Nanoparticle-mediated Intracellular Lipid Accumulation during C2C12 Cell Differentiation

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Abstract

In this report, we sought to elucidate whether multiwall carbon nanotubes are involved in the modulation of the proliferation and differentiation of the skeletal muscle cell line C2C12. Skeletal muscle is a major mass peripheral tissue that accounts for 40% of total body weight and 50% of energy consumption. We focused on the differentiation pathway of myoblasts after exposure to a vapor-grown carbon fiber, HTT2800, which is one of the most highly purified carbon nanotubes. This treatment leads in parallel to the expression of a typical adipose differentiation program. We found that HTT2800 stimulated intracellular lipid accumulation in C2C12 cells. We have also shown by quantified PCR analysis that the expression of adipose-related genes was markedly upregulated during HTT2800 exposure. Taken together, these results suggest that HTT2800 specifically converts the differentiation pathway of C2C12 myoblasts to that of adipoblast-like cells.

Keywords: myoblast, adipoblast, multiwall carbon nanotube, lipid accumulation

Abbreviations: PBS, phosphate-buffered saline; DMEM, Dulbecco's modified Eagle's medium; FABP, Fatty Acid Binding Protein

1. Introduction

Skeletal muscle formation, or myogenesis, is an intricate and highly regulated process [1]. A unique feature of skeletal muscle cell differentiation is the fusion of mononucleated myoblasts to multinucleated myotubes [2] [3]. Myogenesis is regulated by the sequential expression of myogenic regulatory factors (MRFs), including MyoD and myogenin [4]. MyoD is the primary myogenic regulatory factor required for the formation, proliferation, and survival of myoblasts, whereas myogenin acts late during myogenesis and activates the expression of important muscle-specific genes, such as myosin heavy chain and creatine kinase [5] [6]. It has been reported that MyoD is prevalent in fast-twitch muscles whereas myogenin is prevalent in slow-twitch muscles [7]. It has also been noted that myogenic differentiation is regulated at the transcriptional level by a combination of activating and repressing factors [8] [9].

Several reports have indicated that nanoparticles have been functionalized to improve their biocompatibility and promote their interaction with biomolecules [10]. The discovery of carbon nanotubes has greatly advanced this area owing to their peculiar structures and properties. Interestingly, Flahaut et al. [11] reported that carbon nanotubes synthesized by catalytic chemical vapor deposition were found to be nontoxic to human cells. Unpurified carbon nanotubes have been reported to be more toxic than metal-free nanoparticles [12]. HTT2800 removed the iron catalyst by thermally treating the material at a temperature of 2800°C in an argon atmosphere [13]. This suggests that only a very low concentration of the iron-based material remains in HTT2800. Recently, many synthetic or natural compounds have been reported to induce a differentiated phenotype in C2C12 cells [14] [15]. However, the effect of carbon nanotubes on the growth and differentiation of skeletal muscle cells has received little attention. To assess potential mechanisms of carbon nanotube action in skeletal muscle, we examined the effects of HTT2800 on C2C12 cells. C2C12 cells are a well-established and commonly used *in vitro* model for the study of myogenic differentiation [16]. When C2C12 cells become confluent in culture, they fuse into typical multinucleated myotubes. In this study, we report here for the first time that

HTT2800 stimulates intracellular lipid accumulation and adipocyte-related gene expression in C2C12 cells.

2. Materials and methods

2.1. Reagents

We used the highly purified multiwall carbon nanotube abbreviated as HTT2800, which has been previously described in detail [13]. In brief, HTT2800 has a high aspect ratio, diameter in the range of 100–150 nm, and length in the range of 10–20 μm. HTT2800 was dispersed with 0.1% gelatin (Nacalai Tesque, Kyoto, Japan) in phosphate buffered saline (PBS, pH 7.4), and agglomeration of HTT2800 was prevented by applying an ultrasonicator to destroy the agglomerates before use. Tissue culture media were obtained from Nacalai Tesque. Rabbit polyclonal antibody against MyoD (sc-304), Myogenin (sc-576), PPARγ (sc-7196), CD36 (sc-9154) and mouse polyclonal antibody against β-actin (sc-47778) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit polyclonal antibody against FABP4 (#2120) was purchased from Cell Signaling Technology (Danvers, MA, USA).

2.2. Cell culture and HTT2800 exposure

Mouse myogenic C2C12 cells were purchased from the European Collection of Cell Cultures. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS (Sigma-Aldrich, St. Louis, MO, USA), penicillin (100 U/ml), and streptomycin (100 μg/ml). Cells were grown and maintained in 100-mm culture plates (Iwaki, Tokyo, Japan) at 37°C in a 5% CO_2 incubator. To induce myoblast differentiation, C2C12 cells were grown in DMEM supplemented with 2% horse serum (Nichirei Biosciences Inc., Tokyo, Japan).

