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ORIGINAL ARTICLE

The maintenance ability and Ca^{2+} availability of skeletal muscle are enhanced by sildenafil

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Sildenafil relaxes vascular smooth muscle cells and is used to treat pulmonary artery hypertension as well as erectile dysfunction. However, the effectiveness of sildenafil on skeletal muscle and the benefit of its clinical use have been controversial, and most studies focus primarily on tissues and organs from disease models without cellular examination. Here, the effects of sildenafil on skeletal muscle at the cellular level were examined using mouse primary skeletal myoblasts (the proliferative form of skeletal muscle stem cells) and myotubes, along with single-cell Ca^{2+} imaging experiments and cellular and biochemical studies. The proliferation of skeletal myoblasts was enhanced by sildenafil in a dose-independent manner. In skeletal myotubes, sildenafil enhanced the activity of ryanodine receptor 1, an internal Ca^{2+} channel, and Ca^{2+} movement that promotes skeletal muscle contraction, possibly due to an increase in the resting cytosolic Ca^{2+} level and a unique microscopic shape in the myotube membranes. Therefore, these results suggest that the maintenance ability of skeletal muscle mass and the contractility of skeletal muscle could be improved by sildenafil by enhancing the proliferation of skeletal myoblasts and increasing the Ca^{2+} availability of skeletal myotubes, respectively.
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INTRODUCTION

Skeletal muscle is composed of myotubes, which are long-cylindrical and multi-nucleated cells (also called myofibers).[1,2] In the postnatal and adult periods, satellite cells (that is, skeletal muscle stem cells) in skeletal muscle have qualities of mitotic quiescence and self-renewal, and they proliferate in response to regenerative cues, such as injury or exercise, to repair and maintain skeletal muscle.[3] Proliferative skeletal muscle satellite cells, generally called myoblasts, migrate to align closely together, fuse to one another (and/or fuse to immature myotubes) and become multi-nucleated myotubes, a process that is called differentiation.[3] Therefore, migration and fusion of myoblasts are important steps for the differentiation to myotubes and for the regenerative activity of skeletal muscle.[3,4] Cytosolic Ca^{2+} elevation to activate various signaling pathways is required for myoblast migration and fusion.[5,6] In addition, myogenic regulatory factors (MRFs) have key roles in differentiation as follows: primary MRFs such as MyoD are required for the determination of myoblasts and are necessary for retaining the expression of muscle-related genes and secondary MRFs such as myogenin are expressed on differentiation and regulate differentiation.[7,8]

Body posture and locomotion are accomplished by skeletal muscle contraction operated by excitation–contraction (EC) coupling.[9–11] During skeletal EC coupling, α-motor neurons depolarize the transverse (t)-tubule membranes of skeletal myotubes. The dihydropyridine receptor (DHPR, a Ca^{2+} channel in the t-tubule membrane) senses the depolarization and, in turn, activates ryanodine receptor 1 (RyR1, a Ca^{2+} channel on sarcoplasmic reticulum (SR) membrane) by physical interactions. Ca^{2+} ions in the SR are released into the cytosol through the activated RyR1, and these Ca^{2+} ions ultimately activate contractile proteins for skeletal muscle contraction. RyR1 is also activated by Ca^{2+} and releases Ca^{2+} from the SR, which is called Ca^{2+}-induced Ca^{2+} release (CICR); however, CICR is not predicted to contribute significantly to physiological Ca^{2+} supply for volitional skeletal muscle contraction.[9,12,13] Extracellular Ca^{2+} entry in skeletal myotubes via either the Orai1 or the canonical-type transient receptor potential cation channel 3 (TRPC3)

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contributes to maximizing the Ca^{2+} supply.[14–16] During skeletal muscle relaxation, to reduce cytosolic Ca^{2+} levels for rest and to replenish the SR with Ca^{2+}, sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase 1a (SERCA1a) takes up Ca^{2+} from the cytosol to the SR.[9–11] Entry of extracellular Ca^{2+} through the DHPR also contributes to replenishing the SR with Ca^{2+}.[17] A close and efficient arrangement of the proteins described above is maintained by the formation of junctional membrane complexes in which t-tubule and the SR membranes are closely juxtaposed.[10,11,18,19]

Sildenafil, a specific inhibitor of phosphodiesterase type 5 (PDE5), has been clinically used to treat pulmonary artery hypertension and erectile dysfunction because of its vasodilatation effect on vascular smooth muscle by blocking the degradation of intracellular cyclic guanosine monophosphate (cGMP), subsequently amplifying cGMP-dependent signaling in smooth muscle cells.[20–22] However, the effectiveness of sildenafil on skeletal muscle and the benefit of its clinical use have been controversial–positive, negative or no effect. Sildenafil reduces fatigue of the knee extensors in generally healthy males[23] and decreases exaggerated skeletal muscle fatigue in a mouse model of Duchenne muscular dystrophy (DMD).[24] Sildenafil enhances the grip-strength of skeletal muscle in mice[25] and alleviates exercise-induced skeletal muscle ischemia in boys with DMD.[26] However, in addition to these positive effects, some negative and neutral effects of sildenafil on skeletal muscle have been also reported. Sildenafil promoted dystrophic pathology in a mouse DMD model[27] and induced the atrophy of skeletal muscle in rats.[28] Sildenafil does not enhance walking tolerance in patients with intermittent claudication.[29] In addition to 'these discrepancies', most studies on sildenafil in skeletal muscle have been conducted using tissues or organs from 'disease models', and the functional effects of sildenafil on normal skeletal muscle at the cellular level have not been well examined. Therefore, in the present study, the effects of sildenafil on the proliferation and differentiation of skeletal myoblasts and on differentiated skeletal myotubes were examined using primary skeletal myoblasts and myotubes from normal mouse.

MATERIALS AND METHODS

Ethics statement

All surgical interventions and pre- and post-surgical animal care were provided in accordance with the Laboratory Animals Welfare Act, the Guide for the Care and Use of Laboratory Animals and the Guidelines and Policies for Rodent Survival Surgery provided by the Institutional Animal Care and Use Committee of the College of Medicine at The Catholic University of Korea.

