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Mutated *WWP1* Induces an Aberrant Expression of *Myosin Heavy Chain* Gene in C_2C_{12} Skeletal Muscle Cells

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The WW domain containing E3 ubiquitin protein ligase 1 (WWP1), an enzyme to degrade unneeded or damaged proteins, was recently identified as the responsible for chicken muscular dystrophy. Despite of intensive studies on oncogenic characters, the role of WWP1 to muscular diseases has not yet been fully understood. Since it is generally known that the switching of myosin heavy chain (MyHC) isoforms from neonatal isoform to adult one is inhibited in chicken muscular dystrophy, we transfected either of wild and mutated types of *WWP1* gene into C_2C_{12} cells to monitor the expression pattern of muscle-differentiation markers including *MyHC*s by real-time PCR. Excessive *WWP1* expression enhanced the expression of the *MyHC Ia* gene but lowered the expression of the *MyHC IIb* gene. On the other hand, mutated *WWP1* gene transfected into myoblasts was distinct from these cases in that the *MyHC* gene or genes expression inhibited the normal myoblast differentiation. The present data suggest that WWP1 promotes myoblast differentiation from embryonic into fast twitch phase while a mutation in WWP1 results to retain slow and fast twitch isoforms characteristic of dystrophic fast twitch muscles.

Key words: chicken, gene expression, muscular dystrophy, myosin heavy chain, WWP1

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Introduction

The WW domain containing E3 ubiquitin protein ligase 1 (WWP1) is classified into a ubiquitin ligase (E3) which plays an important role in ubiquitin-proteasome pathway (UPP) to degrade unneeded or damaged proteins (Scheffner and Staub, 2007). The WWP1 and similar E3 ligases play important roles in cancer development, bone remodeling and central nervous system regeneration (Chen and Matesic, 2007; Glimcher *et al.,* 2007; Bernassola *et al.,* 2008; Qin *et al.,* 2008).

Recently, we identified the *WWP1* gene as a candidate gene responsible for the chicken muscular dystrophy, and the R441Q missense mutation in the *WWP1* gene was found to be the cause of muscular dystrophic phenotype (Matsumoto *et al.,* 2007, 2008). It is generally known that myosin is the principal protein of the contractile apparatus in muscle, and myosin diversity is primarily produced by the different expression of multiple isoforms of myosin heavy chain (MyHC) subunits which undergo transition during development in a variety of muscle systems (Schiaffino and Reggiani, 1994). The switching of the adult phenotype in fast muscle is inhibited in chicken muscular dystrophy, resulting in the continued expression of a slow twitch MyHC isoform in adult fast muscles (Bandman, 1985; Bandman and Bennett, 1988; Kaprielian *et al.,* 1991; Tidyman *et al.,* 1997).

The *WWP1* gene is expressed strongly in skeletal muscles (Flasza *et al.,* 2002; Komuro *et al.,* 2004; Matsumoto *et al.,* 2009), but the relationship between WWP1 and MyHC proteins has not been examined. To investigate the effects of the overexpression and the expression of the mutated *WWP1* gene on the *MyHC* genes expressions, we transfected the wild and mutated types of the *WWP1* gene into C_2C_{12} cells which were myoblasts derived from the mice skeletal muscle. The expression of muscle-differentiation markers, *Myogenin* (*Myog*), *myogenic differentiation 1* (*MyoD*) and *MyHC*s, was analyzed by real-time PCR.

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Materials and Methods

Cell Culture

The C3H murine skeletal muscle cell line C_2C_{12} (CRL-1722) was commercially obtained from the American Type Culture Collection (ATCC), VA, USA. Cells were cultured in the growth medium: Dulbecco's modified Eagle's medium (DMEM) (Nissui, Tokyo, Japan) containing 0.2% sodium hydrogen carbonate (Nakalaitesque, Kyoto, Japan), 0.008% kanamycin (Wako, Osaka, Japan) and L-glutamine (10 μg/ml) (Nissui), supplemented with 15% fetal bovine serum (FBS) (Gibco, NY, USA). The cells were incubated at 37℃ in humidified 95% air and 5% CO_2 atmosphere. Differentiation of C_2C_{12} cells was initiated by placing 80% confluent cell cultures in the differentiation medium: DMEM supplemented with 1% FBS.

