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1. Title Page

Journal of Pharmacology and Experimental Therapeutics **Article**

Opening of intermediate conductance Ca^{2+}-activated K^+ channels in C2C12 skeletal muscle cells increases the myotube diameter via the Akt/mTOR pathway

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2. Running Title Page

a) Running Title, IK_{Ca} channels in muscle hypertrophy

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Abstract, 224 (< 250) words

Introduction, 529 (<750) words

Discussion, 1297 (<1,500) words

d) Abbreviations

DCEBIO, 5,6-dichloro-1-ethyl-1,3-dihydro-2H-benzimidazol-2-one; IGF-1, insulin-like growth factor; IK_{Ca} channel, intermediate conductance Ca^{2+}-activated K^+ channels; mTOR, mammalian target of rapamycin; S6K, S6 kinase; SK_{Ca} channel, small conductance Ca^{2+}-activated K^+ channel; TRAM-34, 1-[(2-chlorophenyl)diphenylmethyl]-1H-pyrazole.

e) Section

Drug Discovery and Translational Medicine

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3. Abstract

The activation of potassium channels and following hyperpolarization in skeletal myoblasts is essential for myogenic differentiation. However, the effects of K^+ channel opening in myoblasts on skeletal muscle mass are unclear. Our previous study revealed that pharmacological activation of intermediate conductance Ca^{2+}-activated K^+ channels (IK_{Ca} channels) increases myotube formation. In this study, we investigated the effects of DCEBIO, a K_{Ca} channel opener, on the mass of skeletal muscle. Application of DCEBIO to C2C12 cells during myogenesis increased the diameter of C2C12 myotubes in a concentration-dependent manner. This DCEBIO-induced hypertrophy was abolished by gene-silencing of IK_{Ca} channels. However, it was resistant to 1 μM, but sensitive to 10 μM TRAM-34, a specific IK_{Ca} channel blocker. Furthermore, DCEBIO reduced the mitochondrial membrane potential by opening IK_{Ca} channels. Therefore, DCEBIO should increase myotube mass by opening of IK_{Ca} channels distributed in mitochondria. Pharmacological studies revealed that mitochondrial reactive oxygen species (mitoROS), Akt, and mTOR are involved in DCEBIO-induced myotube hypertrophy. An additional study demonstrated that DCEBIO-induced muscle hypertrophic effects are only observed when applied in the early stage of myogenic differentiation. In an *in vitro* myotube inflammatory atrophy experiment, DCEBIO attenuated the reduction of myotube diameter induced by endotoxin. Thus, we concluded that DCEBIO increases muscle mass by activating the IK_{Ca} channel/mitoROS/Akt/mTOR pathway. Our study suggests the potential of DCEBIO in the treatment of muscle wasting diseases.

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4. Significance Statement

Our study shows DCEBIO, a small molecule opener of Ca^{2+} activated K^+ channel, increased muscle diameter via mitochondria ROS/Akt/mTOR pathway. And DCEBIO overwhelms C2C12 myotube atrophy induced by endotoxin challenge. Our report should inform novel role of K^+ channel in muscle development and novel usage of K^+ channel opener such as for the treatment of muscle wasting diseases.

6. Introduction

Skeletal muscle wasting is caused by aging, obesity, drugs, infectious diseases, and genetic disorders (Rosenberg, 1997; Sakamoto and Kimura, 2013; Santilli *et al.*, 2014; Friedrich *et al.*, 2015; Verhaart and Aartsma-Rus, 2019). It not only makes daily life difficult, but also leads to metabolic diseases, such as diabetes and respiratory failure, which can be life-threatening. As skeletal muscle wasting is caused by atrophy and reduction of skeletal muscle fibers, drugs that promote skeletal muscle protein synthesis, inhibit protein degradation, and increase the differentiation capacity of skeletal muscle progenitor cells are promising mechanisms of action for the treatment of skeletal muscle wasting (Watanabe and Miyagoe-Suzuki, 2015). Although selective androgen receptor modulators and inflammatory cytokine inhibitors have been proposed as drug therapies for skeletal muscle wasting, they have not reached clinical application (Papanicolaou *et al.*, 2013; Ono *et al.*, 2020).

In non-excitable and proliferating cells, including skeletal muscle progenitors, opening of K^+ channels leads to hyperpolarization-induced intracellular calcium influx, which activates numerous signaling cascades (Bernheim and Bader, 2002). Stimulation of muscle differentiation increases potassium channel activity in myoblasts, including inward rectifying K^+ channels (Kir2.1), ether-a-go-go-related gene channels (ERG), TWIK-related acid-sensitive K^+ channels (TASK) 2, and TWIK-related K^+ channels (TREK) 1 (Bijlenga *et al.*, 1998; Hinard *et al.*, 2008; Afzali *et al.*, 2016). These cause hyperpolarization of myoblasts during differentiation, leading to Ca^{2+} influx via transient receptor potential canonical (TRPC) 1/4 and T-type Ca^{2+} channels, thereby activating muscle differentiation signals (Bijlenga *et al.*, 2000; Antigny *et al.*, 2013).

The intermediate-conductance calcium-activated potassium channels (IK_{Ca} channels: also known as $K_{Ca}3.1$ channels, KCNN4 channels, and Gardos channels) are also expressed in skeletal myoblasts, and are thought to be involved in the regulation of muscle differentiation (Fioretti *et al.*, 2005; Pietrangelo *et al.*, 2006). The authors previously reported that

administration of the K_{Ca} channel opener 5,6-dichloro-1-ethyl-1,3-dihydro-2H-benzimidazole-2-one (DCEBIO) accelerated muscle differentiation (Tanaka *et al.*, 2017). This was the first report to demonstrate that potassium channel opening agents act on muscle differentiation. IK_{Ca} channels are also distributed on the inner mitochondrial membrane (IMM), and their opening causes depolarization of the mitochondrial membrane potential, leading to regulation of oxidative phosphorylation and an increase in reactive oxygen species (ROS) (De Marchi *et al.*, 2009; Sassi *et al.*, 2010; Leanza *et al.*, 2014), in addition to mitochondrial ATP-sensitive K^+ channels ($mitoK_{ATP}$ channel) and mitochondrial large conductance Ca^{2+}-activated K^+ channels ($mitoBK_{Ca}$ channel) (Singh *et al.*, 2013; Paggio *et al.*, 2019). Of note, IK_{Ca} channels are known to activate Akt, a known skeletal muscle hypertrophic factor, in macrophages and vascular endothelial cells, but the underlying mechanism remains unclear (Kang *et al.*, 2014; Huang *et al.*, 2016).

In this study, we hypothesized that DCEBIO causes muscle hypertrophy not only through its muscle differentiation action, but also through Akt activation. To investigate this hypothesis, we induced muscle differentiation in the presence of DCEBIO in C2C12 mouse myoblasts, which are progenitors of strained skeletal muscle frequently used as a muscle differentiation model, and analyzed the effects of DCEBIO on the myotube diameter and the mechanism of Akt activation by pharmacological and gene silencing. In the present study, we found that pharmacological activation of IK_{Ca} channels activates the mitochondrial ROS/Akt/mTOR pathway to hypertrophic neonatal skeletal muscle.

7. Materials and Methods

Reagents and antibodies

10-(4′-(N-diethylamino) butyl)-2-chlorophenoxazine (Akt Inhibitor-X), apamin, [10-(2,5-dihydroxy-3,4-dimethoxy-6-methylphenyl)decyl]triphenyl-phosphonium, monomethanesulfonate (MitoQ),

(3S,6R,7E,9R,10R,12R,14S,15E,17E,19E,21S,23S,26R,27R,34aS)-9,10,12,13,14,21,22,23,24,25,26,27,32,33,34,34a-hexadecahydro-9,27-dihydroxy-3-[(1R)-2-[(1S,3R,4R)-4-hydroxy-3-methoxycyclohexyl]-1-methylethyl]-10,21-dimethoxy-6,8,12,14,20,26-hexamethyl-23,27-epoxy-3H-pyrido[2,1-c][1,4]-oxaazacyclohentriacontine-1,5,11,28,29(4H,6H,31H)-pentone (rapamycin), and 1-[(2-chlorophenyl)diphenylmethyl]-1H-pyrazole (TRAM-34) were from Cayman Chemical (Ann Arbor, MI). The following primary antibodies were used: Akt (pan) (C67E7) rabbit mAb, Phospho-Akt (Thr308) (244F9) rabbit mAb, p70 S6 Kinase (49D7) rabbit mAb, and Phospho-p70 S6 Kinase (Thr389) (108D2) rabbit mAb (Cell Signaling Technology, Danvers, MA).

Cell culture

C2C12 mice skeletal myoblasts were obtained from the RIKEN cell bank (Tsukuba, Japan), and grown in Dulbecco's modified Eagle's medium (Thermo Fisher Scientific, Inc. Waltham, MA) supplemented with 15% heat inactivated fetal bovine serum (Thermo Fisher Scientific) (growth medium; GM), 100 units/ml of penicillin, and 100 µg/ml of streptomycin (Wako Pure Chemicals, Tokyo, Japan). The cell line was maintained in a 5% CO_2 atmosphere at 37°C. To induce myogenic differentiation, a medium containing 15% fetal bovine serum was substituted with heat-inactivated 2% horse serum (Thermo Fisher Scientific) (differentiation medium; DM) at approximately 80% cell confluency. During differentiation, DM with or without reagent(s) was exchanged every 2 days.

Counting nuclei in the myotubes

Myotubes were visualized by May-Grunwald Giemsa staining. The cells were washed twice with phosphate-buffered saline, fixed, and stained with May-Grunwald solution and Giemsa solution (Wako Pure Chemicals). The stained cells were photographed using the EVOS XL Core system (Thermo Fisher Scientific).