Cell culture and sildenafil treatment

Mouse skeletal satellite cells were isolated from mouse skeletal muscle, and a 'single satellite cell clone' was allowed to proliferate (that is, primary skeletal myoblasts) or differentiate to myotubes, as previously described.[18,30–33] Briefly, primary myoblasts were cultured on 10-cm plates coated with collagen in growth medium (F-10 Nutrient Mixture (HyClone Laboratories, Logan, UT, USA) containing 20% fetal bovine serum (FBS), 100 units ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, 2 mM L-glutamine and 20 nM basic FGF) at 37 °C in a 5% CO_2 incubator. For the differentiation of myoblasts into myotubes, myoblasts were replated either on 10-cm plates (for the quantitative RT-PCR (qPCR) analysis or for the preparation of myotube lysate) or on 96-well plates (for the single myotube Ca^{2+} imaging experiment) coated with Matrigel (BD Biosciences, San Jose, CA, USA). When the myoblasts reached ~70% confluence, the growth medium was replaced with differentiation medium (5% heat-inactivated horse serum and low-glucose DMEM without growth factors instead of 20% FBS and F-10 Nutrient Mixture in the growth medium), and the myoblasts were placed in an 18% CO_2 incubator for 5 days to induce differentiation. For treatment, various amounts of sildenafil (Sigma-Aldrich, St Louis, MO, USA) dissolved in dimethyl sulfoxide (DMSO, <0.05%) were added to the growth or differentiation medium, and the medium was changed to fresh medium once per day. All reagents for cell culture were obtained from Invitrogen or Gibco (Waltham, MA, USA).

Proliferation and migration assay

For the proliferation assay, images of primary skeletal myoblasts on 10-cm culture dishes were randomly captured, and the number of myoblasts in 0.33 mm^2 was counted. The cell migration assay was performed in accordance with previously described methods.[34] Briefly, myoblasts grown on 10-cm plates were scraped off using a pipette tip to produce a long and thin acellular area, and the growth medium was then treated with sildenafil. To evaluate the number of migrated myoblasts into the acellular area, images of the acellular area were obtained immediately after scraping and sildenafil treatment, and images of the same areas were obtained again at 12 h post-scraping. The number of myoblasts that migrated into the acellular area (0.24 mm^2) was counted.

qPCR analysis

The qPCR was performed as previously described.[34] The complimentary DNA (cDNAs) were synthesized using total RNA from primary skeletal myotubes treated with sildenafil. The qPCR was performed using 20 ng of synthesized cDNA, the primers presented in Table 1, a SYBR Green PCR kit (Invitrogen, Waltham, MA, USA) and an i-Cycler PCR thermocycler (Bio-Rad Laboratories, Hercules, CA, USA): denaturation at 95 °C for 15 s, annealing at 58 °C for 30 s and extension at 72 °C for 30 s (in duplicate, eight sets per each). The obtained values were normalized to those from the corresponding α-actin samples.

Single myotube Ca^{2+} imaging experiment

Single myotube Ca^{2+} imaging experiments were performed as previously described.[18,30–33] Primary skeletal myotubes on 96-well

Table 1 List of qPCR primers

	qPCR primers
Myogenin	
Forward	5′–TTGCTCAGCTCCCTCAACCAGGA–3′
Backward	5′–TGCAGATTGTGGGCGTCTGTAGG–3′
Actin	
Forward	5′–CCAGGCATTGCTGACAGGAT–3′
Backward	5′–AGCCACCGATCCACACAGAG–3′

Abbreviation: qPCR, quantitative RT-PCR.

plates were loaded with 5 μM fura-2-acetoxymethyl ester (AM) for the measurement of resting cytosolic Ca^{2+} levels or with 5 μM fluo-4-AM for other measurements in an imaging buffer (25 mM HEPES, pH 7.4, 125 mM NaCl, 5 mM KCl, 2 mM KH_2PO_4, 2 mM $CaCl_2$, 6 mM glucose, 1.2 mM $MgSO_4$ and 0.05% BSA) at 37 °C for 45 min. Each well of the 96-well plate was then washed three times with imaging buffer. The myotubes were transferred to an inverted stage microscope (Nikon Eclipse TS100, Melville, NY, USA) equipped with a 40× oil-immersion objective (NA 1.30). Before starting the single myotube Ca^{2+} imaging experiments, images of myotubes were captured for comparison of myotube formations and for the observation of myotube membranes and the measurement of width. During single myotube Ca^{2+} imaging, images of the myotubes were captured using a high-speed monochromator with a 75 W xenon lamp (FSM150Xe, Bentham Instruments, Verona, VA, USA) and a 12-bit charge-coupled device camera (DVC-340M-OO-CL, Digital Video Camera Company, Austin, TX, USA). The data were displayed and analyzed using image acquisition and analysis software (High-Speed InCyt Im1 for fluo-4 and InCyt Im2 for fura-2, v5.29, Intracellular Imaging, Cincinnati, OH, USA). Either caffeine or KCl was dissolved in imaging buffer and applied via an auto-perfusion system (AutoMate Scientific, Berkeley, CA, USA). To measure the amount of releasable Ca^{2+} from the SR to cytosol, thapsigargin (TG, 2.5 μM) dissolved in DMSO (<0.05%) was manually applied to myotubes in the absence of extracellular Ca^{2+}. DMSO (0.05%) alone had no effect on the release of Ca^{2+}. To analyze the Ca^{2+} release obtained from the Ca^{2+} imaging experiments, the peak amplitude, which exhibited similar increases or decreases in peak areas, was considered. For the long-term Ca^{2+} release induced by TG, both the peak area under the traces and peak amplitude (height) were analyzed. All reagents for Ca^{2+} imaging experiments were obtained from Sigma-Aldrich.

Width measurement

Measurements of the width in primary skeletal myotubes (one criterion that is used to evaluate the degree of skeletal myotube formation (that is, the degree of differentiation)[18,30–32]) were performed as previously described.[30,31] The width at the thickest part of each myotube was measured using the ImageJ program (http://imagej.nih.gov/ij/).

Immunoblot assay

Primary skeletal myotubes were solubilized as previously described,[18,30–33] and the solubilized lysate (10 μg of total protein) was subjected to SDS–PAGE (8, 10 or 12% gel). The proteins on the gel were transferred to a polyvinylidene difluoride membrane at 100 V for 1 h. The membranes were blocked with 5% (w/v) non-fat milk dissolved in PBS, incubated with a corresponding primary antibody, washed three times with PBS containing 0.1% Tween20 and then incubated with horseradish peroxidase-conjugated secondary antibodies (anti-goat (205-035-108), anti-mouse (715-035-151), anti-rabbit (711-035-152), 1:50 000 dilution, Jackson ImmunoResearch Laboratories, West Grove, PA, USA) at room temperature (24 °C). The membranes were washed three times with PBS and developed using a SuperSignal Ultra Chemiluminescent substrate (Pierce, Rockford, IL, USA). Anti-RyR1 (anti-mouse, MA3-925), anti-SERCA1a (anti-mouse, MA3-912), anti-CSQ (anti-rabbit, PA1-913), anti-CaM1 (anti-mouse, MA3-917), anti-Mg29 (also called TRIM29, anti-rabbit, PA5-30488), anti-Mg53 (also called TRIM72, anti-goat, PA5-19398), anti-JP1 (anti-rabbit, PA5-20640) and anti-JP2 (anti-rabbit, PA5-20642) antibodies (1:1000) were obtained from Thermo Scientific (Rockford, IL, USA). Anti-TRPC1 (anti-rabbit, ACC-010), anti-TRPC3 (anti-rabbit, ACC-016), anti-TRPC4 (anti-rabbit, ACC-018) and anti-TRPC6 (anti-rabbit, ACC-017) antibodies (1:800 dilution) were obtained from Alomone Laboratories (Jerusalem, Israel). Anti-DHPR (anti-mouse, ab2864), anti-Orai1 (anti-mouse, ab59330), anti-STIM1 (anti-mouse, ab57834) and anti-α-actin (anti-mouse, ab28052) antibodies (1:1000 dilution) were obtained from Abcam (Cambridge, MA, USA).

