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Effect of thermal manipulation during embryogenesis on the promoter methylation and expression of myogenesis-related genes in duck skeletal muscle

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ABSTRACT

Avian embryos are an ideal system to investigate the effect of incubation temperature on embryonic development, but the characteristics and mechanisms of temperature effects on poultry embryonic myogenesis are unclear. In this study, we investigated the effect of increasing the incubation temperature by 1 °C on the expression of nine myogenesis-related genes in ducks and then explored the correlation between the alteration of promoter methylation and the expression of two of the nine genes under thermal manipulation (**TM**). The qRT-PCR results showed that TM during embryonic days (**ED**) 1–10 promoted ($P < 0.05$) the expression of genes in breast muscle (*PAX3*, *PAX7*, *MYOG*, *MCK*, *SIX1*, *TNNC1*) and leg muscle (*MYOD*, *MYOG*, *MYF5*, *MCK*, *AKIRIN2*, *TNNC1*). TM during ED10-20 promoted the expression of *PAX3*, *MYF5* and *MCK* and inhibited *AKIRIN2* expression in breast muscle ($P < 0.05$); however, it inhibited the expression of *PAX3*, *PAX7*, *MYOD*, *MYOG*, *MYF5*, *SIX1*, *AKIRIN2* and *TNNC1* and promoted *MCK* expression in leg muscle ($P < 0.05$). TM during ED20-27 inhibited the expression of genes in breast muscle (*PAX7*) and leg muscle (*MYOD*, *MYOG*, *MYF5*, *TNNC1*) and promoted *MCK* expression in breast and leg muscle ($P < 0.05$). Furthermore, with the Sequenom MassARRAY platform, it was observed that the average methylation level of *AKIRIN2* (ED10) and *TNNC1* (ED20) in leg muscle decreased ($P < 0.05$) after TM. Notably, we found significant ($P < 0.05$) inverse correlations between the methylation and mRNA levels of *AKIRIN2* under TM during ED1-10 ($r = -0.969$) and ED10-20 ($r = -0.805$). Taken together, TM during ED1-10 was more favorable for improving duck myogenesis-related gene expression than TM during ED10-20 and ED20-27. TM during duck embryogenesis seemed to have a greater effect on the development of leg muscle than breast muscle and might alter *AKIRIN2* expression by changing its promoter methylation status. These findings may be helpful to understand temperature effects on the muscle development of avian embryos and to explore the role of epigenetic regulation during this process.

1. Introduction

Avian embryos develop in the egg and are more easily affected by environmental factors than mammals. Among the environmental factors that impact egg development, incubation temperature is recognized as one of the most important (Deeming, 2002). In chicken and turkey, slight changes in incubation temperature had profound impacts on various phenotypes (Barri et al., 2011; Loyau et al., 2014; Maltby et al., 2004) and even caused prolonged effects on postnatal development (Piestun et al., 2013, 2009). Our previous studies on duck embryos showed that increasing the incubation temperature by 1 °C did not decrease the hatching rate (Liu et al., 2015) but influenced immune organ development (Liu et al., 2013) and lipid metabolism (Wang et al., 2014). Furthermore, some studies suggested that appropriate thermal manipulation (**TM**) during embryogenesis could improve the thermal tolerance of poultry and was beneficial to animal welfare (Loyau et al., 2016, 2014; Piestun et al., 2008).

It is well known that the embryo stage is critical for meat production in most farm animals, including poultry. The total number of muscle fibers is fixed during embryogenesis, and thereafter, muscle mass increases primarily depend on the lengthening and thickening of muscle fibers (Picard et al., 2002). By investigating physiological and

Abbreviations: ED, embryonic days; TFBS, transcription factor binding sites; TM, thermal manipulation

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biochemical indexes, some researches indicated that TM affected embryonic muscle development and postnatal muscle growth (Maltby et al., 2004; Piestun et al., 2015, 2013). TM could also affect the expression of genes closely related to embryonic myogenesis, including *PAX7* and *MYF5* (Al-Musawi et al., 2012; Gabriel et al., 2003). Interestingly, the effects of TM varied at different embryonic stages and in different muscle tissues. TM during embryonic days (**ED**) 8–10 slightly decreased the ultimate pH of chicken breast meat, whereas TM during ED16-18 increased breast meat yield without affecting the ultimate pH (Collin et al., 2007). Our previous study found that TM during ED11-20 caused differential effects on the phenotypes of duck breast and leg muscle (Liu et al., 2015). However, research focusing on the tissue- and developmental stage-specific effects of TM during embryogenesis on myogenesis-related gene expression is limited.

