RESEARCH ARTICLE

Transiently expressed pattern during myogenesis and candidate miRNAs of *Tmem8C* in goose

KE HE, TING REN, SONGHUI ZHU, SHIRI LIANG and AYONG ZHAO*

College of Animal Science and Technology, Zhejiang A&F University, Lin'an, Zhejiang Province, People's Republic of China

Abstract

Transmembrane protein 8C (Tmem8C) is a muscle-specific membrane protein that controls myoblast fusion, which is essential for the formation of multinucleated muscle fibres. As most of the birds can fly, they have enormous requirement for the muscle, but there are only a few studies of *Tmem8C* in birds. In this study, we obtained the coding sequence (CDS) of *Tmem8C* in goose, predicted miRNAs that can act on the 3′UTR, analysed expression profiles of this gene in breast and leg muscles (BM and LM) during the embryonic period and neonatal stages, and identified miRNAs that might affect the targeted gene. The results revealed a high homology between *Tmem8C* in goose and other animals (indicated by sequence comparisons and phylogenetic trees), some conservative characteristics (e.g., six transmembrane domains and two E-boxes in the 5′UTR might be the potential binding sites of muscle regulatory factors (MRFs)), and the d_N/d_S ratio indicated purifying selection acting on this gene, facilitating conservatism in vertebrates. Q-PCR indicated *Tmem8C* had a peak expression pattern, reaching its highest expression levels in stage E15 in LM and E19 in BM, and then dropping transiently in E23 ($P < 0.05$). We examined 13 candidate miRNAs, and negative relationships were detected both in BM and LM (mir-125b-5p, mir-15a, mir-16-1 and mir-n23). Notably, mir-16-1 significantly decreased luciferase activity in dual luciferase reporter gene (LRG) assay, suggesting that it can be identified as potential factors affecting *Tmem8C*. This study investigated *Tmem8C* in water bird for the first time, and provided useful information about this gene and its candidate miRNAs in goose.

Introduction

Myogenesis is a multistep process involving myoblast determination, withdrawal of myoblasts from the cell cycle, the appearance of multinucleated myotubes and the formation of mature myofibres (Buckingham and Rigby 2014). This process is thought to occur both in the embryonic and postnatal stages. During both the embryonic and adult stages, myogenesis is regulated by many genes and factors, e.g. muscle regulatory factors (MRFs) can regulate almost all muscle-related gene transcription through the E-box (Yafe *et al.* 2008); Myostatin can inhibit cell proliferation and cell growth by controlling the G(1)-phase to S-phase transition in the cell cycle, and reducing the hyperplasia of myoblasts (Thomas *et al.* 2000); paired box 3 (*Pax3*) keeps migrated precursor cells nondifferentiated; and paired box 7 (*Pax7*) is a significant regulator of the satellite cell population (Relaix *et al.* 2006). Apart from gene regulation, which appears to be 'on or off', there are also 'fine tuners' that moderately regulate the overall level of their target's expression. Recently, it has been shown that miRNAs also play important roles in skeletal muscle development (Li *et al.* 2011; Gu *et al.* 2014) and regeneration, e.g. miR-1 and miR-206 can control somite myogensis primarily by directly targeting *Pax3* (Goljanek-Whysall *et al.* 2011).

The target gene of this study was *Tmem8C*, which is a membrane activator of myocyte fusion. Fusion of myoblasts means that mononucleated myoblasts exit the cell cycle, start differentiation, and fuse with one another to generate syncytial myofibres (Millay *et al.* 2013). This process is essential for the formation of multinucleated fibres. Several proteins, such as Ras-related C3 botulinum toxin substrate 1 (*RAC1*) (Vasyutina *et al.* 2009), cell division cycle 42 (*cdc42*) (Vasyutina *et al.* 2009), nephrin (Sohn *et al.* 2009), CD9 (Charrin *et al.* 2013), CD81 (Charrin *et al.* 2013) and myoferlin (Doherty *et al.* 2005) have been studied, which may participate in this process. However, *Tmem8C* is the only muscle-specific protein known to be absolutely essential for fusion of development and regeneration of myoblasts in mammals (Millay *et al.* 2013, 2014). Functional

---

studies in mice have shown that loss of function of *Tmem8C* causes perinatal death due to the absence of multinucleated muscle fibres in the development period and complete abolition of muscle regeneration (Millay *et al.* 2013, 2014). It has also been demonstrated that zebrafish derived from embryos injected with a morpholino, targeted against *Tmem8C* are unable to swim (Landemaine *et al.* 2014). It also demonstrated knockdown of *Tmem8C* by siRNA inhibited myoblast fusion in chicken (Luo *et al.* 2015), while detailed information about this crucial gene remains obscure in other birds.

Zhedong goose (*Anser cygnoides* var. domestica) an important meat-type breed in China, has a high production of muscle in the leg and the breast. Flight and swimming creates enormous demands on muscles in birds. *Tmem8C* has a critical role in myogenesis, however, the gene characteristics and expression profile of *Tmem8C* are unknown in waterfowls. In this study, we described the biological characteristics of the *Tmem8C* gene and its differential expression in breast muscle (BM) and leg muscle (LM) tissues during the embryonic to neonatal stages, and the identification of candidate miRNAs which might affect the expression of *Tmem8C*, could provide preliminary data to improve our understanding the roles of *Tmem8C* and its related miRNAs in goose embryonic myogenesis.

