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Expression analysis of *Gli1* and *Gli2* in different tissues and muscle-derived cells of Qinchuan cattle

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ABSTRACT. The Hedgehog (Hh) signaling pathway regulates the differentiation of many kinds of cells and plays a critical role in many embryonic and postnatal developmental processes. Gli1 and Gli2 are two transcription factors of the Hh signaling pathway. In this study, we used quantitative real-time polymerase chain reaction to detect the relative expression of *Gli1* and *Gli2* in 13 tissues from three two-year-old purebred Qinchuan cattle, as well as in different cell populations derived from muscle and different stages of myogenic differentiation of myoblasts. The expression levels of *Gli1* and *Gli2* in muscle were the lowest of the 13 tissues ($P < 0.05$), and they declined predominantly from preplate (pp)1 to pp6 cells. However, the expression of *Gli2* was elevated during myogenic differentiation until the 6th day. We speculated that Hh signaling was negatively activated in myocytes and quiescent myoblasts. The increased expression of *Gli1* and *Gli2* in the early days of myogenic differentiation suggested that Hh signaling would be activated when the quiescent bovine myoblast was stimulated

to initiate myogenic differentiation.

Key words: Hedgehog signaling pathway; Myogenic differentiation; Myoblast; Quantitative real-time polymerase chain reaction

INTRODUCTION

The Hedgehog (Hh) signaling pathway has been thought to be critical to many developmental and growth processes (Varjosalo and Taipale, 2008; Schaefer et al., 2013). Aberrant Hh signaling can cause several kinds of cancer and congenital defects (Fontaine et al., 2008). The ligands of the Hh signaling pathway are secreted proteins, which usually act as morphogens that signal target cells according to a concentration gradient to influence their fate in the development of many parts of the body (Ingham and McMahon, 2001; Hooper and Scott, 2005). Sonic hedgehog (Shh), Indian hedgehog (Ihh), and Desert hedgehog (Dhh) are three Hh proteins that have been identified in mammals.

When the ligand Hh is absent, Patched1 (Ptch1) represses Smoothened (Smo) activity, and the transcription factor Gli enters the nucleus as the repressor form (Gli-R) and blocks the transcription of target genes (Milla et al., 2012). The binding of Hh to Ptch1 can remove the inhibition of Smo. Activated Smo enables the release of Gli from a repressor complex. The activated form of Gli (Gli-A) binds to the regulation region and causes the expression of the target gene. The relative ratio of Gli-A to Gli-R is thought to be crucial for transducing the extracellular Hh signal and for determining concentration-dependent cell fates (Persson et al., 2002; Bai et al., 2004).

Many studies support the hypothesis that activating the Hh signaling pathway by an agonist or overexpressing its positive regulator promoted the differentiation of mesenchymal stem cells (MSCs) into chondrocytes (Warzecha et al., 2006; Li et al., 2012; Steinert et al., 2012) and osteoblasts (Dwyer et al., 2007; Ho et al., 2007; James et al., 2010; Cai et al., 2012a,b; Oliveira et al., 2012), but it inhibited adipocyte differentiation (Fontaine et al., 2008; Cai and Deng, 2010; James et al., 2010). Additionally, the repression of the Hh signaling pathway induced by cyclopamine or a small interfering RNA (siRNA) of its positive component inhibited chondrogenic differentiation (Wu et al., 2013). The Hh signaling pathway promotes myogenesis in zebrafish (Flynt et al., 2007; Lobbardi et al., 2011), *Xenopus* (Martin et al., 2007), mouse (Straface et al., 2009; Hu et al., 2012; Voronova et al., 2013), and chick (Elia et al., 2007).

Although the Hh signaling pathway has been studied in multiple tissues and cells, the relative expression levels in different tissues in many species are still unclear. Cattle are the main type of livestock in the world, and their muscles are an important food for humans. This study aimed to determine the relative expression levels of the two transcription factors Gli1 and Gli2 and their expression patterns in different populations of cells derived from muscle and in different stages of myoblast differentiation.

MATERIAL AND METHODS

Sample collection

Samples of 13 tissues including heart, liver, spleen, lung, kidney, rumen, reticulum, omasum, abomasum, small intestine, large intestine, muscle, and abdominal fat were obtained

from three two-year-old purebred Qinchuan cattle (Experimental Farm of National Beef Cattle Improvement Center, Yangling, Shaanxi, China) after slaughter. All samples for RNA extraction were promptly frozen in liquid nitrogen and stored at -80°C after being washed three times in phosphate-buffered saline (PBS).

