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*Full Length Article*

# MicroRNA Expression Profile Analysis during Myogenic Differentiation in Pigeon *(Columba livia)* Skeletal Muscle Satellite Cells

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## Abstract

Skeletal muscle satellite cells (SMSCs) are essential for the rapid growth of pectoral muscles in newly hatched pigeons. As post-transcriptional modulatory factors, miRNAs can exert vital influence on muscle cell differentiation. Here, we performed a comparative profile of miRNAs in pigeon SMSCs and myotubes using high-throughput sequencing. We identified 297 known pigeon miRNAs, 131 novel miRNAs, and 261 conserved miRNAs. Of these, 193 were differentially expressed (DE)–74 were up-regulated and 119 down-regulated during myogenic differentiation in pigeon SMSCs. A functional enrichment analysis revealed that genes modulated by DE miRNAs (with read counts >1000) were principally enriched in the catergories: developmental process, growth and PI3K/Akt signaling pathway. Dual-luciferase reporter assays indicated that a down-regulated miRNA, cli-miR181a-5p can directly target the 3' untranslated region of *Mef2a*. Our findings demonstrated that miRNAs widely participate in the differentiation of SMSCs in pigeons, and provide valuable information toward a better understanding of muscle differentiation and development in pigeons. © 2020 Friends Science Publishers

**Keywords:** Pigeon; miRNA; Muscle satellite cells; Myogenesis

## Introduction

The domesticated pigeon (*Columba livia*) has been bred for hundreds of years for the production of meat and ornamentals (Sales 2003) and for use as an experimental animal in some research fields such as toxicology (Zeid *et al.* 2019) and behavior (Wilkie *et al.* 1981). Pigeon meat is gaining popularity among consumers in Europe, China and the United States (Pomianowski *et al.* 2009) which has greatly increased the number of farms breeding pigeons for the production of meat and meat products. In China, pigeon has become the fourth-largest domestic poultry by breeding scale, following chicken, duck and goose. Unlike other poultry, pigeons are altricial birds with an extraordinary growth rate during early development. Specifically, reported that the weight of breast meat increased 173.3-fold between 1-day-old and 35-day-old birds (Gao *et al.* 2016). The mechanism underlying this rapid growth in pigeon pectoral muscles has not been fully elucidated. Skeletal muscle growth is a complicated process involving cell proliferation, apoptosis, differentiation and the transformation of muscle fiber types (Gan *et al.* 2018). In post-hatch birds, the development of muscle cells occurs exclusively through an increase in myofiber size (hypertrophy) (Remignon *et al.* 1995). This process is mediated by activated muscle satellite cells (MSCs) (Harding *et al.* 2016), which proliferate and fuse with muscle fibers, ultimately cause an increase in the DNA content and protein synthetic capacity of the developing muscles (Yin *et al.* 2014). MSCs are a multipotential mesenchymal stem cell population (Harding *et al.* 2016). When skeletal muscle suffers an injury, SMSCs become active and begin to fuse to form a new myotube (Relaix and Zammit 2012). In broilers, MSCs are highly proliferative and actively differentiating one-week post-hatch, after which, the MSC population decreases dramatically (Halevy *et al.* 2000, 2001). Moreover, temporarily reducing SMSC activity *via* irradiation of the turkey *Pectoralis* causes a decrease in mature muscle size (Mozdziak *et al.* 1997). Therefore, the mitotic activity of SMSCs in early life governs the ability of muscle to meet its full potential genetic size (Simone and Vieira 2004).

Myogenesis is controlled by myogenic regulatory

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factors including Myf5, MyoD and MRF4 (Pownall *et al.* 2002). As post-transcriptional regulators of myogenic gene expression, miRNAs also have a vital effect on myocyte differentiation (Zhang *et al*. 2017), microRNAs (miRNAs) can influence cellular processes such as proliferation, apoptosis and differentiation (Siengdee *et al*. 2015). As skeletal muscle precursors, SMSCs are also modulated by miRNAs (Zhang *et al.* 2015). For example, miR-192 significantly attenuated SMSCs differentiation by targeting the 3′ untranslated region (UTR) of sheep retinoblastoma 1(*RB1*) (Zhao *et al.* 2016), and miR-143 modulates the differentiation and proliferation of SMSCs by targeting *IGFBP5* (Zhang *et al.* 2017). To date, miRNA expression profiles in SMSCs of some domestic species (*e.g.*, bovine) have been surveyed using high-throughput sequencing (Zhang *et al*. 2016). However, there have been no studies regarding miRNA identification in pigeon SMSCs. Here, we have identified the miRNAs in pigeon SMSCs and differentiated myotubes. Our study will be instrumental in promoting a better understanding of the roles of miRNAs during the differentiation of pigeon MSCs.

