Expression of microRNA and microRNA processing machinery genes during early quail (*Coturnix japonica*) embryo development

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ABSTRACT MicroRNA (miRNA) are small regulatory RNA molecules that are implicated in regulating and controlling a wide range of physiological processes including cell division, differentiation, migration, apoptosis, morphogenesis, and organogenesis. The aim of this study was to determine the expression pattern of 32 miRNA and 18 miRNA processing machinery genes during somite formation in quail embryos. The embryos were collected at stages HH (Hamburger and Hamilton) 4, 6, and 9 of embryo development (19, 24, and 30 h of incubation, respectively). Total RNA including miRNA was isolated from 4 groups of embryos (each group consisting of 6 to 8 embryos) were collected at each of the 3 stages (19, 24, and 30 h). The expression pattern of candidate miRNA and miRNA processing machinery genes was performed using quantitative real-time PCR. The results demonstrated that 7 miRNA (let-7a, mir-122, mir-125b, mir-10b, $P < 0.01$; let-7b, mir-26a, and mir-126, $P < 0.05$) were differentially expressed during early quail embryo development. In addition, the expression profile of 18 miRNA processing machinery genes was not significantly increased at 30 h of incubation compared with both 19 and 24 h. Our results suggest that machinery genes for miRNA biogenetic pathways are functional, and hence, miRNA may be involved in the regulation of early quail development. These 7 differentially expressed miRNA are suggested to play critical roles in quail embryo somite formation.

Key words: microRNA, processing machinery gene, quail, embryo, development

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INTRODUCTION

MicroRNA (**miRNA**) are small (ranging in size approximately 19 to 24 nucleotides), noncoding, highly conserved RNA that account for approximately 1 to 1.5% of all cellular transcripts in animals (Lee and Ambros, 2001; Lewis et al., 2005). They play a fundamental role in regulation of gene expression in eukaryotes at the posttranscriptional level through mRNA cleavage, RNA degradation, translation inhibition, or DNA methylation (Fire et al., 1998; Hwang and Mendell, 2007; Williams et al., 2009). The miRNA normally regulate the expression of genes by guiding the RNA-induced silencing complex to a target sequence, which is usually located at the 3′ untranslated region (**UTR**) of mRNA. It has been estimated that they may target up to 30% of protein coding genes in animals. The latest release of the miRNA registry demonstrates a total of 8,619 miRNA across plant and animal genomes (miRBase release 12.0, September 2008; http://microrna.sanger.ac.uk/sequences, Griffiths-Jones, 2004).

Poultry embryos have been extensively used in developmental studies because the developmental processes of the avian embryos, particularly chicken, are well defined (Hamburger and Hamilton, 1951) and live embryos can be easily manipulated (Brown et al., 2003). Japanese quail is both one of the favored animal models in developmental biology (Ainsworth et al., 2010) and a commercial bird for egg and meat production. However, advanced research developments have been slowed down by the limited information that is available on the genetics of this species (Minvielle et al., 2005; Bonneaud et al., 2008). Embryonic development is continuum of highly complex morphogenetic process-

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es that are coordinated both spatially and temporally by well-orchestrated cellular events.

One of the major morphogenetic processes is the formation of somites during embryo development of animals. Somites are transient mesodermal structures that give rise to the axial skeleton, trunk musculature, muscles of the limbs, connective tissue, endothelial cells, and smooth muscle cells (Stockdale et al., 2000; Smith et al., 2005). The miRNA regulate a range of physiological processes during this period, which include developmental timing, cell division, differentiation, migration, apoptosis, morphogenesis, and organogenesis (Hwang and Mendell, 2007; Williams et al., 2009). Whole embryo development involves the proliferation and differentiation of early embryonic cells. It is thus conceivable that miRNA also play an important role in regulating whole embryonic development in addition to somite formation.

Understanding the functions of miRNA and miRNA machinery genes necessitates detailed studies on their regulation and expression patterns. It is the objective of the current study to provide a comprehensive analysis of 32 miRNA and 18 miRNA processing machinery genes in Japanese quail to establish their potential role during first somite formation. Candidate miRNA were selected from studies in chicken embryos that demonstrated their expression during first somite formation (Darnell et al., 2006; Glazov et al., 2008; Rathjen et al., 2009). The present experiment was designed to profile the expression of miRNA and miRNA processing machinery genes before, during, and after the time of first quail somite formation [Hamburger and Hamilton (**HH**) stages 4, 6, and 9, respectively]. To the best of our knowledge, this is the first study to investigate the expression patterns of miRNA and miRNA processing machinery genes during early quail development.

MATERIALS AND METHODS

Collection and Processing of Quail Embryos

Fertilized eggs (*Coturnix japonica*) were obtained from the experimental research station of Frankenforst at University of Bonn. Embryos were harvested in compliance with an approved Bonn University Animal Care and Use Committee Protocol. Eggs were incubated for set periods of time at 37.8°C in a humidified (65%) incubator (Easy 150 Model, Hemel Brutgerate, Verl, Germany) based on the manufacturer's recommendations. All the embryos were washed free of yolk, albumen, and extra-embryonic membranes by sterile nuclease-free water and were staged according to Hamburger and Hamilton (1951). There is general agreement that there is little developmental difference between the chick and quail embryos during early embryonic development (Sellier et al., 2006; Ainsworth et al., 2010). Additionally, Ainsworth et al. (2010) reported that the early quail stages (stage 4 to 28) correspond directly with HH stages for chick embryos. Whole embryos were collected at 19 h (h) of incubation (stage 4), which was preceded first somite formation, at 24 h of incubation (stage 6), which was during first somite formation and 30 h of incubation, which was at the 10 somite stage (stage 9). Four groups of embryos (each consists of 6 to 8 embryos) were collected at each of the 3 stages (19, 24, and 30 h). Embryos were stored in RNA-Later (Ambion, Austin, TX) at 4°C until isolation of RNA.

Isolation of Total RNA

Embryos from each stage of incubation (19, 24, and 30 h) were initially centrifuged at 300 × *g* for 2 min at 4°C and then washed 2 times with ice-cold PBS. First, an equal volume of ice-cold PBS was added to reduce the density of RNA-Later, centrifuged for 2 min at 4°C with 300 × *g*, and removed the diluted supernatant leaving some solution with the sample. Second, washing with ice-cold PBS was repeated once again, removed the supernatant without disturbing the pellet, and immediately preceded for isolation of RNA. Total RNA from the 4 groups of embryo at each stage of incubation (19, 24, and 30 h) were isolated using miRNeasy mini kit (Qiagen GmbH, Germany) following the manufacturer's protocol. Immediately after isolation, contaminating DNA and divalent cations from RNA samples were removed using the Turbo DNA-free kit (Ambion) according to the manufacturer's instructions. The quality and the concentration of the RNA were assessed using a NanoDrop 8000 spectrophotometer (Thermo-Scientific, Waltham, MA).