Statistical analysis

The results are presented as the means ± s.e. for the number of experiments presented in Table 2 and in the figure legends. Significant differences were analyzed using an unpaired *t*-test (GraphPad InStat, v2.04, GraphPad Software, La Jolla, CA, USA). The differences were considered significant at $P<0.05$. For the myoblast proliferation assay, the number of myoblasts at each concentration was compared with the corresponding DMSO control using *t*-tests, and a Bonferroni correction was conducted. The graphs were prepared using Origin v7 software (OriginLab, Northampton, MA, USA).

RESULTS

The proliferation of primary skeletal myoblasts is enhanced by sildenafil

To examine the effect of sildenafil on the proliferation of skeletal myoblasts, primary skeletal myoblasts (that is, proliferative form of satellite cells) were isolated from mouse skeletal muscle as described in the 'Materials and methods' section. Various concentrations of sildenafil dissolved in DMSO were added to the myoblast culture medium for different periods of time and the number of myoblasts was counted. DMSO treatments were used as controls. For different concentrations of sildenafil from 10 nM to 1 μM, 50 nM sildenafil effectively enhanced the proliferation

Table 2 Properties of primary skeletal myotubes with sildenafil treatments

	Untreated	*DMSO*	*Sildenafil*
KCl response	1.00 ± 0.06 (119)	1.00 ± 0.07 (129)	1.14 ± 0.07[a] (144)
Caffeine response	1.00 ± 0.06 (119)	0.99 ± 0.07 (129)	1.18 ± 0.07[a] (144)
Releasable Ca^{2+} from the SR	1.00 ± 0.05 (68)	0.97 ± 0.05 (62)	1.03 ± 0.05 (64)
Resting $[Ca^{2+}]_{cytosol}$, nM	87.20 ± 5.23 (99)	90.37 ± 7.51 (110)	112.86 ± 8.03[a] (103)

Abbreviations: DMSO, dimethyl sulfoxide; SR, sarcoplasmic reticulum
[a]Significant difference compared with the untreated control ($P<0.05$).
The values, except for those of resting cytosolic Ca^{2+} levels, were normalized to the mean value of those from the untreated controls. The values are presented as the mean ± s.e. for the number of myotubes shown in parentheses.

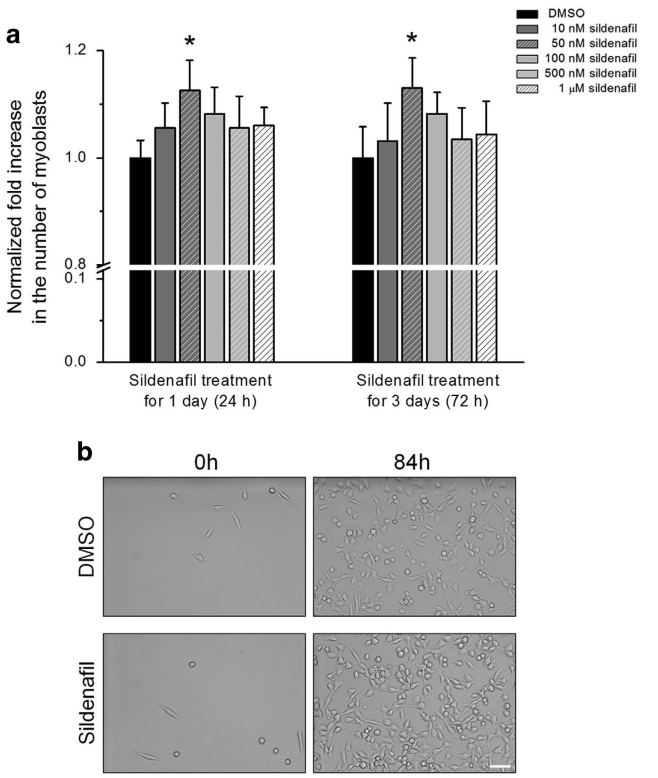

Figure 1 Proliferation of primary skeletal myoblasts. (**a**) A quantitative analysis of myoblast proliferation using various concentrations of sildenafil for 24 or 72 h. The number of myoblasts was normalized to the corresponding number of myoblasts in the DMSO control and is presented as histograms (fold-increase). The number of myoblasts at each concentration was compared with the corresponding DMSO control using *t*-tests (*, significant, $P<0.05$) and a Bonferroni correction was conducted. The results are presented as the means ± s.e. A quantitative analysis of myoblast proliferation using various concentrations of sildenafil for various time periods is presented in Supplementary Figure 1a. The number of places used in the analysis of the sildenafil effect on myoblast proliferation is presented in Supplementary Figure 1b. (**b**) Representative images of myoblasts treated with DMSO or 50 nM sildenafil for 84 h are presented. The scale bar indicates 50 μm. The proliferation of primary skeletal myoblasts is significantly enhanced by 50 nM sildenafil treatment for 24 h or longer periods.

of myoblasts, and the enhancement was observed after 24 h of treatment and after treatments for longer than 24 h (~11.2 or 11.3% higher than the DMSO control in both the 24 or 72 h-treatment, respectively, in Figure 1a and Supplementary Figure 1a). Note that the enhancement of skeletal myoblast proliferation is presented as 'fold-increase'. Concentrations of sildenafil lower or greater than 50 nM did not affect the proliferation of myoblasts, suggesting that the enhancement of skeletal myoblast proliferation by sildenafil was not dose-dependent. Representative images of myoblasts at 84 h after 50 nM sildenafil treatment are shown in Figure 1b. All further experiments were conducted using 50 nM sildenafil.