Preparation of different populations of muscle-derived cell cultures

The mass of fresh longissimus muscle was taken to laboratory from the butchery within 2 h in sterile PBS containing 2% penicillin-streptomycin (Gibco, United States). Muscles were minced into a coarse slurry with a fine scissors, enzymatically dissociated at 37°C in 0.2% collagenase type I (Sigma-Aldrich, United States) for 3 h, and centrifuged at 1500 rpm for 5 min. The supernatant was removed, and the cell pellet was resuspended and washed with Dulbecco's modified Eagle medium (DMEM) (Gibco) containing 10% fetal bovine serum (FBS) (PAA, Germany) and 2% penicillin-streptomycin (Gibco). Resuspended cells were filtered with a 40-μm cell mesh. In order to isolate the different populations of muscle-derived cells, we adopted the method from Qu-Petersen et al. (2002). The filtered muscle cells were centrifuged, resuspended in proliferation medium [PM: DMEM + 20% FBS + 10% horse serum (HS) (Gibco)] containing 1% penicillin-streptomycin, and plated on collagen-coated dishes (collagen type I; Sigma-Aldrich) for 2 h (preplate 1, pp1). The nonadherent cells were then transferred to other dishes (pp2). After 24 h, the suspended cells in pp2 were transferred to new dishes (pp3) to incubate for another 24 h. Then, the suspension was centrifuged, resuspended in fresh medium, and plated on new dishes (pp4). Pp5 and pp6 were obtained in the same way. All dishes were replaced with fresh medium every 48 h, and cells were cryopreserved or collected for RNA extraction when the cells reached 70% confluence.

Differentiation of myoblasts

The pp6 cells were digested with 0.05% trypsin-ethylenediaminetetraacetic acid (Gibco), and centrifuged at 1000 rpm for 5 min. The cells were resuspended in PM and then seeded into Petri dishes. When the cells reached 70% confluence, one of the dishes of cells was collected as the 0 day differentiation sample. At the same time, the medium was changed to differentiation medium (DM: DMEM + 2% HS). Cells from one of the dishes was collected for RNA extraction, and the medium was changed in the other dishes on the 3rd, 6th, 9th, 12th, 15th, and 18th days.

RNA extraction

Total RNA was extracted from tissue and adhered cell samples using Trizol reagent (MRC, USA) or E.Z.N.Z.™ Total RNA Kit (Omega, United States). The RNA samples were digested by DNase I for 30 min to remove the genomic DNA before being reverse transcribed via PrimeScript™ RT Master Mix (Takara, Japan).

Quantitative real-time polymerase chain reaction (PCR)

Quantitative real-time PCR was carried out on the ABI 7500 RT-PCR system (Applied Biosystems, United States). Each 20-μL reaction included 10 μL SYBR® Premix Ex Taq™ II (Takara), 0.8 μL 10 μM PCR forward primer, 0.8 μL 10 μM PCR reverse primer, 0.4 μL ROX

Reference Dye (Takara), and 2 μL template cDNA. The Bovine *GAPDH* (NM_001034034) gene was adopted as an endogenous control. Primers for the *GAPDH* gene and the genes involved in the Hh signaling pathway are listed in Table 1. The PCR protocol was as follows: initial denaturation step at 95°C for 30 s, 40 cycles of denaturation at 95°C for 5 s and extension at 60°C for 34 s to amplify products, and a dissociation stage of 95°C for 15 s, 60°C for 1 min, and 95°C for 15 s to obtain the melting curve. All conditions were performed in triplicate based on a standard curve method. The $2^{-\Delta\Delta Ct}$ method was applied to calculate fold changes of the expression levels of the genes between different tissue samples.

Table 1. Primers used for quantitative real-time polymerase chain reaction.

Gene	Primer (5'→3')
GAPDH	Forward primer: TTCAACGGCACAGTCAAGG Reverse primer: ACATACTCAGCACCAGCATCAC
Gli1	Forward primer: TGCACATGCGCAGACACAC Reverse primer: TTACTGCAGCCCTCATGCTCAC
Gli2	Forward primer: GCAGCAGCAACTGTCTGAGTGA Reverse primer: GACCTTGCTGCGCTTGTGA

Statistical analysis

Statistical analysis was performed using the SPSS 18.0 software. Statistical significance was determined using one-way analysis of variance, and $P < 0.05$ was considered to be statistically significant.