Fractionation of Total RNA into Small and Large RNA

Large (>200 nt) and small RNA (<200 nt) were separated from isolated total RNA using special silica membrane spin column and chemicals of RT^2 qPCR-Grade miRNA isolation kit (SABiosciences, Frederick, MD) according to manufacturer's instructions. For every study, for every experiment a NanoDrop 8000 spectrophotometer (Thermo-Scientific) was used to assess the concentration of both the small and large RNA. Isolated small RNA were used for the study of expression profiling of individual miRNA, whereas the large RNA were in turn used for analysis of the regulatory miRNA processing transcripts at the selected stages of embryo development.

Expression Profiling of miRNA by Quantitative Real-Time PCR

A total of 300 ng of small RNA from each group of embryos at each of the 3 stages (in quadruplet) were synthesized into first strand cDNA using the RT^2 miRNA first strand kit (SABiosciences) according to the manufacturer's instructions.

Table 1. The sequence and accession number microRNA (miRNA) used for the study

Serial no.	miRNA (ID)	Sequence (5′–3′)	Accession (miRBase, ENSEMBL)	Tm[1] (°C)
1.	gga-let-7a	TGAGGTAGTAGGTTGTATAGTT	MIMAT0001101	54.7
2.	gga-let-7b	TGAGGTAGTAGGTTGTGTGGTT	MIMAT0001102	58.4
3.	gga-miR-101	TACAGTACTGTGATAACTGAA	MIMAT0001185	52.0
4.	gga-miR-103	AGCAGCATTGTACAGGGCTATGA	MIMAT0001145	60.6
5.	gga-miR-10b	TACCCTGTAGAACCGAATTTGT	MIMAT0001148	56.5
6.	gga-miR-122	TGGAGTGTGACAATGGTGTTTG	MIMAT0001190	58.4
7.	gga-miR-125b	TCCCTGAGACCCTAACTTGTGA	MIMAT0001105	60.3
8.	gga-miR-126	CGTACCGTGAGTAATAATGCG	MIMAT0001169	57.9
9.	gga-miR-135a	TATGGCTTTTTATTCCTATGTGA	MIMAT0001099	53.5
10.	gga-miR-140	TACCACAGGGTAGAACCACGGA	MIMAT0001159	62.1
11.	gga-miR-146b	TGAGAACTGAATTCCATAGGC	MIMAT0003351	55.9
12.	gga-miR-15a	TAGCAGCACATAATGGTTTGT	MIMAT0001117	54.0
13.	gga-miR-15b	TAGCAGCACATCATGGTTTACA	MIMAT0001154	56.5
14.	gga-miR-17–5p	CAAAGTGCTTACAGTGCAGGTAGT	MIMAT0001114	61.0
15.	gga-miR-184	TGGACGGAGAACTGATAAGGGT	MIMAT0001158	60.3
16.	gga-miR-18a	TAAGGTGCATCTAGTGCAGATA	MIMAT0001113	56.5
17.	gga-miR-19a	TGTGCAAATCTATGCAAAACTGA	MIMAT0001112	55.3
18.	gga-miR-196	TAGGTAGTTTCATGTTGTTGG	MIMAT0001121	54.0
19.	gga-miR-199a	CCCAGTGTTCAGACTACCTGTT	MIMAT0001152	60.3
20.	gga-miR-202	TTCCTATGCATATACTTCTTT	MIMAT0003355	50.1
21.	gga-miR-20a	TAAAGTGCTTATAGTGCAGGTAG	MIMAT0001111	57.1
22.	gga-miR-206	TGGAATGTAAGGAAGTGTGTGG	MIMAT0001139	58.4
23.	gga-miR-21	TAGCTTATCAGACTGATGTTGA	MIMAT0003774	54.7
24.	gga-miR-211	TTCCCTTTGTCATCCTTTGCC	MIMAT0003368	57.9
25.	gga-miR-22	AGTTCTTCAGTGGCAAGCTTTA	MIMAT0007288	56.5
26.	gga-miR-222	AGCTACATCTGGCTACTGGGT	MIMAT0001107	59.8
27.	gga-miR-24	TGGCTCAGTTCAGCAGGAACAG	MIMAT0001188	62.1
28.	gga-miR-26a	TTCAAGTAATCCAGGATAGGC	MIMAT0001118	55.9
29.	gga-miR-27b	TTCACAGTGGCTAAGTTCTGC	MIMAT0001187	57.9
30.	Bta-miR-410	AATATAACACAGATGGCCTGT	MIMAT0009311	54.0
31.	gga-miR-9	TCTTTGGTTATCTAGCTGTATGA	MIMAT0001195	55.3
32.	gga-miR-92	TATTGCACTTGTCCCGGCCTG	MIMAT0001109	61.8
33.	SNORD47	CCGTTCCATTTTGATTCTGAG	ENSBTAT00000059172	55.9
34.	U6 RNA	ATTGGAACGATACAGAGAAGATTAG	ENSGALT00000028603	58.1
35.	5S rRNA	TCTACGGCCATACCACCCTGA	ENSGALT00000035456	60.5

[1]Tm = melting curve.

Quantitative real-time PCR for the selected miRNA (n = 32), along with 3 endogenous controls (5S rRNA, U6 snRNA, and Snord47), was performed using SYBR Green technology (SABiosciences) in an ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA). For this purpose, synthesized cDNA were diluted 10 times with RNase-DNase free water. Twenty microliters of reaction mixture, each consisting of 5 μL of respective diluted cDNA, 0.5 μL of each sequence-specific miRNA forward and reverse primers, and 13.5 μL of SYBR Green, was aliquoted to each well of the 96-well plate and loaded onto an ABI Prism 7000 Real-Time PCR apparatus. Primers were obtained from SABiosciences (Table 1). Thermal cycling was performed as 95°C for 10 min, 40× of 95°C for 15 s, and 60°C for 1 min. The dissociation stage was incorporated at the end of the cycle to assess the melting curve (Tm) of the amplified PCR amplicon to verify the specificity of the amplification reaction. In addition, amplification was verified for selected reactions through 2.5% agarose gel electrophoresis. The quantitative real-time PCR (**qPCR**) data were analyzed by ΔC_t method ($\Delta Ct = Ct_{\text{target gene}} - Ct_{\text{housekeeping gene}}$) (Silver et al., 2006) and normalization performed by geometric mean of the 2 endogenous controls (5S and U6) through SABiosciences' PCR array data analysis online web-based analysis portal.