2.3. Cell viability and proliferation

To determine the viability of C2C12 cells exposed to different concentrations of HTT2800, we performed Alamar Blue [17] and EdU incorporation assays by using alamarBlue cell viability reagent (Invitrogen, Carlsbad, CA) and the Click-iT EdU Microplate Assay Kit (Molecular Probes, Eugene, OR), respectively. Assays were performed following the manufacturers' instructions.

2.4. Oil red O staining

The extent of differentiation was determined by the amount of lipid accumulation after 7 days by using a Lipid Staining Kit (Zen-Bio Inc., Research Triangle Park, NC, USA). Briefly, cells were incubated in a fixative solution for 1 h, stained with 0.6% oil red O solution in isopropyl alcohol/H_2O (60:40, v/v) for 1 h at room temperature, and washed 4 times with distilled water. Differentiation was examined by visual inspection and quantified by elution with isopropyl alcohol and an optical density measurement at 590 nm (Beckman DU-640).

2.5. Quantitative real-time PCR analysis

Total RNA was prepared using a TRIzol reagent (Invitrogen) from C2C12 cells (2×10^5). One microgram of total RNA was used for the subsequent synthesis of cDNA with the ReverTra Ace qPCR RT Kit (TOYOBO, Osaka, Japan) according to the manufacturer's instructions. Quantification of mRNA levels was measured using the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) and the First Start Universal SYBR Green PCR Master mixture (Roche Applied Science, Mannheim, Germany) with the following primer-pair sets: PPARγ_2, 5′-GCTGTTATGGGTGAAACTCTG-3′ (F) and 5′-ATAAGGTGGAGATGCAGGTTC-3′ (R); CD36, 5′-GCCTCCTTTCCACCTTTTGT-3′ (F) and 5′-TCTGTACACGGGGATTCCTT-3′ (R); FABP4, 5′-CTTCGATGATTACATGAAAGAAGTG-3′ (F) and 5′-ACGCCCAGTTTGAAGGAAAT-3′

(R); Sox8, 5′-GACCAGTACTTGCCCCTCAAT-3′ (F) and 5′-TCAGCTGCTCCGTCTTGATCT-3′ (R); KLF15, 5′-ACCGAAATGCTCAGTGGGTTACCTA-3′ (F) and 5′- GGAACAGAAGGCTTGCGAGTCA-3′ (R); Nur77, 5′-TGATGTTCCCGCCTTTGC-3′ (F) and 5′-CAATGCGATTCTGCAGCTCTT-3′ (R); MyoD, 5′-GACCTGCGCTTTTTTGAGGACC-3′ (F) and 5′-CAGGCCCACAGCAAGCAGCGAC-3′ (R); myogenin, 5′-CATCCAGTACATTGAGCGCCTA-3′ (F) and 5′-GAGCAAATGATCTCCTGGGTTG-3′ (R); and GAPDH, 5′-CTGCACCACCAACTGCTTAG-3′ (F) and 5′-GGGCCATCCACAGTCTTCT-3′ (R). The cycling parameters for these assays were 50°C for 2 min, 95°C for 2 min, and 40 cycles of 95°C for 30 s and 60°C for 1 min. Quantification of the cDNA levels of each gene was performed for each of the 3 replicates of cDNA prepared.

2.6. Western blot analysis

C2C12 cells were washed twice with ice-cold PBS and solubilized with whole-cell extraction buffer (20 mM HEPES [pH 7.9], 0.5% NP-40, 15% glycerol, 300 mM NaCl, 1 mM EDTA, 10 mM NaF, 1 mM Na_3VO_4, 1 mM DTT, 1 mg/ml leupeptin, 1 mg/ml pepstatin, 1 mg/ml aprotinin, and 0.5 mM PMSF). The cell lysate was centrifuged at 14,000 × *g* for 5 min, and the protein in the supernatant was quantified using Bradford protein assay reagent (Bio-Rad, Hercules, CA, USA). Total protein was diluted 1:4 with a lane marker reducing sample buffer (ThermoFisher Scientific, Waltham, MA, USA) and boiled for 5 min. The resultant protein was separated on a 10% SDS-PAGE gel and transferred onto a PVDF membrane (GE Healthcare, Piscataway, NJ, USA). The membrane was blocked by Block Ace (DS Pharma Biomedical Co. Ltd., Osaka, Japan) for 1 h at room temperature and probed with primary rabbit MyoD (sc-304), myogenin (sc-576), and mouse β-actin antibodies (Santa Cruz Biotechnology) at 4°C overnight. After

washing, the membrane was incubated with a secondary anti-rabbit antibody for 1 h at room temperature and developed with ECL Plus chemiluminescent detection reagent (GE Healthcare).