## Materials and Methods

### Cell culture

Three 16-day-old pigeon embryo eggs were purchased from the FengMao pigeon breeding farm (Mianyang, China). Isolation of pigeon MSCs based on our previously established method (Lin *et al.* 2019). Briefly, 16-day-old pigeon embryos were dissected, followed by removing bilateral pectoral muscles and soaking in PBS (Hyclone, Utah, U.S.A.). The pectoral muscles were finely minced and dissociated in 0.1% collagenase type IV (Sigma, U.S.A.) for about 45 min. Subsequently, the cell suspension was filtered using a 40-$\mu$m nylon mesh (BD, Falcon™). The cells were collected after centrifugation at 1500 r·min$^{-1}$ for 3 min and resuspended in DMEM (Hyclone, Utah, U.S.A.) containing 20% FBS (Natocor, Argentina) with antibiotics (Solarbio, China). Subsequently, cells were seeded in 96-well or 24-well plate and cultured in $CO_2$ incubator (Thermo, U.S.A.) at 37°C and 5% $CO_2$ with saturating humidity. The culture medium was refreshed every 48 h until the fifth day. Based on our previous finding that pigeon SMSCs can be automatically differentiated into myotubes in common growth medium without addition of horse serum, induction of differentiation was merely to have the SMSCs incubated in DMEM medium containing 20% FBS for 5 days (Lin *et al*. 2019).

### Immunofluorescence staining

Pigeon SMSCs and myotubes were fixed with 4% paraformaldehyde for 15 min. After washing with PBS, cells were permeabilized with 0.5% Triton X-100 for 15 min. Next, cells were blocked by goat serum (Solarbio, China) for 30 min. Subsequently, cells were incubated with primary antibody against mouse Anti-MyHC (1:500, Abcam, U.S.A.) overnight at 4°C. Followed by washing twice with PBS, cells were incubated with the secondary antibody (FITC labeled goat anti-mouse IgG, 1:500, Abcam, U.S.A.) for 1 h and the cell nuclei were counterstained for 10 min with DAPI. Then, digital photomicrographs were taken.

### RNA extraction and high-throughput sequencing

Total RNA was respectively extracted from pigeon SMSCs and myotubes with TRIzol reagent (Invitrogen, U.S.A.). Each differentiation stage had three replicates that came from the three pigeon embryos. The quantity of total RNA was assessed using an Agilent 2100 Bioanalyzer. For samples of high-throughput sequencing, the RNAs from three replicates at each differentiation stage were pooled as one RNA sample. Small RNA ranging from 10–45 nt in length was purified by polyacrylamide gel electrophoresis and ligated using adaptors. The ligated RNA was reverse-transcribed to cDNA and amplified by PCR. Finally, the libraries were sequenced on a BGIseq-500 sequencing platform.

### Identification and differential expression analysis of miRNAs

Raw reads were filtered to remove the low quality-reads, repeated sequences and the adaptors, and the remaining reads was called clean data. Subsequently, filtered sequences were mapped to the pigeon reference genome (ColLiv2, GenBank assembly accession: GCA_001887795.1) with stringent criteria (0 mismatch for full length) using Bowtie software. Next, mappable reads were extended in the reference genome as predicted miRNA precursors. Only candidate precursors that perfectly matched to known pigeon (*Columba livia*) mature miRNAs annotated by miRBase (Release 22.0) were identified as known pigeon miRNAs (Kozomara *et al.* 2019). Subsequently, to identify the conserved miRNAs, we performed alignments between remaining candidate precursors and seed sequences of mature miRNAs from chicken, zebra finch and other mammals, allowing no mismatch. Novel miRNAs were further predicted using miRDeep2 (FriedläNder *et al*. 2008). EdgeR was used for differential expression analysis between SMSCs and myotubes in the OmicShare tools (Robinson *et al.* 2010). The miRNAs with $|\log_2(\text{fold change})| > 1$ and false discovery rate (FDR) <0.001 were identified differentially expressed miRNAs.

