Vitamin D is known to be effective for the prevention of muscle atrophy, such as age-related sarcopenia. However, vitamin D action in skeletal muscle tissue and muscle cells is largely unknown. We previously found that a transcription factor, FOXO1 gene expression, was induced in various muscle atrophy conditions causing muscle atrophy by upregulating atrophy-related genes, including atrogin 1 (ubiquitin ligase) and cathepsin L (lysosomal proteinase). In this study, we found that vitamin D inhibited FOXO1-mediated transcriptional activity in a reporter gene assay. Moreover, vitamin D suppressed the glucocorticoid-induced gene expression of atrogin 1 and cathepsin L in C2C12 myoblasts. Thus, vitamin D may prevent muscle atrophy via the FOXO1-mediated pathway in muscle cells.

**Key Words** vitamin D, sarcopenia, atrophy, skeletal muscle, nuclear receptor

Skeletal muscle is the largest organ in the human body, comprising approximately 40% of the total body weight, and it plays important roles in exercise, energy expenditure, and glucose/amino acid metabolism. Skeletal muscles plastically adapt to their environment, and appropriate exercise with sufficient nutrition increases muscle mass (1). Meanwhile, various life conditions, such as bedrest, aging, and other diseases, as well as a glucocorticoid treatment, cause muscle atrophy and decrease quality of life (2). In aging societies, which is a growing situation in many developed countries, the prevention/cure of atrophy is particularly important (1, 3). Understanding the molecular mechanisms underlying muscle hypertrophy/atrophy is important for developing methods to prevent muscle atrophy/dysfunction, which seriously impairs human health and quality of life.

FOXO1 is a forkhead-type transcription factor that antagonizes insulin-mediated anabolic signals. We have been investigating the role of FOXO1 in metabolic regulation in skeletal muscles. Specifically, based on data that FOXO1 gene expression is induced by energy deprivation (4), we developed transgenic mice that overexpressed FOXO1 (FOXO1 Tg mice) and showed muscle atrophy (5). In addition, we showed that FOXO1 activates the genes involved in protein degradation (6, 7). FOXO1 activates the expression of atrophy-related genes such as atrogin 1 and cathepsin L in various muscle atrophy-related conditions, including a glucocorticoid treatment (6–8). These findings suggest that FOXO1 in skeletal muscles plays an important role in muscle atrophy.

Vitamin D, a fat-soluble vitamin, is well known for its role in regulating bone homeostasis. Evidence indicates that vitamin D plays roles in many other tissues including skeletal muscle (3, 9). An epidemiological study suggested that a vitamin D treatment was effective for the prevention of decreased muscle mass (sarcopenia) (10). Previously, we first reported the cloning of mouse vitamin D receptor (VDR) cDNA (11). VDR is a nuclear receptor-type transcription factor, which is activated in the presence of 1,25(OH)2 vitamin D3, the active form of vitamin D. It has been reported that aged people with sarcopenia show a decreased VDR expression (10). Recent findings showed that VDR knockout mice showed insulin resistance with an increased FOXO1 expression in skeletal muscle (12). In addition, following a 1,25(OH)2 vitamin D3 treatment, C2C12 muscle cells showed a decreased FOXO1 expression (12). However, the atrophy phenotype of 1,25(OH)2 vitamin D3 in FOXO1 action was not examined.

In the present study, we found that 1,25(OH)2 vitamin D3 can suppress FOXO1 transcriptional activity. Thus, we examined the effect of 1,25(OH)2 vitamin D3 for muscle atrophy gene expression, which is the target of FOXO1, in muscle cells.

**Materials and Methods**

**Transcriptional activation assays.** HEK293T cells (Riken Cell Bank, Tsukuba, Japan) were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Transfections and reporter assays were performed as described previously (13). We used amino acids 1–147 of GAL4 (13) that were fused to the full length of FOXO1 cDNA.
HEK293T cells were co-transfected with a reporter gene containing four copies of a GAL4 binding site ((UAS)4-Luc), and pM-FOXO1 (GAL4-FOXO1). The luciferase reporter plasmid (25 ng), expression plasmid (pM-FOXO1: 25 ng), and the phRL-TK vector (2 ng: Promega Co., Madison, WI) as an internal control of transfection efficiency were transfected into HEK293T cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Twenty-five hours after transfection, the medium was replaced with a new medium containing the indicated amount of 1,25(OH)2 vitamin D3 (Calcitriol, No 71820, Cayman, Ann Arbor, MI). After 20 h, cells were lysed and assayed for luciferase activity using the Dual-Glo Luciferase Assay kit (Promega). The activity was calculated as the ratio of firefly luciferase activity to Renilla luciferase activity (internal control) and expressed as an average of triplicate experiments.

