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Comparison and analysis of Wuding and avian chicken skeletal muscle satellite cells

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ABSTRACT. Chicken skeletal muscle satellite cells are located between the basement membrane and the sarcolemma of mature muscle fibers. Avian broilers have been genetically selected based on their high growth velocity and large muscle mass. The Wuding chicken is a famous local chicken in Yunnan Province that undergoes non-selection breeding and is slow growing. In this study, we aimed to explore differences in the proliferation and differentiation properties of satellite cells isolated from the two chicken breeds. Using immunofluorescence,

hematoxylin-eosin staining and real-time polymerase chain reaction analysis, we analyzed the *in vitro* characteristics of proliferating and differentiating satellite cells isolated from the two chicken breeds. The growth curve of satellite cells was S-shaped, and cells from Wuding chickens entered the logarithmic phase and plateau phase 1 day later than those from Avian chicken. The results also showed that the two skeletal muscle satellite cell lines were positive for *Pax7*, *MyoD* and *IGF-1*. The expression of *Pax7* followed a downward trend, whereas that of *MyoD* and *IGF-1* first increased and subsequently decreased in cells isolated from the two chickens. These data indicated that the skeletal muscle satellite cells of Avian chicken grow and differentiate faster than did those of Wuding chickens. We suggest that the methods of breeding selection applied to these breeds regulate the characteristics of skeletal muscle satellite cells to influence muscle growth.

Key words: Avian chicken; Wuding chicken; Skeletal muscle satellite cells; Cell growth curves; Genetic resources

INTRODUCTION

Skeletal muscle satellite cells, also called muscle stem cells, are located between the basal lamina and plasmalemma of myofiber and play an important role in postnatal muscle growth, repair, and maintenance (Zammit et al., 2006a,b; Sacco et al., 2008). These cells were first isolated from frog muscle and were later identified in adult avian and mammalian muscle (Mauro, 1961). In recent decades, research on the function and mechanism of action of satellite cells in muscle development and regeneration has increased markedly. Satellite cells are mitotically quiescent, indifferent mononuclear small spherical cells distributed in normal mature skeletal muscle, but are activated to proliferate and differentiate as part of myonuclear turnover or for the formation of other cell types, such as osteocytes, adipocytes, and neurocytes. Therefore, skeletal muscle satellite cells are considered to be the major source of skeletal muscle fiber regeneration following skeletal muscle growth and injury (Schultz and McCormick, 1994). *Pax7*, *Myf5*, *MyoD*, *MyoG*, and *MRF4* are myocellular proteins, which can be used as markers to determine the proliferation and differentiation status of satellite cells. *Pax7* is only expressed in quiescent satellite cells, and activated satellite cells express *Myf5*, *MyoD*, *Myogenin*, and *MRF4* (Yablonka-Reuveni and Rivera, 1994; Zammit, 2008).

Theoretically, the number of muscle fibers should not vary following embryonic development, with an increase in muscle mass being due to the hypertrophy of skeletal muscle fibers after birth, which is mainly due to the proliferation of muscle satellite cells and their fusion with muscle fiber. Avian broilers (selected chicken for meat production) and Wuding chickens (non-selected chicken) are ideal models to explore the regulatory mechanisms determining the rate of myogenesis and muscle fiber size. During the past few decades, Avian chicken, as broilers, have been genetically selected due to their high growth velocity and large muscle mass; however, the Wuding chicken is a famous local chicken in Yunnan Province with non-selection breeding and slow growth. There are large differences in the skeletal muscle phenotype of the two breeds, and the growth rate of Avian broiler muscle is higher than that of Wuding chickens.

In this study, we used a blood cell counting chamber to analyze the proliferation characteristics of muscle satellite cells isolated from 4-week-old chickens, and determined the expression of related genes during the differentiation phase of satellite cells isolated from both chicken lines by RT-PCR. The objective of this research was to investigate the molecular mechanisms underlying the differentiation and proliferation of skeletal muscle stem cells (satellite cells) in Avian broiler chicken and in Wuding chicken used in selection breeding.