Materials and methods

Animals

Three to four-week-old geese were provided by the Tianhong goose farm (Shaoxing, China), and BM was collected, snap frozen in liquid nitrogen and stored at −80°C. Also 100 fertile eggs of Zhedong goose were obtained from the same place, and incubated in a commercial incubator. Ten eggs were removed at each of seven developmental stages, including six embryonic stages (E7 (7 days after hatching), E11, E15, E19, E23 and E27) and one posthatching stage (P7). Ten one-week-old geese were also obtained from Tianhong goose farm. Samples of BM and LM were collected and were handled as above until RNA isolation.

RNA isolation and cDNA synthesis

Total RNA was obtained using Trizol (Invitrogene, Carlsbad, USA), and reverse translated with two kits for different purposes. The PrimeScript first Strand cDNA synthesis kit (Takara, Dalian, China) was used to synthesise the cDNA of BM from four-week-old geese, which was used to amplify the partial CDS of *Tmem8C*. We also used this kit to produce cDNA from muscles of embryonic and neonatal stages, and these cDNAs were used as templates in quantitative realtime-polymerase chain reaction (qRT-PCR) of *Tmem8C*. The cDNAs were stored at −20°C until use.

5′ and 3′ RACE of Tmem8C

According to the sequences of *Tmem8C* in other species (NM_025376 *Mus musculus*, NM_001080483 *Homo sapiens*, XM_001236918 *Gallus gallus* and XM_005013308 *Anas platyrhynchos*), we designed a set of primers (T-F and T-R) to obtain a partial CDS of *Tmem8c* gene (table 1). The PCR product was purified with a DNA PCR purification kit (Axygen, Hangzhou, China) and sequenced. Based on this sequence, primers for 5′ and 3′ RACE were designed (table 1). The complete CDS of *Tmem8C* was obtained using a SMARTer RACE 5′ and 3′ kit, using BM from four-week-old geese as a template. Since gene expression was not abundant in adults, we used nested PCR to obtain a distinct product (table 1).

Purification and infusion cloning of RACE products were processed with the appending kits in the SMARTer RACE 5′ and 3′ kit. Twelve clones were picked and cultured for 6 h at 280 rpm at 37°C. Three positive clones were selected for sequencing.

Prediction of miRNA

Sequences of 3′ untranslated regions (UTRs) are known to be target sites of miRNAs, so once the UTR sequence was determined, we could predict which miRNA could combine with the target sequence. TargetScan was not used for the analysis because our research was focussed on myogenesis alone during embryonic and neonatal stages. We screened for miRNAs which have abundant expression or significant differential expression during the embryonic stage in Peking ducks and in broiler and layer chickens. The seed sequences were confirmed with miRBase (http://www.mirbase.org/index.shtml), and their ability to bind to 3′UTRs of *Tmem8C* gene was checked. The miRNAs used in this study are listed in table 2.

Quantification of mRNA and miRNA by qRT-PCR and statistical analyses

For quantification of mRNA, qRT-PCR analysis of *Tmem8C* was performed using SYBR Premix Ex Taq™ II (Takara, Dalian, China) on a real-time PCR detection system according to the manufacturer's protocol. The primers were T-QF/T-QR. The thermal cycling parameters were two steps of PCR and the cycle of melting curve analysis was performed after each PCR to confirm product specificity. Each sample was tested in triplicate, and β-action was used as a reference gene (table 1). For quantification of miRNA, cDNA synthesis and qPCR were carried out with Mir-X miRNA First-Strand Synthesis and SYBR qRT-PCR (Takara, Dalian, China), respectively. The reaction cycles were as described in the user manual, and U6 snRNA was used as a control (table 1). Three replicates were taken for each sample. The sequence of miRNA reverse was 5′-CTCAACTGGTGTCGTGGA-3′. The relative expression levels of genes and miRNAs in the BM and LM samples of the geese were calculated using the ΔCt method. The correlation between the expression profiles of *Tmem8C* and

Table 1. Primers used in this study.

Primer	Sequence (5′–3′)
T-F	GGACACATGCATGTGATGGC
T-R	TGGCACAGTGGTAGAAGCTG
GSP1	TCTCCTTGGGCAGCAGCAGCACGAA
NGSP1	TCGAACTCAGCCAGGGCCATCAGGGACAC
GSP2	GGCCCCGGCTTATCAGTGCTGTGCTTCATG
NGSP2	GTGTCCCTGATGGCCCTGGCTGAGTTCG
T-QF	GCCATGGTYTACTTCTTCAC
T-QR	GGCACAGTGGTAGAARCTGT
β-Action forward	GTGGGGCGCCCAGGCACCT
β-Action reverse	CTTCCTTAATGTCACGCACGATTG
U6-forward	CTCGCTTCGGCAGCACA
U6-reverse	AACGCTTCACGAATTTGCGT
3′UTR-F	CCGCTCGAGGCCCCCGGCACCTGCTCCT
3′UTR-R	ATAGTTTAGCGGCCGCGTATTATTTACATCAAGAGA

Table 2. MiRNAs studied and correlation analysis of miRNAs and *Tmem8C* gene.

