Suppression of *WWP1* Gene Via RNAi Induced the Reduction of Proliferation Rate of C₂C₁₂ Myoblasts

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The WW domain containing E3 ubiquitin protein ligase 1 (*WWP1*), which plays an important role in ubiquitin-proteasome pathway to degrade unneeded or damaged proteins, was recently identified as the responsible for chicken muscular dystrophy. Despite intensive studies on oncogenic characters, the role of *WWP1* to muscular diseases has not yet been fully understood. Previously, we transfected either of wild and mutated types of *WWP1* gene into C₂C₁₂ mouse myoblasts to monitor the expression pattern of muscle-differentiation markers, so that excessive *WWP1* expression enhanced the expression of the *myosin heavy chain (MyHC) Ia* gene but lowered the expression of the *MyHC Iib* gene, while mutated *WWP1* gene transfected into myoblasts was distinct from these cases in that the *MyHC* gene or genes expression inhibited the normal myoblast differentiation. However, the mechanism for the mutation to inhibit muscle differentiation remains elucidated. The current study attempted to suppress the *WWP1* expression by RNAi technique and to observe its effect on C₂C₁₂ cells. The effect of *WWP1* suppression was clearly different from that of the R441Q missense mutation in the *WWP1* gene. The *WWP1* suppression reduced the proliferation rate of C₂C₁₂ myoblasts, while clear difference was not observed in the proliferation rate by the transfection of the mutated *WWP1* gene into the cells. Our RT-PCR analysis indicated that the reduction of the *WWP1* expression is the specific effect mediated by RNAi and that the reduction of proliferation rate observed in this study is largely attributed to the suppression of the *WWP1* expression. These data indicated that the mutation responsible for chicken muscular dystrophy does not eliminate the enzymatic activity and provides some new function for the gene.

Key words: C₂C₁₂, chicken, muscular dystrophy, RNAi, *WWP1*

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Introduction

Ubiquitination, addition of ubiquitin (Ub) chains to a target protein, is one of the most common forms of post-translational modification, and it controls various important aspects of cell functions including protein degradation, DNA damage tolerance and signal transduction (Passmore and Barford, 2004; Pickart and Fushman, 2004). In ubiquitin-proteasome pathway (UPP), at least three types of enzymes are required: E1 Ub-activating enzyme, E2 Ub-conjugating enzyme and E3 Ub ligase (Hershko *et al.*, 1983). Comparative genome analysis reveals the existence of few genes encoding E1, tens of E2 encoding genes and hundreds of E3 encoding genes (Semple *et al.*, 2003).

The WW domain containing E3 ubiquitin protein ligase

1 (*WWP1*) is classified into a HECT (homologous to the E6-AP carboxyl terminus)-type E3 which recognizes and catalyzes Ub conjugation to specific protein substrates (Liu, 2004). *WWP1* possesses one C2 domain, multiple WW domains and one HECT domain (Pirozzi *et al.*, 1997; Flaszka *et al.*, 2002). The C2 domain binds to the cellular membranes in a Ca²⁺-dependent manner (Plant *et al.*, 1997) and mediates interactions with other proteins (Plant *et al.*, 2000; von Poser *et al.*, 2000; Augustine, 2001). The WW domain has two conserved tryptophan residues and binds proline-rich region (Sudol *et al.*, 1985). The HECT domain, similar to E2s structurally, has a cysteine residue as an active center that transfers the activated Ub from E2 onto first itself, and then onto its substrates (Jackson *et al.*, 2000).

The relationship between *WWP1* and breast and prostate cancer was intensively studied and some proteins involving cancer development have been elucidated (Chen and Metasic, 2007; Bernassola *et al.*, 2008). Knockdown of *WWP1* induces growth arrest and apoptosis in breast epithelial cancer cell lines, substantiating the concept that

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WWP1 promotes cell proliferation and survival of tumor cells (Chen *et al.*, 2007). Recently, we identified the *WWP1* gene as the responsible gene 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). The *WWP1* gene is expressed strongly in skeletal muscles (Flasza *et al.*, 2002; Komuro *et al.*, 2004; Matsumoto *et al.*, 2009), but the role which WWP1 plays in skeletal muscles is still unclear.

Previously we transfected the wild and mutated types of the *WWP1* gene into C₂C₁₂ cells which were myoblasts derived from the mice skeletal muscle and analyzed its effect on muscle differentiation (Matsumoto *et al.*, 2010). The C₂C₁₂ cells were used instead of chicken myoblasts since they were easily obtained and chicken cultured cells were not available. Excessive *WWP1* expression enhanced the expression of the *myosin heavy chain (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 data suggested 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. But the mechanism for the mutation to inhibit muscle differentiation still remains elucidated. In the current study, we applied RNAi technique to suppress the *WWP1* expression in C₂C₁₂ cells for the comparison of the effect induced by *WWP1*'s suppression with that by the mutation in *WWP1* observed in the previous study (Matsumoto *et al.*, 2010). The information obtained by this study will help understand the mechanism of chicken muscular dystrophy.

