Maternal rodent exposure to di-(2-ethylhexyl) phthalate decreases muscle mass in the offspring by increasing myostatin

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Abstract

Background Di-(2-ethylhexyl) phthalate (DEHP) and its metabolites can cross the placenta and may cause birth defects and developmental disorders. However, whether maternal DEHP exposure affects skeletal muscle development in the offspring and the pathways involved are unknown. This study investigated the effects of maternal DEHP exposure and the contribution of myostatin (MSTN) to skeletal muscle development in the offspring.

Methods Pregnant wild-type and muscle-specific myostatin knockout (MSTN KO) C57BL/6 mice were randomized to receive vehicle (corn oil) or 250 mg/kg DEHP by gavage every other day until their pups were weaned (postnatal day 21 [PND21]). Body weights of the offspring mice were measured longitudinally, and their hindleg muscles were harvested at PD21. Also, C2C12 cells were treated with mono-2-ethylhexyl phthalate (MEHP), the primary metabolite of DEHP, and proteolysis, protein synthesis, and myogenesis markers were measured. The contribution of myostatin to maternal DEHP exposure-induced muscle wasting in the offspring was determined.

Results Maternal DEHP exposure reduced body weight growth, myofibre size, and muscle mass in the offspring compared to controls (Quad: 2.70 ± 0.1 vs. 3.38 ± 0.23, Gastroc: 2.29 ± 0.09 vs. 2.81 ± 0.14, Tibialis: 1.01 ± 0.07 vs. 1.25 ± 0.11, mg/tibial length in mm, all P < 0.01, n = 35). Maternal DEHP exposure significantly increased Myostatin expression (2.45 ± 0.41 vs. 0.03 ± 0.00 DEHP vs. controls, P < 0.01, n = 5), Atrogin-1 (2.68 ± 0.65 vs. 0.63 ± 0.01, P < 0.05, n = 5), MuRF1 (1.56 ± 0.51 vs. 0.31 ± 0.01, P < 0.05, n = 5), and Smad2/3 phosphorylation (4.12 ± 0.35 vs. 0.49 ± 0.18, P < 0.05), and decreased MyoD (0.27 ± 0.01 vs. 1.52 ± 0.01, P < 0.05, n = 5), Myogenin (0.25 ± 0.03 vs. 1.95 ± 0.56, P < 0.05, n = 5), and AKT phosphorylation (4.12 ± 0.35 vs. 1.00 ± 0.06, P < 0.05, n = 5), in skeletal muscle of the offspring in MSTN flox/flox, but not in MSTN KO mice. Maternal DEHP exposure resulted in up-regulation of CCAAT/enhancer-binding protein δ (C/EBPδ, 4.12 ± 0.35 vs. 1.00 ± 0.19, P < 0.05, n = 5) in skeletal muscle of the offspring in MSTN flox/flox and MSTN KO mice (4.12 ± 0.35 vs. 4.35 ± 0.28, P > 0.05, n = 5). In vitro, C/EBPδ silencing abrogated the MEHP-induced increases in Myostatin, MuRF-1, and Atrogin-1 and decreases in MyoD and Myogenin expression.

Conclusions Maternal DEHP exposure impairs skeletal muscle development in the offspring by enhancing the C/EBPδ-myostatin pathway in mice.

Keywords di-(2-ethylhexyl) phthalate; maternal; offspring; skeletal muscle; myostatin

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Introduction

The loss of muscle mass and function is a hallmark of several debilitating musculoskeletal disorders. Previous studies have suggested that maternal exposure to phthalates may lead to myopathies in foetuses, neonates and adults but the pathways involved remain incompletely characterized. Di-(2-ethylhexyl) phthalate (DEHP) is one of the most common phthalates and is commonly found in polyvinyl chloride, cosmetics, perfumes, medical devices, and food packaging. DEHP is highly hydrophobic and not covalently bound in polymers and can be released from plastic products into the environment and to humans. DEHP is also known to cross the placenta into fetal blood circulation. A recent study reports that infants can absorb about 1–10 mg/kg/day of DEHP from breast milk and cow’s milk. Also, occupational and medical exposures can reach much higher levels. For instance, exposure to DEHP from blood transfusions can be as high as 250–300 mg, equivalent to a dose of 3.5–4.3 mg/kg for an adult weighing 70 kg. A longitudinal cohort study in Taiwan revealed that median (range) levels of estimated phthalate daily intake of children (2–18 years old) was 5.81 (0.257–56.5) mg/kg/day. DEHP in the intestinal lumen can then be rapidly metabolized by esterases into mono-2-ethylhexyl phthalate (MEHP) that can be converted into 5-OH-MEHP, 5-oxo-MEHP, and other metabolites. These DEHP metabolites are detected in 85%–99% of urine samples from pregnant women.