RESULTS

Tissue-specific expression profile of *Gli1* and *Gli2*

Of the 13 examined tissues, *Gli1* expression was the highest in abomasum (Figure 1A). Additionally, liver, lung, and small intestine also had high expression of *Gli1* (Figure 1A). In contrast, the *Gli2* expression level was high in abdominal fat and intestine (Figure 1B). No significant differences were observed between *Gli1* and *Gli2* expression in heart, kidney, rumen, reticulum, and omasum ($P > 0.05$) (Figure 1). In the four stomachs, there was no significant difference in *Gli2* expression ($P > 0.05$) (Figure 1B), but *Gli1* transcript levels were much higher in the abomasum than in the other stomachs ($P < 0.05$) (Figure 1A). Interestingly, the expression levels of *Gli1* and *Gli2* were the lowest in the muscle of the 13 examined tissues (Figure 1).

Expression of *Gli1* and *Gli2* in different populations of muscle-derived cells

Most of the cells in pp1 to pp6 were spindle shaped (Figure 2A-F), but the relative expression of *Gli1* and *Gli2* varied distinctively (Figure 2G-H). The relative expression of *Gli1* showed a decreasing trend from pp1 to pp6 (Figure 2G). The *Gli1* expression was lower in muscle tissue than in all muscle-derived cell populations ($P < 0.05$) (Figure 2G). Except pp2 and pp4 having higher *Gli2* expression than pp1 ($P < 0.05$) and pp3 ($P < 0.05$), respectively, the expression of *Gli2* decreased from pp1 to pp6, and the expression of *Gli2* was lower in muscle than in other cell populations ($P < 0.05$) (Figure 2H).

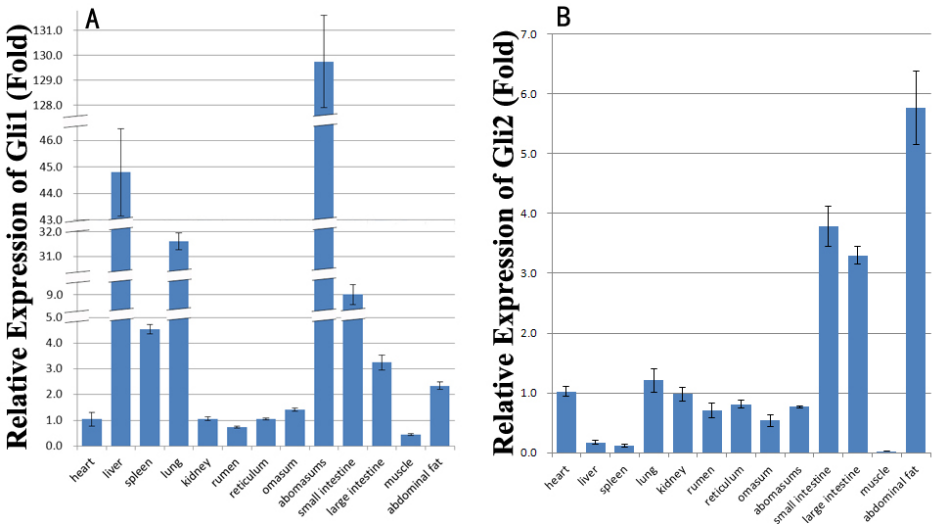

Figure 1. Tissue-specific expression profiles of *Gli1* (**A**) and *Gli2* (**B**).