Reverse-Transcription and SYBR Green-Based Quantification of miRNA Processing Genes

Important candidate genes involved in transcription, processing, and generating mature miRNA were quantified at different stages of quail embryo by qPCR. Primers for qPCR analysis (Table 2) were designed using the Primer Express 2.0 software program (Applied Biosystems) and synthesized by Eurofins MWG Operon (Ebersberg, Germany). The sequences of PCR primers are listed in Table 2. Reverse transcription of 170 ng of isolated large RNA from each sample was performed using Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, CA) in combination with random primers (Invitrogen) and oligo $(dT)_{23}$(Sigma). The cDNA was stored at −20°C until use. All primers used were designed and optimized to ensure optimum reaction efficiencies for target and housekeeping reference genes (*GAPDH*, *ACTB*). Triplicate reactions were performed for each gene using a standard PCR protocol in a 20-μL reaction volume consisting of 10 μL of iTaq

SYBR Green Supermix with ROX (Bio-Rad, Hercules, CA), forward and reverse primers at 200 to 300 n*M* final concentration, and 2 μL of diluted template cDNA. The universal thermal cycling parameter specified for the instrument was 50°C for 10 s, 95°C for 10 min, followed by 40 amplification cycles at 95°C for 15 s and at 60°C for 1 min. In addition, at the end of the last cycle, a dissociation curve was generated by starting the fluorescence acquisition at 60°C and taking measurements at 7-s intervals until the temperature reached 95°C. The same PCR protocol was used for all primers, and data were normalized using ΔCt ($\Delta Ct = Ct_{\text{target gene}} - Ct_{\text{housekeeping gene}}$) (Silver et al., 2006).

Statistical Analysis

The data were analyzed using the SAS software package version 9.2 (SAS Institute Inc., Cary, NC). All values are expressed in the Supplemental Tables S1 and S2 (available online at http://ps.fass.org/) as means of duplicate data ± SEM. A one-way factorial ANOVA with Tukey's test was used to detect any possible effect of time on early embryo development among the different mRNA and miRNA. Significant differences between time groups are indicated with capital letters ($P < 0.01$) and lowercase letters ($P < 0.05$).

RESULTS

Expression of miRNA During Early Quail Embryo Development

Results demonstrated that all the 32 miRNA were expressed during the time periods investigated during the early quail embryo development (Figure 1; Supplemental Table 1, available online at http://ps.fass.org/). From these 32 miRNA, the expression pattern of 14 miRNA, which included mir-122, mir-24–3p, mir-21, mir-26a, mir-126, mir-10b, let-7a, and let-7b, were numerically reduced at 24 and 30 h compared with 19 h

Table 2. The sequence and accession number microRNA processing machinery genes used for the study

Gene	Primer sequence[1] (5′→3′)	Accession number	Product size (bp)
ADAR1	F-AATGGCTTTGCTGCAGAGTT R-GCGCTCTGCTTTCTCTGTTT	XM_001232161	192
ADARB1	F-GAGGATCAAGGGGACAGACA R-CAATACGTTCCACCTTGCT	NM_001111074	187
GEMIN5	F-ATGGCTGCAAAGTGCTTCTT R-CTGCTGCACGTTGTTCTCAT	XM_414574	170
GEMIN6	F-TGATCCAGTTTCTGCCAGTG R-TGGGCTGTAAGCTTTGCTTT	XM_423929	178
SIP1	F-TCACAGGATTTGGACAGCAA R-AGGAGGAAAGCCCACCTTTA	NM_001039302	156
YBX1	F-CAGACCAGTCAGGCAGAACA R-GCGTCTGCGTCTGTAGTTGA	NM_204414	196
RNASEN	F-GGGATTCGACAGCCAAAGTA R-CCACAGCATCTCCCAGAAAT	NM_001006379	166
DGCR8	F-GAAACATGATCCTCCCCTCA R-CAGAATCAGCAGCAGTGGAA	BX933340	185
XPO1	F-GACAAGGGGAAATGGCTGTA R-TTGCACAGGTAAGCAACCAG	NM_001012869	168
XPO4	F-AGAGCAAATCTCACGCACCT R-GGGAACCCTCTGTATCAGCA	NM_001012947	160
XPO5	F-GCACTTGTCCTCATCAGCAA R-TTTGTTGTCTGCACCCACAT	XM_419501.2	162
EIF2C3	F-CTTACCAGCTCTGCCACACA R-TTGCTCTGTCCTGAAACGTG	NM_001030900.1	157
EIF2C4	F-GCAGGGCATTTGAATGAAGT R-TGGAAACCAAACCAGACCTC	NM_001039276.1	167
MOV10	F-GCCCCGTTACATCTACAGGA R-ACCAACCTTCCTGCTGTCAC	NM_001012843.1	177
PIWIL1	F-TCGACAAGCCATAGAACACG R-CGTGCTTCCATCTCAGGATT	NM_001098852.1	150
FMR1	F-GTTTGGCCAGATGACCAGTT R-GGATTTGTTCCAGCCACAGT	XM_420363.2	164
DICER1	F-AGCAAGGCTGTTGAAGAGGA R-CCACAGCTGGTCAGATAGCA	NM_001040465.1	182
SCPEP1	F-CCTGGCTGGATTAGAAGCTG R-GGAGAAATGGGCAGATGAAA	NM_001012803.1	177
ACTB	F-GTCGCACCACTGGTATTGTG R-GCTGTGGTGGTGAAGCTGTA	AF199488	175
GAPDH	F AGTCGGAGTCAACGGATTTG R-ACAGTGCCCTTGAAGTGTCC	NM_204305.1	165
RPL31	F-ACCAGGCTGAACAAAGCAGT R-TCATCCACATTGACTGTCTGC	DQ983817.1	176

[1]F = forward; R = reverse.

