Exercise intolerance is a common clinical manifestation associated with chronic heart failure (CHF). Various morphological, histological, and biochemical changes are observed in the skeletal muscle of patients with CHF. These changes are collectively called skeletal myopathy and are the major cause of exercise intolerance. Fibers of the skeletal muscle are classified into 4 types according to their biochemical characters; slow-twitch type I fatigue-resistant fiber, fast-twitch type IIa fatigue-resistant fiber, fast-twitch type IIX fiber with intermediate fatigue-resistance, and fast-twitch type IIb fatigable fiber. Fiber type shift toward more fatigable type IIb fiber is one of the characteristics observed during the development of skeletal myopathy in patients with CHF.1,5

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Forkhead box O (FoxO) transcription factors play important roles in the regulation of various cellular processes.
such as cell proliferation, stress tolerance, and metabolism.6 FoxO factors have also been reported to regulate skeletal muscle wasting and fiber type specification.7,8 FoxO overexpression in skeletal muscle promotes muscle atrophy and fiber type shift from type I to type II fiber.9 In contrast, muscle wasting and fiber type specification.7,8 FoxO overexpression of either peroxisome proliferator–activated receptor γ coactivator 1α or insulin-like growth factor 1 in skeletal muscle ameliorates skeletal myopathy in heart failure through suppression of FoxO activity.10,11 These studies suggest that FoxO signaling is critically involved in skeletal myopathy associated with CHF, however, it remains elusive how FoxO signaling is activated in patients with CHF.

Wnt signaling is an evolutionarily conserved signaling cascade that plays essential roles in embryonic development through regulating various cellular processes, including cell differentiation and proliferation.12,13 Recent studies have reported that Wnt signaling also plays an important role during adulthood, including stem cell regulation, skeletal muscle regeneration, and cancer progression.14,15 FoxO and β-catenin are key proteins that mediate Wnt signal transduction. In the absence of activation of Wnt receptors, cytosolic β-catenin is usually degraded via proteasome system. However, when Wnt receptors are activated, cytosolic β-catenin becomes stabilized and translocates into the nucleus where it usually binds with T-cell factor (TCF) or lymphoid enhancer factor (LEF) transcription factors and induces Wnt/β-catenin/TCF/LEF target gene transcription.13 Essers et al16 reported that β-catenin also binds to FoxO transcription factors and induces the expression of FoxO target genes. Interaction between Wnt signaling and FoxO signaling is also observed in vivo and plays an important role in the pathophysiology of diabetes mellitus, cancer metastasis, and bone formation.17–19

We have recently reported that serum from CHF model mice activates Wnt signaling more potently than serum from control mice and that complement C1q mediates this activation.20 Here, we demonstrate that activation of Wnt signaling plays a causative role in fiber type shift in skeletal myopathy associated with CHF through functional interaction between FoxO and β-catenin.

Methods

An expanded Methods section is available in the Data Supplement.

Animal Model

All experiments were approved by the Institutional Animal Care and Use Committee of Osaka University. We used knock-in mice with deletion of K210 in cardiac troponin T as a model of CHF.21 These DCM mice exhibited heart failure with marked left ventricular dysfunction and increased heart weight and lung weight at the age of 2 months (Figure 1A and 1B). Weight of the skeletal muscle was not decreased in these mice (Figure 1C). However, the expression of Myh7 and Myh2, which encode the myosin heavy chain gene for type I and type IIa fatigue-resistant fiber, was markedly decreased, whereas the expression of Myh4, which encodes the myosin heavy chain gene for type Iib fatigable fiber, was increased in the quadriceps muscle, a typical muscle, which is mainly consisted with type II fiber (Figure 1D). Decreased expression of Myh7 and increased expression of Myh4 was also observed in the soleus muscle, a typical muscle, which is mainly consisted with type I fiber (Figure 1D). Immunostaining revealed that loss of type Ila fiber in the quadriceps muscle and appearance of type Iib fiber in the soleus muscle were observed in DCM mice (Figure 1E). These results collectively suggest that the early stage skeletal myopathy characterized by fiber type shift toward fatigable fiber is observed in DCM mice.