3. Results

3.1. Effects of HTT2800 on C2C12 cell morphology and proliferation

The C2C12 cells were derived from adult murine myoblasts, and these cells can undergo *in vitro* myogenesis when cultured in low-serum conditions after reaching confluence [2]. As shown in Fig. 1A, we planned a dosing schedule for exposure of C2C12 cells to HTT2800. C2C12 cells displayed an almost complete morphology of multinucleated myotubes when maintained in DMEM containing 2% horse serum from seeding to day 7 after reaching confluence (Fig. 1B). It was suggested that C2C12 cells were induced to differentiate in low-serum conditions. To investigate the effects of HTT2800 on the differentiation of C2C12 cells, the cells were seeded in 24-well plates in DMEM supplemented with 10% FBS. Interestingly, as shown in Fig. 1C, exposure of confluent cells to HTT2800 (10 μg/ml) led to the appearance of round giant cells (indicated by arrows) compared to control (10% FBS). The cell samples were then stained with Hoechst 33342 and viewed under a fluorescence microscope (Olympus IX-71; Olympus, Tokyo, Japan). We found that cell nuclei had high numbers and irregular distributions relative to the control vehicle-treated cells (Fig. 1G–J). Next, to investigate the long-term effect on cell growth, cell viability and proliferation were examined. As shown in Fig. 1K and L, both assays were used to determine any possible adverse effects of HTT2800. The cultured C2C12 cells were not affected by incubation in medium with various concentration of HTT2800 (up to 10 μg/ml).

3.2. HTT2800 treatment induces cellular lipid accumulation in C2C12 cells

As shown in Fig. 2B, we have also found that incubation of C2C12 cells with HTT2800 (10 μg/ml) leads to lipid accumulation in cells, as indicated by the increase in oil red O staining (Fig.

2C–F). After 7 days in culture, cells were filled up with neutral lipid, which was positively stained by oil red O (Fig. 1F and H). The carbon black particle CB-R (Mitsubishi Chemical, Tokyo, Japan), a negative control, failed to induce intracellular lipid accumulation (data not shown). We identified that upon incubation of C2C12 cells with HTT2800, 10–20% of the total cells became round and exhibited lipid accumulation. In contrast, when the cells were cotreated with 0.1% gelatin, a significant conversion to the adipocyte phenotype was not observed (Fig. 2A and B).

3.3. HTT2800 induces adipocyte-related gene expression in C2C12 cells

Quantified PCR analyses were performed to investigate the effect of HTT2800 on the expression of RNA markers characteristic of either muscle or adipose differentiation in C2C12 cells. MyoD was expressed in proliferating myoblasts and involved in their maintenance [4]. As shown in Fig. 3A, when the medium was switched to DMEM with 10% FBS containing 10 μg/ml HTT2800, MyoD mRNA levels were markedly reduced after 24 h. However, after 7 days, MyoD mRNA levels were significantly increased. The expression of myogenin was also reduced in a time-dependent manner after exposure to HTT2800 (Fig. 3B). Fig. 3C shows the western blot analysis of MyoD and myogenin expression after exposure to HTT2800, and the levels of both proteins were decreased in a time-dependent fashion. Next, we investigated the expression of specific markers of adipocyte differentiation after 7 days of exposure of HTT2800. A wide variety of genes was involved in lipid storage and lipid synthesis [18]-[19]. After treatment with HTT2800, we then analyzed the expression of KLF15 [9], Sox8 [20], PPARγ [9,21], CD36 [21], FABP4 [22], and Nur-77 [23]. As shown in Fig. 3D, the expression of the transcription factors Sox8 and KLF15 genes was markedly upregulated by HTT2800 during exposure to C2C12 cells. KLF15 has been reported to positively regulate adipogenesis through the activation of PPARγ [9]. As shown in Fig. 3D and E, PPARγ gene and protein expression was increased; the expression level of PPARγ mRNA was increased up to 2.0-fold compared to the levels after vehicle

treatment. This action of KLF15 is mediated, at least in part, through the induction of PPARγ expression. We examined 2 other PPARγ target genes, FABP4 and CD36. Only FABP4 mRNA and protein levels were increased after exposure to HTT2800.