DEHP exposure is associated with the development of obesity, glucose intolerance, and lower birth weight, all serious public health concerns worldwide. More importantly, foetuses and neonates are particularly sensitive to DEHP, and early-life exposures to DEHP may have lifelong detrimental effects, leading to abnormal sexual development, adverse birth outcomes, and neurodevelopmental and hormonal disturbances. Despite the important role of skeletal muscle dysfunction in obesity, glucose intolerance, insulin resistance, and low birth weight, whether and how maternal DEHP exposure affects skeletal muscle development in the offspring is unknown.

During myogenesis, precursor myoblasts differentiate into myoblasts that then fuse into myotubes. It is known that myoblast differentiation protein D (MyoD) and myogenic factor 5 can initiate myoblast differentiation, and myogenin and myogenic regulatory factor-4 can promote myoblast maturation during myogenesis. DEHP at a high dose can inhibit the expression of MyoD limiting myogenic differentiation, which may be related to a decrease in mitochondria and peroxisome function. Also, myogenesis can be negatively regulated by an imbalance of muscular proteolysis and protein synthesis due to aberrant activation of muscle ring finger 1 (MuRF1) and muscle atrophy F-box (MAFBx/Atrogin-1), two ligases in the ubiquitin–proteasome system, and by activation of myostatin. Myostatin is a cytokine in the transforming growth factor β superfamily that can inhibit skeletal muscle growth, leading to skeletal muscle atrophy.

Whether maternal DEHP exposure affects the expression of these pathways in the offspring has not been fully clarified.

Here, we report the effects of maternal DEHP exposure on skeletal muscle development in the offspring using a mouse model. We found that maternal DEHP exposure impaired myogenesis in the offspring by enhancing myostatin expression. Our findings provide new insights into the mechanisms underlying the skeletal muscle toxicity of environmental phthalates and may aid in the design of new therapies for the prevention and treatment of phthalate-related skeletal muscle disorders in children.

Materials and methods

Animals

Muscle-specific myostatin knockout (MSTN KO) C57BL/6 mice were generated using the muscle creatine kinase (MCK) promoter Cre-LoxP system. Myostatin-loxP+/− (stock# 012685) and MCK-Crefl/fl C57BL/6 mice (stock# 006405) were obtained from Jackson Laboratories (Bar Harbor, ME, USA). These mice were bred to generate MSTN KO mice at the Army Medical University. Their genotypes were determined by PCR using specific primers (Table S1). All MSTNlox/fl mice had the Myostatin-LoxP+/−/genotype and the MSTN KO mice had the MCK-Crefl/fl myostatin-LoxP+/− genotype (Figure S1A,B). The experimental protocols were approved by the Institutional Animal Care and Use Committee of the Army Medical University.

DEHP exposure

Wild-type (WT), MSTN KO, or MSTNlox/fl female C57BL/6 mice were bred with the same strain of male mice. Vaginal plugs were monitored daily, and individual female mice with observed vaginal plugs were considered as on gestation day 0. The same strains of pregnant mice were randomized and administered vehicle (corn oil [cat#C116025, Aladdin], Vehicle group) or 250 mg/kg DEHP (cat# R004097, RHAWN, purity >99.5%, CAS No.: 117-81-7) in corn oil (DEHP group) by oral gavage once every other day (n = 7–10 per group) until weaning (day 21 postnatal, PND21). Previous reports using similar models exposed pregnant female rats or mice to vehicle (corn oil) or DEHP at 10–750 mg/kg by oral gavage. To avoid over-stimulation of pregnant mice, we administered 250 mg/kg via gavage every other day. Pregnant mice and their male mates were kept in the same cages until weaning. Mice were sacrificed, and tissues were harvested on PND21. All animals were ad libitum fed.