Source	mir family	miR-name	Primer (5′–3′)	in BM	in LM
Duck	mir-10	mir-125b-5p	TCCCTGAGACCCTAACTTGTGA	−0.284 (NA)	−0.301 (NA)
GGA	mir-15	mir-15a	GCGTAGCAGCACATAATGGTTTGT	−0.228 (NA)	−0.250 (NA)
GGA	mir-15	mir-15b	TAGCAGCACATCATGGTTTGCA	−0.455 (NA)	0.277 (NA)
GGA	mir-15	mir-16-1	CGTAGCAGCACGTAAATATTGGTG	−0.471 (NA)	−0.126 (NA)
GGA/duck	mir-15	mir-16c	GTAGCAGCACGTAAATACTGGAG	−0.416 (NA)	0.390 (NA)
GGA	mir-17	mir-106	CGAAAAGTGCTTACAGTGCAGGTA	−0.466 (NA)	0.390 (NA)
GGA	mir-17	mir-17	CAAAGTGCTTACAGTGCAGGTAGT	−0.292 (NA)	0.533 (NA)
GGA	mir-17	mir-20a	GCGTAAAGTGCTTATAGTGCAGGTAG	−0.342 (NA)	0.399 (NA)
GGA	mir-17	mir-20b	CAAAGTGCTCATAGTGCAGGTAG	−0.163 (NA)	0.589 (NA)
GGA	mir-184	mir-184	CCTTATCACTTTTCCAGCCCAGC	−0.371 (NA)	−0.445 (NA)
GGA	mir-199	mir-199-1	CCCAGTGTTCAGACTACCTGTTC	−0.435 (NA)	0.302 (NA)
GGA		mir-n18	CGAGAGGAGCTGGGAGGATG	0.052 (NA)	0.508 (NA)
Duck		mir-n23	GGGGATGTGTAAAAGAAGAAGCG	−0.302 (NA)	−0.546 (NA)

miRNAs was analysed using Pearson's correlation, and the *P* value was calculated using student's *t*-test. We took a *P* value <0.05 as statistically significant.

Transfections and dual luciferase reporter assay

The 3′UTRs were amplified from the embryonic leg muscle cDNA (table 1, 3′UTR-F/3′UTR-R) and ligated into the psiCHECK-2vector (Promega, Madison, USA). Three miRNAs (gga-mir-16-1, gga-mir-15a and gga-mir-125b-5p) were selected for this analysis (reason blow). The miRNA mimics were synthesized by GenePharma (Shanghai, China). We took baby hamster kidney (BHK-21) cell lines, and transfection was carried out using Lipofectamine 2000 (Invitrogen) as described in Yang *et al.* (2013). Total RNA was prepared at 24 h after transfection for qPCR analysis. Dual-Glo Luciferase assay system (Promega) was taken to measure firefly and Renilla luciferase activities, from which normalized data were calculated (as the quotient of Renilla/firefly luciferase activities). Three independent experiments were performed in triplicate.

Sequence analysis and statistical analysis

Sequence alignment and splicing were carried out using DNAStar. Once the CDS was confirmed, the *Tmem8C* sequence was analysed using the conserved domain architecture retrieval tool of BLAST (http://www.ncbi.nlm.nih.gov/BLAST). Signal P3.0 server (http://www.cbs.dtu.dk/services/SignalP/), SMART (http://smart.embl-heidelberg.de/) and TMHMM (http://www.cbs.dtu.dk/services/TMHMM/) were used to predict the signal peptides and transmembrane domain. Psort II (http://psort.hgc.jp/) was used to predict protein sorting signals and intracellular localization. The secondary structure of deduced amino acids was analysed using SOPMA (http://npsa-pbil.ibcp.fr/) and the HNN secondary structure prediction method was used.

