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Roles of chicken growth hormone receptor antisense transcript in chicken muscle development and myoblast differentiation

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ABSTRACT Muscle is one of the important economic traits in poultry production, and its production depends on the increased number of muscle fibers during the embryonic stage. Chicken *GHR* gene can transcribe in double directions, possessing not only *GHR-S* but also *GHR-AS*. The 2 kinds of transcripts are partially complementation in sequences and interact with each other. Until now, the roles and mechanisms of *GHR-AS* in myoblast differentiation was still unknown. In this study, we not only analyzed the *GHR-AS* expression patterns in myoblast differentiation phase but also clarified that *GHR-AS* promoted myoblast differentiation via GH-GHR-IGF1 signal pathway. Quantitative PCR analysis indicated that *GHR-AS* was increased during myoblast differentiation. Sub-cellular localization showed that *GHR-AS* and *GHR-S* were expressed at a higher level in the nucleus than that in the cytoplasm. The expression of *MyoD* and *MyHC* and the myoblast differentiation significantly increased after *GHR-AS* overexpression, while the distance between wounds decreased, suggesting that *GHR-AS* repressed myoblast migration and promoted differentiation. Additionally, the expression of *GHR-AS*, *IGF1* and *MyHC* increased after GH protein treated, and the myoblast differentiation also increased. In conclusion, *GHR-AS* promoted myoblast differentiation by enhancing fusion and inhibiting migration possibly via GH-GHR-IGF1 signal pathway.

Key words: chicken, growth hormone receptor, natural antisense transcript, myoblast, differentiation

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INTRODUCTION

The muscle growth is a complex process which depends on the increased number of muscle fibers (Buckingham et al., 2010; Guo et al., 2015). Once the growth phase is completed, the fiber numbers do not increase but the fiber volume increases (Buckingham et al., 2010). These processes influence meat quality of poultry and livestock. During myoblast differentiation from embryonic stem cell to myofiber in skeletal muscle development, genes play important roles in different stages (Luo, 2016). Recently, many researchers identified long non-coding RNAs such as *linc-MD1* and *lnc-Dum*, in the regulation of myogenesis (Legnini et al., 2014; Wang et al., 2015). Natural antisense transcripts (**NATs**), a kind of non-coding RNAs, often overlapped with the protein coding gene transcripts in opposite directions and regulated the mRNA and protein expression (Faghihi and Wahlestedt, 2009).

GHR plays a key role in human and animal growth. Both human Laron-type dwarfism (Godowski et al., 1989) and sex-linked dwarf chicken (Lin et al., 2012) are caused by the mutation of *GHR* gene. Chicken *GHR* gene located in chromosome Z comprises of 8 exons. Growth hormone (**GH**) secreted by pituitary gland can combine with GHR to activate the intracellular insulin-like growth factors (**IGFs**) pathway involved in cell proliferation and differentiation (Sperling, 2016). Previously, we identified a NAT of *GHR* gene (***GHR-AS***), which was overlapped with *GHR* mRNA (***GHR-S***) in the 3'UTR, exons 8, 7, and 6 of *GHR* gene (Zhang et al., 2016).

HuaiXiang (**HX**) chicken is a famous local breed with yellow feathers, skin, and feet mostly found in the Guangdong province of China. Also, it has a high tolerance to roughage feeding and general stress resistance. In this study, we mainly analyzed the roles of *GHR-AS* in HX chicken myoblasts differentiation process, and

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found that *GHR-AS* promoted myoblast differentiation via the GH-GHR-IGF1 pathway.

MATERIALS AND METHODS

Ethics Statement

Animals involved in this research were humanely sacrificed as necessary to ameliorate suffering. It was approved by the Animal Care Committee of Guangdong Ocean University (Zhanjiang, China).

Experimental Animals and Tissue Samples

The fertile eggs were obtained from the HX Chicken Breeding Farm (Yingfu Company, Xinyi, Guangdong province), incubated in an automatic incubator (Baihui, Shandong, China) at 37.8°C, with 60 ± 10% humidity, and the broilers were fed with the normal basal diet, which including 11.51% metabolic energy, 15.23% crude protein, 3.10% calcium, 0.45% available phosphorus, and a few non-essential amino acid. According to our previous research (Zhang et al., 2017), E11 was the best time to isolate and culture chicken myoblast in vitro. Thus, the leg muscles collected from E11 embryos were used to isolate the chicken myoblast.

