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Overexpression of *Syndecan-4* inhibits myogenesis by regulating the expression of myogenic regulatory factors

Sarang Choi[1], Jeong-Woong Park[2], Sang In Lee[1,3], Sangsu Shin[1,3]*

[1]*Department of Animal Science and Biotechnology, Kyungpook National University, Sangju 37224, Korea*
[2]*Center for Industrialization of Agricultural and Livestock Microorganisms, Jeongeup 56212, Korea*
[3]*Research Institute for Innovative Animal Science, Kyungpook National University, Sangju 37224, Korea*

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***Corresponding author**
Sangsu Shin
Department of Animal Science and Biotechnology, Kyungpook National University, Sangju 37224, Korea.
Tel: +82-54-530-1941
E-mail: sss@knu.ac.kr

ORCID
Sarang Choi
https://orcid.org/0000-0002-5488-146X
Jeong-Woong Park
https://orcid.org/0000-0003-0885-3078
Sang In Lee
https://orcid.org/0000-0002-0019-1834
Sangsu Shin
https://orcid.org/0000-0002-5264-9632

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Abstract

Syndecan-4, a type of heparan 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 quail myoblast (QM7) cells, which constituting 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

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]. Quantitative traits such as growth rate and carcass percentage are important economic traits and critical factors to increase poultry meat production [2].

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Availability of data and material
Upon reasonable request, the datasets of this study can be available from the corresponding author.

Authors' contributions
Conceptualization: Shin S.
Data curation: Choi S, Shin S.
Formal analysis: Choi S, Shin S.
Methodology: Choi S, Park JW, Shin S.
Software: Choi S, Park JW.
Validation: Choi S, Lee SI, Shin S.
Investigation: Choi S, Park JW.
Writing - original draft: Choi S, Shin S.
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Ethics approval and consent to participate
This article does not require IRB/IACUC approval because there are no human and animal participants.

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

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

Although previous studies have actively investigated the role of *SDC4* in satellite cells [21,23–25], research regarding its involvement in muscle differentiation is still limited. This study hypothesized that *SDC4* plays a crucial role in muscle growth and development particularly through regulating MRFs. To reveal its effects on quail muscles, either *SDC4* expression vector or an empty vector (EV) was transfected into quail myoblasts (QM7) cells, and the differentiation of the cells was compared by analyzing myotube formation. Subsequently, changes in the expression of MRFs and *MSTN* in the cells overexpressing *SDC4* were analyzed and compared with those factors in the control cells.

MATERIALS AND METHODS

Experimental design

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

SDC4 gene cloning and expression vector construction

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

Cell culture and transfection of cells

QM7 cells (American Type Culture Collection, Manassas, VA, USA) were cultured in medium 199 containing 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA), 10% tryptose phosphate broth (TPB; Sigma-Aldrich, St. Louis, MO, USA), and 1% antibiotic-antimycotic (ABAM; Gibco). The cells were incubated at 37℃ in 5% CO_2 and subcultured before filling in the culture dish to prevent differentiation. To promote cell differentiation, the differentiation medium was prepared by changing the FBS concentration to 0.5%; all remaining compositions and conditions were the same. The cells were seeded at density of 8×10^5 in a culture dish and transfected with EV or *SDC4* expression vector after 18 h using Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocols. For the control, the pcDNA3.1 designated as EV was used. The culture medium was replaced with fresh growth medium at 5 h after transfection. After 16 h, the culture medium was replaced with the differentiation medium. They were differentiated for 4 d, and from day 2, half the culture medium was replaced with fresh differentiation medium every day.

Immunofluorescence staining of cells and myotube area analysis

The cells were washed in phosphate-buffered saline (PBS) and fixed for 15 min using 10% neutral-buffered formalin. Cell membrane permeability was induced using 0.3% NP-40. The non-specific antigens were blocked using PBS mixed with 0.1% Tween-20 (PBST) containing 5% non-fat dry milk. The primary antibody was treated with anti-myosin heavy chain (MF20; Developmental Studies Hybridoma Bank, Iowa City, IA, USA) for 1 h and the secondary antibody with anti-mouse immunoglobulin G (IgG; Santa Cruz Biotechnology, Dallas, TX, USA) conjugated with

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CruzFluor™594. The nuclei were then stained with 4′,6-diamidino-2-phenylindole (DAPI). Photographs were captured using an inverted fluorescent microscope (CKX53; Olympus, Tokyo, Japan). To measure the area of differentiated myotubes, the photographs of immunofluorescence-stained cells were used to measure the length and thickness of myotubes.