The migration of primary skeletal myoblasts is slowed by sildenafil

During the differentiation of skeletal myoblasts to myotubes, myoblasts migrate and fuse to one another to form myotubes. The first step in determining the effect of sildenafil on the degree of differentiation is to evaluate the effect of sildenafil on skeletal myoblast migration, which involves the formation of an acellular area by scraping the myoblasts off a culture plate and then treating the resultant culture medium with sildenafil. After 12 h of sildenafil treatment, the number of myoblasts that migrated into the acellular area was counted (Figure 2). To exclude the proliferative effect of sildenafil and to determine the sole effect of sildenafil on myoblast migration, sildenafil was treated to myoblasts for 12 h (that is, myoblast migration was examined at 12 h (Supplementary Figure 2) because this time point preceded the first observation of significant effects of sildenafil on proliferation at 24 h of treatment). The number of myoblasts that migrated into the acellular area was reduced by sildenafil. Therefore, sildenafil slowed the migration of skeletal myoblasts, suggesting that sildenafil has different effects on differentiation based on its effect on the proliferation of skeletal myoblasts.

The overall differentiation of primary skeletal myoblasts to myotubes is not affected by sildenafil

The second step was to evaluate the effect of sildenafil on skeletal myoblast fusion during differentiation, which involved treatment of the culture medium with sildenafil during differentiation. Interestingly, instead of the typically smooth and cylindrical shape of skeletal myotube membranes, the myotubes treated with sildenafil were characterized by uneven plasma membranes, which can be described as puddles or bumpy regions similar to twisted bread sticks (enlarged images 1 and 2 in Figure 3a). However, the widths (one criterion used to evaluate the degree of myoblast fusion and differentiation, as described in the 'Materials and methods' section) and lengths of myotubes were not changed by sildenafil (Supplementary Figure 3), suggesting that overall differentiation was not affected by sildenafil.

To confirm the lack of an effect of sildenafil on differentiation, the mRNA level of myogenin, a secondary MRF that is expressed on differentiation and regulates differentiation, was examined in myotubes treated with sildenafil using qPCR analysis. There were no changes in the mRNA levels of myogenin, (Figure 3b), which again showed that the overall differentiation of skeletal myoblasts to myotubes was not affected by sildenafil.

In primary skeletal myotubes, RyR1 activity and Ca^{2+} movements for muscle contraction are enhanced by sildenafil

Functional properties of myotubes treated with sildenafil were examined by measuring intracellular Ca^{2+} movement. RyR1 is a key protein for releasing Ca^{2+} from the SR into the cytosol during skeletal muscle contraction, and myotubes were treated with caffeine, which is a direct agonist

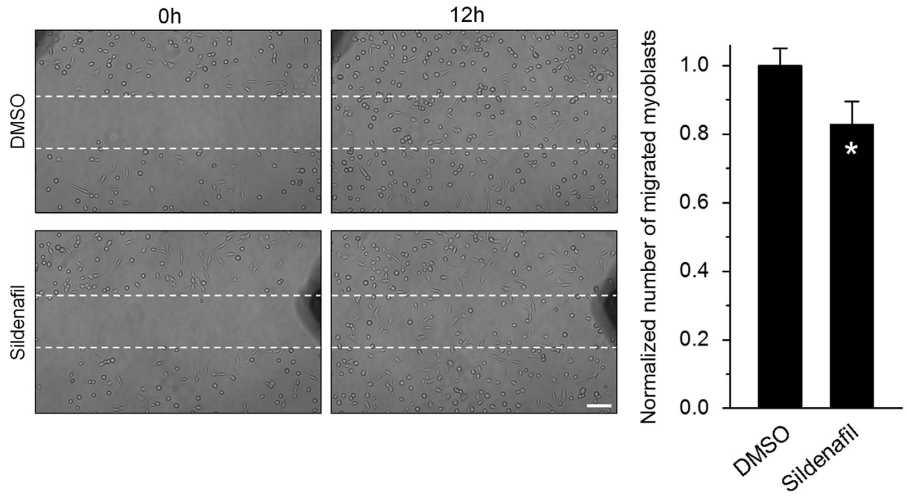

Figure 2 Migration of primary skeletal myoblasts. At 12 h after treatment with sildenafil, the number of myoblasts that migrated into the acellular area was counted. DMSO treatments were used as controls. The scale bar indicates 100 μm. The number of myoblasts in the acellular area was normalized to the DMSO control. Sixteen places per sample were examined, and the results are presented as the means ± s.e. *, and the significant difference was compared with DMSO ($P<0.05$). The migration of primary skeletal myoblasts was significantly slowed by sildenafil.

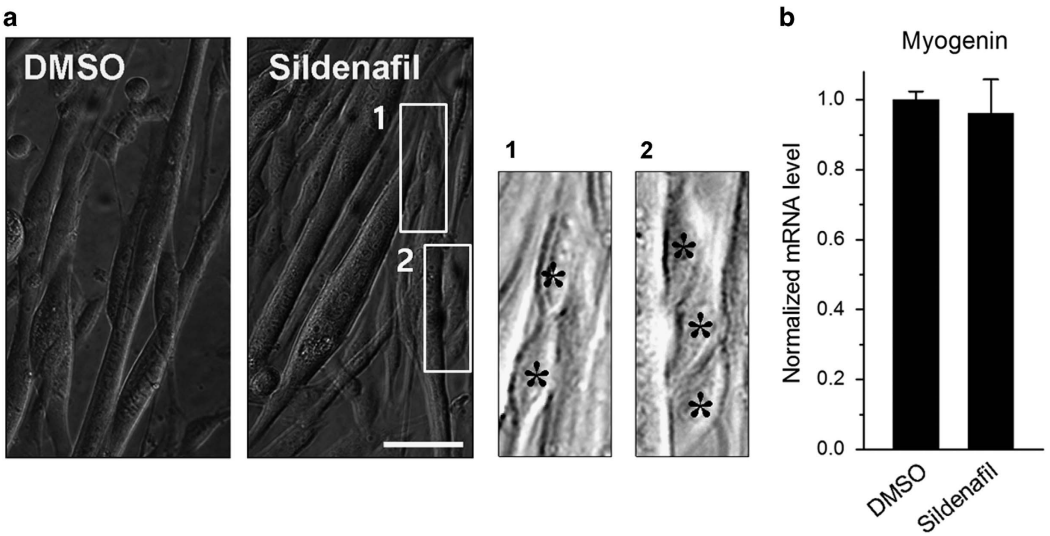

Figure 3 Differentiation of primary skeletal myoblasts to myotubes. (**a**) Representative images of myotubes treated with sildenafil are presented, and the boxed areas in the images are enlarged in the right panel. The scale bar indicates 100 μm. Neither the fusion of myoblasts nor the formation of myotubes was affected by sildenafil. A unique microscopic shape in the myotube membranes was induced by sildenafil (indicated by asterisks). (**b**) The mRNA level of myogenin in myotubes treated with sildenafil was assessed by qPCR analysis. The mRNA levels were normalized to the DMSO control (in duplicate, eight sets). The results are presented as the means ± s.e. *, and the significant difference was compared with DMSO ($P<0.05$). There was no significant change in the mRNA level of myogenin. The overall differentiation of primary skeletal myoblasts to myotubes was not affected by sildenafil.

