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NUDT7 Can Promote Slow Myofiber Formation and Its Endogenous Expression

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Skeletal muscle is a tissue mainly composed of four types of myofibers. They are formed of multi-nucleated myotubes through myogenic differentiation. Myogenic differentiation has several steps and they express specific gene markers sequentially. It was reported that Nucleoside diphosphate linked moiety X–type motif7 (NUDT7) is expressed in muscle. To determine NUDT7 function in myogenesis *in vitro*, we performed the experiments with C2C12 myoblasts. The cells were pre–cultured in the 10% FBS–growth medium for a day and were induced differentiation in 2% HS–DMEM differentiation medium for eight days. They were forced to overexpress NUDT7 in lipofection method. They were harvested and were analyzed for mRNA and protein expression. In the results, NUDT7 mRNA expression was increased during myogenic differentiation. Myf5 was increasingly expressed at the early stage (day 2 and 3) followed by Mrf4, Myogenin and Myoglobin. The expression pattern of NUDT7 is similar to that of Myogenin and Myoglobin. The NUDT7 overexpression activated endogenous NUDT7 expression and MyHC1 mRNA expression, suggesting that NUDT7 overexpression partly promoted slow myofiber formation, which may explain the mechanisms to modulate the types of myofibers.

Key words: Skeletal muscle, Nucleoside diphosphate linked moiety X–type motif7 (NUDT7), C2C12 myoblasts, MyHC1

INTRODUCTION

Myogenic differentiation has several steps, which start from myoblasts (Buckingham and Rigby, 2014). Myoblasts proliferate and fuse to become multi–nucleated myotubes, where specific markers are expressed, such as Pax7 and Myf5 (Bentzinger *et al*., 2012; Buckingham and Rigby, 2014; Rudnicki and Jaenisch, 1995; Seale *et al*., 2000; Weintraub *et al*., 1991; Zammit, 2017). It is known that Pax7 is expressed in myoblasts and its expression level decreases with myogenic differentiation (Buckingham and Rigby, 2014; Olguin and Olwin, 2004). Myf5 is a downstream of Pax7 in the genetic network controlling myogenesis and is expressed early in myogenic differentiation (McKinnell *et al*., 2008). In addition, the myogenic markers such as MRF4, Myogenin and Myoglobin, are expressed late in myogenic differentiation and their expression is maintained in multi–nucleated myotubes (Bentzinger *et al*., 2012; Buckingham and Rigby, 2014; Rudnicki and Jaenisch, 1995; Seale *et al*., 2000; Weintraub *et al*., 1991; Zammit, 2017). These myogenic markers are induced sequentially, which is one of the standards for determining the level of myogenic differentiation. When myogenic differentiation proceeds and when myotubes, the weak myofibers, mature to become myofibers, they are characterized as myofiber types (Schiaffino and Reggiani, 2011). The types are called “slow–twitch”, “intermediate–twitch” and ”fast–twitch” in skeletal muscle tissue (Schiaffino and Reggiani, 2011). The markers of myofiber types are Myosin Heavy Chains (MyHCs) and their expression pattern defines myofiber types in skeletal muscle. Each MyHC is characterized: MyHC1 as slow–twitch marker, MyHC2A and MyHC2X as intermediate–twitch markers and MyHC2B as fast–twitch marker. Thus slow myofibers have a high MyHC1 expression ratio and low MyHC2B expression ratio, in reverse, fast myofibers have a low MyHC1 expression ratio and a high MyHC2B expression ratio (Gea, 1997; Schiaffino and Reggiani, 2011).

Nucleoside diphosphate–linked moiety X–type motif7 (NUDT7) was reported to be located in peroxisome and that it was a specific hydrolase for CoA and CoA derivatives (Cartwright *et al*., 2000; Gasmi and McLennan, 2001; McLennan, 2006; Reilly *et al*., 2008). In mouse and human, NUDT7 expression was confirmed in various organs and tissues including skeletal muscle (Gasmi and McLennan, 2001). The detailed analysis of NUDT7 functions in animals have not been performed yet, while in plants such as *Arabidopsis thaliana*, some researchers have performed comprehensive analysis on Nudix hydrolase (Nudt) (Bartsch *et al*., 2006; Ge *et al*., 2007; Kraszewska, 2008). Therefore, the functions of NUDT7 remained a matter of speculation. NUDT7 among the Nudt superfamily was reported to be expressed in skeletal muscle (Gasmi and McLennan, 2001). M Taniguchi *et al*. reported that rat L6 myoblasts express NUDT7 and that NUDT7 overexpression downregulates heme biosynthesis in L6 myoblasts (Taniguchi *et al*., 2010a, 2010b). However, it is not clear how NUDT7 functions

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in the differentiation of myoblasts into myotubes or myofibers and how NUDT7 contributes to myofiber type determination. Therefore, we studied the effects NUDT7 has on myoblasts differentiation and investigated the contribution of NUDT7 to myofiber type determination *in vitro*.

