Journal of Thermal Biology 84 (2019) 190–199

Contents lists available at ScienceDirect

Journal of Thermal Biology

journal homepage: www.elsevier.com/locate/jtherbio

Maternal manganese activates anti-apoptotic-related gene expressions via miR-1551 and miR-34c in embryonic hearts from maternal heat stress (*Gallus gallus*)

Xiudong Liao[a,1], Yongwen Zhu[b,1], Lin Lu[a], Wenxiang Li[c], Liyang Zhang[a], Cheng Ji[c], Xi Lin[d], Xugang Luo[a,*]

[a] Mineral Nutrition Research Division, Institute of Animal Science, Chinese Academy of Agricultural Sciences, Beijing, 100193, China
[b] Guangdong Provincial Key Laboratory of Animal Nutrition Control, College of Animal Science, South China Agricultural University, Guangzhou, 510642, China
[c] College of Animal Science and Technology, China Agricultural University, Beijing, 100193, China
[d] Department of Animal Science, North Carolina State University, Raleigh, NC, 27695, USA

ARTICLE INFO

Keywords:
Maternal heat stress
Manganese
MicroRNA expression
Anti-apoptotic gene expression
Offspring chick embryo
Heart

ABSTRACT

MicroRNAs (miRNAs) expressions are altered by maternal stresses and nutritional status. Our previous study has demonstrated that maternal manganese (Mn) addition could protect chick embryos against maternal heat stress via enhancing anti-apoptotic ability in embryonic hearts. The objective of this study was to investigate whether this protective effect could be achieved via miRNA mechanisms, and also be sustained in offspring broilers. A completely randomized design with a 2 (maternal normal and high temperatures: 21 and 32 °C) × 2 (maternal control basal diet and the basal diet + 120 mg Mn/kg) factorial arrangement of treatments was adopted. Totally 96 broiler breeder hens were allotted to 4 treatments with 6 replicates. Subsequently, 24 hatched chicks from each maternal treatment were divided into 6 replicates. Maternal supplemental 120 mg Mn/kg reduced the increased expressions of miR-1551 and miR-34c in hearts of offspring embryos but not broilers under maternal heat stress. *B-cell CLL/lymphoma 2* (*BCL2*) and *NF-κB-inducing kinase* (*NIK*) genes related to anti-apoptotic ability were identified as direct targets for miR-1551 and miR-34c, respectively. Under maternal heat stress, maternal supplemental 120 mg Mn/kg activated target BCL2 expression and NIK-dependent NF-κB pathway via mediating miR-1551 and miR-34c expressions in hearts of offspring embryos rather than broilers.

1. Introduction

MicroRNAs (miRNAs) as epigenetic markers in embryonic tissues can be reprogrammed by maternal environmental exposures and nutritional status (Constantinof et al., 2016; Wu et al., 2004). Previous studies have shown that maternal environmental stresses could alter the biogenesis of miRNA and mRNA expressions of target genes in the embryonic tissues of zebrafish and drosophila (Flynt et al., 2009; Leaman et al., 2005; Li et al., 2009). The aberrant miRNAs could further induce the abnormal embryonic development via modulating apoptotic-related gene expressions (Adlakha and Saini, 2011; Carraco et al., 2014). Studies *in vitro* have demonstrated that miRNA-15/16 and miRNA-1/181c directly inhibited target anti-apoptotic protein B-cell CLL/lymphoma 2 (*BCL2*) expression, and then resulted in the increase of apoptosis in lymphocyte (Cimmino et al., 2005) and cardiomyocyte (Tang et al., 2009; Wang et al., 2015), respectively. Besides, miRNAs as the new effectors and regulators play a crucial role in mediating apoptosis indirectly via the noncanonical nuclear factor-kappa B (NF-κB) pathway (Boldin and Baltimore, 2012; Uno et al., 2014). Epigenetic loss of miR-31 increased apoptosis resistance by activating the non-canonical NF-κB pathway in adult T-lymphocytes and other cancers (Yamagishi et al., 2012). The roles of maternal macronutrients (e. g. amino acid and fat) and micronutrients (e. g. vitamins and copper) in modulating the miRNA expressions in mice and minipigs have been reviewed by Chango and Pogribny (2015). Chick embryos are easier to obtain and manipulate than most other vertebrate species. Furthermore, miRNA expressions in chick embryos have been characterized and the functions of miRNAs are conserved across many species (Carraco et al., 2014; Darnell et al., 2006). Manganese (Mn) functioning as a metal cofactor of the metalloenzyme Mn superoxide dismutase

* Corresponding author.
E-mail address: wlysz@263.net (X. Luo).
[1] These authors contributed equally to the present study.

https://doi.org/10.1016/j.jtherbio.2019.07.014
Received 14 May 2019; Received in revised form 2 July 2019; Accepted 2 July 2019
Available online 03 July 2019
0306-4565/

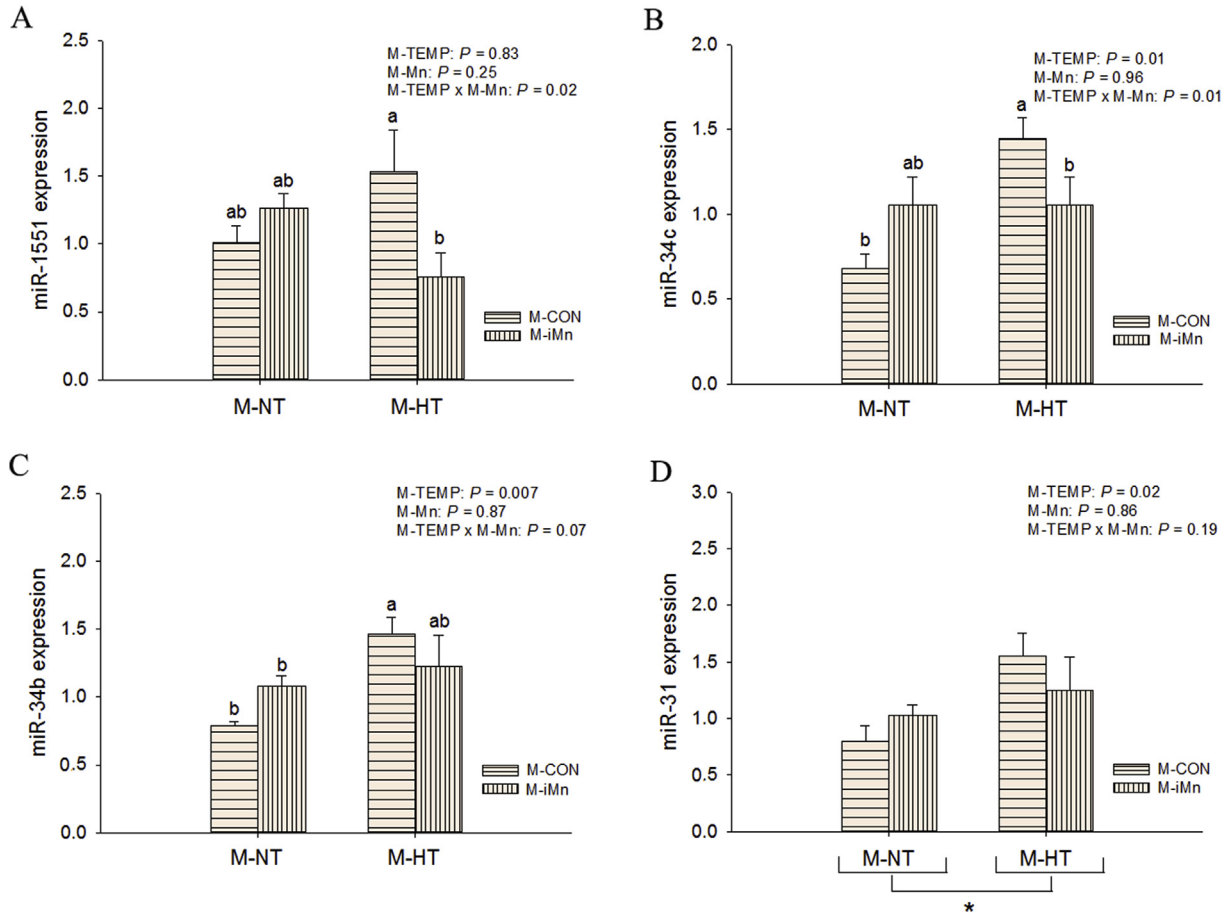

Fig. 1. Verification of the expressions of miR-1551 (A), miR-34c (B), miR-34b (C) and miR-31 (D) obtained in high-throughput sequencing in the embryonic heart by RT-qPCR. Based on the 2-way ANOVA analyses, "*" means significant differences at $P < 0.03$ between M-NT ($n = 12$) and M-HT ($n = 12$) as determined by a main effect of maternal environmental temperature; lacking common letters (a, b) means significant differences ($P < 0.07$) between single bars (individual treatments, $n = 6$) as determined by their interaction. All values are expressed as means ± SE. M-TEMP = maternal environmental temperature; M-Mn = maternal dietary manganese (Mn); M-NT = maternal normal temperature; M-HT = maternal high temperature; M-CON = the maternal Mn-unsupplemented control basal diet; M-iMn = the maternal control basal diet supplemented with 120 mg Mn/kg as the inorganic Mn sulfate.