of RyR1. Responses to caffeine were enhanced in myotubes treated with sildenafil compared with control myotubes (Figure 4a; Table 2). Responses to KCl were also enhanced by sildenafil (Figure 4b; Table 2). Ca^{2+} movements in skeletal myotubes in response to KCl mimic Ca^{2+} movements during EC coupling (that is, membrane depolarization by KCl activates DHPR, and direct interaction between the activated DHPR and RyR1 induces RyR1 activation), suggesting that, indeed, the enhanced Ca^{2+} movement by sildenafil could contribute to the Ca^{2+} supply needed for skeletal muscle contraction. Therefore, in skeletal muscle, sildenafil could enhance Ca^{2+} movement and supply more Ca^{2+} for muscle contraction. In addition, the increment of the response to KCl by sildenafil was similar to that of caffeine, a specific agonist of RyR1, suggesting that RyR1 activity could be increased in a selective manner by sildenafil, and this increased RyR1 activity could be a major reason for the enhanced Ca^{2+} movement.

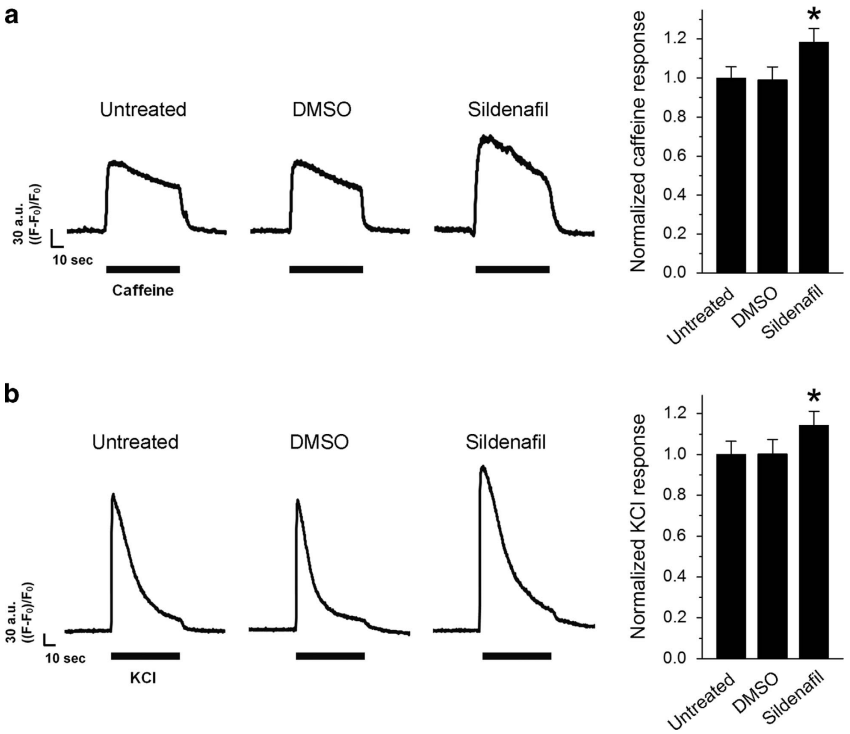

Figure 4 Ca^{2+} movement in primary skeletal myotubes. Caffeine (**a**), which is a direct RyR1 agonist or KCl (**b**), a membrane depolarizer, was applied to myotubes treated with sildenafil, and Ca^{2+} movement from the SR to cytosol in myotubes was measured. Histograms are shown for the normalized peak amplitude to the mean value of those from an untreated control. The results are presented as the means ± s.e. for the number of experiments in the parentheses in Table 2. *, and the significant difference was compared with untreated controls ($P<0.05$). Ca^{2+} release in primary skeletal myotubes in response to both caffeine and KCl as significantly enhanced by sildenafil.

In primary skeletal myotubes, the resting cytosolic Ca^{2+} level is increased by sildenafil

To identify other proteins that participate in the enhancement of Ca^{2+} movement in myotubes treated with sildenafil (Figure 4), 15 proteins that are related to or mediate Ca^{2+} movement during the contraction and maintenance of skeletal muscle were examined by immunoblot assays using myotube lysate from sildenafil treated cells (Figure 5a). There was no significant change in the expression levels of the three main proteins that mediate Ca^{2+} movements between the SR and cytosol during skeletal muscle contraction and relaxation: DHPR, RyR1 and SERCA1a. There was also no significant change in the expression levels of proteins responsible for extracellular Ca^{2+} entry: Orai1, STIM1, TRPC1, TRPC3, TRPC4 and TRPC6. This suggested a low possibility that a change in extracellular Ca^{2+} entry is a direct cause of the enhanced Ca^{2+} movement. There was also no change in the expression levels of proteins that mediate formation of the junctional membrane complex and the handling of Ca^{2+}: JP1, JP2, calsequestrin, calmodulin, mitugumin 29 and mitugumin 53. Therefore, these results suggest that a change in the expression levels of proteins that mediate Ca^{2+} movement in skeletal muscle does not result in an enhancement of Ca^{2+} movement in myotubes treated with sildenafil.

To maintain or increase cytosolic Ca^{2+} levels to a certain level at different time points is the key to evoking skeletal muscle contraction, and the amount of releasable Ca^{2+} from the SR into the cytosol or resting cytosolic Ca^{2+} level was measured in myotubes treated with sildenafil. To measure the amount of releasable Ca^{2+} from the SR to the cytosol (that is, to estimate how much Ca^{2+} ion is stored in the SR), TG was used to treat myotubes in the absence of extracellular Ca^{2+} to avoid extracellular Ca^{2+} entry and exclusively assess the amount of Ca^{2+} stored in the SR. For analyzing TG responses, both the peak area under the traces and amplitude were analyzed. Sildenafil caused no significant change in the amount of releasable Ca^{2+} from the SR to the cytosol (Figure 5b; Table 2; Supplementary Figure 4). To measure the resting cytosolic Ca^{2+}, the same experimental conditions for the measurement of KCl or caffeine response were used (that is, in the presence of extracellular Ca^{2+}). Interestingly, resting cytosolic Ca^{2+} levels were significantly increased by sildenafil (Figure 5c; Table 2), suggesting that resting cytosolic Ca^{2+} levels could be a possible reason for the increase in RyR1 activity and the subsequently enhanced Ca^{2+} movement in myotubes treated with sildenafil.

