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Abstract

Syndecan-4, a type of heparin sulfate proteoglycan, plays an important role in muscle development, regeneration, and maintenance. Although, the important effects of *syndecan-4* on the regulation of myogenesis in mice, turkeys, and bovines have been consistently reported, the molecular mechanisms of *syndecan-4* in myogenesis are not well understood. In this study, the role of *syndecan-4* in regulating myogenesis was investigated in QM7 cells, which constitute a quail myogenic cell line. Overexpression of *syndecan-4* inhibited myogenesis, resulting in reduced myoblast fusion and shorter myotubes than in the control group. Therefore, the cells overexpressing *syndecan-4* showed a smaller total myotube area than did the control cells. Furthermore, these cells had lesser myosin heavy chain proteins, suggesting that muscle differentiation is inhibited by *syndecan-4*. To investigate the inhibitory effect of *syndecan-4* on myogenic differentiation, the mRNA expression levels in several genes known to regulate myoblast proliferation and differentiation were compared. Myogenic regulatory factors, including *myogenic factor 5*, *myogenic differentiation 1*, and *myogenin*, showed significantly different expressions between the groups during myogenesis. *Myostatin*, a negative regulator of muscle growth, showed significantly higher expression on day 4 in cells overexpressing *syndecan-4*. In conclusion, *syndecan-4* could delay and inhibit muscle differentiation by regulating the expression levels of myogenic factors and muscle growth regulator in quail myocytes. This study provides valuable information regarding the role of *syndecan-4* in myogenesis, which may aid in improving the production of poultry meat.

Keywords: Syndecan-4, QM7 cell line, Myogenic regulatory factors, Myogenesis, Muscle differentiation

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Introduction

Owing to the rapid rise in the worldwide demand for poultry meat, the efficient production of poultry meat has been an important issue [1]. The quantitative traits such as growth rate and carcass percentage are important economic traits and critical factors to increase the poultry meat production [2]. Traditionally, those traits were improved by breeding and feeding [3,4], however, their molecular mechanisms were not clear. Recently, numerous genetic and functional studies are being conducted to understand the skeletal muscle development [5,6]. This information could provide insights to improve the efficiency of poultry meat production.

Muscle is an essential tissue that helps in movement and support of the body. It is composed of bundles of multinucleated fusion cells called myofibers. Myogenesis occurs through myoblast fusion, which is initiated and

37 regulated by various transcription factors and mechanisms. Generally, myogenesis is controlled by regulatory
38 factors called paired box (*PAX*) genes and myogenic regulatory factors (MRFs) [7]. The *PAX* genes act as
39 regulators of myoblast or satellite cell proliferation and differentiation during early myogenesis [8]. MRFs are
40 composed of four genes (*myogenic differentiation 1* [*MYOD1*], *myogenic factor 5* [*MYF5*], *myogenin* [*MYOG*],
41 and *myogenic factor 6* [*MYF6*]), which are the major regulators of muscle differentiation. They can even induce
42 myogenic differentiation in non-myogenic cells [9-11]. *Myostatin* (*MSTN*), which belongs to the transforming
43 growth factor-beta superfamily, plays an important role as a negative regulator of skeletal muscle growth in
44 animals [12]. It is mainly expressed in skeletal muscles [13]. Previous studies reported that various species with
45 naturally occurring mutations in *MSTN* and *MSTN* knockout models demonstrated increased skeletal muscle mass
46 [14-17]. Keller-Pinter et al. demonstrated that syndecan-4 (SDC4) affects myoblast proliferation by modulating
47 *MSTN* signaling [18]. Research regarding the relationship between *MSTN* and myogenesis has resulted in the
48 emergence of myoblasts as an important factor related to the development of the poultry industry.

49 SDC4 is a heparin sulfate proteoglycan, composed of a core protein connected to glycosaminoglycan chains,
50 which are one or more long linear carbohydrate chains [19]. It belongs to the syndecan family that regulates
51 integrin by interacting with fibronectin and signaling proteins found in the basement membrane [20]. Like SDC2,
52 SDC4 is a glycosaminoglycan chain that attaches to heparin sulfate [18]. Despite being the smallest protein in the
53 syndecan family, SDC4 has been studied the most. While its molecular mechanisms have not been sufficiently
54 established, *SDC4* is known to affect muscle differentiation and production [19]. Its important role in muscle
55 development, maintenance, and regeneration is being investigated pursued in mice, turkeys, and bovines. More
56 myotubes are formed when the *SDC4* gene is knocked out in bovine muscle cells. Moreover, other studies have
57 shown that *SDC4* knock-out causes muscle damage by reducing the number of satellite cells in case of disease in
58 mice and turkeys [21,22,23]. These results may support that *SDC4* has an effect of inhibiting muscle development.
59 And this inhibitory effect could be mediated by various genes and factors that negatively affect for the
60 differentiation, such as *MSTN* and fibroblast growth factor (FGF) [18,22]. Therefore, the expression of *SDC4*
61 must be decreased during normal muscle differentiation.