MATERIAL AND METHODS

Ethics statement

All of the experiments complied with the requirements of the Directory Proposals on the Ethical Treatment of Experimental Animals in China.

Animals

Avian broiler chickens were purchased from Kunming Zhengda Group and Wuding chickens were obtained from Yunan Agriculture University. All chickens were grown under the same conditions and fed a diet consistent with the Chinese chicken Feeding Standard recommendations and the formulation of NRC 1994. All the experiments complied with the requirements of Yunan Agriculture University's Animal Care. Twenty randomly selected Yunnan Wuding chickens and 20 Avian broiler individuals with 4 weeks old were sacrificed.

Cell culture

Muscle samples were soaked for 15 min in 75% ethanol to sterilize external surfaces, placed in sterilized gauze to isolate skeletal muscles, and cut into ~1-mm^3 pieces using ophthalmic scissors. These pieces were disaggregated by digestion with 0.1% collagenase I for 20 min and 0.25% trypsin for 30 min at 37°C. Next, Dulbecco modified Eagle medium (DMEM) containing 10% horse serum (HS), 5% chicken serum (CS), and 1% amphotericin B (Biochrom, Germany) was added to terminate the reaction. The cell suspensions were filtered through 70- and 40-mm nylon cell strainers, and then centrifuged at 1000 rpm for 5 min with the supernatant discarded. Cells were resuspended in complete medium (DMEM/F12, 10% HS, 5% CS, 1% amphotericin B) and seeded in cell culture bottles. Cells were cultured in a 5% CO_2 incubator at 37°C for 2 h. The cell suspensions were then transferred to a new cell culture bottle, and culture was continued with 5% CO_2 at 37°C (Qu et al., 1998).

When the cells reached 80-90% confluence, 0.25% trypsin containing 0.02% EDTA was added to digest cells, which were then subcultured at a ratio of 1:2.

The cells were subcultured to passage three until they reached 80-90% confluence, and were then digested and centrifuged and the supernatant was discarded. Then, 1.5 mL freezing medium (20% HS + 10% CS + 10% dimethyl sulfoxide + 60% DMEM/F12) was added. The suspension was dispensed into freezing tubes, and incubated at 4°C for 30 min, -20°C for 1 h, and -80°C overnight, before being transferred into a liquid nitrogen tank for long-term preservation. To recover cells, the freezing tubes were removed from the liquid nitrogen and placed rapidly into a 37°C water bath until the contents were 90% melted. Cells were then centrifuged at 1000 rpm for 5 min with the supernatant discarded. Complete medium was added and the cells were cultured in a 37°C, 5% CO_2 incubator.

Identification of skeletal muscle satellite cells

Preliminary identification by RT-PCR

Cells in passage three were collected and total RNA was extracted using TRIzol (Invitrogen, USA). Total RNA was reverse transcribed to cDNA using a SYBR-Green kit (iQTM SYBR-Green® Supermix, Dalian TaKaRa Biotechnology Co. Ltd.). The expression of *Pax7* and *MyoD*, which encode muscle-specific proteins, was determined by RT-PCR to identify and evaluate the purity of satellite cells. Information on the primer pairs used is listed in Table 1. PCR was performed in a 25-μL mixture containing 12.5 μL IQ SYBR Green Supermix, 9.5 μL dH_2O, 1 μL forward and reverse primers, and 1 μL template cDNA. The cycling conditions consisted of an initial 2 min at 94°C for one cycle followed by 40 cycles of 30 s at 94°C for denaturing, 30 s at 50-60°C for annealing, and 30s at 72°C for extension. PCR products were detected by 2.5% agarose gel electrophoresis. Levels of gene expression were determined by expression relative to 18S.

Table 1. Primer sequences used in the RT-PCR assay.

