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Original Article

Expression Pattern of Sulf1 and Sulf2 in Chicken Tissues and Characterization of Their Expression During Different Periods in Skeletal Muscle Satellite Cells

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ABSTRACT

Heparan sulfate proteoglycans (HSPGs) are present on the cell surface and in the extracellular matrix in all metazoans. HSPGs interact with growth factors and receptors through heparan sulfate (HS) chains. The sulfation pattern of heparan sulfate chains influences signaling events mediated by heparan sulfate proteoglycans located on the cell surface. *SULF1* and *SULF2* are two endo-sulfatases that can cleave specific 6-O-sulfate groups within the heparan chains. To determine their possible roles in tissues and satellite cells in vitro, their expression pattern was examined in tissues from 40-day-old chickens and in satellite cells from the breast muscles of 1-week-old and 2-week-old chickens using RT-PCR and immunocytochemistry analyses. The *SULF1* and *SULF2* transcripts were widely distributed in various tissues. Upon increasing culture times in chicken´s primary skeletal muscle satellite cells, *SULF1* and *SULF2* expression in 1-week-old chickens was significantly higher than in 2-week-old chickens, suggesting that sulfatases play a key role in satellite cell development. Therefore, our findings increase our knowledge of sulfatase expression diversity and provide a solid basis for further research concerning this molecular mechanism.

INTRODUCTION

Skeletal muscles are derived from mesodermal precursor cells originating from the somites (Buckingham *et al.*, 2003). Somites are divided into a dorsal epithelial dermomyotome and a ventral mesenchymal sclerotome (Bober *et al.*, 1994). Skeletal myogenesis is then initiated in myogenic cells originating from the dermomyotome lips that differentiate to form primary muscle fibers (Asakura *et al.*, 2002). When skeletal muscle is injured due to physical or chemical insult, a pool of self-renewing muscle stem cells residing within the skeletal muscles, called satellite cells, can give rise to differentiated myofibers to repair injured muscle (Charge & Rudinicki, 2004; Gros et al., 2005; Buckingham, 2006).

When activated by injury, satellite cells reenter the cell cycle and proliferate in response to extracellular growth factors (Relaix & Zammit, 2012; Maltzahn *et al.*, 2013; Yin *et al.*, 2013). The proliferation and differentiation of satellite cells are regulated by a number of extracellular signals (Wang & Rudnicki, 2012; Pasut *et al.*, 2013; Günther *et al.*, 2013; Fry *et al.*, 2015). Heparan sulfate (HS) structural analysis demonstrates that *SULF1* and *SULF2* are regulatory HS-modifying enzymes that control HS 6-O-desulfation of activated satellite cells (Houben *et al.*, 2013; Pang *et al.*, 2015).

HS is a linear polysaccharide that takes part in most of the major cellular processes through its ability to bind and modulate a very large array of proteins (Bernfield *et al.*, 1992). During HS biosynthesis in

the Golgi apparatus, this simple polymer of repeated disaccharide units undergoes a series of modifications, including epimerization and sulfation (Bülow & Hobert, 2004). The sulfation pattern in glucosamines and uronic acids is dynamically regulated during many cellular processes, generating diversity of the chains and thus diversity of binding (Arlov *et al.*, 2014; Thacker *et al.*, 2014). By targeting HS functional sulfated domains, Sulfs dramatically alter the ligand binding properties of HS, thereby modulating a broad range of signaling pathways (Pempe *et al.*, 2012). Therefore, HS plays an important role in numerous biochemical and cellular processes, such as the mature lung homeostasis (Perkins, *et al.*, 2018), inflammatory responses (Pomin, 2015), mitogenic signaling (Nieto *et al.*, 2013), the development of fibrosis (Ferreras *et al.*, 2019) and so on.

SULF1 and *SULF2* are two endosulfatases able to cleave specific 6-O-sulfate groups within the heparan chains (Morinoto-Tomita *et al.*, 2002). The two proteins are highly homologous and highly conserved in sequence and domain organization, but they are differentially expressed throughout the body. Some researches demonstrated that the activity of *SULF1* was outweighed by *SULF2* in modification of lung HSPG sulfation (Nagamine *et al.*, 2012). SULFs have demonstrated to regulate the microenvironment of adult stem cells during regeneration (Esko & Selleck, 2002; Langsdorf *et al.*, 2007; Tran *et al.*, 2012). Furthermore, SULFs can also promote myoblast fusion during skeletal muscle regeneration (Huntington, 2005). However, very little is known about SULF genes expressed in chicken muscle tissues and satellite cells.