Isolation and Mutagenesis of Mouse WWP1 Sequences

Mouse total RNA was isolated from an ICR strain liver using Sepasol RNAI (Nakalaitesque). The mouse was sacrificed according to the guideline of Animal Experimentation of Kobe University. The cDNA was generated by reverse transcription using oligo (dT) primer and SuperScript III Reverse Transcriptase (Invitrogen, CA, USA). The mouse *WWP1* sequence was amplified from the cDNA using primers 5′-ATCGTGTCTTATTCATCTTCGTATCCTCAG-3′ (WWP1-full-F) and 5′-GTGTGTATAAGCTGCTCATTCTGTA-3′ (WWP1-full-R) (NM_177327). The primers were designed to amplify all the CDS of *WWP1* gene (197–3115). *PfuUltra* High-fidelity DNA Polymerase (Stratagene, CA, USA) was applied as the DNA polymerase. The PCR was as follows: initial denaturation and enzyme activation for 120 s at 94℃, followed by 30 cycles of 30 s denaturation at 94℃, 30 s annealing at 58℃, and 180 s elongation at 68℃. The amplified sequence was ligated into a pGEM®-T Easy Vector (Promega, WI, USA), and the sequence was verified.

The mutation homologous to the responsible mutation for chicken muscular dystrophy (R436Q) was introduced by site-directed mutagenesis using the QuikChange site-directed mutagenesis kit (Stratagene) and the following primer (upper strand represented with the mutated nucleotide underlined): M 5′-TTCAACCAACAATACCTCTATTCGG-3′. *PfuUltra* High-fidelity DNA Polymerase (Stratagene) was applied and the PCR condition was as follows: initial denaturation and enzyme activation for 120 s at 94℃, followed by 30 cycles of 30 s denaturation at 94℃, 30 s annealing at 55℃, and 180 s elongation at 68℃. The mutation was verified by sequencing.

Two types of full *WWP1* sequence (WT: wild type and R436Q) were obtained by *Eco*RI (Takara, Tokyo, Japan) digestion and inserted into the unique *Eco*RI site between the CAG promoter and the 3'-flanking sequence of the rabbit *β-globin* gene of the pCAGGS expression vector.

Exogenous WWP1 Expression in C2C12 Cells

Plasmid pCAGGS-empty, pCAGGS-WT and pCAGGS-R436Q were diluted in Opti-MEM® I Reduced Serum Medium (Gibco), Plus reagent (Invitrogen) and Lipofectamine™ LTX (Invitrogen). Cells were incubated in the differentiation medium with each DNA-Lipofectamine™ LTX complex. The day 24 hours after the transfection was counted as day 0.

The vector capacity was assessed by the RT-PCR method using *GAPDH* as an internal standard. *TaKaRa Ex Taq*™ Hot Start Version (Takara, Tokyo, Japan) was used as the DNA polymerase. The primer sequences were 5′-CATAACACCAGAACAACAACC-3′ (WWP1rt-F), 5′-AACATGGAAGCCGAATAGAGG-3′ (WWP1rt-R) (NM_177327), 5′-ATGACAATGAATACGGCTACAGCAA-3′ (GAPDHrt-F) and 5′-GCAGCGAACTTTATTGATGGTATT-3′ (GAPDHrt-R) (NM_008084). The PCR was as follows: initial denaturation and enzyme activation for 120 s at 94℃, followed by 35 cycles of 30 s denaturation at 94℃, 30 s annealing at 60℃, and 30 s elongation at 72℃. PCR products were subjected in electrophoresis in an ethidium bromide-added 1% agarose gel. The band density was calculated with Scion Image (Scion Corporation, MD, USA).