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Gene silencing of IK_{Ca} channels

The siRNA sequence for IK_{Ca} channels ($siIK_{Ca}$) was 5′ GGAAGCUGGAGUUCAACAAtt 3′, which was designed as 'KCa3.1 siRNA-B' (Zhang *et al.*, 2012) and synthesized by FASMAC (Atsugi, Japan). Negative control siRNA (siNC) was Stealth RNAi™ siRNA Negative Control, Med GC (Thermo Fisher). For transfection, trypsinized C2C12 myoblasts (5×10^5 cells) in Opti-MEM (100 μL) containing 300 pmole of siRNA were electroporated in 2-mm gap cuvettes using a NEPA21 type II electroporator (NepaGene, Ichikawa, Japan). The parameters were as follows: voltage, 150 V; pulse length, 5 ms; pulse interval, 50 ms; number of pulses, 2; decay rate, 10%; polarity + as poring pulse and voltage, 20 V; pulse length, 50 ms; pulse interval, 50 ms; number of pulses, 5; decay rate, 40%; and polarity +/− as transfer pulse. Subsequently, the mixtures were transferred into fresh GM and seeded in a well of 24-well plate. Forty-eight hours after transfection, the medium was replaced by DM.

RNA extraction, reverse transcription, conventional RT-PCR, and RT-qPCR

The total cellular RNA was extracted from the C2C12 cells using TRI Reagent (Molecular Research Center, Cincinnati, OH). We synthesized first-strand cDNA and performed real-time RT-PCR (RT-qPCR) as previously reported (Sakamoto *et al.*, 2009). Primers were: 5′-CGTGCACAACTTCATGATGGA-3′ and 5′-CCTTCCTTCGAGTGTGCTTGTAGT-3′ for mouse IK_{Ca} channels, 5′-GGAGGCCCTAGATCTTCTTG-3′ and 5′-CGCGTTAAGACGTTTTGATT-3′ for mouse Casein kinase 2 α2 (Csnk2a2). Csnk2a2 was adopted as an internal control because its expression is stable during myogenic differentiation (Hildyard and Wells, 2014).

Measurement of mitochondrial membrane potential

The mitochondrial membrane potential (ΔΨm) was assessed using JC-1 (Dojindo, Tokyo, Japan) staining according to the manufacturer's protocols and previous reports (Smiley *et al.*, 1991; Namba, 2019). Fluorescence was measured on a fluorescence microplate reader (Wallac ARVO SX 1420, PerkinElmer, Waltham, MA) with filter pairs of 550 nm/590 nm and 485 nm/535 nm. Results are shown as a normalized ratio of fluorescence measured at 550 nm/590 nm to that measured at 485 nm/535 nm.

Western blotting

C2C12 myotubes were lysed in NP-40 buffer (25 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% NP-40, and 5% glycerol; pH 7.4) supplemented with protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific). The protein concentration was measured using a BCA assay (Thermo Fisher Scientific). The protein lysates were separated and transferred to polyvinylidene fluoride membranes. The membranes were incubated with primary and secondary antibodies using the iBind system (Thermo Fischer Scientific). Primary antibodies were diluted 1:1000 and the secondary antibody was diluted 1:5000. Signals were detected using enhanced chemiluminescence (Clarity Western ECL Substrate, Bio-Rad Laboratories, Hercules, CA). The intensities of the immunoreactive protein bands were analyzed using ImageJ software version 1.47 (National Institutes of Health, Bethesda, MD).

Analysis

The data are expressed as means ± S.D. Significant differences between two groups and among multiple groups were evaluated using Student's *t*-tests or Tukey-Kramer's test after the F-test, or one-way ANOVA, respectively. A *P*-value of <0.05 was considered significant. All statistical analyses were carried out using R.

8. Results

Activation of IK_{Ca} channels increases C2C12 myotube diameter

We first examined whether DCEBIO exerts skeletal muscle hypertrophic effects on differentiating C2C12 myoblasts. C2C12 myoblasts were differentiated in the presence of DCEBIO (1, 3, or 10 μM) for 6 days. The diameter of C2C12 myotubes was thickened in a concentration-dependent manner by DCEBIO (Fig. 1B, C). The myotube diameter in the absence of DCEBIO was 11.3 ± 5.8 μm, that in the presence of 10 μM DCEBIO was 21.7 ± 10.5 μm, and the increase in myotube diameter with 10 μM DCEBIO was 10.4 μm (95% CI: 8.1 to 12.7) (Fig. 1).

[Fig. 1 here]

We next examined whether IK_{Ca} channel opening mediates the target of action for the muscle hypertrophic effects of DCEBIO. siIK_{Ca} reduced the mRNA expression level of IK_{Ca} channels by 53.9 ± 16.6% at 48 h post-transfection (n=4). The knock-down of IK_{Ca} channels significantly suppressed the muscle hypertrophic effects of DCEBIO in C2C12 myotubes (Fig. 2A-C). The selective IK_{Ca} channel blocker TRAM-34 did not alter the myotube diameter at 1 μM, but significantly attenuated the effects of DCEBIO when administered at 10 μM (Fig. 2D-F).

[Fig. 2 here]

Hypertrophic effects of DCEBIO are mediated by mitochondrial IK_{Ca} channels

According to the gene-silencing and pharmacological studies, the opening of IK_{Ca} channels is involved in DCEBIO-induced hypertrophy of C2C12 myotubes. However, it is less sensitive to TRAM-34 than DCEBIO-induced membrane hyperpolarization and increased differentiation (Tanaka *et al.*, 2017). IK_{Ca} channels are distributed not only on the plasma membrane, but also on the inner mitochondrial membrane (IMM) (De Marchi *et al.*, 2009; Sassi *et al.*, 2010). To investigate whether DCEBIO affects mitoIK_{Ca} channels, ΔΨm of

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C2C12 myoblasts were monitored using JC-1, a mitochondrial membrane potential-sensitive dye. DCEBIO reduced ΔΨm of C2C12 myoblasts, which were sensitive to 10 μM TRAM-34 (Fig. 3A, B). The reduction of ΔΨm increases mitochondrial reactive oxygen species (mitoROS) (Malinska *et al.*, 2010), which are known to promote muscle hypertrophy (Lee *et al.*, 2011; Kim *et al.*, 2018). To examine whether this pathway is involved in DCEBIO-induced myotube hypertrophy, we applied MitoQ, a mitochondrial ROS scavenger. Compared with the single application of DCEBIO on C2C12 cells, co-application of DCEBIO and MitoQ significantly reduced the myotube diameter by 6.5 μm (95% CI: 4.7 to 8.4) ($p<0.001$, t-test) (Fig. 3C, D).

[Fig. 3 here]

DCEBIO increases the phosphorylation level of Akt and S6 kinase in the differentiating C2C12 cells

A moderate increase in the production of mitochondrial ROS causes muscle hypertrophy via activation of the Akt/mTOR pathway (Kim *et al.*, 2018). Therefore, we examined whether DCEBIO increases the phosphorylation of S6K, a substrate for Akt and mTOR. We noted an increase in the levels of Akt and S6K phosphorylation after 1 day of treatment (Fig. 4A). Next, the involvement of DCEBIO in C2C12 myotube hypertrophy was examined pharmacologically, and the hypertrophic effects of DCEBIO were significantly attenuated when DCEBIO was co-administered with either an Akt (1 μM Akt Inhibitor-X; Fig. 4B, C) or mTOR inhibitor (0.1 nM rapamycin; Fig. 4D, E). Thus, the Akt/mTOR pathway may be involved in the hypertrophic effects of DCEBIO.

[Fig. 4 here]

K^+ efflux increases neither Akt phosphorylation in the differentiating C2C12 cells nor myotube diameter

According to the low sensitivity of TRAM-34 on DCEBIO induced myotube hypertrophy, this study pursues the involvement of IK_{Ca} channel in mitochondria and mitoROS/Akt/mTOR pathway. However, DCEBIO activates IK_{Ca} channel in the plasma membrane of C2C12 cells (Fioretti *et al.*, 2005; Tanaka *et al.*, 2017) and it has been reported that K^+ efflux from cytosol activates Akt in tumor-specific T-cells (Eil *et al.*, 2016). To test if K^+ efflux in C2C12 cells increases the Akt phosphorylation, we treated C2C12 myoblasts with a potassium ionophore gramicidin for 10 min as shown in Eil's paper using tumor-specific T-cells. Gramicidin failed to increase the phosphorylation of Akt in C2C12 myoblasts. Furthermore, to test if K^+ efflux changes myotube diameter, we applied gramicidin from 10 pM to 1 μM to the differentiating C2C12 myoblasts for 6 days. Gramicidin in the concentration range of 10 pM to 10 nM did not change myotube diameter, and gramicidin higher than 100 nM completely abolished myotube formation (Fig. 5B).

[Fig. 5 here]

Change in IK_{Ca} channel expression during myogenic differentiation

Are the muscle hypertrophic effects of DCEBIO observed in mature muscle fibers? IK_{Ca} channels reduce membrane currents from skeletal muscle progenitor cells as they differentiate and mature. As IK_{Ca} channels are rarely expressed in skeletal muscle tissue, we predicted that the muscle hypertrophic effects of DCEBIO decrease with maturity. We first analyzed IK_{Ca} channel gene expression in C2C12 cells during muscle differentiation. As the number of days of differentiation increased, IK_{Ca} channel expression decreased (Fig. 6A). Next, we analyzed the effects of DCEBIO on myotube diameter by changing the duration and timing of DCEBIO administration. After 6 days of differentiation, the myotube diameter increased by 7.9 μm (95% CI: 3.4 to 12.4) after the first 2 days of DCEBIO, by 16.4 μm (95% CI: 3.4 to 12.4) after the first 4 days of DCEBIO administration (Fig. 6A) (95%CI: 12.0 to 20.8), and by 15.3 μm (95% CI: 10.9 to 19.7) when administered continuously for 6 days. There was no

difference in myotube diameter after 6 days of differentiation between the cessation of DCEBIO treatment on day 4 and continued administration for 6 days. Second, the increase in myotube diameter was 2.5 μm (95% CI: -1.9 to 6.8) when treated for 4 days after day 2 of differentiation (D2-6), and no significant myotube hypertrophy was observed. Thus, DCEBIO strongly affects the early stage of muscle differentiation of C2C12 cells.

[Fig. 6 here]

DCEBIO attenuates LPS-induced myotube atrophy

Lastly, to examine the effects of DCEBIO on an *in vitro* model of pathological muscle wasting, we treated differentiating C2C12 cells with LPS, an endotoxin, to induce inflammatory muscle wasting. Application of 0.1-1 μg/mL of lipopolysaccharide (LPS) reduced myotube formation and diameter in a concentration-dependent manner (Fig. 7A, B). Co-application of 10 μM DCEBIO with 1 μg/mL of LPS caused these parameters to increase (Fig. 7B). This supports the idea that DCEBIO overcomes inflammatory muscle wasting.