Isolation, Culture, and Differentiation of Chicken Primary Myoblast

Myoblasts were isolated from chicken leg muscles at E11 according to the methods described by Zhang et al. (2017). Briefly, differential attachment was performed 3 times at every 40 min after the muscles were cut into pieces. During the third time, the adherent cells were collected and directly grown on a plastic plate. The cells were cultured in growth medium with DMEM-F12 (Gibco, New York, USA), 15% FBS (Gibco, New York, USA) and 1% penicillin/streptomycin (Gibco, New York, USA) at 37°C in 5% CO2, then induced to differentiation in differentiation medium (DM), DMEM-F12 containing 2% horse serum (Gibco, New York, USA), until 90% confluent were reached.

Subcellular Location of GHR-AS in Chicken Myoblast

Nucleus and cytoplasm RNA were individually extracted from chicken myoblast during proliferation or differentiation phase according to the guide of PARIS Kit Protein and RNA Isolation System (Invitrogen, Carlsbad, CA, USA). The PARIS method is based on differential lysis of plasma and nuclear cell membranes by nonionic detergents. Cells are first separated into nuclear and cytoplasmic fractions then RNA is isolated. Briefly, the cell pellet was suspended in a buffer of RNA purification and then centrifuged twice at 4°C for 5 min. The supernatant was the cytoplasmic fraction and the pellet was the nucleus fraction. RNAs were extracted from both fractions using Trizol (Mange, Guangzhou, China). The effectiveness of cell fractionation can be easily checked by Quantitative PCR (**qRT-PCR**) or western blot with antibodies for proteins found predominantly in the nucleus or cytoplasm of cells such as *sno-U6* (predominantly in the nucleus) or *GAPDH* (predominantly in the cytoplasm). In this study, we analyzed the *sno-U6* and *GAPDH* expression with qRT-PCR to indicate the methodology was successful. The relative levels of *GHR-AS* and *GHR-S* were analyzed by qRT-PCR.

Construction of Chicken GHR-AS Plasmids and Cell Transfection

To construct the *GHR-AS* plasmid (**p*GHR-AS***), the full length of *GHR-AS* was amplified by PCR with forward and reward primers including *KpnI* and *XbaI* restriction enzyme site at 5′-ends, respectively (Table 1). The PCR was performed with a 20 μL reaction mixture containing 1 μL of cDNA from chicken myoblast, 10 μL of TaKaRa Taq Version 2.0 plus dye (TaKaRa, Kyoto, Japan), 1 μL each of the forward and reward primer (10 μM), and double-distilled water. The PCR reaction procedure involved: 94°C 2 min, 40 cycles at 94°C 30 s, 57°C 30 s, and 72°C 30 s; followed by 72°C for 5 min. The PCR products were digested with *KpnI* and *XbaI* restriction enzyme and the purified fragment was ligated into pcDNA3.1(+) plasmid to obtain the *GHR-AS* recombinant plasmid. *GHR-AS* overexpression experiment were carried out in a 12-well plate by transient transfecting p*GHR-AS* into myoblast during differentiation using Lipofectamine3000 (Invitrogen, Carlsbad, CA).

Wound-Healing Assay to Detect the Effects of GHR-AS on Myoblast Migration

A wound healing assay was carried out to compare the myoblast migration after being transfected with p*GHR-AS* or pcDNA3.1(+) plasmids. The transfected cells were cultured in a 12-well plate until confluent. A straight scratch was created in the center of each well using a micropipette tip. Cell migration was assessed by measuring the movement of the cells into the scratch in the well. After being incubated for 6 h, cells were photographed under an inverted microscope (Olympus, Tokyo, Japan). The distance between the 2 edges of the scratch (wound width) was measured at 9 sites (100 × magnification) in each image. The cell migration distance was measured using ImageJ software by measuring the wound width, and then normalized to the length at 0 h. Experiments were carried out 3 times.

Myoblast Treatment with Chicken GH Protein

After inducing myoblast differentiation, we added recombinant chicken GH (Abcam, Cambridge, London,

Table 1. Primers used in this study.

Application	Primer name	Primer sequence	Product/bp	Tm/°C
Constructed *GHR-AS* vector	*GHR-AS*-F	CTTggtaccGTATGAAGAGTCCCAACCAAC	4,184	57.0
	GHR-AS-R	CCCtctagaTACACGGACTACAAAGGGGAC		
GHR-AS quantitative PCR	*GHR-AS*-F	TTGCTAATGTTTCTGTTCTGTG	117	59.9
	GHR-AS-R	GGGTCAATCCCTTTAATCTTT		
GHR-S quantitative PCR	*GHR-S*-F	AGTCCGATCAAGACAACGTAC	128	59.9
	GHR-S-R	CTAAGAACCAGGGAAACTCG		
IGF1 quantitative PCR	*IGF1*-F	TGTGCTCCAATAAAGCCACCT	125	57.0
	IGF1-R	TTTCTGTTTCCTGTGTTCCCTCTAC		
MyoD quantitative PCR	*MyoD*-F	GCTACTACACGGAATCACCAAAT	200	58.0
	MyoD-R	CTGGGCTCCACTGTCACTCA		
MyHC quantitative PCR	*MyHC*-F	CTCCTCACGCTTTGGTAA	213	58.0
	MyHC-R	TGATAGTCGTATGGGTTGGT		
Myomaker quantitative PCR	Myomaker-F	TGGGTGTCCCTGATGGC	135	58.0
	Myomaker-R	CCCGATGGGTCCTGAGTAG		
Internal control in qPCR analysis	*GAPDH*-F	AGGACCAGGTTGTCTCCTGT	153	60.0
	GAPDH-R	CCATCAAGTCCACAACACGG		
	Sno-U6-F	CTCGCTTCGGCAGCACA	94	53.0
	Sno-U6-R	AACGCTTCACGAATTTGCGT		