Quantitive RT-PCR

Total RNA was isolated from cells and cDNA was generated as mentioned above. The cDNA was used as a template in the subsequent PCR analysis. Gene expression levels were detected by real-time PCR with SYBR® Premix *Ex Taq*™ II (Perfect Real Time) (Takara) for five genes; *WWP1, Myog, MyoD, MyHC Ia* and *MyHC IIb*. In addition, *GAPDH* was used as internal standard. All of real-time PCR primer pairs, designed by Primer Bank (http: //pga.mgh.harvard.edu/primerbank/), are shown in Table 1. The PCR was achieved with initial denaturation and enzyme activation for 20 s at 95℃, followed by 40 cycles of 20 s denaturation at 95℃, 8 s annealing at 60℃, and 30 s elongation at 72℃. The last cycle was performed at 95℃ for 10 s, 60℃ for 30 s and 72℃ for 60 s. Reactions were run on a TaKaRa PCR Thermal Cycler Dice® Real Time System (Takara) using cycling parameters defined by the manufacturer.

Each assay included a standard curve for each gene with five serial dilution points of a standard cDNA and a no-template control. Expression levels for each gene were calculated as relative expression levels toward *GAPDH* mRNA expression. For each gene, three individual samples were prepared and reactions were repeated two times for each sample. Statistical analysis was carried out using Statcel2 (Oms-publishing, Tokyo, Japan).

Statistical Analysis

Values in this paper represent means ± S.D. Differences between two groups were examined for statistical significance using Student's *t* test. *P* value less than 0.05 denoted the presence of a statistically significant difference.

Results and Discussion

We transfected the *WWP1* gene into C_2C_{12} cells to analyze the expressions of the muscle-differentiation markers. To evaluate the vector capacity, *WWP1* expression of each

Table 1. **Primers used for real-time PCR**

Name	GenBank accession #	Sequence (5′→3′)	PrimerBank ID #	Amplicon size (bp)
WWP1	NM_177327	CCTTGGAGTTCCGAGTTTGGA AGTTCCCCAGTTTGCACTATTC	28893447a2	175
Myog	NM_031189	GGTGTGTAAGAGGAAGTCTGTG TAGGCGCTCAATGTACTGGAT	13654247a2	184
MyoD	NM_010866	CCACTCCGGGACATAGACTTG AAAAGCGCAGGTCTGGTGAG	6996932a1	109
MyHC Ia	AF009960	CCTGGAGCCCCTAGATGAGG GGGGTTCATTGAGATCACCAC	3378046a1	106
MyHC IIb	AJ278733	AAACCACCTCAGAGTTGTGGA GTTCCGAAGGTTCCTGATTGC	9581821a2	172
GAPDH	NM_008084	AGGTCGGTGTGAACGGATTTG TGTAGACCATGTAGTTGAGGTCA	6679937a1	123

group (pCAGGS-empty-; control, pCAGGS-WT-; WT and pCAGGS-R436Q-transfected cells; R436Q) was analyzed by RT-PCR using cells at day 0 (Fig. 1A). Although the WT and R436Q groups expressed 3.83 and 5.47 fold higher than control level, clear difference was not observed in the myotube morphology and the proliferation rate of myoblasts (data not shown).

Figure 1B shows the diachronic analysis of the *WWP1* expression in control cells during myogenic differentiation. Since *in vitro* myogenesis is completed within a week (Sultan *et al.,* 2006), *WWP1* expressions analyzed by real-time PCR were made at day 0, 2, 4, 6 and 8. The *WWP1* gene seems to be expressed stably in each time point, though upward trend was observed in day 2 and 4.