[Fig. 7 here]

9. Discussion

Our pharmacological analysis demonstrated that a SK_{Ca}/IK_{Ca} channel opener, DCEBIO, activated mitoIK_{Ca} channels in C2C12 cells and activated the mitoROS/Akt/mTOR pathway to increase the diameter of regenerating, but not existing, myotubes. This effect enables myotubes to resist inflammation-induced atrophy.

In our previous report, TRAM-34 significantly inhibited the myogenic differentiation-promoting effects of DCEBIO and abolished DCEBIO-induced hyperpolarization at 1 μM (Tanaka *et al.*, 2017). However, in the present study, a higher concentration (10 μM) of TRAM-34 was required for DCEBIO-induced muscle hypertrophy.

This suggests that different mechanisms of action are involved in the muscle differentiation-promoting effects and muscle hypertrophic effects of DCEBIO. In general, potassium channel blockers are required at higher concentrations for potassium channels distributed on the mitochondrial inner membrane than for channels on the plasma membrane, consistent with the target molecule for DCEBIO-induced muscle hypertrophy being mitoIK_{Ca} channels. The concentrations used in this study were considered to be in the appropriate range because TRAM-34 was used at 2-80 μM in previous cell functional analyses using human colon cancer cells, rat bone marrow-derived macrophages, and human melanoma cells (Sassi *et al.*, 2010; Quast *et al.*, 2012; Kang *et al.*, 2014). The possibility that the muscle hypertrophic effects of DCEBIO by TRAM-34 are nonspecific cannot be excluded, and these effects are likely due to IK_{Ca} channel opening because the muscle hypertrophic effects of DCEBIO were inhibited by both siRNA and IK_{Ca} channel-specific blockers in this study.

In the present study, DCEBIO reduced the ΔΨm of C2C12 cells in a concentration-dependent manner, which was inhibited by TRAM-34. Furthermore, DCEBIO-induced muscle hypertrophy was suppressed by MitoQ. Taken together, mitoIK_{Ca} channel opening by DCEBIO may cause muscle hypertrophy via ΔΨm reduction and subsequent mitochondrial ROS generation. Excessive ROS production causes cytotoxicity, whereas low concentrations of ROS have beneficial effects on cell signaling (Rhee, 2006). Mitochondrial ROS promote muscle differentiation and hypertrophy, especially in skeletal muscle differentiation (Lee *et al.*, 2011; Hong *et al.*, 2014). These findings are consistent with the results of this study. There are two possible mechanisms for mitochondrial ROS production by the opening of K^+ channels in IMM. One is ΔΨm homeostasis. To recover the low ΔΨm induced by K^+ channel openers, the electron transfer system is activated and the proton-mediated membrane potential compensation mechanism is activated (Malinska *et al.*, 2010). Another is the maintenance of mitochondrial K^+ concentration homeostasis. To

counteract K^+ channel opener-induced K^+ influx into the matrix, the mitochondrial K^+/H^+ exchanger acts to extrude K^+ into the mitochondrial intermembrane lumen, which activates the electron transfer system to compensate for the H^+ influx (Heinen, Aldakkak, *et al.*, 2007; Heinen, S Camara, *et al.*, 2007). Both mechanisms cause increased mitochondrial ROS production due to activation of the electron transfer system.

In this study, AI-X abolished DCEBIO-induced muscle hypertrophy, suggesting that Akt is an important molecule in DCEBIO-induced muscle hypertrophy. The activation of Akt by selective openers of mitoK_{ATP} channels has been reported in rat ventricular muscle, rat hippocampal neurons, and human CD34+ hematopoietic stem cells, but the mechanism has not been fully elucidated (Ahmad *et al.*, 2006; Xue *et al.*, 2011; Joshi and Jarajapu, 2018). In human CD34+ hematopoietic stem cells, diazoxide-induced $\Delta\Psi m$ reduction causes an increase in mitoROS and promotes eNOS production via Akt and Ca^{2+}-dependent pathways (Joshi and Jarajapu, 2018). However, the mechanism leading to the increase in mitoROS and Akt activation has not been analyzed. Recently, mitoROS were reported to activate Akt/mTOR via PTEN/PI3K in C2C12 cells (Kim *et al.*, 2018), and DCEBIO may activate this pathway. To date, there have been two reports on the association between IK_{Ca} channels and Akt. In rat and human macrophages, IK_{Ca} promotes macrophage osteoclast differentiation (cell fusion) by activating Akt via Ca^{2+} signaling (Kang *et al.*, 2014). In human proximal tubular cells, IK_{Ca} channels are required for TGF-β-induced PI3K/Akt/mTOR pathway activation, but the mechanism has not been investigated (Huang *et al.*, 2016). We cannot exclude the possibility that Ca^{2+} signaling associated with KCa3.1 opening is involved in Akt activation in skeletal myoblasts. However, the relationship between Ca^{2+} signaling and Akt activation remains unclear.

In the present study, we found reduction of IK_{Ca} channel mRNA expression during muscle

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differentiation. The IK_{Ca} current disappears upon differentiation (Fioretti *et al.*, 2005), and our study suggests that the reduction in gene expression associated with differentiation is a factor in the reduction of the IK_{Ca} current. Our timing experiment revealed that the efficiency of DCEBIO on muscle hypertrophy is strong in the early stages of differentiation, but it becomes poor in the late stages of differentiation. This may be due to the decreased expression of the target molecule IK_{Ca} channels in C2C12 cells by DCEBIO. IK_{Ca} channel expression is cell-cycle dependent, being high in proliferative stages in T cells and cardiac fibroblasts (Ghanshani *et al.*, 2000; Wang *et al.*, 2013). If this were true for skeletal muscle progenitor cells, the pro-myogenic hypertrophic effects of DCEBIO should be evident when skeletal muscle progenitor cells are in a proliferative phase such as after physical trauma or muscular dystrophy. Although this study does not unveil the mechanism of IK_{Ca} channel mRNA reduction during myogenic differentiation, it is an important topic. It has been reported that the expression of IK_{Ca} channel is modulated by the repressor element-1 silencing transcription factor/neuron-restrictive silencer factor (REST/NRSF) in vascular smooth muscle cells (Cheong *et al.*, 2005). While REST/NRSF is downregulated in the proliferating stage of vascular smooth muscle cells, it is upregulated in proliferating skeletal myoblasts (Iannotti *et al.*, 2013). Interestingly, IK_{Ca} channel expression is upregulated both in proliferating smooth muscle cells and skeletal myoblasts. These suggest the difference in the role of REST/NRSF in IK_{Ca} channel regulation in both cell types.

In the present study, DCEBIO inhibited LPS-induced muscle atrophy. We investigated whether DCEBIO is effective an *in vitro* model of skeletal muscle pathology. Activation of TLR4 in skeletal muscle is caused by fatty acids and endotoxins, and induces skeletal muscle inflammatory responses, including muscle atrophy (Doyle *et al.*, 2011; Ono and Sakamoto, 2017; McKenzie *et al.*, 2020). Administration of the TLR4 ligand LPS into myocytes has been used as a reproducible model for septic muscle atrophy and obese sarcopenia. We previously

reported that this muscle atrophy involves the inactivation of the Akt/mTOR pathway (Ono *et al.*, 2020), and we hypothesized that DCEBIO, which has Akt-activating effects, is effective against inflammatory muscle atrophy. Mitochondrial K^+ channel openers generally have organ protective effects (Ohya *et al.*, 2005; Sakamoto *et al.*, 2008). However, the mechanism of DCEBIO-inhibitory effects on inflammatory muscle atrophy requires further investigation.

A limitation of this study is that we were unable to conduct animal studies. Although our *in vitro* results suggest that the pharmacological activation of IK_{Ca} channels is useful to prevent muscle atrophy, it is difficult to conclude the usefulness of DCEBIO in the treatment of patients with muscle wasting diseases. The systemic effects of IK_{Ca} channel modifiers are controversial. IK_{Ca} channel blockers, such as TRAM-34 and senicapoc, were reported to have anti-inflammatory effects in the bowel, heart, lung, and brain (Ohya *et al.*, 2014; Staal *et al.*, 2017; Xie *et al.*, 2018; She *et al.*, 2019). On the other hand, the IK_{Ca} channel opener SKA-31 improved age-related cardiac function without inflammatory responses such as cytokine increase or organ damage (John *et al.*, 2020). These paradoxical effects of IK_{Ca} channel blockers and openers may be due to the distribution of IK_{Ca} channels in the cell membrane and inner mitochondrial membrane.

In conclusion, our study demonstrated that DCEBIO increased differentiating myotube diameters and prevented inflammatory myotube atrophy through the mitoIK_{Ca} channel-dependent activation of the mitoROS/Akt pathway, thereby improving muscle wasting. Thus, it is likely that mitoIK_{Ca} channels in muscle stem cells are novel regulators of skeletal muscle development. Our observations also suggest that pharmacological activation of mitoIK_{Ca} channels by small molecules provides protective effects for skeletal muscle.

10. Acknowledgments

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11. Authorship Contributions

Participated in research design: Iseki, Ono, Hibi, Tanaka, Maejima, Kurokawa, Murakawa, Shimomura and Sakamoto.

Conducted experiments: Iseki, Ono, Hibi, Tanaka, Takeshita, Maejima, Kurokawa, and Sakamoto.

Contributed new reagents or analytic tools: Ono and Sakamoto.

Performed data analysis: Iseki, Ono, Hibi, Tanaka, Takeshita, Maejima, and Sakamoto

Wrote or contributed to the writing of the manuscript: Iseki, Ono, Maejima, Kurokawa, Shimomura, and Sakamoto.

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13. Footnotes.

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14. Figure legends

Figure 1. DCEBIO increases the diameter of C2C12 myotubes.

(A) C2C12 cells were cultured for 6 days in DM plus vehicle (Control) and DM plus DCEBIO 10 μM (DCEBIO), fixed, and subjected to May–Grünwald–Giemsa staining. Upper images are at a low magnification (Scale bar = 1 mm) and lower images are at a higher magnification (Scale bar = 200 μm). Yellow lines indicate the maximum myotube diameter. (B) Summary of myotube diameters in the absence or presence of DCEBIO (1, 3, and 10 μM). *P*-values were calculated by Dunnett's test. (C) Change in myotube diameter due to the application of DCEBIO (1, 3, and 10 μM).

