Wnt/β-catenin Signaling Contributes to Skeletal Myopathy in Heart Failure via Direct Interaction with FoxO

Okada et al: Wnt/FoxO and Myopathy in Heart Failure

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Animal models of human disease
Abstract

Background—There are changes in the skeletal muscle of patients with chronic heart failure (CHF), such as volume reduction and fiber type shift toward fatigable type IIb fiber. FoxO signaling plays a critical role in the development of skeletal myopathy in CHF, and functional interaction between FoxO and the Wnt signal mediator β-catenin was previously demonstrated. We have recently reported that serum of CHF model mice activates Wnt signaling more potently than serum of control mice and that complement C1q mediates this activation. We therefore hypothesized that C1q-induced activation of Wnt signaling plays a critical role in skeletal myopathy via the interaction with FoxO.

Methods and Results—Fiber type shift toward fatigable fiber was observed in the skeletal muscle of dilated cardiomyopathy (DCM) model mice, which was associated with activation of both Wnt and FoxO signaling. Wnt3a protein activated FoxO signaling and induced fiber type shift toward fatigable fiber in C2C12 cells. Wnt3a-induced fiber type shift was inhibited by suppression of FoxO1 activity, whereas Wnt3a-independent fiber type shift was observed by overexpression of constitutively active FoxO1. Serum of DCM mice activated both Wnt and FoxO signaling and induced fiber type shift toward fatigable fiber in C2C12 cells. Wnt inhibitor and C1-inhibitor attenuated FoxO activation and fiber type shift both in C2C12 cells and in the skeletal muscle of DCM mice.

Conclusions—C1q-induced activation of Wnt signaling contributes to fiber type shift toward fatigable fiber in CHF. Wnt signaling may be a novel therapeutic target to prevent skeletal myopathy in CHF.

Key Words: Wnt, FoxO, chronic heart failure, skeletal myopathy, fiber type shift
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 four 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.

Forkhead box O (FoxO) transcription factors play important roles in the regulation of various cellular processes such as cell proliferation, stress tolerance and metabolism. FoxO factors have also been reported to regulate skeletal muscle wasting and fiber type specification. FoxO overexpression in skeletal muscle promotes muscle atrophy and fiber type shift from type I to type II fiber. In contrast, 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. 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. Recent studies have reported that Wnt signaling also plays an important role during adulthood, including stem cell regulation, skeletal muscle regeneration and cancer progression. β-catenin is the key protein that mediates 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 al. reported that β-catenin also binds to FoxO transcription factors and induces the expression of FoxO target genes.16 Interaction between Wnt signaling and FoxO signaling is also observed in vivo and plays an important role in the pathophysiology of diabetes, 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 mutation K210 in cardiac troponin T gene as dilated cardiomyopathy (DCM) model mice.21

Cell culture

C2C12 mouse myoblasts were maintained in growth medium (Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 20% fetal bovine serum, 100 U/mL penicillin and 100
5 μg/mL streptomycin). For myotube formation, growth medium was replaced with differentiation medium (DMEM supplemented with 2% horse serum, 100 U/mL penicillin and 100 μg/mL streptomycin). Plasmid DNA transfection was performed using Lipofectamine 2000 (Invitrogen). siRNAs were transfected at a concentration of 200 nM using Lipofectamine RNAiMAX (Invitrogen).