FoxO Signaling and Wnt Signaling are Both Activated in Skeletal Muscle of DCM Mice

Activation of FoxO signaling is known to play a causative role in skeletal muscle atrophy. We found that the expression of FoxO target genes and the nuclear amount of FoxO1 were increased in the skeletal muscle of DCM mice (Figure 2A and 2B). The purity of the nuclear fraction in the skeletal muscle was confirmed by Western blot analysis (Figure 1A and 1B). Immunofluorescence staining revealed that FoxO1 was localized in the nuclei of skeletal muscle cells.22 However, the phosphorylation levels of FoxO1 in skeletal muscle were comparable between DCM mice and wild-type mice (Figure 2B), and similar changes in localization and phosphorylation were also observed in FoxO3a (Figure 1B in the Data Supplement). These results suggest the presence of phosphorylation–independent mechanisms regulating FoxO activity in the skeletal muscle of DCM mice.

Wnt signaling has also been implicated in the development of muscle atrophy.22 We found that the expression of a Wnt/β-catenin/TCF/LEF target gene Axin2 and the nuclear amount of β-catenin were increased in the skeletal muscle of DCM mice (Figure 2C and 2D), suggesting that Wnt signaling as well as FoxO signaling is activated in the skeletal muscle of DCM mice.

Statistical Analysis

All values are reported as mean±SD. Statistical analyses were performed with JMP Pro 10.0.2 (SAS Institute Inc). We analyzed the data using Wilcoxon rank sum test to compare 2 groups. For comparing ≥3 groups, we used the Kruskal–Wallis procedure followed by Steel–Dwass multiple comparisons test. Significant differences were defined as P<0.05.

Results

Skeletal Muscle Fiber Type Shift in Heart Failure Model Mice

We used DCM model mice with knock-in mutation containing deletion of K210 in cardiac troponin T as a model of CHF.21 These DCM mice exhibited heart failure with marked left ventricular dysfunction and increased heart weight and lung weight at the age of 2 months (Figure 1A and 1B). Weight of the skeletal muscle was not decreased in these mice (Figure 1C). However, the expression of Myh7 and Myh2, which encode the myosin heavy chain gene for type I and type IIa fatigue-resistant fiber, was markedly decreased, whereas the expression of Myh4, which encodes the myosin heavy chain gene for type Iib fatigable fiber, was increased in the quadriceps muscle, a typical muscle, which is mainly consisted with type II fiber (Figure 1D). Decreased expression of Myh7 and increased expression of Myh4 was also observed in the soleus muscle, a typical muscle, which is mainly consisted with type I fiber (Figure 1D). Immunostaining revealed that loss of type Ila fiber in the quadriceps muscle and appearance of type Iib fiber in the soleus muscle were observed in DCM mice (Figure 1E). These results collectively suggest that the early stage skeletal myopathy characterized by fiber type shift toward fatigable fiber is observed in DCM mice.
Functional Interaction Between β-Catenin and FoxO in Cultured Skeletal Myocytes

A previous report suggests that the direct interaction of β-catenin and FoxO prompts FoxO activation downstream of Wnt signaling. Among FoxO subfamily members, FoxO1 plays a major role in the regulation of skeletal muscle fiber type. We, therefore, tested whether activation of Wnt signaling promotes the binding between β-catenin and FoxO1 and cross-activates FoxO-dependent gene transcription in skeletal muscle. Wnt3a treatment promoted the binding of FoxO1 and β-catenin and increased the level of nuclear localized FoxO1 in C2C12 cells overexpressing wild-type FoxO1 (Figure 3A and 3B; Figure IIA in the Data Supplement). Expression of fiber type–specific myosin heavy chain genes in the quadriceps and soleus muscles from WT and DCM mice was analyzed by quantitative reverse transcription polymerase chain reaction (quadriceps, n=8 each and soleus, n=6 each). Expression of FoxO target genes as well as a Wnt/β-catenin/TCF/LEF target gene Axin2 was increased by Wnt3a treatment in C2C12 cells overexpressing wild-type FoxO1 and these changes were blocked by Dkk1. In contrast, Wnt3a-induced FoxO1/β-catenin interaction and nuclear accumulation of FoxO1 were blocked by Dkk1, an endogenous inhibitor of Wnt signaling (Figure 3A and 3B; Figure IIA in the Data Supplement). Expression of FoxO target genes as well as a Wnt/β-catenin/TCF/LEF target gene Axin2 was increased by Wnt3a treatment in C2C12 cells overexpressing wild-type FoxO1 and these changes were blocked by Dkk1 (Figure 3C, left), whereas the expression of Axin2 but not FoxO target genes was increased by Wnt3a treatment in C2C12 cells.
Figure 2. Activation of Wnt signaling and forkhead box O (FoxO) signaling in the skeletal muscle of dilated cardiomyopathy (DCM) mice. A, Expression of FoxO target genes in the quadriceps and soleus muscles of wild-type (WT) and DCM mice was analyzed by quantitative reverse transcription polymerase chain reaction (RT-PCR; quadriceps, n=8 each and soleus, n=6 each). *P<0.05 vs WT mice. B, Nuclear protein and total protein were extracted from the quadriceps muscles of WT and DCM mice and the expression of FoxO1 and phosphorylated FoxO1 was analyzed by Western blotting and quantified by densitometry analysis. Histone H3 and GAPDH were used as internal controls for nuclear protein and total protein, respectively (FoxO1/Histone H3, n=6 each and p-FoxO1/FoxO1, n=5 each). *P<0.05 vs WT mice. C, Expression of a Wnt/β-catenin/T-cell factor/lymphoid enhancer factor target gene Axin2 in the quadriceps and soleus muscles of WT and DCM mice was analyzed by quantitative RT-PCR (quadriceps, n=8 each and soleus, n=6 each). *P<0.05 vs WT mice. D, Nuclear protein and total protein were extracted from the quadriceps muscles of WT and DCM mice and the expression of β-catenin was analyzed by Western blotting and quantified by densitometry analysis. Histone H3 and GAPDH were used as internal controls for nuclear protein and total protein, respectively (β-catenin/Histone H3, n=6 each and β-catenin/ GAPDH, n=5 each). *P<0.05 vs WT mice.