DISCUSSION

The effectiveness of sildenafil on skeletal muscle has been controversial, unlike its effect on smooth muscle, as discussed in the Introduction section, displaying positive, negative, or neutral effects.[23,25–29] In addition, unlike smooth muscle, most sildenafil studies on skeletal muscle were conducted at macroscopic levels, such as physiological aspects of tissues or

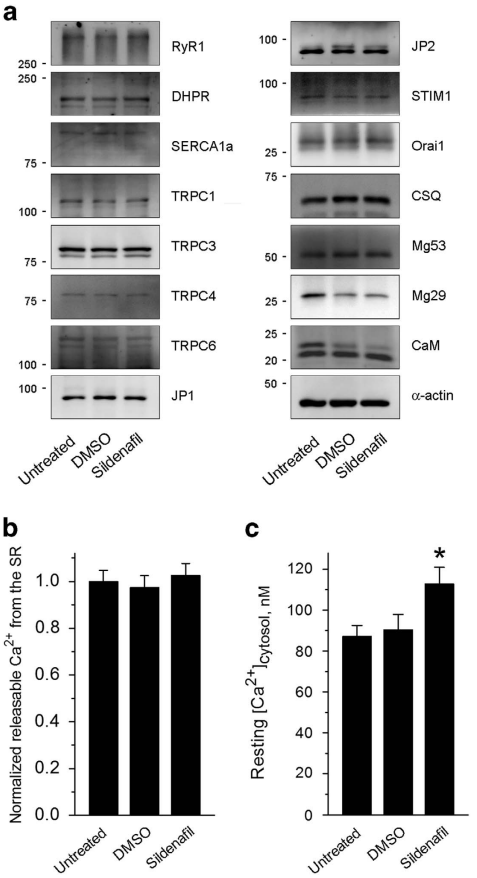

Figure 5 Expression level of skeletal muscle proteins, the amount of releasable Ca^{2+} from the SR to the cytosol, and the resting cytosolic Ca^{2+} level in primary skeletal myotubes. (**a**) The immunoblot analysis of proteins mediating Ca^{2+} movements and handling in skeletal muscle was conducted using the lysate of myotubes treated with sildenafil. Fifteen proteins were examined, and no expression levels were changed by sildenafil (the expression level of proteins is presented as bar graphs in Supplementary Figure 5). α-Actin was used as a loading control. At least three independent experiments per protein were conducted and a representative result is presented. JP, junctophilin; CSQ, calsequestrin; Mg, mitsugumin; CaM, calmodulin. The amount of releasable Ca^{2+} from the SR to the cytosol in response to TG (2.5 μM) (**b**) or resting cytosolic Ca^{2+} level (**c**) was examined in myotubes treated with sildenafil, and histograms are shown for the normalized peak amplitude or area under the peak to the mean value of those from untreated controls as described in the 'Materials and methods' section. TG was applied to myotubes in the absence of extracellular Ca^{2+} to avoid extracellular Ca^{2+} entry. The results are presented as the means ± s.e. for the number of experiments presented in the parentheses of Table 2. *, and the significant difference was compared with the untreated control ($P<0.05$). There was no significant change in the amount of releasable Ca^{2+} from the SR to the cytosol caused by sildenafil, and the resting cytosolic Ca^{2+} levels in primary skeletal myotubes were significantly increased by sildenafil.

organs, but not at the cellular level that constitutes tissues and organs. Crucially, the studies were conducted using different methods for sildenafil administration (oral administration (gavage or as tablet forms), subcutaneous injection, drinking water, intraperitoneal injection and so on), which resulted in various working doses of sildenafil in the tissues or organs that researchers examined. Therefore, uncontrolled concentrations of sildenafil (usually doses higher than those used in clinical prescriptions) could be misleading about the effects of sildenafil on skeletal muscle. Various periods and frequencies for sildenafil administration in disease models[26–29] are also another key reason for controversies surrounding the effect of sildenafil on skeletal muscle.

In the present study, concentrations of sildenafil greater than 50 nM had no effect on myoblast proliferation on the cellular level. There are two possible explanations for the lack of effects with higher doses of sildenafil than 50 nM. One is that higher concentrations of sildenafil may be harmful to myoblasts. Indeed, floating and dead myoblasts were found in the culture medium with the sildenafil treatment of 0.1 mM or more in the present study, which is consistent with the observation that sildenafil aggravates the pathology of DMD[27] and induces skeletal muscle atrophy.[28] The other possibility is that 50 nM of sildenafil may simply be the maximally effective concentration under the given culture conditions. However, trials of cell culture using different sized culture plates (6- or 96-well, or 10-cm culture plates) ruled out the latter possibility. Sildenafil ameliorates age-related dysfunction in the cardiac muscle of a mouse DMD model by inhibiting PDE5 ($IC_{50} = 10$ nM).[35] Skeletal muscle could be less sensitive to sildenafil than cardiac muscle, and a metabolite of sildenafil and/or other types of PDE could mediate the proliferative effect of sildenafil on skeletal myoblasts, although the cellular effects of sildenafil on skeletal myoblasts from normal mice in the present study is not directly comparable to those on cardiac muscle function in a mouse disease model.

Nitric oxide promotes skeletal myoblast proliferation and muscle regeneration.[36] In the present study, sildenafil effectively enhanced skeletal myoblast proliferation, and the effects of sildenafil were observed in the absence of the overt manipulation of the nitric oxide level, suggesting that sildenafil could amplify the downstream responses to nitric oxide endogenously produced in skeletal myotubes. Therefore, sildenafil could be an effective way to avoid the superfluous and sometimes deleterious side effects of nitric oxide and provide a shorter path to the proliferation of skeletal myoblasts in adults. Postnatal skeletal muscle growth or regeneration after damage is accomplished by fusions among pre-existing/intact muscle fibers, fusions of pre-existing/intact muscle fibers with satellite cells (that is, precursor cells of myoblasts), or fusions among satellite cells (*de novo* formation).[37–42] In adults, satellite cells account for <5% of the total nuclei in skeletal muscle mass, which is much less than the ~32% seen at birth.[43] A reduction in skeletal muscle mass usually occurs during the course of healthy aging as well as with diverse diseases.[44,45] On the basis of our results, an adequate amount of sildenafil could be beneficial for the maintenance of skeletal muscle mass in healthy people as well as for patients or people with special situations, such as air force pilots or astronauts, who experience high-altitude or non-gravitation situations. A drug

repositioning (extended application) of sildenafil to skeletal muscle would have significant advantages because a significant number of toxicity and other test results have already established its safety.