### Prediction and functional annotation of target genes

The TargetScan (Garcia *et al.* 2011) and RNAhybrid (Jan and Marc 2006) were used to predict the target genes of

differentially expressed miRNA. The R package ClusterProfiler was used for GO enrichment and KEGG pathway analysis (Yu *et al.* 2012).

**Dual-luciferase reporter assay**

To validate the miRNA-target interactions between representative DE miRNA and its target gene. Fagments (50 bp each) of 3′-UTR of *Mef2a* (XM_005514073.2) containing the wild-type or mutant binding sites for cli-miR-181a-5p were synthesized (Tsingke, China). The sequences were cloned into the pmirGLO plasmid (Promega, USA). 100 ng recombinant pmirGLO vector were co-transfected with 50 nM of the cli-miR-181a mimics or NC miRNA (GenePharma, China) into HeLa cells by Lipofectamine 3000 (Invitrogen, U.S.A.). After 48h of cell culture, dual-luciferase activity was measured using the Dual-Luciferase Reporter Assay System kit (Promega, U.S.A.) according to the manufacturer's protocols.

**qRT-PCR**

miRNAs were reverse-transcribed using Mir-X miRNA First-Strand Synthesis Kit, and qPCR assays were carried out using SYBR® Premix Ex Taq™ II on the CFX96 Real-Time PCR System (Bio-Rad, USA). Relative miRNA levels were normalized against U6 snRNA, and calculated using the $2^{-\Delta\Delta Ct}$ method. Primer sequences are listed in Table S1.

**Statistical analysis**

Statistical analyses were performed by using the SPSS 19.0 software. Student's t-test was applied to compare the means between two groups. $P < 0.05$ was regarded as statistically significant.

## Results

**Evaluation of pigeon SMSCs differentiation**

Terminal differentiation and myotube formation were evaluated by morphology and Myosin heavy chain (MHC) immunofluorescence staining. SMSCs were successfully isolated from pectoral muscle tissue of 16-day pigeon embryos. After incubation in growth medium (without horse serum) for 5 days, SMSCs were automatically differentiated into myotubes (Fig. 1A). Furthermore, immunofluorescence staining showed MHC protein was normally expressed in myotubes, but almost not in undifferentiated SMSCs (Fig. 1B).

**Overview of miRNA sequencing data**

To explore miRNA profiles in pigeon SMSCs and differentiated myotube, two pooled total RNA samples (from three biological replicates, respectively) at each differentiation stage were used to construct sequencing

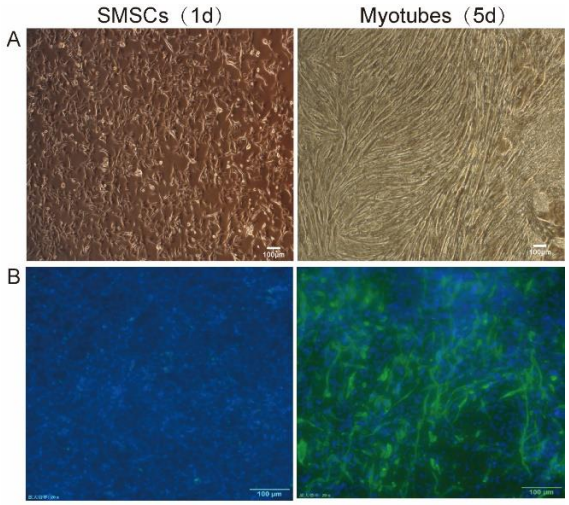


**Fig. 1:** Morphology and immunofluorescence identification of pigeon SMSCs. **A**. Morphological changes during differentiation of pigeon SMSCs (40×). **B**. Immunofluorescence staining of MHC in SMSCs and myotubes (100×)

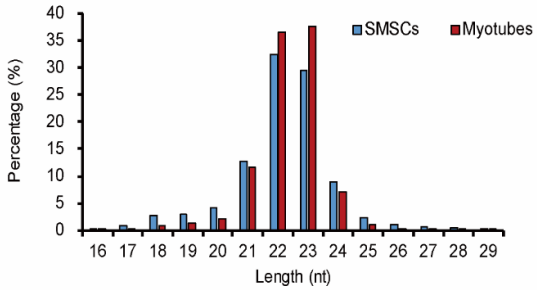


**Fig. 2:** Length distributions of the pigeon SMSCs and myotubes miRNA libraries. Blue columns represent length distributions of reads in the SMSCs library; red columns represent length distributions of reads in the myotubes library