The lowercase part is the enzyme site.

UK) at 200 ng/mL in DM. Then we harvested cells for the analysis of *IGF1* expression at 0, 3, 6, 9, 12, and 24 h, respectively.

Quantitative Real Time PCR (qRT-PCR)

The total RNA was extracted from myoblast by Trizol reagent (Mange, Guangzhou, China) according to the manufacturer's protocol. The RNA integration and concentration were detected using electrophoresis at 1.5% agarose gel and Nanodrop 2000 spectrophotometry.

The cDNA was synthesized with PrimeScript RT Reagent Kit (TaKaRa, Kyoto, Japan). In addition, the cDNA was diluted 4 times with RNase free water and stored at −20°C. Real time PCR was conducted in a Bio-rad CFX96 Real-Time Detection System (Bio-rad, Hercules, CA, USA) using TransStart Green qPCR SuperMix (Transgen Co., Ltd., Beijing, China). The *GHR-AS*, *GHR-S* and *IGF1* qRT-PCR primers used are listed in Table 1. The expressions were normalized with *GAPDH*, whiles gga-*sno-U6* was used as the internal control of nucleus RNA. We performed qRT-PCR with 20 μL mixture containing 1 μL of cDNA, 10 μL of 2 × TransStart Green qPCR SuperMix, 0.5 μL each of the forward and reward primers (10 μM), and double-distilled water. The qRT-PCR reaction procedure is as follows: 95°C for 30 s; 40 cycles at 95°C for 5 s, annealing for 30 s, 72°C for 30 s; followed by 72°C for 5 min. The expression of gene was quantified using comparative threshold cycle ($2^{-\Delta\Delta Ct}$) methods.

Western Blot Analyzing the IGF1 Protein

For Western blotting, cells were collected from different groups, then pelleted by centrifugation and lysed in RIPA lysis buffer (P0013B, Beyotime, Shanghai, China, https://www.beyotime.com/ripa-lysis-bufferh.htm) containing 1% Phenylmethanesulfonyl fluoride (ST506, Beyotime, Shanghai, China). Total protein was prepared and its concentration was determined using the BCA method (P0010, Beyotime, Shanghai, China). Protein was then separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto the polyvinylidene fluoride (**PVDF**) membrane. After blocking for 1.5 h at room temperature with 5% skimmed milk, the PVDF membrane was incubated with anti-IGF1 (Cloud-Clone Corp, Wuhan, China), and anti-GAPDH antibodies (Bioworld, Nanjing, China), respectively. After washing 3 times with Tris-Buffered Saline and Tween-20, the membrane was incubated with horseradish peroxidase-conjugated secondary goat anti-rabbit IgG (**H&L**) antibody (Bioworld, Nanjing, China) at room temperature for 1.5 h, followed by visualization using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific, Karlsruhe, Germany). An ImageMaster DVS system was used to calculate the relative mean gray values (**A**) of the target product and GAPDH. Expression index (**I**) of the target product was calculated using the formula of I = A product/A GAPDH.

Immunofluorescence Assay

Chicken primary myoblast at 95% confluence were washed 3 times with PBS (pH 7.4), and permeabilized for 15 min in PBS with 0.5% Triton X-100 before fixation in 4% paraformaldehyde for 30 min. Immunostaining was carried out as follows: the cells were incubated at 4°C overnight with primary anti-MyHC, diluted in 1% BSA. The cells were washed and then incubated at room temperature for 2 h