Western blotting

The cells were washed with PBS and extracted in 1× lysis buffer. Cellular debris was removed by centrifuging at 14,000×g for 10 min at 4℃. Subsequently, the same amount of 2 × Laemmli buffer, containing 2-mercaptoethanol, as that of the supernatant, excluding the pellets, was added. The protein was denatured at 95℃ for 5 min. Protein concentrations were measured using Coomassie staining, and they were separated by polyacrylamide gel electrophoresis. The protein samples were transferred onto a polyvinylidene fluoride (PVDF) membrane. Subsequently, the non-specific antigens were blocked using 5% non-fat dry milk in Tris-buffered saline mixed with Tween-20 (TBST). After processing the primary antibodies overnight at 4℃, the secondary antibodies were processed at room temperature. During treatment with antibodies, the concentration of skim milk was lowered to 2.5%. After processing the enhanced chemiluminescence (ECL) solution, images were obtained using the ImageQuant LAS 500 imager (GE Healthcare, Chicago, IL, USA).

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

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

Statistical analysis

All data were expressed as the mean ± SEM and analyzed using Student's t-test and two-way analysis of variance (ANOVA) in R packages (R Foundation for Statistical Computing, Vienna, Austria). Multiple groups showing significance in the groups were compared using the least

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 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 TCC 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

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PAX, paired box; MYF5, myogenic factor 5; MYOD1, myogenic differentiation 1; MYOG, myogenin; MSTN, myostatin.

significant difference (LSD) test. The results were considered significant if $p < 0.05$. All experiments were independently conducted in triplicate.

RESULTS AND DISCUSSION

SDC4 overexpression reduces myotube formation in quail muscle cells

An overexpression vector of *SDC4* was designed and transfected into QM7 to verify the effect of *SDC4* on muscle cell differentiation. *SDC4*-OE cells showed lesser muscle cell differentiation than did the EV cells, which were used as the control cells (Fig. 1). Differentiation was observed at four time points: day 0, the start of differentiation; day 1, the change of myogenic gene expression; day 2, the active myotube formation; and day 4, the sufficient myotube formation. Distinctive differences in myotube formation were observed during days 2–4 after differentiation between the groups. On day 2, while the EV cells started forming myotubes, the *SDC4*-OE cells did not. Myotube formation in *SDC4*-OE cells was observed on day 4, and its rate was as low as that observed in the EV cells on day 2. The myotubes in the *SDC4*-OE cells were shorter and thinner than those in the EV cells on day 4. Therefore, *SDC4* is a negative regulator of myotube formation.

To observe and measure the relative area of the myotubes, they were stained with anti-myosin heavy chain (MyHC) antibodies and DAPI on day 4 (Fig. 2). As shown in Fig. 1, the myotubes of the *SDC4*-OE cells were narrower and shorter than those in the control cells (Fig. 2A). Among the stained cells, the number of mononucleated cells was higher in the *SDC4*-OE cells than that of those in the control cells, suggesting that fusion of the myocytes is inhibited by SDC4. The myotube area of *SDC4*-OE cells was reduced by less than half of that of the control cells ($p < 0.001$) (Fig. 2B).

To confirm the degree of differentiation between the groups at a molecular level, western blotting was performed using MyHC, which is the most abundant protein responsible for muscle contraction in the skeletal muscles [26]. MyHC protein was not observed on day 0 in both groups, suggesting that the cells had not started differentiating at that time (Fig. 3). However, MyHC protein was detected on day 4 at different levels in both groups. The amount of MyHC protein