4. Discussion

Applications of nanoparticles to biomedicine include cellular drug delivery [10] and biochemical marker [24]. Carbon nanotubes have greatly advanced these fields. Similarly as other nanoparticles, carbon nanotubes can be readily taken up by cells and are noncytotoxic within a certain concentration limit if suitably functionalized [25] [26]. The main finding of this study was that HTT2800 has a significant effect on intracellular lipid accumulation in C2C12 cells. Intracellular lipid accumulation is a feature of adipocyte development. It has been reported that cellular differentiation can be considered a shift in gene expression patterns and morphological changes [27]. In this report, we found that when the HTT2800 was added to C2C12 myocytes, it was incorporated into the cells and promoted their differentiation into adipose-like cells. Then, oil red O staining was performed to assess the effects on the cellular lipid content. The determination of lipids with oil red O can be used for any *in vitro* cultured cells [28]. Compared with vehicle-treated cells, HTT2800-exposed cells contained greater amounts of lipid. In an attempt to further demonstrate the inhibition of C2C12 differentiation by HTT2800, quantified real-time PCR analyses were performed to investigate the effects of HTT2800 on the expression of mRNA markers characteristic of either muscle or adipose differentiation. Generally, after HTT2800 exposure, the levels of genes were 0.9- to 4-fold of the levels in vehicle-treated cells. The up- and downregulated transcripts after HTT2800 exposure in C2C12 cells are described in Fig. 3. We first examined the expression of muscle-specific genes including MyoD and myogenin. MyoD is a master regulator of muscle cell differentiation and the major regulator of myogenin expression [7]. Cells maintained in medium with vehicle were clearly differentiated into myotubes. By contrast, exposure to HTT2800 significantly reduced the expression of myogenin and led to the

emergence of adipocyte-related gene markers. In addition, the expression of muscle-specific genes associated with myogenesis is also controlled by other myogenic transcription factors including Sox8 and Nur-77. Sox8 was upregulated by more than 2.5-fold after exposure to HTT2800. It has been reported that Sox8 acts as a specific negative regulator of skeletal muscle cell differentiation, and many Sox proteins play fundamental roles in vertebrate development and differentiation processes [20]. Interestingly, Nur-77 expression was significantly increased up to 3.8-fold after HTT2800 exposure. Nur-77 encodes an orphan nuclear receptor that is classified as a transcriptional modulator protein. A recent report suggested that Nur77 is modulated and induced in a variety of cells in response to signals for growth and differentiation [23].

Another interesting result of our study was that HTT2800 upregulated KLF15 gene expression and increased the expression level of KLF15 mRNA up to 4.0-fold. It has been reported that overexpression of KLF15 induces adipocyte maturation and the expression of the nuclear receptor PPARγ [9]. We then examined the expression level of PPARγ in C2C12 cells by using quantified real-time PCR. HTT2800 upregulated PPARγ gene expression and increased the expression level of PPARγ mRNA by as much as 2.0-fold compared to that induced by vehicle treatment. The expression of PPARγ mRNA in C2C12 cells increased accompanying the differentiation into adipose-like cells after exposure of HTT2800. As shown in Fig. 3D, the expression of CD36 had no obvious difference after cells were treated with HTT2800. It has been reported that a PPARγ target gene, CD36, is a scavenger receptor expressed in the skeletal muscle plasma membrane of C2C12 myocytes and that overexpression of this transport protein increased fatty acid uptake and utilization by myotubes [29]. Our study suggested that HTT2800 significantly inhibits myotube formation and the expression of muscle-specific genes. The inhibition of myotube formation and the reduced expression of muscle-specific genes were caused by in part CD36 expression. Overall, these findings demonstrate that HTT2800 promotes the transition from myogenic lineage to an adipose-like lineage. It has also been reported that C2C12 cells have pluripotent mesenchymal precursor cell characteristics. Similar features emerge from reports describing the

conversion of the differentiation pathway of C2C12 myoblasts into adipocytes [30] [31] or osteoblasts [32] respectively, although we do not yet understand the precise reason for these results. However, this was the first study to report the association between carbon nanotubes and adipose-like cell differentiation in C2C12 cells (Fig.4). A more well-designed prospective study of more specified HTT2800-related genes with C2C12 cells would give more clues to clarify the mechanisms related with the clear link between HTT2800 and cell differentiation. These studies support the potential use of carbon nanotubes as a targeted delivery agent for cell differentiation program.