Materials and Methods

shRNA Design and Plasmid Construction

The RNAi vectors were constructed with BLOCK-iT U6 RNAi Entry Vector Kit (Invitrogen, CA, USA). The cDNA sequence of *WWP1* was obtained from GenBank (accession number NM_177327). The siRNA target design tool from Invitrogen (RNAi Designer) was used to design WWP1-shRNA sequences. WWP1-shRNA sequences were designed and synthesized as follows (sense target sequence underlined): WWP1, sense: 5'-CACCGCTGA-TAATACTTCAACCATGCGAACATGGTTGAAGTA-TTATCAGC-3' and antisense: 3'-CGACTATTATGAA-GTTGGTACGCTTGTACCAACTTCATAATAGTCG-AAAA-5'. The oligonucleotides were annealed and inserted into pENTRTM/U6 using T4 DNA Ligase. Inserted sequences were verified by DNA sequencing.

In order to exclude the possibility of off-target effects, three other WWP1-shRNA sequences were prepared and their effects were analyzed: WWP1-O1, sense: 5'-CACC-GAGTTGATGATCGTGGAAGCGAACTTCCACGATCATCAACTC-3' and antisense: 3'-CTCAACTACTA-

GCACCTTCGCTTGAAGGTGCTAGTAGTTGAGAAA-5', WWP1-O2, sense: 5'-CACCGACACGGTTA-ATGGAGAATCACGAATGATTCTCCATTAACCG-TGTC-3' and antisense: 3'-CTGTGCCAATTACCTC-TTAGTGCTTACTAAGAGGTAATTGGCACAGAAA-5' and WWP1-O3, sense: 5'-CACCGCATCCTCA-GAACACAGTGAACGAATTCAGTGTGTTCTGAG-GATGC-3' and antisense: 5'-CGTAGGAGTCTTGTG-TCACTTGCTTAAGTGACACAAGACTCCTACGAAA-3'.

Cell Culture and Transfection Conditions

The C3H murine skeletal muscle cell line C₂C₁₂ (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 10% fetal bovine serum (FBS) (Gibco, NY, USA). The cells were incubated at 37°C in humidified 95% air and 5% CO₂ atmosphere.

C₂C₁₂ cells were cultured overnight to 80% confluence prior to transfection. Transfection was performed using Opti-MEM[®] I Reduced Serum Medium (Gibco), Plus reagent (Invitrogen) and Lipofectamine[™] LTX (Invitrogen). The ratio of the plasmids and the transfection reagent was 1 mg: 2 ml. Following the protocol's specifications, cells were transfected with different vectors: pENTR[™]/U6-WWP1-shRNA, pENTR[™]/U6-WWP1-O-1-3-shRNA and pcDNA[™] 3.1/His/lacZ control vector of β-Gal Staining Kit (Invitrogen). Transfection efficiency of the RNAi was > 60%, as determined by β-Gal Staining Kit (Invitrogen).

Semi-Quantitative RT-PCR Analysis

Total RNA was isolated from cells using Sepasol RNAI (Nakalaitesque). The cDNA was generated by reverse transcription using oligo (dT) primer and SuperScript III Reverse Transcriptase (Invitrogen). The expressions of *WWP1* and its structurally similar genes (*WWP2*, *Itchy1*, *Nedd4-2*, *Nedd4-1*, *Smurf2*, *Smurf1*) were analyzed by the semi-quantitative RT-PCR method using *GAPDH* as an internal standard. Gene similarity was assessed by Protein BLAST (<http://www.ncbi.nlm.nih.gov/BLAST/>) and genes with > 400 bits (score) were regarded as the similar genes. The PCR was done for 35, 37 and 39 cycles at 94°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec using *TaKaRa Ex Taq[™]* Hot Start Version (Takara, Tokyo, Japan) as the DNA polymerase. All of the primer pairs in this procedure, designed by OLIGO 4.0 program (National Biosciences, MN, USA), are shown in Table 1. 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). Expression levels for each gene were calculated as relative expression levels toward *GAPDH* mRNA expression using the band of 37 cycles. For each gene, three

