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ORIGINAL ARTICLE

KLF5 functions in proliferation, differentiation, and apoptosis of chicken satellite cells

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Abstract

KLF5 is an important regulator of cell proliferation, differentiation, and apoptosis in mammals. Little is known about the function of *KLF5* in the regulation of chicken. Hence, qPCR was used to detect the expression of *KLF5* in different tissues of chicken. And chicken skeletal muscle satellite cells (SMSCs) were transfected *KLF5*-specific small interfering RNA (siRNA) to assay SMSCs' proliferation, differentiation, and apoptosis. The results showed that *KLF5* expressed higher in skeletal muscle than in the other tissues of chicken. Knockdown of *KLF5* significantly inhibited the differentiation and increased apoptosis of chicken SMSCs, but it had no significant effect on proliferation of SMSCs. These results indicate that *KLF5* plays an essential role during myogenesis, which will affect muscle repair and muscle regeneration, and may ameliorate muscle aging or sarcopenia.

Keywords *KLF5* · Chicken satellite cells · Proliferation · Differentiation · Apoptosis

Introduction

Skeletal muscle not only meets the physiological need of animals, but also relates to muscle aging and a variety of common diseases, such as cancer, heart failure, and kidney failure. The number of muscle fibers remains constant basically after animals birth (Stickland et al. 2004); repaired muscle fibers rely on skeletal muscle satellite cells (SMSCs. SMSCs are pluripotent stem cells with differentiation, when adult myofibers are damaged or necrotic, SMSCs will be activated, and then proliferated, differentiated, and fused to form new myofibers to repair damaged one (Buckingham et al. 2014). SMSCs can also differentiate into nerve cells and adipocytes, which can increase the fat content and provide nutrition for new muscles, and, thus, improve the effect of repair in injured muscle. Myogenesis of satellite cells is partially regulated by an orchestration of several major factors, the extracellular matrix (Shin et al. 2012; Velleman 1999), growth factors (Allen et al. 2010; Florini et al. 1991), myogenic regulatory transcription factors (Cornelison et al. 2000; Meadows et al. 2008), and apoptosis (Song et al. 2013).

KLF5 (Krüppel-like factor 5) is a member of the Krüppel-like factor subfamily of zinc-finger transcription proteins; it is a basic transcription factor which regulates genes transcription by binding to GC boxes of gene promoters. Like other KLFs, the *KLF5* has three tandem Cys_2His_2 zinc-finger motifs located at the extreme C-terminus of the protein; the zinc-finger motifs play functions in protein–protein interactions that modulate DNA-binding specificity (Kaczynski et al. 2003). *KLF5* was first identified from a human placenta library using a homology screening strategy (Sogawa et al. 1993). It mediates the signaling functions in cell proliferation, cell cycle, apoptosis, migration, differentiation, and stemness by regulating gene expression in response to environmental stimuli. In NIH3T3 cells, ectopic expression of *KLF5* significantly increased the rate of cell proliferation (Sun et al. 2001), *KLF5* is upregulated in oncogenic H-Ras-transformed NIH3T3 cells, knockdown of *KLF5* expression leads to a decreased proliferation rate and a significant reduction in colony formation (Nandan et al. 2004). In adipocytes, *KLF5* is induced at an early stage of differentiation by C/EBPβ/δ, and followed binding to the promoter of *PPARγ* gene, which activates *PPARγ* gene expression to promote adipocyte differentiation (Oishi et al. 2005). It has also been reported that *KLF5* has an anti-apoptosis function.

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Liu et al. suggest that *KLF5* can promote breast epithelial cell survival by increasing the MKP-1 protein levels. *KLF5* can also make smooth muscle cell resistant to apoptosis, in vascular lesions; *KLF5* confers apoptotic resistance through interacting with PARP-1, which is a nuclear enzyme important in DNA repair and apoptosis (Suzuki et al. 2007).

In summary, there are lots of evidences to show that *KLF5* was essential for the proliferation, differentiation, and apoptosis in different cell types. However, these studies have only been carried out in mammals, no relevant studies have been reported in poultry. In this study, we examined the role of *KLF5* in proliferation, differentiation, and apoptosis of chicken SMSCs by determining the effects of *KLF5* knockdown by *KLF5*-specific small interfering RNA (siRNA).

Materials and methods

Isolation, culture, and transfection of chicken SMSCs

Twenty male Ross-308 chickens with 5 days old were sacrificed to isolate SMSCs. Primary chicken SMSCs were isolated as described by Bai et al. (2012) Chicken SMSCs were initially cultured in growth medium composed of 1% blue streptomycin mixture, 10% gestational horse serum, 10% fetal bovine serum (FBS), and Dulbecco's Modified Eagle Medium (DMEM) of 0.5% chicken embryo extract at 37 °C under 5% CO_2 with saturating humidity. Differentiation was induced by replacing the original growth medium with DMEM containing 2% of gestational horse serum, 5% of FBS, and 1% of blue streptomycin mixture.