Figure 2. Gene-silencing and pharmacological blocking of IK_{Ca} channels suppress DCEBIO-induced myotube hypertrophy.

(A) May-Grünwald-Giemsa stained C2C12 cells differentiated for 6 days. Cells were transfected with negative control siRNA (siNC) or IK_{Ca} siRNA ($siIK_{Ca}$) 2 days before medium change by electroporation. (B) Summary of the siRNA transfected myotube diameter in the absence or presence of DCEBIO. *P*-values were calculated by the Student's t-test. (C) Change in DCEBIO-treated myotube diameter due to $siIK_{Ca}$ transfection. (D) May-Grünwald-Giemsa-stained C2C12 cells differentiated for 6 days with or without indicated reagent(s). (E) Summary of the myotube diameters after DCEBIO and/or TRAM-34 treatment. (F) Change in DCEBIO-treated myotube diameter due to TRAM-34.

Figure 3. Opening of IK_{Ca} channels in mitochondria is involved in DCEBIO-induced myotube hypertrophy.

(A and B) The mitochondrial membrane potential (ΔΨm) of C2C12 myoblasts was monitored by the JC-1 fluorescence ratio between red fluorescence of polarized mitochondria and green

fluorescence of deporalized mitochondria.

(A) The concentration-dependence of DCEBIO based on the JC-1 fluorescence ratio in C2C12 myoblasts. *P*-values were calculated by Dunnett's test. (B) The effects of TRAM-34 on DCEBIO-induced decreases in ΔΨm in C2C12 myoblasts. FCCP was used as a negative control of ΔΨm. *P*-values were calculated by the t-test. (C and D) Effects of MitoQ on DCEBIO-induced myotube hypertrophy. (C) Summary of myotube diameters in the absence or presence of DCEBIO (10 μM) and/or MitoQ (0.1 μM). *P*-values were calculated by the t-test. (D) Change in DCEBIO-treated myotube diameter due to the application of MitoQ.

Figure 4. Involvement of the Akt/mTOR pathway in DCEBIO-induced myotube hypertrophy. (A) Representative Western blots for phosphorylated Akt (pAkt), total Akt (tAkt), phosphorylated S6K (pS6K), and total S6K (tS6K). Quantitative summaries were shown below. (B) Summary of the myotube diameter in the absence or presence of DCEBIO and/or Akt Inhibitor-X (AI-X). *P*-values were calculated by the Student's t-test. (C) Change in DCEBIO-treated myotube diameter due to the application of AI-X. (D) Summary of the myotube diameter in the absence or presence of DCEBIO and/or rapamycin. *P*-values were calculated by the Student's t-test. (E) Change in DCEBIO-treated myotube diameter due to the application of rapamycin.

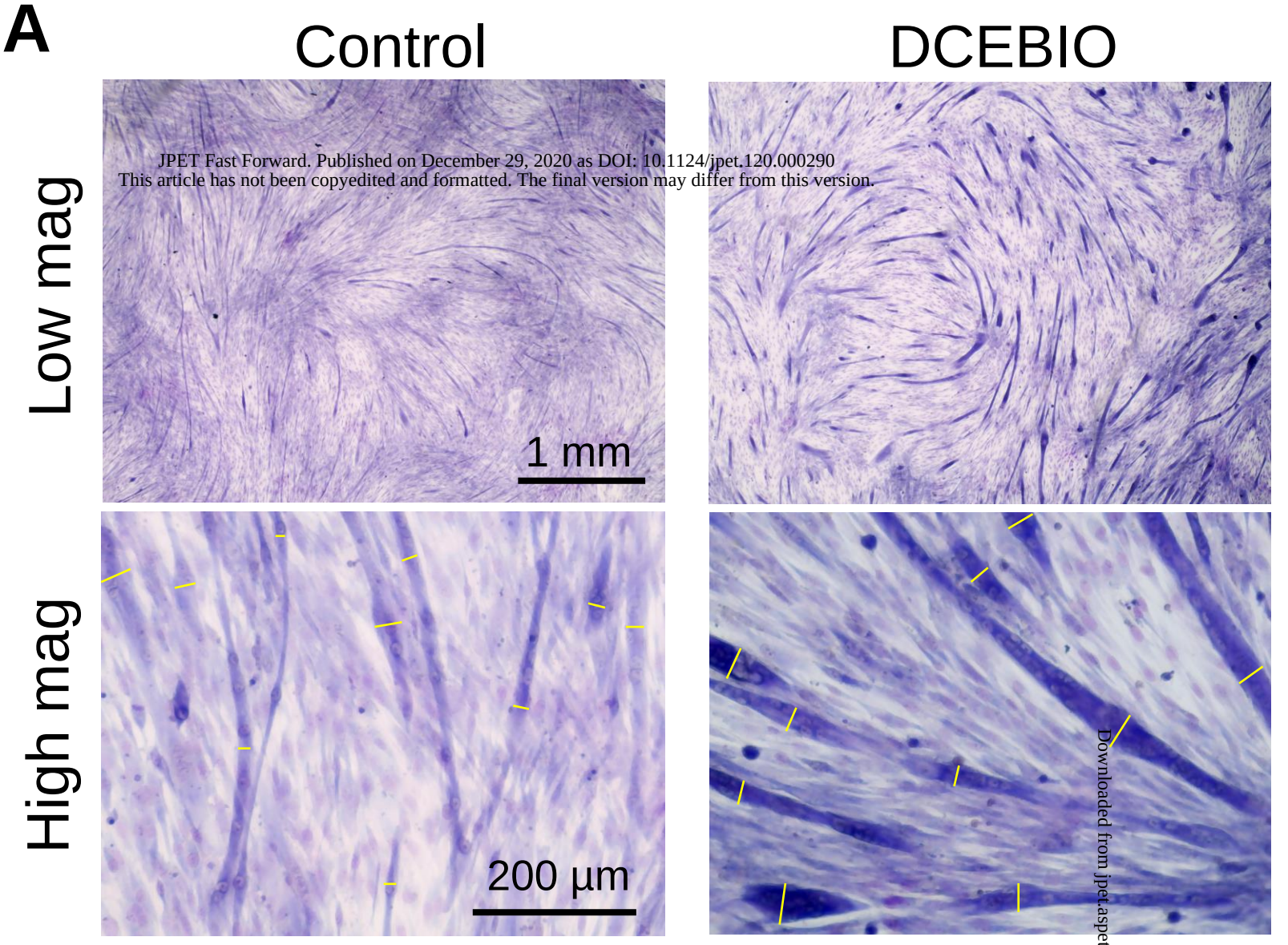
Figure 5. Involvement of the Akt/mTOR pathway in DCEBIO-induced myotube hypertrophy. (A) Representative Western blots for phosphorylated Akt (pAkt) and total Akt (tAkt). Quantitative summary was shown below. (B) Summary of myotube diameters in the absence or presence of gramicidin (10 pM -1 μM). *P*-values were calculated by Dunnett's test.

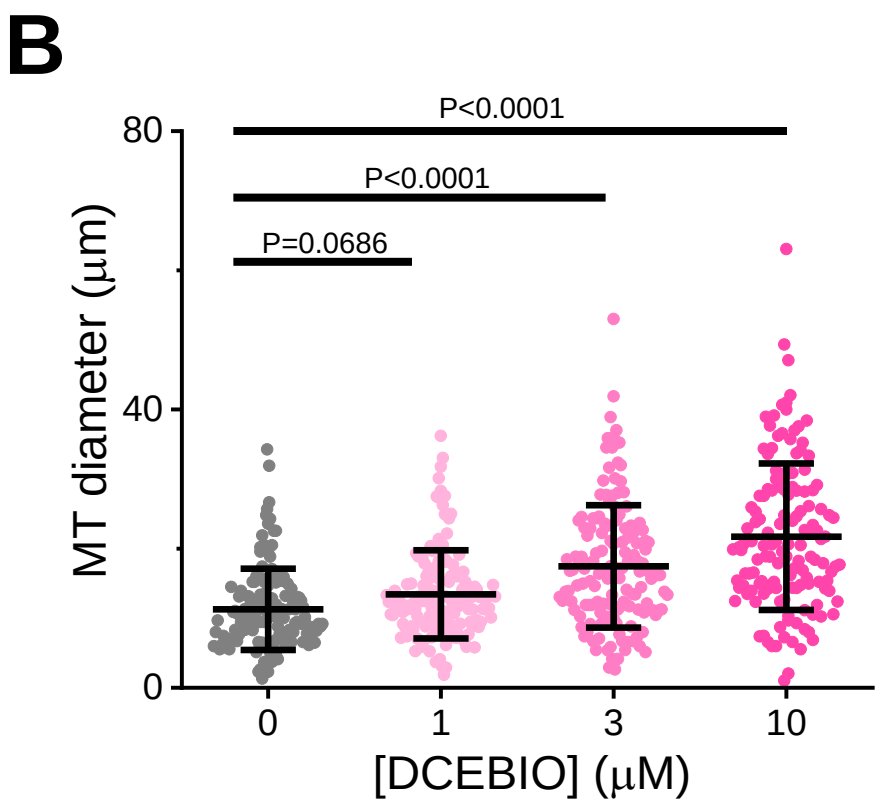
Figure 6. Myotube hypertrophic effects of DCEBIO are limited in the early stage of myogenic differentiation of C2C12 cells.

(A) Expression of the IK_{Ca} channels decreased during myogenic differentiation. qRT-PCR results obtained using samples extracted from C2C12 cells in DM from days 0 to 6. The expression levels of mRNA were quantified by real-time PCR and mRNA products were divided by the product of Csnk2a2. The numbers in parentheses indicate independent experiments. *P*-values were calculated by Dunnett's test. (B and D) Summary of the myotube diameters. C2C12 myoblasts were differentiated for 6 days with DCEBIO from day 0 to day 2 (D0-2), from day 0 to day 4 (D0-4), day 0 to day 6 (D0-4), and day 2 to day 6 (D2-6). *P*-values were calculated by Dunnett's test (B) and one-way ANOVA with Tukey-Kramer's test (D). (C and E) Change in myotube diameter due to the application of DCEBIO in the indicated periods.

Figure 7. DCEBIO overcomes LPS-induced myotube atrophy.