62 Although previous studies have actively investigated the role of *SDC4* in satellite cells [21,23,24,25],
63 research regarding its involvement in muscle differentiation is still limited. This study hypothesized that *SDC4*
64 plays a crucial role in muscle growth and development particularly through regulating MRFs. To reveal its effects
65 on quail muscles, each of *SDC4* expression vector or an empty vector (EV) was transfected into quail myoblasts
66 (QM7) cells, and the differentiation of the cells was compared by analyzing myotube formation. Subsequently,

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67 changes in the expression of MRFs and *MSTN* in the cells overexpressing *SDC4* were analyzed and compared
68 with those factors in the control cells.
69
70 # Materials and Methods

71 **Experimental design**

72 The effect of *SDC4* on muscle differentiation was investigated by performing a series of experiments with
73 three replicates. Each replicate consisted of two groups: *SDC4*-overexpressed (OE) and control. QM7 cells from
74 different passages were used for each replicate. Each of 14 Φ35-mm dishes was seeded with 8×10^5 cells 1.5 d
75 before starting differentiation (day 0). On the day before differentiation, the cells were transfected with either
76 *SDC4*-expression vector or empty vector. Differentiation was observed at four time points: day 0, the start of
77 differentiation; day 1, the change of myogenic gene expression; day 2, the active myotube formation; and day 4,
78 the sufficient myotube formation. mRNA samples were collected at four time points (day 0, 1, 2, 4), protein
79 samples at two time points (day 0, 4), and immunofluorescence samples at one time point (day 4). Each sample
80 was prepared at each time point for further analysis.
81

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82 ***SDC4* gene cloning and expression vector construction**

83 The coding sequence of *SDC4* was amplified with a primer set, SDC4-f (5'-GTT CCG TTC TGA TTC AGC
84 GC-3') and SDC4-r (5'-CTC ATT GGA GGG CAC AGT GT-3'), by PCR and cloned into the pGEM-T easy
85 vector (Promega, Madison, WI, USA). After confirming the sequence of *SDC4* gene by Sanger sequencing, the
86 hemagglutinin (HA)-tag sequence was added in the front of the stop codon of the *SDC4* gene by PCR with a
87 primer set, the SDC4-f and SDC4-HA-r (5'-TTA CAA GCT GTA ATC TGG ACC ATC GTA TGG GTA AGC
88 ATA GAA CTC ATT TGT AGG-3') Then, the amplified sequence was inserted into the EcoRV site of the
89 pcDNA3.1 vector (Invitrogen, Grand Island, NY, USA) to generate the final *SDC4* expression vector.
90

91 **Cell culture and transfection of cells**

92 QM7 cells (American Type Culture Collection, Manassas, VA, USA) were cultured in medium 199
93 containing 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA), 10% tryptose phosphate broth (TPB;
94 Sigma-Aldrich, St. Louis, MO, USA), and 1% antibiotic-antimycotic (ABAM; Gibo). The cells were incubated
95 at 37 °C in 5% CO_2 and subcultured before filling in the culture dish to prevent differentiation. To promote cell