**Statistical analysis**

All values are reported as mean ± S.D. Statistical analyses were performed with JMP Pro 10.0.2 (SAS Institute Inc.). We analyzed the data using Wilcoxon rank sum test to compare two groups. For comparing three or more 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. 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, 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 IIa 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. Activation of FoxO signaling is known to play a causative role in skeletal muscle atrophy.9 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, 2B). The purity of the nuclear fraction in the skeletal muscle was confirmed by western blot analysis (Supplemental Figure 1A). FoxO activity is mainly regulated by its subcellular localization and phosphorylation of FoxO by Akt and other kinases regulates its nuclear translocation.6 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 (Supplemental Figure 1B). 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, 2D), suggesting that Wnt signaling as well as FoxO signaling is activated in the skeletal muscle of 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.16 Among FoxO subfamily members, FoxO1 plays a major role in the regulation of skeletal muscle fiber type.8, 9 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, 3B, Supplemental Figure 2A). Wnt3a-induced FoxO1/β-catenin interaction and nuclear accumulation of FoxO1 were blocked by Dkk1, an endogenous inhibitor of Wnt signaling (Figure 3A, 3B, Supplemental Figure 2A). In contrast, Wnt3a-induced FoxO1/β-catenin interaction and nuclear accumulation of FoxO1 were totally prevented in C2C12 cells overexpressing dominant negative FoxO1, which lacks the C-terminal transactivation domain and the binding site with β-catenin (Figure 3A, 3B, Supplemental Figure 2A).16, 23 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 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 (Supplemental Figure 2B, 2C) also prevented Wnt3a-induced activation of FoxO signaling without affecting Wnt3a-induced activation of Wnt signaling (Supplemental Figure 2D). 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, 3E). The purity of the nuclear fraction in
C2C12 cells was confirmed by western blot analysis (Supplemental Figure 2E). 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 trans-activation 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 Myh2, 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 Dkk-1 (Figure 4B, left).

On the other hand, 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 (Supplemental Figure 3A). 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 (Supplemental Figure
3B, 3D). 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 Mef2c.\textsuperscript{8, 9} Overexpression of constitutively active FoxO1 suppressed the expression of Mef2c, while knockdown of FoxO1 enhanced the expression of Mef2c (Supplemental Figure 3C, 3E). These results suggest that Wnt signaling affects the fiber type shift from fatigue-resistant fiber to fatigable fiber by co-activation 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.\textsuperscript{20} 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, 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, 5B). All of these changes were blocked by treatment with Dkk1 (Figure 5A, 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 C1-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 the present 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. Since 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. Muscle wasting is one of the major abnormalities in skeletal myopathy associated with CHF. However, we did not observe skeletal muscle wasting in this study, in part due to the differences in the animal models. 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. 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. Recent studies have revealed its contribution during skeletal myopathy associated with CHF. 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. However, recent studies revealed that FoxO activity is also regulated by other mechanisms, including its interaction with β-catenin. Wnt signaling is also an evolutionarily conserved signaling cascade which plays essential roles during embryonic development. Recent studies have indicated that Wnt signaling also plays an important role in skeletal muscle atrophy and regeneration. In the present 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. 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 more potently than serum from control mice and that complement C1q mediates this activation. In the present study, we have demonstrated that increased C1q in the serum from DCM mice activates 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 up-regulation of C1q biosynthesis leads to an increase in serum C1q concentration in aged mice. 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 CHF patients or in animal models of CHF.

Overexpression of constitutively active β-catenin has been reported to promote the fiber type shift toward type I fatigue-resistant fiber. 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, and that β-catenin is diverted from TCF/LEF to FoxO under certain conditions such as nutritional starvation and oxidative stress. Oxidative stress promotes the utilization of active β-catenin by FoxO and increases the expression of FoxO target genes whereas it suppresses the utilization of active β-catenin by TCF/LEF and decreases expression of Wnt/β-catenin/TCF/LEF target genes. 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. Activation of Wnt signaling in the skeletal muscle of DCM mice may be somewhat modified by increased oxidative stress; oxidative stress may promote utilization 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

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Disclosures

None.
References


**Figure Legends**

**Figure 1.** Fiber type shift in the skeletal muscle of DCM mice.

(A) M-mode echocardiographic images from WT and DCM mice. Yellow scale bar; 1mm. White scale bar; 100 msec. (B) Heart weight (HW) and lung weight (LW) normalized by tibial length (TL) in WT and DCM mice (n=30 each). *P<0.05 vs. WT mice. (C) Skeletal muscle weight (MW) of quadriceps, gastrocnemius and soleus muscles normalized by tibial length in WT and DCM mice (n=10 each). (D) Expression of fiber type specific myosin heavy chain genes in the quadriceps and soleus muscles from WT and DCM mice was analyzed by quantitative RT-PCR (quadriceps; n=8 each, soleus; n=6 each). *P<0.05 vs. WT mice. (E) Immunostaining of the quadriceps and soleus muscles from WT and DCM mice using antibodies for fiber type specific myosin heavy chain (blue; anti-MYH7 (type I fiber), red; anti-MYH2 (type IIa fiber), black; no staining (type IIx fiber), green; anti-MYH4 (type IIb fiber)). Scale bar; 100 μm.