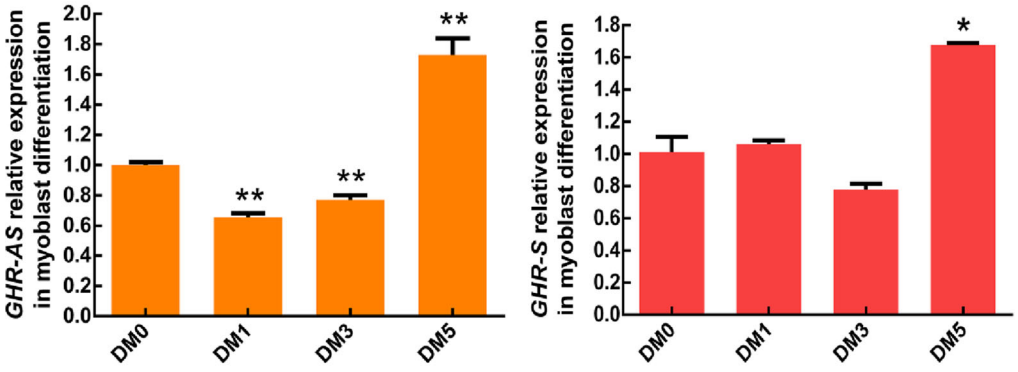

Figure 1. The relative expression of GHR-AS and GHR-S gene expression during chicken myoblast differentiation. Each value represented the mean of 3 batches ± SEM.*$P \leq 0.05$, **$P \leq 0.01$.

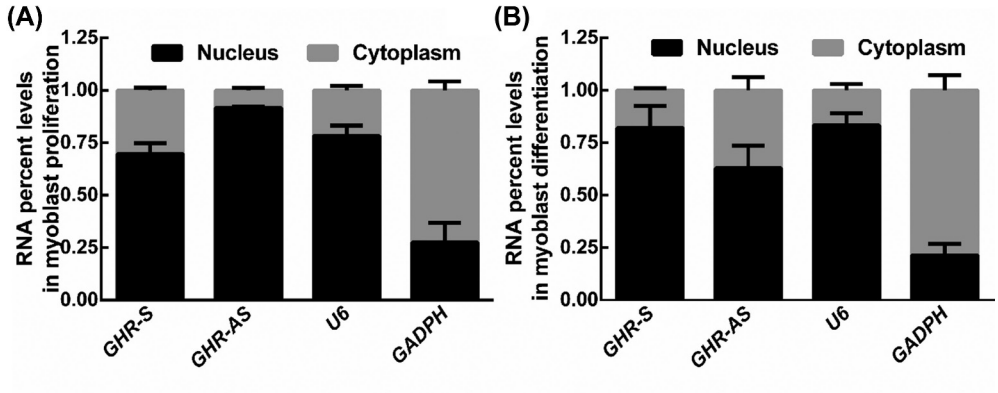

Figure 2. Subcellular localization of GHR-AS and GHR-S in myoblast proliferation (A) and differentiation (B).

with the corresponding secondary antibody goat anti-rabbit IgG H&L (1:1,000; Abcam, Cambridge, MA) diluted in 1% BSA. DNA was visualized using 5 mg/mL DAPI. Finally, the cells were washed 3 times with PBS and observed with fluorescence microscope (DM5000B, Leica, Solms, Germany).

Statistical Analysis

Each experiment was repeated 3 times, and all the data were expressed as the mean±SE and processed using the statistical software SAS 9.1.3 (SAS Institute Inc., NC). $P < 0.05$ was considered significant differences between groups.

RESULTS

GHR-AS Increasingly Expressed During Myoblast Differentiation

To investigate the expression of *GHR-AS* in chicken myoblast, primary myoblast was isolated from HX chicken leg muscles at E11, and induced differentiation by adding 2% horse serum to the DM. Quantitative PCR analysis indicated that *GHR-AS* was increased during myoblast differentiation. Also, the *GHR-S* has the same trend with *GHR-AS*, suggesting they might function during myoblast differentiation (Figure 1).

GHR-AS was Expressed at a Higher Level in the Nucleus than in Cytoplasm

To understand the possible roles and mechanisms of *GHR-AS* during myoblast proliferation and differentiation, we analyzed the location of *GHR-AS* using the PARIS RNA Isolation System Kit. Results showed that *GHR-AS* was rich in nucleus than cytoplasm in both myoblast proliferation (Figure 2A) and differentiation (Figure 2B). *GHR-S* possesses the same trend with *GHR-AS*. The nucleus co-localization of both *GHR-AS* and *GHR-S* demonstrated that they could interact with each other.