Although reports have described temperature effects on avian embryonic myogenesis, the underlying mechanisms have not been clarified. DNA methylation is one of the most common epigenetic events that can modify gene activity (Das and Singal, 2004) and repress transcription (Bird, 1992). Palacios and Puri (2006) reported that DNA methylation also regulates myogenesis. Burgerhout et al. (2017) found that epigenetic variation in Atlantic salmon might be involved in the local adaptation to fluctuating temperatures. Recently, we raised the incubation temperature from 37.8 °C to 38.8 °C during ED1-10, ED10-20 and ED20-27 to examine the effect of TM on DNA methylation (Yan et al., 2015). The results showed that TM had effects on the expression of *DNMTS* and *MBPS* gene families and on the activities of corresponding enzymes in embryonic duck breast and leg muscle. The *DNMTS* and *MBPS* gene families were proven to play a key role in the establishment and maintenance of genomic DNA methylation patterns (Geiman and Muegge, 2010).

Thus, the aim of the present study was to explore the effects of TM during different embryonic stages on the expression of nine myogenesis-related genes in duck breast and leg muscle and then to evaluate the correlation between the methylation and mRNA expression levels of two of the nine genes under TM. These experiments may provide clues to how TM affects muscle development and knowledge for future investigations regarding poultry production.

2. Materials and methods

2.1. Thermal treatments and sample collection

According to our previous study (Yan et al., 2015), the embryonic development of duck is divided into three phases: early embryonic stage (ED1-10), middle embryonic stage (ED10-20) and late embryonic stage (ED20-27). In this study, we performed TM during the above three stages. More than 100 Peking duck eggs with a weight within the range of 84.0 ± 2.0 g were obtained from the Sichuan Agricultural University Waterfowl Breeding Experimental Farm. All eggs were randomly divided into two groups: eggs in the treatment group were incubated at 38.8 °C, while eggs in the control group were incubated at 37.8 °C. Subsequently, 15 eggs in the control group were transferred to the treatment group at ED10 and ED20. The detailed conditions of incubation and TM have been previously described (Yan et al., 2015).

At ED10, ED20 and ED27, six ducklings were randomly selected from each group, and the breast and leg muscle samples were isolated in these 36 individuals. All samples were immediately frozen in liquid nitrogen and stored in a − 80 °C freezer until the further extraction of genomic DNA and total RNA. The procedures in this study were conducted in compliance with the requirements of the Institutional Animal Care and Use Committee of Sichuan Agricultural University.

2.2. DNA preparation, RNA extraction and cDNA synthesis

Genomic DNA was extracted from leg muscle samples using a TIANamp Genomic DNA Kit (Tiangen Biotech, Beijing, China) and stored at − 20 °C until cloning and quantitative DNA methylation assays. The total RNAs of each sample were isolated using Trizol Reagent (Takara, Dalian, China) and quantified using a Nanodrop2000 spectrometer. The first-strand cDNAs were synthesized from the total RNAs by using a reverse transcript system (Takara) and finally stored at − 20 °C.

Table 1
Primer sets used for qRT-PCR.

Gene	Primer sequence (5′-3′)	Product length (bp)	Tm (°C)
β-actin	Forward: GCTATGTCGCCCTGGATTTC Reverse: CACAGGACTCCATACCCAAGAA	168	60
GAPDH	Forward: AAGGCTGAGAATGGGAAAC Reverse: TTCAGGGACTTGTCATACTTC	254	60
PAX3	Forward: GTCAATCAGCTCGGAGGAGT Reverse: TCTCCTGGTACCTGCAGAGA	133	59
PAX7	Forward: GAGTTCAGGTGTGGTTCAGCA Reverse: GAAATGGTGGTGGTTGGGTAG	169	60
MYOD	Forward: GCAACGCCATCCGCTACAT Reverse: GCAATCAAGGCTGGAAACAACA	85	55
MYOG	Forward: CGGATCACCTCCTGCCTGA Reverse: CGTCCTCTACGGCGATGCT	87	63
MYF5	Forward: AGGAGGAGGCTGAAGAAAGTGA Reverse: GCTCTGTCTCGGCAGGTGATA	180	60
MCK	Forward: GATCTCCTCGATCTTCTTGAGG Reverse: GCATCTGGCACAATGACAAC	143	60
SIX1	Forward: GATGCTGCCGTCGTTTGG Reverse: AGGACGCTCTCGTTCTTGTG	144	60
AKIRIN2	Forward: TCTACTGATGCACAGCCACAT Reverse: TTCACGCTCTTTCAGCAAAC	153	60
TNNC1	Forward: CGGTAGAGCAGTTGACAGAAGA Reverse: CAGCATCCTCATCACCTTCC	122	61

2.3. Quantitative real-time PCR

In this study, the mRNA expression levels of nine genes (*PAX3*, *PAX7*, *MYOD*, *MYOG*, *MYF5*, *MCK*, *SIX1*, *AKIRIN2*, *TNNC1*) were detected. These genes were selected due to their important roles in the different steps of myogenesis (Pajcini et al., 2008; Palacios and Puri, 2006; Sousa et al., 2006; Sun et al., 2016; Yajima et al., 2010). At ED10, ED20 and ED27, the mRNA expression levels of the nine myogenesis-related genes were measured in each group using SYBR PrimerScript RT-PCR kit (Takara) and an iQ5 system (Bio-Rad, Hercules, USA). All reactions were repeated three times. The identity of the amplified products was confirmed by sequencing (Huada Gene, Beijing, China). The primers were designed using Primer Premier 5 software and synthesized by Huada Gene, and the primer information is listed in Table 1.