**Figure 2.** Activation of Wnt signaling and FoxO signaling in the skeletal muscle of DCM mice.

(A) Expression of FoxO target genes in the quadriceps and soleus muscles of WT and DCM mice was analyzed by quantitative RT-PCR (quadriceps; n=8 each, 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, p-FoxO1/FoxO1; n=5 each). *P<0.05 vs. WT mice. (C) Expression of a Wnt/β-catenin/TCF/LEF target gene Axin2 in the quadriceps and...
soleus muscles of WT and DCM mice was analyzed by quantitative RT-PCR (quadriceps; n=8 each, 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, β-catenin/GAPDH; n=5 each). *P<0.05 vs. WT mice.

Figure 3. Interaction of Wnt signaling and FoxO signaling.

(A) C2C12 cell lines which stably harbor cumate-inducible FLAG-WT-FoxO1 or FLAG-DN-FoxO1 were established as described in the Supplemental Methods section. Cumate (300 μg/mL) was added to the culture medium for 48 hours to induce gene expression. Wnt3a (50 ng/mL), Dkk1 (200 ng/mL) or 0.1% BSA/PBS (control) was added to the culture medium for the last 24 hours. Total protein samples extracted from C2C12 cells were immunoprecipitated with an antibody against FLAG or control IgG and analyzed by western blotting. (B) Immunostaining of C2C12 myoblasts transiently expressing FLAG-WT-FoxO1 or FLAG-DN-FoxO1. C2C12 myoblasts were treated with either 0.1% BSA/PBS (control), Wnt3a (50 ng/mL) or Dkk1 (200 ng/mL) for 24 hours and stained with anti-FLAG antibody (red). Actin filament and nuclei were labeled with phalloidin (green) and TO-PRO-3 (blue), respectively. Scale bar; 50 μm. (C) Expression of a Wnt/β-catenin/TCF/LEF target gene Axin2 and FoxO target genes in C2C12 cell lines which stably harbor cumate-inducible WT-FoxO1 or DN-FoxO1 was analyzed by quantitative RT-PCR (n=8 each). Cumate (300 μg/mL) was added to the culture medium from day 6 of differentiation to induce gene expression. Wnt3a (50 ng/mL), Dkk1 (200 ng/mL) or 0.1% BSA/PBS (control) was added to the culture medium from day 7 to day 9 of differentiation.
Activation of Wnt signaling induces fiber type shift in C2C12 cells. Wnt3a (50 ng/mL) or 0.1% BSA/PBS (control) was added to the culture medium from day 6 to day 8 of differentiation (n=10 each). *P<0.05 vs. control. (D) Expression of FoxO target genes in C2C12 cells. Wnt3a (10 ng/mL) from day 6 to day 8 of differentiation. (n=8 each). *P<0.05 vs. control. (E) Nuclear protein and total protein were extracted from C2C12 cells 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 (n=4 each). C2C12 cells were treated as indicated in (D). †P<0.05 vs. control.

Figure 4. Activation of Wnt signaling induces fiber type shift in C2C12 cells.