Table 1. Primers used for semi-quantitative RT-PCR

Name	GenBank accession #	Sequence (5'→3') ¹	Scores (bits) ²	Identities (%) ³
<i>WWP1</i>	NM_177327	CCTTGGAGTTCGAGTTTGGA AGTTCCCCAGTTTGCACTATTC	1917	100
<i>WWP2</i>	NM_025830	ATATGCTGGGAAGAACAATTACT AATGGAGTTGTAAAACTCAGGGT	1094	60
<i>Itchy1</i>	NM_008395	AAACAACGCCTTAACCCTAAGAA GACTCCACACACGAAAACATAAT	1088	60
<i>Nedd4-2</i>	NM_031881	ACCATTGTCTTATTTACCTTC TTACCATTATTTCTGACCCGTTG	517	45
<i>Nedd4-1</i>	NM_010890	AGTTGTGAGAGTGAAAGGTTATAG AGGAAATCATCTCTTGTCAAACG	489	45
<i>Smurf2</i>	NM_025481	TGACCATAACAACAGAACAACAC TTCCTCAAAAATCTCTCCCTAG	483	44
<i>Smurf1</i>	NM_001038627	AGATCACAATAATAGGACAACCC ACAAAGTGGAAGTATGACAGATG	467	63
<i>GAPDH</i>	NM_008084	AGGTCGGTGTGAACGGATTTG TG TAGACCATGTAGTTGAGGTCA	—	—

¹ Each column's upper primers are forward primers and lower ones reverse primers.

² Genes with >400 bits in BLAST were regarded as the similar genes.

³ Identities of amino acid sequence are shown.

individual samples were prepared.

Proliferation Rate of the Cell

C₂C₁₂ cells were observed by optical microscope and the number of cells was counted manually immediately after and 24 hours after transfection. Five observation sites were randomly selected for one condition. Proliferation rates were calculated as relative levels toward the cell numbers of just after transfection. For each calculation, three individual samples were prepared.

Statistical Analysis

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

Results and Discussion

We previously suggested the mutation in the *WWP1* gene disrupts the myoblasts' differentiating process to result in muscular dystrophic phenotype (Matsumoto *et al.*, 2010). However, the mechanism for the mutation to inhibit muscle differentiation remains elucidated. The current study attempted to suppress the *WWP1* expression by RNAi technique and to observe its effect on C₂C₁₂ mouse myoblasts. For this purpose, pENTR™/U6-WWP1-shRNA vector (shWWP1) was constructed and its effect on C₂C₁₂ cells was analyzed and compared with pcDNA™ 3.1/His/lacZ control vector (shLacZ)-transfected and untransfected cells. The capacity of shWWP1 vector was assessed by semi-quantitative RT-PCR using *GAPDH* gene as an internal control (Fig. 1). Though the transfection efficiency was not high enough (>60%, not shown), the *WWP1* expression of shWWP1-transfected cells was successfully reduced to 54 ± 0% compared with

that of shLacZ-transfected control cells.

Unintended effects on gene expression mediated by RNAi, off-target effects, should be concerned when applying RNAi technique. Off-target effects are divided into two types: specific and nonspecific off-target effects. Specific off-target effects are mediated by partial sequence complementarity of the RNAi construct to mRNAs other than the intended target (Rao *et al.*, 2009). In order to confirm the specificity in RNAi mediated gene silencing, not only the *WWP1* expression but the expressions of its structurally similar genes were analyzed. The *WWP2*, *Itchy1*, *Nedd4-2*, *Nedd4-1*, *Smurf2* and *Smurf1* were selected as the genes similar to *WWP1* according to the BLAST score (Table 1), although these genes did not have homologue sequence with the RNAi sequence. Their expressions in shWWP1-transfected cells were 92 ± 7% (*WWP2*), 83 ± 5% (*Itchy1*), 92 ± 1% (*Nedd4-2*), 93 ± 0% (*Nedd4-1*), 90 ± 2% (*Smurf2*) and 92 ± 3% (*Smurf1*) (Fig. 1). Though the expressions of all genes examined were reduced, their downward trends were not as sharp as that of *WWP1* and the differences of *WWP1* expression with the expressions of the other genes were significant, suggesting the reduction of the *WWP1* expression in shWWP1-transfected cells is the specific effect mediated by the RNAi.

Twenty-four hours after transfection, the proliferation rate of shWWP1-transfected cells was significantly reduced to 89 ± 35%, though that of shLacZ-transfected cells was 161 ± 21% and untransfected cells 263 ± 53% (Fig. 2, 3). The *WWP1* suppression reduced the proliferation rate of C₂C₁₂ myoblasts, while clear difference was not observed in the proliferation rate by the transfection of the mutated *WWP1* gene into the cells (Matsumoto *et al.*, 2010). The effect of *WWP1* suppression was clearly dif-

Fig. 1. Confirmation of the specificity in RNAi mediated gene silencing. Expression of *WWP1* and its structurally similar genes, *WWP2*, *Itchy1*, *Nedd4-2*, *Nedd4-1*, *Smurf-2* and *Smurf-1*, in sh*WWP1*-transfected (W) and shLacZ-transfected cells (L) was analyzed by the semi-quantitative RT-PCR method using *GAPDH* as an internal standard. The PCR was performed for 35, 37 and 39 cycles. Though the *WWP1* expression in W was reduced to $54 \pm 0\%$ compared with L, expressions of the others were not significantly altered. Three individual samples were prepared for each gene and the representative images were shown.

ferent from that of the R441Q missense mutation in the *WWP1* gene, indicating that the mutation responsible for chicken muscular dystrophy does not eliminate the enzymatic activity and provides some new function for the gene.