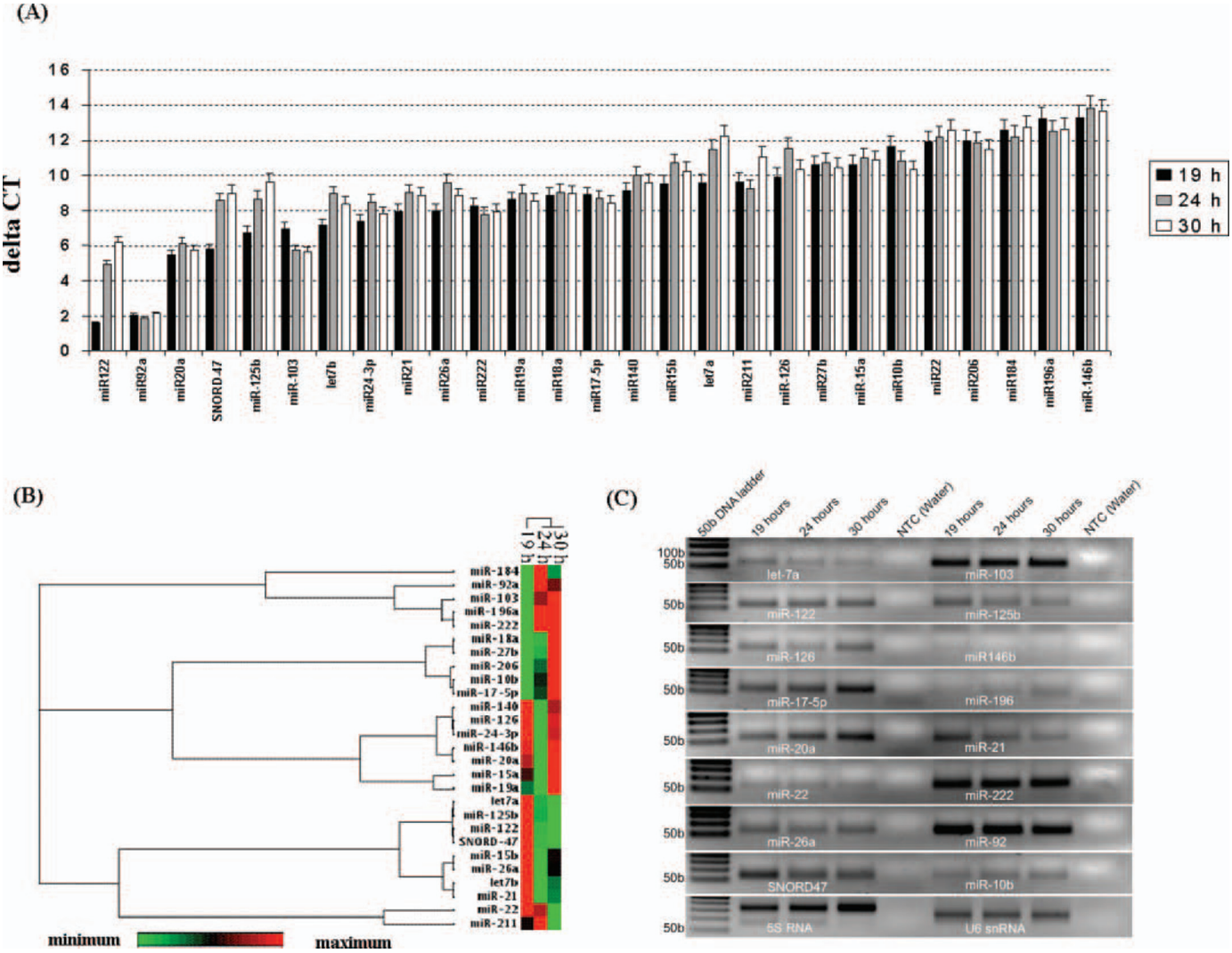

Figure 1. The expression pattern of 32 candidate microRNA (miRNA) during the different stages of quail embryo development. The average delta cycle threshold (Ct) value of each miRNA during the different stage of embryo development (A). The heatmap showing the expression pattern of 32 miRNA in different stage of quail embryo development (B). The agarose gel electrophoresis picture of miRNA (C). Color version available in the online PDF.

after incubation. Alternatively, the expression pattern of mir-103, mir-10b, mir-196a, and mir-20 was numerically increased at 24 and 30 h. Further statistical analysis of these miRNA revealed that 3 of these miRNA (let-7a, mir-122, mir-125b) were significantly increased ($P < 0.01$) from 19 to 30 h, whereas the expression pattern of let-7b, mir-26a, and mir-126 was significantly ($P < 0.05$) reduced from 19 to 24 h of incubation (Figure 2). In contrast, mir-10b expression was significantly increased ($P < 0.01$) as embryo development proceeded from 19 to 24 h of incubation (Figure 2).

Molecular Pathways Affected by miRNA During Quail Embryo Development

Among the 32 miRNA included in the current study, the expression patterns of 7 miRNA were differentially expressed during early quail development ($P < 0.05$; Figure 2). To characterize the function of these miRNA, the genes targeted by these miRNA were identified using the mirecords (http://mirecords.biolead.org/), which integrates 11 miRNA-target interaction prediction tools, namely DIANA-microT, MicroInspector, miRanda, MirTarget2, miTarget, NBmiRTar, PicTar, PITA, RNA22, RNAhybrid, and TargetScan/TargertScanS. The target genes identified by at least 3 or more target predicting tools were selected as potential target genes. Thus, a total of 1,046 genes were found to be the potential target genes of 7 differentially expressed miRNA (Supplemental Table 3, available online at http://ps.fass.org/) of which 59 genes, including *ABCC5*, *CPEB3*, *GALNT7*, *SEMA4D*, *GAS7*, *DAPK1*, *ELAVL2*, *LGR4*, *P4HA1*, *PLCL1*, *ULK1*, *YPEL1*, *BZW1*, and *BZW2*, were targeted by 2 or more miRNA. Gene ontological annotation of the miRNA target genes was then assessed by DAVID Functional Annotation Bioinformatics Microarray Analysis (http://david.abcc.ncifcrf.gov/). Accordingly, several developmentally relevant biological processes were identified