overexpressing dominant negative FoxO1 (Figure 3C, right). Dominant negative FoxO1 inhibits the transcriptional activity of FoxO1, FoxO3a, and FoxO4.24 Therefore, we examined the influence of FoxO1 knockdown on C2C12 cells. Knockdown of FoxO1 in C2C12 cells (Figure IIB and IIC in the Data Supplement) also prevented Wnt3a-induced activation of FoxO signaling without affecting Wnt3a-induced activation of Wnt signaling (Figure IID in the Data Supplement). We also found that Wnt3a treatment increased the nuclear level of FoxO1 and the expression of FoxO target genes without affecting its phosphorylation level in C2C12 cells (Figure 3D and 3E). The purity of the nuclear fraction in C2C12 cells was confirmed by Western blot analysis (Figure IIE in the Data Supplement). These results suggest the novel mechanism for regulation of FoxO activity in skeletal muscle; activation of Wnt signaling promotes the binding of β-catenin and FoxO1, leading to nuclear localization of FoxO1, and activates FoxO signaling in skeletal muscle.

**Wnt Signaling Promotes Fiber Type Shift Through Transactivation of FoxO Signaling**

We next assessed whether Wnt signaling and FoxO signaling affect the fiber type of skeletal muscle using C2C12 cells. Activation of Wnt signaling by Wnt3a increased the expression of Myh1 and Myh4 and decreased the expression of Myh7, suggesting that Wnt signaling affects the fiber type of skeletal muscle at the cellular level (Figure 4A). We next tested how the interaction between Wnt signaling and FoxO signaling acts on the fiber type shift of skeletal muscle. We established inducible C2C12 cell lines that harbor cumate-inducible wild-type FoxO1, dominant negative FoxO1, or constitutively active FoxO1 and differentiated them into myotubes with or without Wnt3a. We found that Wnt3a treatment increased the expression of Myh1 and Myh4 and decreased the expression of Myh7 in myotubes overexpressing wild-type FoxO1, which was reversed by addition of Dkk1 (Figure 4B, left). However, Wnt3a treatment decreased the expression of Myh4 and increased the expression of Myh2 in myotubes overexpressing dominant negative FoxO1 (Figure 4B, right). Wnt3a treatment also decreased the expression of Myh1 and Myh4 and increased the expression of Myh7 and Myh2 in myotubes with FoxO1 knockdown (Figure IIIA in the Data Supplement). In addition, induction of constitutively active FoxO1 dramatically increased the expression of Myh1 and Myh4 and decreased the expression of Myh7 in myotubes (Figure 4C). Knockdown of FoxO1 increased the expression of Myh7 and decreased the expression of Myh4 in myotubes (Figure 4D). Protein level of MYH4 was also increased by overexpression of constitutively active FoxO1 and decreased by knockdown of FoxO1 (Figure IIIB and IIID in the Data Supplement). FoxO1 has been reported to promote fiber type shift toward type II fatigable fiber by suppressing the expression of type I fiber–related genes, including...
Overexpression of constitutively active FoxO1 suppressed the expression of Mef2c, whereas knockdown of FoxO1 enhanced the expression of Mef2c (Figure III C and III E in the Data Supplement). These results suggest that Wnt signaling affects the fiber type shift from fatigue-resistant fiber to fatigable fiber by coactivation of FoxO signaling.