GHR-AS Repressed Myoblast Migration and Promoted its Differentiation

To evaluate roles of *GHR-AS* in myoblast differentiation and fusion, the myoblast was transfected with p*GHR-AS* during differentiation. Compared to the

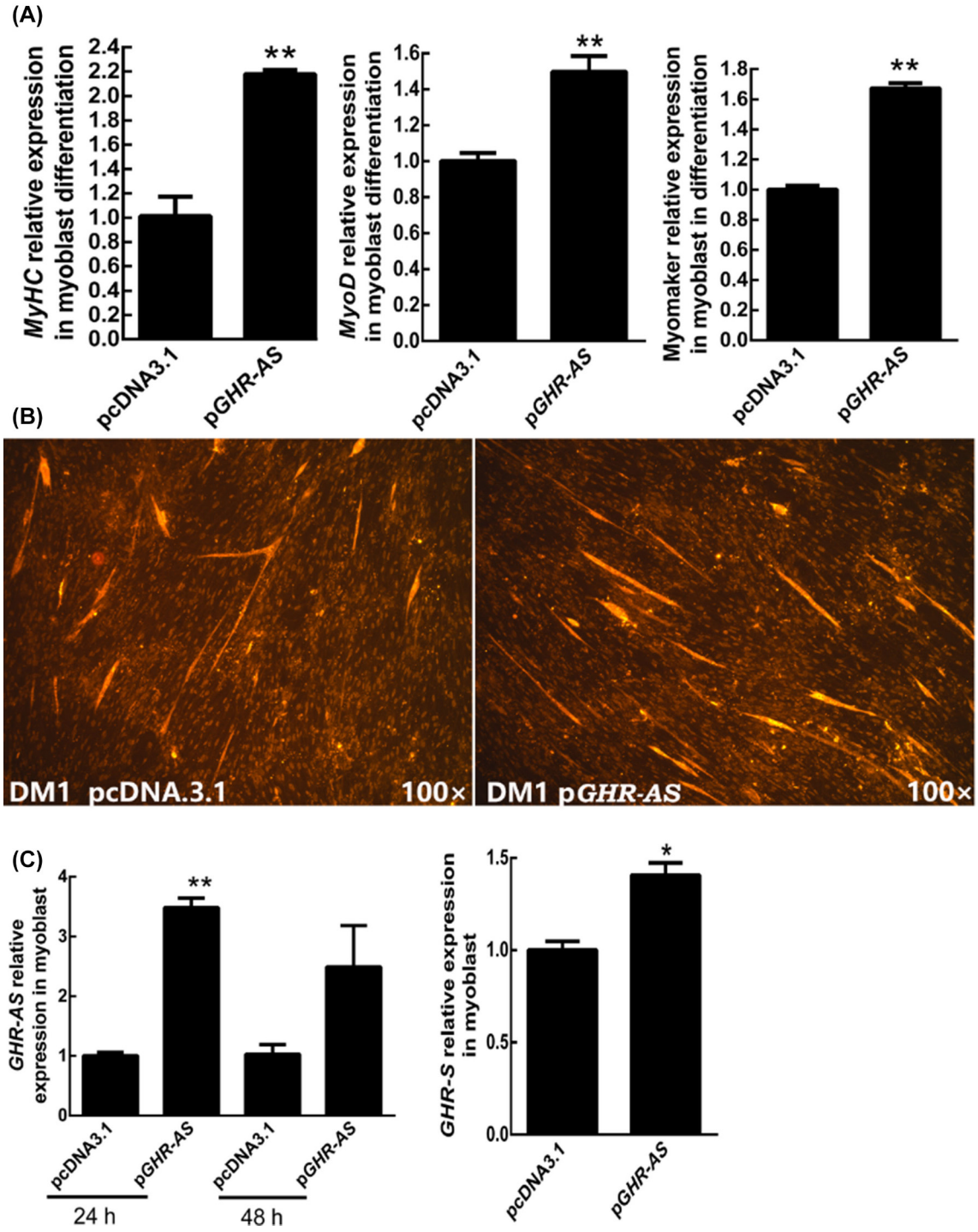

Figure 3. The effects of *GHR-AS* on myoblast differentiation. (A): the relative expression of *MyHC*, *MyoD*, and Myomaker in myoblast differentiation after *GHR-AS* overexpression; (B): *MyHC* immunofluorescence assay of chicken primary myoblast after *GHR-AS* overexpression; (C): the relative expression of *GHR-AS* and *GHR-S* in myoblast differentiation after *GHR-AS* overexpression. Each value represented the mean of 3 batches ± SEM. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$.

control group, the myoblast differentiation and the expression of *MyHC*, *MyoD*, and *Myomaker* significantly increased (Figure 3A). Immunofluorescence assay for MyHC indicated that the differentiation of chicken myoblast were promoted (Figure 3B). Also, *GHR-S* was increased during the process (Figure 3C).

Migration is the important character to measure the ability of cell differentiation. Scratch wound healing assay showed that the distance between wounds in *GHR-AS* overexpression group was larger than the control group (Figures 4A and 4B), indicating that *GHR-AS* inhibited myoblast migration.