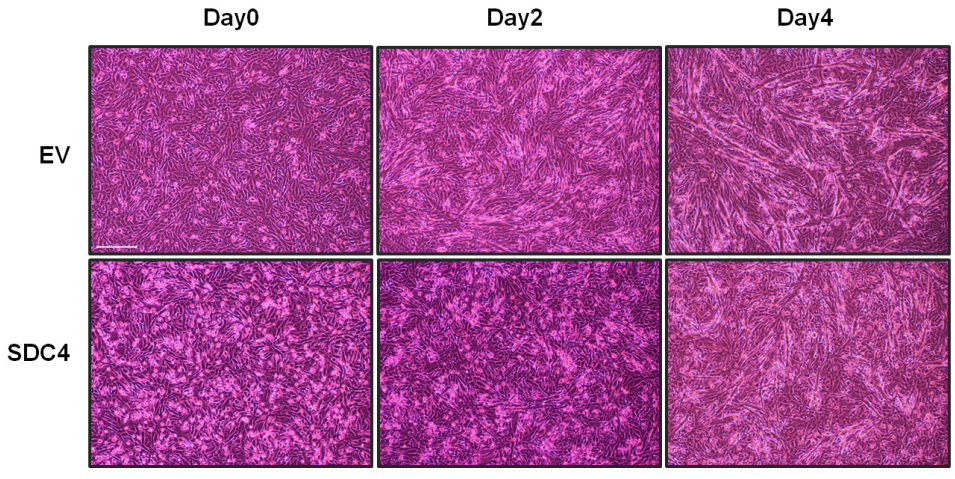

Fig. 1. Effect of transfection of overexpression vector of *SDC4* on differentiation of QM7 cells (magnification, 100×). The differentiation was conducted on day 0, day 2, and day 4. On day 4 of differentiation, *SDC4*-OE cells demonstrate lesser extent of differentiation than do the EV cells. Additionally,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

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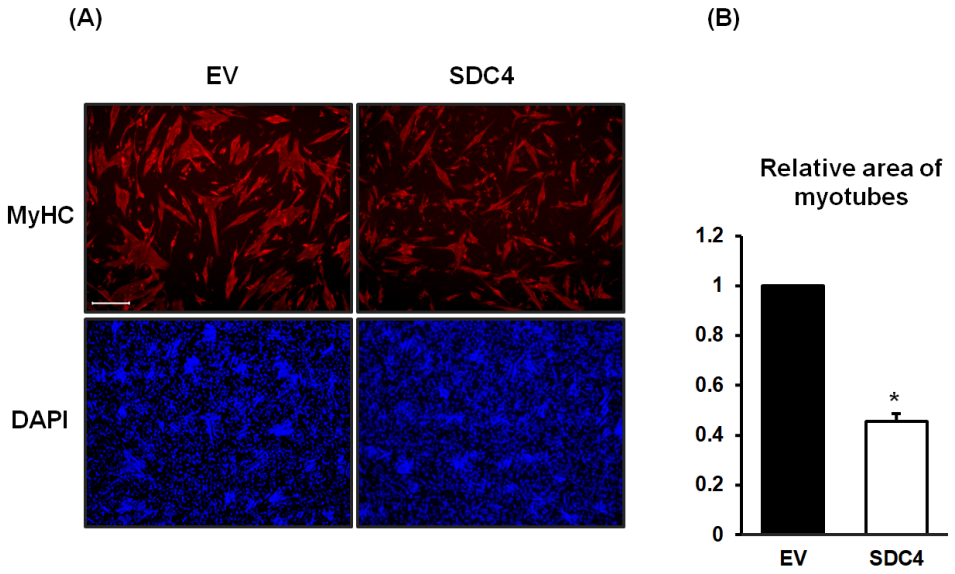

Fig. 2. Myotube staining and measurement of myotube area (magnification, 100×). (A) Myotubes (red) and nuclei (blue) were stained with MyHC antibodies and DAPI, respectively on day 4 of differentiation. The myotubes are shorter and thinner in the *SDC4*-OE cells than those in the control cells. (B) The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

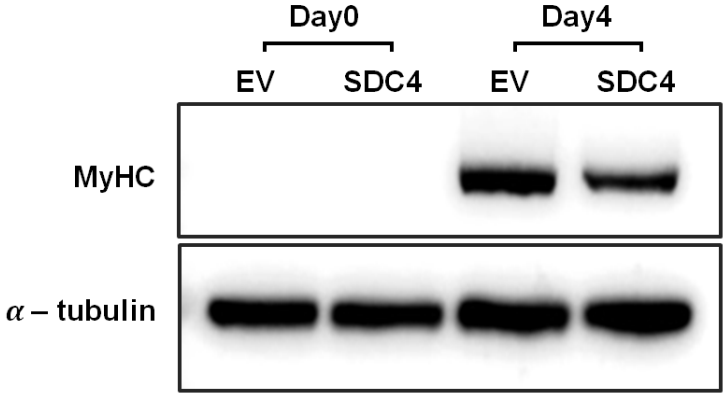

Fig. 3. Quantitative comparison of MyHC protein between control and *SDC4*-OE groups. The difference in MyHC protein expression was compared between day 0 and day 4. MyHC proteins were not observed before differentiation in both groups. On day 4, MyHC proteins were detected, but the level of MyHC protein is lower in the *SDC4*-OE cells than that in the control cells. EV, empty vector; *SDC4*, *Syndecan-4*; MyHC, anti-myosin heavy chain; OE, overexpressed.