In this study, we examined the expression profiles of sulfatases in chicken, specifically, 12 tissue types from 40-day-old chickens were examined. We isolated satellite cells from the breast muscle of 1- to 2-week-old chickens and cultured the cells for 96 h. After being purified from the primary satellite cells that were cultured in vitro for 48 h, a desmin protein that was specific to these cells was identified via the analysis of the immunocytochemistry of satellite cells. *SULF1* and *SULF2* mRNA expression were examined in relation to different culture times.

MATERIAL AND METHODS

All experimental procedures were conducted in conformity with the institutional guidelines for the care and use of experimental animals in the Sichuan Agricultural University, permit number 2014-18.

Animals and sample collection

In this study, a total of twenty 1-day-old healthy Avian chickens (Wang, 2009) were randomly selected as test samples (purchased from Wenjiang Zhengda Co. Ltd , China). These chickens were maintained under natural conditions of light and temperature at the Experimental Poultry Breeding Farm of Sichuan Agriculture University (Sichuan, Ya'an, China). Birds were provided with free access to feed and water. At 40 days of age (n=4), 12 kinds of tissues including heart, liver, leg muscle, pectoralis muscle, lung, cecum, testicle, brain, spleen, kidney, muscular stomach and abdominal fat tissues were immediately collected after slaughter. Tissue samples were frozen in liquid nitrogen and then stored at -80 °C for total RNA extraction.

Isolation of satellite cells

Satellite cells (SCs) were isolated from breast muscle of 1- to 2-week-old chickens with the following modifications. Briefly, pieces of breast muscles that were approximately 1 mm^3 in size were minced with scissors and digested in 5 ml 0.1% collagenase I (Sigma, USA) and in phosphate buffered saline (PBS) at 37 °C for 30 min, which was followed by a second digest in 5 ml 0.25% trypsin (HyClone, USA) at 37 °C for 30 min. After digestion, the cellular supernatant was sequentially filtered through sieves of 200 μm and 500 μm and was then collected by centrifugation for 10 min at 2,000 r/min. Satellite cells were cultured on collagen-coated tissue culture dishes in satellite cell growth medium [DMEM/F12, 15% FBS (Gibco, USA), 15% horse serum (HyClone, USA), 3% chick embryo extraction and 1% penicillin/streptomycin (Solarbio, Beijing, China)].

Immunocytochemistry

To determine whether the isolated cells were muscle satellite cells, immunocytochemistry identification with specific antibodies was performed. Briefly, when the cells proliferated to 70%-80% confluence, they were washed three times with PBS, fixed in 4% paraformaldehyde for 20 min at room temperature, and then washed three times with cold PBS (5 min per wash). Cells were permeabilized with 0.5% Triton X-100 (Biotopped, Shanghai) for 15 min at room temperature. For the blocking step, 5% bovine serum albumin (BSA) was used for blocking liquid (Beyotime, Shanghai), and then the cells were incubated at room temperature for 40 min. The primary antibody, mouse anti-chicken desmin (1:30 dilution, Abcam, USA), was added (diluted in PBS, 0.2%) at 4°C overnight.

Then, PBS was added to wash the samples, and the secondary antibody goat anti-mouse IgG-Alexa Fluor 488 (1:250 dilutions, Beyotime, Shanghai) was added and incubated in the dark at 37 °C for 2 h. The cells were also counterstained with 1 μg/ml 4',6-diamidino-2-phenylindole (DAPI) (Beyotime, Shanghai) for 15 min and washed three times with PBS. Images were captured with a fluorescence microscope (Ecliope E400, Nikon, Japan).