(MnSOD) could enhance the antioxidant ability by scavenging excessive reactive oxygen species (Fridovich, 1995). One study from our laboratory has showed that supplemental 120 mg Mn/kg might alleviate oxidative stress induced by high temperature in broiler breeders compared to Mn-unsupplemented control (Zhu et al., 2015). Furthermore, another our previous study has demonstrated that maternal supplemental 120 mg Mn/kg could protect offspring chick embryos against maternal heat stress by enhancing the anti-apoptotic ability in chick embryonic hearts compared to Mn-unsupplemented control (Zhu et al., 2017). However, it is unknown whether maternal Mn nutrition could enhance the anti-apoptotic ability in the embryonic hearts from maternal heat stress by mediating related miRNA expressions and then regulating the target BCL2 expression and noncanonical NF-κB pathway.

It is now recognized that maternal epigenetic factors that do not alter the sequence of DNA base pairs could cause the acquired changes of offsprings at the different developmental stages by modulating the expressions of related miRNAs and target genes (Smythies et al., 2014). In mice, Rodgers et al. (2013) found that increased expressions of 9 specific sperm miRNAs resulting from parental stress were observed in the offsprings throughout puberty and in adulthood. Kawano et al. (2012) also found that the 2 novel miRNAs (spR-12 and spR-14) providing resistance to the periodate-oxidation-mediated reaction in one-cell embryos could be maintained at the preimplantation stage, but not at the later differentiation stages. Although anti-maternal heat-stressed effects of maternal Mn nutrition via the enhancement of anti-apoptotic ability were proved in the chick embryonic hearts in our previous study (Zhu et al., 2017), it is unclear whether this protective effect could be sustained in the hearts of offspring broilers via miRNA mechanisms. Therefore, in the present study, it was hypothesised that maternal Mn nutrition could modulate the expressions of related miRNAs and target genes to enhance the anti-apoptotic ability in the hearts of offspring embryos and broilers from maternal heat stress. To test the above hypothesis, the expressions of miRNAs and target anti-apoptotic genes induced by maternal heat stress and Mn nutrition were investigated in the hearts of both offspring embryos and broilers in the present study.

2. Materials and methods

2.1. Birds and diets

All experimental procedures were approved by the Animal Management Committee (in charge of animal welfare issue) of the Institute of Animal Science, Chinese Academy of Agricultural Sciences (IAS-CAAS, Beijing, China) and performed in accordance with the guidelines. Ethical approval on animal survival was given by the animal ethics committee of IAS-CAAS. This experiment included 3 consecutive phases with maternal laying broiler breeder hens, their offspring embryos and broilers. Experimental design and treatments for the maternal laying broiler breeder hen and offspring embryo phases were described in details in our previous studies (Zhu et al., 2015, 2017). Briefly, a completely randomized design with a 2 [maternal normal and

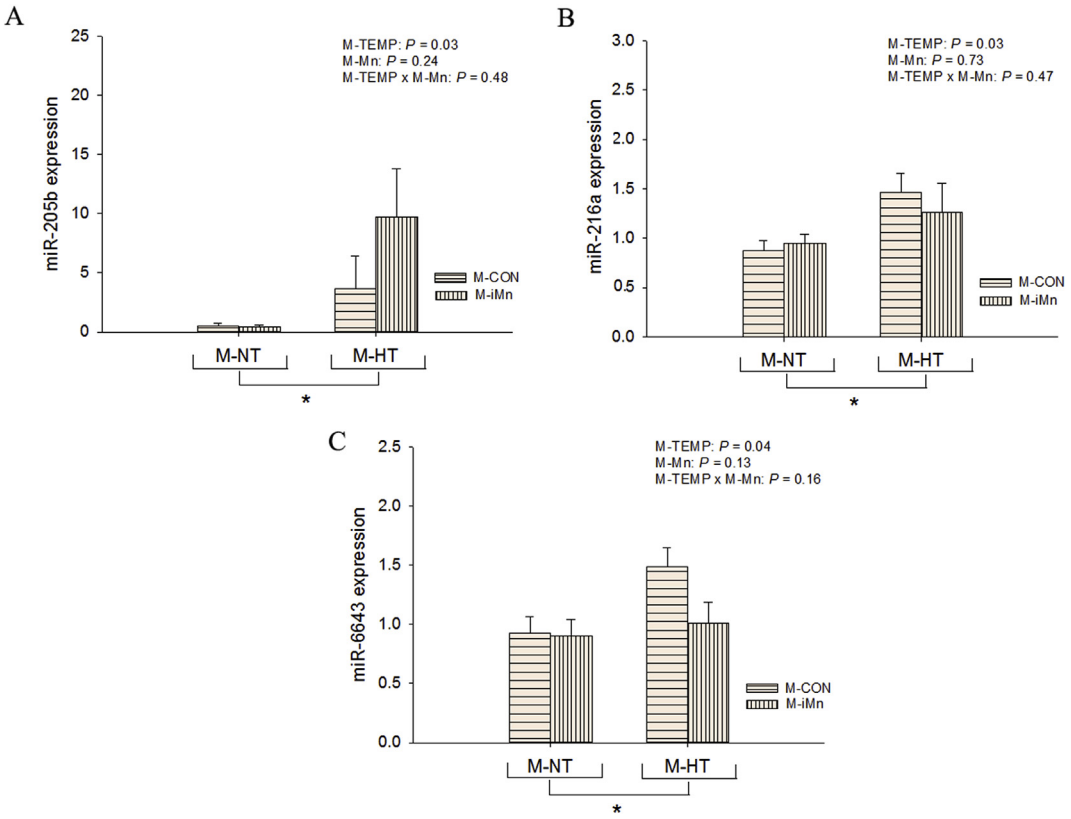

Fig. 2. Verification of the expressions of miR-205b (A), miR-216a (B) and miR-6643 (C) obtained in high-throughput sequencing in the embryonic heart by RT-qPCR. Based on the 2-way ANOVA analyses, "*" means significant differences at $P < 0.05$ between M-NT ($n = 12$) and M-HT ($n = 12$) as determined by a main effect of maternal environmental temperature. All values are expressed as means ± SE. M-TEMP = maternal environmental temperature; M-Mn = maternal dietary manganese (Mn); M-NT = maternal normal temperature; M-HT = maternal high temperature; M-CON = the maternal Mn-unsupplemented control basal diet; M-iMn = the maternal control basal diet supplemented with 120 mg Mn/kg as the inorganic Mn sulfate.