MATERIALS AND METHODS

Cell culture

Mouse C2C12 skeletal myoblasts were used for this research. Myoblasts were maintained in the following growth medium (GM): Dulbecco's Modified Eagle Medium (DMEM with high glucose, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Invitrogen), 1% antibiotic–antimycotic mixture (Invitrogen, Massachusetts, USA) and 0.5% gentamicin (Invitrogen) at 37.0°C in 5% CO_2 atmosphere. To induce differentiation, myoblasts were incubated with the following differentiation media (DM): Dulbecco's Modified Eagle Medium (DMEM with high glucose, Invitrogen) with 2% horse serum (HS, Invitrogen), 1% antibiotic–antimycotic mixture and 0.5% gentamicin. For overexpression experiments, transfection reagent was poured into the well with differentiation media.

Plasmid vector construct and transient transfection

Full–length mouse NUDT7 expression plasmid vector was constructed in pcDNA3.1. Five–myc tag was inserted at 5' end of Nudt7 ORF. The myoblasts were transfected using Trans IT LT–1 reagent (TAKARA, Kyoto, Japan) with serum–free OPTIMEM (Gibco, Massachusetts, USA) and the pcDNA3.1 vector or the Mouse NUDT7 expression vector according to the manufacturer's protocol. Reverse transcription–polymerase chain reaction (RT–PCR)

Total RNA was isolated from cultured myoblasts using RNeasy Micro kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. cDNA was synthesized from total RNA by a reverse–transcriptase SuperScript Ⅲ (Invitrogen) using an oligo(dT) primer. mRNA expression of NUDT7, Myf5, MRF4, Myogenin, Myoglobin, and MyHCs (MyHC1, MyHC2A, MyHC2X, MyHC2B) were monitored by real–time quantitative PCR (RT–qPCR) using Roche LightCycler1.5 (Roche, Mannheim, Germany) run under the TaqMan probe detection format standardized with hypoxanthine guanine phosphoribosyl transferase (HPRT). The primer sets were designed using ProbeFinder (version 2.52 and 2.53 for mouse, Roche) with an intron–spanning assay for mouse Nudt7, Myf5, MRF4, Myogenin, Myoglobin, and MyHCs (MyHC1, MyHC2A, MyHC2X, MyHC2B) as shown in Table 1. Annealing temperature was set to 60°C in all cases.

Whole cell extracts and enhanced chemiluminescence western blotting

Whole cell extracts were harvested in 1× SDS–PAGE sample buffer, electrophoresed on 10% or 12% polyacrylamide gels under reducing conditions and transferred to PVDF membranes. The membranes were blocked with 5% skim milk in 0.1% (T–TBS) for 1 h at room temperature, followed by incubation overnight at 4°C in a dilution of primary antibodies against NUDT7 (polyclonal, Abnova, Taipei, Taiwan), MyHC–slow (monoclonal, Sigma–Aldrich, St. Louis, USA) or β–actin (monoclonal, Abcam, Cambridge, UK). Antibodies were diluted in CanGetSignal solution 1 (Toyobo, Osaka, Japan) containing 0.05% NaN_3. The membranes were washed three times (10 min each) with T–TBS and further incubated with HRP–conjugated goat anti–mouse IgG (DAKO, Tokyo, Japan) or HRP–conjugated swine anti-rabbit IgG (DAKO) secondary antibodies in CanGetSignal solution 2 (Toyobo, Osaka, Japan) for 1 h at 25°C. The membranes were washed as above and HRP activity was detected using an enhanced chemiluminescence (ECL) detection kit (GE Healthcare, Little Chalfont, UK) read in a Fusion SL4 system (M&S Instruments Trading Inc., Osaka, Japan). Band intensities of immunoblots were assessed using ImageJ software (National Institute of Health, Bethesda, MD, USA).