For analysis of the evolutionary relationships of *Tmem8C*, Mega 5.0 (Tamura *et al.* 2011) was used (i) to align multiple sequences between species, (ii) search the best evolutionary models, and (iii) construct phylogenetic trees using the neighbour-joining (NJ) method (Saitou and Nei 1987). The bootstrap levels were calculated based on 10,000
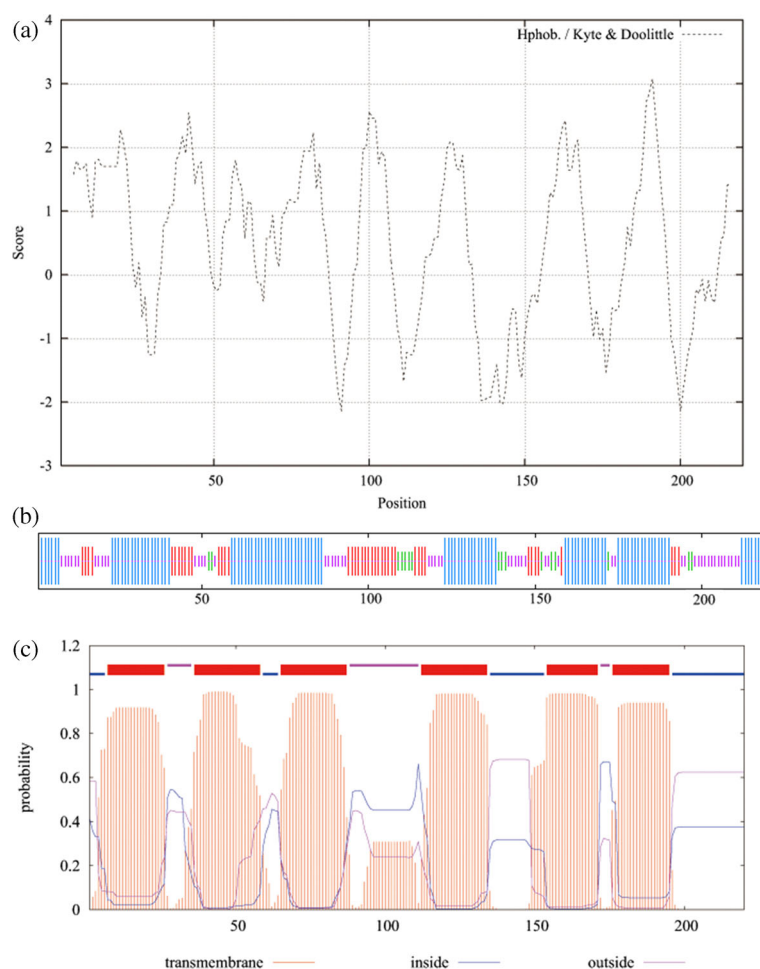
Figure 1. (a) Hydrophobicity profile, (b) predicted secondary structure and (c) TMHMM posterior probabilities of *Tmem8C*. Alpha helices, extended strands, beta turns, random coils are indicated, respectively, in blue, red, green and purple in (b).

replications. We also calculated the number of synonymous (d_S) and nonsynonymous (d_N) substitutions per site using the Jukes–Cantor method in Mega 5.0.

Results and discussion

Sequence analysis of Tmem8C gene

The full-length cDNA of *Tmem8C* of goose was reported in this study (GenBank: KT751177). We obtained the 1412 bp sequence of *Tmem8C* by cloning and splicing. This sequence contained a 663-bp CDS, 283-bp 5′UTR and 466-bp 3′UTR. Alignment of nucleotides suggested homology of this sequence with *Tmem8C* in mouse and human. No promoter was found in 5′UTR with Promoter 2.0 (http://www.cbs.dtu.dk/services/Promoter/). A conserved domain (DUF3522 superfamily starting from the 3rd amino acid and ending at the 185th amino acid) was found using BLAST. Hydrophobicity profiles indicated that the *Tmem8C* protein contained more hydrophobic amino acids than hydrophilic amino acids (figure 1a). The results of TMHMM revealed that there

were six obvious transmembrane domains in this protein (figure 1b), which was consistent with the analysis in the Signal P3.0 server. A signal peptide was also predicted from positions 1 to 25, with no applicable E-value. However, von Heijne's method for signal sequence recognition in PSort II suggested there was no N-terminal signal peptide. The prediction of secondary structure by SOPMA suggested 107 (48.64%) alpha helices, 55 (25.00%) random coils, 42 (19.09) extended strands and 16 (7.27%) beta turns in this protein (figure 1c). Reinhardt's method for cytoplasmic/nuclear discrimination suggested that *Tmem8C* functions in the cytoplasm. We tried to predict the protein 3D conformation using the SWISS-MODEL server (http://www.expasy.org/swissmod/SWISSMODEL.html). However, the templates covered only 5.88–17.24% of *Tmem8C*, and we obtained the predicted 3D structure from only a small part of this gene. Thus, the result was not adopted.

We compared *Tmem8C* gene sequence from goose with *Mus musculus* (Mumu), *Homo sapiens* (human), *Gallus gallus* (Gaga), *Danio rerio* (Dare), *Taeniopygia guttata* (Tagu), *Meleagris gallopave* (Mega) and *Anas platyrhynchos* (Anpl).
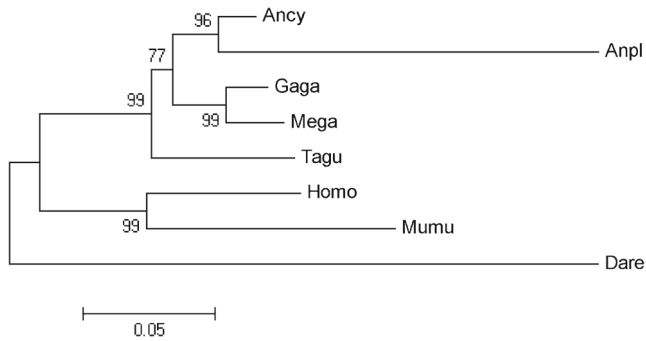
Figure 2. Phylogenetic tree of *Tmem8C*. The following sequences were included for tree building: NM_001002088 (*Danio rerio*, Dare), XM_010570827 (*Meleagris gallopavo*, Mega), XM_005013308 (*Anas platyrhynchos*, Anpl), XM_002199251 (*Tarnipygia guttata*, Tagu), KP230536 (*Gallus gallus*, Gaga), NM_001080483 (*Homo sapiens*, Homo) and NM_025376 (*Mus musculus*, Mumu).