2.4. Sequence and quantitative DNA methylation analysis

Considering the extent of changes in mRNA expression, two out of the nine myogenesis-related genes (*AKIRIN2* and *TNNC1*) were chosen for further analysis. Based on two reference sequences in GenBank (NW_004678519.1 for *AKIRIN2* and NW_004677655.1 for *TNNC1*), the transcription start sites of duck *AKIRIN2* and *TNNC1* were predicted using the Neural Network Promoter Prediction (http://www.fruitfly.org/seq_tools/promoter.html). We determined two sequences that contained 5000 bp upstream and 1000 bp downstream of the transcription start sites and then searched for potential CpG islands using the CpG Island Searcher (http://cpgislands.USC.edu/). Finally, one CpG island of each gene was selected for the DNA methylation analysis.

By using MethPrimer (http://www.urogene.org/methprimer/), we designed primers (Table 2) for *TNNC1* and *AKIRIN2* to cover the regions with most CpG sites in the selected CpG islands. For PCR

Table 2
Primer sets used for the methylation assay.

Gene	Primer sequence (5′-3′)	Product length (bp)	Tm (°C)
AKIRIN2	Forward: AGAGGAGGCTGAAGTAGGGCACTGC Reverse: GGTAACAAGGCAAACAATTATTTTC	407	56
TNNC1	Forward: AATAGGTGGTAGTGATATGGTGGTA Reverse: CAACCATCCTCATATCCTCAATTAC	438	58