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Figure legends

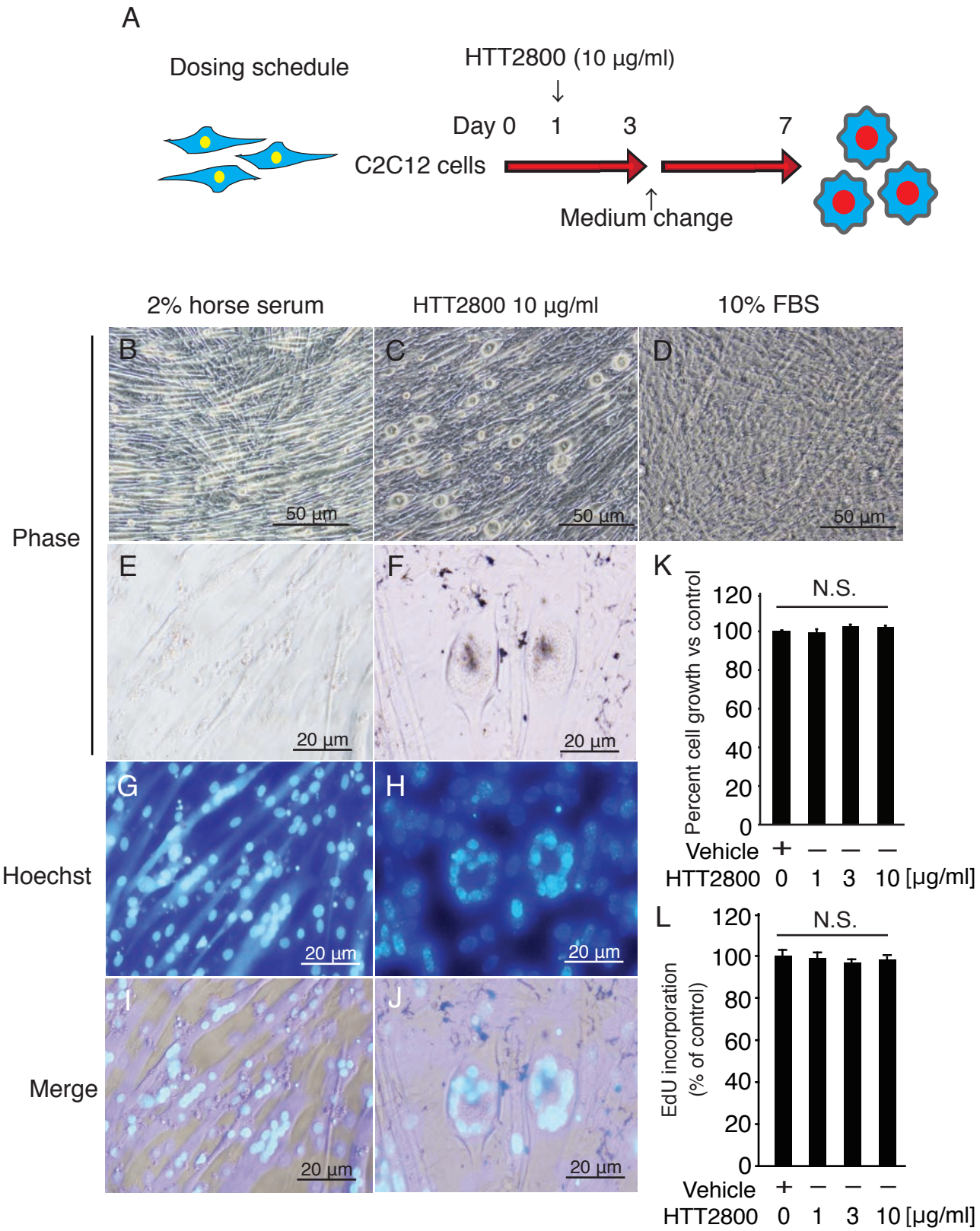
Fig. 1. Effect of HTT2800 on the morphology of C2C12 skeletal muscle cells. (A) Schematic showing the protocol for HTT2800-induced cell morphology. Cells were cultured in growth medium until 80–90% confluency and then in medium with or without HTT2800 (10 μg/ml). (B) Seven days of C2C12 cell differentiation under a low-serum condition (2% horse serum) led to changes in morphology from myoblasts to myotubes as observed by light microscopy. (C and D) Cells were maintained to day 7 after reaching confluence in DMEM with 10% FBS without additions or in the presence of 10 μg/ml HTT2800. Cellular morphology was visualized on day 7 by using light microscope. (G and H) Cell nuclei stained with Hoechst 33342 were visualized on day 7 by using a fluorescent microscope. (I and J) Merge image of phase contrast and fluorescence.

Fig. 2. HTT2800 treatment induces cellular lipid accumulation in C2C12 cells. Cells were treated with 2% horse serum (HS) (A) and 10% FBS (C) or 10 μg/ml HTT2800 (E and G) for 7 days after cells reached 70–80% confluence. Oil red O staining was performed after 7 days of exposure to the growth medium. The arrows indicate cells positive for lipid droplet content. Cell nuclei (indicated as black arrows) stained with Hoechst 33342 (blue) were visualized on day 7 by using a fluorescent microscope (B, D, F, and H). (I) Quantitative analysis of lipid accumulation was assayed with a lipid-staining reagent to evaluate the lipid content.