96 differentiation, the differentiation medium was prepared by changing the FBS concentration to 0.5%; all
97 remaining compositions and conditions were the same. The cells were seeded a density of 8×10^5 in a culture dish
98 and transfected with EV or *SDC4* expression vector after 18 h using Lipofectamine 3000 reagent (Invitrogen,
99 Carlsbad, CA, USA) according to the manufacturer's protocols. For control, the pcDNA3.1 designated as EV was
100 used. The culture medium was replaced with fresh growth medium at 5 h after transfection. After 16 h, the culture
101 medium was replaced with the differentiation medium. They were differentiated for 4 d, and from day 2, half the
102 culture medium was replaced with fresh differentiation medium every day.
103
104 **Immunofluorescence staining of cells and myotube area analysis**
105 The cells were washed in phosphate-buffered saline (PBS) and fixed for 15 min using 10% neutral-buffered
106 formalin. Cell membrane permeability was induced using 0.3% NP-40. The non-specific antigens were blocked
107 using PBS mixed with 0.1% Tween-20 (PBST) containing 5% non-fat dry milk. The primary antibodies were
108 treated with anti-myosin heavy chain (MF20; Developmental Studies Hybridoma Bank, Iowa city, IA, USA) for
109 1 h and the secondary antibodies with anti-mouse immunoglobulin G (IgG; Santa Cruz Biotechnology, Dallas,
110 TX, USA) bound to CruzFluor™594. The nuclei were then stained with 4′,6-diamidino-2-phenylindole (DAPI).
111 Photographs were captured using an inverted fluorescent microscope (CKX53; Olympus, Shinjuku, Tokyo, Japan).
112 To measure the area of differentiated myotubes, the photographs of immunofluorescence-stained cells were used
113 to measure the length and thickness of myotubes.
114
115 **Western blotting**
116 The cells were washed with PBS and extracted in 1× lysis buffer. Cellular debris was removed by
117 centrifuging at 14 000 × g for 10 min at 4 °C. Subsequently, the same amount of 2 × Laemmli buffer, containing
118 2-mercaptoethanol, as that of the supernatant, excluding the pellets, was added. The protein was denatured at
119 95 °C for 5 min. Protein concentrations were measured by Coomassie staining, and they were separated by
120 polyacrylamide gel electrophoresis. The protein samples were transferred onto a polyvinylidene fluoride (PVDF)
121 membrane. Subsequently, the non-specific antigens were blocked using 5% non-fat dry milk in Tris-buffered
122 saline mixed with Tween-20 (TBST). After processing the primary antibodies overnight at 4 °C, the secondary
123 antibodies were processed at room temperature. During treatment with antibodies, the concentration of skim milk
124 was lowered to 2.5%. After processing the enhanced chemiluminescence (ECL) solution, images were obtained

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125 using the Image Quant LAS 500 imager (GE Healthcare, Chicago, IL, USA).

126

127 **RNA isolation and quantitative real-time polymerase chain reaction (qRT-PCR)**

128 When the cultured cells reached a density of 8×10^5 QM7 cells in the culture dish, they were differentiated
129 after 42 h to ensure sufficient growth. The differentiated mRNA samples were extracted on days 0, 1, 2, and 4
130 using RNAiso plus (Takara Bio Inc., Shiga, Japan). Total RNA was identified and 1 μg was quantified using the
131 P200 Micro-volume spectrophotometer (Biosis Design, Gwangmyeong-si, Republic of Korea) and by
132 electrophoresis. cDNA was prepared using the DiaStar RT Kit (SolGent, Daejeon, Republic of Korea) according
133 to the manufacturer's instructions. Additionally, cDNA was subjected to qRT-PCR using the Bio Rad CFX
134 Connect™ Real-Time PCR Detection System (Bio Rad Hercules, CA, USA). Target genes were normalized to
135 glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) that was used as the housekeeping gene. Table 1 shows
136 the primer pairs designed for qRT-PCR. The expression level of the target gene was calculated using the $2^{-\Delta\Delta Ct}$
137 method.

138

139 **Statistical analysis**

140 All data were expressed as mean ± standard error of the mean (SEM) and analyzed using Student's t-test and
141 two-way analysis of variance (ANOVA) in R packages (R Foundation for Statistical Computing, Vienna, Austria.
142 Multiple groups showing significance in the groups were compared using the least significant difference (LSD)
143 test. The results were considered significant if $p < 0.05$. All experiments were independently conducted in
144 triplicate.

145

146 # Results and Discussion

147 ***SDC4* overexpression reduces myotube formation in quail muscle cells**

148 An overexpression vector of *SDC4* was designed and transfected into QM7 to verify the effect of *SDC4* on
149 muscle cell differentiation. *SDC4*-OE cells showed lesser muscle cell differentiation than did the EV cells, which
150 were used as the control cells [Fig. 1]. Differentiation was observed at four time points: day 0, the start of
151 differentiation; day 1, the change of myogenic gene expression; day 2, the active myotube formation; and day 4,
152 the sufficient myotube formation. Distinctive differences in myotube formation were observed during days 2–4
153 after differentiation between the groups. On day 2, while the EV cells started forming myotubes, the *SDC4*-OE