(A) May-Grünwald-Giemsa-stained C2C12 cells differentiated for 6 days in the absence or presence of DCEBIO with or without LPS (Scale bar = 200 μm). (B) Summary of myotube diameters under the indicated conditions. *P*-values were calculated by the t-test.





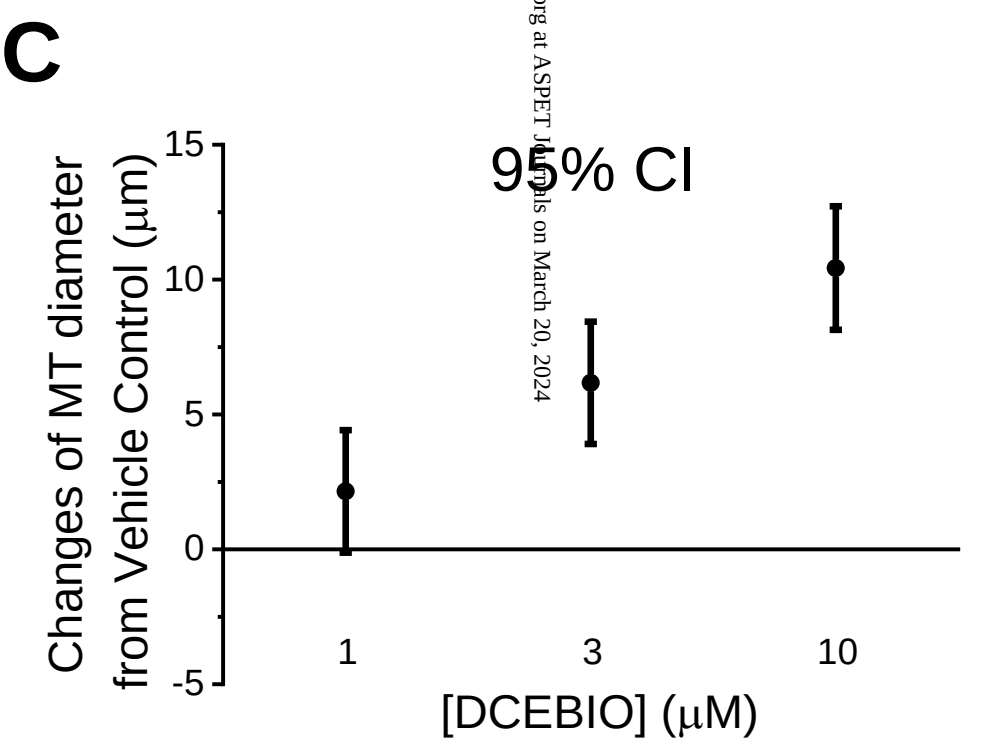
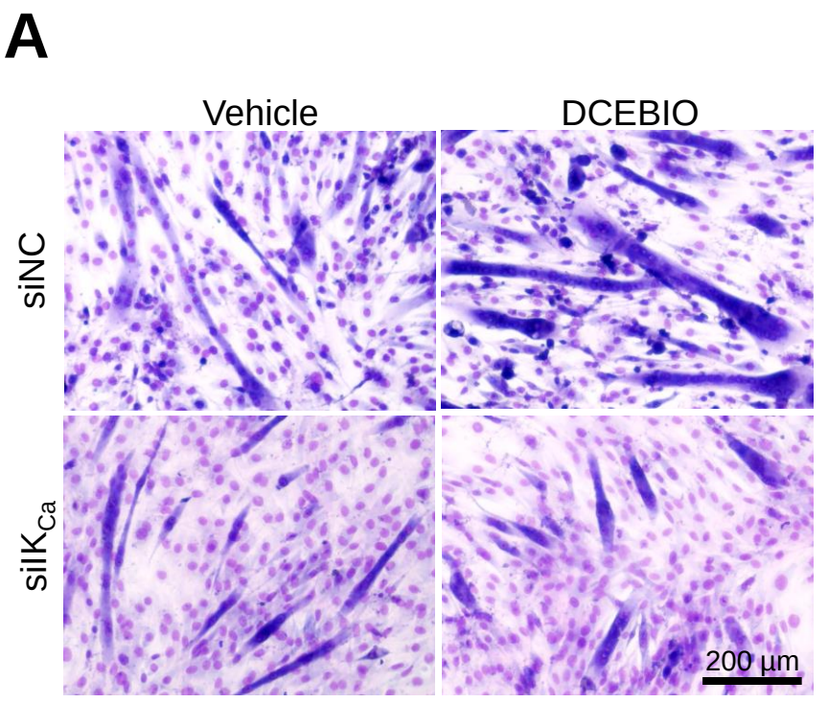
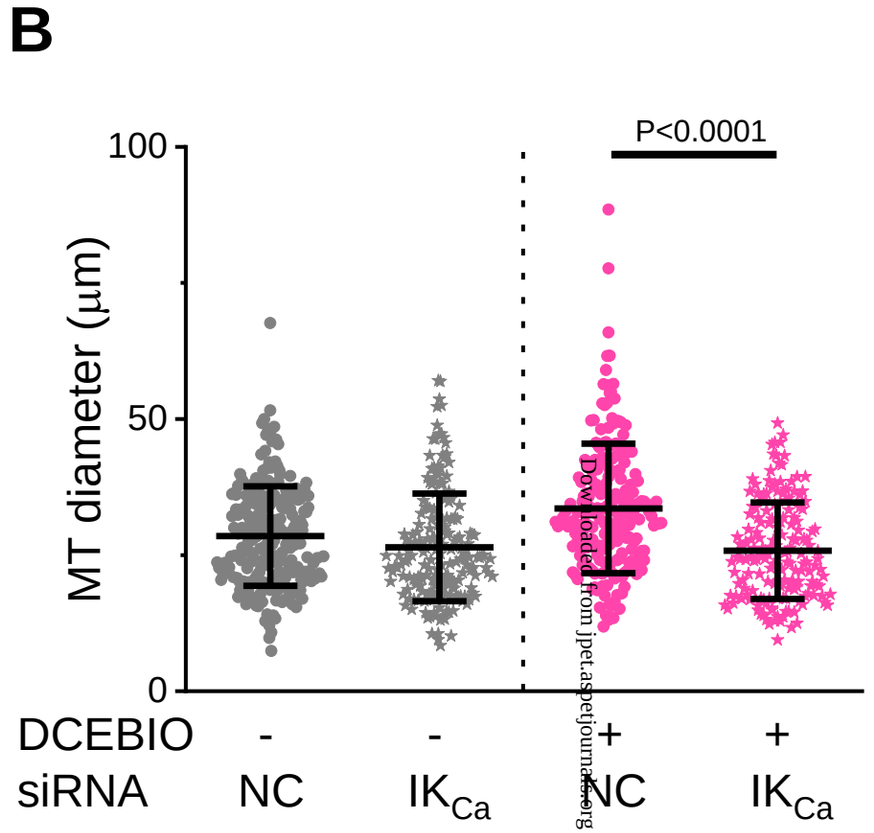
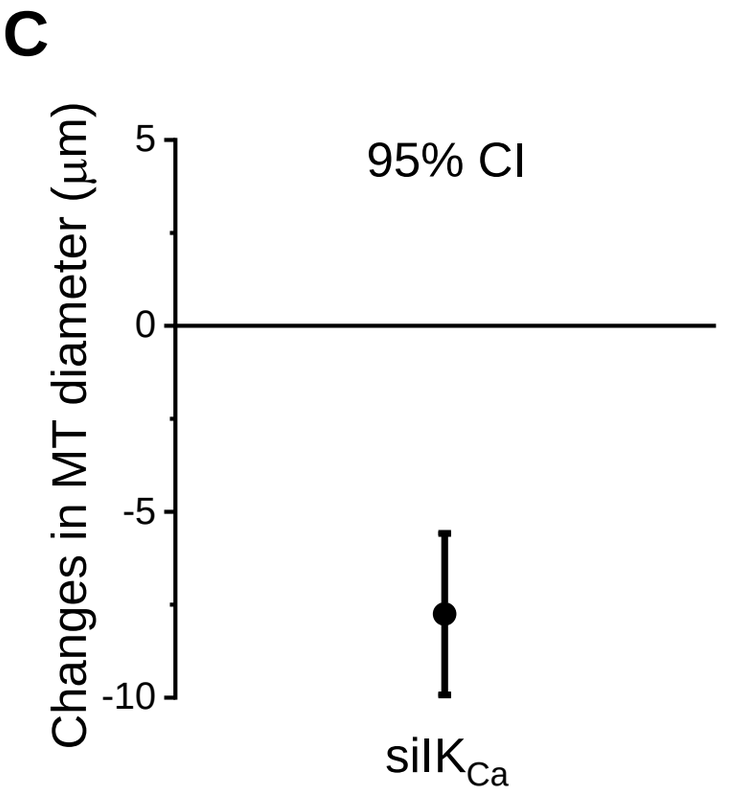
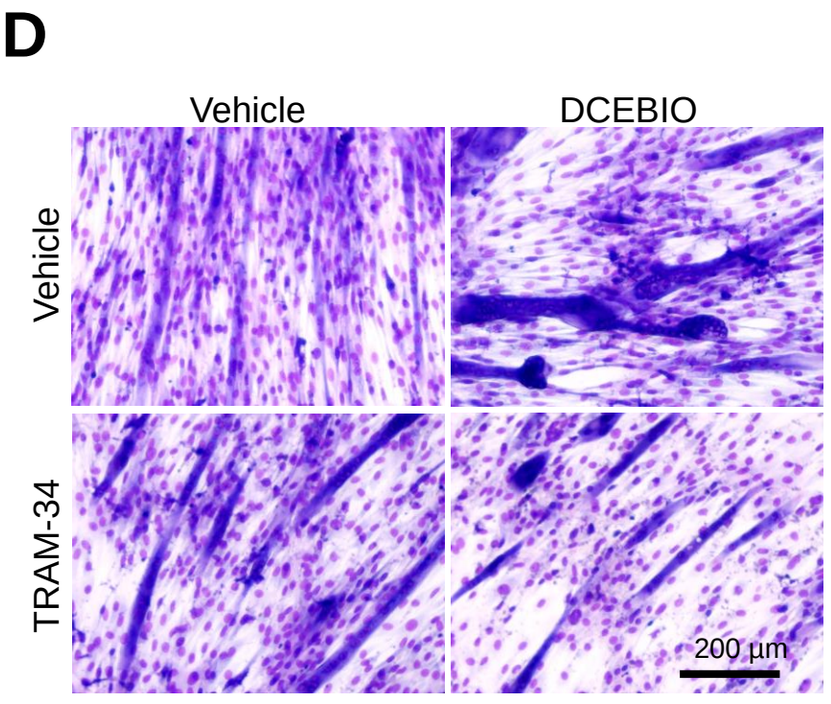