Quantitative real-time RT-PCR

Total RNA was isolated from the tissues by using the RNAiso Plus reagent (Takara, Dalian, China), and concentration and purity was assessed by a NanoVue Plus™ spectrophotometer (Thermo Scientific, Wilmington, DE, USA). cDNA was synthesized using the PrimeScript™ RT Reagent Kit with gDNA Eraser (Takara, Dalian, China) according to the manufacturer's instructions. RT-PCR was performed using gene-specific primers (Table 1) that were designed based on the mRNA sequences of the chicken *GAPDH* gene (No: NM-204305.1), SULF1 gene (No: XM-004939905.1) and SULF2 gene (No: XM-417386.4).

qPCR was performed using the CFX96-Touch TM machine (Bio-Rad, USA). A single PCR reaction of 11 μl contained 6 μl of SYBR Premix Ex Taq II, 0.5 μl each of forward and reverse primers (10mM), 1 μl of cDNA template and 3 μl of ddH_2O. PCR amplifications were carried out at 95 °C for 3 min, which was followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 40 s. A final extension step was then carried out for 3 min at 72 °C. To verify that there was no non-specific amplification, following the completion of the qPCR, a melting curve analysis was performed. Amplifications were performed in replicate for each gene.

Statistical analysis

The relative expression levels were calculated by the comparative Ct ($2^{-\Delta\Delta Ct}$) method using the glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) gene as an endogenous reference gene. Gene expression levels were quantified relatively to the expression of the GAPDH according to the formula as followed (Livak & Schnittgen, 2001). The statistical analysis (one-way ANOVA) was performed using SAS 9.0. The means ± SEM results were plotted using GraphPad Prism 5 software (GraphPad Software, La Jolla, CA, USA). The comparisons were considered significant at $p<0.05$ and extremely significant at $p<0.01$.

RESULTS

Standard curves and melting curves of the SULF1, SULF2 and GAPDH genes

Serial dilutions (10^{-3}-10^{-8}) of the PCR products for the *SULF1*, *SULF2* and *GAPDH* genes in the breast muscle tissue were tested by RT-PCR. The crossing point, where the sample's fluorescence curve turns sharply upward, indicating exponential amplification, was automatically determined by the qPCR software as 5.57-24.55 for *SULF1*and 7.61-26.22 for *SULF2*, and the range of the Ct values for the *GAPDH* gene was 5.31-21.17. Plotting the obtained Ct values relative to the serial dilutions of *SULF1*, *SULF2* and *GAPDH* resulted in a linear correlation with square regression coefficients of 0.998, 0.998 and 0.999, respectively, suggesting that quantification of the target DNA was possible. The average slopes of the *SULF1*, *SULF2* and *GAPDH* genes were 3.829, 3.731 and 3.940. According to the formula log E=slope^{-1}, the current PCR reaction efficiencies are above 105.4% for the *SULF1* gene, 105.1% for the *SULF2* gene and 103.2% for the *GAPDH* gene.

Expression of the SULF1 and SULF2 mRNA in different chicken tissues

The expression of *SULF1* and *SULF2* mRNAs was detected in the 40-day-old chicken tissues analyzed in this study. Relative to the *GAPDH* gene, the expression levels of the *SULF1* (Fig. 1) and *SULF2* (Fig. 2) mRNAs varied considerably in different tissues. Compared with the expression pattern of the *SULF1* mRNA in other tissues, the *SULF1* transcript had a relatively higher expression in spleen, lung, brain, stomach muscle, and abdominal fat tissues and a relatively lower expression

Table 1 – Primer pairs for the quantification of *GAPDH*, *SULF1* and *SULF2* mRNAs.

Primer name	Primer sequence (5'⟶3')	Annealing temperature (° C)	Product length (bp)
SULF1-F	CATCCTTCATCAATGCCTTCG	60	128
SULF1-R	CCAGGAGGGAGAAGAGCAGTT		
SULF2-F	CGCTCTACCCGCTCTGTATCTG	60	115
SULF2-R	TCTGCATCTTGTGCCGCTTG		
GAPDH-F	AGGACCAGGTTGTCTCCTGT	60	153
GAPDH-R	CCATCAAGTCCACAACACGG		

in the liver, kidney, leg muscle, and pectoralis muscle tissues. The *SULF2* transcript had a higher relative expression in the lung, spleen, and abdominal fat tissues and a lower relative expression in leg muscle and pectoralis muscle tissues.