In order to exclude the possibility of the nonspecific off-target effects, we designed three other *WWP1*-shRNA sequences (sh*WWP1*-O1-3) and observed their effects on C₂C₁₂ cells (Fig. 3). Nonspecific off-target effects include a wide variety of immune and toxicity related effects that are intrinsic to the RNAi construct itself or its delivery vehicle (Rao *et al.*, 2009). All cells with sh*WWP1*-O1-3 vectors showed similar downward tendencies to sh*WWP1*-transfected cells in terms of proliferation rate (sh*WWP1*-O1: $101 \pm 22\%$, sh*WWP1*-O2: $83 \pm 16\%$ and sh*WWP1*-O3: $110 \pm 22\%$). Though all transfected cells exhibited downward trends compared with untransfected cells, sig-

nificant difference did exist only between shLacZ-transfected cells and sh*WWP1*s-transfected cells. The reduction of proliferation rate observed in this study might be largely attributed to the suppression of the *WWP1* expression.

The suppression of the *WWP1* expression might induce apoptosis and/or cell-cycle arrest. One of known proteins interacting with *WWP1* is Notch (Flasza *et al.*, 2006), which regulates various proteins involved in apoptosis and cell-cycle such as NF- κ B, Akt, Cyclin D1 and CDK2 (Oswald *et al.*, 1998; Katso *et al.*, 2001; Ronchini *et al.*, 2001; Palomero *et al.*, 2008). The *WWP1* suppression might lead the aberration of these proteins to reduce the proliferation rate of C₂C₁₂ cells. In addition, the ablation of Notch 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). In chicken muscular dystrophy, the switching of the adult phenotype in fast muscle is inhibited (Bandman, 1985; Bandman and Bennett, 1988; Kaprielian *et al.*, 1991; Tidyman *et al.*, 1997), suggesting the *WWP1*-Notch interaction plays an important role for muscle to develop normally. Therefore, *WWP1* suppressed chickens via RNAi could exhibit muscular dystrophic phenotype, since accumulation of *WWP1*'s substrates is expected to result in the phenotype. However, the chickens might not show the phenotype because of the functional redundancy among members of the NEDD4 family including *WWP1* (Chen and Metasic, 2007).

In the current study, we suppressed the *WWP1* expression and observed its effect on C₂C₁₂ myoblasts. The reduction of proliferation rate was observed due to the *WWP1* suppression, which was an effect apart from the mutation in *WWP1*. Elucidating the mechanism of the reducing proliferation rate via the *WWP1* suppression and the characteristic of the mutation responsible for chicken muscular dystrophy will contribute to the understanding of the role that *WWP1* gene plays in skeletal muscle.

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Fig. 2. Reduction of proliferation rate in *WWP1* suppressed C₂C₁₂ cells. Twenty-four hours after transfection (24 H), C₂C₁₂ cells were observed by optical microscope and the number of cells was counted manually. The proliferation rate of shWWP1-transfected cells was significantly reduced to $89 \pm 35\%$, though that of shLacZ-transfected cells was $161 \pm 21\%$ and untransfected cells $263 \pm 53\%$. Proliferation rates were calculated as relative levels toward the cell numbers of immediately after transfection (0 H). Three individual samples were prepared for each calculation and the representative images were shown.

Fig. 3. The effects of four WWP1-shRNA sequences on C₂C₁₂ cells. In addition to the original one (shWWP1), three other WWP1-shRNA sequences (shWWP1-O1-3) were prepared and their effects on the proliferation rate of C₂C₁₂ cells were observed. All cells with shWWP1-O1-3 vectors showed significant downward tendencies as shWWP1-transfected cells twenty-four hours after transfection (24 H) (shWWP1: $89 \pm 35\%$, shWWP1-O1: $101 \pm 22\%$, shWWP1-O2: $83 \pm 16\%$ and shWWP1-O3: $110 \pm 22\%$). Y-axis indicates relative proliferation rate based on the cell numbers of immediately after transfection (0 H). Three individual samples were prepared for each calculation. Definition of proliferation rate was depicted in Fig. 2.

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