In this study, three siRNAs for *KLF5* were designed and synthesized (synthesized and provided by GenePharma, China) based on the mRNA sequence of Gallus gallus *KLF5* gene (Gene ID: 418818) (Table 1). SMSCs were plated in 6-well plates and grown to approximately 70–80% confluence, and cells were transfected with *KLF5* siRNAs and negative siRNA. Cell transfection was performed using the reagent protocol Lipofectamine 3000 (Invitrogen, USA). The Lipofectamine 3000 and siRNA were diluted with optim-MEM culture medium. The diluted siRNA and TransEasy were mixed uniformly and placed at room temperature for 15 min. The composite was added to the cell culture plate and mixed in the culture plate. Knocking down efficiency was estimated by quantitative qPCR of *KLF5* mRNA.

All experimental procedures involving animals were approved by the Animal Care and Use Committee of College of Animal Science and Technology, Sichuan Agricultural University (No. S20163637), and were carried out in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals.

Quantitative qPCR

Total RNA from tissue or cells was isolated by Total RNA Isolation Kit reagent (ROREGENE, China). RNA quality and concentration were evaluated by an Agilent 2100 Bioanalyzer (Agilent Technologies, USA). qPCR primers (Table 2) were designed by Primer Premier 5 software. A 15 µL reaction containing 6.5 µL of SYBR premix Ex Taq TM (TaKaRa Biotechnology, China), 1.5 µL of cDNA, 0.3 µL of forward primer, 0.3 µL of reverse primer, and 6.4 µL of RNasefree H_2O (Tiangen, China) was used for qPCR. The reaction was carried out with the following amplification conditions: 95 °C for 30 s, followed by 40 cycles of 95 °C for 10 s, primer-specific annealing temperature for 10 s, and 72 °C for 10 s (Yu et al. 2018). Each experiment was biologically replicated three times. The qPCR data were normalized relative to the expression of *β-actin* (endogenous control) and the $2^{-\Delta\Delta Ct}$ method was applied to quantify mRNA expression levels.

Cell proliferation assay

SMSCs viability was analyzed by cell counting kit-8 (CCK-8) (Bestbio Biotechnology, China). SMSCs were seeded at a density of 5×10^3 cells/well in a 96-well plate; after transfection negative control siRNA or KLF5 siRNA, cells were incubated with 10 µl CCK-8 for additional 3 h at 37 °C; the absorbance at 450 nm was measured using a Microplate Reader (Thermo, USA). The samples from each treatment at each point had ten replicates.

Western blot analysis

Total cellular proteins from cells were isolated by lysing cells in ice-cold radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, and 1 mM EDTA). Protein samples from individual experiments were pooled

Table 1 Design of siRNA target on chicken *KLF5* CDS

siRNA	Sense strands (5′→3′)	Anti-sense strands (5′→3′)
KLF5 siRNA 1	GAAGUACAGAAGAGACAGUTT	ACUGUCUCUUCUGUACUUCTT
KLF5 siRNA 2	UUCACAACCCGAAUUUACCTT	GGUAAAUUCGGGUUGUGAATT
KLF5 siRNA 3	GUAACCCAGAUUUGGAGAATT	UUCUCCAAAUCUGGGUUACTT
Negative siRNA	UUCUCCGAACGUGUCACGUTT	ACGUGACACGUUCGGAGAATT

Table 2 Primers for qPCR

Gene	Primer sequences (5′→3′)	Annealing temperature (°C)	Product (bp)
KLF5	F: CGCGCTCGGATGAATTAACG	60	164
	R: GATACAGAACAGCCTCGGCA		
PCNA	F: AACACTCAGAGCAGAAGAC	54	225
	R: GCACAGGAGATGACAACA		
CCND1	F: CTCCTATCAATGCCTCACA	54	165
	R: TCTGCTTCGTCCTCTACA		
MyoD	F: GCCGCCGATGACTTCTATGA	60	66
	R: CAGGTCCTCGAAGAAGTGCAT		
MYHC	F: GAAGGAGACCTCAACGAGATGG	60	138
	R: ATTCAGGTGTCCCAAGTCATCC		
Caspase-3	F: CGGACTGTCATCTCGTTCA	57	186
	R: TGGCTTAGCAACACACAAAC		
Survivin	F: GCCTATGCTGAAATGCTGCC	60	246
	R: CGCGGAGTGCTTTTTGTGTT		
β-actin	F: GTCCACCGCAAATGCTTCTAA	59	78
	R: TGCGCATTTATGGGTTTTGTT		

for western blotting analysis. The following primary antibodies were used: anti-MYHC (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:500 dilution), anti-MyoG (Abcam, San Francisco, CA, USA; 1:500 dilution), and anti-β-actin (N21; Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:1000 dilution). The membranes were incubated with antibodies at 4 °C overnight and then washed in washing buffer [10 mM Tris-HCI, pH 7.5, 100 mM NaCI, and 0.1% (v/v) Tween 20]. Next, the membranes were treated with horseradish peroxidase-conjugated IgG antibody (Santa Cruz Biotechnology; 1:2000 dilution) for 2 h at room temperature, making use of chemiluminescence as a result. Each experiment was biologically replicated three times. Densitometric analysis of the bands, relative to β-actin, was performed using ImageJ software (National Health Institute, Bethesda, MD, USA). The relative expression of protein was analyzed with Quantity One software.