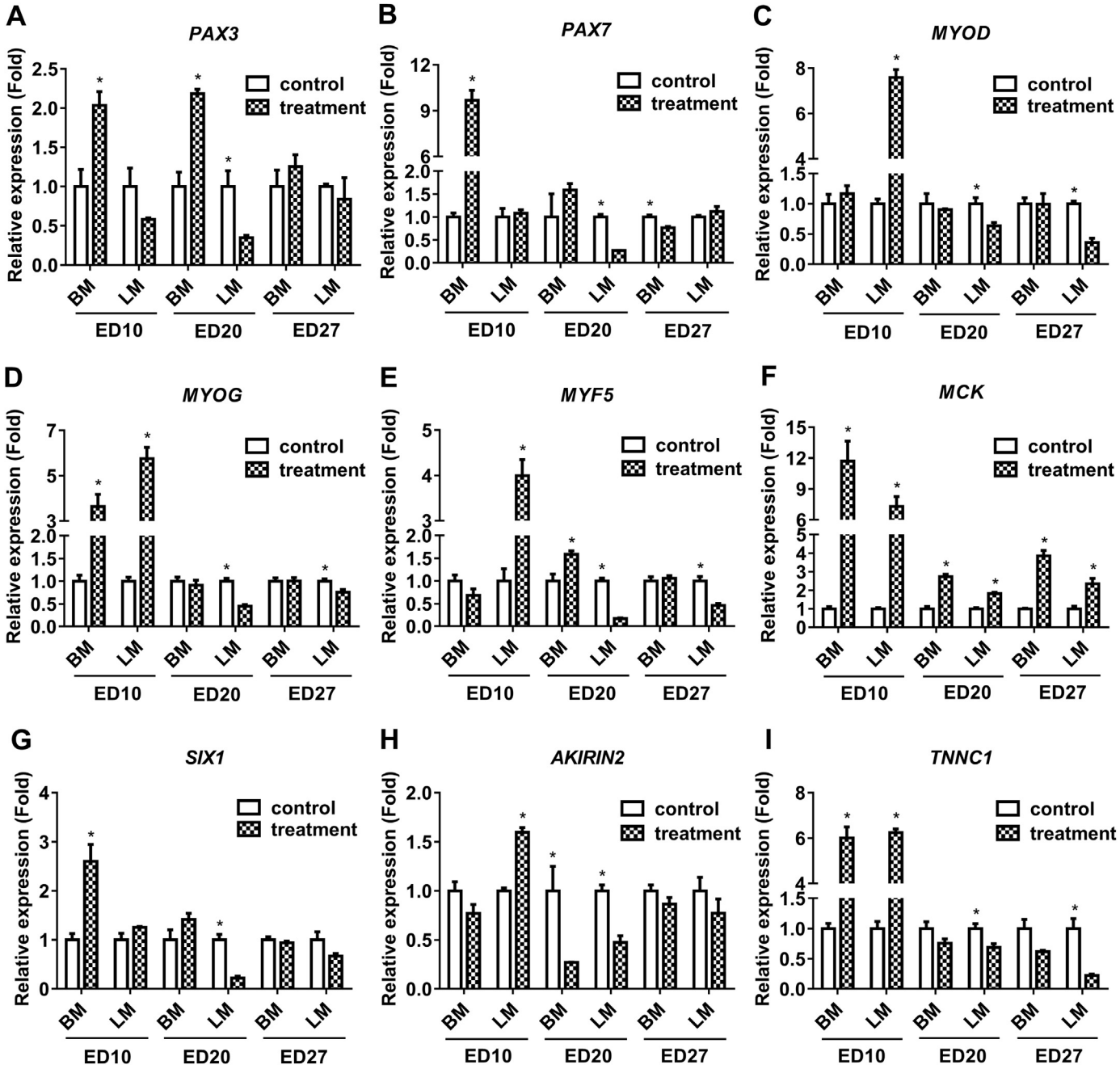

Fig. 1. The mRNA expression profiles of the nine myogenesis-related genes under TM. The asterisk on the bar indicates a significant difference at the level of $P < 0.05$. BM represents the breast muscle, and LM represents the leg muscle.

amplicons from the genomic DNA, quantitative methylation analysis was performed with the Sequenom MassARRAY platform. The detailed operation principles and processes have been described (Ehrich et al., 2005; Wong et al., 2008). Additionally, potential transcription factor binding sites (**TFBS**) within the selected CpG islands were analyzed using PATCH (http://www.gene-regulation.com/cgi-bin/pub/programs/patch/bin/patch.cgi), and the lower score boundary was set as 100 to avoid false positive results.

2.5. Statistical analysis

All statistical analyses were performed with SPSS 19.0 software

Table 3
The changes in expression of the nine myogenesis-related genes under TM.

Tissue	ED1-10	ED10-20	ED20-27
Breast muscle	*PAX3*↑*PAX7*↑*MYOG*↑*MCK*↑*SIX1*↑*TNNC1*↑	*PAX3*↑*MYF5*↑*MCK*↑*AKIRIN2*↓	*PAX7*↓*MCK*↑
Leg muscle	*MYOD*↑*MYOG*↑*MYF5*↑*MCK*↑*AKIRIN2*↑*TNNC1*↑	*PAX3*↓*PAX7*↓*MYOD*↓*MYOG*↓*MYF5*↓*MCK*↑*SIX1*↓*AKIRIN2*↓*TNNC1*↓	*MYOD*↓*MYOG*↓*MYF5*↓*MCK*↑*TNNC1*↓

The up and down arrows represent significantly upregulated and downregulated mRNA levels, respectively.