high environmental temperatures of 21 (M-NT) and 32 °C (M-HT)] × 2 [the maternal control basal diet without Mn supplementation (M-CON; 14.3 mg Mn/kg by analysis) and the M-CON diet + 120 mg Mn/kg as the inorganic Mn sulfate (M-iMn; 133 mg Mn/kg by analysis)] factorial arrangement of treatments was adopted. Therefore, a total of 96 18-wk-old Arbor Acres laying broiler breeder hens were randomly allotted to 4 maternal treatments with 6 replicates of 4 birds per replicate for each treatment. The whole experimental period lasted for 23 weeks, including the adaption (18–29 weeks of age), Mn-depletion (30–31 weeks of age) and experimental (32–40 weeks of age) stages. The composition and nutrient levels of the Mn-unsupplemented corn-soybean meal basal diet for the maternal laying broiler breeder hens during the Mn-depleting and the experimental stages, as well as lighting and feeding management throughout the experiment, were the same as described in our previous study (Zhu et al., 2015). All hatched eggs collected from the above 4 maternal treatments during the last 2 weeks of the experimental period were incubated at a normal incubation temperature of 37.8 °C and a relative humidity of 55–60% for the 21-day offspring embryonic phase. The details of incubation manipulation were the same as described in our previous study (Zhu et al., 2017). After hatching, 24 hatched chicks from each maternal treatment group were divided into 6 replicates, with 4 chicks (2 males and 2 females) per replicate based on the average birth weight of each maternal treatment, resulting in a total of 96 offspring broilers. The offspring broilers from all maternal treatments were kept in the whole room where initial temperatures were 33 ± 1 °C at the first week and then reduced by 2–3 °C every week gradually to 23 ± 1 °C until the end of the experiment. A constant 24 h-lighting was maintained. The birds were allowed *ad libitum* access to the same offspring diets and tap water according to the guidelines approved by Arbor Acres Farm (Arbor Acres, Huadu Broiler Company, Beijing, China). The corn-soybean meal diets for offspring broilers (Supplementary Table S1) were formulated to meet or exceed the NRC (1994) requirements including Mn during 1–42 days of age. Body weight, feed intake and mortality of birds in each replicate cage were recorded during days 1–42. On day 42, 2 fasted birds (1 male and 1 female) from each replicate cage were randomly selected, weighed, and killed by cervical bleeding. The carcasses were eviscerated and weighed to determine the dressing and eviscerated yield percentages. The left breast and leg muscles and abdominal fat were removed and weighed to determine the percentages of the breast and leg muscles and abdominal fat.

2.2. Sample collections and preparations

On day 18.5 of incubation, 24 offspring embryos (6 replicates of 4 embryos per replicate) from each treatment were killed by cervical dislocation. On day 42 after hatching, the rest 2 fasted offspring broilers (1 male and 1 female) from each replicate were killed by cervical dislocation. Heart samples from the offspring embryos and broilers were immediately dissected and frozen in liquid nitrogen, and then stored at −80 °C for further analyses. The samples with equal weight of individual offspring embryos or broilers per replicate cage were pooled prior to analyses.

2.3. MiRNA library preparation and sequencing

Total RNA of 24 samples from 4 maternal groups was extracted using the standard Trizol method according to the manufacturer's instructions. Equal amount sub-samples of the embryonic heart RNA samples from 6 replicates in each treatment were pooled into 1 mixed

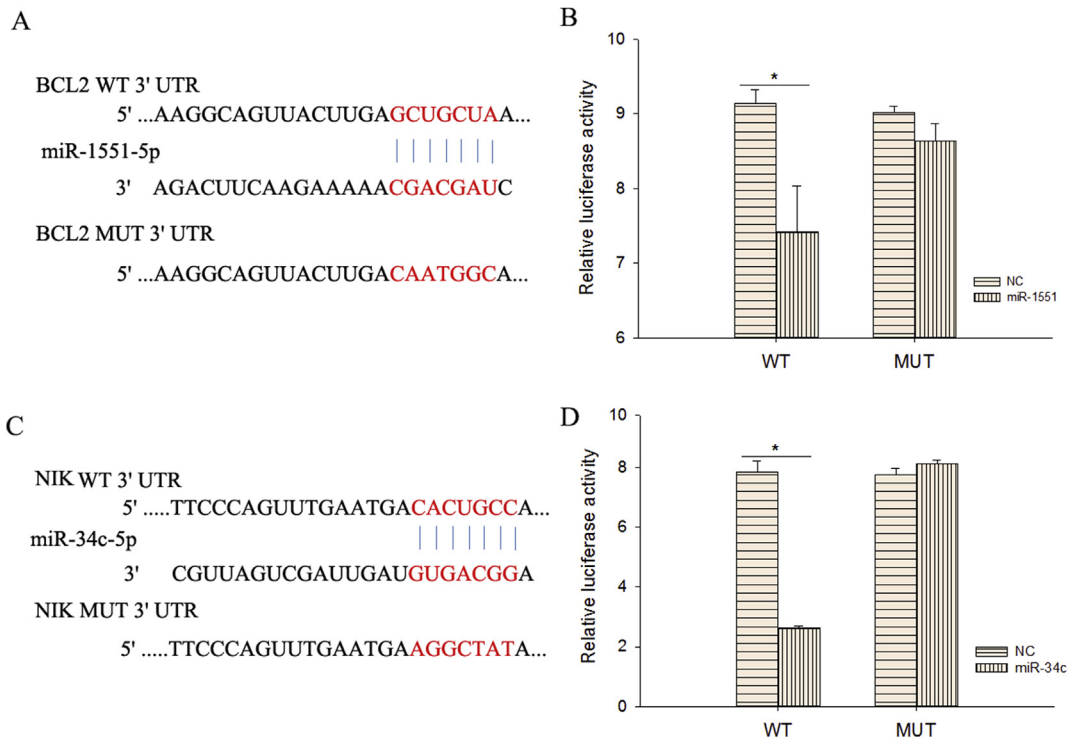

Fig. 3. Validation of the predicted target BCL2 and NIK for miR-1551 and miR-34c. Putative binding sequences of miR-1551 and miR-34c were predicted for the mRNA 3'-UTR of BCL2 (A) and NIK (C), respectively. Mutation was generated on the mRNA 3'-UTR of BCL2 and NIK sequences in the complementary site for the seed regions of miR-1551 and miR-34c, respectively. Dual-luciferase reporter assays were performed to test luciferase activity of the interaction between miR-1551 and BCL2 mRNA site (B) or between miR-34c and NIK mRNA site (D). "*" means significant differences at $P < 0.05$ between the negative control (NC, n = 6) and miRNA constructs (n = 6) for WT. All values are expressed as means ± SE. WT = wild-typle; MUT = mutant; BCL2 = B-cell CLL/lymphoma 2; NIK = nuclear factor-kappa B-inducing kinase.

Table 1
Summary of 7 differentially expressed candidate miRNAs in embryonic hearts subjected to maternal environmental temperature and dietary Mn.

MicroRNAs	Chromosomal position	Fold change					
		T2/T1	T3/T1	T4/T1	T3/T2	T4/T2	T4/T3
miR-1551	chr14_11115	1.70	1.30	0.10	0.76	0.06	0.08
miR-34c	chr24_22696	0.63	1.31	0.13	2.10	0.20	0.10
miR-34b	chr24_22694	0.62	1.57	0.48	2.54	0.77	0.30
miR-31	Z_46241	2.00	4.00	1.58	2.09	0.83	0.40
miR-205b	chr1_3711	1.72	1.13	3.84	0.66	2.23	3.39
miR-216a	chr3_26875	0.33	6.67	3.67	20.00	11.00	0.55
miR-6643	chr14_11720	2.50	1.50	2.00	0.60	0.80	1.33

The miRNAs displaying fold-change ≥ 2.0 up-regulation or ≤0.5 down-regulation were selected as the differentially expressed miRNAs of the mixed embryonic heart samples. The above 7 differentially expressed miRNAs were screened according to the occurrence of miRNAs in the 6 sets of differentially expressed miRNAs after pairwise comparisons among the 4 maternal treatments. T1 = the maternal Mn-unsupplemented control basal diet (M-CON) under maternal normal temperature (M-NT); T2 = the maternal control basal diet supplemented with 120 mg Mn/kg as the inorganic Mn sulfate (M-iMn) under maternal normal temperature (M-NT); T3 = the maternal Mn-unsupplemented control basal diet (M-CON) under maternal high temperature (M-HT); T4 = the maternal control basal diet supplemented with 120 mg Mn/kg as the inorganic Mn sulfate (M-iMn) under maternal high temperature (M-HT).