libraries. As a result, we totally obtained 23.98 million raw reads. The adaptor sequences, contamination and low-quality reads were then trimmed and the remaining reads were regarded as high-quality clean reads. The proportion of high-quality clean reads account for 93.12 and 87.30% in the SMSCs and myotubes libraries, respectively. As shown in Fig. 2, the majority (≥ 83.42%) of the small RNAs have a length within the range between 21 and 24 nt, which is a common length for miRNAs. 65.97 and 79.30% of the high-quality clean reads in the two libraries were mapped to the pigeon genome, respectively (Table 1). The mappable reads were used for further miRNA identification.

**Identification of miRNAs and expression profiles in pigeon SMSCs**

We totally identified 689 mature miRNAs in two differentiation stages of pigeon SMSCs (Table 2). These miRNA candidates were classified into three types:

**Table 1:** Mapping the clean reads to the reference pigeon genome

| Mapping statistics | SMSCs | Myotubes |
|---|---|---|
| Raw Reads | 11,932,387 | 12,049,737 |
| High-quality clean Reads | 11,111,427 | 10,519,865 |
| Mapped Reads | 7,330,625 | 8,341,898 |
| Unmapped Reads | 3,780,802 | 2,177,967 |
| Mapped Ratio (%) | 65.97 | 79.30 |
| Unmapped Ratio (%) | 34.03 | 20.70 |

**Table 2:** Pigeon miRNAs identified in two sRNA libraries

| Group (number of pre-miRNA/miRNA) | SMSCs | Myotubes | Total |
|---|---|---|---|
| Pigeon known miRNAs | 162/290 | 155/272 | 166/297 |
| Pigeon conserved miRNAs | 156/185 | 159/201 | 219/261 |
| Pigeon putative novel miRNAs | 80/99 | 75/104 | 99/131 |

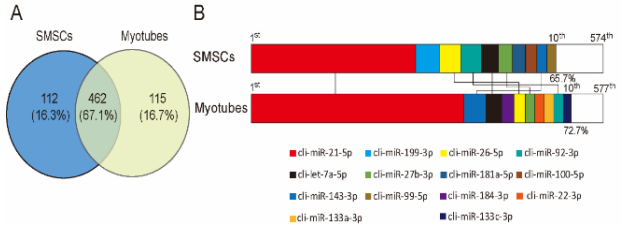


**Fig. 3:** miRNAs expression profiles in pigeon SMSCs and myotubes. **A**. The number of expressed miRNAs at the two differentiation stages. **B**. Top 10 unique miRNAs with the highest expression during SMSC differentiation. Plot with different color represents the percentage of each miRNA expression in each library. The 6 overlapped miRNAs in the top 10 miRNAs in the two libraries are connected by lines

known miRNAs, conserved miRNAs and putative novel miRNAs. There are 297 pigeon known miRNAs corresponded to 166 pigeon known pre-miRNAs (Table S2). 261 pigeon conserved miRNAs corresponded to 219 other species pre-miRNAs (Table S3). 131 putative novel miRNAs corresponded to 99 candidate pre-miRNAs (Table S4). Besides, 462 miRNAs were shared by the two libraries, while 112 miRNAs only expressed in SMSCs, and 115 miRNAs merely in myotubes (Fig. 3A).

To unveil the potential roles of miRNAs in pigeon SMSCs and myotubes, identified miRNAs were ranked by expression abundance (Fig. 3B). The miRNAs expression profile was different between SMSCs and myotubes. Of note, the expression abundance of top 10 unique miRNAs accounts for 65.7 and 72.7% of the total counts in these two libraries, respectively. Also, the top 10 unique miRNAs across two SMSCs differentiation stages involves 14 kinds of unique miRNAs. Among these miRNAs, six miRNAs (miR-21-5p, miR-26-5p, miR-92-3p, let-7a-5p, miR-27b-3p, miR-143-3p) overlapped.

### Differentially expressed (DE) miRNAs analysis and qPCR validation

To screen the differential miRNAs between pigeon SMSCs and myotubes libraries, differential expression analysis by taking $|\log_2(\text{fold change})| > 1$ and FDR <0.001 as criteria were performed after removing miRNAs of less than 11 count reads. We totally identified 193 miRNAs that were differentially expressed during SMSCs differentiation (Table S5). Among these DE miRNAs, 74 miRNAs were up-regulated, while another 119 miRNAs were down-regulated (Fig. 4).