Increased Serum Wnt Activity in DCM Mice Induces Fiber Type Shift Toward Fatigable Fiber

We have recently reported that serum from heart failure model mice activates Wnt signaling more potently than the serum from wild-type mice and that complement C1q is responsible for its activity. As expected, serum from
DCM mice increased the level of nuclear β-catenin and the expression of *Axin2* in C2C12 cells compared with the serum from wild-type mice (Figure 5A and 5B). The serum from DCM mice also increased the level of nuclear FoxO1 and the expression of FoxO target genes and promoted the fiber type shift toward fatigable fiber (Figure 5A and 5B). All these changes were blocked by treatment with Dkk1 (Figure 5A and 5B). Phosphorylation of FoxO1 and Akt was not affected by the serum from DCM mice (Figure 5A). We also found that intramuscular injection of Dkk1 blocked the activation of FoxO signaling and the fiber type shift toward fatigable fiber in the skeletal muscle of DCM mice (Figure 5C). These results suggest that activation of Wnt signaling by substances in the serum plays a causative role in the activation of FoxO signaling and fiber type shift in the skeletal muscle of DCM mice.

Complement C1q Mediates Serum-Induced Activation of Wnt Signaling in DCM Mice

Given that complement C1q is at least one of the substances in the serum that activate Wnt signaling, we tested whether C1q is responsible for fiber type shift in DCM mice. ELISA analysis revealed that serum C1q concentration was increased in the serum from DCM mice (Figure 6A). Stimulation with C1q increased the expression of *Myh1* and *Myh4* as well as FoxO target genes in myotubes (Figure 6B), whereas C1q-inhibitor, an endogenous inhibitor of C1r and C1s, blocked the effect of the serum from DCM mice (Figure 6C). We also found that intravenous injection of C1-inhibitor blocked the activation of both Wnt signaling and FoxO signaling and prevented the fiber type shift toward fatigable fiber in the skeletal muscle of DCM mice (Figure 6D). These results suggest that increased C1q in the serum activates FoxO signaling and plays a causative role in fiber type shift toward fatigable fiber in DCM mice.

Discussion

In this study, we found that activation of Wnt signaling and functional interaction between β-catenin and FoxO play a critical role in the development of skeletal myopathy associated with CHF. We showed that activation of Wnt signaling in skeletal muscle induces nuclear translocation of FoxO transcription factors and promotes fiber type shift from fatigue-resistant fiber to fatigable fiber. We also elucidated that C1q increased in the serum of heart failure model mice might play a causative role in the fiber type shift toward fatigable fiber by activating both Wnt and FoxO signaling. Our findings suggest the novel molecular mechanisms underlying the regulation of fiber type shift during CHF. Because fiber type shift of the skeletal muscle is one of the most important qualitative changes during
progression of skeletal myopathy in patients with CHF, our findings may lead to the development of new therapeutic strategies targeting skeletal myopathy that would eventually improve the daily activities and quality of life in patients with CHF.

In patients with CHF, skeletal myopathy is frequently observed and recognized as a major cause of exercise intolerance.\(^5\)\(^,\)\(^25\) Muscle wasting is one of the major abnormalities in skeletal myopathy associated with CHF.\(^1\) However, we did not observe skeletal muscle wasting in this study, in part because of the differences in the animal models.\(^10\)\(^,\)\(^26\) The fiber type shift toward fatigable fiber is another major abnormality in CHF-associated skeletal myopathy and accounts for the exercise intolerance in patients with CHF.\(^2\)\(^,\)\(^5\) Our observations suggest that CHF-associated fiber type shift of the skeletal muscle might occur independently of or earlier than muscle wasting.