Cell apoptosis assay

Cells cultured in 6-well plates and the control group was incubated with 0.5 μM staurosporine (STS) at 37 °C with 5% CO_2 for 12 h. Apoptosis was measured using an eBioscience™ Annexin V-FITC Apop Kit apoptosis detection kit (Invitrogen, AU). Briefly, cells cultured in 6-well plates were trypsinized, washed, and stained with Annexin V-FITC under darkness for 10 min, and then stained with PI under darkness for 5 min at room temperature, analyzed by flow cytometry (Backman; USA).

Statistical analyses

Comparisons between two groups were analyzed using two-tailed student's *t* test. Differences among more than two groups were analyzed using one-way ANOVA followed by Tukey–Kramer post hoc tests. All these analyses were performed using the JMP Pro software (SAS, NC, USA). Values of $p < 0.05$ were considered statistically significant. All data are shown as mean ± SEM.

Results

Expression of *KLF5* gene in different tissues of chickens

According to qPCR, the expression of *KLF5* mRNA was mainly detected in breast muscle and leg muscle among 12 different chicken tissues or organs (Fig. 1). Relative expression levels followed the intestine, kidney, fat, and glandular stomach. The heart, liver, spleen, lung, muscular stomach, and brain were extremely low.

Confirmation of interference efficiency of KLF5 gene

To determine the potential role of *KLF5* in chicken SMSCs proliferation, differentiation, and apoptosis, three siRNA molecules were transfected in chicken SMSCs. Cells were transfected with three siRNAs for 24 h and the interference efficiency was quantitatively detected by qPCR, and the interference efficiency was 65.8, 32.6, and 34.2%

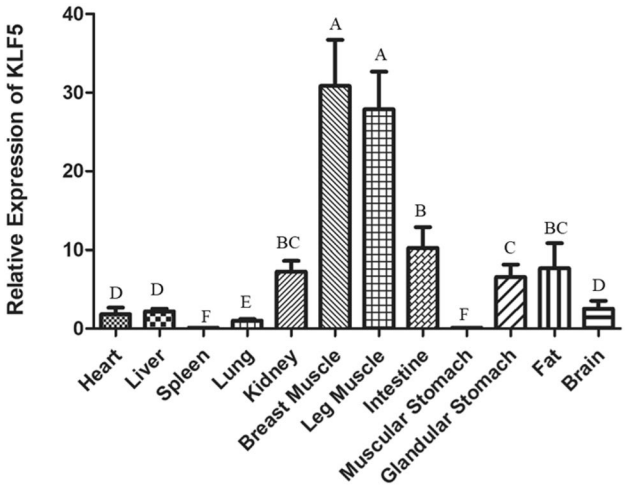


Fig. 1 The *KLF5* mRNA express in different tissues and organs of chicken. The mRNA expression level was measured by qPCR and results are averaged from three independent replicates at 4 days old. The expression level was normalized to β-actin and measured with $2^{(-\Delta\Delta Ct)}$ value. Data are expressed as the mean ± SEM. $n = 3$

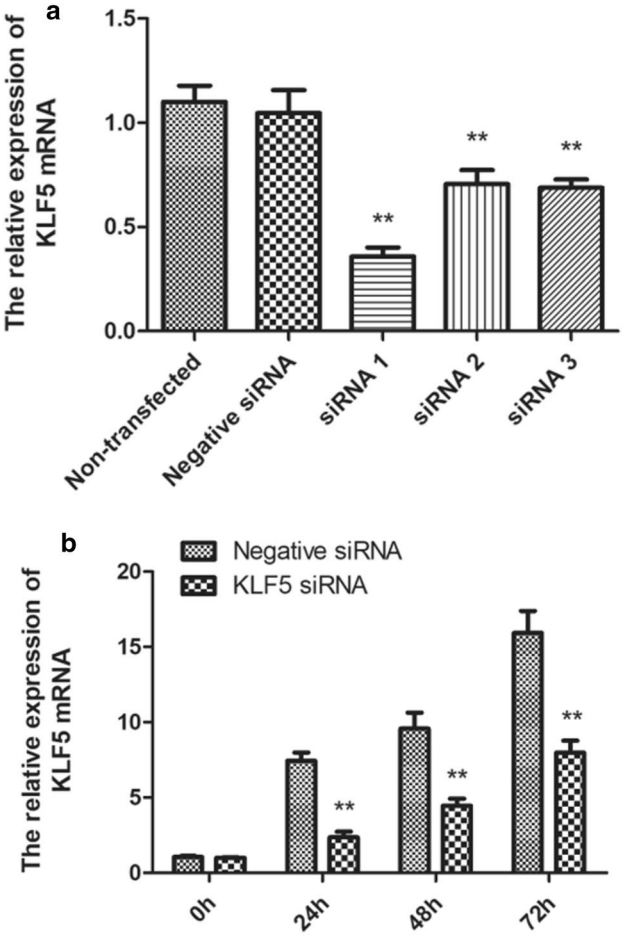


Fig. 2 The siRNA molecules interfere with mRNA expression of *KLF5* in the chicken SMSCs. **a** Interference efficiency among the three different *KLF5*-siRNA on 24 h. **b** Expression of *KLF5* at 0, 24, 48, and 72 h after transfection of siRNA1 and negative siRNA. The mRNA expression level was measured by qPCR, normalized to *β*-actin and measured with $2^{(-\Delta\Delta Ct)}$ value. Data are expressed as the mean ± SEM. $n = 3$. $^{**}p < 0.01$ vs the negative siRNA groups

respectively (Fig. 2a). We chose siRNA1 to subsequent research because of its highest interference efficiency. Based on the results, the expression levels of *KLF5* showed gradually increasing at 0 h, 24 h, 48 h, and 72 h (Fig. 2b).