RESULTS

While NUDT7 is reported as a molecule related to

Table 1. PCR primer sets for mouse Nudt7, Myf5, MRF4, Myogenin, Myoglobin, MyHC1, MyHC2A, MyHC2X, MyHC2B and HPRT

Primer	Accession number	Left Primer	Right Primer
Nudt7	NM_024437.3	agccactgctctccgtga	ggggttaccagtgcatcatt
Myf5	NM_008656.5	ctgctctgagcccaccag	gacagggctgttacattcagg
Mrf4	NM_008657.2	gggcctcgtgataactgct	aagaaaggcgctgaagactg
Myogenin	NM_031189.2	ccttgctcagctccctca	tgggagttgcattcactgg
Myoglobin	NM_001164047.1	tagacccctggagggttga	gggcttttataccttcccagat
MyHC1	NM_080728.2	agatccgaaagcaactggag	ggatcttgccctcctcgt
MyHC2A	NM_001039545.2	aactccaggcaaaagtgaaatc	tggatagatttgtgttggattgtt
MyHC2X	NM_030679.1	aatcaaaggtcaaggcctacaa	gaatttggccaggttgacat
MyHC2B	NM_010855.2	tgcctccttcttcatctggt	ccatctcagcgtcggaac
HPRT	NM_013556.2	cctcctcagaccgcttttt	aacctggttcatcatcgctaa

the immune system of Arabidopsis thaliana, it is known as a peroxisomal nudix hydrolase having specificity for coenzyme A and its derivatives (Cartwright *et al*., 2000; Gasmi and McLennan, 2001; Reilly *et al*., 2008). According to these studies, NUDT7 is located in many organs and tissues including skeletal muscle, however, the importance of the role of NUDT7 in skeletal muscle is unclear. Therefore, we investigated the functions of NUDT7 in myogenesis.

NUDT7 expression is elevated late in myogenesis.

We first induced the myogenic differentiation of the mouse C2C12 myoblast cell line and analyzed the expression pattern of NUDT7. The C2C12 myoblast began elongation on d1 and fusion on d2 (Fig. 1A). We then observed multi–nucleated myotubes on d4. From d5, myotubes were lengthened and become more hypertrophic. The number of myotubes significantly increased from d6 to d8. We then examined the expression of myogenic markers during myogenic differentiation to determine whether differentiation was successful at the molecular level (Fig. 1B). The expression of Myf5, the early myogenic marker, began to rapidly increase. The expression then gently decreased from d4 and continued until d8. MRF4 reached the peak on differentiation day 5 (d5). It has been reported that MRF4 expression begins to increase after Myf5 increases (Bentzinger *et al*., 2012). Myogenin, a late myogenic marker, was only slightly expressed on d1 and began to increase slightly on d3 and continued to elevate until d8. The expression on d8 was one hundred–fold that of d1. Myoglobin was also induced the expression late in myogenic differentiation. It started to increase on d4 and continued to increase until d8. NUDT7 also increased their expression during myogenic differentiation as same as Myogenin and Myoglobin. The expression of NUDT7 peaked on d8, compared with the expression on d1, it was 17.6 times. To our knowledge, this study is the first to elucidate NUDT7 expression during myogenic differentiation.

NUDT7 overexpression led to elevate MyHC1 expression in myotubes.

We introduced 5myc–tagged mouse NUDT7 expression vector into C2C12 myoblasts using lipofection method to perform overexpression experiments. We cultured myoblasts in DM for 8 days and forced them to overexpress NUDT7 in the cells. Cell morphological characteristics were not significantly different between control and NUDT7+ C2C12 myoblasts (Fig. 2A). Myoblasts began to elongate from d2 as shown in Fig. 1A, and we observed fused myotubes on d6. Next, we performed real time RT–qPCR to observe the mRNA expression. NUDT7+ C2C12 myoblasts showed that NUDT7 expression peaked on d2, after which the expression decreased on d4 and d6 (Fig. 2B). These data confirmed that the transfection of NUDT7 was successful. One report indicated that pig NUDT7 would affect heme content in rat L6 myoblasts (M Taniguchi *et al*., 2010b). Heme is a substrate of myoglobin, which has previously been reported to be differently expressed in fast–twitch muscle and in slow twitch muscle (Schiaffino and Reggiani, 2011). Therefore, we investigated the skeletal muscle fiber type marker, MyHCs. MyHC1 as a slow–twitch muscle marker, MyHC2A and 2X as intermediate–twitch muscle markers, MyHC2B as a fast–twitch skeletal muscle marker in real time RT–qPCR (Fig. 2C). C2C12 myoblasts expressed MyHC1, 2A, 2X and 2B and the expression of MyHCs increased on d6. This happened because the number of myotubes fused with one another increased (Fig. 2A). While MyHC2A and MyHC2B was not differently expressed between control C2C12 myoblasts and NUDT7+ C2C12 myoblasts, the expression of slow–twitch fiber marker MyHC1 and intermediate–twitch fiber marker MyHC2X was significantly elevated in NUDT7+ C2C12 myoblasts on d6 (Fig. 2C).