To confirm the small RNA-seq results, we selected 8 miRNAs to conduct a qPCR assay. As shown in Fig. 4C, 6 miRNAs (miR-181-5p, miR-429-3p, miR-119-5p, miR-126-3p, miR-200a-3p, miR-214-3p) were down-regulated in myotubes, while the other 2 miRNAs (miR-184a-3p and miR-133a-3p) were up-regulated when compared with SMSCs. These results of qPCR assay were highly consistent with small RNA-seq.

### Functional enrichment analysis

Differentially expressed miRNAs with reading numbers higher than 1000 were selected to predict the target genes. There is a total of 5284 genes having target sites for these miRNAs. GO categorization of these target genes were enriched in 237 terms including regulation of the biosynthetic process, cell differentiation, cell development, anatomical structure morphogenesis, embryo development, tube development (Fig. 5A, Table S6). KEGG pathways analysis indicated these target genes were principally enriched in 25 pathways, such as Endocytosis, Regulation of actin cytoskeleton, MAPK, Wnt, mTOR and TGF-beta signal pathway (Fig. 5B, Table S6). Above results were closely linked to the differentiation of skeleton muscle satellite cells.

### Target verification of miR-181a-5p

Based on *in silico* analysis, 3'-UTR region of *Mef2a* has potential binding sites for cli-miR-181a-5p (Fig. 6A–B), we speculated that cli-miR-181a-5p can directly target 3'UTR region of *Mef2a* and a dual-luciferase reporter assay was carried out. As shown in Fig. 6C, cli-miR-181a-5p conspicuously decreased luciferase activity of wild-type reporter of *Mef2a* 3′-UTR, whereas luciferase activity with *Mef2a* 3′-UTR mutant construct exhibited no statistically significant difference between the cells transfected with cli-miR-181-5p mimics and NC. These findings indicated that cli-miR-181a-5p can directly target *Mef2a*.

## Discussion

The establishment of a cellular research model through the isolation of muscle satellite cells from different animal species is critical approach for understanding the mechanisms of myogenic differentiation. Based on our previously established method, we here isolated from the eggs of 16-day-old pigeon embryo. Interestingly, after being cultivated in growth medium without horse serum for 5 days,

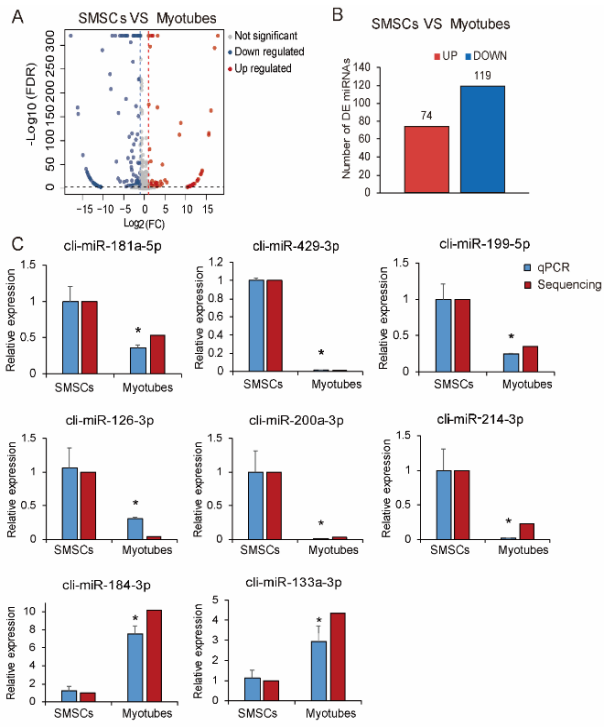


**Fig. 4:** Differential miRNAs analysis and qPCR validation. **A**. Volcano plot of miRNA in SMSCs versus mytubes. **B**. The upregulated and downregulated number of the DE miRNAs. **C**. qRT-PCR validation of 8 DE miRNAs. Red bars represent the miRNA relative expression abundance determined by the miRNA sequencing reads (normalized). Blue bars represent the miRNA relative expression abundance determined by qRT-PCR (mean ± SE). * above the error bars for each miRNA show significant differences at $P < 0.05$

