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

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 & Rudnicki, 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, Sults 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 endo-sulfatases 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 expressed in chicken muscle tissues and satellite cells. We isolated 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 mm³ 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 ºC for 30 min, which was followed by a second digest in 5 ml 0.25% trypsin (HyClone, USA) at 37 ºC for 30 min. After digestion, the cellular supernatant was sequentially filtered through sieves of 200 μm 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.

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 ºC 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 mm³ 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 ºC for 30 min, which was followed by a second digest in 5 ml 0.25% trypsin (HyClone, USA) at 37 ºC for 30 min. After digestion, the cellular supernatant was sequentially filtered through sieves of 200 μm and then stored at -80 ºC for total RNA extraction.

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.

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.
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RESULTS

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

Serial dilutions ($10^{-2}$-$10^{-8}$) 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, 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 in different chicken tissues.
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.

**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.
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](image)

**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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