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154 cells did not. Myotube formation in *SDC4*-OE cells was observed on day 4, and its rate was as low as that observed
155 in the EV cells on day 2. The myotubes in the *SDC4*-OE cells were shorter and thinner than those in the EV cells
156 on day 4. Therefore, *SDC4* is a negative regulator of myotube formation.
157 To observe and measure the relative area of the myotubes, they were stained with anti-myosin heavy chain
158 (MyHC) antibodies and DAPI on day 4 [Fig. 2]. As shown in Fig. 1, the myotubes of the *SDC4*-OE cells were
159 narrower and shorter than those in the control cells [Fig. 2A]. Among the stained cells, the number of
160 mononucleated cells was higher in the *SDC4*-OE cells than that of those in the control cells, suggesting that fusion
161 of the myocytes is inhibited by SDC4. The myotube area of *SDC4*-OE cells was reduced by less than half of that
162 of the control cells ($p < 0.001$) [Fig. 2B].
163 To confirm the degree of differentiation between the groups at a molecular level, western blotting was
164 performed using MyHC, which is the most abundant protein responsible for muscle contraction in the skeletal
165 muscles [26]. MyHC protein was not observed on day 0 in both the groups, suggesting that the cells had not started
166 differentiating at that time [Fig. 3]. However, MyHC protein was detected on day 4 at different levels in both the
167 groups. The amount of MyHC protein in the *SDC4*-OE cells was less than half of that in the control cells. Myotube
168 staining showed similar results, indicating that myotube formation is reduced by *SDC4* overexpression. Similarly,
169 a previous study showed that myotube formation, which is increased by *SDC4* knockout in bovine cells and the
170 cytoplasmic domain of *SDC4*, inhibits myoblast fusion [22]. Szabo et al. reported that *SDC4* expression gradually
171 decreases during muscle differentiation, while high expression of *SDC4* hinders myogenesis [27]. Decreasing
172 *SDC4* expression during myogenesis is crucial for myotube formation. In summary, *SDC4* plays a role in
173 inhibiting myocyte fusion and myotube formation, which leads to reduced myogenesis.
174
175 ***SDC4* regulates the expression of MRFs during differentiation**
176 The effect of *SDC4* on the expression of genes known to regulate myotube formation was investigated via
177 qRT-PCR [Fig. 4]. *PAX3* and *PAX7*, which are expressed in the early stages of muscle development and are
178 involved in the proliferation of myoblasts and initiation of myogenic differentiation [28], showed no differences
179 between the groups. However, they were significantly reduced during differentiation ($p < 0.01$) [Fig. 4A and 4B],
180 indicating that *SDC4* does not affect the initiation of differentiation by regulating *PAX3* and *PAX7* expression.
181 Subsequently the expressions of MRFs were analyzed. *MYF5* showed a continuous, significant reduction in
182 expression during differentiation in both the groups ($p < 0.001$) [Fig. 4C]. *MYF5* expression was significantly
183 lesser in the *SDC4*-OE cells than that in the control cells on days 1 and 2 ($p < 0.001$). Among the MRFs, *MYF5*

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184 is expressed first and initiates differentiation of muscle progenitor cells into myoblasts. It is turned off when
185 *MYOD1* is expressed in the myoblasts [29]. These results suggest that decreased expression of *MYF5* by *SDC4*
186 cannot adequately induce differentiation of progenitor cells and would induce insufficient expression of *MYOD1*.
187 The expression of *MYOD1* was significantly affected by the interaction between the day of differentiation
188 and treatment ($p < 0.01$) [Fig. 4D]. The expression level of *MYOD1* was the same between the groups before
189 differentiation but differed significantly after initiation of differentiation. In the control group, the expression of
190 *MYOD1* increased on day 1 and then decreased from day 2 onward. In *SDC4*-OE cells, *MYOD1* expression
191 decreased slightly on day 1 and increased from day 2. *MYOD1* expression in the control group was significantly
192 higher than that in the *SDC4*-OE cells on day 1, whereas it was significantly higher in the *SDC4*-OE cells from
193 day 2. *MYOD1* expression is known to increase in highly proliferative myoblasts; it then decreases after inducing
194 the expression of *MYOG* to initiate myotube formation [30,31]. In this study, *SDC4* altered the expression of
195 *MYOD1* in a different pattern, maintaining higher expression in the mid and late stages of differentiation, which
196 resulted in delayed myoblast differentiation and myotube formation.
197 *MYOG* expression gradually increased during myogenesis in both the groups ($p < 0.001$); however, its
198 expression in the *SDC4-OE* cells was lower than that in the control cells during differentiation ($p < 0.01$) [Fig.
199 4E]. Increased *MYOG* expression is known to enhance myotube formation during differentiation. During
200 differentiation, *MYOG* is upregulated to induce differentiation of myoblasts into myotubes, which is accompanied
201 by the downregulation of *MYOD1* [32,33]. Sustained expression of *MYOD1* during differentiation may aid in
202 proliferation of the myoblasts rather than differentiation in *SDC4*-OE cells. Additionally, the previous study
203 reported that a defect in *MYOG* results in a severe reduction of all skeletal muscles [34]. In this study, the lower
204 expression of *MYOG* due to *SDC4* overexpression may also delay myoblast differentiation. *SDC4* plays a role in
205 recruiting FGF to transmit signals across the cell membrane and acts as a co-receptor for FGF receptor (FGFR).
206 FGF2 is known to reduce the expression of *MYOG*, thus acting as an inhibitor of skeletal muscle differentiation
207 [35]. Hence, decreased *MYOG* expression and myotube formation may be caused by elevated FGF2 signaling
208 owing to *SDC4* overexpression.
209 Expression pattern analysis of the myostatin (*MSTN*) gene, which does not belong to MRF family but is
210 closely related to the regulation of muscle development, was conducted [36]. A significant change in *MSTN*
211 expression was observed in both the groups depending on the day of differentiation but not on treatment ($p <$
212 0.001) [Fig. 4F]. *MSTN* expression increased dramatically on day 1 and then decreased quickly in the control cells
213 but not in the *SDC4*-OE cells. The interaction between the day of differentiation and treatment had a significant