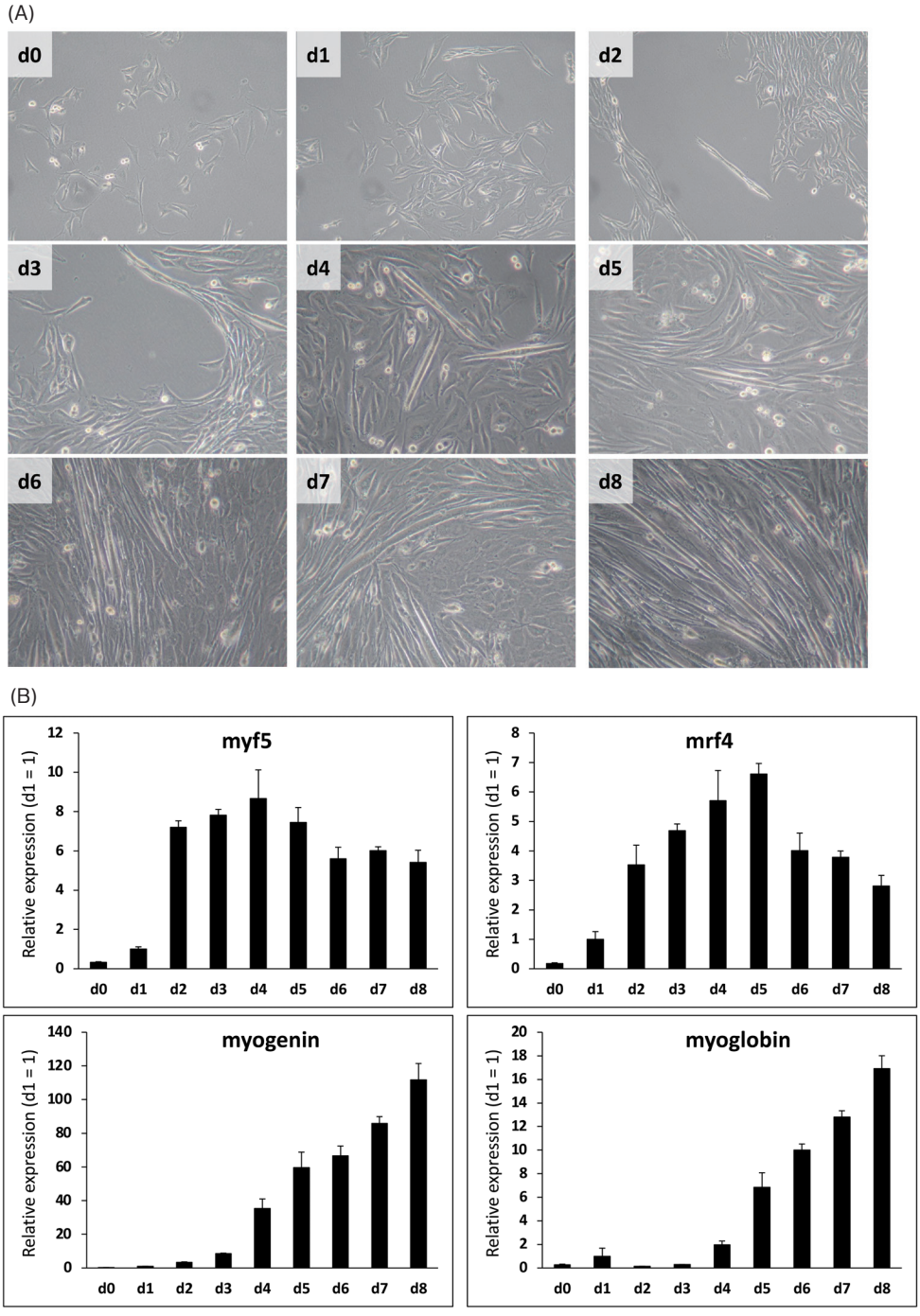
NUDT7 overexpression during C2C12 myoblast differentiation induced the elevation of endogenous NUDT7 in C2C12 myoblasts.