in the *SDC4*-OE cells was less than half of that in the control cells. Myotube staining showed similar results, indicating that myotube formation was reduced by *SDC4* overexpression. Similarly, a previous study showed that myotube formation, which is increased by *SDC4* knockout in bovine cells and the cytoplasmic domain of *SDC4*, inhibits myoblast fusion [22]. Szabo et al. reported that *SDC4* expression gradually decreases during muscle differentiation, while high expression of *SDC4* hinders myogenesis [27]. Decreasing *SDC4* expression during myogenesis is crucial for myotube formation. In summary, *SDC4* plays a role in inhibiting myocyte fusion and myotube formation,

which leads to reduced myogenesis.

SDC4 regulates the expression of myogenic regulatory factors during differentiation

The effect of *SDC4* on the expression of genes known to regulate myotube formation was investigated via qRT-PCR (Fig. 4). *PAX3* and *PAX7*, which are expressed in the early stages of muscle development and are involved in the proliferation of myoblasts and initiation of myogenic differentiation [28], showed no differences between the groups. However, they were significantly reduced during differentiation ($p < 0.01$) (Figs. 4A and 4B), indicating that *SDC4* does not affect the initiation of differentiation by regulating *PAX3* and *PAX7* expression.

Subsequently, the expression of MRFs was analyzed. *MYF5* showed a continuous, significant reduction in expression during differentiation in both groups ($p < 0.001$) (Fig. 4C). *MYF5* expression was significantly lesser in the *SDC4*-OE cells than in the control cells on days 1 and 2 ($p < 0.001$). Among the MRFs, *MYF5* is expressed first and initiates differentiation of muscle progenitor cells into myoblasts. It is turned off when *MYOD1* is expressed in the myoblasts [29]. These results suggest that decreased expression of *MYF5* by *SDC4* cannot adequately induce differentiation of progenitor cells and may lead to insufficient expression of *MYOD1*.

The expression of *MYOD1* was significantly affected by the interaction between the day of differentiation and treatment ($p < 0.01$) (Fig. 4D). The expression level of *MYOD1* was the same