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effect on *MSTN* expression ($p < 0.05$); it was higher in the *SDC4*-OE cells on day 4. *SDC4* was recently reported to reduce the formation of mature MSTN by binding with proMSTN [18], which usually gets reduced because of conversion into mature MSTN through a proteolytic process. However, SDC4 blocks this process, thus reducing the amount of mature MSTN, which induces the proliferation of myoblasts during the early stage of skeletal muscle regeneration. In this study, the higher level of *MSTN* expression maintained during differentiation in *SDC4*-OE cells might have inhibited myoblast proliferation and differentiation. The inhibitory effect of *MSTN* may get gradually elevated because of reduced transiently expressed *SDC4* during differentiation.

SDC4 is highly expressed during satellite cell proliferation in turkeys [37]. In an *SDC4*-knockout mouse model, muscle recovery abilities were distinguishably decreased, and the muscle fibers were smaller than those in the control group [38]. Moreover, the model exhibited defects in satellite cell activation, proliferation, and *MYOD1* expression [23]. These studies showed that *SDC4* is closely associated with regeneration through activation and proliferation of the satellite cells. This finding along with our data show that an appropriate level of SDC4 is required for normal proliferation and differentiation of myoblasts during muscle development. MSTN is also known to inhibit myoblast differentiation by reducing *MYOD1* synthesis and activity [39]. In this study, both *MSTN* and *MYOD1* were upregulated in *SDC4*-OE cells on day 4. These results suggest that there could be another pathway, including *SDC4* signaling cascades, affecting *MSTN* and *MYOD1* regulation.

In conclusion, this study demonstrates the effect of *SDC4* on muscle cell differentiation and elucidates the various changes in gene expression. In quail muscle cells, *SDC4* reduces myotube formation and negatively affects muscle formation. This inhibitory effect of *SDC4* on muscle formation is mediated by regulation of the expression of MRFs and *MSTN*. This finding may help to elucidate the intricate molecular mechanisms of *SDC4* and to comprehend the muscle development, disease, and regeneration. Furthermore, it could enhance poultry production, which would be advantageous for poultry breeders. However, the further studies are necessary to unveil the intricate mechanisms underlying the modulation of the expression of MRFs and *MSTN* by *SDC4*.

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Acknowledgments

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331 # Figure legends

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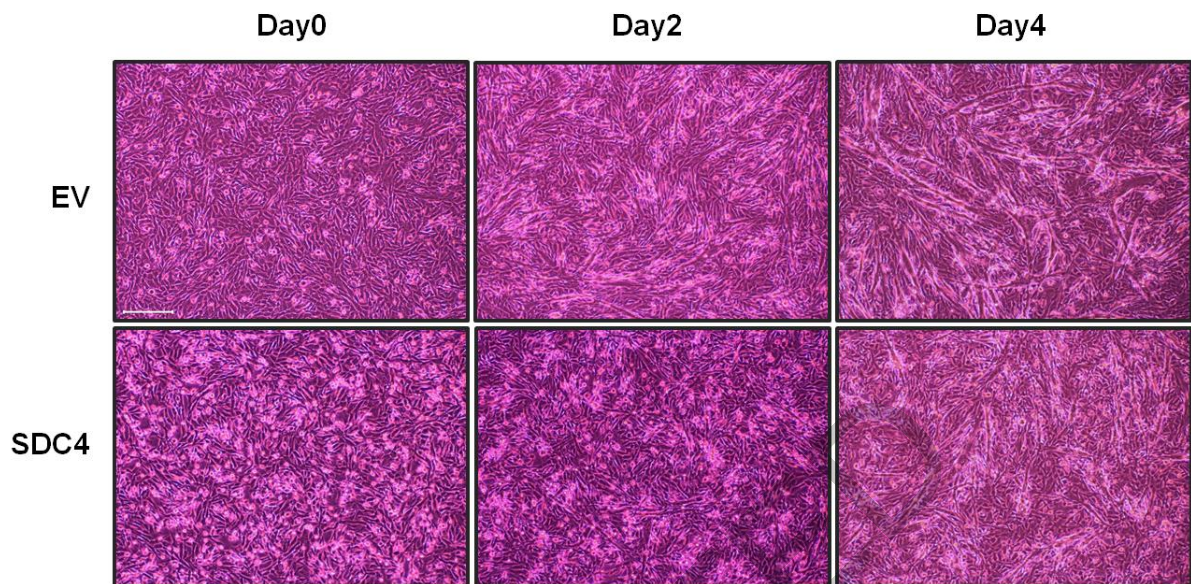