Next, we investigated the protein expression of NUDT7 by western blotting. Five–myc–tagged NUDT7 (32 kDa), the transfected NUDT7, showed strong signal intensity at d2, and it continued until d4 (Fig. 3). These data coincided with mRNA result of NUDT7 (Fig. 2B). Interestingly, we found that endogenous NUDT7 (27 kDa) was increasingly expressed on d2, 3 and 4 (Fig. 3). According to these data, we could confirm NUDT7 was overexpressed in C2C12 myoblasts in terms of mRNA level and in protein level.

DISCUSSION

It is reasonable to think that NUDT7 had several functions. NUDT7 is known as the nudix hydrolase specific to acyl–CoA and its derivatives (Cartwright *et al*., 2000; Gasmi and McLennan, 2001; Reilly *et al*.,., 2008; Shumar *et al*., 2015), and it is known that CoA has various functions (Hunt *et al*., 2014). Because NUDT7 was able to interfere in such pathways related to CoA, NUDT7 could have important and various roles in cells. Our study revealed that NUDT7 is increasingly expressed in myogenic differentiation, which suggests that NUDT7 can be a late myogenic marker such as Myogenin and Myoglobin (Fig. 1B). Then we saw the expression of Myf5 and MRF4 increased earlier than that of NUDT7 (Fig. 1B). It is reported that Myf5 binds to DNA and acts as a transcription factor (Wang *et al*., 2004). Therefore, there is a possibility that Myf5 binds to NUDT7 promoter and modulates NUDT7 expression. While it has been reported that Myf5 can regulate the expression of Desmin, we hypothesize that Myf5 is related to the expression of NUDT7.

In our experiments, NUDT7 overexpression did not result in any morphological significant difference (Fig. 2A). The mRNA expression pattern of myogenic markers was not significantly changed (data not shown). This suggests that NUDT7 is not a factor that affects the myogenic differentiation rate, and it is unlikely that



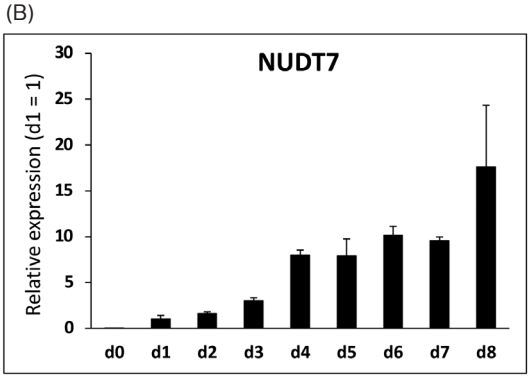
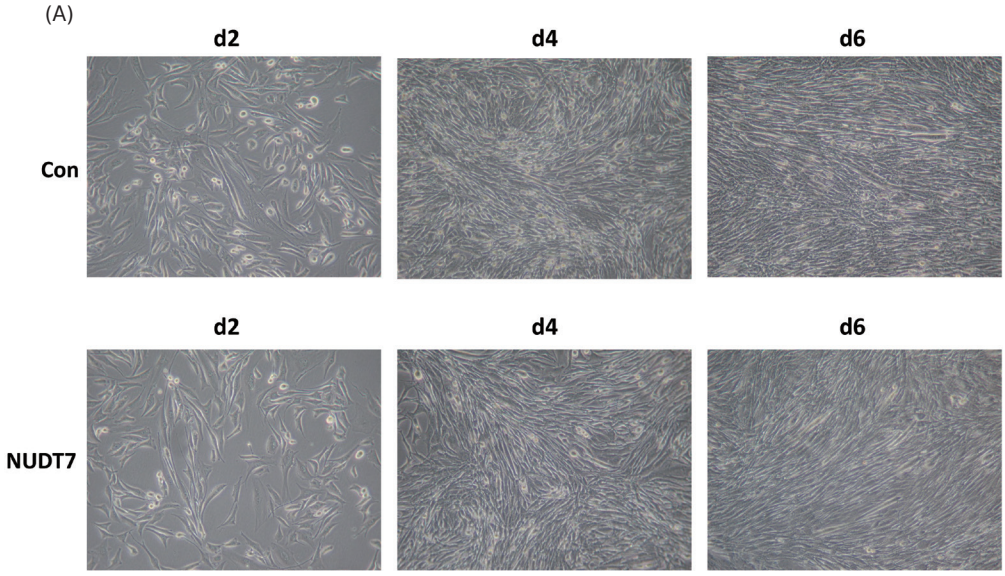