Effects of *KLF5* gene knockdown on the proliferation of chicken SMSCs cells

To determine the effects of *KLF5* knockdown on cell proliferation, SMSCs were transfected with *KLF5* siRNA and negative siRNA after SMSCs achieving 30% cell confluence for 0 h, 24 h, 48 h, and 72 h. Interference effects of *KLF5* on SMSCs proliferation rate were detected by CCK-8. The number of cells displayed almost linearly growth during the 72 h period, but there was no significant difference between *KLF5* siRNA group and negative siRNA group (Fig. 3a). The regulated factors of *CCND1* and *PCNA* were closely related with cell proliferation (Chen et al. 2017; Guzińska-Ustymowicz et al. 2009), their expression levels were detected by qPCR, and the results also showed that the mRNA expression levels of *CCND1,* aznd *PCNA* were no significant difference between *KLF5* siRNA group and negative siRNA group (Fig. 3b). These results showed that *KLF5* had no effect on the proliferation of chicken SMSCs.

Effects of *KLF5* gene knockdown on the differentiation of chicken SMSCs cells

To investigate the effects of *KLF5* knockdown on cell differentiation, SMSCs were transfected with negative siRNA and *KLF5* siRNA, respectively, after SMSCs achieving 80% cell confluence. As shown in Fig. 4a, morphologically, in negative siRNA group, the SMSCs began to differentiate into myotubes at 24 h after transfecting negative siRNA. At 48 h, the SMSCs were well differentiated into larger myotubes, and the number of myotubes was increased dramatically at 72 h. In contrast, the cells transfected with *KLF5* siRNA did not appear to undergo myotube formation. The bright-field images confirmed that differentiation in the SMSCs transfected with *KLF5* siRNA was decreased significantly at 24 h, 48 h, and 72 h fusion compared with negative siRNA group. After immunofluorescence staining, we found that the knockdown of *KLF5* gene inhibited the differentiation of SMSCs and significantly reduced the total areas of myotubes (Fig. 4b).

To further test the differentiation effect of *KLF5* on SMSCs, the mRNA and protein expression levels of *MYHC*