sample for analyses. Then, high-throughput sequencing for miRNAs was performed as described previously. Briefly, a miRNA sequencing library was prepared by ligating sequentially to 3' and 5' RNA adapters using RNA ligase (Promega, Madison, WI, USA). Ligation products were then amplified in situ and finally sequenced for 36 cycles on Illumina HiSeq2000 according to the manufacturer's instruction (Illumina, San Diego, CA, USA). Subsequently, 3' adapter sequences were deleted from clean reads and the reads that were shorter than 15 nt were excluded from further analysis. The miRNA expression levels were measured and normalized as transcripts per million of total aligned miRNA reads (TPM) aligned to known *Gallus gallus* pre-miRNAs in miRBase 21.0. The lengths and composition of all sequenced sRNAs were listed in Supplementary Fig. S1 and Supplementary Table S2. Firstly, fold-change ≥ 2.0 or ≤0.5 and false discovery rate (FDR) < 1% were selected to screen the differentially expressed miRNAs by multiple comparisons of 4 maternal groups. Secondly, differentially expressed miRNAs were further screened and identified by pairwise comparisons among the 4 maternal groups. Then 6 sets of differentially expressed miRNAs were obtained after the pairwise comparisons. Finally, differentially expressed miRNAs were ordered and narrowed according to the occurrence rates of miRNAs in the above screened 6 miRNAs sets. Top 7 differentially expressed miRNAs were screened according to the occurrence of miRNAs in the above 6 sets and then validated using RT-qPCR method.

2.4. RT-qPCR for miRNA

Total RNA from each heart sample was reversely transcribed to generate cDNA with a gene-specific primer (for miRNAs) and oligo dT primer (for TFs) using a Superscript™III reverse transcriptase kit (Cat # 218161, Qiagen, Carlsbad, CA, USA) in accordance with the manufacturer's instructions. The sequences of RT primers for miRNA are presented in Supplementary Table S3. The qPCR reaction was performed with QuantiTect SYBR Green PCR Master Mix (Cat # 218161, Qiagen, Carlsbad, CA, USA) by ABI 7500 real-time PCR system (Applied Biosystems) using the procedure: 95 °C for 15 min to denature DNA templates, followed by 40 cycles of 95 °C for 15 s, 60 °C for 34 s. The *Gallus* U6 gene was used as the internal control. Expression fold-changes were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

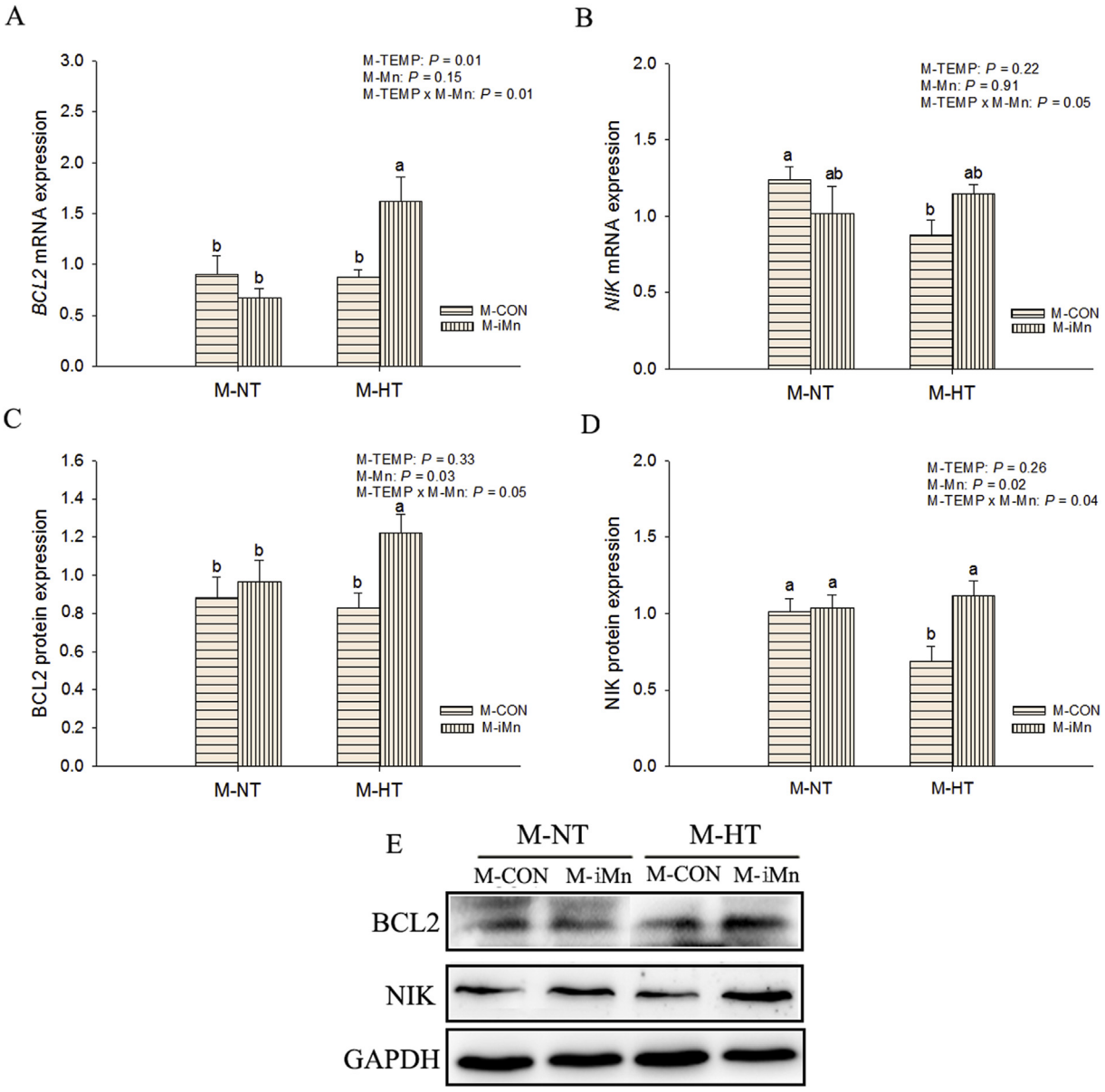

Fig. 4. Target BCL2 and NIK expressions in the embryonic heart. The geometric mean of internal references, *β-actin* and *GAPDH*, was used to normalize the mRNA expressions of target *BCL2* (A) and *NIK* (B). The GAPDH was selected to normalize the protein expressions of target BCL2 (C) and NIK (D). Representative immunoblots of the indicated proteins were shown (E). Based on the 2-way ANOVA analyses, lacking common letters (a, b) means significant differences at $P < 0.007$ between single bars (individual treatments, $n = 6$) as determined by their interaction. All values are expressed as means ± SE. M-TEMP = maternal environmental temperature; M-Mn = maternal dietary manganese (Mn); M-NT = maternal normal temperature; M-HT = maternal high temperature; M-CON = the maternal Mn-unsupplemented control basal diet; M-iMn = the maternal control basal diet supplemented with 120 mg Mn/kg as the inorganic Mn sulfate; BCL2 = B-cell CLL/lymphoma 2; NIK = nuclear factor-kappa B-inducing kinase.

2.5. RT-qPCR for mRNA expression

MiRDB was used to predict the target genes of the differentially expressed miRNAs. In order to validate the expressions of predicted targets for dysregulated miRNAs in the chick embryos and offsprings, RT-qPCR of sub-samples from heart samples of 4 maternal treatments was performed for target gene mRNA expression, according to previously reported methods (Qin et al., 2017). The PCR primers for mRNAs are listed in Supplementary Table S3.

2.6. Western blotting for protein expression

Total protein was extracted with ice-cold RIPA lysis buffer (Cat #P0013B, Beyotime Institute of Biotechnology, Haimen, China). The procedure following the preparation of the protein sample and SDS-PAGE, blotting transfer and detection of the protein specific antibodies were performed as described previously (Qin et al., 2017). The following primary antibodies are listed in Supplementary Table S4.