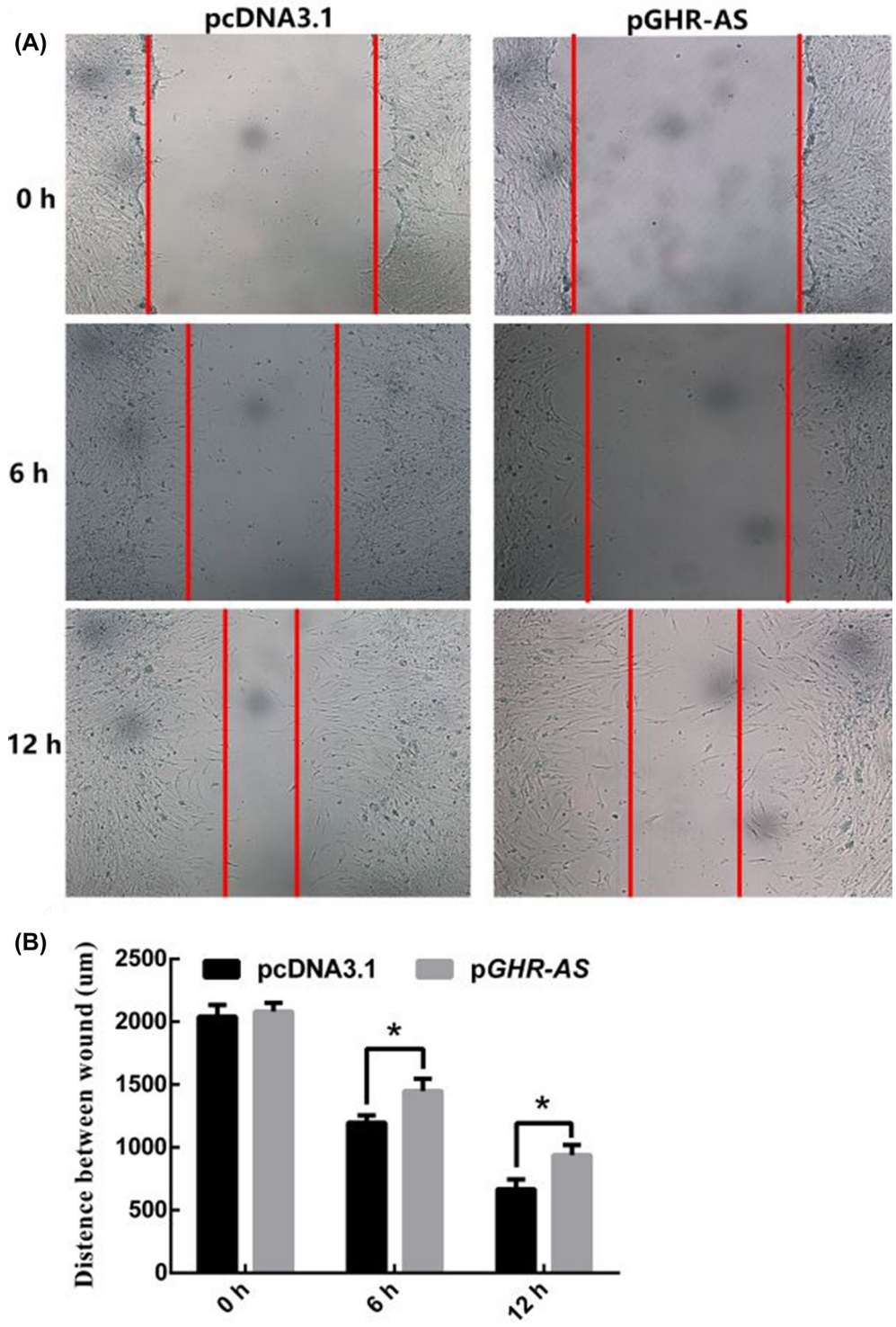

Figure 4. *GHR-AS* reduced chicken myoblast migration. (A): migration of myoblast was detected after transfection with pcDNA3.1 or p*GHR-AS*, respectively, for 6 h and 12 h; (B): the statistical date of distance between wound after *GHR-AS* overexpression. Each value represented the mean of 3 batches ± SEM. * $^{*}P \leq 0.05$, ** $P \leq 0.01$.

GHR-AS May Regulate the Myoblast Differentiation via GH-GHR-IGF1 Signal Pathway

Animal growth and development are affected by GH-GHR-IGF1 signal pathway: GH protein secreted from the pituitary gland briefly binds to the GHR in cytomembrane and activates the GH-GHR-IGF1 signaling pathway. Other tissues, like genital gland, also secrete GH protein, promoting *IGFs* expression by paracrine or autocrine fashion (Luna et al., 2014; Marisela et al., 2016).