Gene	Sequence	Tm (°C)	Length (bp)	Cycles
MyoD	F:gatttccacagacaactccaca	61.7	188	40
	R:cttggtagattggattgctgct			
Pax7	F:gctgacttctccatctctcctc	61.7	155	40
	R:ctgtaactggtggtgctgtagg			
IGF-1	F:agacgaggcttctacttca	54	153	40
	R:gcagatttaggtggcttt			
18S	F:cgcgtgcatttatcagacca	58	148	40
	R:acccgtggtcaccatggta			

Immunofluorescence

Cells in passage two were collected and seeded on 6-well plates at a density of 1 x 10^5 cells/mL. Pax7 Immunofluorescence was detected during proliferation at 2 and 4 days. The cells were washed with phosphate buffered saline (PBS), fixed with 4% paraformaldehyde for 10 min at room temperature, and washed three times with PBS (3 min each). Then, the samples were incubated for 20 min with 0.5% Triton X-100

for permeabilization and washed three times with PBS (3 min each). To prevent non-specific binding of antibodies, the samples were blocked in 5% bovine serum albumin for 20 min at room temperature. After incubation overnight at 4°C with anti-pax7 (1:50, Santa Cruz Technology), the primary antibody was removed and the cells were washed three times with PBS (3 min each). Fluorescein isothiocyanate goat anti-mouse IgG secondary antibody (1:100, Zhongshan Golden Bridge, Beijing) was added and samples were incubated for 1 h at room temperature in a darkroom. The secondary antibody solution was decanted and samples were washed three times with PBS (5 min each) in the dark. Finally, the cells were coated with anti-fluorescence attenuator for microscopic analysis.

Growth curves for Wuding chicken and Avian broiler cells

Passage three cells were digested with 0.25% trypsin, and seeded on 24-well plates at a density of 2.25 x 10^4 cells/mL. Every 24 h two wells were counted with a blood-cell counting

chamber until the twelfth day, to ensure accuracy, each well was counted three times and the average number was taken. If cells overlapped, the original cell suspension was diluted and the cells recounted. Finally, time was taken as the abscissa and cell density as the vertical to generate the cell growth curve.

Differentiation of skeletal muscle satellite cells

Passage three cells grew to 70-80% confluence in culture, were washed three times with PBS, and then induced to differentiate into muscle cells by culturing in DMEM with 2% HS and 1% antibiotic for 8 days. The induction medium was changed every day (Katagiri et al., 1994). We collected induced cells at 0, 1, 3, 5, and 7 days and RNA was extracted with TRIzol. Total RNA was reverse transcribed for use in RT-PCR. Information on specific primer pairs is listed in Table 1.

Statistical analysis

The primers were designed by Primer 5 and the results are reported as means ± standard deviation. The treatment effect was assessed using non-parametric Kruskal-Wallis analysis of variance (ANOVA; SPSS 19.0, StatSoft). When the ANOVA revealed a significant effect, a Mann-Whitney test was performed. The cycle threshold (CT) values for each treatment group were calculated as the $\Delta CT = CT$ (target gene) - CT (18S). All the experiments were performed at least twice. Within an experiment, results for each time point were obtained from three replicates.

RESULTS

Morphology of skeletal muscle satellite cells

Under an inverted microscope, primary chicken satellite cells were found to be round with strong refraction before adhering (Figure 1). After 24 h of culture, the cells began to adhere and were spindle shaped or fusoid (Figure 1). Their proliferative capacity increased over 48 h of culture and the cells were arranged regularly in parallel (Figure 1). In this experiment, the adherence capability of skeletal muscle satellite cells from Avian chicken was found to be stronger than that of cells from Wuding chicken. The proliferation rate of cells from Avian chicken is faster than that of cells from Wuding chicken.

