect was observed (supplemental Fig. 3S). These data indicate that in cell culture GM conditions, FOXO signaling does not significantly contribute to p27kip1 expression in MPCs of either age. These data contribute to our understanding of the impaired MPC activation and proliferation that occurs in aged skeletal muscle during regeneration (13).

Here, we report that overexpression of exogenous FOXO3a resulted in a 50% greater increase in p27kip1 promoter activity in MPCs isolated from old animals compared with young animals (Fig. 7). This could be partly explained by an age-associated decrease in Akt signaling in MPCs, which is indicated by the decreased basal Akt phosphorylation in MPCs isolated from old animals compared with young animals (supplemental Fig. 2S). However, the role of Akt in aged skeletal muscle is not certain. Kimball et al. (21) found no significant change in basal Akt activity between 12 and 27 mo of age in the rat gastrocnemius muscle, whereas Edstroem et al. (15) observed that basal Akt phosphorylation was higher in the gastrocnemius muscle from 30-mo-old rats compared with 12-mo-old rats. To examine the effect of FOXO3a on p27kip1 independent of Akt, we overexpressed the triple mutants FOXO1 A3 and FOXO3a A3, in which the three Akt serine phosphorylation sites have been mutated to alanines so that Akt could not phosphorylate and exclude FOXO1 and FOXO3a from the nucleus. MPCs isolated from young animals exhibited a 10-fold induction of p27kip1 promoter activity in the presence of the triple mutant FOXO3a, whereas MPCs from old rats displayed a 21-fold induction. Since the FOXO3a A3 mutant is not responsive to the inhibitory effects of Akt signaling, an alternate signaling pathway(s) may be responsible for the age effect. For example, FOXO activities have been shown to be regulated via phosphorylation by several different kinases, acetylation, and forming interactions with coactivators (37). Therefore, it is possible that even though FOXO3a A3 would be sequestered in the nucleus in MPCs isolated from both young and old animals, other regulatory mechanisms do exist that may account for the age-associated increase in FOXO3a-induced p27kip1 promoter activity.

In summary, we demonstrated that FOXO1- and FOXO3a-induced p27kip1 transcriptional activity is regulated through a promoter construct that contained 400 bp of the 5′-UTR. We identified a positive DBE regulatory element in the 5′-UTR of the rat p27kip1 gene that is responsive to both FOXO1 and FOXO3a. Moreover, MPCs isolated from aged animals exhibited a greater FOXO3a-induced p27kip1 promoter activity compared with MPCs isolated from young animals. Interestingly, site-directed mutagenesis of the DBE site in the 5′-UTR eliminated the majority of the age-related difference in FOXO3a activation of the rat p27kip1 gene. Together, these findings suggest that aging is associated with increased signaling through a FOXO/p27kip1 pathway, at least in part, via a DBE found in the 5′-UTR of the p27kip1 gene.

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