2.7. Luciferase reporter assay

The wild-type *BCL2* and *NIK* 3' untranslated regions (3'-UTR) containing the predicted miR-1551 and miR-34c target sequence A and C), respectively were amplified from genomic DNA (293T cells) and cloned into the pGL3 firefly luciferase control vector (Promega Madison, WI, USA) at the XbaI restriction site immediately downstream of the luciferase reporter gene. Each of these vectors was co-transfected with Renilla plasmid psiCHECK and miR-1551/miR-34c mimics

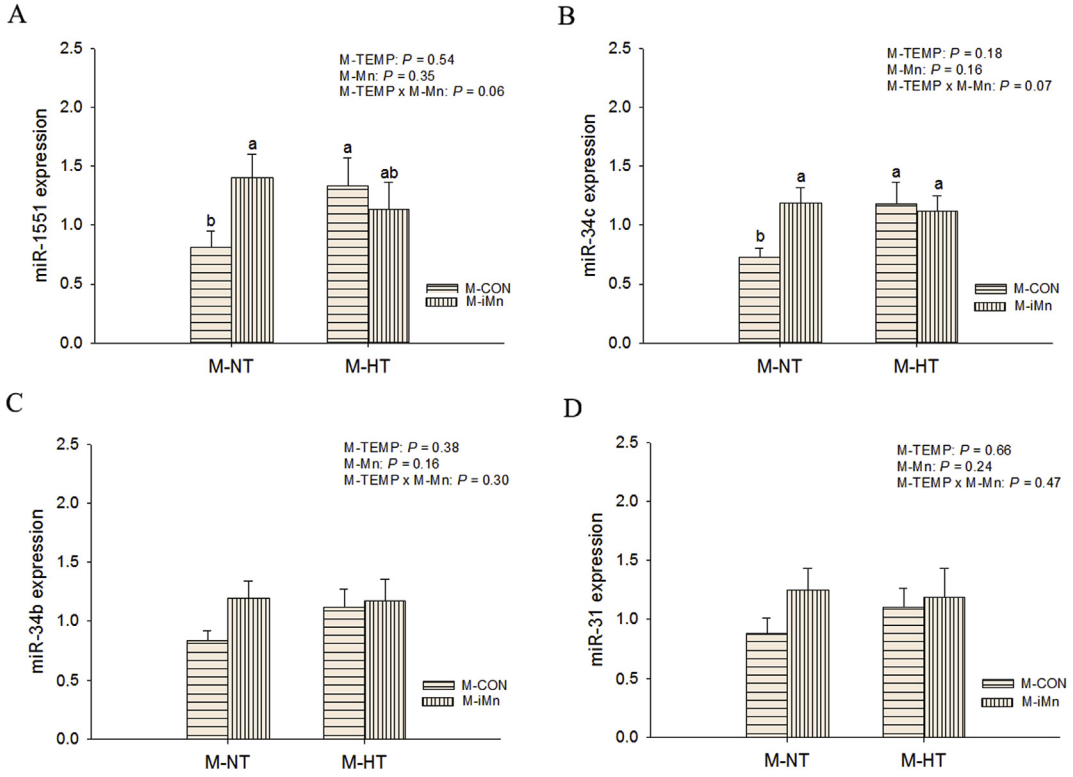

Fig. 5. Expressions of miR-1551, miR-34c, miR-34b and miR-31 in the heart of offspring broilers. According to the most differentially expressed miRNAs with an interaction between maternal environmental temperature and dietary Mn screened in the chick embryonic heart, the expressions of miR-1551 (A), miR-34c (B), miR-34b (C) and miR-31 (D) were also validated in the heart of offspring broilers by RT-qPCR. Based on the 2-way ANOVA analyses, lacking common letters (a, b) means significant differences ($P < 0.09$) between single bars (individual treatments, $n = 6$) as determined by their interaction. All values are expressed as means ± SE. M-TEMP = maternal environmental temperature; M-Mn = maternal dietary manganese (Mn); M-NT = maternal normal temperature; M-HT = maternal high temperature; M-CON = the maternal Mn-unsupplemented control basal diet; M-iMn = the maternal control basal diet supplemented with 120 mg Mn/kg as the inorganic Mn sulfate.

(Genepharma, Shanghai, China) into 293T cells in 48-well plates using Lipofactamine 2000 (Invitrogen, Carlsbad, CA, USA). The 2 ng Renilla reporter was mixed with either miRNA negative control or precursor miRNA at a 100 nmol/L final concentration using the Lipofectamine 2000 reagent according the manufacturer's instructions (Invitrogen Carlsbad, CA, USA). After 24-h post-transfection, luciferase activity was measured with Dual-Glo (Promega, Madison, WI, USA) according to the manufacturer's instructions. Mutant constructs of *BCL2* and *NIK* 3'-UTRs with a mutant target sequences were made by site-directed mutagenesis to replace seed sequence with BglII cleavage site. The cloned wild-type and mutant target fragments were sequenced following sequence alignment algorithms (Supplementary Fig. S2).

2.8. Statistical analyses

Data were analyzed by 2-way ANOVA using the general linear model procedure of the SAS 9.4 (SAS Institute Inc., Cary, NC, USA), and the model included the main effects of maternal environmental temperature, maternal dietary Mn, and their interaction. Treatment comparisons for significant differences were tested using the least significant difference method. Each replicate served as the experimental unit for all statistical analyses. Significant differences were set at $P < 0.10$ (Li et al., 2004; Liao et al., 2017; Luo et al., 2007). The FDR was estimated using the Benjamini-Hochberg method (1995), and the statistical significance for differential expressions of miRNAs was set at FDR < 1%.

3. Results

3.1. Identification of miRNAs regulated by maternal environmental temperature and dietary Mn

The sequencing data were analyzed using miRBase 21.0 (Kong et al., 2015). A total of 237 candidate miRNAs were identified (Supplementary Dataset S1), and each identified in the maternal treatments was at more than 7.5 transcripts per million (TPM) in total. We found that 109 miRNAs were significantly overexpressed (fold-change ≥ 2.0 and FDR < 1%) or underexpressed (fold-change ≤ 0.5 and FDR < 1%) after pairwise comparisons among the 4 maternal treatments. Seven differentially expressed miRNAs were screened, consisting of miR-1551, miR-34c, miR-34b, miR-31, miR-205b, miR-216a, and miR-6643 (Table 1). To validate the results of miRNA sequencing, the above mentioned 7 miRNAs (either up-regulated or down-regulated) were selected for analysis by RT-qPCR. Maternal environmental temperature had an effect ($P < 0.05$) on expressions of miR-31, miR-205b, miR-216a, and miR-6643 (Figs. 1D and 2A-2C). In addition, the interaction between maternal environmental temperature and dietary Mn affected ($P < 0.08$) miR-1551, miR-34c and miR-34b expressions (Fig. 1A–C). However, maternal dietary Mn did not affect ($P > 0.12$) the 7 miRNA expressions. Compared to M-NT, M-HT increased ($P < 0.05$) the transcript abundances of miR-31, miR-205b, miR-216a and miR-6643 in the embryonic heart. Under M-NT, no differences ($P > 0.10$) were observed in miR-1551 and miR-34c expressions between M-CON and M-iMn; however, under M-HT, M-iMn decreased the miR-1551 ($P < 0.03$) and miR-34c ($P < 0.07$) expressions in the embryonic heart compared to M-CON (Fig. 1A and B).