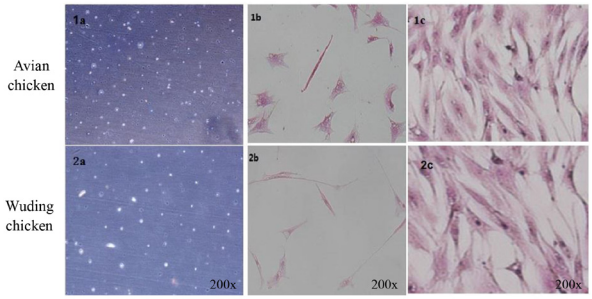
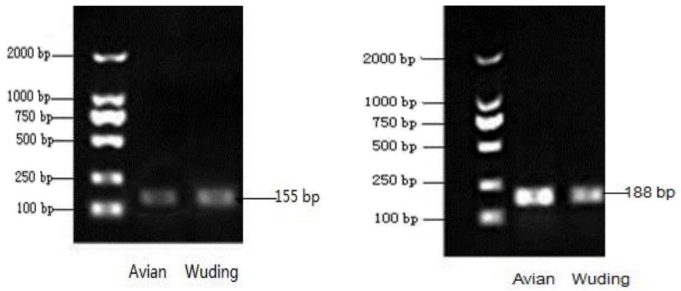


Figure 1. Morphology of cultured skeletal muscle satellite cells. **1a. 2a.** Satellite cells were round and strong refraction. **1b. 2b.** Cells were stained by hematoxylin eosin (H&E), adherent cells were spindle-shaped or fusoid. **1c. 2c.** With increase in cell density, cells were arranged in parallel, as shown by H&E staining.

Identification of skeletal muscle satellite cells

Preliminary identification of RT-PCR

In this study, we analyzed the mRNA expression of *Pax7* and *MyoD* genes, which are specific markers of satellite cells, in skeletal muscle satellite cells by RT-PCR. All skeletal muscle satellite cells isolated from the two chicken lines were positive for *Pax7* and *MyoD* (Figure 2A-B). This confirms that the isolated cells are pure skeletal muscle satellite cells; these provided material for the further study of muscle satellite cell characteristics.

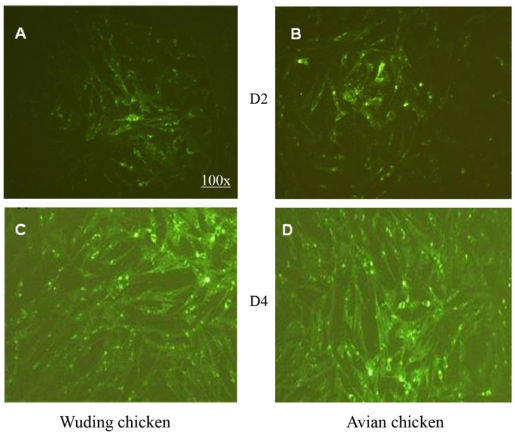


Pax7-A MyoD-B

Figure 2. RT-PCR analysis of skeletal muscle satellite cells. **A.** Samples from Avian and Wuding chickens were positive for *Pax7*. **B.** Avian and Wuding chickens were positive for *MyoD*.

Immunofluorescence

Pax7 is a specific marker of skeletal muscle satellite cells. In this experiment, *Pax7* was found to be distributed in the nucleus, and was expressed in samples from Wuding and Avian chicken. Over time, an increasing number of satellite cells were positive for *Pax7* (Figure 3A-D). This confirmed that the cultured cells were satellite cells.



Wuding chicken Avian chicken

Figure 3. Immunofluorescence detection of satellite cells. **A.-C.** Immunofluorescence analysis of Pax7 in myogenic cells from Wuding chicken following 2 and 4 days of proliferation. **B.-D.** Immunofluorescence analysis of Pax7 in myogenic cells from Avian chicken following 2 and 4 days of proliferation.

Growth characteristics of skeletal muscle satellite cells

Passage-three cells were used to generate growth curves of the two chicken lines (Figure 4). There were differences in the development of skeletal muscle satellite cells between Avian chicken and Wuding chicken, and the standard curve was almost S-shaped. Avian chicken cells entered the logarithmic phase after 2-days proliferation and myoblasts proliferated rapidly from days 2 to 8. The rate of proliferation then slowed after 8 days and cells entered the plateau phase. However, cells from Wuding chickens entered these phases one-day later than those from Avian chickens, and the number of satellite cells was lower than in Avian chicken at day 7 and 8 ($P < 0.05$).