Adequate migration of myoblasts is a prerequisite for favorable myoblast fusion to form myotubes during differentiation.[3] In the present study, sildenafil led to slower myoblast migration, but the differentiation of myoblasts to myotubes remained normal. Because the fusion of myoblasts during differentiation requires elevation of the cytosolic Ca^{2+} level,[5,6] the higher cytosolic Ca^{2+} levels in myotubes treated with sildenafil (Figure 5c) could be a link between the slower migration and normal differentiation in the present study, such that the higher ability to fuse due to the increase in resting cytosolic Ca^{2+} level (a type of ready-to-go kit) could compensate for the slower migration of myoblasts and normalize differentiation. The unique microscopic shape of the plasma membranes and no reduction in the time required for differentiation (no temporal delay) in myotubes treated with sildenafil could be evidence for a higher ability of the myoblasts to fuse, albeit with a slower myoblast migration.

Because an increase in the cytosolic Ca^{2+} level activates RyR1 via a CICR mechanism and accelerates Ca^{2+} movements through RyR1 by increasing RyR1 sensitivity to agonists by the presence of higher cytosolic Ca^{2+} level,[9,12] the enhanced RyR1 activity and Ca^{2+} movement in myotubes treated with sildenafil could be caused by the increase in the resting cytosolic Ca^{2+} level (Figure 5c). Extracellular Ca^{2+} entry via Orai1 or TRPC channels is a possible reason for an increase in the cytosolic Ca^{2+} level.[14–16] However, this is not the case here because the expression levels of Orai1 (with its main regulatory protein, STIM1) and four types of TRPCs that are expressed in skeletal muscle (TRPC1, TRPC3, TRPC4 and TRPC6)[10,11] were not changed by sildenafil. Another possible reason for the enhanced RyR1 activity and Ca^{2+} movements in myotubes treated with sildenafil could be the unique microscopic shape in the myotube membranes (Figure 3a), similar to caveolae structures in other types of cells.[46] The unique plasma membranes (with puddles and bumpy regions) may be more appropriate for capturing external signals by mimicking the conditions for higher concentrations of signals. This is the first report to suggest that Ca^{2+} movements via RyR1 and membrane shape (that is, the morphology of myotubes) in skeletal muscle could be correlated. Accordingly, sildenafil could enhance RyR1 activity and Ca^{2+} movement through a more efficient spatial capturing of KCl or caffeine. This is consistent with no change in the expression levels of RyR1 in myotubes treated with sildenafil. In either case, sildenafil could enhance skeletal muscle contractility by increasing Ca^{2+} availability.

Sildenafil has an anti-proliferative effect on smooth muscle cells by decreasing TRPC1 expression and extracellular Ca^{2+} entry.[20,47] In the present study, however, sildenafil had a proliferative effect on skeletal myoblasts with no change in TRPC1 expression. In addition, unlike the relaxation effect of sildenafil on smooth muscle cells,[20–22] sildenafil induced greater contraction in skeletal muscle myotubes by enhancing Ca^{2+} movement in this study. Therefore, sildenafil effects in skeletal muscle could be the opposite of what occurs in smooth muscle. Sildenafil lowers blood pressure by dilating blood vessels,[20–22] it improves skeletal muscle oxygenation during exercise in patients with intermittent claudication,[29] and it ameliorates skeletal muscle fatigue in a mouse DMD model and in healthy men.[23,24]

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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1 Mauro A. Satellite cell of skeletal muscle fibers. *J Biophys Biochem Cytol* 1961; **9**: 493–495.
2 Bischoff R. Regeneration of single skeletal muscle fibers *in vitro*. *Anat Rec* 1975; **182**: 215–235.
3 Wagers AJ, Conboy IM. Cellular and molecular signatures of muscle regeneration: current concepts and controversies in adult myogenesis. *Cell* 2005; **122**: 659–667.
4 Charge SB, Rudnicki MA. Cellular and molecular regulation of muscle regeneration. *Physiol Rev* 2004; **84**: 209–238.
5 Hindi SM, Tajrishi MM, Kumar A. Signaling mechanisms in mammalian myoblast fusion. *Sci Signal* 2013; **6**: re2.
6 Constantin B, Cognard C, Raymond G. Myoblast fusion requires cytosolic calcium elevation but not activation of voltage-dependent calcium channels. *Cell Calcium* 1996; **19**: 365–374.
7 Megeney LA, Rudnicki MA. Determination versus differentiation and the MyoD family of transcription factors. *Biochem Cell Biol* 1995; **73**: 723–732.
8 Fong AP, Tapscott SJ. Skeletal muscle programming and re-programming. *Curr Opin Genet Dev* 2013; **23**: 568–573.
9 Zucchi R, Ronca-Testoni S. The sarcoplasmic reticulum Ca^{2+} channel/ryanodine receptor: modulation by endogenous effectors, drugs and disease states. *Pharmacol Rev* 1997; **49**: 1–51.
10 Lee EH. Ca^{2+} channels and skeletal muscle diseases. *Prog Biophys Mol Biol* 2010; **103**: 35–43.
11 Lee EH, Kim do H, Allen PD. Interplay between intra- and extracellular calcium ions. *Mol Cells* 2006; **21**: 315–329.
12 Endo M. Calcium release from the sarcoplasmic reticulum. *Physiol Rev* 1977; **57**: 71–108.
13 Endo M. Calcium-induced calcium release in skeletal muscle. *Physiol Rev* 2009; **89**: 1153–1176.
14 Kurebayashi N, Ogawa Y. Depletion of Ca^{2+} in the sarcoplasmic reticulum stimulates Ca^{2+} entry into mouse skeletal muscle fibres. *J Physiol* 2001; **533**: 185–199.
15 Stiber J, Hawkins A, Zhang ZS, Wang S, Burch J, Graham V *et al.* STIM1 signalling controls store-operated calcium entry required for development and contractile function in skeletal muscle. *Nat Cell Biol* 2008; **10**: 688–697.
16 Lee EH, Cherednichenko G, Pessah IN, Allen PD. Functional coupling between TRPC3 and RyR1 regulates the expressions of key triadic proteins. *J Biol Chem* 2006; **281**: 10042–10048.
17 Lee CS, Georgiou DK, Dagnino-Acosta A, Xu J, Ismailov II, Knoblauch M *et al.* Ligands for FKBP12 increase Ca^{2+} influx and protein synthesis to improve skeletal muscle function. *J Biol Chem* 2014; **289**: 25556–25570.