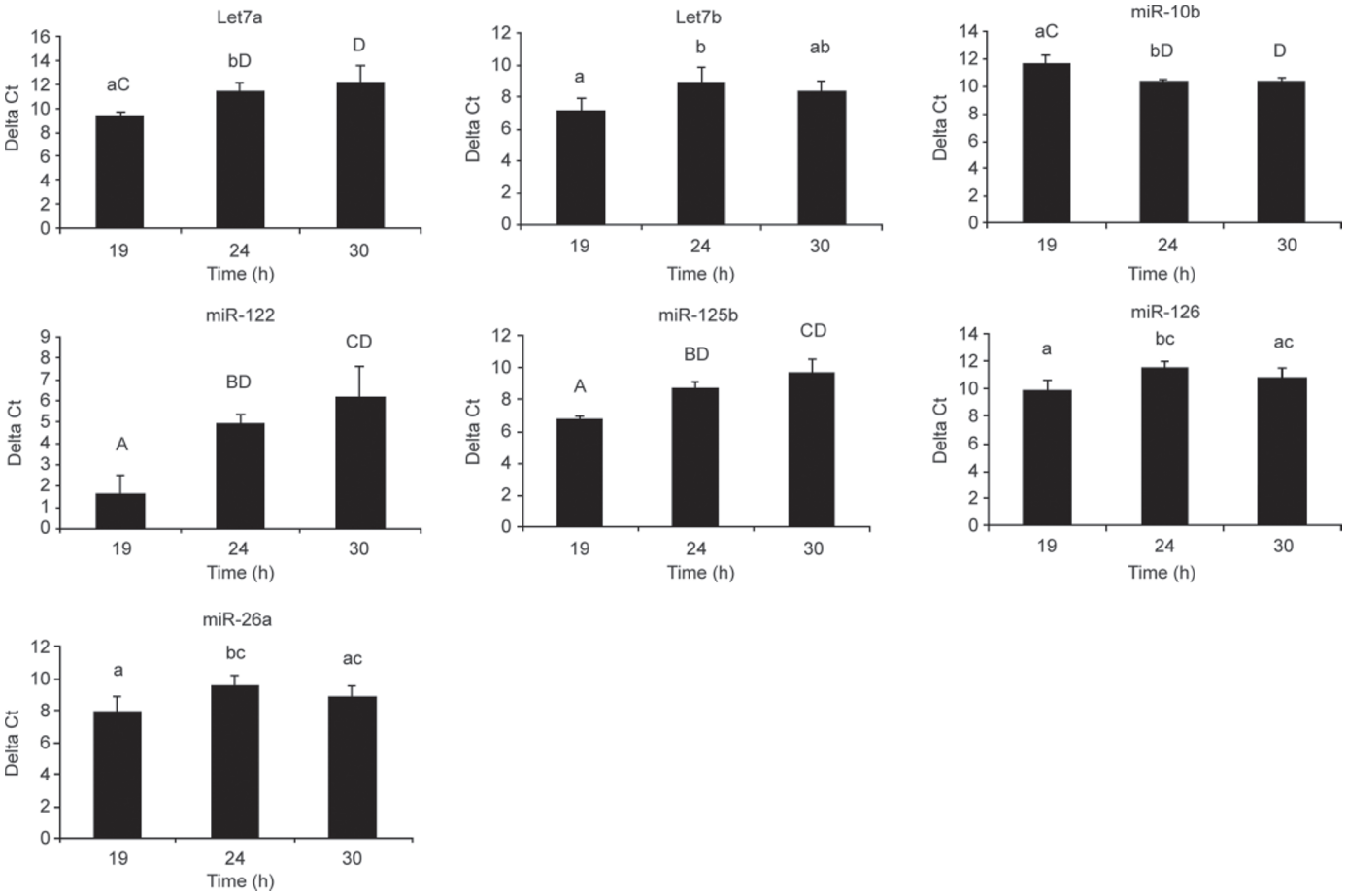

Figure 2. The expression pattern of 7 differentially expressed microRNA (miRNA) during the early development of the quail embryo. Different letters (a–c; A–D) on the bars represent significant differences ($P < 0.05$, capital letters represent $P < 0.01$).

from the target genes. Among those, the target genes of mir-26a, which was increased at 24 h of incubation, were found to be involved in several biological processes including phosphate metabolic process, protein localization, regulation of cell death, cell motion, immune system development, hemopoiesis, cellular component morphogenesis, axonogenesis, and extracellular structure organization. Apart from mir-26, the target genes of mir-125b, Let-7a, mir-10b, Let-7b, mir-10b, mir-26a, mir-122, and Let-7b were found to be involved in proteolysis, protein transport, response to abiotic stimulus, intracellular protein transport, Rho protein signal transduction, polysaccharide biosynthetic process, glycerolipid biosynthetic process, and transition metal ion transport.

Expression Profile of miRNA Processing Machinery Genes During Quail Embryo Development

In addition to the miRNA selected for analysis, the expression profile of 16 miRNA processing machinery genes was profiled at 19, 24, and 30 h during quail embryo development. The results revealed that the expression profile of the selected machinery genes was numerically increased at 30 h of incubation compared with both 19 and 24 h but the differences were not statistically significant (Figure 3; Supplemental Table 1, available online at http://ps.fass.org/). However, the expression pattern of the genes remains similar at 19 and 24 h of embryo development.

DISCUSSION

The relative expression of miRNA and miRNA processing genes at the time of first somite formation have been investigated in quail. We are aware of the fact that RNA were prepared from whole embryos at the stages of HH 4, 6, and 9 (19, 24, and 30 h of incubation, respectively) and is thus difficult to discuss observed changes in abundance of specific miRNA in the context of somite formation. However, certain trends in gene expression were observed. For instance, there was a significant decrease in the abundance of 3 miRNA (let-7a, mir-122, mir-125b; Figure 2), which suggests a progressive restriction in the expression of these respective miRNA as embryonic development proceeded from 19 to 30 h. Nevertheless, even though there was a significant reduction in the abundance of 3 miRNA (let-7b, mir-26a, and mir-126; Figure 2) as embryo development