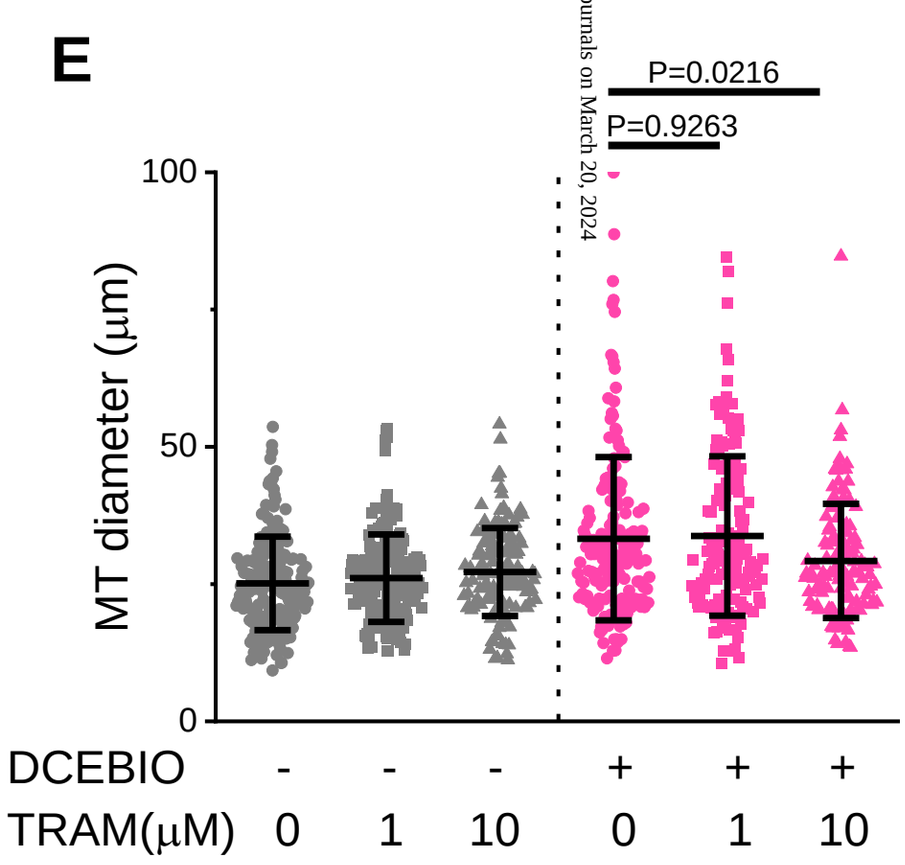
Figure 1

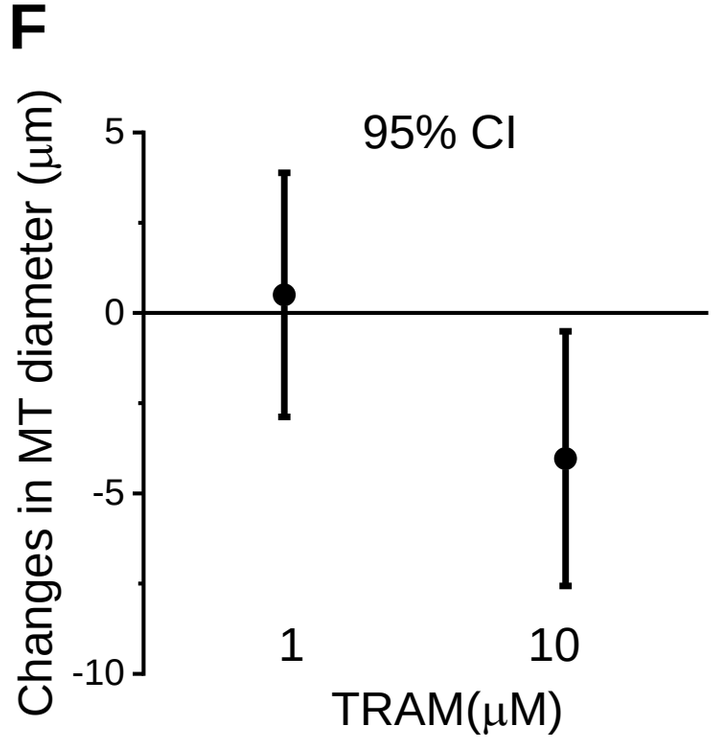












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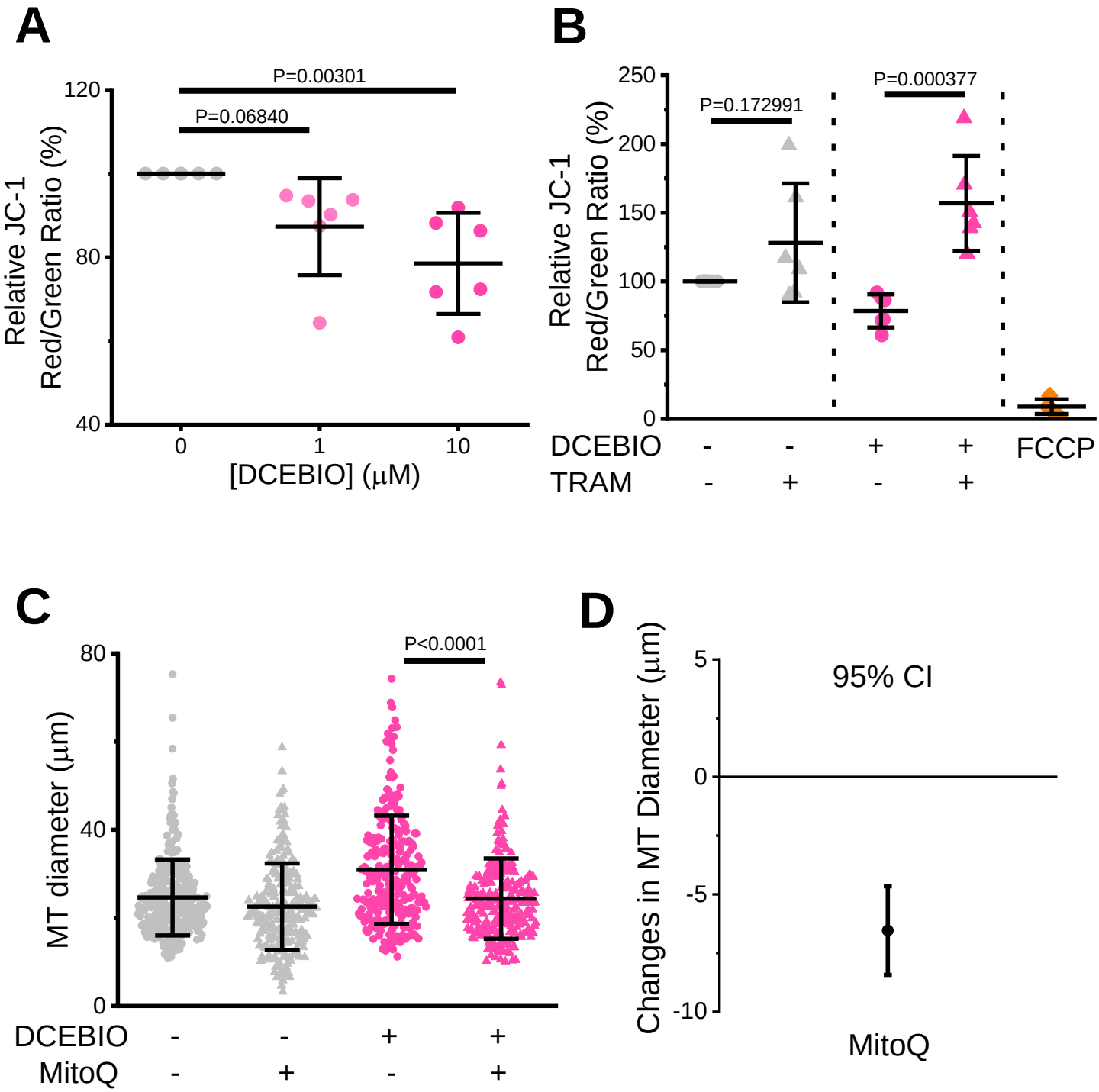
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1
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[DCEBIO] (μM)
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250
200
150
100
50
0
P=0.172991
P=0.000377
Relative JC-1
Red/Green Ratio (%)
DCEBIO - - + + FCCP
TRAM - + - +
C
P<0.0001
80
40
0
MT diameter (μm)
DCEBIO - - + +
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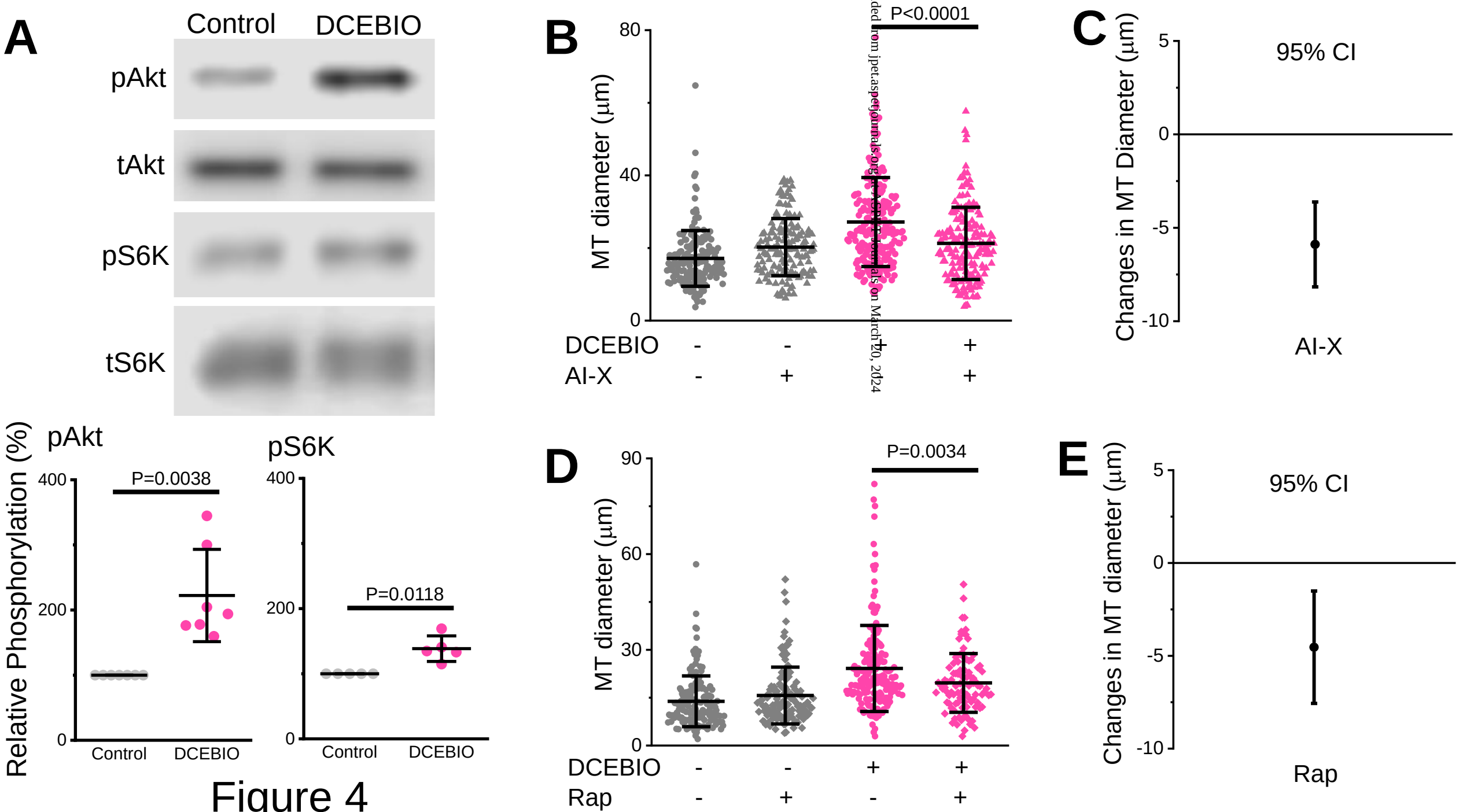
D
95% CI
5
0
-5
-10
Changes in MT Diameter (μm)
MitoQ