333
334 Figure 1. Effect of transfection of overexpression vector of *SDC4* on differentiation of QM7 cells (magnification,
335 100×). The differentiation was conducted on the day 0, day 2, and day 4. On day 4 of differentiation, *SDC4*-
336 OE cells demonstrate lesser extent of differentiation than do the EV cells. Additionally, myotubes in the *SDC4*-
337 OE cells are shorter and thinner than those in the EV cells on day 4. Scale bar means 200µm. *SDC4, syndecan-*
338 *4*; EV, empty vector; OE, overexpressed

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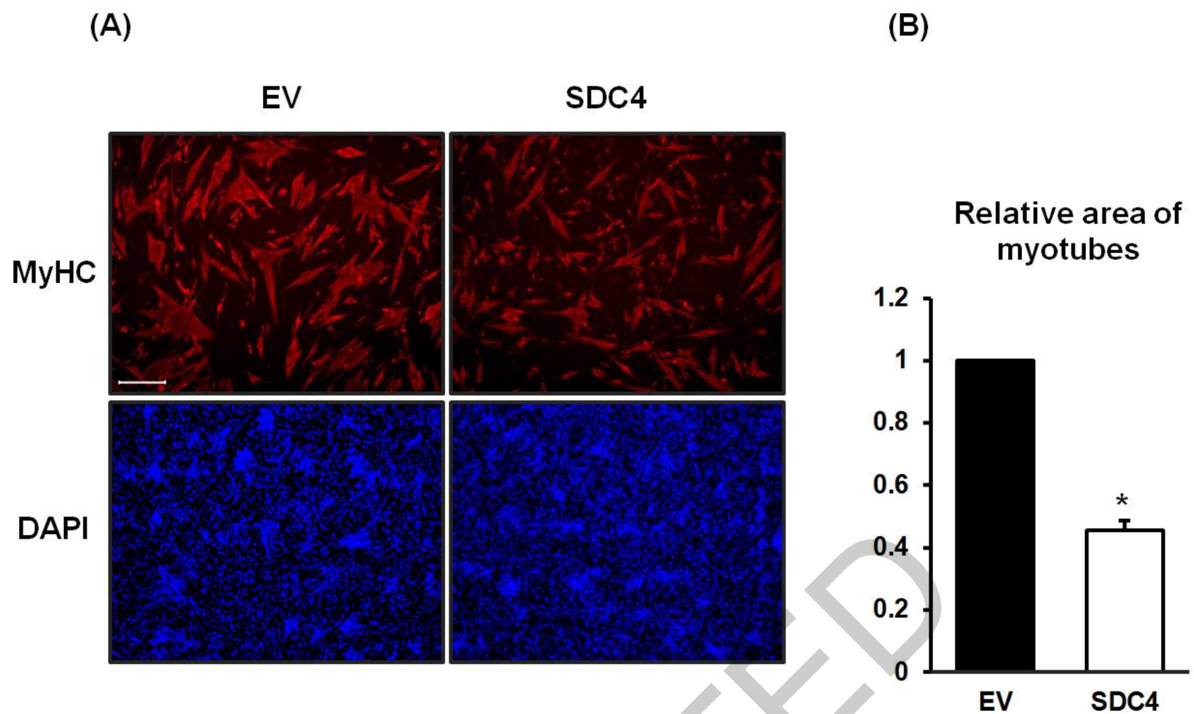