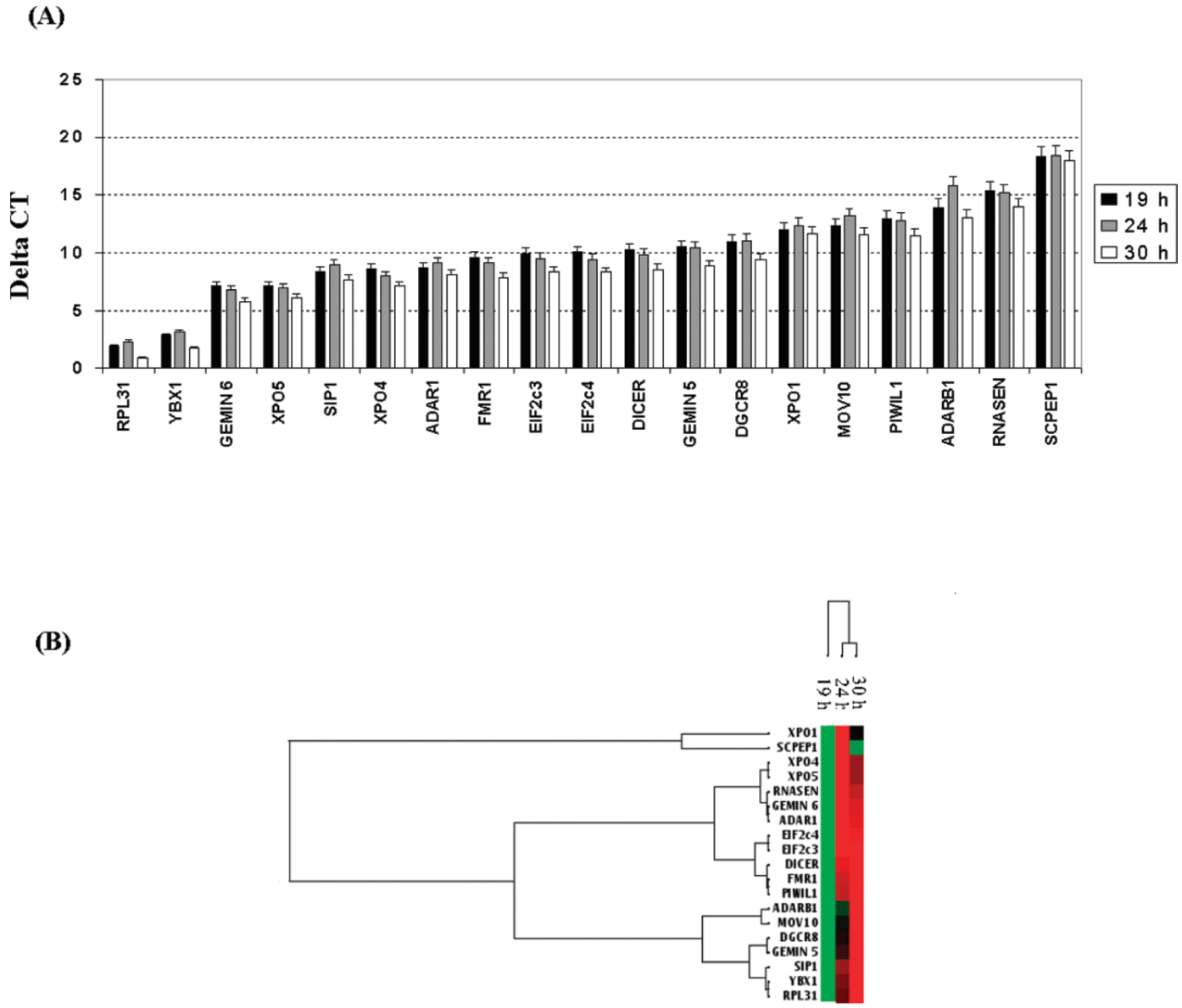

Figure 3. The expression pattern of microRNA (miRNA) processing genes during the different stages of quail embryo development (A). The average delta cycle threshold (Ct) value of each gene during the different stages of embryo development. (B) The heatmap showing the expression pattern of miRNA processing machinery genes during the different stages of early quail embryo development. Color version available in the online PDF.

proceeded from 19 to 24 h of incubation, this reduction was not observed at 30 h of development.

The let-7 miRNA gene family codes for at least 11 miRNA that differ by up to 4 nucleotides. Darnell et al. (2006) demonstrated that let-7a expression was mainly localized in the dorsal half of the early chicken embryo brain and limb primordia, whereas let-7b was detected in hindbrain, spinal cords, and notochord (around HH stage 14). In the current study, we found that the expression of let-7a was significantly reduced at 30 h of incubation, whereas let-7b expression was decreased 24 h of incubation and stayed in that level until 30 h of development. The differences observed between the 2 studies might be due to the complexity of the factors contributing to the control of inducible and repressible miRNA expression. The let7 miRNA is known to downregulate Hmga2 (Helland et al., 2011), which is a small, nonhistone, chromatin-associated protein that is believed to influence chromatin remodeling (Nishino et al., 2008). The Hmga2 is intensively expressed in undifferentiated proliferating cells, embryogenesis, and in a variety of benign and malignant tumors (Nishino et al., 2008). The Hmga2 expression and its interaction with the let7 miRNA family should be of interest to determine in quail embryos.

Darnell et al. (2007) reported that 75 miRNA were expressed at one or more stages, following screening of chicken embryos between 18 and 50 h of incubation (HH stages 3 to 25). Expression of some miRNA is highly cell-type-specific, whereas many of the miRNA were expressed ubiquitously throughout the embryos. The mir-126 was one of the cell-type-specific miRNA in those embryos. The mir-126 is an example of a cell-specific minas; it is only detected in vascular endothe-

lial cells (Darnell et al., 2007). Additionally, mir-126 has been shown to regulate developmental angiogenesis in vivo (Selvamani et al., 2012). Given the fact that the vascular system is one of the first organ systems to develop and blood islands are established after 1 d of incubation in chicken embryos, it should be of interest to investigate whether significantly decreased expression of miRNA-126 at 24 h incubation of quail embryo is also found in vascular endothelial cells, blood islands, or both, as in chick embryos.

Homeobox (***Hox***) genes encode homeodomain-containing transcription factors that control segmental patterning and establish the identification of embryonic regions along the antero-posterior axis of mouse embryos (Durston et al., 2010; Mallo et al., 2010). These genes are highly conserved and the precise spatio-temporal control of their expression is very important for normal development in several species (Duboule, 2007). The miRNA-10b located on Hox gene cluster was reported to be directly targeting the *Hoxd10* gene (Foley et al., 2011). Additionally, miRNA-10b was found to be positively regulating cancer cell migration and retinoic acid-induced neural cell differentiation (Meseguer et al., 2011). In the current study, miRNA-10b expression was found to be significantly increased at 24 h of incubation, which is in the time frame associated with the first somite formation of quail embryos, suggesting that miRNA-10b may play a significant role on somite formation and patterning. Target prediction analysis in this study demonstrated that miRNA-10b targeted *Hoxa3* gene instead of *Hoxd10*, and thus it would be of interest to investigate the interaction between miRNA-10b and *Hoxa3* gene as well as the possible involvement of *Hoxa3* gene function in quail somite formation.