FoxO transcription factors are highly conserved in evolution and play important roles in various cellular processes, including skeletal muscle homeostasis and fiber type specification.\(^6\)\(^,\)\(^27\) Recent studies have revealed its contribution during skeletal myopathy associated with CHF.\(^10\)\(^,\)\(^31\) FoxO activity is mainly controlled by its subcellular localization. Phosphorylation of FoxO is the most widely known mechanism that controls the localization of FoxO protein.\(^6\) However, recent studies revealed that FoxO activity is also regulated by other mechanisms, including its interaction with β-catenin.\(^5\)\(^,\)\(^16\) Wnt signaling is also an evolutionarily conserved signaling cascade, which plays essential roles during embryonic development.\(^13\) Recent studies have indicated that Wnt signaling also plays an important role in skeletal muscle atrophy and regeneration.\(^14\)\(^,\)\(^22\) In this study, we have demonstrated that both Wnt signaling and FoxO signaling are activated in skeletal muscle of DCM mice. We have also shown that Wnt signaling promotes FoxO1 nuclear translocation without affecting its phosphorylation level and increases the expression of FoxO target genes in cultured skeletal muscle cells. A recent report showed that interaction between Wnt signaling and FoxO signaling in hepatocytes plays an important role in regulating hepatic metabolism.\(^17\) In this study, we have identified another role of Wnt-FoxO interaction in regulating the homeostasis of skeletal muscle fiber.

We have recently reported that serum from heart failure model mice activates Wnt signaling and forkhead box O (FoxO) signaling and induces fiber type shift in vitro and in vivo. A, C2C12 myotubes were treated with serum from wild-type mice (10% final concentration), serum from DCM mice (10% final concentration), or serum from DCM mice plus Dkk1 (200 ng/mL) from day 6 to day 8 of differentiation. Nuclear protein and total protein were extracted from C2C12 cells and the expression of β-catenin, FoxO1, phosphorylated FoxO1, Akt, and phosphorylated Akt was analyzed by Western blotting and quantified by densitometry analysis. Histone H3 and GAPDH were used as internal controls for nuclear protein and total protein, respectively (n=5 each). *\(P<0.05\) between groups. B, C2C12 cells were treated as indicated in (A). Expression of fiber type–specific myosin heavy chain genes, a Wnt/β-catenin/T-cell factor (TCF)/lymphoid enhancer factor (LEF) target gene Axin2 and FoxO target genes was analyzed by quantitative reverse transcription polymerase chain reaction (RT-PCR; n=8 each). *\(P<0.05\) between groups. C, Dkk1 (500 ng in 25 μL solution) or equal amount of control solution (0.1% BSA/PBS) was injected into tibialis anterior muscles. Tibialis anterior muscles were harvested 2 days after injection. Expression of fiber type–specific myosin heavy chain genes, a Wnt/β-catenin/TCF/LEF target gene Axin2 and FoxO target genes in tibialis anterior muscles was analyzed by quantitative RT-PCR (n=8 each). *\(P<0.05\) between groups.
FoxO signaling through activation of Wnt signaling in skeletal muscle cells. C1q is known to be produced from the cells of a monocyte/macrophage lineage and upregulation of C1q biosynthesis leads to an increase in serum C1q concentration in aged mice.20,28 Although we do not know the mechanism by which C1q level is increased in the serum of DCM mice, we speculate that it reflects the chronic inflammation observed in patients with CHF or in animal models of CHF.29

Overexpression of constitutively active β-catenin has been reported to promote the fiber type shift toward type I fatigue-resistant fiber.30 Then why the activation of Wnt signaling changed the fiber type from type I to type II in the skeletal muscle of DCM mice? It has been proposed that TCF/LEF and FoxO transcription factors compete for the limited pool of active β-catenin,31 and that β-catenin is diverted from TCF/LEF to FoxO under certain conditions, such as nutritional starvation and oxidative stress.16,17,31 Oxidative stress promotes the use of active β-catenin by FoxO and increases the expression of FoxO target genes, whereas it suppresses the use of active β-catenin by TCF/LEF and decreases expression of Wnt/β-catenin/TCF/LEF target genes.31 It has been reported that oxidative stress plays an important role in the pathogenesis of CHF and that oxidative stress is increased in the skeletal muscle as well as in the heart during CHF.32,33 Activation of Wnt signaling in the skeletal muscle of DCM mice may be somewhat modified by increased oxidative stress; oxidative stress may promote use of β-catenin by FoxO that promotes the expression of type II fatigable fiber at the expense of TCF/LEF-mediated classical Wnt signaling that promotes the expression of type I fatigue-resistant fiber (Figure 7).9,30