341
342 Figure 2. Myotube staining and measurement of myotube area (magnification, 100×). (A) Myotubes (red) and
343 nuclei (blue) were stained with MyHC antibodies and DAPI, respectively on day 4 of differentiation. The
344 myotubes are shorter and thinner in the *SDC4*-OE cells than those in the control cells. (B) The myotube area
345 in the *SDC4*-OE cells is approximately half of that in the control cells. Thus, the overexpression of *SDC4*
346 inhibits myotube formation. The level of significance is denoted as follows: * $p < 0.001$. Scale bar means
347 200μm. MyHC, anti-myosin heavy chain; DAPI, 4′,6-diamidino-2-phenylindole; OE, overexpressed; SDC4,
348 syndecan-4

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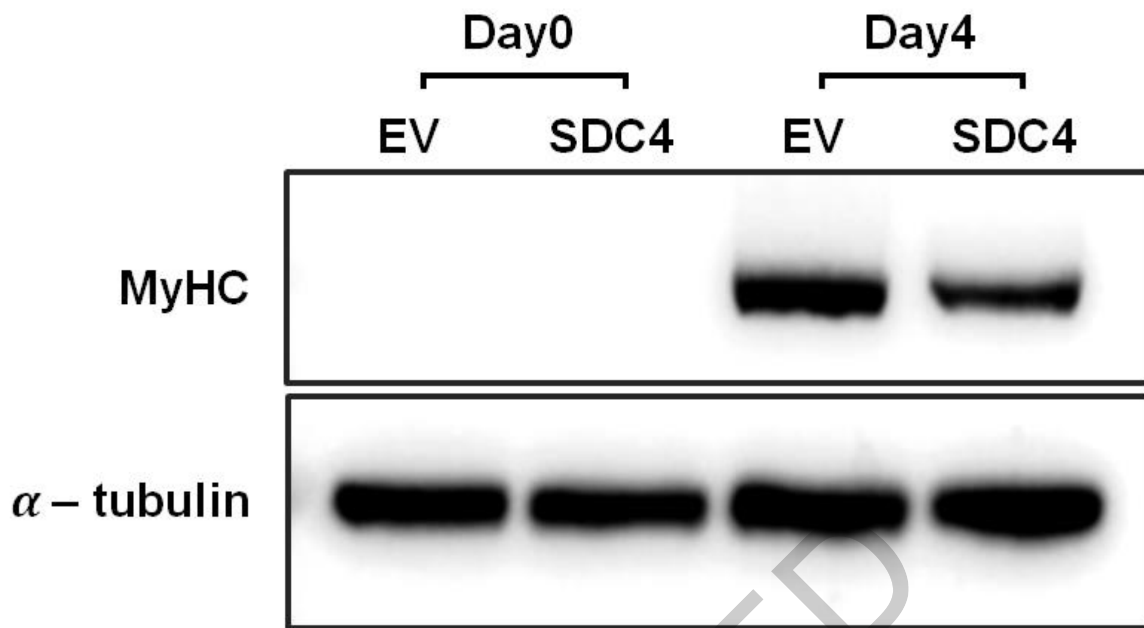

351
352 Figure 3. Quantitative comparison of MyHC protein between control and *SDC4*-OE groups. The difference in
353 MyHC proteins expression was compared between day 0 and day 4. MyHC proteins are not observed before
354 differentiation in both groups. On day 4, On day 4, MyHC proteins were detected, but the level of MyHC
355 protein is lower in the *SDC4*-OE cells than that in the control cells. MyHC, anti-myosin heavy chain; OE,
356 overexpressed; *SDC4*, *syndecan-4*