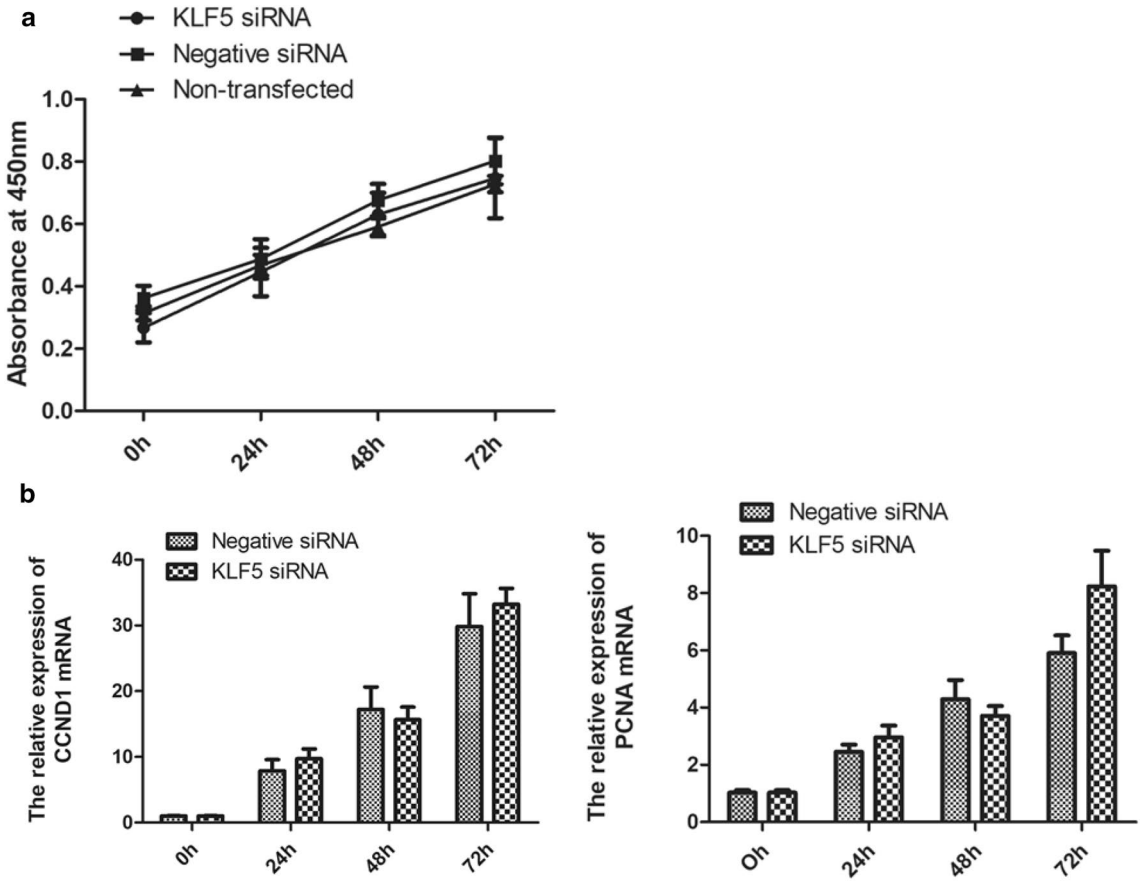


Fig. 3 Effect of *KLF5* knockdown on the proliferation of chicken SMSCs. **a** Proliferation rate of SMSCs' cells transfected with *KLF5* siRNAs, negative siRNA, or non-transfected. Cell proliferation rate was estimated by CCK-8 at 0 h, 24 h, 48 h, and 72 h after transfection. The absorbance at 450 nm on the *y*-axis represents the number of viable cells. The proliferation rate was not different between cells transfected with *KLF5* siRNAs and negative siRNA or non-transfected. **b** Relative mRNA expression levels of *CCND1* and *PCNA* at 0 h, 24 h, 48 h, and 72 h detected by qPCR. Data are expressed as the mean ± SEM. $n = 3$

and *MyoG* related to muscle differentiation were detected in 0 h, 24 h, 48 h, and 72 h after transfected with *KLF5* siRNA and negative siRNA. Results showed that the mRNA and protein expression levels of *MYHC* and *MyoG* in SMSCs with *KLF5* siRNA were significantly lower than negative siRNA groups in 24 h, 48 h, and 72 h ($p < 0.01$) (Fig. 4b, c). These results indicated that *KLF5* had a positive relationship with the differentiation of chicken SMSCs.

Effects of *KLF5* gene knockdown on the apoptosis of chicken SMSCs' cells

To explore the effects of *KLF5* knockdown on cell apoptosis, flow cytometry was performed to detect the cell apoptosis rate after transfected with *KLF5* siRNA and negative siRNA on 48 h, and the control group was transfected STS which can induce apoptosis before 12 h of detection. The results showed that the apoptotic rate of *KLF5* siRNA group was significantly higher than negative siRNA group whether STS was transfected or not (Fig. 5a). *Caspase-3* is widely believed to be a pro-apoptotic factor, the *survivin* is a survival factor making cells resistant to apoptosis, and their expression levels are closely related with the apoptosis. In the present study, the mRNA levels of *caspase-3* were significantly upregulated and *survivin* were significantly downregulated in the *KLF5* knockdown group ($p < 0.01$) in 24 h, 48 h, and 72 h after transfected with *KLF5* siRNA (Fig. 5b). Altogether, knockdown of *KLF5* could induce the apoptosis in chicken SMSCs.

Discussion

The previous studies have shown that *KLF5* played an essential role for the cell proliferation, differentiation, and apoptosis in mammals (Dong and Chen 2009), but its regulatory role in poultry is not clear. In this study, we determined the expression pattern of *KLF5* gene in chicken. *KLF5* was