Fig. 3. Effects of HTT2800 on the expression of myogenic or adipose differentiation markers in C2C12 cells. C2C12 cells were exposed to HTT2800 (10 μg/ml) for 7 days, and RNA was isolated. mRNA levels for the downregulated (MyoD and Myogenin) and upregulated (PPARγ2, FABP4, Sox8, KLF15 and Nur-77) gene targets were determined by real-time PCR (Fig. 3A, B, and D). Genes mRNA levels were normalized using GAPDH (mean ± SEM, n = 3, $^{**}p < 0.01$ based on Student's *t*-test). (C and E) Western blotting analysis after exposure of C2C12 cells to HTT2800. β-Actin was used as a loading control.

Fig. 4. Schematic representation of the proposed mechanism of HTT2800 action in C2C12 cells. A, HTT2800 enters the C2C12 cells. B and C, HTT2800 suppresses myogenic differentiation of myoblast from C2C12 cells, and also promotes their differentiation into adipose-like cells. D, These combined effects may contribute to facilitating the intracellular lipid accumulation.

Figure


A
Dosing schedule
HTT2800 (10 μg/ml)
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C2C12 cells
Medium change
2% horse serum
HTT2800 10 μg/ml
10% FBS
Phase
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Percent cell growth vs control
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120
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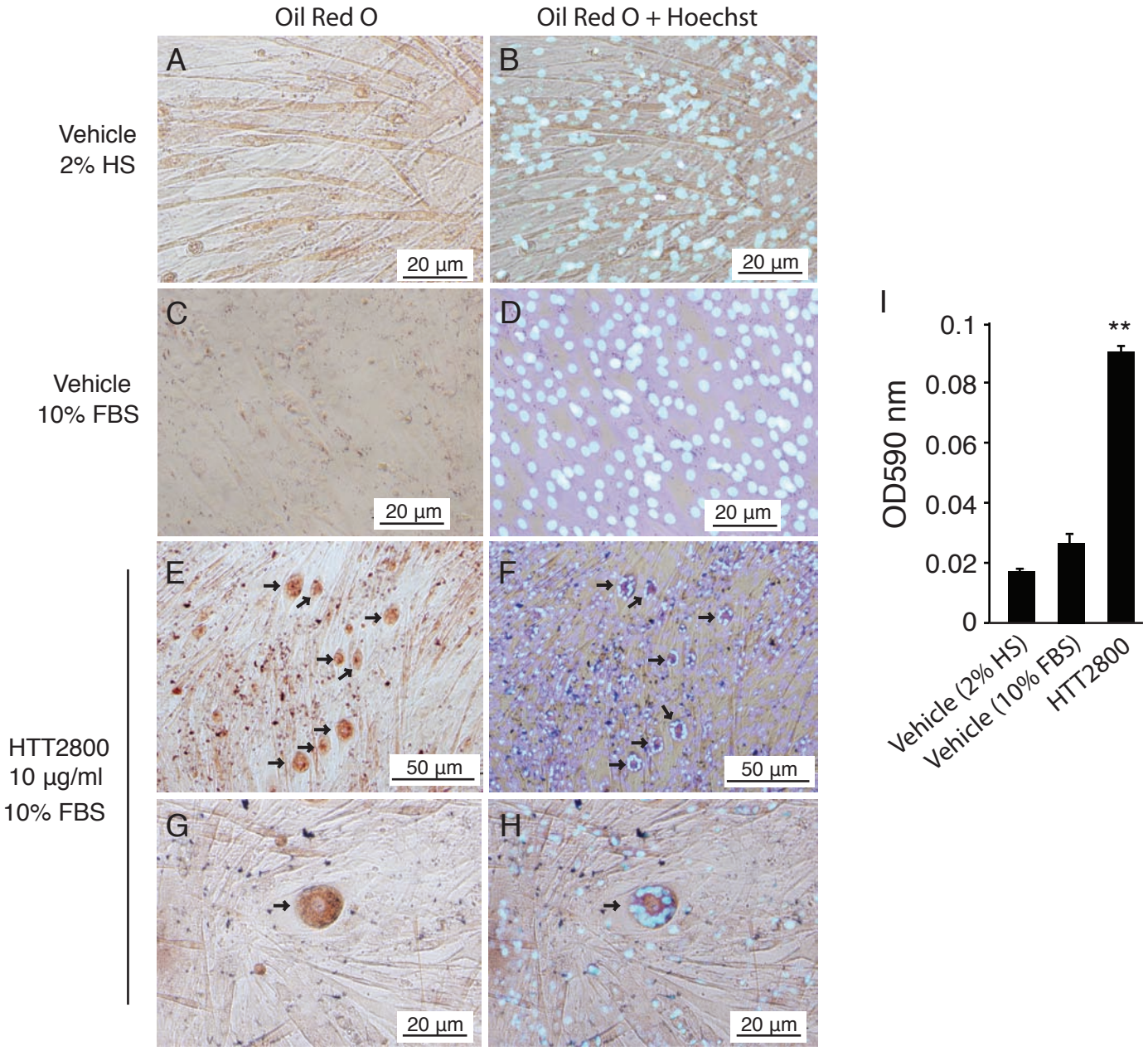
Fig.1