**Figure 6.** Serum C1q activates Wnt signaling and forkhead box O (FoxO) signaling and induces fiber type shift in vitro and in vivo. A, Concentration of C1q in the serum of wild-type (WT) and dilated cardiomyopathy (DCM) mice was assessed by ELISA (n=12 each). *P<0.05 vs WT mice. B, C2C12 cells were stimulated with C1q (100 μg/mL) from day 6 to day 8 of differentiation. Expression of fiber type–specific myosin heavy chain genes, a Wnt/β-catenin/ T-cell factor (TCF)/lymphoid enhancer factor (LEF) target gene Axin2 and FoxO target genes in C2C12 cells was analyzed by quantitative reverse transcription polymerase chain reaction (RT-PCR; n=8 each). †P<0.05 vs control. C, C2C12 cells were treated with serum from WT mice (10% final concentration), serum from DCM mice (10% final concentration), or serum from DCM mice plus C1-inhibitor (C1-INH, 100 μg/mL) from day 6 to day 8 of differentiation. Expression of fiber type–specific myosin heavy chain genes, a Wnt/β-catenin/TCF/LEF target gene Axin2 and FoxO target genes in C2C12 cells was analyzed by quantitative RT-PCR (n=8 each). ‡P<0.05 between groups. D, C1-INH (15U in 150 μL solution) or equal amount of PBS (as a control) was administered intravenously. Tibialis anterior muscles were harvested 2 days after administration. Expression of fiber type–specific myosin heavy chain genes, a Wnt/β-catenin/TCF/LEF target gene Axin2, and FoxO target genes in tibialis anterior muscles was analyzed by quantitative RT-PCR (WT, n=6; DCM, n=4; and DCM+C1-INH; n=6). ‡‡P<0.05 between groups.
Figure 7. Hypothetical model of Wnt–forkhead box O (FoxO) interaction in skeletal muscle during chronic heart failure. Serum C1q activates Wnt signaling and induces β-catenin translocation into the nucleus with FoxO. Activated FoxO1 promotes fiber type shift toward type IIb fatigable fiber. LEF indicates lymphoid enhancer factor; and TCF, T-cell factor.

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Disclosures

None.

References


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**CLINICAL PERSPECTIVE**

Skeletal myopathy, characterized by muscle wasting and fiber type shift toward type II fatigable fiber, is frequently observed and recognized as a major cause of exercise intolerance in patients with chronic heart failure. Better understanding of mechanisms underlying skeletal myopathy in heart failure and specific therapies targeting this myopathy might contribute to the improvement of exercise intolerance. It has been reported that forkhead box O signaling plays a critical role in the development of skeletal myopathy in chronic heart failure and that there is the functional interaction between forkhead box O and the Wnt signal mediator β-catenin. In this study, we have shown that the complement C1q increased in the serum of heart failure mice activates Wnt signaling, leading to the activation of forkhead box O signaling and fiber type shift toward fatigable fiber. These results propose the novel molecular mechanism underlying the regulation of fiber type shift in skeletal myopathy associated with chronic heart failure. Our findings may lead to the development of new therapeutic strategies targeting skeletal myopathy that would eventually improve the daily activities and quality of life in patients with chronic heart failure.
Wnt/β-Catenin Signaling Contributes to Skeletal Myopathy in Heart Failure via Direct Interaction With Forkhead Box O

Katsuki Okada, Atsuhiko T. Naito, Tomoaki Higo, Akiti Nakagawa, Masato Shibamoto, Taku Sakai, Akihito Hashimoto, Yuki Kuramoto, Tomokazu Sumida, Seitaro Nomura, Masamichi Ito, Toshihiro Yamaguchi, Toru Oka, Hiroshi Akazawa, Jong-Kook Lee, Sachio Morimoto, Yasushi Sakata, Ichiro Shiojima and Isssei Komuro

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SUPPLEMENTAL MATERIAL

Supplemental Methods

Reagents
Cumate solution was from System Biosciences. Human complement C1q and complement C1-esterase inhibitor (C1-INH) were from Calbiochem. C1-INH (Berinert) for in vivo administration was from CSL Behring. Mouse recombinant Wnt3a and human recombinant Dkk-1 were from R&D Systems. The antibodies were from Cell Signaling Technology (FoxO1, Phospho-FoxO1 (Ser256), FoxO3a, Phospho-FoxO3a (Ser253), Akt, Phospho-Akt (Ser473)), Developmental Studies Hybridoma Bank (BA-F8, BF-F3, SC-71), BD Transduction Laboratories (β-catenin), Abcam (GAPDH), Sigma (FLAG-M2, Histone H3) and Proteintech (MYH4). Pre-designed siRNAs were from Ambion Silencer-Select (Negative Control #1, Foxo1 (siRNA ID: s80621)).

