Vitamin D/VDR signaling attenuates skeletal muscle atrophy by suppressing renin-angiotensin system

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Running title: VDR protects muscle *via* RAS

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ABSTRACT

The nutritional level of vitamin D may affect musculoskeletal health. We have reported that vitamin D is a pivotal protector against tissue injuries by suppressing local renin-angiotensin system (RAS). This study aimed to explore the role of vitamin D receptor (VDR) in the protection against muscle atrophy and the underlying mechanism. A cross-sectional study on participants (n = 1034) in Shanghai (China) was performed to analyze the association between vitamin D level and the risk of low muscle strength as well as to detect the circulating level of angiotensin II (Ang II). In animal studies, dexamethasone (Dex) was applied to induce muscle atrophy in wild-type (WT) and VDR-null mice, and the mice with the induction of muscle atrophy were treated with calcitriol for 10 days. The skeletal muscle cell line C2C12 and the muscle satellite cells were applied in *in vitro* studies. The increased risk of low muscle strength was correlated to a lower level of vitamin D (adjusted OR, 0.58) accompanied by an elevation in serum Ang II level. Ang II impaired the myogenic differentiation of C2C12 myoblasts as illustrated by the decrease in the area of myotubes and the down-regulation of myogenic factors (MHC & MyoD). The phenotype of muscle atrophy induced by Dex and Ang II was aggravated by VDR ablation in mice and in muscle satellite cells, respectively, and mediated by RAS and its downstream PI3K/Akt/FOXO1 signaling. Calcitriol treatment exhibited beneficial effects on muscle function as demonstrated by the increased weight-loaded swimming time, grip strength and fiber area, and improved fiber type composition via regulating ubiquitin ligases and their substrates MHC and MyoD through suppressing renin/Ang

II axis. Taken together, VDR protects against skeletal muscle atrophy by suppressing RAS. Vitamin D could be a potential agent for the prevention and treatment of skeletal muscle atrophy.

KEY WORDS: Muscle atrophy; Vitamin D; Myogenesis; Angiotensin II; Vitamin D receptor

Introduction

Skeletal muscle atrophy, which is triggered by an unbalance between synthesis and degradation of muscle structural proteins, contributes to the reduction in muscle strength and muscle mass.[1,2] It leads to a higher risk of chronic musculoskeletal pain, sarcopenia and associated falls.[3] Muscle atrophy could be induced by lack of exercise, glucocorticoid, ageing, bacterial infection, chronic diseases and nutritional deficiency.[1,4]

Vitamin D deficiency is one of common health problems worldwide. Studies support that vitamin D regulates the physiologies and functions of multiple human systems, such as the maintenance of calcium homeostasis and the promotion of mineralization in bone tissue, *via* binding to vitamin D receptor (VDR). The relevance of vitamin D to skeletal muscle health has been highlighted in recent years.[5] The interest in vitamin D arises from research findings from biological, clinical and epidemiological studies that demonstrate a causal association between vitamin D deficiency and the increased risk of muscle atrophy in the elderly.[6,7]

It has been reported that the active vitamin D, like 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), has a direct regulatory action in promoting myogenesis and protein synthesis as well as in regulating mitochondrial metabolism of skeletal muscles through activation of various intracellular biological events.[3] However, the molecular mechanisms of the vitamin D signaling involved in the regulation of skeletal muscles are not fully understood.

Local renin-angiotensin system (RAS) plays a crucial role in the pathogenesis of

tissue injuries. Renin, a rate-limiting enzyme, is involved in the classical RAS for the formation of angiotensin II (Ang II), which is an active peptide that binds to G-protein-coupled receptor, Ang II type 1 receptor (AT1R), and displays multiple biological effects. We have previously revealed the involvement of tissue RAS in the pathological process of chronic kidney diseases,[8,9] osteoporosis[10,11] and neuroinflammation.[12,13] More importantly, our previous studies have demonstrated that vitamin D and its analogues exert protection against tissue damages by negatively regulating RAS *via* binding to VDR.[8,9,12] Several lines of evidence have demonstrated that RAS participates in the pathology of skeletal muscle atrophy[14] and Ang II appears to be negative on myogenesis.[1] The deletion of VDR would result in hyperreninemia and RAS activation,[15] but the role of VDR in skeletal muscle atrophy and in the regulation of local RAS in muscle tissue has not been well characterized.

It was hypothesized that vitamin D could protect against muscle atrophy by suppression RAS in skeletal muscle. This study aimed to explore the role of VDR in managing muscle health and decipher the underlying mechanism behind the beneficial effects of vitamin D on skeletal muscle. A cross-sectional study was carried out to investigate the association between circulating total 25-hydroxyvitamin D (25(OH)D), Ang II levels, and muscle strength in populations. The molecular mechanisms of vitamin D/VDR signaling were characterized by using VDR(-/-) mice and cell culture studies of the myoblast cell line C2C12 and muscle satellite cells. The therapeutic efficacy of calcitriol on skeletal muscle atrophy was also studied.

Materials and Methods

Human study

Participants

Participants of the present cross-sectional study were enrolled from September 2016 to February 2017 from two subdistricts (Longhua community and Lujiazui community) in Shanghai (China). The study protocol (2014LCSY12) was approved by the Ethics Committee of Longhua Hospital affiliated to Shanghai University of Traditional Chinese Medicine. A written informed consent was provided and signed by the participants prior to participation.

The criteria for participation were permanent residents (women aged ≥45 years and men aged ≥50 years) of the aforementioned communities, and the exclusion criteria were as previously described[16]: (1) residents with acute infectious diseases or severe mental diseases or those who were unable to complete the survey; (2) women during lactation or pregnancy; (3) those who had severe physical diseases that influenced gastrointestinal absorption and/or bone metabolism; (4) those who had been taking vitamin D or multivitamin supplements in the three months prior to the study.

Data collection

Face-to-face interviews were conducted by physicians from the Longhua Hospital (Shanghai University of Traditional Chinese Medicine) who were responsible for getting participants' basic characteristics and medical history, such as, but not limited to, age, sex, height, weight, daily tobacco use and alcohol consumption. There were a

total of 1226 residents who took part in the survey. Body mass index (BMI) was calculated as the ratio of weight (kg) to height squared (m^2). Among the 1226 residents, 192 residents were excluded because they either took vitamin D supplements, or failed to complete the test on handgrip strength or the test on 5-time chair stand due to limb fracture, waist injury, blindness, vertigo or other reasons during the study. A total of 1034 participants were enrolled for the final analysis (Fig. 1A).

Serum 25(OH) D and Ang II measurements

Fasting blood samples were harvested from the participants in the morning on their day of clinic visit. The serum total 25(OH)D (D2 and D3) levels were measured by HPLC (Shimadzu Corporation, Japan) and LC-MS/MS system (API 5500, Applied Biosystems Inc., USA). The assay for measuring 25(OH)D level had a sensitivity of less than 3.75 nmol/L, the intra-assay coefficient of variation (CV) was less than 5.0%, and the inter-assay CV was less than 12.0%. To study the relationship of serum 25(OH)D and Ang II levels, 40 participants (cases) in the group with the highest serum 25(OH)D level (quartile 4, ≥54.7 nmol/L) and 40 participants (controls) in group with the lowest serum 25(OH)D level (quartile 1, <34.0 nmol/L) were randomly chosen. The controls were matched with the cases on a 1:1 ratio for age (±1 year) and sex. Serum Ang II level was measured by using Human/Mouse/Rat Angiotensin II Enzyme Immunoassay kit (RayBiotech, Norcross, GA, USA). All assays were measured in duplicate.

Handgrip strength measurement

Muscle strength was measured by an electronic hand dynamometer (EH101, CAMRY, Guangdong, China). Handgrip strength was recorded as the mean grip strength of both hands of the participants separately measured in three readings. Low muscle strength was defined as handgrip strength <28 kg for men and <18 kg for women.[17]

Mouse study

Animals and treatments

The animals were housed in environmentally controlled SPF animal facilities, which were kept at 22°C with an alternation of light:dark (12h:12h) and humidity (45-55%)-controlled condition. All animal procedures were performed in accordance with NIH Guide for Care and Use of Laboratory Animals. The animal study protocol was reviewed and approved by the Animal Care and Use Committee of Longhua Hospital affiliated to Shanghai University of Traditional Chinese Medicine. VDR(-/-) mice in C57BL/6 background were described previously.[18] To induce muscle atrophy, 8-week-old male VDR(-/-) and wild-type (WT) mice (n = 6/group) were daily injected (i.p.) with dexamethasone (Dex, 25 mg/kg) dissolved in PEG-400:normal saline (3:7) solution for 10 days. The groups of age-matched VDR(-/-) and WT mice (n = 6/group) were designed as controls without Dex treatment.