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Cell culture and treatments

Mouse myoblast C2C12 cells (American Type Culture Collection [ATCC], Manassas, USA) were cultured in Dulbecco’s modified Eagle medium (DMEM) (cat# E600003–0500, Sangon Biotech, Shanghai, China) containing 10% of fetal bovine serum (cat# SH30406.05, HyClone, Logan, USA), penicillin (10 000 U/ml)–streptomycin (10 mg/ml) solution, 10X, (cat# C0222, Beyotime, Jiangsu, China). Passages between 3 and 7 were used for experiments.

C2C12 myoblasts were plated out in 6-well or 96-well plates at a density of 500 cells per cm². After 24 h, cells were cultured in medium with DEHP or MEHP (cat# HY-W018392/ CS-W019178, MCE, purity ≥ 97.0%, CAS No.: 4376-20-9) or without supplements (controls). Images in the DEHP treatment group (100 μM) were obtained after 1, 2, 3, and 4 days post incubation. Images in DEHP or MEHP treatment groups at different doses were obtained after 1 or 3 days post incubation. Myoblasts treated with MEHP were harvested 48 h later for total RNA or protein isolation, and other assays.

Apoptosis assay

The impact of MEHP on apoptosis of C2C12 myoblasts was determined by flow cytometry. Cells were treated in triplicate with or without MEHP for 48 h and stained with Annexin V-FITC and Propidium iodide using the Annexin V-FITC Apoptosis Detection Kit (cat#1062, Beyotime, Jiangsu, China), followed by flow cytometry analysis in a flow cytometer (BD Biosciences, San Jose, CA, USA). The percentages of viable, necrotic, and apoptotic cells were analysed using FlowJo software v10 (BD Biosciences, San Jose, CA, USA).

Quantitative real-time PCR

Total RNA was extracted from mouse gastrocnemii or C2C12 cells using Trizol reagent (cat# 15596–026, Life Technologies, Grand Island, USA) and reversely transcribed into cDNA using specific kit (cat# RR047A, PrimeScript™ RT reagent Kit with gDNA Eraser, TaKaRa, Japan). The relative levels of gene mRNA transcript to the control glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were quantified by RT-qPCR in a Bio-Rad CFX-96 using specific primers (Table S2), and data were analysed by 2−ΔΔCt.

Immunofluorescence staining

Frozen TA muscle sections (5 μm) were fixed in 4% paraformaldehyde (cat# Z2902, Sigma). After being washed with Tris-buffered saline Tween-20 (cat# T8220, Solarbio, Beijing, China) sections were blocked with blocking buffer for 1 h and incubated with the primary antibody (1:200 dilution) overnight at 4°C. The bound antibodies were detected with Alexa Fluor 488 (cat# A-11060) or 546 (cat#A-11081)-labelled secondary antibodies (1:1000 dilution, Invitrogen) 1 h at RT, followed by nuclear staining with 4’6-diamidino-2-phenylindole (cat# 4083, CST). The TA muscle sections (5 μm) were stained with anti-laminin antibody (cat# L0663, Sigma) for analysis of CSAs of at least 500 myofibres (>100 magnification) per animal.

Western blot analysis

Western blots analyses were performed as previously described. Briefly, gastrocnemii were homogenized in lysis buffer, and myoblasts were lyzed in buffer (cat# V900854, Sigma-Aldrich, St. Louis, USA) containing protease inhibitors (cat# A32955, Thermo Scientific) and phosphorylation protease inhibitors (cat# 4906845001, Roche). After quantification of protein concentrations using a BCA Protein Assay Kit (cat# T9300A, TaKaRa), their nuclear proteins were extracted using the EpiQuik Nuclear Extraction Kit (cat# OP-0002-1, Epigentek Farmingdale, NY, USA). The lysates or nuclear proteins (20 μg/lane) were separated by SDS-PAGE on 4%–12% gels and transferred onto polyvinylidene fluoride (PVDF) membrane (cat# IPFL00010, Millipore, Bedford, Mass). After being blocked with 5% bovine serum albumin (BSA, cat# ST025, Beyotime, Jiangsu, China), the membranes were probed with primary antibodies overnight at 4°C. The bound antibodies were detected with Dylight 680/800-labelled anti-rabbit/mouse/goat IgG (cat#: 35568, Thermo Scientific) and visualized in LI-COR Odyssey (LI-COR, Lincoln, Dearborn, USA). The signals were quantified using the Image-Pro plus.