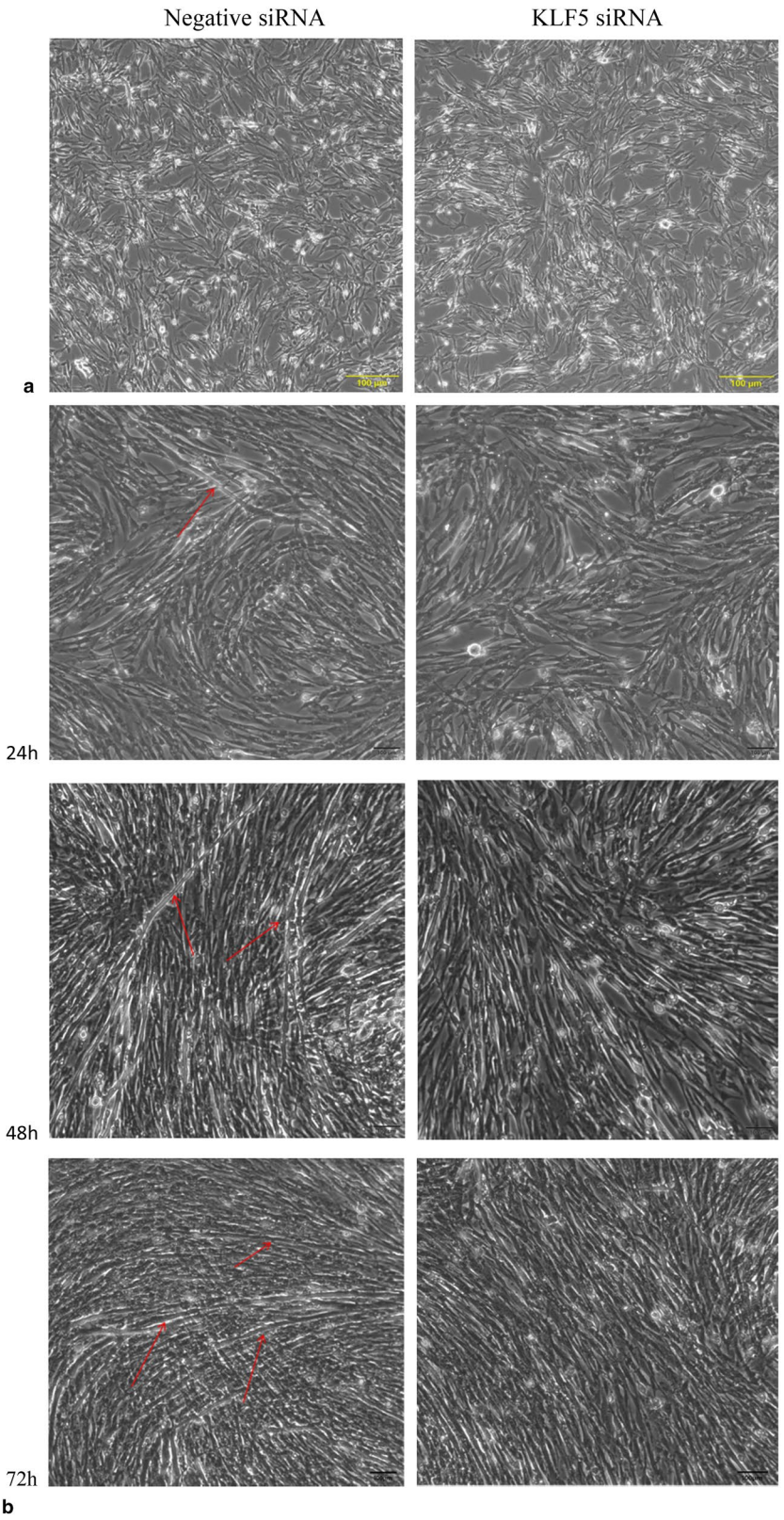
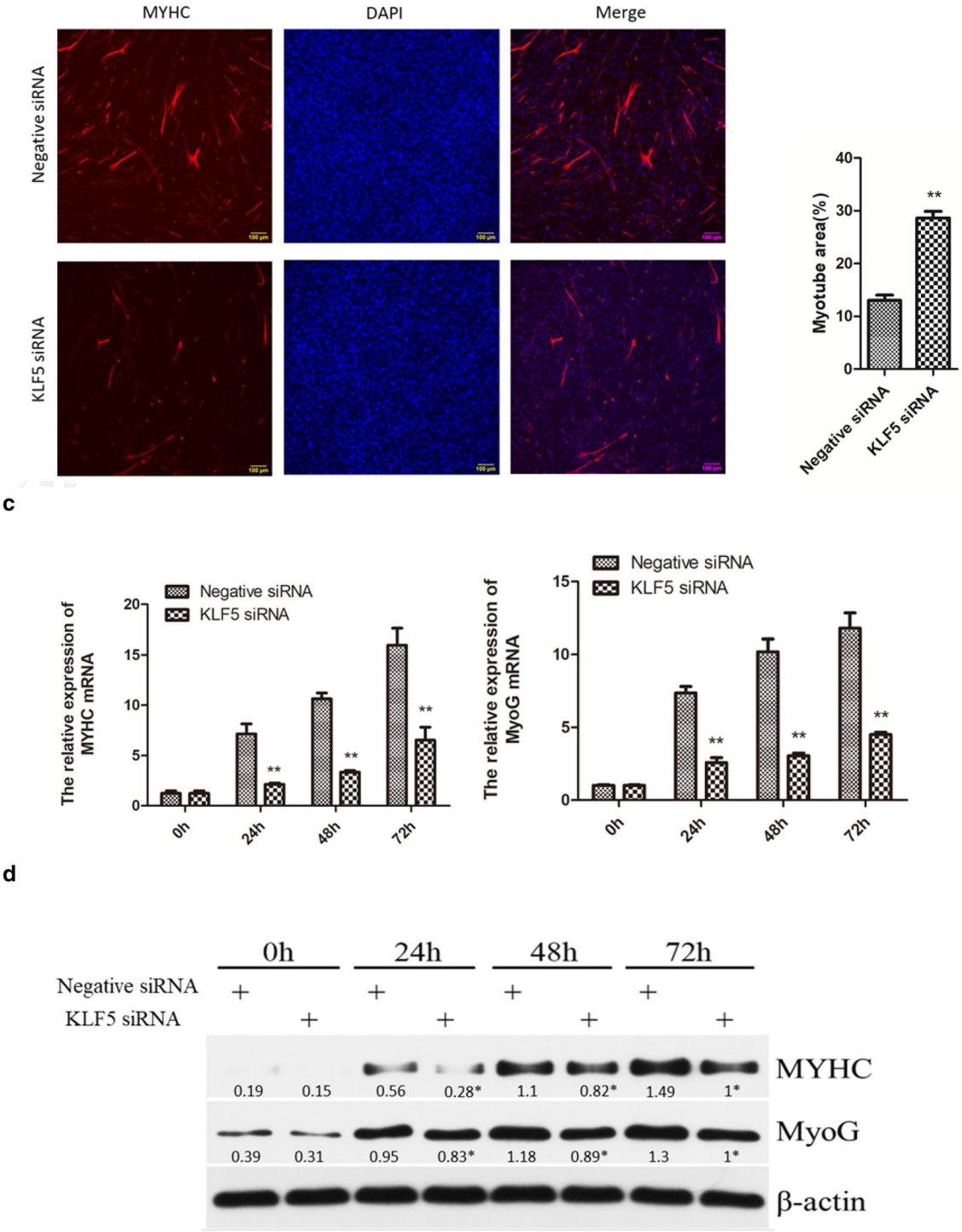