Oil Red O
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2% HS
Vehicle
10% FBS
HTT2800
10 µg/ml
10% FBS
20 µm
50 µm
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0.1
0.08
0.06
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0.02
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Vehicle (2% HS)
Vehicle (10% FBS)
HTT2800

Fig.2

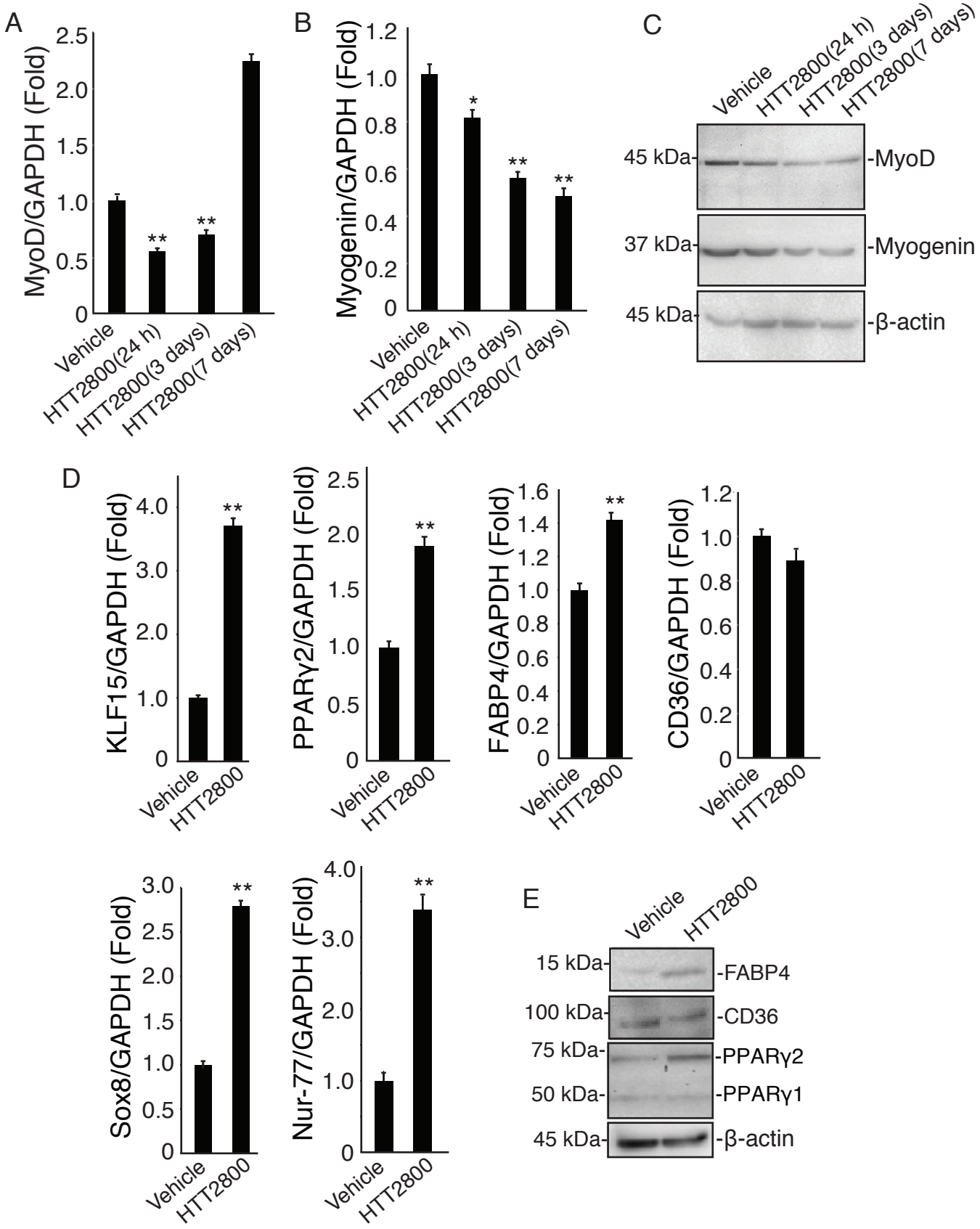
A
MyoD/GAPDH (Fold)
2.5
2.0
1.5
1.0
0.5
0
**
**
Vehicle
HTT2800(24 h)
HTT2800(3 days)
HTT2800(7 days)
B
Myogenin/GAPDH (Fold)
1.2
1.0
0.8
0.6
0.4
0.2
0
*
**
**
Vehicle
HTT2800(24 h)
HTT2800(3 days)
HTT2800(7 days)
C
Vehicle
HTT2800(24 h)
HTT2800(3 days)
HTT2800(7 days)
45 kDa
-MyoD
37 kDa
-Myogenin
45 kDa
-β-actin
D
KLF15/GAPDH (Fold)
4.0
3.0
2.0
1.0
0
**
Vehicle
HTT2800
PPARγ2/GAPDH (Fold)
2.5
2.0
1.5
1.0
0.5
0
**
Vehicle
HTT2800
FABP4/GAPDH (Fold)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
**
Vehicle
HTT2800
CD36/GAPDH (Fold)
1.2
1.0
0.8
0.6
0.4
0.2
0
Vehicle
HTT2800
Sox8/GAPDH (Fold)
3.0
2.5
2.0
1.5
1.0
0.5
0
**
Vehicle
HTT2800
Nur-77/GAPDH (Fold)
4.0
3.0
2.0
1.0
0
**
Vehicle
HTT2800
E
Vehicle
HTT2800
15 kDa
-FABP4
100 kDa
-CD36
75 kDa
-PPARγ2
50 kDa
-PPARγ1
45 kDa
-β-actin