Figure 3. Developmental expression profiles of the *Tmem8C* gene in BM and LM. Values with the same letter are not significantly different for the same gene ($P > 0.05$), values with different capital letters (A–C) were significantly different ($P < 0.01$), and values with different lower case letters (a–c) had different mean values ($P < 0.05$).

The similarity of the CDS in goose with those in Anpl, Gaga, Tagu, Mega, Mumu, human and Dare were 85.82, 94.42, 91.40, 93.51, 81.00, 84.16 and 73.60%, respectively. The similarities of amino acids were 97.27, 95.45, 97.27, 84.55, 87.27 and 78.18% to Gaga, Tagu, Mega, Mumu, human and Dare, respectively. Anpl had a stop codon in the CDS, thus it was excluded from the amino acid analysis. The phylogenetic tree of *Tmem8C* was constructed linking several species (figure 2). The results showed goose *Tmem8C* was grouped with Anpl with a bootstrap of 96%. Some insertions and deletions were evident in Anpl's sequence and, as a consequence, were also found in goose. The d_N/d_S ratios can be used as an indicator of selective pressure acting on a protein-coding gene. Homologous genes with d_N/d_S ratios of >1, 1 and <1 are usually said to be evolving under positive selection, neutral selection and purifying selection, respectively. The result of nucleotide substitutions in genes coding for proteins suggested purifying selection in this gene, with a d_N/d_S value of 0.10.

Tmem8C was highly conserved across vertebrates. The *Tmem8C* of goose shared several features with other vertebrate, including the six tranmembrane domain and the hydrophobicity showing extensive regions of hydrophobic amino acids. It was suggested that this protein maybe localized in a cellular membrane (Millay *et al.* 2013). Two E-boxes were also found in positions 83–88 and 144–149 in our sequence, which might be potential binding sites of MRFs. Study in chicken suggested that E-box 1 in the *Tmem8C* promoter was essential for its transcription (Luo *et al.* 2015). The high homology between the goose and other species in terms of both sequence (73.60–94.42%) and amino acid levels (78.18–95.45%) were found, d_N/d_S ratio indicated that purifying selection may be acting on this gene, and the phylogenetic tree of *Tmem8C* show the cluster of birds (figure 2), all these suggested conservatism of this gene in vertebrates. Because of the swimming and flying skills of geese, their
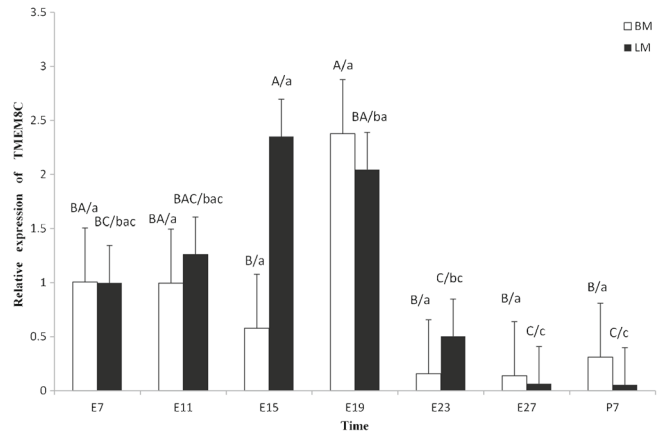
myogenesis related genes are more likely to undergo selective pressure during evolution (Scott *et al.* 2009). Negative selection was found to be acting on the bHLH domains of all MRF family members (Liu *et al.* 2011). Thus, we suppose that its important function makes the gene highly conserved in these species.

Developmental expression profile of the goose Tmem8C in BM and LM

The developmental expression profile of *Tmem8C* in goose is shown in figure 3. The mRNA expression level at E7 was regarded as a control and assigned a value of 1. During the development of the embryo, the expression of *Tmem8C* gene was variable, but there was a similar expression pattern in BM and LM. *Tmem8C* reached its highest expression levels in E15 in LM and E19 in BM. For LM, the expression levels showed a peak model: rising from E7 to E15 (the expression level in E15 was significantly higher than those in E7, E23, E27 and P7 ($P<0.05$)), then decreasing slightly, but maintaining a relatively high expression in E19, and finally declining significantly after E19 ($P<0.05$). A similar peak trend appeared in BM, but expression peaked at E19, at which point it was significantly higher than in E15 ($P<0.05$). After E19, the expression level decreased significantly ($P<0.05$). In summary, the expression dropped transiently.