The primary antibodies were against p-Akt (Ser 473, cat#4060), C/EBP-δ (cat#2318), and Smad2/3 (D7G7, cat#8685); p-Smad2 (Ser465/467)/Smad3 (Ser423/425, D27F4, cat#8828, from Cell Signalling Technology; GAPDH (cat# 60004-1-Ig, Proteintech, Chicago, USA); myostatin (GDF8, cat#AF788, R&D system), myoD (cat#12344), and Lamin A/C (cat#L9393) from Sigma-Aldrich; myogenin (cat#ab1835) from Abcam (Cambridge, UK); Akt (cat#8312), and atrogin-1/MAFbx (cat#sc-166806) from Santa Cruz Biotechnology (Dallas, Texas, USA).

Dual-luciferase reporter assay

C2C12 myoblasts were transfected with the recombinant plasmids for myostatin luciferase reporter, Renilla luciferase or control phRLTK-luc using Transfectin Lipid Reagent (cat# 1703351, Bio-Rad, Hercules, CA, USA). Six hours later for total RNA or protein isolation, and other assays.
say system (cat# E2940, Dual-Glo® Luciferase Reagent and Dual-Glo® Stop & Glo® Reagent, Promega).

**RNAi**

RNA interference was performed with small interfering RNA (siRNA). The siRNA oligonucleotides were synthesized and purified by GenePharma (Shanghai, China), which also provided the scrambled negative control (Table S3). C2C12 myoblasts were cultured in a 24-well plate for 48 h and transfected with 20 μmol/L of control siRNA or C/EBPδ-specific siRNA in 1% FBS culture medium by using Lipo2000® (Beyotime, C0526FT). After incubation for 6 h, the culture media were replaced with regular siRNA-free culture medium. Media were replaced every day, and C2C12 myoblasts were harvested for Real-time PCR after 3 days.

**Statistical analysis**

All data were expressed as the mean ± standard deviation (SD). The difference between groups was analysed by the independent-samples Student’s t-test (two-tailed). Two-way analysis of variance (ANOVA) was performed to identify differences between genotypes (MSTN KO vs. MSTN KO mice) across treatments (Vehicle, DEHP) followed by Fisher’s least significant difference (LSD) post hoc test ($P < 0.05$). All statistical testing was performed using GraphPad Prism 8.3.1 (GraphPad Software, San Diego, CA). A P-value of $< 0.05$ was considered statistically significant.

**Results**

**Maternal DEHP exposure causes low skeletal muscle development in the offspring**

To test the impact of maternal DEHP exposure on skeletal muscle development of the offspring, WT pregnant C57BL/6 mice were randomized and treated with vehicle or DEHP up to the weaning day. Body weight change of pups in the DEHP group was significantly lower than that in vehicle-treated controls (Figure 1A). Also, the ratios of quadriceps (Quad), gastrocnemius (Gastro) and TA muscle weights to tibia lengths of the DEHP-exposed group at PND21 were significantly lower than that in the control group ($P < 0.05$ for all, Figure 1B). Histological analyses showed that the mean TA myofibre CSA in the DEHP group was also smaller than that in the control group ($P < 0.05$, Figure 1C-D). Analysis of myofibres revealed that the distribution was left-shifted as compared with the normal curve (Figure 1E).

**Maternal DEHP exposure increases muscle proteolytic markers and decreases markers of myogenesis in the offspring**

We tested how maternal DEHP exposure affected proteolysis and myogenesis in the offspring. In comparison with vehicle-treated controls, RT-qPCR indicated the relative levels of MuRF1 and atrogin 1 mRNA transcripts increased significantly while the markers of myogenesis MyoD and Myogenin mRNA transcripts significantly decreased in DEHP-exposed mice ($P < 0.05$ for all, Figure 2A). These results suggest that maternal DEHP exposure promoted proteasome activity and attenuated myoblast differentiation in offspring mice.