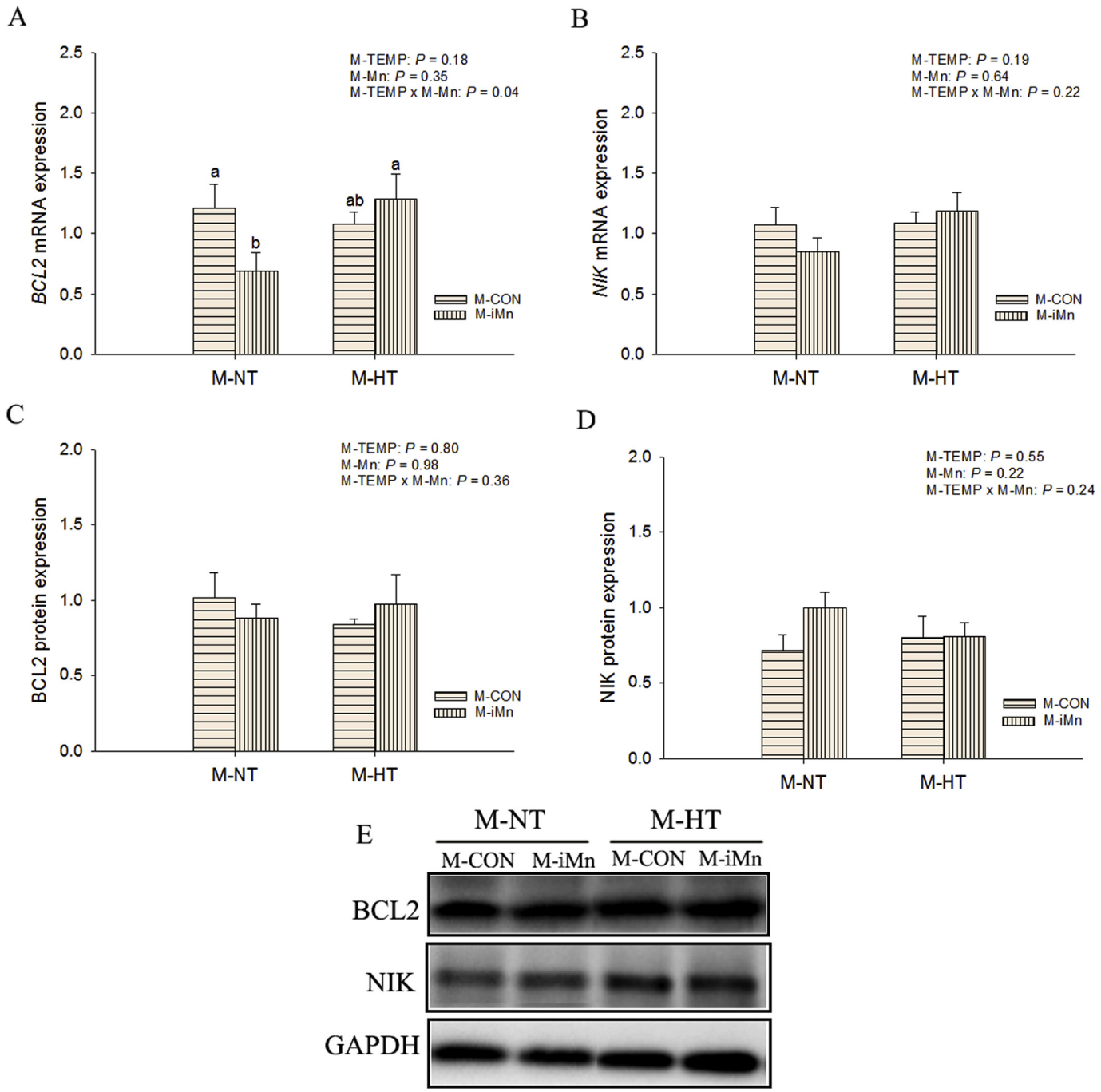

Fig. 6. Expressions of target BCL2 and NIK in the heart of offspring broilers. The geometric mean of internal references, *β-actin* and *GAPDH*, was used to normalize the mRNA expressions of target *BCL2* (A) and *NIK* (B). The GAPDH was selected to normalize the protein expressions of target BCL2 (C) and NIK (D). Representative immunoblots of the indicated proteins were shown (E). Based on the 2-way ANOVA analyses, lacking common letters (a, b) means significant differences ($P < 0.04$) between single bars (individual treatments, n = 6) as determined by their interaction. All values are expressed as means ± SE. M-TEMP = maternal environmental temperature; M-Mn = maternal dietary manganese (Mn); M-NT = maternal normal temperature; M-HT = maternal high temperature; M-CON = the maternal Mn-unsupplemented control basal diet; M-iMn = the maternal control basal diet supplemented with 120 mg Mn/kg as the inorganic Mn sulfate; BCL2 = B-cell CLL/lymphoma 2; NIK = nuclear factor-kappa B-inducing kinase.

3.2. BCL2 and NIK are direct targets of miR-1551 and miR-34c

To identify candidate miRNA-regulated target genes, the most differentially expressed miRNAs (miR-1551 and miR-34c) with an interaction between maternal environmental temperature and dietary Mn were predicted by miRDB and the target genes were then submitted to Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway for analysis. The KEGG pathway database is copyrighted by Kanehisa laboratories (Kanehisa et al., 2017, 2019; Kanehisa and Goto, 2000). We found that the dysregulated miRNAs significantly affected the pathway of apoptosis by targeting 7 genes via this analysis (Supplementary Fig. S3). The key genes of *BCL2* and *NIK* related to anti-apoptotic ability were predicted to be targeted by miR-1551 and miR-34c, respectively. The results of Luciferase-3' UTR reporter assays demonstrated that miR-1551 and miR-34c significantly decreased ($P < 0.05$) the relative luciferase activities for WT containing targeting site in *BCL2* and *NIK* as compared with the negative control, respectively (Fig. 3B and D). However, either miR-1551 or miR-34c binding sites in *BCL2* and *NIK*

3'UTR were mutated, and no differences ($P > 0.14$) in the relative luciferase activities were observed between miRNAs (either miR-1551 or miR-34c) treatment and the negative control (Fig. 3B and D).

3.3. MiRNA-mediated target gene mRNA and protein expressions in the chick embryonic heart

To investigate the relationship between miR-1551 and target BCL2 *in vivo* as well as the epigenetic transmission of the miR-1551-mediated BCL2 expressions between the embryonic stage and offspring broiler stage, the related data on *BCL2* mRNA and protein expressions in the embryonic heart published in our previous study (Zhu et al., 2017) were used in the present study. Firstly, we studied the relationship between transcript abundances of miR-1551 and miR-34c and their target mRNA expressions of *BCL2* and *NIK* in the embryonic heart. Maternal environmental temperature affected ($P < 0.02$) *BCL2* mRNA expression and had no effect ($P > 0.21$) on *NIK* mRNA expression in the embryonic heart (Fig. 4A and B). Embryonic heart *BCL2* and *NIK* mRNA expressions were affected ($P \leq 0.05$) by the interaction between maternal environmental temperature and dietary Mn, but were not affected ($P > 0.14$) by maternal dietary Mn (Fig. 4A and B). Under M-NT, no difference ($P > 0.26$) in *BCL2* mRNA expression was observed between M-CON and M-iMn, but under M-HT, M-iMn increased ($P < 0.007$) the *BCL2* mRNA expression in the embryonic heart compared with the M-CON (Fig. 4A). Compared to M-NT, M-HT decreased ($P < 0.05$) the *NIK* mRNA expression from the M-CON group, but had no effect ($P > 0.46$) on it from the M-iMn group (Fig. 4B). We subsequently investigated the protein expressions of BCL2 and NIK regulated by miR-1551 and miR-34c, respectively. Protein expressions of BCL2 and NIK in the embryonic heart were affected ($P \leq 0.05$) by maternal dietary Mn and the interaction between maternal environmental temperature and dietary Mn, but were not affected ($P > 0.25$) by maternal environmental temperature (Fig. 4C and D). Under M-NT, no differences ($P > 0.32$) in BCL2 and NIK protein expressions were observed between M-CON and M-iMn; however, under M-HT, M-iMn increased ($P < 0.004$) the protein expressions of BCL2 and NIK in the embryonic heart compared with the M-CON (Fig. 4C and D). The change of target BCL2 protein expression was consistent with that of the target mRNA expressions in the embryonic hearts from M-CON and M-iMn under maternal heat stress.