Tmem8C is a key factor in the fusion of development and regeneration in myogenesis. The expression profile in BM and LM of geese indicated that there was a peak expression pattern both in BM and LM. The same sharply downregulated expression pattern was also reported in mouse, zebrafish and chicken (Millay *et al.* 2013; Landemaine *et al.* 2014). In chicken, *Tmem8C* mRNA expression was upregulated from E10 to E14 and sharply downregulated after E16 (Luo *et al.* 2015). The morphological analysis of muscle
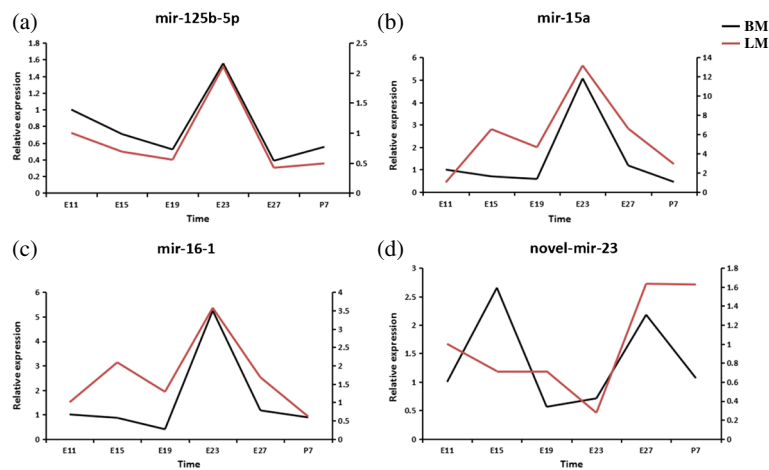
Figure 4. Developmental expression profiles of the 4 miRNAs in BM and LM. The vertical coordinate of BM and LM were listed in right and left, respectively. The miRNAs basically did not express in E7, so we took the expression level at E11, which was regarded as a control and assigned a value of 1.

development in LM of ducks suggested interfusion cells could be found at stage E14, and myolin was formed at stage E18. While this phenomenon lagged in BM, obvious interfusion of cells can be seen at stages E14 and E18, and myolin could be seen at stage E22 (Li *et al.* 2010a). Gu *et al.* (2014) considered E19 as the fastest growth stage of embryonic breast muscle of Peking duck. Based on the transient expression pattern of *Tmem8C* in goose, it appears that infusion occurred in the E15 and E19 periods in LM and E19 in BM, which is a few days later than in chicken and duck. Li *et al.* (2010b) proposed that the development of BM lags behind that of LM in embryonic phases in duck, and suggested this might be related to the environmental needs of ducklings. This was confirmed by the studies of Liu *et al.* (2011) and Zhu *et al.* (2014). This hypothesis was also consistent with the expression profiles found in goose: expression in LM peaked before BM. This phenomenon might be due to the different tissue content in BM and LM. In poultry, BM and LM tissues consist mainly of fast muscle fibres and slow muscle fibres, respectively. After the stage of fusion, the expression of *Tmem8C* decreased significantly in both BM and LM.

Correlation of miRNA and Tmem8C expression during myogenesis and mir-16-1 may inhibit targeted gene

Based on the skeletal muscle miRNA transcriptome of chickens and ducks and the 3′UTR of the *Tmem8C* gene, we selected 13 miRNAs (table 2) and found nearly 50% of miRNAs were negatively correlated with *Tmem8C* (except mir-n18 in BM and nine miRNAs in LM), however, no correlations were significant (table 2). Overall, four miRNAs (mir-125b-5p, mir-15a, mir-16-1 and novel-mir-23) were negatively correlated with *Tmem8C* both in BM and LM (figure 4). Similar expression patterns of BM and LM were
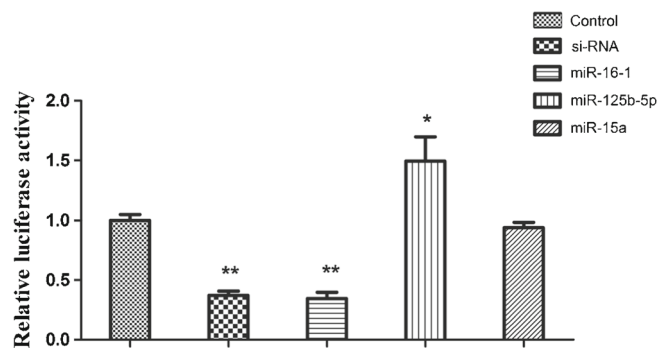

Figure 5. The luciferase assay of BHK-21 cells cotransfected with luciferase reporter vectors containing the 3′-UTR of *Tmem8C* gene and several miR mimics or miR-negative control. Renilla luciferase values were normalized against firefly luciferase values, **$P < 0.01$, *$P < 0.5$.

observed in mir-125b-5p, mir-15a and mir-16-1, excepting the fold which was slightly different (figure 4). However, in novel-mir-23, different expression pattern was found (figure 4), and it was species-specific in duck, thus we took other three miRNAs (mir-125b-5p, mir-15a and mir-16-1) for further study.