The miRNA-122 is found to be highly expressed in developing and adult liver (Chang et al., 2004) and regulates metabolic functions such as lipid metabolism (Esau et al., 2006). The mir-122 is highly expressed during early stages of bovine embryo development and declines after the 8-cell stage to blastocyst (Abd El Naby et al., 2013). The miRNA-122 has been observed to be upregulated in human embryonic stem cells that have differentiated into endoderm (Laurent et al., 2008; Tzur et al., 2008). In our study, miRNA-122 expression was significantly downregulated in quail embryos from 19 to 30 h of incubation. Additional experiments should be carried out to clarify whether miRNA-122 plays a role during endodermal differentiation in the quail embryos.

The mir-125b has been shown to regulate both proliferation and differentiation of breast cancer cells (Iorio et al., 2005) and neuronal differentiation (Sempere et al., 2004). It has also been reported that mir-125b inhibited cell proliferation of human breast cancer cells and differentiation in mouse mesenchymal cells by the inhibition of *ErbB2/3* genes (Scott et al., 2007; Mizuno et al., 2008). Moreover, it has been demonstrated that miRNA-125b regulates the differentiation of tissues from mesodermal precursors, including osteoblasts from mesenchymal stem cells (Mizuno et al., 2008), skeletal muscle from C2C12 myoblasts (Ge et al., 2011), and skin elements from stem cells (Zhang et al., 2011). Wong et al. (2012) suggested that differentiation of mesodermal cells likely occurs through miRNA-125b targeting of the pluripotency factor Lin 28. The Lin 28 has been identified as a marker of undifferentiated human embryonic stem cells and also implicated in promoting the formation of specific tissues (Darr and Benvenisty, 2009). In the present study, mir-125b expression was significantly reduced from 19 to 30 h of incubation, suggesting that downregulation of mir-125b expression contributes to somite formation. Our miRNA target prediction analysis did not identify the Lin 28 gene as a target (Supplemental Table 1; available online at http://ps.fass.org/), and thus, further investigation is necessary to elucidate whether there are other mechanisms involved in quail embryos.

It has been demonstrated that miRNA-26a expression levels are upregulated during myogenesis, coinciding with the decline in the expression of chromatin-modifying enzyme, Ezh2, a known suppressor of myogenesis (Caretti et al., 2004; Wong and Tellam, 2008). The miRNA-26a also promotes carcinoma growth through the activation of β-catherin (Zhang et al., 2012) and as well as stimulating apoptosis in rat cardiomyocytes by repressing GSK-3β protein expression (Suh et al., 2012). The miRNA-26a is highly expressed in early cattle embryos (Coutinho et al., 2007). In contrast to these findings, results from the current study demonstrate that miRNA-26a expression was downregulated at 24 h of incubation and remained lower until 30 h of incubation, which coincided with the intense proliferation and apoptosis occurring during this time of embryo development (Table 3). It would thus be of interest to determine whether miRNA-26a plays a comparable role in quail embryo development.

The relative expression pattern of miRNA processing genes influences the function of miRNA during development. Programs that regulate gene expression are very dynamic during embryonic development, analyzing expression profiles during early quail embryonic development from 19 to 30 h incubation may help elucidate the regulatory processes of miRNA biogenesis and function. There were no significant changes in the selected miRNA processing machinery genes investigated in the present study, although there was a tendency for them to be increased at the 30 h time point. It should be of interest to determine whether the corresponding protein levels associated with these genes follow the same expression pattern. García-López and del Mazo (2012) have reported a general decrease in the expression of machinery genes in later stages of preimplantation of mouse embryonic development (from zygote to blastocyst). On the other hand, Mtango et al. (2009) examined 25 miRNA processing machinery gene expression patterns in rhesus monkey oocytes and embryos, and found that genes related to miRNA splicing (such as GEMIN 5, 6, and 7) were downregulated dur-

Table 3. The molecular pathways and list of predicted genes targeted by differentially expressed micro RNA (miRNA) during the early quail embryonic development