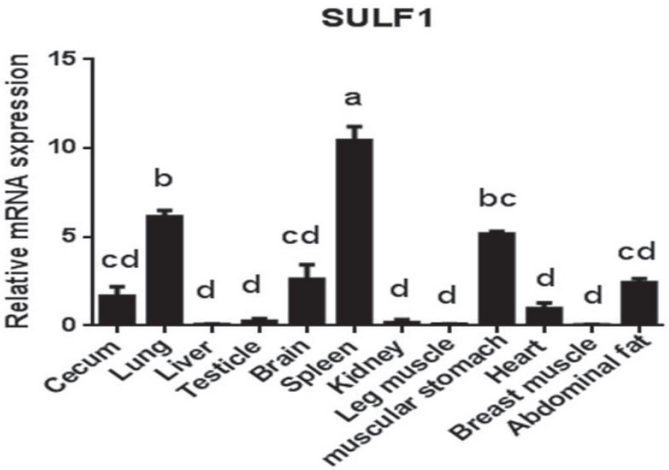

Figure 1 – The expression level of *SULF1* relative to *GAPDH* in different tissues of 40-day-old chickens. The relative levels of expression for *SULF1* were calculated relative to *GAPDH* using the $2^{-\Delta\Delta Ct}$ method. Values are mean±SEM, n=4. The significance of differences in the levels of expression of *SULF1* mRNA was determined by ANOVA. Means with the same letter are not significantly different (p<0.05)

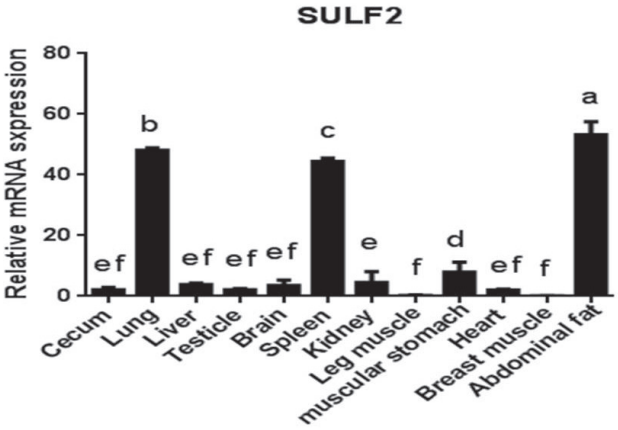

Figure 2 – The expression level of *SULF2* relative to *GAPDH* in different tissues of 40-day-old chickens. The relative levels of expression for *SULF2* were calculated relative to *GAPDH* using the $2^{-\Delta\Delta Ct}$ method. Values are mean±SEM, n=4. The significance of differences in the levels of expression of *SULF2* mRNA was determined by ANOVA. Means with the same letter are not significantly different (p<0.05)

Comparison between the gene expression patterns of SULF1 and SULF2

To further characterize the expression of *SULF1* and *SULF2*, we analyzed the expression level of these two genes in different tissues. Fig. 3 showed that there was no significant difference in the *SULF1* and *SULF2* mRNAs in cecum, liver, testicle, brain, kidney, leg muscle, stomach muscle, heart or pectoralis muscle tissues (p>0.05). However, we found that the *SULF2* mRNA levels in lung, spleen and abdominal fat tissues were much higher than *SULF1* mRNA levels in those tissues.

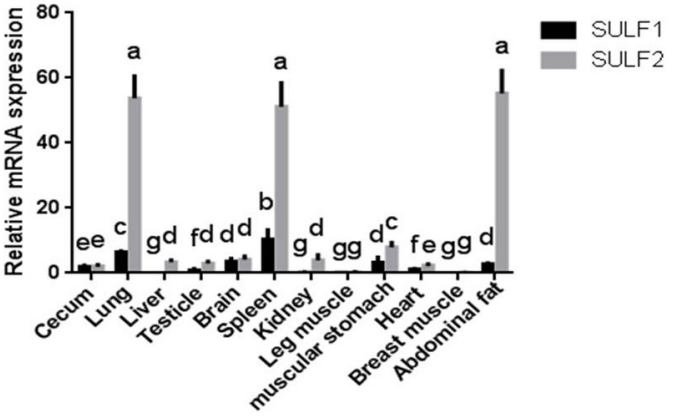

Figure 3 – The expression level of *SULF1* and *SULF2* relative to *GAPDH* in different tissues of 40-day-old chickens. The relative levels of expression for *SULF1* and *SULF2* were calculated relative to *GAPDH* using the $2^{-\Delta\Delta Ct}$ method. Values are mean±SEM, n=4. The significance of differences in the levels of expression of *SULF1* and *SULF2* mRNA was determined by ANOVA. Means with the same letter are not significantly different (p<0.05)