Subsequently, we analyzed the expressions of the muscle-differentiation markers by real-time PCR to access the influence of the *WWP1* overexpression and *WWP1* with R436Q mutation on the gene expressions of *Myog, MyoD, MyHC Ia* and *MyHC IIb* using C_2C_{12} cells at day 6 (Fig. 2). The former two were analyzed as the markers for early stages (Langlands *et al.,* 1997) and the latter two for later stages of muscle differentiation (Silberstein *et al.,* 1986).

The expressions of *Myog* in the WT and R436Q groups were 1.04 fold (±0.02) higher and 0.64 fold (±0.26) lower compared to the control group. The R436Q group was significantly lower than other groups. These results indicate that R436Q mutation in the *WWP1* gene affects early stages of muscle differentiation through the reduction of the *Myog* expression. There was no clear difference in the *MyoD* gene expression among three groups.

It was, however, interesting to indicate that the *WWP1* gene overexpression influenced upon the *MyHC* genes expression. The MyHCs are among muscle proteins increasing during the course of myogenesis (Silberstein *et al.,* 1986), and are divided into two classes, type I composed of fast twitch fibers and type II of slow twitch fibers (Larsson and Salviati, 1989). The *MyHC Ia* expression levels in the WT and R436Q groups increased 2.81 (± 0.95) and 2.18 (±0.24) fold compared to the control group, respectively.

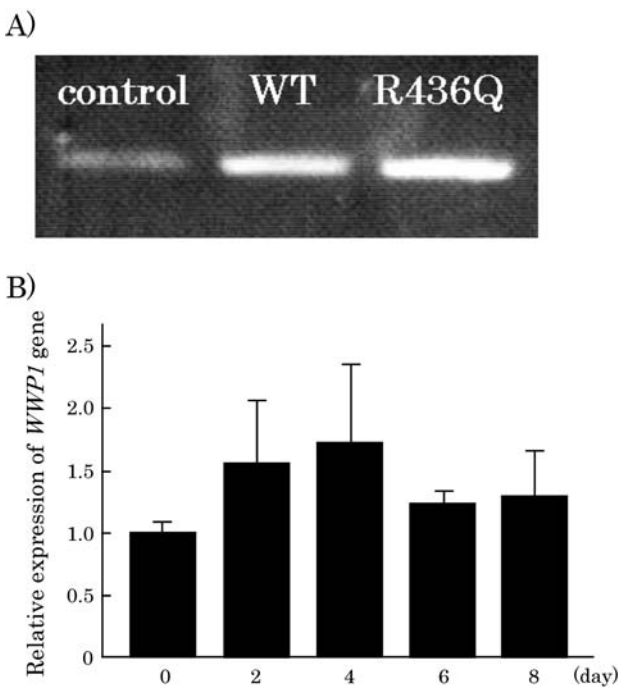

Fig. 1. **Exogenous and endogenous expression of *WWP1* gene in C_2C_{12} cells at day 0.** A) The vector capacity was assessed by RT-PCR method using *GAPDH* as internal standard. Each expression of *WWP1* gene in *WWP1*-transfected cells (WT and R436Q) was greater than empty vector-transfected cells (control). B) Endogenous expression of the *WWP1* gene in differentiating control cells. The *WWP1* expression levels analyzed by real-time PCR were not significantly changed through day 0 to 8. Y-axis indicates relative expression level of *WWP1* gene to the *GAPDH* gene expression. Bars indicate standard deviations.