Two groups of 8-week-old male C57BL/6 mice (n = 10/group) were randomly allocated, and injected daily (i.p.) with Dex (25 mg/kg) and orally co-administrated

with calcitriol (CP Pharmaceutical Qingdao Co., Ltd., China) at low (1 μg/kg) or high dosages (3 μg/kg) for 10 days. A group of age-matched mice without any treatment was designed as control (n = 8) and the mice with Dex treatment alone were designed as disease model group (n = 8).

Animal grip strength test

The grip strength of mice limbs was measured by a grip strength meter (Model YLS-13A, China) as previously described.[19] The operator would hold a mouse gently by the base of the tail, allowing it to grasp the metal bar with its limbs. While the mouse was grasping the metal bar, the operator would flatly pull the mouse backwards by the tail until its grip was lost. The grip strength meter would automatically record the peak force of the gripping in grams (g). The grip strength of each mouse was tested in triplicate.

Weight-loaded swimming test (WLST)

The endurance performance of the mice was evaluated by WLST as previously described with minor modifications.[20] After one hour following the last dosing, the mice were loaded with lead sheets (5% of mouse body weight) that were attached to the same position of the tail. The weight-loaded mice were individually forced to swim in a columnar swimming pool (50 cm high, 20 cm in diameter and 40 cm deep), which was filled with water at a temperature of 25 ± 1°C and a depth of 30 cm. The swimming time was recorded until the mice were exhausted, which was judged by their loss of coordinated movements and failure to rise to the water surface within 7

seconds.

Collection of animal samples

After the grip strength and swimming tests, the mice were sacrificed by overdose anesthesia (pentobarbital, i.p., 70 mg/kg) and blood was collected by cardiac puncture. The tibialis anterior muscles and gastrocnemius of the mice were harvested for a variety of biochemical, histological and molecular analyses.

Skeletal muscle histology and cross-sectional area

After embedding in optimal cutting temperature (OCT) compound (Leica Biosystems) and freezing in isopentane cooled in liquid nitrogen, the frozen transverse sections (10 μm) of the tibialis anterior muscles and gastrocnemius isolated from mice were prepared by a cryostat (CM3050S Leica Biosystems, Germany) at -20°C and mounted on gelatin-coated glass slides. Hematoxylin and eosin (H&E) staining and anti-dystrophin immunostaining were conducted for observation of muscle fibers and for determination of cross-sectional area of myofibers using the *Image J* program.

RT-PCR and quantitative RT-PCR

Total tissue RNA was extracted according to the TRIzol manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). cDNAs synthesis was performed by reverse transcription reactions with 4 μg of total RNA using SuperScript II reverse transcriptase (Invitrogen, USA) with oligo $dT_{(15)}$ primers (Fermentas). The first strand

cDNAs served as the template for the regular PCR performed using a DNA Engine (ABI), and for quantitative PCR performed in Applied Biosystems 7900 Real Time PCR System using a SYBR green PCR reagent kit (Applied Biosystems, Foster City, CA, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control to determine the relative expression of the target genes. The PCR primers (5'-3') used in this study were as follows: MSTN-forward: TTGAAGCCTTTGGATGGGAC, and MSTN-reverse: GCACAAGATGAGTATGCGGA; MyoD-forward: CTAAGCGACACAGAACAGGG, and MyoD-reverse: GCAGTCGATCTCTCAAAGCA. The primer sequences for angiotensinogen (AGT), renin and GAPDH were referred in our published paper.[10]

Cell culture work

Cell culture and treatment

The skeletal muscle cell line C2C12 (ATCC® CRL-1772™) was grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S). Cells were differentiated into myotubes cultured in differentiation medium (DM) consisting of DMEM containing 2% heat-inactivated horse serum (Biological Industries, Kibbutz Beit Haemek, Israel) for 5 days. The cells were cultured at 37°C and in a humidified atmosphere of 95% air and 5% CO_2. The myotubes were incubated with angiotensin II (Ang II, 100 nM, Peptide Institute, Japan) in the absence or presence of olmesartan (1 μM, Sigma), one

of the Ang II type 1 receptor blockers, for 48 hours.

Isolation, differentiation and treatment of primary skeletal muscle satellite cells

Six-week-old WT and VDR(-/-) mice were sacrificed for isolation of hind limb muscles, which were transferred to a fresh Petri dish and minced to a size of 1 mm^3 with ophthalmic scissors. After washing with PBS, the minced muscle tissues were digested with 0.2% collagenase II (Sigma) in a 37°C water bath for 1 h. The cells were collected after centrifuging at 1000 rpm for 10 min at 4°C, and were incubated with neutral protease II (1 mg/mL, Sigma) for 1 h. After repeated pipetting and mixing, the suspension was filtered twice through a cell strainer (100 μm) in sequence and centrifuged at 300-400 rpm for 5 min. The precipitate was lysed with red blood cell lysis buffer (Sangon Biotech, China) at room temperature for 10 min after the supernatant was removed. After centrifuging at 1000-1500 rpm for 6 min, the cells were resuspended with DMEM containing 20% FBS, 10% horse serum, 0.5% chicken embryo extract (Absin Bioscience, China) and 1% P/S. After culturing for 2 h at 37°C in 5% CO_2, the non-adherent cells were transferred to a new culture dish. The muscle satellite cell clusters were detected by immunostaining for specific protein Pax 7.[21] After the cells reached to 80% confluence, the satellite cells were differentiated into myotubes cultured in DM for 5 days, then incubated with vehicle or Ang II (100 nM) for 48 hours. The protein expression of muscle regulatory factors and the components involved in PI3K/Akt/FOXO1 signaling was determined in myotubes derived from the isolated satellite cells from the VDR(-/-) and WT mice by immunoblotting.

Measurement of myotube area

Photographs of immunofluorescent myotubes with MHC staining in different sites on the cell coverslips were used to measure the cross-sectional area of myotubes analyzed by NIH ImageJ software. Myotube diameter was measured at three random points of individual myotubes. The percentage for frequency distribution of cross-sectional area and the mean area of myotubes in each group were calculated.

Immunofluorescence staining

The frozen sections of the tibialis anterior muscle and gastrocnemius were prepared as stated above. The C2C12-differentiated myoblasts were seeded onto glass coverslips. Cells and muscle sections were washed with PBS and immediately fixed with 4% paraformaldehyde for 15 min at room temperature and permeabilized with 0.2% Triton X-100. After blocking with 1% BSA, the cells or tissues were incubated with specific primary antibodies mouse anti-myosin heavy chain (MHC, 1:200, cells) or rabbit anti-dystrophin (1:100, tissue) overnight at 4°C. For determining muscle fiber types, the frozen slides of gastrocnemius were incubated with the following antibodies: BA-F8 for MHC type I (1:50), SC-71 for MHC type IIa (1:600), and BF-F3 for MHC type IIb (1:100) (Developmental Studies Hybridoma Bank, IA, USA). After several washes with PBST (0.1% Tween-20 in PBS), the primary antibodies were probed with 1:200 Alexa Fluor 488-conjugated AffiniPure goat anti-mouse IgG or donkey anti-rabbit IgG (Jackson ImmunoResearch, PA, USA). The mice muscle sections that

had been used for fiber type measurement were incubated with Alexa Fluor conjugated secondary antibodies 350, 488, and 555 (1:250) (Invitrogen, CA, USA) at room temperature for 1 h, followed by staining with regular mounting medium without DAPI (Beyotime, China). Fluorescence images were captured by a fluorescence microscope (VS120, Olympus, Japan).

Protein extraction and immunoblotting

The protein of mouse tibialis anterior tissue was extracted by homogenization in Laemmli buffer supplemented with protease inhibitor cocktail (Roche, Mannheim, Germany). The primary satellite cells and the myotube cells differentiated from C2C12 myoblasts were lysed in RIPA buffer (Beyotime, China) containing protease inhibitor cocktail, 2 mM sodium orthovanadate, 1 mM sodium fluoride and 1 mM PMSF. The protein concentration of all lysates was determined using Bradford assay (Beyotime, China). Lysates containing 30 μg of protein were separated on SDS-PAGE gel, and transferred onto PVDF membrane (Merck Millipore, Darmstadt, Germany). After saturation with 5% (w/v) nonfat dry milk in TBS and 0.1% (w/v) Tween 20 (TBST), the membranes were incubated with primary antibodies (MHC, MyoD, MURF1, MAFbx, Renin, Ang II, p-PI3K, PI3K, p-Akt, Akt, p-FOXO1, FOXO1) with dilutions ranging between 1:1000 and 1:2000 at 4°C overnight (Supplement Table 1). After washing with TBST, membranes were incubated with secondary antibodies and enhanced chemiluminescence (ECL) solution (Bio-Rad, USA). Band intensities were densitometrically assessed by the Lumi-Imager using

Lumi-Analyst version 3.10 software (Roche, Mannheim, Germany) and normalized to the GAPDH expression.