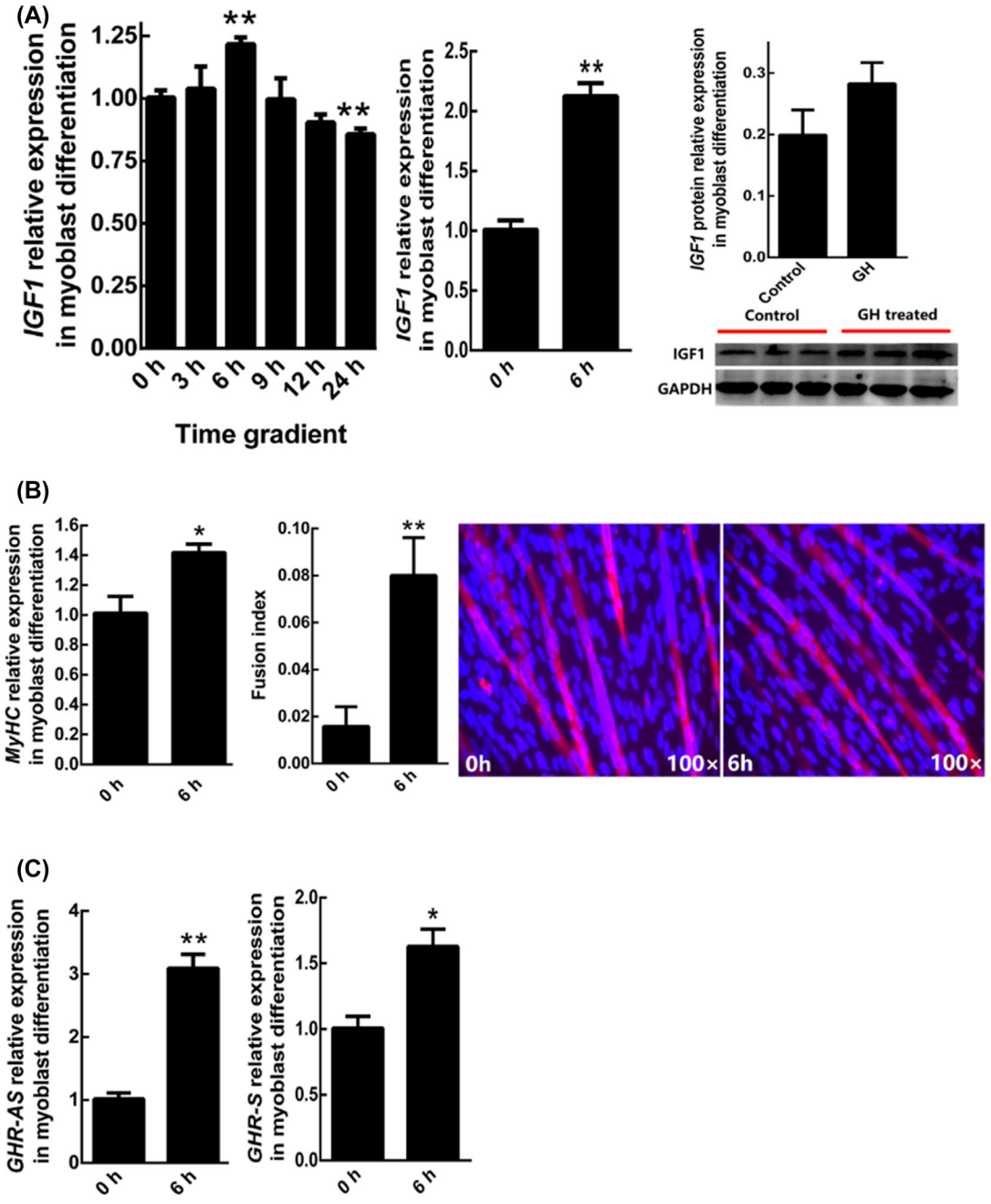

Figure 5. The effects of chicken GH protein on myoblast differentiation. (A): the relative expression of *IGF1* mRNA and protein in myoblast differentiation phase after GH treated; (B): the relative expression of *MyHC* mRNA, the variation of myoblast fusion index, and immunofluorescence of *MyHC* in myoblast differentiation phase after GH treated; (C): the relative expression of *GHR-AS* and *GHR-S* in myoblast differentiation phase after GH treated. Each value represented the mean of 3 batches±SEM. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$.