(A) Expression of fiber type specific myosin heavy chain genes in C2C12 cells was analyzed by quantitative RT-PCR. C2C12 cells were stimulated with 0.1% BSA/PBS (control) or Wnt3a (10 ng/mL) from day 6 to day 8 of differentiation. (n=8 each). *P<0.05 vs. control. (B) Expression of fiber type specific myosin heavy chain genes in C2C12 cell lines which stably harbor cumate-inducible WT-FoxO1 or DN-FoxO1 was analyzed by quantitative RT-PCR (n=8 each). Cumate (300 μg/mL) was added to the culture medium from day 6 of differentiation to induce gene expression. Wnt3a (50 ng/mL), Dkk1 (200 ng/mL) or 0.1% BSA/PBS (control) was added to the culture medium from day 7 to day 9 of differentiation (n=8 each). †P<0.05 between groups, *P<0.05 vs. control. (C) Expression of fiber type specific myosin heavy chain genes in a C2C12 cell line which stably harbors cumate-inducible CA-FoxO1. 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 (n=4 each). *P<0.05 vs. control. (D) Expression of fiber type specific myosin heavy chain genes in C2C12 cells at day 9 of differentiation. Foxo1 or negative
control siRNA (200 nM) was transfected at day 6 of differentiation (n=6 each). *P<0.05 vs. control.

**Figure 5.** Serum from DCM mice activates Wnt signaling and FoxO signaling and induces fiber type shift in vitro and in vivo.

(A) C2C12 myotubes were treated with serum from WT 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/TCF/LEF target gene Axin2 and FoxO target genes was analyzed by quantitative 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.

**Figure 6.** Serum C1q activates Wnt signaling and FoxO signaling and induces fiber type shift in vitro and in vivo.

(A) Concentration of C1q in the serum of WT and 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/TCF/LEF target gene Axin2 and FoxO target genes in C2C12 cells was analyzed by quantitative 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 ul 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, DCM + C1-INH; n=6). ‡P<0.05 between groups.

Figure 7. Hypothetical model of Wnt-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.
Figure 1.

A

[Images of tissue sections labeled WT and DCM]

B

[Bar graphs showing HW/TL and LW/TL for WT and DCM]

C

[Graphs showing MW/TL for Quadriceps, Gastrocnemius, and Soleus muscles]

D

[Bar graphs showing relative mRNA expression for Myh7, Myh2, Myh1, and Myh4 in WT and DCM for Quadriceps and Soleus muscles]

E

[Images of stained tissue sections labeled WT and DCM for Quadriceps and Soleus muscles]
Figure 2.

A

Quadriceps

Soleus

Relative mRNA expression

MAFbx/atrogen-1

Pdk4

WT

DCM

WT

DCM

WT

DCM

* * *

B

Nuclear fraction

Whole tissue lysate

FoxO1

Histone H3

WT

DCM

WT

DCM

WT

DCM

* * *

C

Quadriceps

Soleus

Relative mRNA expression

Axin2

WT

DCM

WT

DCM

WT

DCM

* * *

D

Nuclear fraction

Whole tissue lysate

β-catenin

Histone H3

WT

DCM

WT

DCM

WT

DCM

* * *

β-catenin/GAPDH

WT

DCM

WT

DCM

WT

DCM

* * *
**Figure 3.**

A  Control Wnt3a  Wnt3a+Dkk1  Control Wnt3a

<table>
<thead>
<tr>
<th>FLAG-WT-FoxO1 C2C12</th>
<th>FLAG-DN-FoxO1 C2C12</th>
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<tr>
<td>WB:</td>
<td>WB:</td>
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<tr>
<td>Catenin</td>
<td>Catenin</td>
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<tr>
<td>GAPDH</td>
<td>GAPDH</td>
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<td>Input</td>
<td>Input</td>
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B

<table>
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<tr>
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<th>FLAG-DN-FoxO1 C2C12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Wnt3a</td>
</tr>
<tr>
<td>Wnt3a+Dkk1</td>
<td>Control</td>
</tr>
</tbody>
</table>

C

WT-FoxO1 C2C12 myotube

WT-FoxO1 C2C12 myotube

DN-FoxO1 C2C12 myotube

D

C2C12 myotube

E

C2C12 myotube

Nuclear fraction

Whole cell lysate

Relative mRNA expression

Pdk4

GAPDH

Relative expression

FoxO1

Histone H3

p-FoxO1

FoxO1/ Histone H3

p-FoxO1/ FoxO1
Figure 4.