Statistical analysis

Statistical significance of differences in baseline characteristics was assessed between the participants with normal muscle strength and those with low muscle strength using *t*-tests, Kruskal-Wallis rank sum tests or chi-square tests. We explored the association between serum 25(OH)D level and low muscle strength using thin plate regression splines in generalized additive models implemented by the R package *mgcv*. Logistic regression models were performed to determine this association both with and without adjusting for age, sex, BMI, smoking and drinking habits. To investigate the relation between serum 25(OH)D and Ang II, 40 participants (cases) with serum 25(OH)D levels in quartile 4 (≥54.7 nmol/L) were randomly chosen, and 40 participants with serum 25(OH)D levels in quartile 1 (<34.0 nmol/L) were chosen as controls, which were matched with the cases on a 1:1 ratio for age (±1 year) and sex. A two-tailed $P < 0.05$ was considered to be statistically significant in all analyses. Data were analyzed using the statistical package R version 3.6.0 (http://www.R-project.org/).

The data from the cell and animal experiments were expressed as mean ± SEM of the values obtained from individual experiments. Statistical comparisons between groups were performed by one-way analysis of variance (ANOVA) followed by Tukey *post hoc* test. A difference of $P < 0.05$ was considered statistically significant. GraphPad Prism 8.0 was used for statistical analysis (GraphPad software, Inc., USA).

Results

The association between serum 25(OH)D level and low muscle strength

The workflow of the cross-sectional study was illustrated in Figure 1A. The analysis was based on 200 participants with low muscle strength and 834 participants with normal muscle strength (Table 1). In univariate analysis, there were no significant differences in the BMI, the smoking habit, or the alcohol use, between the low muscle strength group and the normal muscle strength group. Participants with low muscle strength were significantly older ($P < 0.001$) as shown by the increase in age from 64.6 years old in normal muscle strength to 68.9 years old in low muscle strength. Additionally, the participants with low muscle strength were mainly male. 25(OH)D level in serum was lower ($P < 0.05$) in the low muscle strength group than in the normal muscle strength group.

There was a significant negative association between serum 25(OH)D level and the risk of low muscle strength. In the analysis that had been adjusted for age, sex, BMI, smoking and drinking habits, an increase in serum 25(OH)D level was associated with reduced odds of low muscle strength (adjusted OR, 0.84; 95% CI: 0.71-0.99, $P = 0.043$) (Fig. 1B, Table 2). When serum 25(OH)D level was assessed as quartiles, the adjusted ORs and 95% CI in the second, third and fourth quartile were 0.70 (0.45, 1.08), 0.48 (0.30, 0.77), 0.58 (0.37, 0.90), respectively, as compared with that in quartile 1 (P for trend, 0.005). The participants in serum 25(OH)D quartiles 2-4 (≥34.0 nmol/L) had a significant lower risk of low muscle strength (adjusted OR, 0.58;

95% CI, 0.41-0.83) than that in quartile 1 (<34.0 nmol/L). Similar trends were found in both male and female participants (Table 2). Furthermore, the serum level of Ang II (Fig. 1C, 0.725 ± 0.271) in participants in the lowest quartile of serum 25(OH)D level (<34.0 nmol/L) was significantly higher (P = 0.009) than that (0.545 ± 0.314) in participants in the highest quartile of serum 25(OH)D level (≥54.7 nmol/L).

Furthermore, the serum level of Ang II was categorized as low or high with the cut-point of 0.639 (the median of Ang II level). When the participants were grouped by the levels of both vitamin D (Q1 vs. Q4) and Ang II (low vs. high), the prevalence of low muscle strength was 16.7% in group A (higher vitamin D and lower Ang II) and 41.7% in group B (lower vitamin D and higher Ang II). Compared with group A, a significantly higher prevalence of low muscle strength was found in group B (adjusted OR, 5.44; 95% CI: 1.14-33.35, P = 0.045).

Ang II influenced the formation of myotubes and expression of regulators in C2C12 myoblasts

To elucidate the effects of Ang II on skeletal muscle, the formation of myotubes was determined in mouse C2C12 myoblasts (Fig. 2A). Ang II reduced the area of myotubes (Fig. 2B, P < 0.001) and the myotubes became smaller (Fig. 2C). These were markedly reversed by Ang II type 1 receptor blocker, olmesartan. The protein expressions of muscle-specific ubiquitin ligases including MURF1 and MAFbx (Fig. 2D) and myogenesis factors including MHC and MyoD (Fig. 2F) were assessed. Treatment of olmesartan significantly suppressed the up-regulation of MURF1 (Fig.

2E, $P < 0.01$) and MAFbx (Fig. 2E, $P < 0.05$) and the down-regulation of MHC (Fig. 2G, $P < 0.05$) and MyoD (Fig. 2G, $P < 0.05$) induced by Ang II.

VDR KO mice developed more severe skeletal muscle atrophy with Dex treatment

To determine the role of VDR in skeletal muscle atrophy, WT and VDR(-/-) mice were subjected to Dex injection for 10 consecutive days. The body weight of the Dex-treated VDR(-/-) mice decreased dramatically compared with the Dex-treated WT mice (Fig. 3A). The muscle index (Fig. 3B) showed that Dex treatment significantly reduced the weights of tibialis anterior muscles ($P < 0.01$) and gastrocnemius ($P < 0.01$), diminished muscle function with the decrease in weight-loaded swimming time (Fig. 3C, $P < 0.05$) and grip strength (Fig. 3C, $P < 0.05$). A greater degree of decrease in muscle weight ($P < 0.01$), weight-loaded swimming time ($P < 0.05$) and grip strength ($P < 0.001$) was detected in the Dex-treated VDR(-/-) mice than in the WT mice.

The results of H&E staining showed the cross-sectional area of tibialis anterior of Dex-induced atrophic groups was decreased compared with that of the vehicle control (Fig. 3D&E, $P < 0.05$) and a greater degree of reduction was seen in the VDR(-/-) mice than that in the WT mice ($P < 0.01$). Furthermore, immunofluoresence staining with anti-dystrophin antibody (Fig. 3F&G) demonstrated there was more reduction in muscle-fiber size of tibialis anterior of the Dex-treated VDR(-/-) mice than that of the WT mice ($P < 0.05$).

VDR ablation influenced fiber type composition

To determine whether the fiber types of the skeletal muscle switched after VDR ablation, the immunofluorescence staining was performed to indicate type I (blue), type IIa (green) and type IIb (red) fibers on the frozen sections of gastrocnemius. Dex diminished the ratio of the type I fibers and type IIa fibers (Fig. 3H, $P < 0.001$), the percentages of which were both reduced in VDR(-/-) mice with statistical significance ($P < 0.01$ & $P < 0.001$, respectively), compared with those of the WT mice. On the other hand, the ratio of type IIb fibers was dramatically elevated ($P < 0.001$) in the Dex-treated WT mice compared with that of the vehicle-treated WT mice, and the percentage of type IIb fibers in the Dex-treated VDR(-/-) mice was increased ($P < 0.001$) in comparison to that of the Dex-treated WT mice.

VDR inactivation affected the expressions of muscle regulatory factors (MRFs)

The expressions of regulators for muscle mass in WT and VDR(-/-) mice were detected (Fig. 4A&C). Dex treatment significantly up-regulated MSTN mRNA expression in both WT and VDR(-/-) mice (Fig. 4B, $P < 0.001$) and a higher expression level of MSTN was detected in VDR(-/-) mice ($P < 0.001$) compared with WT mice. The mRNA and protein expressions of MyoD at both mRNA level (Fig. 4B) and protein level (Fig. 4D) as well as the protein expression of MHC (Fig. 4D) were significantly down-regulated in the tibialis anterior of mice by Dex treatment compared with the vehicle control ($P < 0.001$); lower expression levels of these

factors were detected in VDR(-/-) mice compared with WT mice.

A decrease in muscle mass is usually associated with the up-regulation of ubiquitin-protein ligases (E3), such as MURF1 and MAFbx, which was further measured in mice tibialis anterior (Fig. 4C). Dex treatment significantly increased the protein expressions of MURF1 and MAFbx (Fig. 4D), and these up-regulations were much higher in VDR(-/-) mice than in WT mice.

PI3K/Akt/FOXO signaling was attenuated in the Dex-treated VDR(-/-) mice

The protein expression of upstream signaling pathway PI3K/Akt/FOXO, which regulates the expression of MURF1 and MAFbx, was further determined in mice tibialis anterior (Fig. 4E). Dex treatment (Fig. 4F) inhibited the phosphorylation of PI3K ($P < 0.001$), Akt ($P < 0.05$) and FOXO1 ($P < 0.01$). Those protein expression levels were much lower in VDR(-/-) mice than in WT mice ($P < 0.05$).