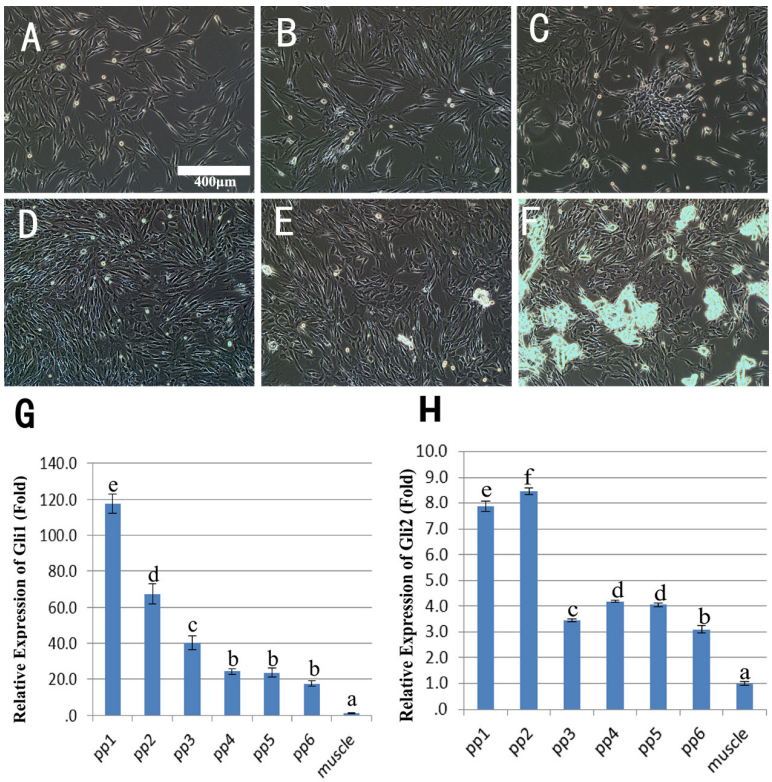

Figure 2. Different populations of muscle-derived cells and the relative expression levels of *Gli1* and *Gli2*. **A.** preplate (pp)1; **B.** pp2; **C.** pp3; **D.** pp4; **E.** pp5; **F.** pp6; **G.** and **H.** Relative expression of *Gli1* and *Gli2* in different cell populations and muscle tissues. Different letters at the top of the columns indicate significant differences ($P < 0.05$).

Expression of *Gli1* and *Gli2* during the myogenic differentiation of myoblasts

Except on the 9th day of induced myogenic differentiation, the expression of *Gli1* was lower in myoblasts than in muscle ($P < 0.05$) (Figure 3A). During the process of myogenic differentiation, the expression of *Gli1* fluctuated but was still elevated on the 3rd and 9th days and peaked on the 9th day (Figure 3A). *Gli2* expression increased from the beginning to the 6th day of induced differentiation, and it declined from the 6th day to the 18th day except the 15th day (Figure 3B). The expression of *Gli2* was much lower in muscle tissue than in all stages of myogenic differentiation of myoblasts ($P < 0.05$), but the expression of *Gli1* was relatively high in muscle tissue compared to its expression in myoblasts during the differentiation process (Figure 3).

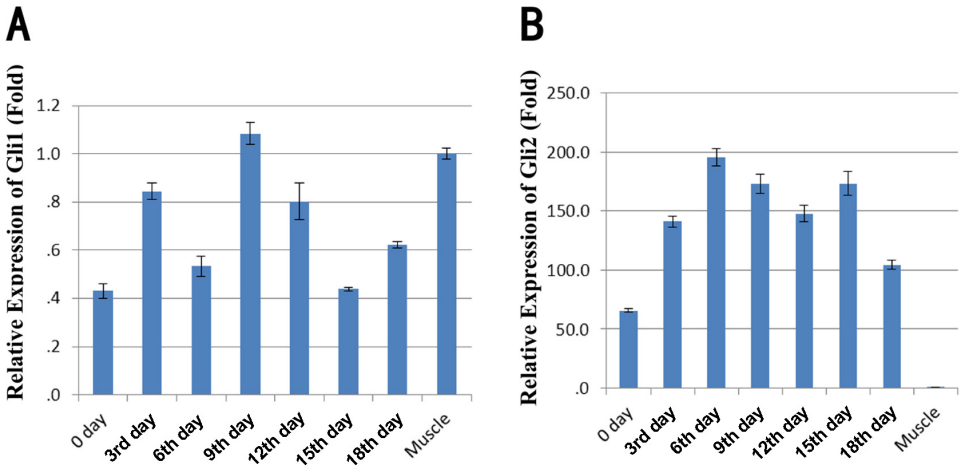

Figure 3. Expression of *Gli1* and *Gli2* in myoblasts during myogenic differentiation.

DISCUSSION

In vertebrates, there are three members of the Gli family, Gli1, Gli2, and Gli3, which are orthologs of the segment polarity gene Cubitus interruptus (Ci), a zinc-finger gene in *Drosophila*. Hh signaling modulates many kinds of cell activation by acting on Gli proteins to prevent the formation of repressor forms of Gli2 and Gli3 and to promote the activator forms of Gli1, Gli2, and Gli3 (Ribes and Briscoe, 2009). In *Drosophila*, Ci is a 155-kDa protein that contains five zinc fingers. The full-length Ci is the activator form, and the repressor form of Ci is a truncation of 75 kDa (Cohen, 2003). The Gli family in vertebrates is less understood than Ci in *Drosophila*. Unlike Ci, the repressor form of Gli1 does not undergo a proteolytic cleavage, and Gli1 does not appear to require binding with CBP for its activator form (Dai et al., 1999). It has been revealed that Gli1 does not have a repressor form, Gli2 has both activator and repressor forms, and Gli3 is a transcriptional repressor that balances and refines the activation of Gli1 and Gli2 (Ingham and McMahon, 2001; Nakajima et al., 2012).