Fig. 1. NUDT7 expression is elevated at late stage of myogenesis.
(A) These images are mouse C2C12 myoblasts during induction of differentiation. Post differentiation day is shown as d0–d8. C2C12 myoblasts were morphologically changed on d1, fused on d2, and formed multi-nuclear cells (myotubes) on d4. From d5, the myotubes lengthened and became more hypertrophic, and the number of myotubes increased from d6 to d8.
(B) Bar graphs of showing mRNA expression in mouse C2C12 myoblasts during induction of differentiation (NUDT7, Myf5, MRF4, Myogenin, Myoglobin). n=3. The values are relative expression (d1= 1). Error bar is standard error. Post-differentiation day is shown as d0-d8. Expression of the early myogenic markers Myf5 and MRF4 peaked on d3 and on d4, and the late myogenic markers, Myogenin and Myoglobin continued to increase their expression during myogenic differentiation. The expression of NUDT7 also continued to increase during myogenic differentiation.



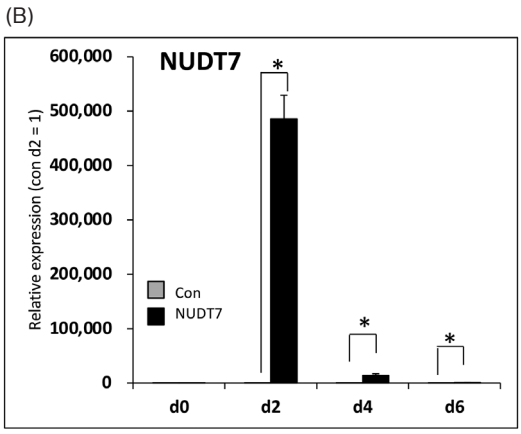
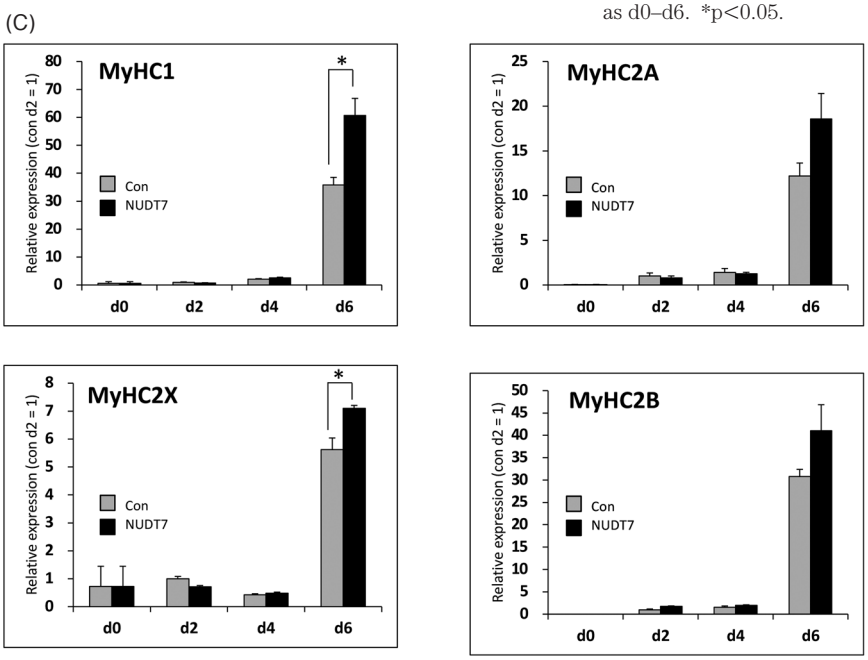