Local renin-angiotensin system (RAS) in skeletal muscle was involved in the regulation of muscle atrophy mediated by VDR

The RAS components angiotensinogen (AGT), renin, and Ang II were measured in the tibialis anterior muscles of mice (Fig. 5A&C). The mRNA (Fig. 5B, $P < 0.001$) and protein (Fig. 5D, $P < 0.01$) expressions of renin, mRNA expression of AGT (Fig. 5B, $P < 0.001$), and protein expression of Ang II (Fig. 5D, $P < 0.001$) were markedly up-regulated and more significant in the Dex-treated VDR(-/-) mice than in the Dex-treated WT mice. However, there were no significant differences in the serum

level of Ang II among all groups (Fig. 5E).

VDR knockout enhanced the destructive effects of Ang II in muscle satellite cells

To clarify the role of VDR in myogenesis, the effects of Ang II on myogenic differentiation and myogenic factors were evaluated in muscle satellite cells (SCs) isolated from mice hind limbs (Fig. 6A). Western blotting showed that Ang II induced higher expression of MURF1 (Fig. 6B, $P < 0.01$) and MAFbx (Fig. 6B, $P < 0.001$) in comparison to those of the respective vehicle-treated group, and more profound in VDR(-/-) mice. Similarly, the protein expressions (Fig. 6B) of MHC and MyoD were markedly down-regulated ($P < 0.05$) in SCs by Ang II treatment in VDR(-/-) mice. The involvement of PI3K/Akt/FOXO1 signaling was also evaluated in SCs (Fig. 6C). Ang II treatment inhibited the phosphorylation of PI3K ($P < 0.001$), Akt ($P < 0.01$) and FOXO1 ($P < 0.01$) in SCs (Fig. 6D), and a lower protein expression ratio of p-FOXO1/FOXO1 was detected in VDR(-/-) mice ($P < 0.05$).

Calcitriol improved skeletal muscle function in mice with muscle atrophy

To further explore the role of VDR in skeletal muscle function of mice with Dex-induced muscle atrophy, the protective effects of calcitriol were examined. Calcitriol did not affect the body weight of mice (Fig. 7A), but it significantly increased their skeletal muscle mass (Fig. 7B, $P < 0.05$) as demonstrated by the elevation in muscle index of tibialis anterior and gastrocnemius, and improved muscle function (Fig. 7C) as shown by the enhancement in weight-loaded swimming time (*P*

< 0.05) and grip strength ($P < 0.001$). Moreover, the myofiber area of tibialis anterior (Fig. 7D&E, $P < 0.05$) and dystrophin-positive myotubes (Fig. 7F&G, $P < 0.01$) were dramatically increased in the calcitriol-treated groups compared with those in the vehicle-treated model group.

Calcitriol improved fiber type composition in mice with muscle atrophy

The beneficial effects of vitamin D on skeletal muscle were also illustrated by the changes in the distribution of fiber type in gastrocnemius (Fig. 7H). The quantitative data clearly showed that calcitriol enhanced the density of type I fibers ($P < 0.001$) and type IIa fibers ($P < 0.01$) and dose-dependently attenuated the distribution of type IIb fibers ($P < 0.001$) compared with those in the muscle atrophy group.

Calcitriol regulated the MRFs in mice with muscle atrophy

The mRNA expressions (Fig. 8B) of MSTN ($P < 0.001$) and MyoD ($P < 0.001$) were increased and decreased, respectively, in tibialis anterior of mice with Dex-induced skeletal muscle atrophy (Fig. 8A). Calcitriol down-regulated MSTN mRNA expression by 52% ($P < 0.001$) and up-regulated MyoD mRNA expression by 50% ($P < 0.001$). The effects of calcitriol on protein expression of ubiquitin ligases and their substrates were evaluated by immunoblotting (Fig. 8C). Calcitriol at both low and high dosages significantly down-regulated protein expressions of MURF1 ($P < 0.001$) and MAFbx ($P < 0.01$) compared with those treated by vehicle (Fig. 8D). Furthermore, the substrate proteins of ubiquitin ligases for myogenesis, including MHC and MyoD,

were significantly up-regulated ($P < 0.001$) by calcitriol.

Calcitriol ameliorated the Dex-induced effects on expression of RAS components

Expressions of RAS components, including mRNA level (Fig. 8E) of AGT and renin and protein level (Fig. 8G) of renin and Ang II, were detected in tibialis anterior. Gene expressions of AGT and renin were significantly lower (Fig. 8F, $P < 0.001$) in the calcitriol-treated group than those in the vehicle-treated atrophic group. Calcitriol treatment down-regulated the protein level (Fig. 8H) of renin ($P < 0.05$) and Ang II ($P < 0.001$) in the tibialis anterior of mice with muscle atrophy induced by Dex in a dose-dependent manner.

Discussion

Sarcopenia is characterized by a progressive and involuntary loss of skeletal muscle mass and muscle strength and/or reduced physical performance.[17] Interest in clinic and basic research on skeletal muscle health has burgeoned internationally.[22] A wealth of studies and academic consensuses about sarcopenia from Asia[17] and Europe[23] have demonstrated that lower vitamin D level is associated with skeletal muscle atrophy, increased risk for chronic musculoskeletal pain, and sarcopenia.[3,5,24] The active vitamin D molecule, $1,25(OH)_2D_3$, displays multiple biological actions via binding to vitamin D receptor (VDR) expressed in multiple tissues and cells. However, the molecular mechanisms of vitamin D signaling involved in maintaining muscle homeostasis have not been fully clarified.

The present data from a cross-sectional analysis of participants from two communities in Shanghai (China) in our study demonstrated a close positive association between circulating total 25(OH)D level and handgrip strength with the findings that those in the highest forth of serum 25(OH)D concentrations had approximately 40% reduced odds of low muscle strength, suggesting a clinical link between *in vivo* vitamin D nutritional level and muscle strength. Though the group assignment of low muscle strength in this study was based only on low grip strength, the evidence from the human part of the study is correlative in nature. More importantly, in agreement with the fact that vitamin D is a negative endocrine regulator of renin-angiotensin system (RAS)[15] and that vitamin D deficiency was followed by hyperactivity of RAS as observed in metabolic syndrome,[25] we

demonstrated in this study that the concentration of circulating Ang II apparently increased associated with the reduction in serum vitamin D level of subjects with decreased muscle strength. These results pointed out the possibility that the vitamin D insufficiency-induced elevation in Ang II level might be one of the risk factors of low muscle strength.

The mouse C2C12 myoblasts were used to further elucidate the *in vitro* effects of Ang II on myogenesis. Our study verified that angiotensin II type 1 receptor blocker (ARB) effectively blocked the Ang II-evoked decrease in formation and size of myotubes. It was well reported that the ubiquitin-proteasome system plays a crucial role in the regulation of skeletal muscle metabolism,[26] and a reduction in muscle mass is associated with the up-regulation of ubiquitin ligases, which could be activated by Ang II.[27] MAFbx and MURF1, two muscle-specific ubiquitin-protein ligases (E3), have been identified to be strongly up-regulated in different atrophy models.[27] MAFbx promotes the degradation of MyoD, a key muscle transcription factor regulating myoblast transition from proliferation to differentiation, and MURF1 acts on the protein substrate MHC, one of the sarcomeric proteins, to accelerate muscle proteolysis.[26,28] This study delineated that ARB successfully suppressed the Ang II-induced up-regulation of MAFbx and MURF1, leading to an elevation of protein expression of MyoD and MHC, and confirmed that Ang II could trigger muscle atrophy through affecting muscle protein synthesis and degradation via interfering with myogenic differentiation.

Together the clinical findings and the *in vitro* results from the present study implied

that the high production of Ang II due to vitamin D deficiency might account for the loss of muscle mass and the impairment of muscle strength. To further confirm that, VDR-null mice were applied to evaluate the sensitivity of skeletal muscle to the induction of dexamethasone (Dex), which was used for developing a classical model of muscle atrophy.[19] The similar studies using mice with systemic genetic ablation of VDR were previously carried out to explore the role of VDR in diseases like acute lung injury,[29] acute kidney injury[30] and colonic injury.[31] As expected, treatment with Dex resulted in the decrease in muscle mass (muscle weight of tibialis anterior and gastrocnemius), muscle function (weight-loaded swimming time and grip strength) and muscle fiber size as shown by H&E staining and anti-dystrophin immunostaining. Most importantly, the results clearly showed that VDR-null mice developed much more severe injuries in skeletal muscle after challenging with Dex, strongly suggesting that VDR signaling might be beneficial to skeletal muscle.

The type and composition of muscle fibers directly influence skeletal muscle fitness and function.[32] The distinct myofiber types, such as type I (slow oxidative myofiber), type IIa (fast oxidative myofiber), and type IIb (fast glycolytic myofiber), are clarified by specific staining on MHC isoforms. This study discovered that Dex stimulated a decrease in the density of type I and type IIa muscle fibers and an enhancement in the density of type IIb muscle fibers, suggesting an inhibition on glycolytic-to-oxidative (GTO) fiber type switch, which was remarkably robust in the muscle of VDR(-/-) mice in comparison to WT mice. Therefore, VDR signaling appeared to manage skeletal muscle oxidative capacity by regulating muscle metabolic phenotype as

shown by the alteration in the composition of muscle fibers.