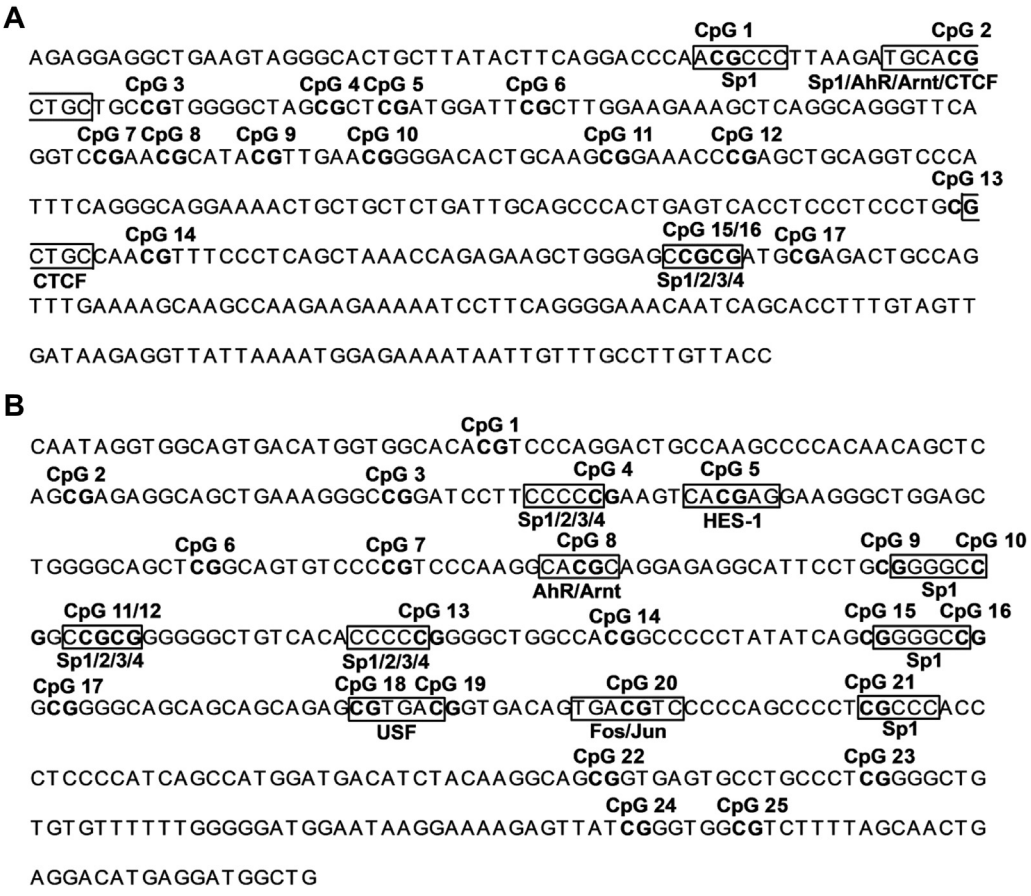

Fig. 2. Sequence analysis of the selected promoter CpG islands in *AKIRIN2* (A) and *TNNC1* (B). All individual CpG sites in the sequences are bolded, and the predicted TFBS containing one or more single CpG sites are boxed with the corresponding transcription factors listed below.

(SPSS Inc., Chicago, USA), and statistical significance was defined as $P < 0.05$. The results are presented as the mean ± SD. The sample size for the mRNA expression analysis was n = 6, and the sample size for the quantitative DNA methylation analysis was n = 3. The raw qRT-PCR data were normalized to *β-actin* (GenBank: EF667345) and *GAPDH* (GenBank: AY436595) by using the $2^{-\Delta\Delta}$Ct method (Livak and Schmittgen, 2001). One-way analysis of variance was used to determine the statistical significance of gene expression levels and methylation levels between the treatment and control groups. The correlation between methylation and mRNA expression levels was evaluated by Pearson correlation coefficient.

3. Results

3.1. Effects of TM on the mRNA expression levels of nine myogenesis-related genes

As shown in Fig. 1A and B, TM promoted *PAX7* expression during ED1-10 and *PAX3* expression during ED1-10 and ED10-20 in breast muscle but inhibited the transcription of *PAX7* during ED20-27 in breast muscle and the transcription of *PAX3* and *PAX7* during ED10-20 in leg muscle ($P < 0.05$). The gene expression patterns of *MYOD*, *MYOG* and *MYF5* were similar (Fig. 1C, D and E). Under TM, the expression levels of the above three genes increased during ED1-10 but decreased during ED10-20 and ED20-27 in leg muscle ($P < 0.05$). In addition, in breast muscle, *MYOG* and *MYF5* expression levels increased under TM during ED1-10 and ED10-20, respectively ($P < 0.05$). *MCK* expression was higher under TM than control conditions at each embryonic stage and in each muscle tissue ($P < 0.05$; Fig. 1F). TM promoted *SIX1* expression during ED1-10 in breast muscle but inhibited *SIX1* expression during ED10-20 in leg muscle ($P < 0.05$; Fig. 1G). TM promoted *AKIRIN2* expression during ED1-10 in leg muscle but inhibited *AKIRIN2* expression during ED10-20 in breast and leg muscle ($P < 0.05$; Fig. 1H). TM promoted *TNNC1* expression during ED1-10 in breast and leg muscle but inhibited *TNNC1* expression during ED10-20 and ED20-27 in leg muscle ($P < 0.05$; Fig. 1I). To create an integrated overview of these results, all the significant changes are summarized in Table 3. These data showed that the effects of TM on myogenesis-related gene expression were different between the two muscle tissues and among the three embryonic stages.