Fig. 2. NUDT7 overexpression led to the elevation of MyHC1 expression of myotubes.
(A) Images show mouse C2C12 myoblasts transfected pcDNA3.1 (control: Con) or Myc–NUDT7 (NUDT7) after which differentiation was induced. Post–differentiation day is shown as d2–d6. C2C12 myoblasts were morphologically changed and fused, and formed multi–nuclear cells (myotubes). The myotubes lengthened and became more hypertrophic. The number of myotubes significantly increased.
(B) Bar graphs showing NUDT7 mRNA expression in transfected (control and NUDT7+ cells)–mouse C2C12 myoblasts. HPRT was used for internal control, n=3. The values are relative expression (control d2=1). Error bar is standard error. Post differentiation day is shown as d0~d6. *p<0.05. NUDT7 expression of NUDT7+ C2C12 myoblasts peaked on d2 and then decreased while NUDT7+ C2C12 myoblasts significantly expressed NUDT7 on d2, d4, d6 compared with control C2C12 myoblasts.
(C) Bar graphs showing MyHCs mRNA expression in transfected (control and NUDT7+ cells)–mouse C2C12 myoblasts. HPRT was used for internal control, n=3. The values are relative expression (control d2=1). Error bar is standard error. Post–differentiation day is shown as d0–d6. *p<0.05.



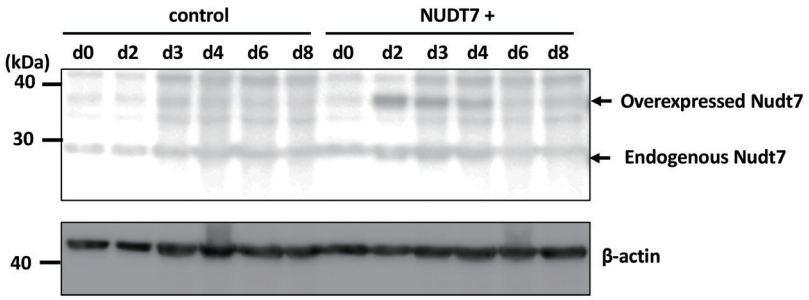


Fig. 3. NUDT7 overexpression during C2C12 myoblasts differentiation induced the elevation of endogenous NUDT7 in C2C12 myoblasts.
Membrane images show western blotting of mouse C2C12 myoblasts transfected with pcDNA3.1 (control: con) or Myc–NUDT7 (NUDT7+: nud) after which differentiation was induced. Each lane is Control C2C12 myoblasts (control) and NUDT7 transfected C2C12 myoblasts (NUDT7+). Post-differentiation day is shown as d0–d8. We observed expression of overexpressed NUDT7 (32 kDa), endogenous NUDT7 (27 kDa) and β–actin (46 kDa).

NUDT7 is necessary for myogenesis such as MyoD (Davis *et al*., 1987). However, we found that NUDT7 overexpression affected muscle–twitch fiber type (Fig. 2C). These data suggest that NUDT7 promoted the formation of slow–twitch fiber. Interestingly, Peroxisome proliferator–activated receptor δ (PPARδ) overexpression induces a shift toward slow–twitch fiber formation, and this has been reported as a factor to improve mitochondrial biosynthesis (Ehrenborg and Krook, 2009; Wang *et al*., 2004). According to these reports, activation of mitochondrial biosynthesis could lead to the shift toward slow–twitch fiber formation. As reported, NUDT7 downregulated heme concentration in differentiating rat L6 myoblast (Taniguchi *et al*., 2010b). In addition to it, our result suggests that NUDT7 can be involved in determining myofiber type. NUDT7 overexpression activated the formation of slow–type myotubes. To elucidate NUDT7 functions, more research is required into the effects of NUDT7 on mitochondrial activity and about the possibility pathways where myoblasts determine the myofiber types through NUDT7.

AUTHOR CONTRIBUTIONS

S–K. Choe and M. Nakamura designed the study and supervised the work. R. Ebihara, S. Bhandari and M. Qahar performed the experiments, analyzed the data. R. Ebihara and M. Nakamura wrote the paper. W. Mizunoya, R. Tatsumi provided facilities and resources. All the authors assisted editing the manuscript and approved the final version.

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