To evaluate the function of *GHR-AS* via GH-GHR-IGF1 signal pathway, chicken GH protein was added during myoblast differentiation. Firstly, the cells underwent serum starvation for 10 h, then, 200 ng/mL chicken GH protein was added into the DM to induce *IGF1* expression. We found that *IGF1* mRNA significantly increased at the 6 h after adding GH protein (Figure 5A). Also, the IGF1 protein showed an ascending trend in the GH treated group (Figure 5A). Accompanied with this, the myoblast fused into multinucleate cells and the differentiation biomarker *MyHC* was highly expressed than the control group (Figure 5B). Interestingly, we found that both *GHR-AS* and *GHR-S* showed upward trends during the process, suggesting that *GHR-AS* may be involved in the myoblast differentiation via GH-GHR-IGF1 signal pathway (Figure 5C).

DISCUSSION

Amount of chicken lncRNAs involved in muscle development have been found by RNA sequencing analysis (Lin et al., 2014; Li et al., 2016). In the

previous study, we identified a non-coding transcript of *GHR* gene, named *GHR-AS*, overlapping with *GHR-S* in a tail-to-tail manner. Previous research showed that *GHR-AS* had slight effects on myoblast proliferation (Zhang, 2016). During the period of E12 to E17, the vital time for muscle formation, the number of myocytes grow fast and lay the foundation for growing and thickening after birth (Li et al., 2017). Here, we explored the effects of *GHR-AS* on myoblast differentiation. In the myoblast differentiation progress, *GHR-AS* appeared in an ascendant tendency. This suggests that *GHR-AS* may be involved in myoblast differentiation.

RNA distribution analysis showed that both *GHR-AS* and *GHR-S* were rich in nucleus than cytoplasm in myoblast proliferation as well as differentiation. Other research showed that *GHR-AS* could change the chromatin status from a closed and inactive state to an open and active state, regulating methylation of histone H3K9me2 on its promoter in LMH cell line (Zhang et al., 2016). The nucleus co-localization of both *GHR-AS* and *GHR-S* in myoblast demonstrated that *GHR-AS* may regulate *GHR-S* in the same way in myoblast.

The fusion of myoblast with the growing multinucleated muscle cell is essential in skeletal muscle growth and development (Sotiropoulos et al., 2006). When *GHR-AS* was overexpressed in chicken myoblast, *MyHC*, *MyoD*, and *Myomaker* increased significantly. Previous report indicated that the myoblast fusion was inhibited after the *GHR* gene being knocked down in mouse, and the cross-sectional area and the number of nucleus in myofiber were noted to be lower than the control group (Mavalli et al., 2010). In the *GHR* knocked down mouse, the power of gripping and staying in limbs was decreased (Mavalli et al., 2010). All these research indicated that *GHR* played vital roles in animal myoblast differentiation. It was noteworthy that *GHR-S* increased when *GHR-AS* was overexpressed. This reminds us that *GHR-AS* might function in myoblast differentiation via *GHR-S* and GH-GHR-IGF1 signal pathway.

Migration and fusion are essential parts in myoblast differentiation. Cell movement capacity in migration is higher than any stage of differentiation, and fusion is the progress of recognition and conglutination among cells (Tomé et al., 2011). Scratch wound healing assay revealed a significant delay in the migration of myoblast after overexpressing of *GHR-AS*, indicating its inhibitory action on cell migration. Collectively, these data suggested that *GHR-AS* could promote myoblast differentiation by accelerating fusion and inhibiting migration.

Animal growth and development are regulated by the endocrine growth axis. GH, secreted by pituitary gland, affects tissues and organ development via the GH-GHR-IGF1 signal pathway (Wu et al., 2007). In addition, tissues themselves, like genital gland, also secrete GH protein, promoting *IGFs* expression to regulate cell proliferation, tissue generation, and organism development by paracrine or autocrine manner (Luna et al., 2014; Marisela et al., 2016). Previous studies exhibited that GH protein can promote *IGF1* expression in mouse C2C12 cell (Vincent et al., 2011) and chicken primary hepatocytes (Wang et al., 2013) in vitro. In this study, we added 200 ng/mL GH protein in differentiation medium to stimulate *IGF1* expression. After treatment, we found that *IGF1*, *MyHC*, and the fusion index of myoblast were significantly higher than the control, indicating that the *IGF1* promotes myoblast differentiation. Interestingly, we found that *GHR-AS* and *GHR-S* also increased after adding GH protein in DM. The results demonstrated that *GHR-AS* can promote myoblast differentiation via GH-GHR-IGF1 signal pathway.

The secretion of hormone, like GH, is impulse type in vivo, and treating myoblast cultured in vitro with GH in impulse type can promote *IGF1* expression more than in continuous mode (Frost et al., 2002), in which there are negative feedback in their regulation pattern. In this study, the *IGF1* expressed increased in 6 h but decreased in 12 h, which may be related to the negative feedback regulation.

All together, we concluded that chicken *GHR-AS* promotes myoblast differentiation possibly via GH-GHR-IGF1 signal pathway and interacting with *GHR-S*.

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