3.2. Sequence analysis and CpG island selection of AKIRIN2 and TNNC1

Based on changes in the mRNA expression levels under TM,

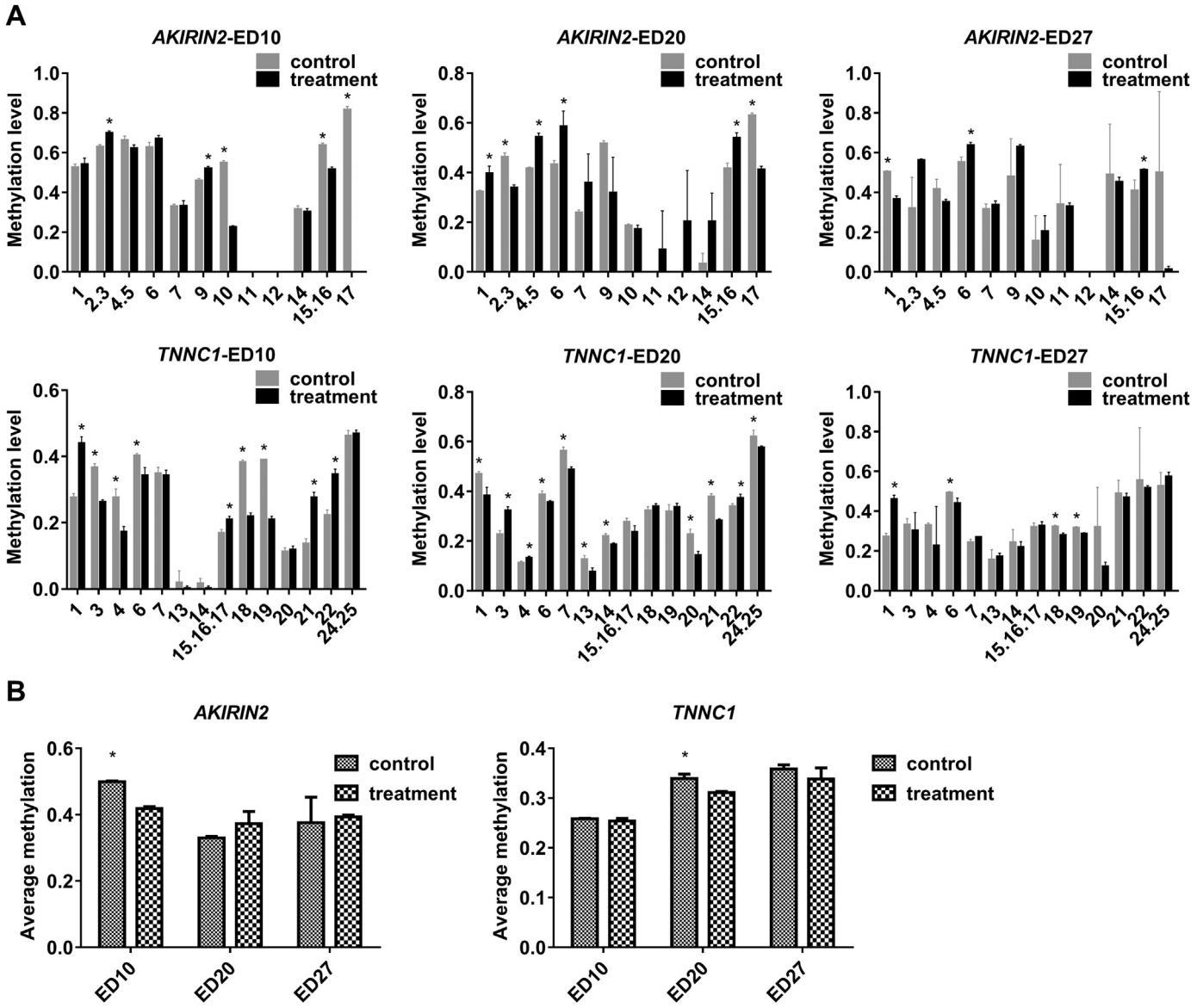

Fig. 3. The methylation levels of the selected CpG islands in the promoters of *AKIRIN2* and *TNNC1* under TM. Methylation level 1 represents 100% methylated CpG dinucleotides on this site. The asterisk indicates a significant difference at the level of $P < 0.05$. (A) Differences in the quantitative methylation levels at each CpG site under TM. (B) Average methylation level measured by calculating the mean of the detected CpG sites.

AKIRIN2 and *TNNC1* were selected for further DNA methylation analysis. Two probable CpG islands were found in the *AKIRIN2* promoter region, and the CpG island located between − 496 bp ∼ + 30 bp relative to the transcription start site was chosen (Fig. 2A). However, only one CpG island (− 211 bp ∼ + 92 bp) was found in the *TNNC1* promoter region (Fig. 2B). Then, the possible single CpG sites and TFBS were predicted in the two chosen CpG islands. The results showed that there were 17 CpG sites in the *AKIRIN2* CpG island and 25 CpG sites in the *TNNC1* CpG island (Fig. 2). Furthermore, four CpG sites in the *AKIRIN2* CpG island and 10 CpG sites in the *TNNC1* CpG island were located within a TFBS (Fig. 2).

3.3. Effects of TM on the promoter methylation status of AKIRIN2 and TNNC1

The methylation levels of individual CpG sites were detected except for CpG sites 8 and 13 in *AKIRIN2* and CpG sites 2, 5, 8–12 and 23 in *TNNC1*. Some individual CpG sites were detected as CpG units, including CpG 2.3, CpG 4.5 and CpG 15.16 in *AKIRIN2* and CpG 15.16.17 and CpG 24.25 in *TNNC1*.

The results showed that changes in methylation level occurred in a few sites (Fig. 3A). Under TM during ED1-10, the methylation levels of CpG 2.3, CpG 9, CpG 10, CpG 15.16 and CpG 17 in *AKIRIN2* and CpG 1, CpG 3, CpG 4, CpG 6, CpG 15.16.17, CpG 18, CpG 19, CpG 21 and CpG 22 in *TNNC1* were significantly changed ($P < 0.05$). Under TM during ED10-20, the methylation levels of CpG 1, CpG 2.3, CpG 4.5, CpG 6, CpG 15.16 and CpG 17 in *AKIRIN2* and CpG 1, CpG 3, CpG 4, CpG 6, CpG 7, CpG 13, CpG 14, CpG 20, CpG 21, CpG 22 and CpG 24.25 in *TNNC*1 were significantly changed ($P < 0.05$). Under TM during ED20-27, the methylation levels of CpG 1, CpG 6, CpG 15.16 in *AKIRIN2* and CpG 1, CpG 6, CpG 18 and CpG 19 in *TNNC1* were significantly changed ($P < 0.05$). Moreover, we also analyzed the global methylation status of the two genes and found that the average methylation levels of *AKIRIN2* and *TNNC1* were significantly decreased ($P < 0.05$) under TM during ED1-10 and ED10-20, respectively (Fig. 3B).