The following results from the molecular analysis on VDR KO mice skeletal muscle tissue and VDR KO mice muscle satellite cells simultaneously showed that the expressions of ubiquitin ligases (MURF1 and MAFbx) and the expressions of myogenic factors MyoD and MHC were increased and decreased, respectively, more in VDR(-/-) mice than in WT mice in both the Dex-induced atrophic muscle tissue and the Ang II-incubated muscle satellite cells. The muscle satellite cells, also called muscle stem cells possessing myogenic activities, could differentiate into myoblasts and myotubes accompanied by the expression of differentiation marker like MyoD, by which they maintain muscle homeostasis under pathological condition and external stimuli.[33,34] Together, the present data revealed that the ablation of VDR led to more severe muscle atrophy partially due to more damages in myogenesis.

The main mechanism behind the over expression of ubiquitin ligases in VDR(-/-) mice appeared to be the mediation of PI3K/Akt/FOXO1 on skeletal muscle. FOXO1, a transcription factor among the FOXO family members in muscle, could activate the expression of MURF1 and MAFbx. Akt phosphorylates FOXO1 protein, promotes its export from the nucleus to the cytoplasm, and consequently blocks the up-regulation of MURF1 and MAFbx.[26] Our results supported the view that the inactivation of VDR exacerbated the suppression of PI3K/Akt/FOXO1 signaling in both the Dex-induced damaged muscle tissue and Ang II-stimulated muscle satellite cells, in accordance with an earlier study which showed that VDR-dependent activation of PI3K/Akt contributed to the anti-apoptotic effects of $1,25(OH)_2D_3$ in osteoblasts.[35] Most striking

in the present study was the finding that the expression of RAS components like renin and its down-stream peptide Ang II was dramatically elevated in the Dex-treated group, and more prominent in the VDR KO group. The accumulating data have revealed that Ang II could trigger down-stream biological events, especially the eliciting of local tissue injuries via regulating PI3K/Akt signaling pathway.[36,37] Collectively, VDR seemed to ameliorate muscle atrophy in a large possibility via repressing tissue RAS, sequentially activating PI3K/Akt/FOXO1 pathway.

To finally confirm the role of VDR in the development of muscle atrophy, the protective effects of $1,25(OH)_2D_3$ calcitriol were evaluated in Dex-induced muscle atrophy model. The present *in vivo* study clearly elucidated that calcitriol could raise muscle weight, enhance muscle strength, and improve skeletal muscle phenotypes including myotube size and myofiber type. Notably, the improvement on swimming was particularly pronounced in the group that received low dose of calcitriol, and for other parameters the same as in low and high dose. This suggested that at even higher doses the benefits of calcitriol in skeletal muscle might disappear. Treatment with calcitriol could facilitate the GTO myofiber switch as shown by the increased proportion of type I and type IIa oxidative myofibers. This sheds new light on the potency of vitamin D in improving endurance in a manner similar to exercise.[32] While, a meta-analysis summarized that vitamin D supplementation produced mild positive action on muscle strength,[38] and one RCT study showed that vitamin D-fortified foods did not change muscle strength.[39] The supplemental dose, mode of administration, and intervention time using vitamin D might explain the contradictory clinical findings.

Thus, the more evidence-based studies with accurate design are required to explore the optimal vitamin D treatment modalities.

The molecular analysis reconfirmed that the administration of calcitriol could remarkably mitigate the stimulation of Dex on the expression of RAS components in muscle. Combined with our previous studies, we have explored the suppressive effects of vitamin D and its analogue on local RAS in the hypothalamus,[12] kidney[8,9] and in muscle as shown in this study, therefore vitamin D exerted protection against neuroinflammation, renal fibrosis and muscle atrophy, respectively. Furthermore, the regulation of calcitriol on muscle RAS might account for its effects on the ubiquitin ligases and the down-stream myogenic factors MHC and MyoD. Taken together, calcitriol exhibited beneficial effects on skeletal muscle via attenuating the activity of muscle tissue RAS.

Our study is the first to delineate the role of VDR in maintaining muscle homeostasis by using VDR KO mice. We demonstrated that VDR signaling exerted beneficial effects on muscle mass and function by affecting the ubiquitin ligases and myogenic differentiation through locally suppressing renin-angiotensin system in skeletal muscle, and calcitriol, the active vitamin D, could be a potential agent for the prevention and treatment of skeletal muscle atrophy, and even sarcopenia.

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Authors' roles: YZ and YW designed the study; YZ wrote the manuscript; CP and MW revised the manuscript; WL, RF and SL performed the study; WL, XQ, JW, FL and YS analyzed and interpreted the data; and all authors read and approved the final version of the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

DATA Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Figure legends

Fig 1. Selection of participants in this study and association analysis and angiotensin II content in serum. (A) flowchart of inclusion of study participants. (B) the association between serum vitamin D level and low muscle strength in total population after adjustment for age, sex, BMI, smoking and drinking habits. (C) the serum level of Ang II in those with quartile 1 of serum 25(OH)D level (<34.0 nmol/L) and with quartile 4 of serum 25(OH)D level (≥54.7 nmol/L). Data are presented as violinplot (with median and interquartile ranges) (Fig. 1C). *P* value was obtained by paired two-tailed *t* test.

Fig 2. Effects of Ang II on myotubes differentiated from murine myoblasts C2C12. The cultured C2C12 cells were differentiated to myotubes by horse serum and then treated with Ang II (100 nM) in the absence or presence of Ang II type 1 receptor blocker (ARB) olmesartan (1 μM). (A) MHC-positive immunofluorescence images. (B) cross-sectional area (CSA) of myotubes. (C) frequency distribution of myotubes CSA. (D & F) immunoblotting detection of protein expression of muscle regulatory factors (MRFs). (E & G) the quantitative data for targeted proteins. Data are presented as boxplots (with median and interquartile ranges). *P* value was obtained by one-way and two-way ANOVA.

Fig 3. VDR ablation led to severe skeletal muscle atrophy and influenced the

distribution of muscle fiber types after dexamethasone challenge. (A) changes in percentage of body weight during experimental period. The muscle mass (B), shown by muscle weight divided by body weight, was measured in tibialis anterior and gastrocnemius. The weight-loaded swimming time and the grip strength were determined to evaluate skeletal muscle strength (C). The sections of the tibialis anterior underwent H&E staining (D) and anti-dystrophin immunostaining (F), and the respective quantitative data on myofibers size were shown in (E) and (G). (H) the immunofluorescence staining on the frozen sections of the gastrocnemius showed type I (blue), type IIa (green) and type IIb (red) myofibers, and the respective percentage of muscle fiber types. Data are presented as boxplots (with median and interquartile ranges). *P* value was obtained by one-way and two-way ANOVA.

Fig 4. VDR knockout produced more profound effects on MRFs and PI3K/Akt/FOXO1 signaling. (A) mRNA expression of MSTN and MyoD. (B) the quantitative data on target genes. (C) protein expression of MURF1, MAFbx, MHC and MyoD. (D) the quantitative data on target proteins. (E) protein expression of molecular components of PI3K/Akt/FOXO1 signaling. (F) the expression ratio of p-PI3K/PI3K, p-Akt/Akt and p-FOXO1/FOXO1 was quantitatively displayed. Data are presented as boxplots (with median and interquartile ranges). *P* value was obtained by two-way ANOVA.

Fig 5. VDR ablation increased the expression of RAS components in tibialis anterior

of Dex-treated mice. (A) mRNA expression of AGT and renin. (B) the quantitative data on target genes. (C) protein expression of renin and Ang II. (D) the quantitative data on target proteins. (E) serum Ang II level. Data are presented as boxplots (with median and interquartile ranges). *P* value was obtained by one-way and two-way ANOVA.

Fig 6. VDR knockout produced more robust effects on MRFs and PI3K/Akt/FOXO1 signaling during myogenic differentiation of muscle satellite cells isolated from hind limbs of mice. The satellite cells were activated and differentiated by horse serum and treated with vehicle or Ang II (100 nM). (A) protein expression of MURF1, MAFbx, MHC and MyoD. (B) the quantitative data on target proteins. (C) protein expression of molecular components of PI3K/Akt/FOXO1 signaling. (D) the expression ratio of p-PI3K/PI3K, p-Akt/Akt and p-FOXO1/FOXO1 was quantitatively shown. Data are presented as boxplots (with median and interquartile ranges). *P* value was obtained by one-way and two-way ANOVA.