Plasmids
For transient expression, pcDNA3 vector with FLAG-tagged mouse FoxO1 wild-type (FLAG-WT-FoxO1), its dominant negative form (Δ256 mutant, FLAG-DN-FoxO1) or its constitutively active form (T24A/S253A/S316A (3A) mutant, FLAG-CA-FoxO1) was used (FLAG-WT-FoxO1 and FLAG-CA-FoxO1; generous gift from Dr. Fukamizu,1 FLAG-DN-FoxO1; constructed from FLAG-WT-FoxO1 with PCR-based subcloning). For inducible expression, FLAG-WT-FoxO1, FLAG-DN-FoxO1 or FLAG-CA-FoxO1 was cloned into the lentiviral vector plasmid with cumate inducible expression system (System Biosciences).

Immunostaining
Frozen sections of skeletal muscles were stained with antibodies against fiber type specific myosin heavy chain (MYH) (anti-MYH2 (SC-71), anti-MYH4 (BF-F3) and anti-MYH7 (BA-F8), Developmental Studies Hybridoma Bank). Fiber type was determined as described in the literature.2 Cultured C2C12 cells were immunostained with FLAG-M2 antibody (Sigma) and counterstained with
Phalloidin and TO-PRO-3 (Molecular Probes). Images were taken using an inverted microscope (FSX 100, Olympus or LSM 700, Carl Zeiss).

**Inducible C2C12 cell lines**

Lentiviral particles were produced according to the manufacturer’s instruction (System Biosciences) and concentrated with PEG-it Virus Precipitation Solution (System Biosciences). Titration of the viral particle was performed with Lenti-X qRT-PCR Titration Kit (Clontech) according to the manufacturer's instruction. C2C12 myoblasts were transduced at a multiplicity of infection of 50. C2C12 clones that stably harbor cumate-inducible system were selected with 4 µg/mL puromycin for 10 days.

**Protein analysis**

Total cell lysate was collected in radio immunoprecipitation assay (RIPA) buffer with protease and phosphatase inhibitor cocktails. To prepare nuclear fraction, nuclear pellet was extracted in the hypotonic buffer (10 mM HEPES, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, 0.05% NP-40, pH7.9) and lysed in RIPA buffer. Densitometry analysis on the bands was calculated using ImageQuant TL (GE Healthcare).

**Immunoprecipitation**

C2C12 cells were lysed in the lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 0.5% Triton-X100, pH8.0) with protease and phosphatase inhibitor cocktails. Protein lysate was immunoprecipitated with FLAG-M2 antibody (Sigma) or its isotype control (Cell Signaling Technology) using Immunoprecipitation Kit Dynabeads Protein G (Invitrogen) according to the manufacturer’s instruction.

**Quantitative real-time PCR**

RNA of the skeletal muscle and C2C12 cells was extracted using TRIzol (Invitrogen) or PureLink RNA Kit (Mini Kit or Micro Kit, Ambion). After elimination of genomic DNA using TURBO
DNA-free kit (Ambion), reverse transcription was performed using SuperScript VILO cDNA synthesis kit (Invitrogen). Quantitative real-time PCR was performed using Universal Probe Library (UPL) system and LightCycler 480 (Roche). Relative levels of target gene expression were calculated with the comparative Ct method. Primer sequences and their corresponding UPL numbers were designed with online program provided by Roche.

**ELISA**

Serum C1q concentrations were measured by using C1q Mouse ELISA kit (Hycult Biotech) according to the manufacturer’s instruction.

**Statistical analysis**

Category distributions were compared using Chi-square test. For comparing three groups with Chi-square test, Bonferroni’s adjustment was applied. Significant differences were defined as $P<0.05$ or $P<0.017$ (Bonferroni’s adjustment).
Supplemental Figure 1.

A

<table>
<thead>
<tr>
<th>Quadriceps</th>
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B

![Image of Western Blot and Bar Graph]

- **Western Blot:**
  - WT and DCM conditions
  - Proteins: GAPDH, Histone H3, FoxO3a, p-FoxO3a

- **Bar Graph:**
  - Relative expression
  - FoxO3a and p-FoxO3a
  - Nuclear fraction and Whole tissue lysate
  - WT vs DCM
Supplemental Figure 2.