A. C2C12 myotube

B. WT-FoxO1 C2C12 myotube

C. CA-FoxO1 C2C12 myotube

D. C2C12 myotube

Relative mRNA expression

- Control
- Wnt3a
- Wnt3a + Dkk1
- Cumate
- siFoxo1
Figure 5.

A) Western blot analysis showing the expression levels of β-catenin, FoxO1, Histone H3, FoxO1, p-FoxO1, Akt, p-Akt, and GAPDH in the nuclear fraction and whole cell lysate of WT serum, DCM serum, and DCM serum + Dkk1. The graph on the right displays the relative expression of the proteins in both fractions. * indicates significant difference from WT serum, ** indicates significant difference from DCM serum, and *** indicates significant difference from DCM serum + Dkk1.

B) Bar graph showing the relative mRNA expression of Axin2, MAFbx/atrogin-1, Pdk4, Myh7, Myh2, Myh1, and Myh4 in WT serum, DCM serum, and DCM serum + Dkk1. The expression levels are compared in both nuclear fraction and whole cell lysate. * indicates significant difference from WT serum, ** indicates significant difference from DCM serum, and *** indicates significant difference from DCM serum + Dkk1.

C) Bar graph showing the relative mRNA expression of Axin2, MAFbx/atrogin-1, Pdk4, Myh7, Myh2, Myh1, and Myh4 in WT, DCM, and DCM + Dkk1. The expression levels are compared in both nuclear fraction and whole cell lysate. * indicates significant difference from WT, ** indicates significant difference from DCM, and *** indicates significant difference from DCM + Dkk1.
Figure 6.

(A) Serum C1q concentration (µg/mL) for WT and DCM mice.

(B) Relative mRNA expression of Axin2, MAFbx/atrogen-1, Pdk4, Myh7, Myh2, Myh1, and Myh4 in WT, DCM, and DCM + C1-INH sera.

(C) Relative mRNA expression of Axin2, MAFbx/atrogen-1, Pdk4, Myh7, Myh2, Myh1, and Myh4 in WT, DCM, DCM serum, and DCM serum + C1-INH sera.

(D) Relative mRNA expression of Axin2, MAFbx/atrogen-1, Pdk4, Myh7, Myh2, Myh1, and Myh4 in WT, DCM, and DCM + C1-INH sera.
Figure 7.

Serum C1q
Activation of Wnt signaling

β-catenin

FoxO1

TCF/LEF

Fiber type shift toward
Type I fiber

Fiber type shift toward
Type IIb fiber

Cytoplasm

Nucleus
Wnt/β-catenin Signaling Contributes to Skeletal Myopathy in Heart Failure via Direct Interaction with FoxO

Katsuki Okada, Atsuhiro T. Naito, Tomoaki Higo, Akio 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 Issei 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, 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. 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>
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<tr>
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B

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<td>GAPDH</td>
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Graph:

- **Nuclear fraction**
- **Whole tissue lysate**

Relative expression:

- FoxO3a
- p-FoxO3a

Legend:

- WT
- DCM
Supplemental Figure 2.

A. FLAG-tagged FoxO1 localization

B. C2C12 myotube

C. C2C12 myotube

D. siFoxo1 C2C12 myotube

E. C2C12 myotube

relative mRNA expression

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<tr>
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relative expression

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Nuclear fraction

C2C12 myotube

GAPDH

Histone H3

Cytoplasmic fraction

GAPDH

Histone H3
Supplemental Figure 3.

A

Relative mRNA expression

Myh7 Myh2 Myh1 Myh4

Type I Type Iia Type Iix Type Iib

Control siFoxo1

* * *

B

CA-FoxO1 C2C12 myotube

Control Cumate

MYH4

GAPDH

Relative expression

MYH4/GAPDH

C

CA-FoxO1 C2C12 myotube

Control Cumate

Mef2c

Relative expression

D

C2C12 myotube

Control siFoxo1

MYH4 GAPDH

Relative expression

MYH4/GAPDH

E

C2C12 myotube

Control siFoxo1

Mef2c

Relative expression

* * *
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