3.4. MiRNAs and target gene expressions in the heart of offspring broilers

Firstly, we found that maternal heat stress, maternal dietary Mn and their interaction had no effects ($P > 0.11$) on growth performance (Supplementary Fig. S4) and carcass traits (Supplementary Fig. S5) of offspring broilers during d 1–42. And no dead birds were observed in all treatments throughout the offspring broiler stage. The interaction between maternal environmental temperature and dietary Mn affect ($P < 0.08$) the expressions of miR-1551 and miR-34c (Fig. 5A and B), and had no effect ($P > 0.29$) on the expressions of miR-34b and miR-31 in the heart of offspring broilers (Figs. 5C and 4D). The above 4 miRNA expressions were not affected ($P > 0.15$) by maternal environmental temperature or maternal dietary Mn (Fig. 5A–D). Compared to M-NT, M-HT up-regulated ($P < 0.09$) the miR-1551 and miR-34c expressions from the M-CON group, but had no effect ($P > 0.31$) on those from the M-iMn group (Fig. 5A and B). As for target gene expressions in the heart of offspring broilers, the mRNA and protein expressions of BCL2 and NIK were not influenced ($P > 0.17$) by maternal environmental temperature and maternal dietary Mn (Fig. 6A–D). The interaction between maternal environmental temperature and dietary Mn affected ($P = 0.04$) *BCL2* mRNA expression (Fig. 6A) and had no effect ($P > 0.21$) on the expressions of *NIK* mRNA as well as BCL2 and NIK proteins (Fig. 6B–D) in the heart of offspring broilers. Compared to the M-NT, M-HT increased ($P < 0.04$) *BCL2* mRNA expression in the heart of offspring broilers from the M-iMn group, but had no effects ($P > 0.25$) on that from M-CON group.

4. Discussion

Responding to maternal stresses, miRNA expression patterns could be restored or reprogramed to modulate the expressions of mRNA targets in the tissues of offsprings (Constantinof et al., 2016). In the present study, the expressions of 6 miRNAs (miR-1551, miR-34c, miR-34b, miR-31, miR-205b, miR-216a, and miR-6643) were up-regulated in the embryonic heart in response to maternal heat stress. As reported previously in lymphoblast cells, exposure to cellular stress from sodium arsenite led to global increases in miRNA expressions (Marsit et al., 2006). Environmental stresses up-regulated miRNA biogenesis with the increase of the transcription of the primary miRNAs and the processing of a population of pri-miRNAs via the induction of p53 (Corney et al., 2007; Raver-Shapira et al., 2007; Suzuki et al., 2009). The aberrant miRNA expressions due to maternal stresses could further delay the development of embryos (Carraco et al., 2014; Riley et al., 2013). For instance, inhibiting mir-125a caused improper segmentation during chick somitogenesis by the translation repression and mRNA degradation of the lunatic fringe gene (Riley et al., 2013). As reported in zebrafish, miR-430 mutation delayed the maternal-to-zygote transition and gastrulation (Bazzini et al., 2012; Giraldez et al., 2006). Our results suggested that the abnormal miRNA expression pattern induced by maternal heat stress could be associated with the impaired embryonic development as reported in our previous study (Zhu et al., 2017).

It has been primarily acknowledged that miRNA expression patterns could be reprogrammed to modulate both the level of mRNA stability by conducting mRNA degradation and the level of translation by inhibiting protein translation or degrading the polypeptides through binding complementarily to 3'-UTR of the target mRNAs (Guo et al., 2010; Valinezhad et al., 2014). Effects of maternal macronutrients (e. g. amino acid and fat) and micronutrients (e. g. vitamins and copper) on miRNA expressions in mice and minipigs have been reviewed by Chango and Pogribny (2015). Therefore, understanding the contribution of nutritionally-modulated miRNA expression during development may open up new avenues for reducing the negative effects of maternal environmental stress. Chick embryos are much easier to obtain and manipulate than fetuses in most other vertebrate species. In addition, the miRNA expressions and functions in chick embryos have been characterized (Darnell et al., 2006). In the current study, the differentially expressed miRNA was identified in the hearts of chick embryos from heat stressed broiler breeder hens with dietary Mn supplementations using large-scale sequencing and RT-qPCR for miRNAs. Our results showed that maternal dietary Mn addition could reduce the increased expressions of miR-1551 and miR-34c induced by maternal heat stress. It is suggested that maternal Mn nutrition could potentially alter miRNA expressions to protect the embryos against maternal heat stress by targeting gene expressions. Subsequently, the target genes of miR-1551 and miR-34c were predicted and confirmed by pairing with the 3'-UTR using the dual-luciferase reporter assay (Jin et al., 2013). In the present study, the results of Luciferase-3' UTR reporter assays *in vivo* indicated that the endogenous miR-1551 and miR-34c inhibit *BCL2* and *NIK* mRNA expressions, respectively. However, as for the mutation of miR-1551 and miR-34c binding sites in BCL2 and NIK 3'UTR, the inhibitory effects were abolished. It was confirmed that BCL2 and NIK should be direct targets for miR-1551 and miR-34c, respectively.

The anti-apoptotic family protein BCL2 acts as a critical life-death decision point within the common pathway of apoptosis (Kowaltowski et al., 2000). Many studies have demonstrated that miRNAs are involved in the regulation of apoptosis pathways by targeting BCL2 (Cimmino et al., 2005; Tang et al., 2009; Wang et al., 2015). *In vivo*, up-regulation of miR-1 facilitated the H_2O_2-induced apoptosis in cardiomyocytes by targeting BCL2 down-regulation (Tang et al., 2009). As reported in DLD1 colon cancer cell line, miR-491 induced apoptosis and decreased cell viability by targeting BCL2 directly (Yu et al., 2016). In

addition, miR-15a and miR-16 were reported to inhibit cell proliferation, induce cellular apoptosis, and suppress tumorigenesis by targeting oncogene BCL2 (Bonci et al., 2008). In the current study, when chick embryos were subjected to maternal heat stress, *BCL2* mRNA and protein expressions were decreased in the embryonic heart from maternal Mn-deficient diet as the miR-1551 expression was increased. This inhibition on target gene expressions induced by miR-1551 might be associated with the blocked translation and elevated mRNA degradation. It is implied that maternal Mn nutrition could enhance the anti-apoptotic ability in the embryonic heart from maternal heat stress by activating the miR-1551 and target BCL2 expressions, which presented the protective effects on embryos against the maternal heat stress as reported in our previous study (Zhu et al., 2017).

MiRNAs as the new effectors and regulators play a crucial role in mediating apoptosis via noncanonical NF-κB (Boldin and Baltimore, 2012). The noncanonical NF-κB activation contributes to enhancing the anti-apoptotic responses in cancer cells (Hoesel and Schmid, 2013). Li et al. (2010) reported that the decreased expressions of miR-15a, miR-16, and miR-223 resulted in the induction of noncanonical NF-κB target genes in cancer cells. The NIK plays a pivotal role in the noncanonical NF-κB pathway as a crucial kinase in receptor-initiating signaling by phosphorylation of Inducible IκB Kinase α (Iκκα), including the responses of oxidative stress and apoptosis (Uno et al., 2014). Epigenetic loss of miR-31 increased apoptosis resistance by activating the non-canonical NF-κB pathway in adult T-lymphocytes and other cancers (Yamagishi et al., 2012). In the present study, maternal dietary Mn supplementation decreased miR-34c expression and activated NIK expression in the embryonic heart subjected to the maternal heat stress. It is suggested that maternal Mn nutrition might activate miR-34c-mediated NIK-dependent noncanonical NF-kB pathway to enhance the anti-apoptotic ability in the embryos from maternal heat stress. However, we failed to further detect the magnitude of the non-canonical NF-κB signaling cascades mediated by miRNA expression level because of a lack of the specific Iκκα and phosphorylated Iκκα proteins for *Gallus gallus*.

MiRNAs as epigenetic markers in embryonic tissues could be reprogrammed by maternal environmental exposures and nutritional status (Constantinof et al., 2016; Wu et al., 2004). Maternal epigenetic factors could cause the acquired changes in tissues of offsprings at the different developmental stages by modulating the expressions of related miRNAs and target genes (Smythies et al., 2014). In mice, the increased expressions of 9 specific sperm miRNAs resulting from parental stress were identified in the offspring from puberty to adulthood (Rodgers et al., 2013). In the present study, the similar aberrant expression patterns of miR-31 and miR-34c, but not miR-1551, in hearts were observed in chick embryos and offspring broilers from maternal heat stress and maternal Mn deficiency. It is suggested that the maternal heat stress caused some heritable changes of miRNA expressions during offspring embryos that could be transmitted to the offspring broilers. After maternal heat stress, the target *BCL2* mRNA expression in the heart of offspring broilers from the M-iMn group was up-regulated as described in the embryonic heart. However, the expression patterns of *NIK* mRNA as well as BCL2 and NIK proteins were inconsistent between the 2 stages of offspring embryos and broilers. Therefore, as for the chicken mode used in the present study, the mechanisms of miRNAs as epigenetic-signaling molecules in regulating target gene expressions to influence the subsequent offspring stages need to be further studied.