The expression level of the *MyHC Ia* gene in the WT group was significantly higher (2.81 fold±0.95) compared to the control group, but the *MyHC IIb* gene expression was significantly lower (0.26±0.04 fold) in the WT group, indicating that the WWP1 promotes to trans-

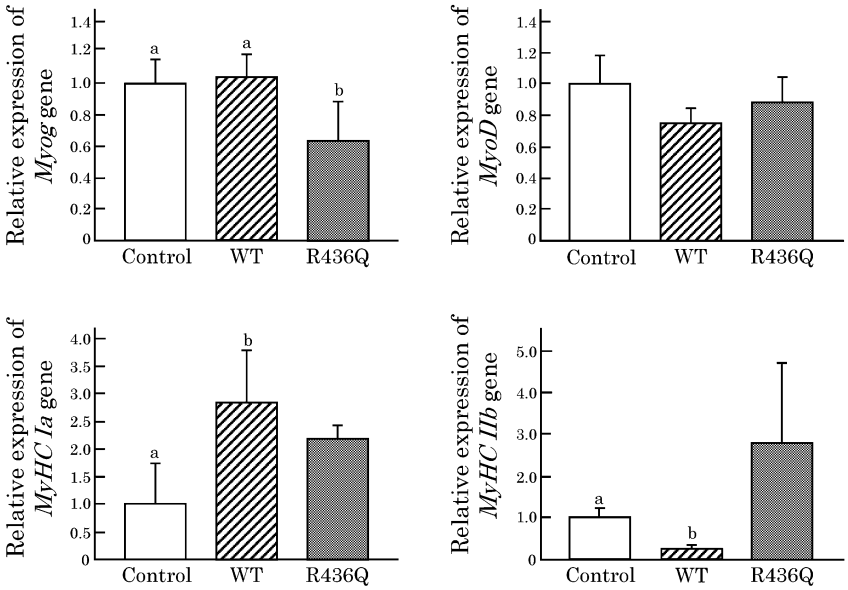

Fig. 2. **Muscle-differentiation markers in *WWP1*-transfected (WT and R436Q) and empty vector-transfected (control) C_2C_{12} cells.** Expression levels of muscle-differentiation markers (*Myog, MyoD, MyHC Ia* and *MyHC IIb*) in differentiating day 6 C_2C_{12} cells were analyzed by real-time PCR. The expression of *Myog* in R436Q group was significantly lower than other groups, while no significant difference was seen in the expression level of *MyoD* gene. However, the expression of fast *MyHC Ia* in *WWP1*-transfected cells was significantly higher than in control cells, while that of slow *MyHC IIb* was significantly lowered in *WWP1*-transfected cells compared to control cells. The R436Q-transfected cells retained the high expression of both fast *MyHC Ia* and slow *MyHC IIb* isoforms compared to control cells. Y-axis indicates relative expression level of each gene to the *GAPDH* gene expression. Bars indicate standard deviations. Different letters indicate significantly differences ($P<0.05$) among column graphs.

form C_2C_{12} cells into fast twitch characteristics. However, the R436Q-transfected cells persisted in the high expression of both fast *MyHC Ia* and slow *MyHC IIb* isoforms compared to the control cells, suggesting retainment in slow and fast twitch isoforms characteristic.

One of known proteins interacting with WWP1 is Notch (Flasza *et al.,* 2006), whose ablation in a skeletal muscle results in increased formation of fast twitch fibers and altered fiber type distribution at the expense of slow twitch fibers (Kitamura *et al.,* 2007). The WWP1 might control skeletal muscle fiber types via the regulation of the *MyHC* genes expression by the Notch signaling. The R436Q-transfected cell group showed the highest *MyHC IIb* expression (2.80 ± 1.92 fold than control level) among three groups, suggesting that C_2C_{12} cells persisted in the slow twitch character. Taken together, the R436Q mutation in *WWP1* gene seems to inhibit the normal fiber type differentiation and to induce atrophy. We assume that the transfection of mutated *WWP1* gene into normal chickens will inhibit the switching of the adult phenotype in their fast muscles and exhibit muscular dystrophic phenotypes such as degenerating fibers with many vacuoles in cytoplasm, as observed in muscular dystrophic chickens (Kikuchi *et al.,* 1981; Bandman, 1985). Our results suggest that WWP1 plays an important role in myoblasts' differentiating process. The mechanism to regulate MyHCs and other related-molecules by WWP1 needs elucidating in the future.

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