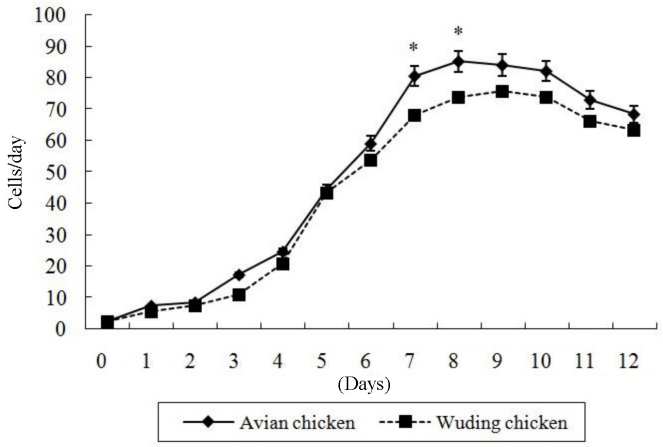


Figure 4. Growth curve of skeletal muscle satellite cells from Avian chicken and Wuding chicken. *Significantly ($P < 0.05$) different means. Significant differences were determined by the Mann-Whitney rank test. Results were obtained from three replicates.

Differentiation of skeletal muscle satellite cells

Cells proliferated slowly when cultured in the induction medium, and anastomosis began to develop between cells. The ability of cells from Avian chicken to fuse into myotubes increased over time as compared with that those from Wuding chicken. The myotubes were observed by day 3 (Figure 5 1a-1b), and after 5 days, many mononucleate myoblasts were fused with centrally located nuclei, forming long bamboo-shaped primary myoblasts (myotubule) with a regular parallel arrangement (Figure 5 2a-2b).

RT-PCR

In this experiment, the expression of *Pax7*, *MyoD*, and *IGF-1* in skeletal muscle satellite cells during differentiation was assessed by RT-PCR. The results showed that the skeletal muscle satellite cells from the two chicken lines were positive for *Pax7*, *MyoD*, and *IGF-1* (Figure 6). The expression of 18S was used as an internal control. Over time, the expression of *Pax7* began to decrease, and the expression of *MyoD* and *IGF-1* first increased and then decreased in samples from the two chickens.

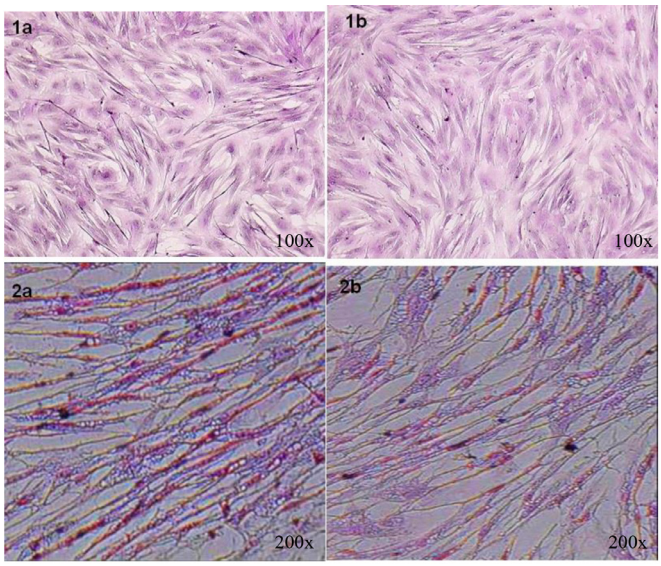


Figure 5. Skeletal muscle satellite cells differentiate into myogenic cells. **1a. 1b.** Myotubes were formed after 3 days of differentiation and observed by H&E staining in samples from Avian chicken and Wuding chicken, respectively. **2a. 2b.** Skeletal muscle satellite cells initiated terminal differentiation to form multinucleated myotubes on day 5.