Characterization and identification of chicken skeletal muscle satellite cells

Chicken primary skeletal muscle satellite cells isolated form pectoralis muscle demonstrated a typical morphology (circular) after 0 h, growing as an even layer of single cells (Fig. 4A). After 24 h, the cells grew denser, aligned with each other, and changed into fusiform cells (Fig. 4B). When the cell density reached 70%-80% confluence, the cells were in a regular parallel arrangement (Fig. 4C). Desmin is the specific marker of skeletal muscle satellite cells. Thus, we examined the expression of this specific marker in chicken skeletal SCs using a confocal fluorescence microscope. We found that 95% of cells were positive for the expression of desmin, which suggests that more than 95% of the cells were muscle satellite cells (Fig. 4D-F).

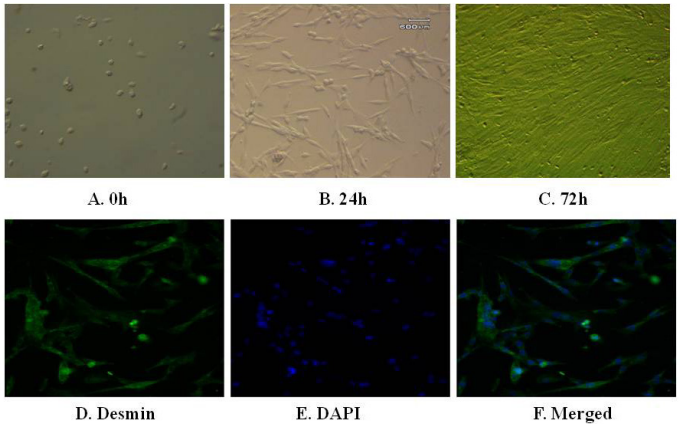

Figure 4 – Morphology of cultured skeletal muscle satellite cells and identification of satellite cell-specific marker by immunofluorescence in chicken. A. Satellite cells were round before adhering; B: Adherent cells were spindle-shaped; C: With an increase in cell density, the cells became regularly arranged in parallel; D. Skeletal muscle satellite cells expressed Desmin in the cytoplasm; E. DAPI staining of the nuclei of skeletal muscle satellite cells; F. The merged image of A and B.

Expression of Sulfatases in chicken muscle satellite cells

To determine if sulfatases are involved in chicken muscle cell development, the expression levels of *SULF1* and *SULF2* at different culture time points in skeletal muscle cells of 1-week-old and 2-week-old chickens were determined by qRT-PCR. As shown in Fig. 5, in 1-week-old chicken SCs, the expression of *SULF1* presented a unimodal distribution pattern with increasing culture time, with a peak at 96 h. Significant differences were observed at various time points (p<0.05). However, in 2-week-old chicken SCs, *SULF1* mRNA expression exhibited a "decline-rise" developmental change, and its expression at 48 h was significantly lower than at other times (p<0.05). Different from the pattern observed for *SULF1*, the *SULF2* expression level in 1-week-old chicken SCs at 48 h was low, then increased to a peak at 72 h and declined at 96 h. Contrary to *SULF1* gene expression, in 2-week-old chicken SCs, the *SULF2* expression level exhibited a "rise-decline" developmental change, but there were no significant differences among time points (p>0.05).