To understand the toxicity of DEHP in myoblasts we examined the effects of DEHP on C2C12 myoblasts. Treatment with different doses of DEHP for varying periods did not obviously change the growth and morphology of C2C12 cells (Figure S2A-B), which may be due to the deficiency of relevant esterases in C2C12 cells (Figure 2B). We subsequently tested the impact of MEHP, the primary metabolite of DEHP, on C2C12 myoblasts. Compared with DMSO-treated control cells, treatment with different doses of MEHP for 3 days decreased the cell viability in C2C12 cells in a dose-dependent manner (Figure S2C). Treatment with MEHP (250 μM) resulted in obvious atrophy of C2C12 cells (Figure 2C) and significantly increased the percentages of apoptotic C2C12 cells ($P < 0.01$, Figure 2D). Western blot analyses showed that treatment with different doses of MEHP significantly increased the ratio of cleaved caspase 3 to total caspase 3 and myostatin expression in C2C12 cells in a dose-dependent manner ($P < 0.05$ for all, Figure 2E).

**Myostatin mediates the effects of maternal DEHP exposure on skeletal muscle development in the offspring**

We tested the effects of maternal DEHP exposure on muscle development in the offspring of MSTN<sup>flx/flx</sup> and MSTN KO C57BL/6 mice to determine the contribution of myostatin in this setting. Maternal DEHP exposure significantly increased myostatin expression in the muscle of MSTN<sup>flx/flx</sup>, but not in MSTN KO mice ($P < 0.05$ for all, Figure 3A-B). Maternal DEHP exposure also decreased body weight, TA CSA and muscle weights in the offspring of MSTN<sup>flx/flx</sup>, but not in MSTN KO mice ($P < 0.05$, Figure 3C-F). These data demonstrated that the effects of maternal DEHP exposure on muscle development in the offspring are dependent on the up-regulation of myostatin.
Maternal DEHP exposure alters muscle proteolysis and myogenesis in the offspring of MSTN\(^{fl^{ox}/fl^{ox}}\), but not in MSTN KO mice

Maternal DEHP exposure increased skeletal muscle Myostatin, Atrogin-1 and MuRF-1, and decreased MyoD and Myogenin mRNA transcripts in MSTN\(^{fl^{ox}/fl^{ox}}\) but not in MSTN KO mice (\(P < 0.05\) for all, Figure 4A). Similarly, treatment with DEHP also increased the relative levels of Atrogin-1 and MuRF-1 and decreased myogenin and MyoD protein expression in MSTN\(^{fl^{ox}/fl^{ox}}\), but not MSTN-KO mice (\(P < 0.05\) for all, Figure 4B). Given that AKT/FoxO1–3 signalling and Smad2/3 activation are crucial for regulating Atrogin-1 and MuRF-1 expression and are downstream of myostatin, we further examined the impact of maternal DEHP exposure on the levels of AKT and Smad2/3 phosphorylation in muscles from the offspring in MSTN\(^{fl^{ox}/fl^{ox}}\) and MSTN KO mice. As shown in Figure 4C, maternal DEHP exposure significantly decreased the ratio of phosphorylated AKT to total AKT expression and increased the ratio of phosphorylated Smad2/3 to total Smad2/3 expression in MSTN\(^{fl^{ox}/fl^{ox}}\) but not in MSTN KO mice. Thus, myostatin deletion prevented changes in muscle proteolysis and protein synthesis, Smad, and AKT signalling induced by maternal DEHP exposure.

**DEHP promotes MSTN expression and inhibits skeletal muscle development by up-regulating C/EBP\(\delta\) expression in myoblasts**

Finally, we investigated how DEHP up-regulated myostatin expression in myoblasts. First, C2C12 cells were transfected with a plasmid expressing luciferase under the control of the myostatin promoter (Figure 5A) and subsequently treated with different doses of MEHP for 48 h before luciferase activity was measured. As shown in Figure 5B, treatment with MEHP increased luciferase activity in a dose-dependent manner.