C2C12 cells and cell cultures. C2C12 mouse myoblasts (Riken Cell Bank) were cultured in DMEM supplemented with 10% FBS until the cells reached confluence. Indicated concentration of dexamethasone (DEX, Nacalai Tesque, Inc., Kyoto, Japan) and 1,25(OH)2 vitamin D3 were added to the medium without FBS, cells were harvested 24 h after the treatment and used for the RNA analysis.

Quantitative real time-polymerase chain reaction analysis. Total RNA was prepared using TRizol (Thermo Fisher, Waltham, MA). cDNA was synthesized from 500 ng of total RNA using the ReverTra Ace qPCR RT MasterMix with a gDNA Remover (Toyobo, Osaka, Japan). Gene expression levels were measured on a CFX Connect Real Time System (Biorad, Tokyo, Japan) using the Thunderbird SYBR qPCR Mix (Toyobo). The primers used were as follows. 36B4: Fw 5′-GGCCCTGCACCTCTCGCTTTTC-3′, Rv 5′-TGCCAGCACGGCTTGT-3′; VDR: Fw 5′-GGCTTCCACTTCAACGCTATG-3′, Rv 5′-TCGTCGCCGTCGAAAGACCC-3′; FOXO1: Fw 5′-GGCGGTGCTGGAAGAATCTCAAT-3′, Rv 5′-TCCGTTCTTCTACCTACCGTA-3′; Atrogin 1: Fw 5′-TGCGAAGTCGCTGCCTGTG-3′, Rv 5′-CCATCGGATACCACCCCATGT-3′; Cathepsin L: Fw 5′-TTCACGGTCAAGCCCATCA-3′, Rv 5′-AAGCGAAATCTCAGGCTTC-3′.

Statistical analyses. We used an analysis of variance followed by a Tukey's test. Data are expressed as the mean±SE, and p<0.05 was considered statistically significant.

Results and Discussion

1,25(OH)2 vitamin D3 suppressed FOXO1 transcriptional activity

We used a GAL-FOXO1 reporter system to evaluate FOXO1 transcriptional activity. FOXO1 fused to GAL transfection in HEK293T cells showed an increased reporter luciferase activity, indicating that FOXO1 has a strong transcriptional activity (Fig. 1). Thus, we examined the 1,25(OH)2 vitamin D3 effect on FOXO1-mediated transcriptional activity. As shown in Fig. 1, a 1,25(OH)2 vitamin D3 treatment suppressed the reporter activity caused by FOXO1 in a dose-dependent manner. Thus, 1,25(OH)2 vitamin D3 appears to suppress FOXO1 transcriptional activity.

1,25(OH)2 vitamin D3 suppresses glucocorticoid-induced FOXO1 target atrophy gene expression

C2C12 cells were treated with DEX and 1,25(OH)2 vitamin D3. In this experiment, FOXO1 expression tended to be increased by a DEX treatment, and a DEX treatment was also reported to increase FOXO1 transcriptional activity (8, 14). Consistently, DEX markedly increased FOXO1 target genes such as atrogin 1 (ubiquitin ligase) and cathepsin L (lysosomal proteinase) (5, 6) (Fig. 2). As we previously showed in another cell (3T3-L1 cells) (15), a 1,25(OH)2 vitamin D3 treatment increased the VDR mRNA level (Fig. 2). In the absence of DEX, a 1,25(OH)2 vitamin D3 treatment did not show any marked effect on the target genes. Interestingly, 1,25(OH)2 vitamin D3 suppressed DEX-induced atrogin 1 and cathepsin L gene expression. FOXO1 expression was not suppressed, but rather increased, by a 1,25(OH)2 vitamin D3 treatment. The physiological significance of increased FOXO1 expression by DEX and 1,25(OH)2 vitamin D3 is currently unclear.

Possible interaction between FOXO1 and 1,25(OH)2 vitamin D3 signaling

From the data in this study, 1,25(OH)2 vitamin D3 appears to suppress FOXO1 transcriptional activity and its target atrophy-related gene expression. There are reports that FOXO1 interacts with multiple nuclear receptors, modifying their transcriptional activity (16). Thus, VDR in the presence of 1,25(OH)2 vitamin D3 may interact with FOXO1 in muscle cells and repress atrophy gene expression. Vitamin D is known to be a promising agent to prevent aged atrophy (sarcopenia), but its molecular mechanism has been largely unclear.
This study may shed light on the mechanism of preventing sarcopenia by supplementing with oral vitamin D. Possible application for screening of agents that inhibit FOXO1 transcriptional activity.

We consider the GAL-FOXO1 reporter system used in this study useful for searching for food components/agents that suppress FOXO1 transcriptional activity. Indeed, we have screened 400 commercially available food components and found several agents that suppressed FOXO1 transcriptional activity (data not shown). Among them, 1,25(OH)2 vitamin D3 most strongly suppressed FOXO1 transcriptional activity. Screening details will be reported elsewhere. These compounds are candidates for functional foods for prevention of skeletal muscle atrophy, including sarcopenia.

Author contributions
Y. Hirose and T. Onishi contributed equally to this work.

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