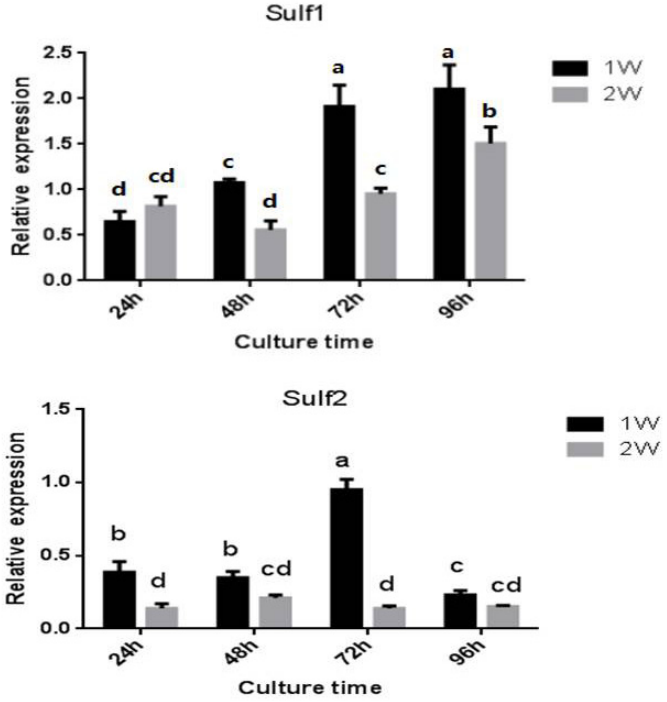

Figure 5 – Relative expression of *SULF1* and *SULF2* mRNA of primary skeletal muscle satellite cells isolated from the 1-week-old and 2-week old broilers during in vitro culture. The expression levels calculated by the relative standard curve method are presented in arbitrary units (AU). Values are presented as the mean ± SEM. The significance levels of the differences in the levels of expression of *SULF1* and *SULF2* mRNA were determined by ANOVA. Means with the same letter are not significantly different (p<0.05).

DISCUSSION

In this study, *SULF1* mRNA had a relatively higher expression in spleen, lung, brain, stomach muscle, and abdominal fat tissues and a relatively lower expression in liver, kidney, leg muscle, and pectoralis muscle tissues. Previous studies showed that the expression of *SULF1* mRNA can be detected in several normal human tissues. In a panel of 24 tissue types, the highest levels were found in testes, stomach, skeletal muscle, lung, and kidney tissues (Morimoto-Tomita *et al.*, 2002). *SULF2* transcripts had higher relative expression in lung, spleen, and abdominal fat, a pattern coincident with previous studies in mouse (Lum *et al.*, 2007). Furthermore, *SULF2* transcripts had a lower relative expression in leg muscle and pectoralis muscle. In murine models, simultaneous disruption of both *SULF1* and *SULF2* leads to perinatal lethality and developmental defects, suggesting overlapping and essential roles of these genes during development (Holst *et al.*, 2007). *SULF1* and *SULF2* mRNAs were shown to be expressed at high levels in regions of developing cartilage and bone (Zaman *et al.*, 2016). Therefore, we cannot eliminate the possibility that *SULF1* and *SULF2* have different expression patterns in species-, gender- or temporal-specific profiles in different tissues.

Previous studies showed that mouse SULFs selectively regulate HS-dependent growth factor-mediated repression of myogenic differentiation during muscle regeneration (Langsdorf *et al.*, 2007). SULFs promote canonical Wnt signaling to antagonize noncanonical signaling, thereby enhancing myoblast fusion (Tran *et al.*, 2012). However, satellite cells are essential for skeletal muscle regeneration (Relaix & Zammit, 2012). To determine if sulfatases are involved in chicken muscle cell development, the expression levels of *SULF1* and *SULF2* at different culture time points in skeletal muscle satellite cells of 1-week-old and 2-week-old chickens were determined by qRT-PCR. *SULF1* mRNA expression gradually increased as culture times increased, and the *SULF1* transcript had a relatively higher expression in 1-week-old cells. *SULF2* mRNA expression also increased with culture time, it increased in 1-week-old cells, but did not increase significantly in 2-week-old cells. *SULF2* mRNA also had a relatively higher expression in 1-week-old cells. The results of the *SULF1* and *SULF2* expression at different culture time points in skeletal muscle satellite cells suggest that sulfatases play a key role in chicken satellite cell development.

CONCLUSIONS

In brief, we detected the expression profiles of sulfatases in chicken tissues. *SULF1* and *SULF2*

transcripts were widely distributed in various tissues. In avian broiler primary skeletal muscle satellite cells, *SULF1* and *SULF2* gene expression gradually increased with increasing culture duration, and *SULF1* and *SULF2* expression levels in 1-week-old cells were significantly higher than in 2-week-old cells, suggesting that sulfatases play a key role in chicken satellite cell development. Therefore, our findings increase our knowledge of sulfatase expression diversity and provide a solid basis for further molecular mechanism research.

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CONFLICTS OF INTEREST

The authors declare that they have no competing interests.

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