Figure 3



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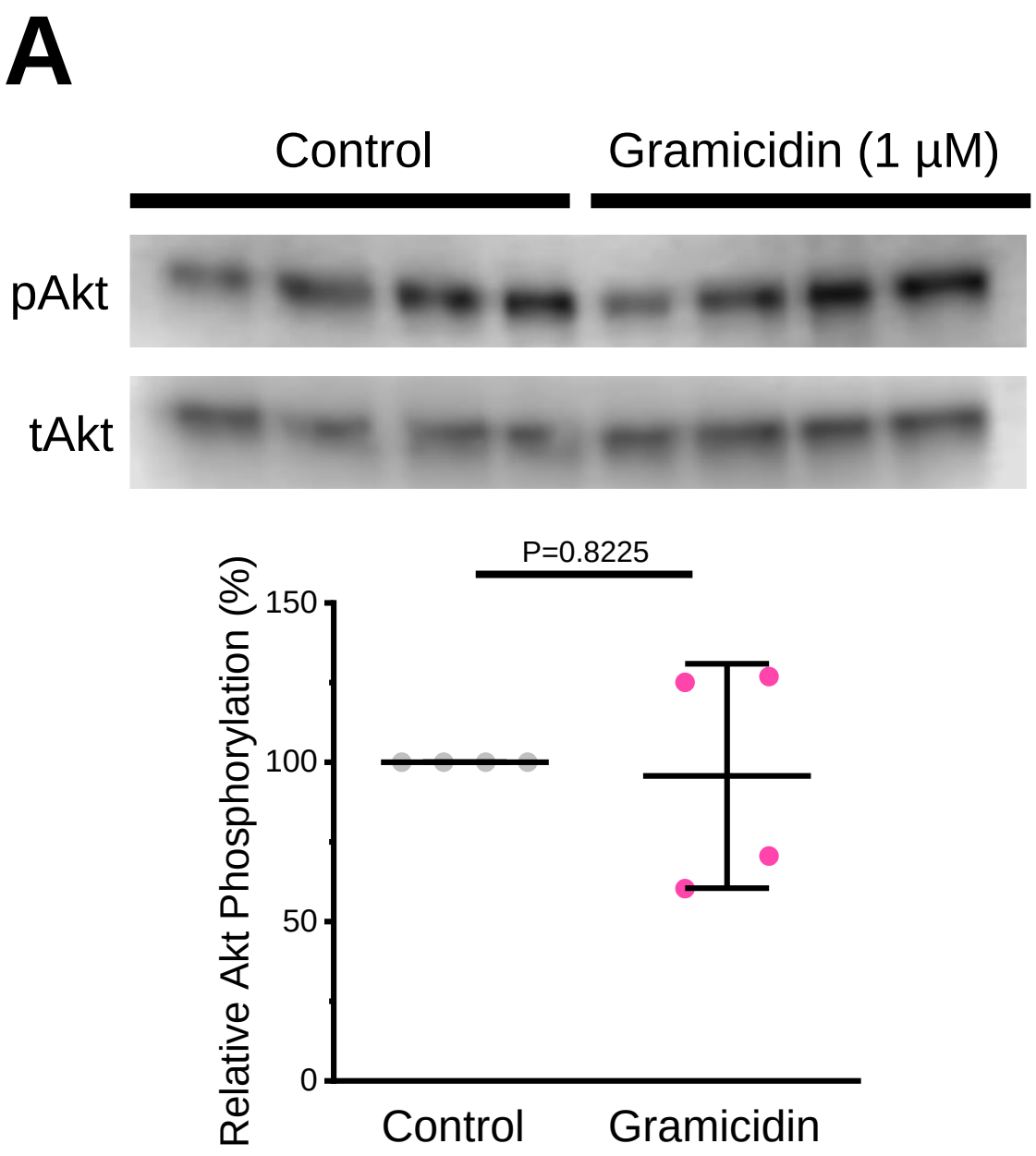


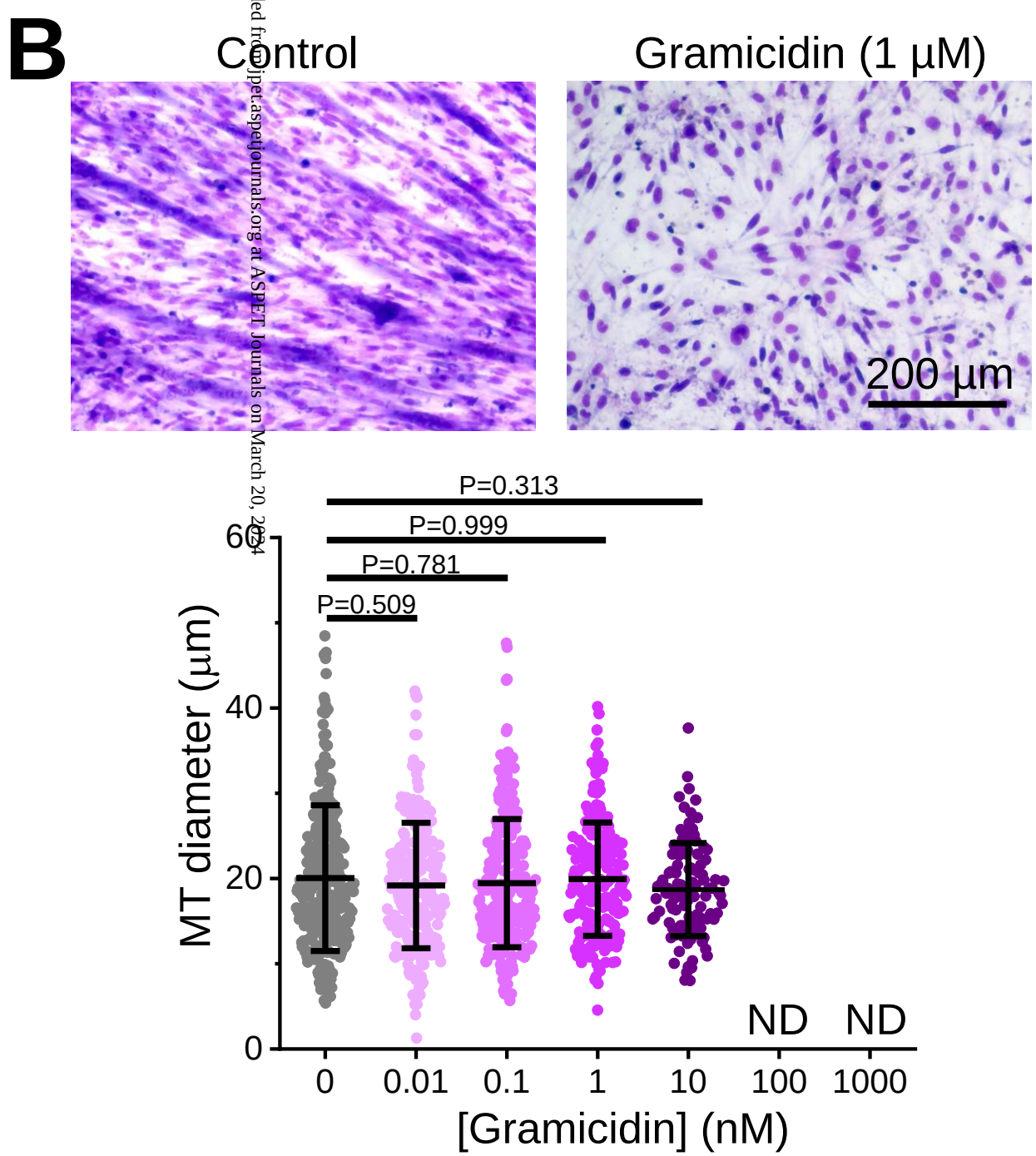
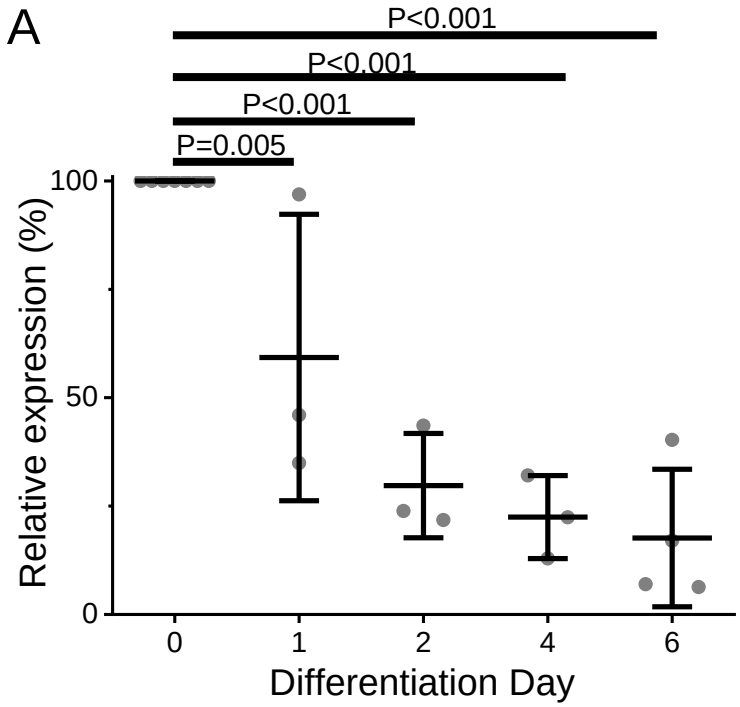