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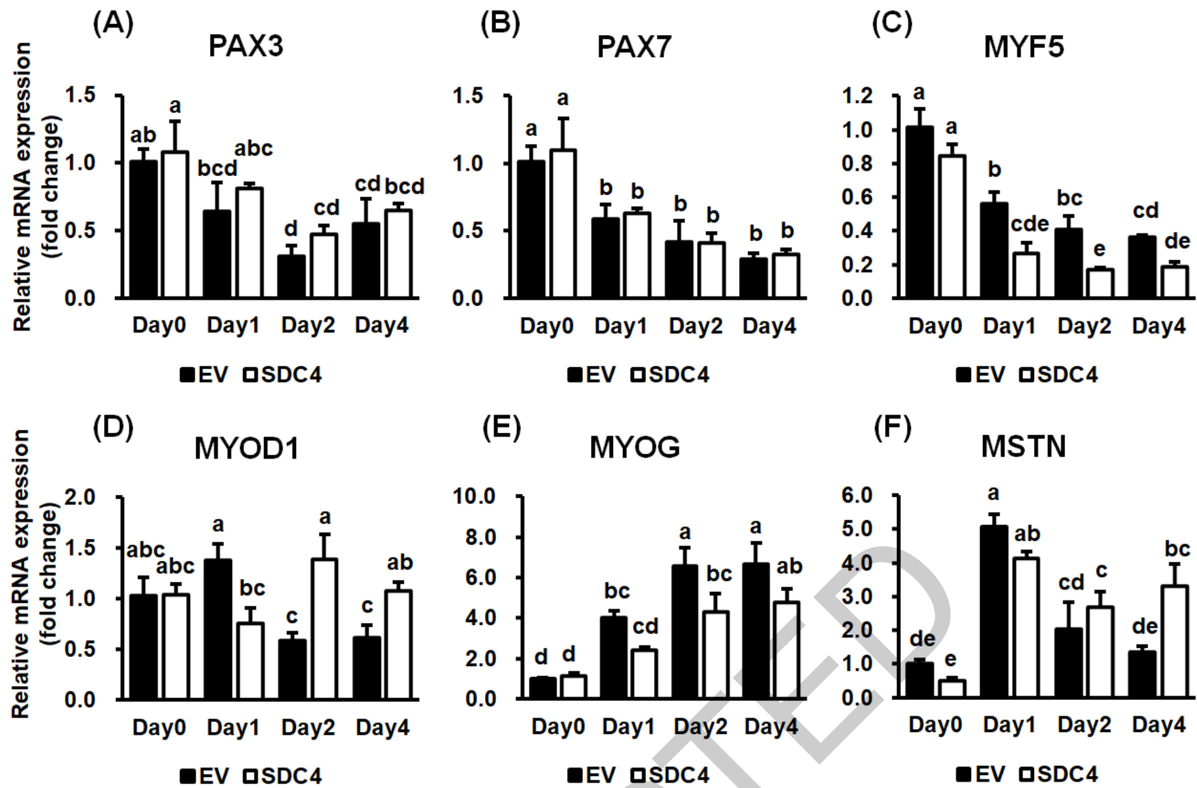

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360 Figure 4. Analysis of expression of genes affecting myogenesis. (A, B) Expression of *PAX* genes show no
361 significant difference between the two groups. (C) *MYF5* expression shows reduction from day 1 to day 4 in
362 *SDC4*-OE cells. The reduction in *MYF5* expression was significant on both day 1 and day 2. (D) *MYOD1*
363 expression is significantly lower on day 1 but significantly higher on days 2 and 4 in *SDC4*-OE cells. (E)
364 *MYOG* expression is low in *SDC4*-OE cells. The reduction in *MYOG* expression was significant on day 2.
365 (F) The *MSTN* expression, decreasing until day 1, increases from day 2 in *SDC4*-OE cells. However, a
366 significant difference is observed on day 4. PAX, paired box; *MYF5, myogenic factor 5; MYOD1, myogenic*
367 *differentiation 1; MYOG, myogenin; MSTN, myostatin; SDC4, syndecan-4*

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Tables

371 Table 1. List of primers designed for quantitative real-time polymerase chain reaction

Primer	Forward (F)	Reverse (R)	Annealing temperature
GAPDH	5'-GAG GGT AGT GAA GGC TGC TG-3'	5'-ACC AGG AAA CAA GCT TGA CG-3'	58.0 ℃
PAX3	5'-AGC AAC TGG AAG AAG AGC TGG AAA G-3'	5'-CTC CTG GGA TCA GGT GGT TAA A-3'	64.0 ℃
PAX7	5'-AGC TGG CAG AGA TGG AGT TG-3'	5'-CTA GTG GTG GTG GTG GCA AA-3'	64.0 ℃
MYF5	5'-AGG AGG CTG AAG AAA GTG AAC C-3'	5'-TAG TTC TTC ACC TGT TCC CTC A-3'	60.0 ℃
MYOD1	5'-CAG CTA CTA CAC AGA ATC ACC AA-3'	5'-TCC CTT CAG CAA CAG CTT CA-3'	63.2 ℃
MYOG	5'-TGC CCA AGG TGG AGA TCC TA-3'	5'-GGG TTG GTG CCA AAC TCC AG-3'	63.2 ℃
MSTN	5'-GGT ATC TGG CAG AGT ATT GAT GTG AA-3'	5’-CAA AAT CTC TGC GGG ACC GT-3’	57.4 ℃

372 GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PAX, paired box; *MYF5, myogenic factor 5; MYOD1*

373 *myogenic differentiation 1; MYOG, myogenin; MSTN, myostatin*

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