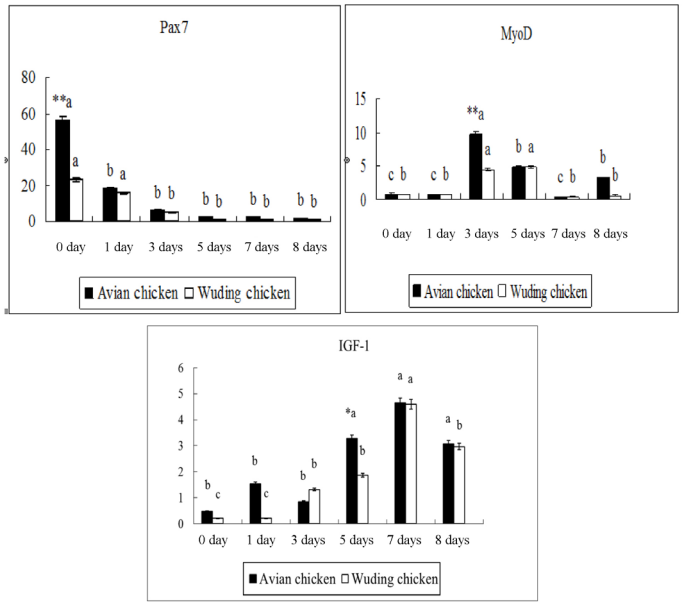


Figure 6. Differentiation of myogenic cells at days 0, 1, 3, 5, 7, and 8. The expression of *Pax7*, *MyoD*, and *IGF-1* in skeletal muscle satellite cells was determined by RT-PCR. **Differences among different chickens at the same time are significant ($P < 0.01$). *Significant difference ($P < 0.05$). Different letters indicate significantly different means ($P < 0.05$).

The expression of *Pax7* in skeletal muscle satellite cells from Avian chicken at day 0 was significantly higher than at any other time assessed ($P < 0.01$). The expression of *Pax7* in cells from Wuding chicken on day 0 and day 1 was significantly higher than at any other time ($P < 0.01$). Over the course of the experiment, gene expression in cells from Avian chicken was higher than in those from Wuding chicken, and maximum expression in samples from both chicken was maximal at day 0.

Expression of the *MyoD* gene in skeletal muscle satellite cells from Avian chicken and Wuding chicken on day 3 was significantly higher than on any other day ($P < 0.01$). The expression of this gene in Wuding chicken was significantly lower than in Avian chicken ($P < 0.01$), compared with the other days. Overall, expression of this gene was higher in Avian chicken than in Wuding chicken.

Expression of the *IGF-1* gene in skeletal muscle satellite cells from Avian chicken at days 5, 7, and 8 was significantly higher than on other days ($P < 0.01$). Expression of this gene in cells from Wuding chicken on days 7 and 8 was significantly higher than on any other days ($P < 0.01$). The maximum expression in the two chicken lines occurred on day 7; expression of *IGF-1* in cells from Avian chicken was higher than that in cells from Wuding chicken on all days, with the exception of day 3.

DISCUSSION

Satellite cells are a type of adult muscle stem cells and are located between the basal lamina and the basement membrane of mature myofibers. They play a key role in the growth of skeletal muscle and in the regulation of muscle growth and development in the post-hatch stage, which is the first stage of muscle growth in adults.

In the present study, the method used to isolate skeletal muscle satellite cells from Wuding and Avian chickens was similar to that used in studies on other animals. We adopted a modified two-step method using collagenase and trypsin to isolate skeletal muscle satellite cells. These cells were then purified using the differential time attachment method, which utilizes the differential adhesive abilities of fibroblasts and satellite cells (Lee et al., 2000). Basal keratinocytes were isolated from skin based on their differential adhesive abilities (Häkkinen et al., 2001; Spichkina et al., 2008).