Figure 5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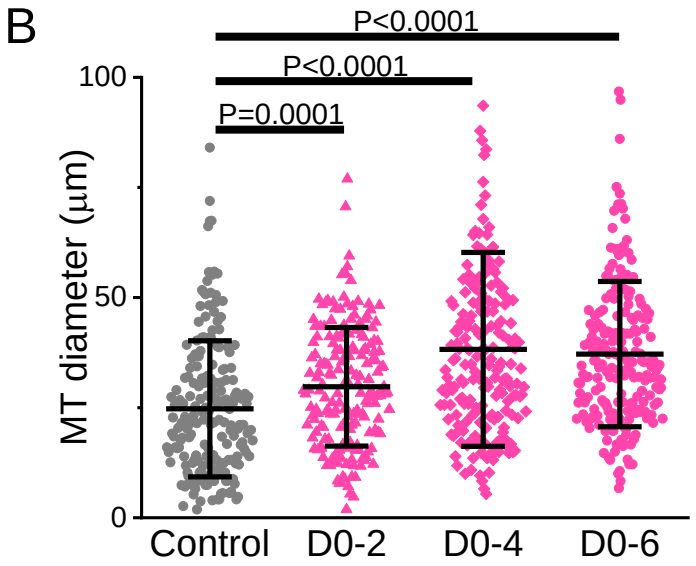
1
2
4
6
Differentiation Day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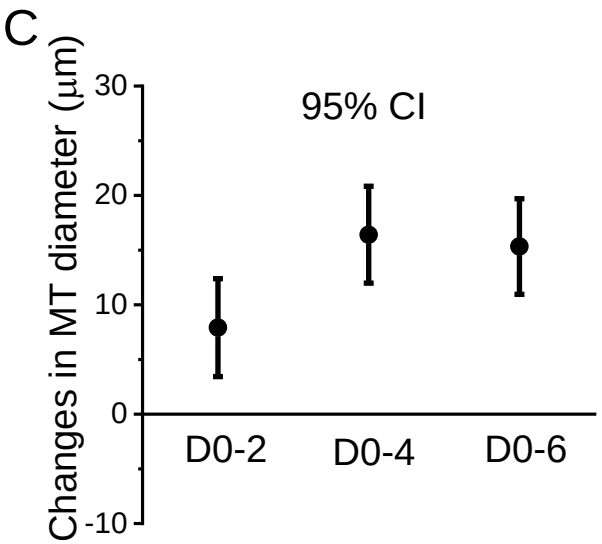
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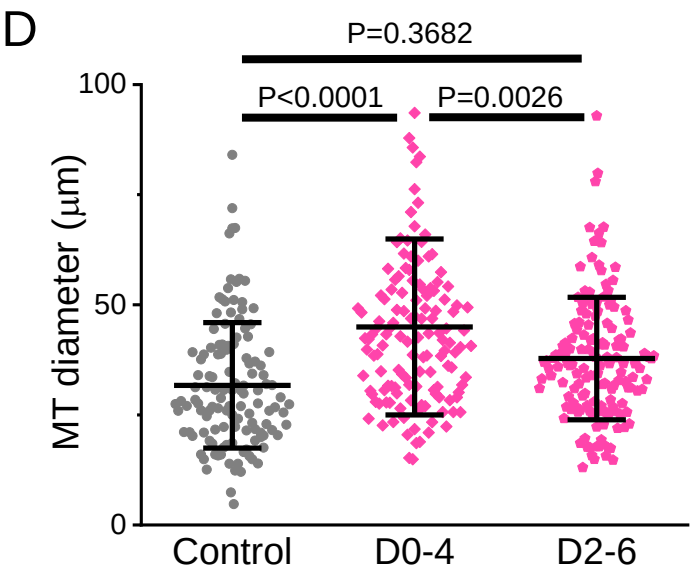
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D0-4
D2-6

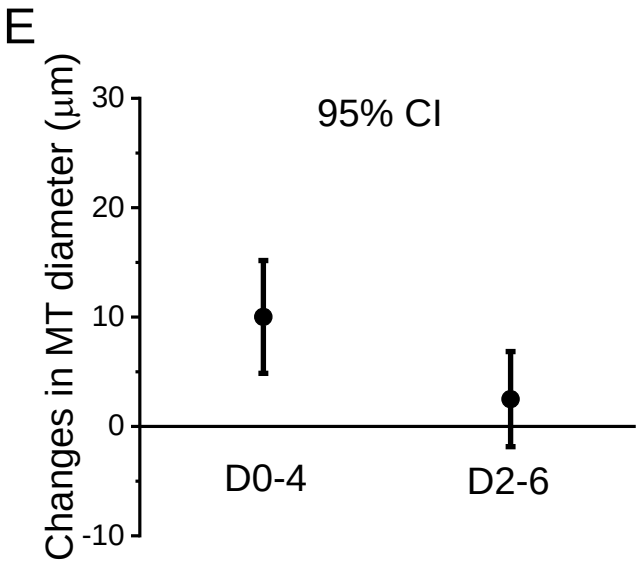
E
95% CI
Changes in MT diameter (μm)
30
20
10
0
-10
D0-4
D2-6

Figure 6

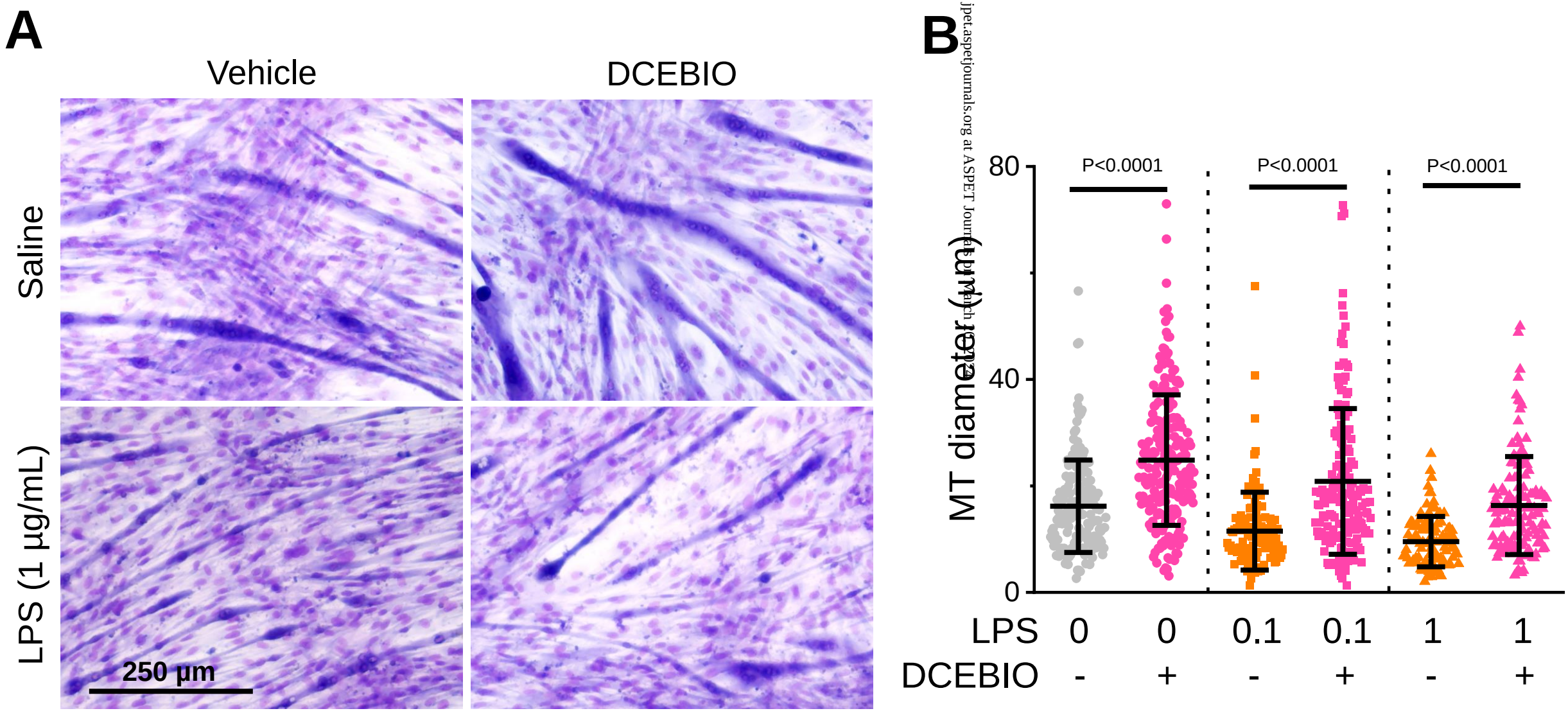

Figure 7