A. FLAG-tagged FoxO1 localization

B. C2C12 myotube

C. C2C12 myotube

D. siFoxo1 C2C12 myotube

E. C2C12 myotube
Supplemental Figure 3.

A

B

C

D

E
Supplemental Figure Legends

Supplemental Figure 1.

(A) Nuclear protein and cytoplasmic protein were extracted from the quadriceps muscles and the expression of GAPDH (as a cytoplasmic marker) and Histone H3 (as a nuclear marker) was analyzed by western blotting. (B) Nuclear protein and total protein were extracted from the quadriceps muscles of WT and DCM mice and the expression of FoxO3a and phosphorylated FoxO3a was analyzed by western blotting and quantified by densitometry analysis. Histone H3 and GAPDH were used as internal controls for nuclear protein and total protein, respectively (FoxO3a/Histone H3; n=4 each, p-FoxO3a/FoxO3a; n=6 each). *P<0.05 vs. control.

Supplemental Figure 2.

(A) The distribution of FoxO1 subcellular localization in immunostaining of C2C12 myoblasts transiently expressing FLAG-WT-FoxO1 or FLAG-DN-FoxO1. C2C12 myoblasts were treated as indicated in Figure 3B (WT-FoxO1 control; n=188, WT-FoxO1 Wnt3a; n=185, WT-FoxO1 Wnt3a + Dkk1; n=191, DN-FoxO1 control; n=209, DN-FoxO1 Wnt3a; n=219). *P<0.017 between groups (Bonferroni’s adjustment). (B) Expression of Foxo1, Foxo3a and Foxo4 genes in C2C12 cells at day 9 of differentiation (n=6 each). Foxo1 or negative control siRNA (200 nM) was transfected at day 6 of differentiation. †P<0.05 vs. control. (C) Total protein was extracted from C2C12 cells at day 9 of differentiation and the expression of FoxO1 was analyzed by western blotting and quantified by densitometry analysis. GAPDH were used as internal control (n=4 each). Foxo1 or negative control siRNA (200 nM) was transfected at day 6 of differentiation. †P<0.05 vs. control. (D) Expression of a Wnt/β-catenin/TCF/LEF target gene Axin2 and FoxO target genes in Foxo1 siRNA treated C2C12 cells was analyzed by quantitative RT-PCR (n=8 each). Foxo1 siRNA (200 nM) was transfected to C2C12 cells at day 6 of differentiation. Wnt3a (50 ng/mL) or 0.1% BSA/PBS (control) was added to the culture medium from day 7 to day 9 of differentiation. †P<0.05 vs. control. (E) Nuclear protein and cytoplasmic protein were extracted from C2C12 cells at day 8 of differentiation and the
expression of GAPDH (as a cytoplasmic marker) and Histone H3 (as a nuclear marker) was analyzed by western blotting.

**Supplemental Figure 3.**

(A) Expression of fiber type specific myosin heavy chain genes in Foxo1 siRNA treated C2C12 cells was analyzed by quantitative RT-PCR (n=8 each). Foxo1 siRNA (200 nM) was transfected to C2C12 cells at day 6 of differentiation. Wnt3a (50 ng/mL) or 0.1% BSA/PBS (control) was added to the culture medium from day 7 to day 9 of differentiation. *P<0.05 vs. control. (B) Total protein was extracted from a C2C12 cell line which stably harbors cumate-inducible CA-FoxO1 and the expression of MYH4 was analyzed by western blotting and quantified by densitometry analysis. GAPDH was used as internal control (n=4 each). Cumate (300 μg/mL) or an equal volume of 95% ethanol (as a control) was added to the culture medium from day 6 to day 9 of differentiation to induce gene expression. *P<0.05 vs. control. (C) Expression of Mef2c gene in a C2C12 cell line which stably harbors cumate-inducible CA-FoxO1 (n=6 each). C2C12 cells were treated as indicated in (B). *P<0.05 vs. control. (D) Total protein was extracted from C2C12 cells at day 9 of differentiation and the expression of MYH4 was analyzed by western blotting and quantified by densitometry analysis. GAPDH was used as internal control (n=4 each). Foxo1 or negative control siRNA (200 nM) was transfected at day 6 of differentiation. *P<0.05 vs. control. (E) Expression of Mef2c gene in C2C12 cells at day 9 of differentiation (n=6 each). C2C12 cells were treated as indicated in (D). *P<0.05 vs. control.
Supplemental References