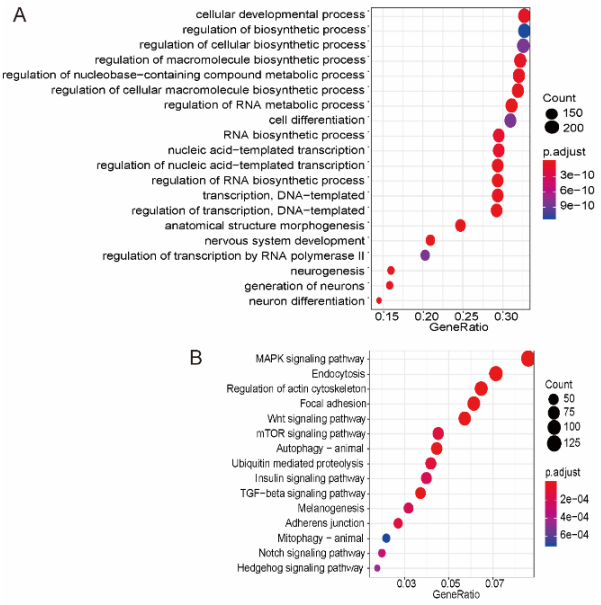


**Fig. 5:** Go functional enrichment and KEGG analysis of target genes of high expressed DE miRNAs in SMSCs and myotubes. **A**. Gene ontology enrichment (top20) analyzed by clusterProfiler. GeneRatio: the ratio of the number of target genes in the GO category to that of the annotated genes in the GO database. **B**. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis (top15). GeneRatio: the ratio of the number of target genes in the KEGG category to that of the annotated genes in the KEGG database

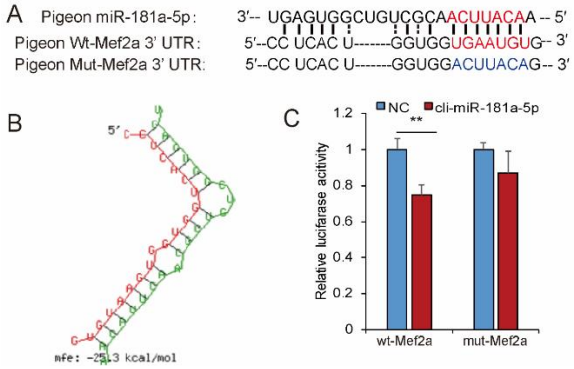


**Fig. 6:** Cli-miR-181a-5p bioinformatics analyses and dual-luciferase reporter assay. **A**. Prediction of cli-miR-181a-5p binding sites in 3'UTR of *Mef2a*.Luciferase reporter plasmids contain WT or MUT putative miR-181a-5p target sites. **B**. Schematic alignment of the free energy scores (RNAhybrid) for miRNA-181a-5p–*Mef2a* hybridization. **C**. A dual-luciferase reporter assay was performed by co-transfecting luciferase reporter containing the 3'UTR of *Mef2a* (wild-type or mutant) with miR-181a-5p mimics or miR-control into HeLa cells. Luciferase activity was determined 48 h after transfection. Three independent experiments were performed in triplicate and all data were expressed as mean ± SE. * $P < 0.05$

we observed numerous fused myotubes. Combined with our results from MHC immunofluorescence staining, this confirmed that the pigeon SMSCs had successfully differentiated. It is notable that horse serum was not essential for pigeon SMSC differentiation, as it is close to indispensable for the differentiation of SMSCs isolated from some other species (*e.g.*, bovine, mouse) into myotubes. Reported that "spontaneous" differentiation of skeletal myoblast (*e.g.*, Sol8 cells) is associated with autocrine secretion of IGF -II (Florini *et al.* 1991). We speculate that this factor may also be associated with the "spontaneous" differentiation of pigeon SMSCs; this will need to be confirmed by further studies.

MiRNAs play crucial roles in myogenic differentiation by participating in an orchestrated process of gene regulation (Horak *et al.* 2016). Certain miRNAs exclusively expressed in the striated muscle are called myomiRs (McCarthy 2008). While miRNAs expressed in SMSCs and muscle tissues of some species (*e.g.*, bovine (Zhang *et al.* 2016), chicken (Li *et al.* 2011), duck (Gu *et al.* 2014)) have been identified, none have been reported in pigeons. Here, we totally identified 689 miRNAs from two differentiation stages of pigeon SMSCs, which is more than that previously reported (617) in bovine (Zhang *et al.* 2016), Among the top

10 unique miRNAs with the highest expression during pigeon SMSC differentiation, three miRNAs (miR-21-5p, miR-199-3p and let-7a-5p) are also ranked within the top ten in bovine SMSCs (Zhang *et al.* 2016).