Fig 7. Treatment of mice with calcitriol alleviated muscle atrophy and improved the distribution of muscle fiber types. (A) body weight. Muscle mass (B), shown by muscle weight divided by body weight, was measured in tibialis anterior and gastrocnemius. Weight-loaded swimming time and grip strength were determined to evaluate skeletal muscle strength (C). The sections of the tibialis anterior underwent H&E staining (D) and anti-dystrophin immunostaining (F), and the respective

quantitative data on myofibers size were shown in (E) and (G). (H) the immunofluorescence staining on the frozen sections of the gastrocnemius showed type I (blue), type IIa (green) and type IIb (red) fibers, and the respective percentage of muscle fiber types. Data are presented as boxplots (with median and interquartile ranges). *P* value was obtained by one-way and two-way ANOVA.

Fig 8. Treatment with calcitriol reversed the effects of Dex on MRFs and repressed the Dex-induced up-regulation in expression of RAS components in tibialis anterior of mice. (A) mRNA expression of MSTN and MyoD. (B) the quantitative data on target genes. (C) protein expression of MURF1, MAFbx, MHC and MyoD. (D) the quantitative data on target proteins. (E) mRNA expression of AGT and renin. (F) the quantitative data on target genes. (G) protein expression of renin and Ang II. (H) the quantitative data on target proteins. Data are presented as boxplots (with median and interquartile ranges). *P* value was obtained by two-way ANOVA.

Table 1 Characteristics of participants with and without low muscle strength

	normal muscle strength (n = 834)	low muscle strength (n = 200)	*P* value
Age, year	64.6 ± 6.9	68.9 ± 7.7	<0.001
Sex			0.018
Female	607 (72.8%)	128 (64.0%)	
Male	227 (27.2%)	72 (36.0%)	
BMI, kg/m^2	24.1 ± 3.3	24.2 ± 3.4	0.844
Muscle strength, kg	26.7 ± 6.9	18.1 ± 5.3	<0.001
Vitamin D, nmol/L	45.7 ± 16.7	43.2 ± 16.8	0.012
Ang II, pg/mL[a]	0.61 ± 0.30	0.73 ± 0.33	0.135
Smoking			0.408
Never	714 (85.6%)	164 (82.0%)	
Current	76 (9.1%)	24 (12.0%)	
Ever	44 (5.3%)	12 (6.0%)	
Drinking alcohol			0.793
Never	729 (87.4%)	174 (87.0%)	
Current	83 (10.0%)	19 (9.5%)	
Ever	22 (2.6%)	7 (3.5%)	

Data are presented as means ± standard deviation (SD) and proportion for continuous and categorical variables, respectively.

[a] Ang II, angiotensin II. Only 80 participants were tested for Ang II.

Table 2 Association of **s**erum 25(OH)D level with muscle strength

Vitamin D (nmol/L)	N	Events (%)	Crude model		Adjusted model*	
			OR (95%CI)	*P*	OR (95%CI)	*P*
Continuous, per SD (16.7) increment	1034	200 (19.3)	0.85 (0.73,1.00)	0.055	0.84 (0.71,0.99)	0.043
Quartiles						
Q1(<34.0)	249	65 (26.1)	1 (Reference)		1 (Reference)	
Q2(34.0-<43.3)	261	50 (19.2)	0.67 (0.44,1.02)	0.061	0.70 (0.45,1.08)	0.108
Q3(43.3-<54.7)	265	40 (15.1)	0.50 (0.32,0.78)	0.002	0.48 (0.30,0.77)	0.002
Q4(≥54.7)	259	45 (17.4)	0.60 (0.39,0.91)	0.018	0.58 (0.37,0.90)	0.017
P for trend			0.007		0.005	
Categories						
Q1(<34.0)	249	65 (26.1)	1 (Reference)		1 (Reference)	
Q2-4(≥34.0)	785	135 (17.2)	0.59 (0.42,0.82)	0.002	0.58 (0.41,0.83)	0.003
Male						
Q1(<34.0)	63	20 (31.7)	1 (Reference)		1 (Reference)	
Q2-4(≥34.0)	236	52 (22.0)	0.61 (0.33,1.12)	0.111	0.58 (0.30,1.11)	0.099
Female						
Q1(<34.0)	186	45 (24.2)	1 (Reference)		1 (Reference)	
Q2-4(≥34.0)	549	83 (15.1)	0.56 (0.37,0.84)	0.005	0.57 (0.37,0.88)	0.010

* Adjusted for age, sex, BMI, smoking, alcohol drinking

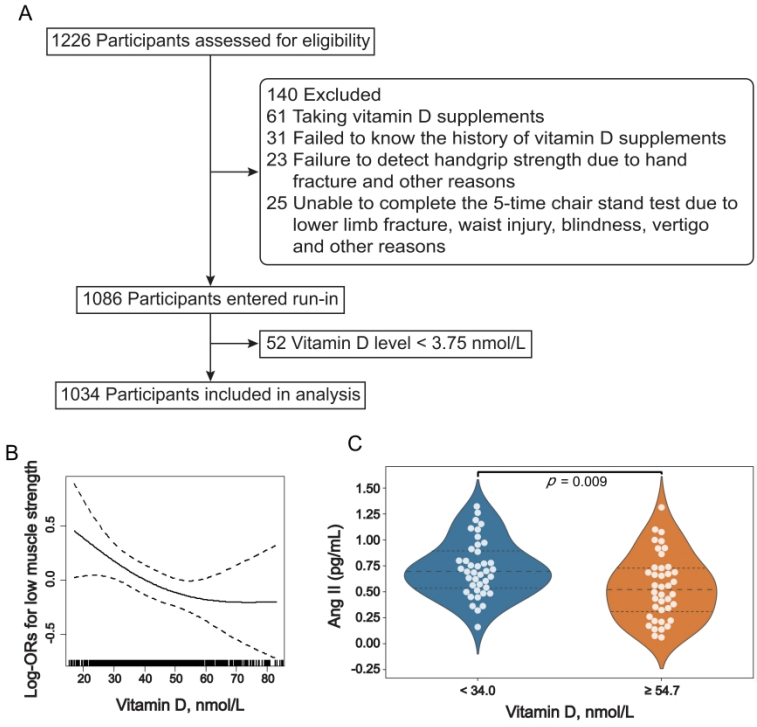

Figure 1

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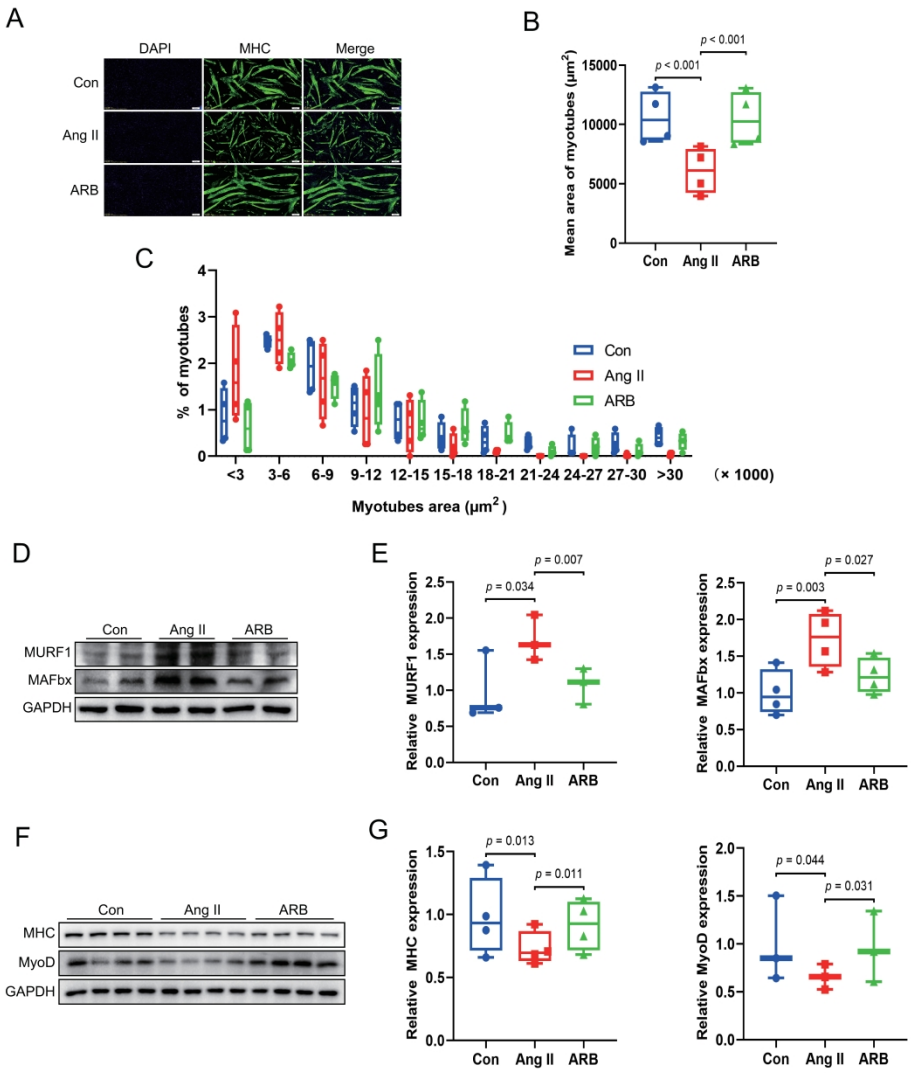