Given that C/EBP can bind to the myostatin promoter,\(^{32}\) we tested whether DEHP could enhance myostatin expression in myoblasts by up-regulating C/EBP\(\delta\). Western blots revealed that maternal DEHP exposure significantly increased skeletal muscle nuclear C/EBP\(\delta\) in the offspring regardless of the presence of myostatin (Figure 5C,D). Also, C/EBP\(\delta\) silencing

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**Figure 1** Maternal DEHP exposure reduces body weight and skeletal muscle mass in offspring mice. WT C57BL/6 pregnant mice were randomized and orally treated with vehicle or DEHP daily until offspring were weaned on postnatal day 21. (A) Body weights in mice (\(n = 35\) per group). (B) Tissue weights of quadriceps (Quad), Gastrocnemius (Gastroc) and tibialis anterioris (TA) muscles normalized to tibial length. (C) Average myofibre cross-sectional areas (CSA). (D) Immunofluorescence analysis of TA muscle stained with an anti-laminin antibody (red). (E) The size distribution of TA muscular fibres in the offspring. Data are representative images or expressed as the mean ± SD of each group (\(n = 35\)) from three separate experiments. *\(P < 0.05\) vs. the vehicle.
abrogated the MEHP-induced increases in myostatin, MuRF-1, and Atrogin-1 expression, as well as the decreases in MyoD and Myogenin expression in C2C12 cells (Figure 5E,F). Collectively, this data showed that maternal DEHP exposure induced myostatin expression by up-regulating C/EBPδ expression.

**Discussion**

Previously, our group and others have shown that prenatal exposure to phthalates is associated with lower birth weight. In this study, we explored the effects of maternal DEHP exposure on muscle development in the offspring mice. Our results indicate that maternal DEHP exposure caused low skeletal muscle development in the offspring at least in part by enhancing C/EBPδ-mediated myostatin expression, thereby inhibiting myogenesis and altering the balance between protein synthesis and proteolysis. These data provide novel insights into the molecular mechanisms underlying the effects of maternal phthalate exposure on skeletal muscle development in the offspring.

Foetuses and neonates are particularly sensitive to phthalate metabolites because they have a deficiency in DNA repair and detoxification enzymes, and an underdeveloped blood–brain barrier. In addition, phthalates can inhibit placental cell
proliferation, disrupt the endocrine system, impair glucose metabolism, and induce cell cycle dysregulation. Our findings that maternal DEHP exposure caused poor muscle development in the offspring could explain the mechanisms behind a previous observation of DEHP exposure-related sarcopenia.

A balance between protein synthesis and degradation is crucial for muscle development and its disruption is associated with the loss of muscle mass and strength. Previous studies have shown that aberrant activation of proteolysis via the ubiquitin-proteasome system (UPS) and/or autophagy pathways can lead to sarcopenia. We found that maternal DEHP exposure significantly increased the expression of MuRF1 and atrogin 1, two muscle-specific ubiquitin ligases commonly overexpressed in sarcopenia and other muscle wasting disorders. We also found a DEHP-induced downregulation of the myogenic transcription factors MyoD and myogenin, which are involved in the withdrawal of myoblasts from the cell cycle and subsequent myogenic differentiation. Similarly, exposure to MEHP reduced MyoD and myogenin expression and inhibited myogenic differentiation.
differentiation in C2C12 cells. The lack of response to DEHP may stem from the deficiency in esterases that are needed to metabolize DEHP into MEHP in C2C12 cells.

Myostatin inhibits skeletal muscle development by negatively regulating myoblast proliferation and differentiation.1,40,41 Hence, it is an important protein for embryogenic myogenesis as skeletal muscle-specific MSTM-deleted mice show muscular hypertrophy.40 Here we report that maternal DEHP exposure significantly increased myostatin expression, and that muscle-specific MSTM deletion prevents the effects of maternal exposure to DEHP on skeletal muscle proteolytic and myogenic markers as well as on muscle mass and CSA. Interestingly, we detected a small amount of myostatin in the KO, which is thought to come from other tissues such as cardiac muscle through circulation.42 To our knowledge, this is the first evidence that myostatin plays a central role in mediating the deleterious effects of maternal DEHP exposure on skeletal muscle development in the offspring. Our data also suggests that myostatin may be a therapeutic target valuable for the prevention and treatment of the DEHP-related muscle disorders in neonates.