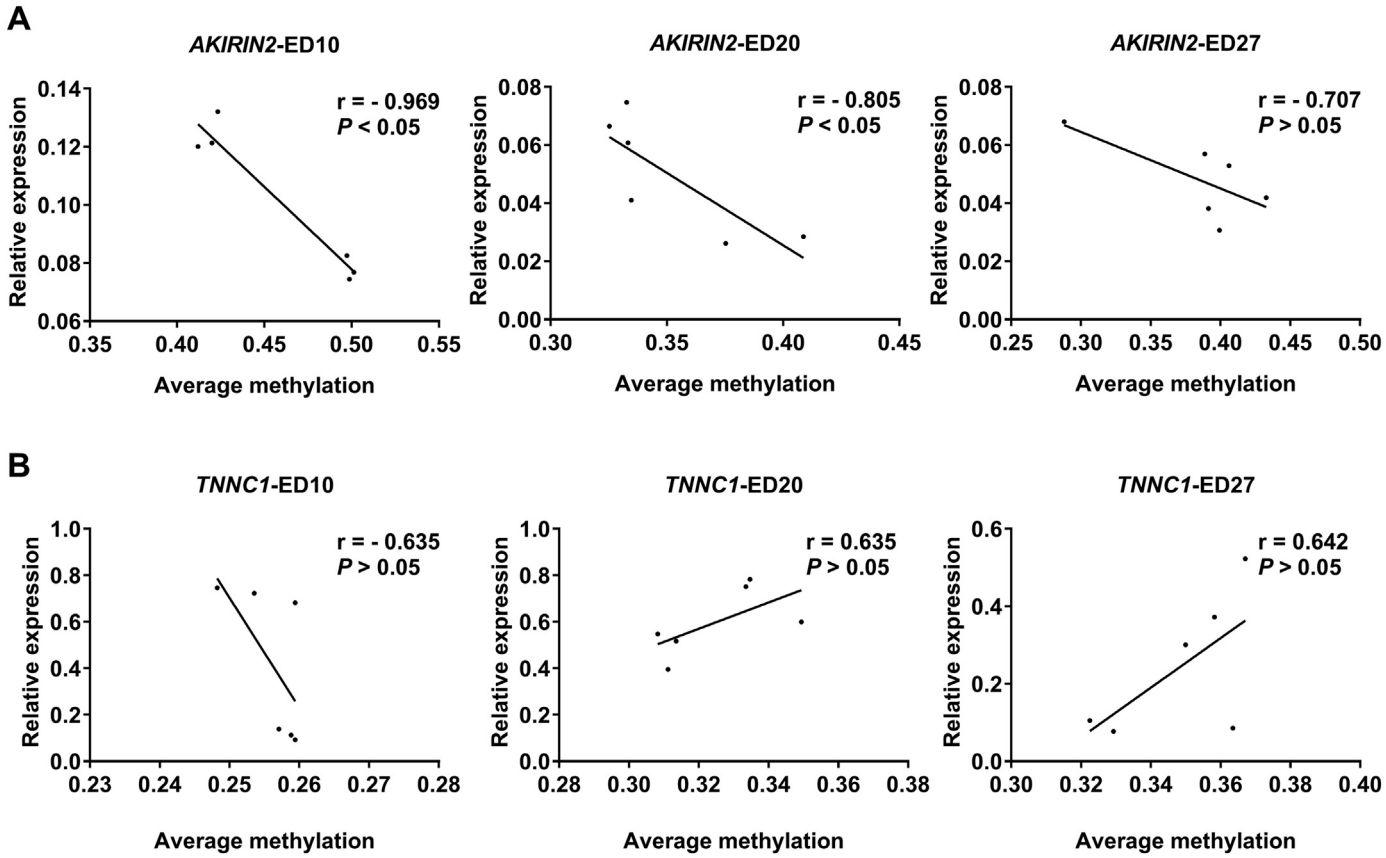

Fig. 4. The correlation between the methylation and mRNA expression levels of *AKIRIN2* (A) and *TNNC1* (B).

3.4. Association between the DNA methylation and mRNA expression of Akirin2 and TNNC1

Our results displayed an inverse correlation of *AKIRIN2* methylation and mRNA expression, and the Pearson correlation coefficients were significant at ED10 (r = − 0.969) and ED20 (r = − 0.805) ($P < 0.05$; Fig. 4A). However, the correlation coefficients of *TNNC1* methylation and mRNA expression were nonsignificant during the three embryonic stages (Fig. 4B).