18 Woo JS, Cho CH, Lee KJ, Kim do H, Ma J, Lee EH. Hypertrophy in skeletal myotubes induced by junctophilin-2 mutant, Y141H, involves an increase in store-operated Ca^{2+} entry via Orai1. *J Biol Chem* 2012; **287**: 14336–14348.
19 Ito K, Komazaki S, Sasamoto K, Yoshida M, Nishi M, Kitamura K *et al.* Deficiency of triad junction and contraction in mutant skeletal muscle lacking junctophilin type 1. *J Cell Biol* 2001; **154**: 1059–1067.
20 Wharton J, Strange JW, Moller GM, Growcott EJ, Ren X, Franklyn AP *et al.* Antiproliferative effects of phosphodiesterase type 5 inhibition in human pulmonary artery cells. *Am J Respir Crit Care Med* 2005; **172**: 105–113.
21 Ghofrani HA, Osterloh IH, Grimminger F. Sildenafil: from angina to erectile dysfunction to pulmonary hypertension and beyond. *Nat Rev Drug Discov* 2006; **5**: 689–702.
22 Kukreja RC, Salloum F, Das A, Ockaili R, Yin C, Bremer YA *et al.* Pharmacological preconditioning with sildenafil: basic mechanisms and clinical implications. *Vasc Pharmacol* 2005; **42**: 219–232.
23 Sheffield-Moore M, Wiktorowicz JE, Soman KV, Danesi CP, Kinsky MP, Dillon EL *et al.* Sildenafil increases muscle protein synthesis and reduces muscle fatigue. *Clin Transl Sci* 2013; **6**: 463–468.
24 Percival JM, Anderson KN, Gregorevic P, Chamberlain JS, Froehner SC. Functional deficits in nNOSmu-deficient skeletal muscle: myopathy in nNOS knockout mice. *PLoS ONE* 2008; **3**: e3387.
25 Nieoczym D, Luszczki JJ, Czuczwar SJ, Wlaz P. Effect of sildenafil on the anticonvulsant action of classical and second-generation antiepileptic drugs in maximal electroshock-induced seizures in mice. *Epilepsia* 2010; **51**: 1552–1559.
26 Nelson MD, Rader F, Tang X, Tavyev J, Nelson SF, Miceli MC *et al.* PDE5 inhibition alleviates functional muscle ischemia in boys with Duchenne muscular dystrophy. *Neurology* 2014; **82**: 2085–2091.
27 Percival JM, Siegel MP, Knowels G, Marcinek DJ. Defects in mitochondrial localization and ATP synthesis in the mdx mouse model of Duchenne muscular dystrophy are not alleviated by PDE5 inhibition. *Hum Mol Genet* 2013; **22**: 153–167.
28 Rinaldi B, Donniacuo M, Sodano L, Gritti G, Signoriello S, Parretta E *et al.* Effects of sildenafil on the gastrocnemius and cardiac muscles of rats in a model of prolonged moderate exercise training. *PLoS ONE* 2013; **8**: e69954.
29 Roseguini BT, Hirai DM, Alencar MC, Ramos RP, Silva BM, Wolosker N *et al.* Sildenafil improves skeletal muscle oxygenation during exercise in men with intermittent claudication. *Am J Physiol-Regul Integr Comp Physiol* 2014; **307**: R396–R404.
30 Lee KJ, Woo JS, Hwang JH, Hyun C, Cho CH, Kim do H *et al.* STIM1 negatively regulates Ca^{2+} release from the sarcoplasmic reticulum in skeletal myotubes. *Biochem J* 2013; **453**: 187–200.
31 Lee KJ, Hyun C, Woo JS, Park CS, Kim do H, Lee EH. Stromal interaction molecule 1 (STIM1) regulates sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase 1a (SERCA1a) in skeletal muscle. *Pflugers Arch* 2014; **466**: 987–1001.
32 Woo JS, Hwang JH, Ko JK, Weisleder N, Kim do H, Ma J *et al.* S165F mutation of junctophilin 2 affects Ca^{2+} signalling in skeletal muscle. *Biochem J* 2010; **427**: 125–134.
33 Woo JS, Kim do H, Allen PD, Lee EH. TRPC3-interacting triadic proteins in skeletal muscle. *Biochem J* 2008; **411**: 399–405.
34 Lee EH, Woo JS, Hwang JH, Park JH, Cho CH. Angiopoietin 1 enhances the proliferation and differentiation of skeletal myoblasts. *J Cell Physiol* 2013; **228**: 1038–1044.
35 Adamo CM, Dai DF, Percival JM, Minami E, Willis MS, Patrucco E *et al.* Sildenafil reverses cardiac dysfunction in the mdx mouse model of Duchenne muscular dystrophy. *Proc Natl Acad Sci USA* 2010; **107**: 19079–19083.
36 Buono R, Vantaggiato C, Pisa V, Azzoni E, Bassi MT, Brunelli S *et al.* Nitric oxide sustains long-term skeletal muscle regeneration by regulating fate of satellite cells via signaling pathways requiring Vangl2 and cyclic GMP. *Stem Cells* 2012; **30**: 197–209.
37 Tedesco FS, Dellavalle A, Diaz-Manera J, Messina G, Cossu G. Repairing skeletal muscle: regenerative potential of skeletal muscle stem cells. *J Clin Invest* 2010; **120**: 11–19.
38 Lepper C, Partridge TA, Fan CM. An absolute requirement for Pax7-positive satellite cells in acute injury-induced skeletal muscle regeneration. *Development* 2011; **138**: 3639–3646.
39 Yin H, Price F, Rudnicki MA. Satellite cells and the muscle stem cell niche. *Physiol Rev* 2013; **93**: 23–67.
40 Relaix F, Zammit PS. Satellite cells are essential for skeletal muscle regeneration: the cell on the edge returns centre stage. *Development* 2012; **139**: 2845–2856.
41 Adams GR. Satellite cell proliferation and skeletal muscle hypertrophy. *Appl Physiol Nutr Metab* 2006; **31**: 782–790.
42 Siegel AL, Kuhlmann PK, Cornelison DD. Muscle satellite cell proliferation and association: new insights from myofiber time-lapse imaging. *Skelet Muscle* 2011; **1**: 7.
43 Seale P, Rudnicki MA. A new look at the origin, function, and 'stem-cell' status of muscle satellite cells. *Dev Biol* 2000; **218**: 115–124.
44 Degens H. The role of systemic inflammation in age-related muscle weakness and wasting. *Scand J Med Sci Sports* 2010; **20**: 28–38.
45 Peake J, Della Gatta P, Cameron-Smith D. Aging and its effects on inflammation in skeletal muscle at rest and following exercise-induced muscle injury. *Am J Physiol Regul Integr Comp Physiol* 2010; **298**: R1485–R1495.
46 Isshiki M, Anderson RG. Function of caveolae in Ca^{2+} entry and Ca^{2+} -dependent signal transduction. *Traffic* 2003; **4**: 717–723.
47 Wang C, Wang J, Zhao L, Wang Y, Liu J, Shi L *et al.* Sildenafil inhibits human pulmonary artery smooth muscle cell proliferation by decreasing capacitative Ca^{2+} entry. *J Pharmacol Sci* 2008; **108**: 71–78.

Supplementary Information accompanies the paper on Experimental & Molecular Medicine website (http://www.nature.com/emm)