Figure 2

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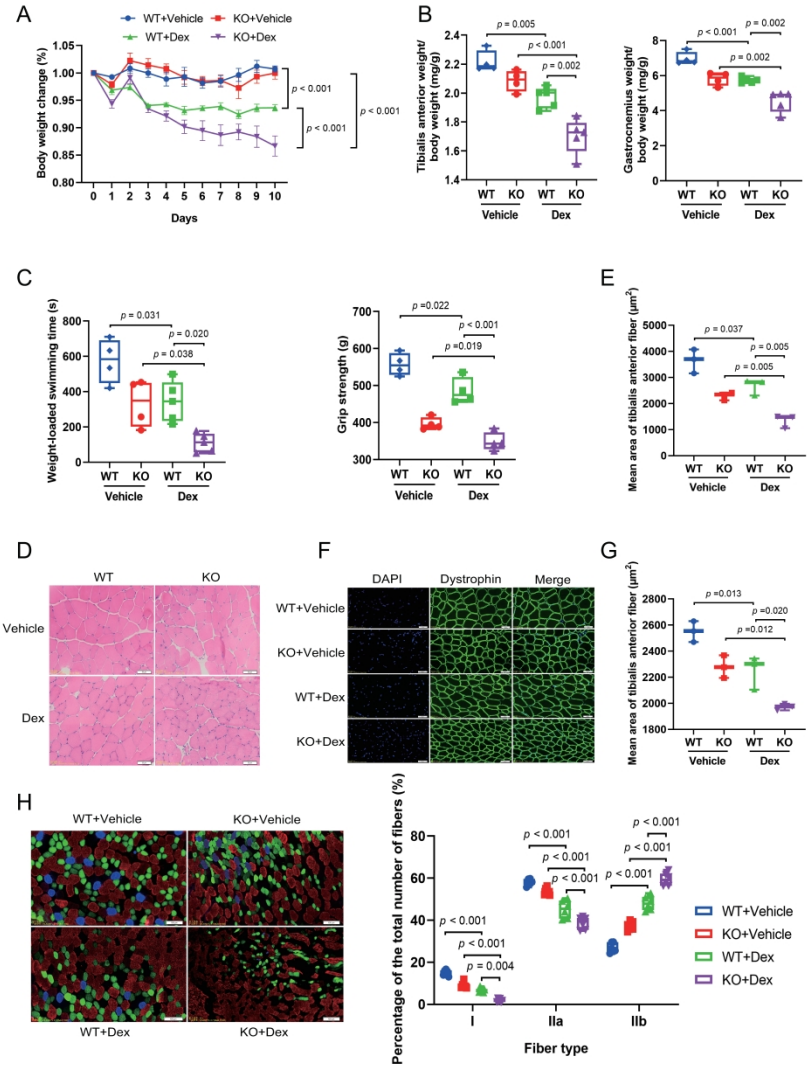

Figure 3

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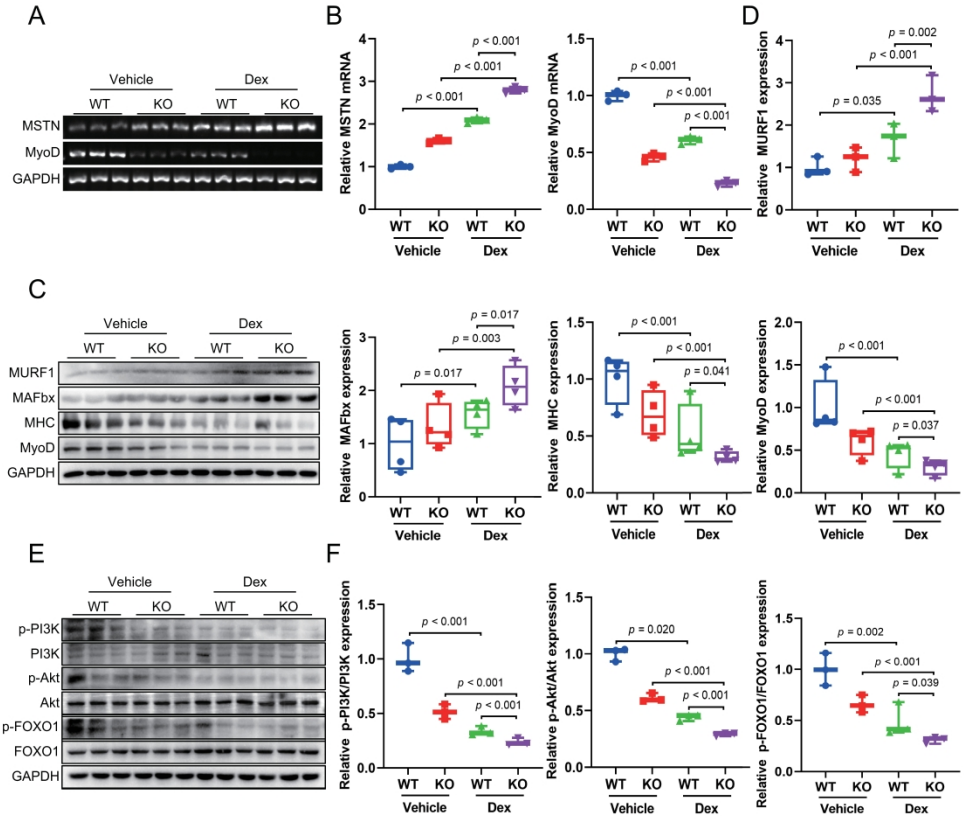

Figure 4

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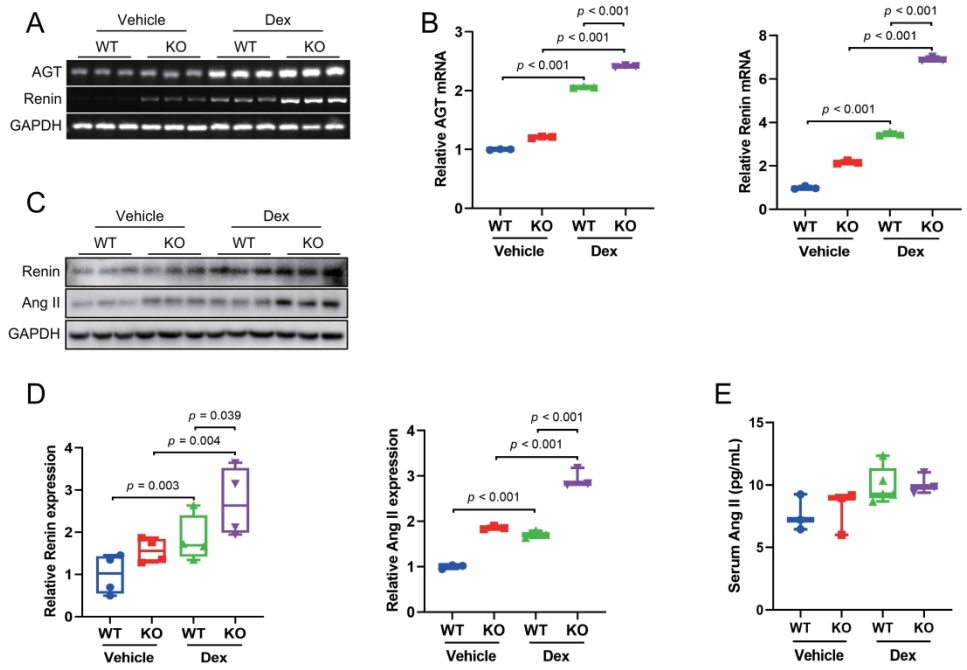