Some of identified pigeon miRNAs have been previously reported to participate in skeletal myogenesis and muscle development, for example, miR-21, miR-133, miR-1, miR-27b, miR-499, miR-26 and miR-181 (Luo *et al.* 2013; Bai *et al.* 2015). Among these, miR-21 exhibited the highest abundance in both two libraries and its expression level was higher in myotubes than in SMSCs. Reported that the miRNA-21 facilitates myogenesis by targeting TGFβI (Bai *et al.* 2015). These results suggest that miRNA-21 also plays a crucial role during skeletal myogenesis in pigeons. In addition, miR-133, miR-1 and miR-499 are muscle-specific miRNAs. Of these, miR-133a suppresses myoblast proliferation and promotes myoblast differentiation (Horak *et al.* 2016); miR-1 can directly target HDAC4 to promote myogenesis (Chen *et al*. 2006); overexpression of miR-499 reduces *Mstn* 3′UTR activity (Bell *et al.* 2010). Our results indicated that expression levels of miR-133a-3p, miR-1 and miR-499 are conspicuously up-regulated during myogenic differentiation in pigeons, which confirms the functional conservation of these myomiRNAs between pigeons and other species. Additional miRNAs with a high abundance in SMSCs and myotubes of pigeons have also been implicated in a variety of physiological processes. For example, miR-184 regulated cell proliferation by targeting *SOX7* (Wu *et al*. 2014) and *AKT2* (Foley *et al.* 2010), let-7a down-regulates MYC and reverses MYC-induced cell growth (Sampson *et al*. 2007), and miR199a-5p inhibits insulin sensitivity via the suppression of ATG14-mediated autophagy (Li *et al*. 2018). Notably, a previous report documented that miR-181a paticipated in muscle regeneration (Naguibneva *et al.* 2006); however, there is also evidence that miR-181 negatively regulates myotube size (Soriano-Arroquia *et al.* 2016). In our study, the expression level of miR-181a-5p was reduced during pigeon SMSCs differentiation, which is consistent with research on bovine SMSCs (Zhang *et al.* 2016). Furthermore, we found that cli-miR-181a-5p may directly target *Mef2a* which interacts with MRF family members to promote myogenic differentiation (Luo *et al.* 2013). Hence, the down-regulation of cli-miR-181a-5p in myotubes may reflect the establishment of a differentiated phenotype via the enhancement of of *Mef2a* expression.

The target genes of highly abundant DE miRNAs were enriched in the GO categories ‘cellular developmental process’, ‘cell differentiation’, ‘anatomical structure morphogenesis’ and ‘tube development’. These results are in line with our morphological observations and immunofluorescence analysis of pigeon SMSC differentiation. Moreover, a KEGG pathway analysis revealed an enrichment of these target genes mainly in the MAPK, Wnt, mTOR and TGF-beta signaling pathways. Studies in other species have also demonstrated that these pathways play crucial roles in myogenic differentiation (Liu *et al*. 2004; Keren *et al*. 2006; Tanaka *et al*. 2011). These findings imply that pigeon miRNAs regulate SMSC differentiation also *via* similar signaling pathways as other species.

## Conclusion

In this study, we identified 297 known miRNAs, 261 conserved miRNAs and 131 novel miRNAs in pigeon SMSCs and myotubes using small RNA sequencing and proved that *Mef2a* is a direct target of cli-miR-181a-5p. We infer that these identified miRNAs could play vital roles during the myogenic differentiation of pigeon SMSCs, and these findings improve our understanding of muscle differentiation and development in pigeons.

## Acknowledgements

Differentially expressed miRNAs were determined using the OmicShare tools, a free online platform for data analysis (http://www.omicshare.com/tools).

## Author Contributions

Xun Wang, Xuewei Li and Mingzhou Li designed the experiments. Zhenhao Lin, Lei Liu, Peiqi Yan and Anan Jiang performed experiments. Yi Luo, Siyuan Feng, Qianzi Tang and Keren Long conducted bioinformatics analysis. Xun Wang, Ling Zhao, Haifeng Liu, Long Jin and Jideng Ma statistically analyzed the data and made illustrations.

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