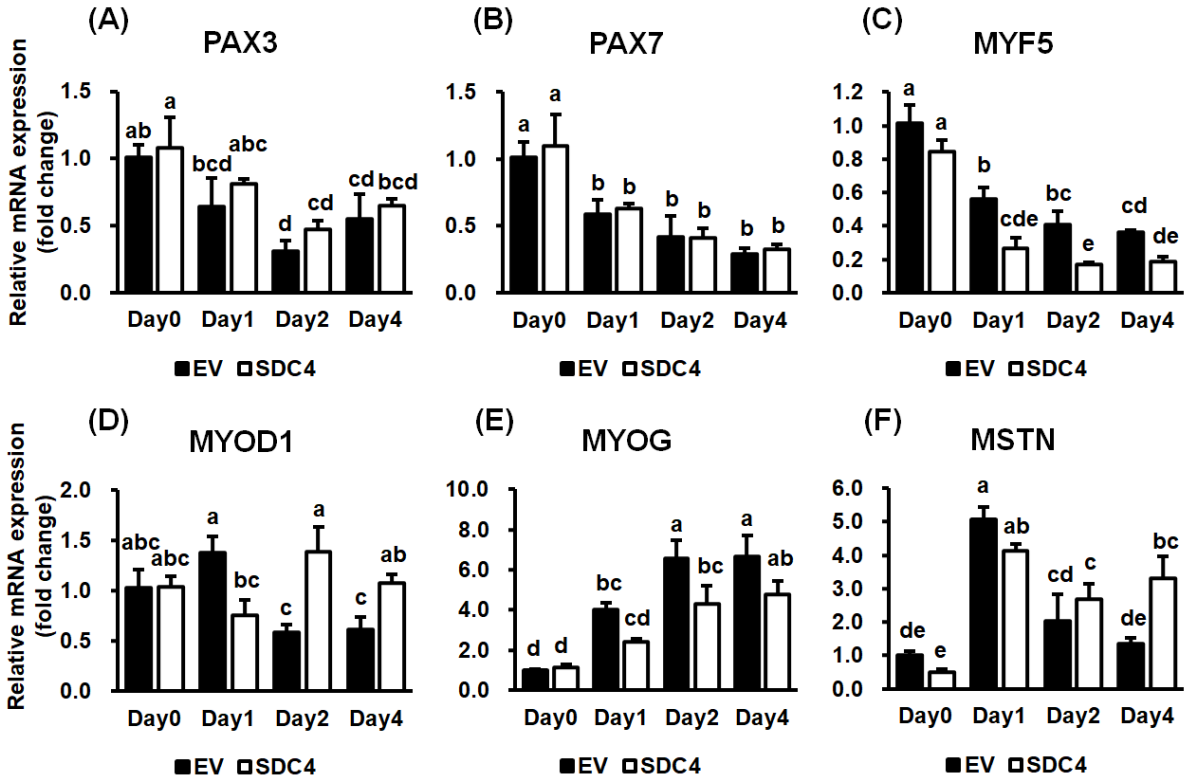

Fig. 4. Analysis of expression of genes affecting myogenesis. (A, B) Expression of *PAX* genes shows no significant difference between the two groups. (C) *MYF5* expression shows reduction from day 1 to day 4 in *SDC4*-OE cells. The reduction in *MYF5* expression was significant on both day 1 and day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

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between the groups before differentiation but differed significantly after initiation of differentiation. In the control group, the expression of *MYOD1* increased on day 1 and then decreased from day 2 onward. In *SDC4*-OE cells, *MYOD1* expression decreased slightly on day 1 and increased from day 2. *MYOD1* expression in the control group was significantly higher than that in the *SDC4*-OE cells on day 1, whereas it was significantly higher in the *SDC4*-OE cells from day 2. *MYOD1* expression is known to increase in highly proliferative myoblasts; it then decreases after inducing the expression of *MYOG* to initiate myotube formation [30,31]. In this study, *SDC4* altered the expression of *MYOD1* in a different pattern, maintaining higher expression in the mid and late stages of differentiation, which resulted in delayed myoblast differentiation and myotube formation.

MYOG expression gradually increased during myogenesis in both groups ($p < 0.001$); however, its expression in the *SDC4-OE* cells was lower than that in the control cells during differentiation ($p < 0.01$) (Fig. 4E). Increased *MYOG* expression is known to enhance myotube formation during differentiation. During differentiation, *MYOG* is upregulated to induce differentiation of myoblasts into myotubes, which is accompanied by the downregulation of *MYOD1* [32,33]. Sustained expression of *MYOD1* during differentiation may aid in proliferation of the myoblasts rather than differentiation in *SDC4*-OE cells. Additionally, the previous study reported that a defect in *MYOG* results in a severe reduction of all skeletal muscles [34]. In this study, the lower expression of *MYOG* due to *SDC4* overexpression may also delay myoblast differentiation. *SDC4* plays a role in recruiting FGF to transmit signals across the cell membrane and acts as a co-receptor for FGF receptor (FGFR). FGF2 is known to reduce the expression of *MYOG*, thus acting as an inhibitor of skeletal muscle differentiation [35]. Hence, decreased *MYOG* expression and myotube formation may be caused by elevated FGF2 signaling owing to *SDC4* overexpression.

Expression pattern analysis of the *MSTN* gene, which does not belong to the MRF family but is closely related to the regulation of muscle development, was conducted [36]. A significant change in *MSTN* expression was observed in both groups depending on the day of differentiation but not on treatment ($p < 0.001$) (Fig. 4F). *MSTN* expression increased dramatically on day 1 and then decreased sharply in the control cells but not in the *SDC4*-OE cells. The interaction between the day of differentiation and treatment had a significant 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 to 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 satellite cells. This finding along with our data shows 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 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 muscle development, disease, and regeneration. Furthermore, it could enhance poultry production, which would be advantageous for poultry breeders. However, 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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