Figure 5

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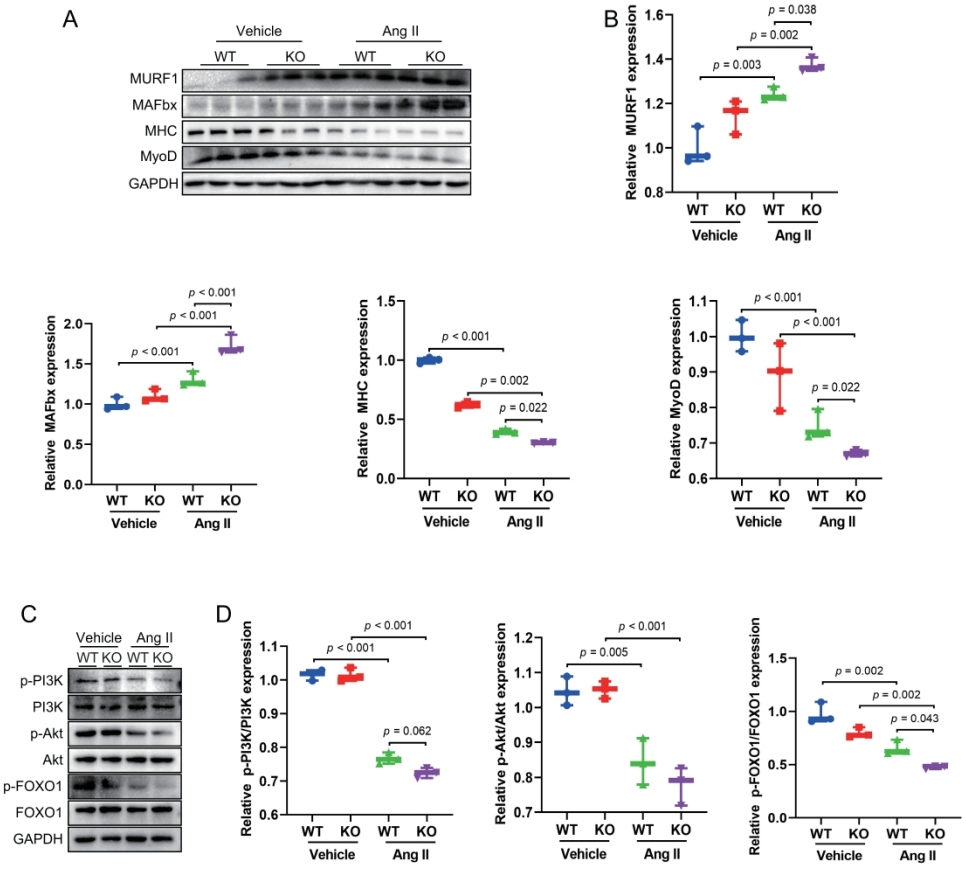

Figure 6

447x396mm (600 x 600 DPI)

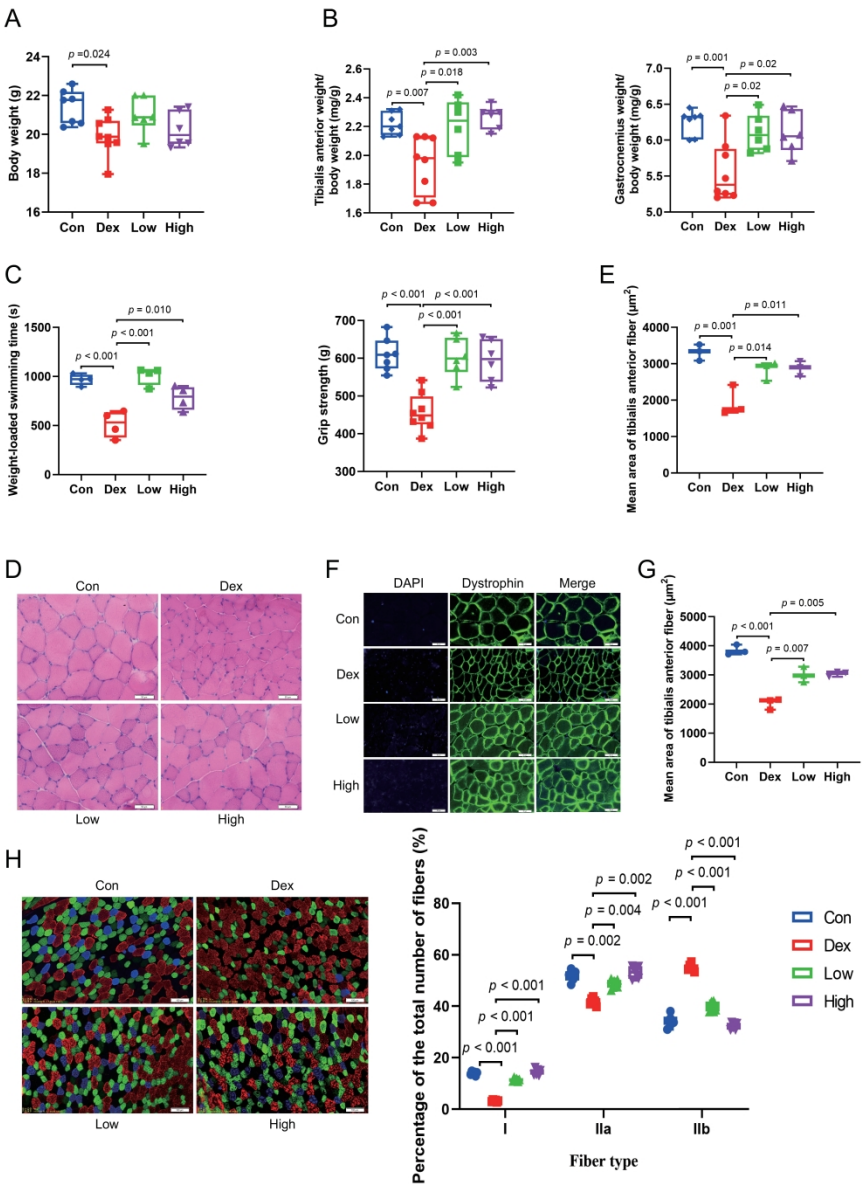

Figure 7

436x579mm (600 x 600 DPI)

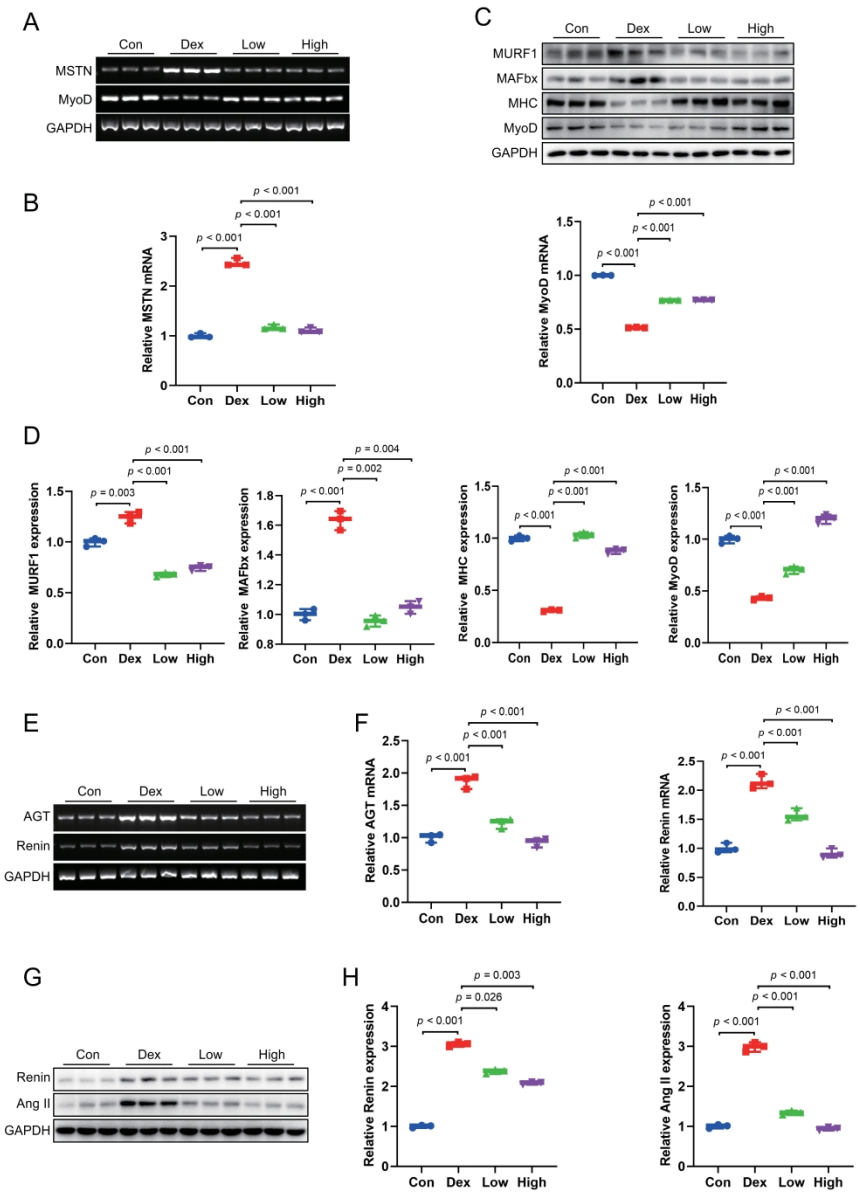

Figure 8

431x580mm (600 x 600 DPI)