Fig. 4 Effect of *KLF5* knockdown on the differentiation of chicken SMSCs. SMSCs were transfected with *KLF5* siRNA or negative siRNA, and cultured in differentiation medium for the following experiments. **a** The myotube formation of cells transfected at 0 h, 24 h, 48 h, and 72 h was observed under the light microscope. Red arrows indicate myotube. Yellow scale bar = 100 μm. **b** Immunofluorescence SMSCs transfected with *KLF5* siRNA or negative siRNA were induced to differentiate for 72 h, and then, cells were stained with MYHC antibody and DAPI (nuclei). Scale bars = 100 μm. **c** Relative mRNAs' expression levels of *MyoG* and *MYHC* at 0 h, 24 h, 48 h, and 72 h of differentiation. **d** Western blots of *MyoG*, *MYHC,* and *β-actin* (loading control) proteins at 0 h, 24 h, 48 h, and 72 h of differentiation. Densitometric analysis was determined with ImageJ and average values from three separate experiments. $*p < 0.05$, $** p < 0.01$ v.s. the negative siRNA groups. Data are expressed as the mean ± SEM. $n = 3$

highly expressed in skeletal muscle compared with other tissues in chicken, which indicated that *KLF5* is important in the growth, maintenance, and function of chicken skeletal muscle. Hence, we knocked down *KLF5* to identify the potential regulation mechanisms of *KLF5* in chicken SMSCs proliferation, differentiation, and apoptosis.

Fig. 4 (continued)



After *KLF5* knockdown, the proliferation rate of SMSCs had no significant difference compared with control group ($p > 0.05$), and both *CCND1* and *PCNA* related to SMSCs proliferation also had no significant difference ($p > 0.05$) on mRNA levels compared with the control group. Although the previous reports have shown the involvement of *KLF5* in controlling cell proliferation in several cancer cell lines (Chen et al. 2010; Dong et al. 2012; Yang et al. 2005), but Hayashi et al. (2016) found that *KLF5* did not affect C2C12 myoblasts cell proliferation. Combined with our present results suggests that *KLF5* has no direct regulatory effect on the proliferation in SMSCs of chicken. And we speculate that *KLF5* is not involved in the proliferation of muscle cells.

Knockdown *KLF5* in differentiated SMSCs, myotube formation was blocked. In addition, muscle differentiation-related genes *MYHC* and *MyoG* exhibited significantly lower