mirRNA	Target genes	Biological process
miR-26a	*PFTK1*, *ERBB4*, *SSH2*, *MKNK2*, *ADRBK1*, *PTEN*, *TRIB2*, *ACVR1C*, *PAK2*, *MAP3K2*, *PPP3CB*, *STK39*, *TNKS*, *PAK1*, *TLK2*, *YES1*, *CAMK2A*, *ADAM9*, *TWF1*, *BCR*, *PAN3*, *PTPN3*, *CSNK1G1*, *RYK*, *NLK*, *TGFBR2*, *RPS6KC1*, *CDK6*, *HGF*, *PRKCD*, *SRPK1*, *EPHA2*, *WEE1*, *DUSP5*, *RPS6KA6*, *EPHA7*, *ICK*, *MAPK6*, *ULK1*, *RPS6KA2*, *EIF2S1*, *GSK3B*, *PTP4A1*, *DYRK1A*, *HIPK2*, *PDGFRA*, *NEK6*	Phosphate metabolic process
miR-26a	*WNT5A*, *PIP5K3*, *MRAS*, *RHOQ*, *RHOU*, *LINGO1*, *PLCL1*, *RAB11A*, *BCL6*, *DEPDC1B*, *PLCB1*, *RAB21*, *ADAM9*, *BCR*, *RAP2C*, *SOCS6*, *IGF1*, *SMAD1*, *DEPDC1*, *HGF*, *SOCS5*, *ARHGEF12*, *PRKCD*, *RPS6KA6*, *ARF1*, *RAB18*, *RPS6KA2*, *HIPK2*, *COL1A2*, *RAP1A*, *STMN1*	Intracellular signaling cascade
miR-26a	*BID*, *EXOC8*, *MTX2*, *CELSR1*, *CLTC*, *SRP19*, *YWHAE*, *TNKS2*, *ZNF236*, *RAB18*, *RAB11A*, *KPNA6*, *BCL6*, *TNKS*, *KPNA3*, *COX18*, *KPNA2*, *RAB21*, *GGA3*, *TOB1*	Protein localization
miR-26a	*B4GALT1*, *BID*, *EEF1A1*, *MAEA*, *MCL1*, *CEBPG*, *MITF*, *IGF1*, *HGF*, *SGMS1*, *EPHA2*, *BAK1*, *EPHA7*, *BTG1*, *KIAA1333*, *HIPK2*, *BCL6*, *APC*, *TP53INP1*	Regulation of cell death
miR-26a	*ERBB4*, *TGFBR2*, *RHOQ*, *SMAD1*, *HGF*, *EPHA2*, *EPHA7*, *GRB10*, *EREG*, *CTGF*, *HIPK2*, *COL1A2*, *PDGFRA*, *ADAM9*, *TOB1*	Enzyme-linked receptor protein signaling pathway
miR-26a	*B4GALT1*, *CDH2*, *YWHAE*, *CDH4*, *COL5A1*, *EPHA2*, *EPHA7*, *ITGA6*, *CTGF*, *STRBP*, *SRGAP1*, *APC*, *MYH10*	Cell motion
miR-26a	*MAEA*, *EPAS1*, *CEBPG*, *MITF*, *TGFBR2*, *CDK6*, *CBFB*, *BAK1*, *BCL11A*, *PPP3CB*, *HOXA9*, *BCL6*, *APC*	Immune system development
miR-26a	*MAEA*, *EPAS1*, *CEBPG*, *MITF*, *TGFBR2*, *CDK6*, *CBFB*, *BAK1*, *BCL11A*, *PPP3CB*, *HOXA9*, *BCL6*, *APC*	Hemopoiesis
miR-26a	*RAP2C*, *ARF1*, *RAB18*, *MRAS*, *COL1A2*, *RAB11A*, *RAP1A*, *RHOQ*, *BCL6*, *RHOU*, *SRGAP1*, *RAB21*	Small GTPase mediated signal transduction
miR-26a	*BAK1*, *EPHA7*, *ACTA1*, *RYK*, *LEF1*, *BCL6*, *STMN1*, *HGF*, *CDH4*, *EPHA2*, *APC*, *MYH10*	Cellular component morphogenesis
miR-26a	*BAK1*, *CEBPG*, *TGFBR2*, *BCL11A*, *PPP3CB*, *BCL6*, *PRKCD*, *CBFB*, *ADAM9*, *APC*	Leukocyte activation
miR-26a	*LINGO1*, *EPHA7*, *RYK*, *MAP1B*, *STMN1*, *CDH4*, *EPHA2*, *APC*, *MYH10*	Neuron projection development
miR-26a	*MAP1B*, *CAPZA1*, *HECTD3*, *IGF1*, *TNKS*, *STMN1*, *CAPZB*, *NEK6*, *APC*	Regulation of organelle organization
miR-26a	*B4GALT1*, *PTP4A1*, *PDGFRA*, *IGF1*, *BCL6*, *STMN1*, *PTEN*, *ADAM9*, *APC*	Regulation of cell motion
miR-26a	*BAK1*, *CEBPG*, *TGFBR2*, *BCL11A*, *MITF*, *PPP3CB*, *BCL6*, *CBFB*, *APC*	Leukocyte differentiation
miR-26a	*BID*, *MTX2*, *KPNA6*, *BCL6*, *KPNA3*, *SRP19*, *KPNA2*, *YWHAE*, *TOB1*	Protein targeting
miR-26a	*BAK1*, *CEBPG*, *BCL11A*, *PPP3CB*, *BCL6*, *PRKCD*, *CBFB*, *APC*	Lymphocyte activation
miR-26a	*EPHA7*, *RYK*, *STMN1*, *CDH4*, *EPHA2*, *APC*, *MYH10*	Axonogenesis
miR-26a	*B4GALT1*, *P4HA1*, *PDGFRA*, *COL1A2*, *NID1*, *COL5A1*, *MYH10*	Extracellular structure organization
miR-26a	*ITGA6*, *CDK6*, *BCL6*, *NID1*, *PTEN*, *ADAM9*, *APC*	Regulation of cell adhesion
miR-26a	*B4GALT1*, *P4HA1*, *PDGFRA*, *COL1A2*, *NID1*, *COL5A1*, *MYH10*	Extracellular matrix organization
miR-26a	*MAEA*, *EPAS1*, *CEBPG*, *TGFBR2*, *MITF*, *BCL6*, *CBFB*	Myeloid cell differentiation
miR-26a	*BID*, *MTX2*, *KPNA6*, *BCL6*, *KPNA3*, *KPNA2*, *TOB1*	Protein import
miR-26a	*ITGA6*, *CTGF*, *ITGB8*, *ACTN2*, *NID1*, *ADAM9*	Cell-substrate adhesion
miR-26a	*ITGA6*, *CTGF*, *ITGB8*, *ACTN2*, *NID1*, *ADAM9*	Cell-matrix adhesion
let-7a	*DDI2*, *TMPRSS2*, *USP38*, *PRCP*, *YOD1*	Proteolysis
miR-10b	*TOM1L2*, *RAB5A*, *RFFL*, *RIMS2*	Protein transport
let-7b	*DCLRE1C*, *NRAS*, *CCDC98*	Response to abiotic stimulus
miR-10b	*TOM1L2*, *RFFL*, *RIMS2*	Intracellular protein transport
miR-26a	*COL1A2*, *BCL6*, *SRGAP1*	Rho protein signal transduction
miR-122	*GLT25D2*, *AGL*	Polysaccharide biosynthetic process
let-7b	*CHPT1*, *PIGA*	Glycerolipid biosynthetic process
miR-125b	*SLC39A9*, *FTD*	Transition metal ion transport
let-7b	*CHPT1*, *PIGA*	Glycerophospholipid biosynthetic process

ing oocyte maturation, whereas those related to miRNA processing (such as DGCR8) were upregulated in embryos. Based on the results of these studies, it would be reasonable to conclude that miRNA processing is differentially regulated between species.

One miRNA could potentially target several hundred mRNA (Krek et al., 2005), whereas 1 gene can be regulated by several miRNA. Because of these complex interactions, research efforts need to be directed toward improving our knowledge of the regulatory pathways that manage miRNA-miRNA as well as miRNA-mRNA interactions. Nevertheless, to characterize the function of 7 differentially expressed miRNA in the present study, 11 miRNA-target interaction prediction tools were used to identify the potential mRNA targets for selected candidate miRNA. Potential target genes were identified by at least 3 or more target predicting tools. It is noteworthy that some miRNA did not have any predicted targets that were common to all 3 programs. However, further analysis of the miRNA genes used in the present study and other miRNA candidate genes with respect to temporal and tissue expression will be more beneficial to understanding the biological function of miRNA during quail embryo development. In summary, we have established the machinery gene expression patterns of 32 miRNA and 18 miRNA processing genes in quail embryos. Our results suggest that machinery genes for miRNA biogenetic pathways exist and hence, miRNA may be involved in the regulation of early quail development. The 7 differentially expressed

miRNA are suggested to play critical roles in quail embryo development, which would include somite formation. Future studies should focus on specialized cells associated with early quail development. These functional studies could potentially use the deep sequencing approach, which should provide more insight into somite formation and subsequent patterning of those somites into tissues.

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