To investigate whether the three selected miRNAs target the 3′UTR of *Tmem8C*, we inserted a partial 3′UTR (including the targeting region) into the psiCHECK2 vector. The luciferase reporter gene and either selected miRNAs mimic or negative control oligonucleotides were cotransfected into BHK-21 cells. The luciferase activity responses for siRNA was significantly downregulated, compared with the control, thus it was an appropriate positive control for normalizing luciferase activity data (figure 5). Transfection of mir-16-1 induced the same effect as positive control, which was significantly different with black control (only psiCHECK2 vector, no siRNA) (figure 5). No significant induction was observed

in cells transfected with mir-15a, but luciferase activity was significantly decreased in constructs transfected with mir-125b-5p (figure 5). Above all, mir-16-1 was inferred as potential inhibitor of *Tmem8C* in goose.

MiRNAs play a role at the posttranscriptional level by interacting with the 3′-UTR of the targeted mRNA (Bartel 2004; Brennecke *et al.* 2005). Various studies had proved miRNAs can influence myogenesis through impacting mRNA stability and translation. For example, mir-206 can target mRNA of *Pax3* and *Pax7* (Goljanek-Whysall *et al.* 2011; Liu *et al.* 2012) and mir-27 and mir486 can also affect the expression of *Pax3* and *Pax7*, respectively (Boutet *et al.* 2012). Thus, posttranscriptional control of myogenesis by miRNAs plays an important role in the formation of muscles and affects their properties (Nie *et al.* 2015). The few reports about miRNA and *Tmem8C* genes was miR-140-3p, which can inhibit *Tmem8C* expression and myoblast fusion *in vitro* (Luo *et al.* 2015). The 13 miRNAs studied presently were not significantly and negatively associated with *Tmem8C*, however, further study of LRG suggested mir-16-1 was identified as a potential factor affecting the targeted gene in the goose. MiR-16-1 has been implicated in various aspects of carcinogenesis including cell proliferation and regulation of apoptosis (Campos-Juanatey *et al.* 2015). It belongs to miR-16 family, which was thought to be associated with protection against cold in wood frogs because transcripts were 1.5-fold higher in the liver, but 50% lower in the skeletal muscle of frozen frogs compared with normal frogs (Biggar *et al.* 2009). In conclusion, mir-16-1 was identified as a potential factor affecting the *Tmem8C* gene in the goose. Further analysis, such as functional studies *in vitro* and *in vivo*, are needed.

Conclusions

In this study, we obtained the full-length sequence of *Tmem8C* in the goose, analysed sequence characteristics and homologies, detected a peak expression model in both BM and LM in E15–E19 and E15, respectively, and suggested mir-16-1 as a potential factor affecting the *Tmem8C* gene in the goose by LRG assay. This is the first report of a *Tmem8C* gene in goose, and can provide preliminary understanding of the roles of *Tmem8C* and its related miRNAs in goose embryonic myogenesis.

References

Bartel D. P. 2004 MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* **116**, 281–297.

Biggar K. K., Dubuc A. and Storey K. 2009 MicroRNA regulation below zero: differential expression of miRNA-21 and miRNA-16 during freezing in wood frogs. *Cryobiology* **59**, 317–321.

Boutet S. C., Cheung T. H., Quach N. L., Liu L., Prescott S. L., Edalati A., Iori K. and Rando T. A. 2012 Alternative polyadenylation mediates microRNA regulation of muscle stem cell function. *Cell Stem Cell* **10**, 327–336.

Brennecke J., Stark A., Russell R. B. and Cohen S. M. 2005 Principles of microRNA-target recognition. *PLoS Biol.* **3**, e85.

Buckingham M. and Rigby P. W. 2014 Gene regulatory networks and transcriptional mechanisms that control myogenesis. *Dev. Cell* **28**, 225–238.

Campos-Juanatey F., Gutierrez-Baños J. L., Portillo-Martin J. A. and Zubillaga-Guerrero S. 2015 Assessment of the urodynamic diagnosis in patients with urinary incontinence associated with normal pressure hydrocephalus. *Neurourol. Urodynam.* **34**, 465–468.

Charrin S., Latil M., Soave S., Polesskaya A., Christien F., Boucheix C. and Rubinstein E. 2013 Normal muscle regeneration requires tight control of muscle cell fusion by tetraspanins CD9 and CD81. *Nat. Commun.* **4**, 1674.

Doherty K. R., Cave A., Davis D. B., Delmonte A. J., Posey A., Earley J. U. *et al.* 2005 Normal myoblast fusion requires myoferlin. *Development* **132**, 5565–5575.

Goljanek-Whysall K., Sweetman D., Abu-Elmagd M., Chapnik E., Dalmay T., Hornstein E. and Monsterberg A. 2011 MicroRNA regulation of the paired-box transcription factor Pax3 confers robustness to developmental timing of myogenesis. *Proc. Nat. Acad. Sci.* **108**, 11936–11941.

Gu L., Xu T., Huang W., Xie M., Sun S. and Hou S. 2014 Identification and profiling of microRNAs in the embryonic breast muscle of Pekin duck. *PLoS One* **9**, e86150.