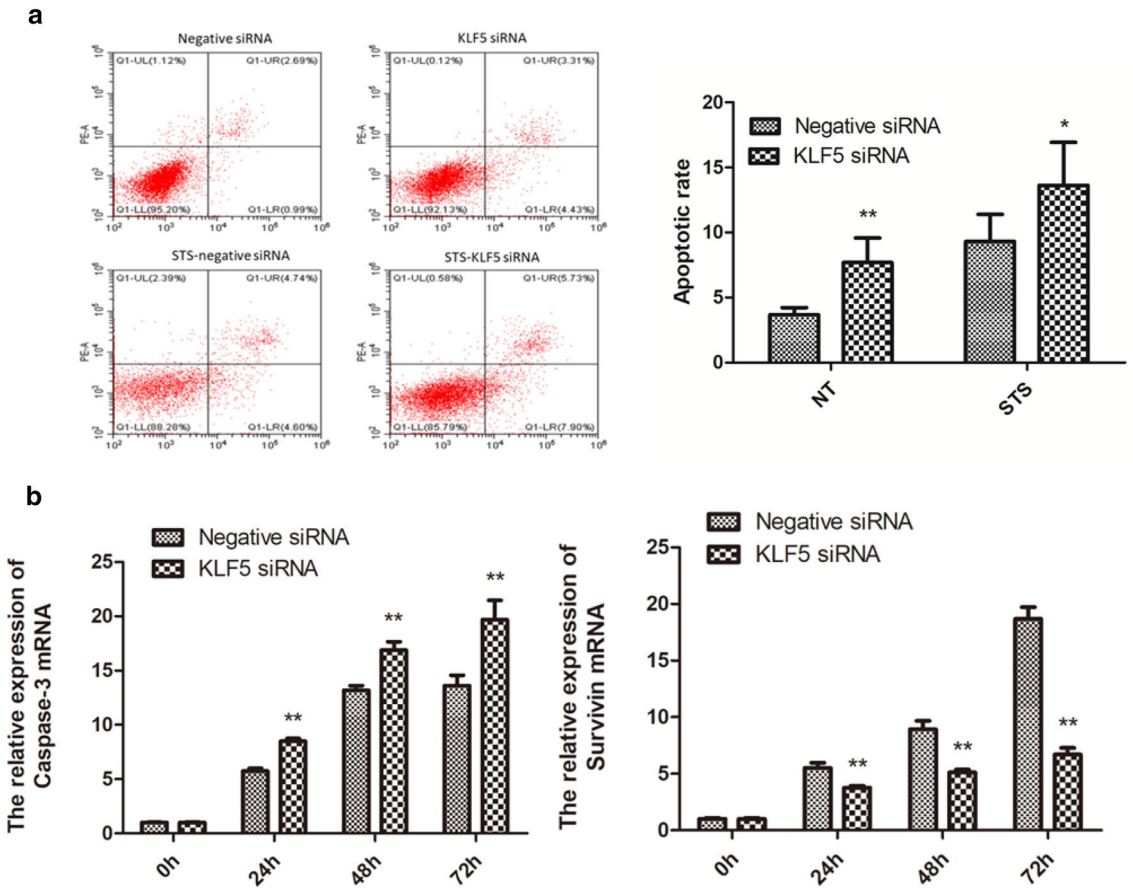


Fig. 5 Effect of *KLF5* knockdown on the apoptosis of chicken SMSCs. **a** Apoptosis in SMSCs was measured by flow cytometry following *KLF5* siRNA or negative siRNA transfection at 48 h; the control groups were treated with 0.5 μM STS for 12 h. **b** Relative mRNA expression levels of *caspase-3* and *survivin* at 0 h, 24 h, 48 h, and 72 h $*p<0.05$, $**p<0.01$ vs the negative siRNA groups. Data are expressed as the mean ± SEM. $n=3$

expression levels of mRNA and protein compared with the control group. These results were consistent with Hayashi et al. (2016), they found that myotube formation was significantly impaired in the KLF5-null C2C12 cells of mice using CRISPR-Cas 9 system compared with the control group, and expression of *MYHC* and *MyoG* was significantly reduced in *KLF5*-null group. To determine whether forced expression of *KLF5* could rescue the compromised differentiation of *KLF5*-null cells, they exogenously introduced a retrovirus harboring murine *KLF5*, found *KLF5*-null cells successfully formed myotubes when stimulated to differentiate, and the expression of *MYHC* and *MyoG* was partially rescued by the forced expression of *KLF5*. These evidences indicate that *KLF5* is essential for myogenic differentiation. *KLF5* regulates skeletal muscle differentiation acting in concert with myogenic transcription factors such as *MyoD* and *Mef2*, and *MyoD* recruitment will greatly reduce in the absence of *KLF5* (Hayashi et al. 2016). In the other hand, *KLF5* is also a regulator of many others' cell differentiation, such as embryonic stem cells (Parisi et al. 2008), smooth muscle cell (Nagai et al. 2003), and epithelial cell (Yang et al. 2007). In summary, *KLF5* plays an important role in promoting differentiation of many kinds of cells including skeletal muscle cells.

Knockdown *KLF5* in chicken SMSCs, the apoptotic rate was significantly higher than negative siRNA group, the same as the results with transfecting STS which can induce apoptosis, and the mRNA expression levels of *survivin* were significantly downregulated and *caspase-3* were significantly upregulated compared with negative siRNA group. The results were similar to the previous studies. Zhu et al. (2006) found that *KLF5* could induce the expression of *survivin*, and *KLF5* binded to the promoter of *survivin* and interacted with *p53* to abrogate *p53*-repressed *survivin* expression. *KLF5* can also regulate apoptosis independent of *p53*, knockdown *KLF5* would induce apoptosis, the apoptotic phenotype was associated with reduced bad phosphorylation, and downregulation of *Pim1* survival kinase, and transfection of wild-type *Pim1* was sufficient to rescue the phenotype (Zhao et al. 2008). And Xiaochen et al. (2014)

found that *KLF5* knockdown inhibited hypoxia-induced cell survival and promoted cell apoptosis by direct interaction with *HIF-1α* to actively downregulate *cyclinB1* and *survivin,* and upregulate *caspase-3*. Combined with these studies, we suggest that *KLF5* negatively regulates SMSCs' apoptosis, which promoted cell survival.

In summary, this study analyzed the effects of *KLF5* on proliferation, differentiation, and apoptosis in SMSCs of chicken. We found that knockdown *KLF5* suppressed SMSCs differentiation and induced SMSCs apoptosis, but there was no direct effect on SMSCs proliferation, suggesting that *KLF5* plays an essential role during myogenesis, which will affect muscle repair and regeneration of muscle, and may ameliorate muscle aging or sarcopenia. Further studies will be needed to investigate the application of *KLF5* as a skeletal muscle repair factor in chicken muscle development. Verification of the anti-apoptotic function of *KLF5* in future studies may allow the development of new therapies for poultry disease.

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Compliance with ethical standards

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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