The three Gli proteins in vertebrates make the Hh signaling more complex than in *Drosophila*, and Gli1 is not only the transcription factor but also the target gene of the Hh signaling pathway. Thus, cellular responses to Hh signaling might be modulated by the ex-

pression of Gli genes, as well as the ligand concentration (Ingham and McMahon, 2001). The expression profiles of *Gli1* and *Gli2* in different tissues are quite distinct (Figure 1). Cohen (2003) reported that *Gli1* has widespread expression in the mesoderm, especially in the ventral neural tube, and *Gli2* also has widespread expression in the mesoderm, especially in the posterior neural tube, which overlaps with the expression of *Gli3*. Three Gli genes in vertebrates may have a complex relationship of competition and cooperation in different tissues and cells (Cohen, 2003). Although Hh signaling has been proven to influence almost all parts of the vertebrate body, in our research, the expression of *Gli1* and *Gli2*, genes encoding two transcription factors of the Hh signaling pathway, are less identical in different tissues of cattle. Our results suggested that the response to Hh signaling was quite different in each tissue. We found that the expression levels of *Gli1* and *Gli2* were lower in muscle than all other endodermal organs. This corresponded with the general opinion that the Hh pathway is silent in postnatal animals (Straface et al., 2009).

Skeletal muscle is a structurally unique tissue consisting predominantly of highly specialized myofibers, which can generate force rapidly but lose the ability to proliferate. Therefore, a reserve pool of undifferentiated cells, which is normally quiescent but would be activated in response to turnover, growth demands, or damage of satellite cells, is important to the function of muscles. On the basis of the adhesion characteristics of different cell populations, a method of preplating cells onto collagen-coated plastic, which was mentioned previously, was established for primary cultures of muscle-derived stem cells (Qu et al., 1998). Fibroblasts and epithelial cells attach a short time after the heterogeneous cell suspension is plated on a Petri dish (Yaffe, 1968; Qu-Petersen et al., 2002). Almost 30 to 40% of the cells in pp2 and pp3 are known to be nonmyogenic, whereas up to 95% of the cells in pp4 and pp5 express desmin (Qu et al., 1998). In our research, from pp1 to pp6, the expression of *Gli1* and *Gli2* decreased predominantly. Therefore, the decreased expression of *Gli1* and *Gli2*, accompanied with the declining percentage of myoblasts, indicated that the *Gli1* and *Gli2* expression levels in myoblasts were lower than in fibroblasts or other cell populations derived from muscle.

Although the expression levels of *Gli1* and *Gli2* in muscle are both the lowest of the 13 tissues we investigated, many previous researches have testified that Hh signaling is crucial in muscle formation during embryonic and postnatal development of vertebrates (Flynt et al., 2007; Martin et al., 2007; Straface et al., 2009; Lobbardi et al., 2011; Hu et al., 2012). At the cellular level, the Hh signaling pathway promotes the proliferation and myogenic differentiation of myoblasts (Pola et al., 2003; Li et al., 2004; Koleva et al., 2005; Elia et al., 2007; Madhala-Levy et al., 2012). Several muscle regulatory factors, such as MyoD (Madhala-Levy et al., 2012), Myf5 (McDermott et al., 2005; Anderson et al., 2012), Pax7 (Madhala-Levy et al., 2012), and MyoG (Madhala-Levy et al., 2012), have been found to be regulated by Hh signaling. In bovine myoblasts, we observed elevated expression levels of *Gli2* on the 3rd and 6th day of induced differentiation by serum starvation. The expression of *Gli1* was elevated on the 3rd and 9th days, but its expression declined on the 6th day. A similar expression profile of *Gli1* and *Gli2* was observed in dimethyl sulfoxide-induced P19 cells (Voronova et al., 2013).

Gli1 and Gli2 are transcription factors of the Hh signaling pathway, and *Gli1* is a target gene of this pathway as well. We speculated that Hh signaling was activated in neither myocytes nor quiescent myoblasts; instead, Hh signaling was activated in fibroblasts or other early attached cells of bovine muscle tissue. The elevated expression of *Gli1* and *Gli2* after serum starvation supports the reactivation of the Shh pathway after ischemia in the adult skeleton and myocardium (Pola et al., 2003; Kusano et al., 2005). Therefore, it suggested that Hh

signaling would be activated while the quiescent bovine myoblast was stimulated to initiate myogenic differentiation.

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