Fig.3

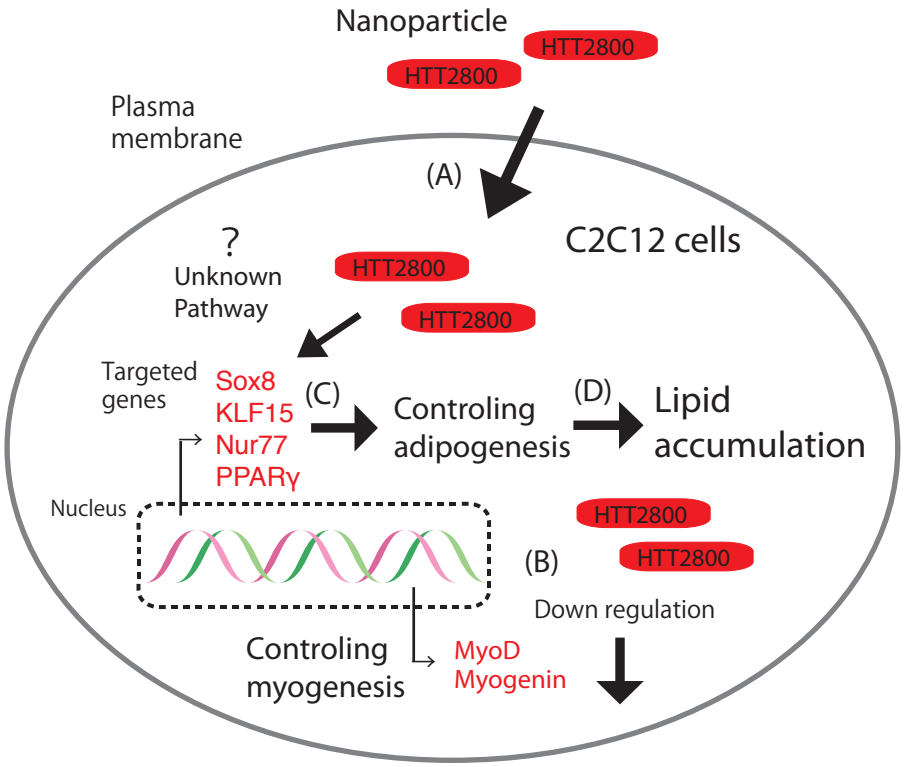
Nanoparticle
HTT2800
HTT2800
Plasma membrane
(A)
?
Unknown Pathway
C2C12 cells
HTT2800
HTT2800
Targeted genes
Sox8
KLF15
Nur77
PPARγ
(C)
Controling adipogenesis
(D)
Lipid accumulation
Nucleus
HTT2800
HTT2800
(B)
Down regulation
Controling myogenesis
MyoD
Myogenin

Fig.4