C/EBPδ activation leads to increased myostatin expression given that the myostatin promoter contains C/EBPδ binding sites.32 Here, we show that C/EBPδ is up-regulated by DEHP and MEHP leading to increased myostatin expression and activation of its downstream mediators, p-Smad2 and p-Smad3,43 inhibiting myoblast differentiation into mature muscle fibres and decreasing p-Akt leading to impaired muscle development.
These changes were ameliorated in both MSTN KO mice and in siRNA-C/EBPδ C2C12 myoblasts suggesting that the skeletal muscle effects of exposure to DEHP are mediated through myostatin via C/EBPδ.32 The up-regulation of myostatin may also inhibit AKT phosphorylation, which may activate caspase-3 to promote myoblast cell apoptosis and proteolysis through the 26S proteasome.44 Down-regulation of AKT can also induce FoxO transcription factor activation to stimulate the expression of Atrogin-1/MAFbx and MuRF-1, increasing proteolysis.45 Furthermore, up-regulation of myostatin can activate Smad2/3 inhibiting myoblast differentiation and myofibre maturation.46

Taken together, the data suggest that C/EBPδ/myostatin signaling is critical for DEHP-induced impaired myogenesis in mice.

There are several limitations in our study. Previous reports using similar models exposed pregnant female rodents to DEHP at doses ranging between 10 and 750 mg/kg by oral gavage.26–30 We tested a single dose of 250 mg/kg of DEHP every other day to minimize the stress in the mothers, accounting for the relative resistance to the effects of phthalates seen in rodents.47 Hence, the effects of other doses in rodents and in humans remain to be determined. Mechanistically, more studies are needed to determine how

Figure 5 DEHP enhances MSTN expression and inhibits skeletal muscle development through up-regulating C/EBPδ expression in offspring mice and C2C12 cells. (A) A diagram of the myostatin promoter structure. (B) Luciferase assay indicated that MEHP enhanced the transcription activity of the myostatin promoter. (C) Western blot revealed that maternal DEHP exposure increased the contents of nuclear C/EBPδ in muscles of MSTNfl/fl mice, and MSTN KO offspring mice (n = 5 per group). (D) RT-qPCR demonstrated C/EBPδ silencing in C2C12 cells (n = 5). (E) RT-qPCR indicated that C/EBPδ silencing abrogated the MEHP-regulated Myogenin, MyoD, Myostatin, MuRF-1, and Atrogin-1 expression in C2C12 cells (n = 5). Data are representative images or expressed as the mean ± SD of each group from three separate experiments. *P < 0.05 vs. MSTNfl/fl-vehicle or Con. #P < 0.05 vs. MSTN KO-vehicle. NS, no significant difference.
Maternal DEHP exposure can up-regulate C/EBPδ expression, the effects of DEHP in differentiated myotubes, and to distinguish the effects of prenatal and postnatal exposure of DEHP on skeletal muscle development. Also, the relative contribution of food intake changes induced by DEHP in the neonates could not be established in the current model. Lastly, future studies should address indicators of maternal health in this model, and explore other pathways that may be contributing, such as autophagy and mitochondrial function, fatty acid oxidation, and glucose metabolism pathways. Measuring total number of fibre muscles will be needed to confirm the effects of DEHP on myogenesis.

In summary, our results demonstrate that (1) maternal DEHP exposure during pregnancy and lactation causes neonatal skeletal muscle growth retardation associated with up-regulation of C/EBPδ, Myostatin, Smad2–3, UPS, and down-regulation of myogenic and protein synthesis markers; (2) targeted knockout of myostatin in skeletal muscle prevents muscle wasting induced by DEHP by rebalancing muscle protein synthesis and degradation; and (3) C/EBPδ down-regulation inhibits myostatin expression and suppresses the expression of its downstream mediators. Taken together, this data indicates that maternal DEHP exposure impaired skeletal muscle development in the offspring at least partially by enhancing the C/EBPδ-mediated myostatin expression and its downstream mediators to promote proteolysis and inhibiting myogenesis (Figure 6). Our findings shed light on the molecular mechanisms underlying the effects of DEHP maternal exposure on skeletal muscle development and may aid in the design of therapies for the prevention and treatment of this and other muscle wasting disorders.

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Online supplementary material

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Conflicts of interest

The authors declare no current or potential conflicts of interest.

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