Landemaine A., Rescan P.-Y. and Gabillard J.-C. 2014 Myomaker mediates fusion of fast myocytes in zebrafish embryos. *Biochem. Biophys. Res. Commun.* **451**, 480–484.

Li G., Miskimen K. L., Wang Z., Xie X. Y., Brenzovich J., Ryan J. J., Tse W. *et al.* 2010a STAT5 requires the N-domain for suppression of miR15/16, induction of bcl-2, and survival signaling in myeloproliferative disease. *Blood* **115**, 1416–1424.

Li L., Liu H.-H., Xu F., Si J.-M., Jia J. and Wang J.-W. 2010b MyoD expression profile and developmental differences of leg and breast muscle in Peking duck (*Anas platyrhynchos* Domestica) during embryonic to neonatal stages. *Micron* **41**, 847–852.

Li T., Wu R., Zhang Y. and Zhu D. 2011 A systematic analysis of the skeletal muscle miRNA transcriptome of chicken varieties with divergent skeletal muscle growth identifies novel miRNAs and differentially expressed miRNAs. *BMC Genomics* **12**, 186.

Liu H.-H., Wang J.-W., Li L., Han C.-C., Huang K.-L., Si J.-M. *et al.* 2011 Molecular evolutionary analysis of the duck MYOD gene family and its differential expression pattern in breast muscle development. *Brit. Poultry Sci.* **52**, 423–431.

Liu N., Williams A. H., Maxeiner J. M., Bezprozvannaya S., Shelton J. M., Richardson J. A., Bassel-Duby R. and Olson E. N. 2012 MicroRNA-206 promotes skeletal muscle regeneration and delays progression of Duchenne muscular dystrophy in mice. *J. Clin. Invest.* **122**, 2054–2065.

Luo W., Li E., Nie Q. and Zhang X. 2015 Myomaker, regulated by MYOD, MYOG and miR-140-3p, promotes chicken myoblast fusion. *Int. J. Molec. Sci.* **16**, 26186–26201.

Millay D. P., O'Rourke J. R., Sutherland L. B., Bezprozvannaya S., Shelton J. M., Bassel-Duby R. and Olson E. N. 2013 Myomaker is a membrane activator of myoblast fusion and muscle formation. *Nature* **499**, 301–305.

Millay D. P., Sutherland L. B., Bassel-Duby R. and Olson E. N. 2014 Myomaker is essential for muscle regeneration. *Genes and Devel.* **28**, 1641.

Nie M., Deng Z.-L., Liu J. and Wang D.-Z. 2015 Noncoding RNAs, emerging regulators of skeletal muscle development and diseases. *BioMed. Res. Int.* **2015**, 676575.

Relaix F., Montarras D., Zaffran S., Gayraud-Morel B., Rocancourt D., Tajbakhsh S. *et al.* 2006 Pax3 and Pax7 have distinct and overlapping functions in adult muscle progenitor cells. *J. Cell Biol.* **172**, 91–102.

Saitou N. and Nei M. 1987 The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4**, 406–425.

Scott G. R., Richards J. G. and Milsom W. K. 2009 Control of respiration in flight muscle from the high-altitude bar-headed goose and low-altitude birds. *Am. J. Phys.-Regul. Integrat. Compar. Phys.* **297**, R1066–R1074.

Sohn R. L., Huang P., Kawahara G., Mitchell M., Guyon J., Kalluri R. *et al.* 2009 A role for nephrin, a renal protein, in vertebrate skeletal muscle cell fusion. *Proc. Nat. Acad. Sci.* **106**, 9274–9279.

Tamura K., Peterson D., Peterson N., Stecher G., Nei M. and Kumar S. 2011 MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molec. Biol. Evol.* **28**, 2731–2739.

Thomas M., Langley B., Berry C., Sharma M., Kirk S., Bass J. and Kambadur R. 2000 Myostatin, a negative regulator of muscle growth, functions by inhibiting myoblast proliferation. *J. Biol. Chem.* **275**, 40235–40243.

Vasyutina E., Martarelli B., Brakebusch C., Wende H. and Birchmeier C. 2009 The small G-proteins Rac1 and Cdc42 are essential for myoblast fusion in the mouse. *Proc. Nat. Acad. Sci.* **106**, 8935–8940.

Yafe A., Shklover J., Weisman-Shomer P., Bengal E. and Fry M. 2008 Differential binding of quadruplex structures of muscle-specific genes regulatory sequences by MyoD, MRF4 and myogenin. *Nucleic Acids Res.* **36**, 3916–3925.

Yang S., Liu X., Li X., Sun S., Sun F., Fan B. and Zhao S. 2013 MicroRNA-124 reduces caveolar density by targeting caveolin-1 in porcine kidney epithelial PK15 cells. *Molec. Cell. Biochem.* **384**, 213–219.

Zhu C., Gi G., Tao Z., Song C., Zhu W., Song W. and Li H. 2014 Development of skeletal muscle and expression of myogenic regulatory factors during embryonic development in Jinding ducks (*Anas platyrhynchos domestica*). *Poultry Sci.* **93**, 1211–1216.

Corresponding editor: INDRAJIT NANDA