4. Discussion

Numerous reports have demonstrated that incubation temperature is the most important environmental factor for avian embryogenesis, including embryonic muscle development. However, the tissue- and developmental stage-specific characteristics of temperature effects on the expression of critical genes have rarely been studied. In addition, the investigation of the underlying mechanisms involved in the response of avian embryos to TM is still in the early stages. Our previous study suggested that DNA methylation may be involved in the thermal epigenetic regulation of duck embryo development (Yan et al., 2015). Therefore, the present study was designed to provide more evidence about the expression patterns of myogenesis-related gene under TM and to explore the role of DNA methylation during this process.

To explore the effect of TM on embryonic myogenesis, the expression levels of nine myogenesis-related genes were analyzed in duck breast and leg muscle. *MYOD*, *MYOG* and *MRF5* can control myoblast proliferation and differentiation (Palacios and Puri, 2006). *PAX3* and *PAX7* can activate *MYOD* and *MRF5* expression and regulate the formation and maintenance of satellite cells (Palacios and Puri, 2006). *SIX1* is involved in the control of satellite cell proliferation and differentiation (Yajima et al., 2010). *AKIRIN2* promotes the proliferation of myoblasts in duck (Sun et al., 2016). *MCK* (Pajcini et al., 2008) and *TNNC*1 (Sousa et al., 2006) are genetic markers of myotubes and muscle fibers, respectively. As shown in Fig. 1 and Table 3, under TM during ED10-20 and ED20-27, there were more genes with significantly altered expression in leg muscle than in breast muscle. We previously found that TM during ED10-20 influenced the relative weight of leg muscle more than breast muscle (Liu et al., 2015). This may be due to the faster development and presence of multiple muscle fiber types in leg muscle in duck (Li et al., 2010). Similarly, when subjected to TM, chicken muscle satellite cells isolated from anaerobic pectoralis major were more predisposed to adipogenic conversion than those from mixed fiber biceps femoris (Harding et al., 2015). Additionally, our results showed that TM during ED1-10 increased myogenesis-related gene expression in duck breast and leg muscle, but the TM effects were gradually reversed during ED10-20 and ED20-27. These data suggested a stage-specific influence, which agreed with previous studies evaluating muscle phenotypes in chicken (Collin et al., 2007). Interestingly, *MCK* expression was notably increased under TM in each tissue and at each stage. This may be connected with the increase in ER stress (Li et al., 2017) and embryonic motility (Hammond et al., 2007) under TM.

Next, we were interested in revealing the potential link between the methylation status of the chosen CpG islands and mRNA expression of the corresponding genes. We found that the changes in methylation levels were concentrated at a few sites, while the methylation status in the other CpG sites was unaltered during embryonic development. Additionally, sequence analysis showed that five CpG sites (29%) in *AKIRIN2* and 14 CpG sites (56%) in *TNNC1* were located within TFBS, which are responsible for the interaction with transcription factors, including SP1, CTCF, HES1, etc. DNA methylation may affect transcription by disturbing the interactions between transcription factors and their DNA binding sites (Jones, 2012). A previous study showed a tight link between DNA methylation and the genome-wide occupancy

patterns of CTCF (Wang et al., 2012). This further supports that TM during embryogenesis may affect gene expression partly through regulating the methylation levels of CpG sites that are within TFBS and thus disturbing the activity of transcription factors.

DNA methylation is a type of gene regulatory mechanism that links genotype and phenotype. Our results displayed significant ($P < 0.05$) negative correlations between the average methylation level of *AKIRIN2* and its mRNA expression level under TM during ED1-10 and ED10-20. Consistent with this, promoter methylation status was generally negatively correlated with mRNA expression (Zilberman, 2007). *AKIRIN* was found to have an important role in the immune response, and subsequent studies described its functions in skeletal myogenesis (Chen et al., 2013). In the absence of *AKIRIN1*, the function of *AKIRIN* in skeletal muscle might be fulfilled by *AKIRIN2* in avian species (Macqueen and Johnston, 2009). A recent study in duck revealed that *AKIRIN2* does not regulate myoblast differentiation but does promote myoblast proliferation (Sun et al., 2016). This suggests that thermal epigenetic regulation during embryogenesis may induce changes in the methylation status of the avian *AKIRIN2* promoter that can affect embryonic myogenesis and even cause phenotypic differences.

In conclusion, the present study shows that embryonic duck leg muscle development is more sensitive to TM than breast muscle and that TM during ED1-10 increases myogenesis-related gene expression in duck skeletal muscle more than TM during ED10-20 and ED20-27. Moreover, TM may affect the promoter methylation status of duck *AKIRIN2* with possible influences on embryonic myogenesis.

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Conflict of interest statement

The authors declare no conflicts of interest.

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