Pax7 (Seale et al., 2000), *M-Cadherin*, *Desmin*, *c-Met*, *MNF*, *MyoD* (Musarò and Barberi, 2010; Wu et al., 2012), *MyoG*, and *Myf5* are specific markers of skeletal muscle satellite cells. *Pax7*, *M-Cadherin*, and *c-Met* can be used to identify quiescent satellite cells and the molecular markers *Myf5*, *MyoD*, and *Desmin* are used to identify proliferating satellite cells. In the present study, *Pax7* and *MyoD* were used to identify skeletal muscle satellite cells by RT-PCR and immunofluorescence. Cultured cells were shown to be pure skeletal muscle satellite cells and were positive for *Pax7* and *MyoD*.

Previous studies demonstrated that the proliferating ability of satellite cells varies in chicken. In our study, the growth curves for skeletal muscle cells from Wuding chicken and Avian chicken were "S" type, and included incubation, growth, platform, and apoptosis stages. Compared with Avian chicken, skeletal muscle satellite cells from Wuding chicken growing in the corresponding logarithmic and platform phase was possibly delayed and their growth rate was sluggish. For the differentiation of satellite cells, different breeds adopt different inducing medium. In a previous study, induction medium containing DMEM with 5% HS was added to differentiate Beijing fatty chicken skeletal muscle cells (Bai et al., 2012). In another study,

muscle cells were cultured in DMEM containing 5% FBS and 1 μM cytosine arabinoside for pig satellite cells (Perruchot et al., 2012). In the present study, we used induction medium containing DMEM/F12 and 2% HS, and multinucleated myotubes were observed clearly on day 5. This is important for skeletal muscle hypertrophy and fiber-type transformation, and ultimately affects meat quality. In addition, the Avian chicken cells formed stronger myotubes than Wuding chicken cells. These data indicate that the skeletal muscle of Avian chicken grows faster than that of Wuding chicken and this is related to the characteristics of the satellite cells.

The myogenic potential of skeletal muscle satellite cells depends on the expression of *Pax3/7* and on myogenic regulatory factors (including *MyoD*, *Myf5*, and *MRFs*). *IGFs* also play a key role in the growth of satellite cells. Changes in gene expression and in the activity of IGF have been studied during cell culture progression (Castillo et al., 2002, 2004, 2006; Codina et al., 2008). Expression of the *IGF-1* gene was reported to be upregulated, when the satellite cells were activated by weight-bearing exercise or trauma (Adams and McCue, 1998). Nevertheless, cell differentiation has been reported to start at the peak of the proliferation curve (Fauconneau and Paboeuf, 2000; Bower and Johnston, 2010). Therefore, we analyzed the expression of *Pax3, MyoD*, and *IGF-1* in differentiating cells. The results showed that the *Pax3* gene was expressed at the highest levels in cells from the two chickens on day 0. For *MyoD*, the highest gene expression as observed on day 3. These results are consistent with those of previous reports (Chen, et al., 2010). When the muscle satellite cells are stimulated, *Pax7* is expressed, leading to the activation of quiescent satellite cells. Skeletal muscle progenitor cells were formed following the proliferation of satellite cells, and *Pax3*, *Myf5*, and *MyoD* were upregulated while *Pax7* was expressed at lower levels. Overall, the expression of *Pax7*, *MyoD*, and *IGF-1* in cells from Wuding chicken was lower than that in cells from Avian chicken. This is reflected in higher growth rate of Avian chicken muscle compared with Wuding chicken muscle.

In conclusion, we established a technology platform involving the isolation and *in vitro* induction of skeletal muscle satellite cell differentiation. The characteristics of Wuding and Avian chicken skeletal muscle satellite cells were compared and analyzed in terms of their proliferation and differentiation abilities, and the skeletal muscle cells from Avian chicken were found to grow and differentiate faster than those from Wuding chicken. Furthermore, these cells differ in their sensitivity to related genes. These results provide references for the further study of skeletal muscle growth and development in selection and non-selection chicken. We have suggested a new method to preserve the valuable genetic resources of Wuding chicken. Wuding chicken is part of the abundant poultry genetic resources in Yunnan, further studies should expand the number of samples to include large numbers of chicken samples of different genetic backgrounds and related screening candidate genes to further investigate the function of skeletal muscle satellite cells in muscle growth and development of native chickens.

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

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