In conclusion, maternal heat stress increased the transcript abundances of miR-31, miR-205b, miR-216a and miR-6643 in chick embryonic hearts. Maternal supplemental 120 mg Mn/kg reduced the increased expressions of miR-1551 and miR-34c in hearts of offspring chick embryos but not broilers induced by maternal heat stress. The BCL2 and NIK related to anti-apoptotic ability were direct targets for miR-1551 and miR-34c, respectively. Under maternal heat stress, maternal supplemental 120 mg Mn/kg activated target BCL2 expression and the NIK-dependent NF-κB pathway via mediating the expressions of the miR-1551 and miR-34c in hearts of offspring embryos rather than broilers.

Conflicts of interest

All authors declare that there is no conflict of interest.

Acknowledgements

This study was supported by the Special Funds of CAAS for Distinguished Scientists, the National Nonprofit Institute Research Grant (2019-YWF-YTS-09), the Agricultural Science and Technology Innovation Program (ASTIP-IAS09), the Key International Cooperation Program of the National Natural Science Foundation of China (31110103916), and the China Agriculture Research System (CARS-41). In addition, we would highly appreciate Dr. David Masters in the University of Western Australia for assisting with the English editing of this manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jtherbio.2019.07.014.

References

Adlakha, Y.K., Saini, N., 2011. MicroRNA-128 downregulates Bax and induces apoptosis in human embryonic kidney cells. Cell. Mol. Life Sci. 68, 1415–1428.
Bazzini, A.A., Lee, M.T., Giraldez, A.J., 2012. Ribosome profiling shows that miR-430 reduces translation before causing mRNA decay in zebrafish. Science 336, 233–237.
Benjamini, Y., Hochberg, Y., 1995. Controlling the false discovery rate: A practical and powerful approach to multiple testing. J. R. Stat. Soc. B. 57, 289–300.
Boldin, M.P., Baltimore, D., 2012. MicroRNAs, new effectors and regulators of NF-kappaB. Immunol. Rev. 246, 205–220.
Bonci, D., Coppola, V., Musumeci, M., Addario, A., Giuffrida, R., Memeo, L., D'Urso, L., Pagliuca, A., Biffoni, M., Labbaye, C., Bartucci, M., Muto, G., Peschle, C., De Maria, R., 2008. The miR-15a-miR-16-1 cluster controls prostate cancer by targeting multiple oncogenic activities. Nat. Med. 14, 1271–1277.
Carraco, G., Goncalves, A.N., Serra, C., Andrade, R.P., 2014. MicroRNA processing machinery in the developing chick embryo. Gene Expr. Patterns 16, 114–121.
Chango, A., Pogribny, I.P., 2015. Considering maternal dietary modulators for epigenetic regulation and programming of the fetal epigenome. Nutrients 7, 2748–2770.
Cimmino, A., Calin, G.A., Fabbri, M., Iorio, M.V., Ferracin, M., Shimizu, M., Wojcik, S.E., Aqeilan, R.I., Zupo, S., Dono, M., Rassenti, L., Alder, H., Volinia, S., Liu, C.G., Kipps, T.J., Negrini, M., Croce, C.M., 2005. miR-15 and miR-16 induce apoptosis by targeting BCL2. Proc. Natl. Acad. Sci. U.S.A. 102, 13944–13949.
Constantinof, A., Moisiadis, V.G., Matthews, S.G., 2016. Programming of stress pathways: A transgenerational perspective. J. Steroid Biochem. Mol. Biol. 160, 175–180.
Corney, D.C., Flesken-Nikitin, A., Godwin, A.K., Wang, W., Nikitin, A.Y., 2007. MicroRNA-34b and MicroRNA-34c are targets of p53 and cooperate in control of cell proliferation and adhesion-independent growth. Cancer Res. 67, 8433–8438.
Darnell, D.K., Kaur, S., Stanislaw, S., Konieczka, J.H., Yatskievych, T.A., Antin, P.B., 2006. MicroRNA expression during chick embryo development. Dev. Dynam. 235, 3156–3165.
Flynt, A.S., Thatcher, E.J., Burkewitz, K., Li, N., Liu, Y., Patton, J.G., 2009. miR-8 microRNAs regulate the response to osmotic stress in zebrafish embryos. J. Cell Biol. 185, 115–127.
Fridovich, I., 1995. Superoxide radical and superoxide dismutases. Annu. Rev. Biochem. 64, 97–112.
Giraldez, A.J., Mishima, Y., Rihel, J., Grocock, R.J., Van Dongen, S., Inoue, K., Enright, A.J., Schier, A.F., 2006. Zebrafish MiR-430 promotes deadenylation and clearance of maternal mRNAs. Science 312, 75–79.
Guo, H., Ingolia, N.T., Weissman, J.S., Bartel, D.P., 2010. Mammalian microRNAs predominantly act to decrease target mRNA levels. Nature 466, 835.
Hoesel, B., Schmid, J.A., 2013. The complexity of NF-kappaB signaling in inflammation and cancer. Mol. Cancer 12, 86.
Jin, Y., Chen, Z., Liu, X., Zhou, X., 2013. Evaluating the microRNA targeting sites by luciferase reporter gene assay. Methods Mol. Biol. 936, 117–127.
Kanehisa, M., Furumichi, M., Tanabe, M., Sato, Y., Morishima, K., 2017. KEGG: New perspectives on genomes, pathways, diseases and drugs. Nucleic Acids Res. 45, D353–D361.
Kanehisa, M., Goto, S., 2000. KEGG: Kyoto encyclopedia of genes and genomes. Nucleic Acids Res. 28, 27–30.
Kanehisa, M., Sato, Y., Furumichi, M., Morishima, K., Tanabe, M., 2019. New approach for understanding genome variations in KEGG. Nucleic Acids Res. 47, D590–D595.
Kawano, M., Kawaji, H., Grandjean, V., Kiani, J., Rassoulzadegan, M., 2012. Novel small noncoding RNAs in mouse spermatozoa, zygotes and early embryos. PLoS One 7, e44542.

Kong, Y., Liang, X., Liu, L., Zhang, D., Wan, C., Gan, Z., Yuan, L., 2015. High throughput sequencing identifies microRNAs mediating alpha-synuclein toxicity by targeting neuroactive-ligand receptor interaction pathway in early stage of drosophila Parkinson's disease model. PLoS One 10, e0137432.

Kowaltowski, A.J., Vercesi, A.E., Fiskum, G., 2000. Bcl-2 prevents mitochondrial permeability transition and cytochrome c release via maintenance of reduced pyridine nucleotides. Cell Death Differ. 7, 903–910.

Leaman, D., Chen, P.Y., Fak, J., Yalcin, A., Pearce, M., Unnerstall, U., Marks, D.S., Sander, C., Tuschl, T., Gaul, U., 2005. Antisense-mediated depletion reveals essential and specific functions of microRNAs in Drosophila development. Cell 121, 1097–1108.

Li, T., Morgan, M.J., Choksi, S., Zhang, Y., Kim, Y.S., Liu, Z.G., 2010. MicroRNAs modulate the noncanonical transcription factor NF-kappaB pathway by regulating expression of the kinase IKKalpha during macrophage differentiation. Nat. Immunol. 11, 799–805.

Li, X., Cassidy, J.J., Reinke, C.A., Fischboeck, S., Carthew, R.W., 2009. A microRNA imparts robustness against environmental fluctuation during development. Cell 137, 273–282.

Li, S., Luo, X., Liu, B., Crenshaw, T.D., Kuang, X., Shao, G., Yu, S., 2004. Use of chemical characteristics to predict relative bioavailability of supplemental organic manganese sources for broilers. J. Anim. Sci. 82, 2352–2363.

Liao, X.D., Ma, C.Y., Lu, L., Zhang, L.Y., Luo, X.G., 2017. Determination of dietary iron requirements by full expression of iron-containing cytochrome c oxidase in the heart of broilers from 22 to 42 d of age. Br. J. Nutr. 118, 493–499.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\triangle\triangle CT}$ method. Methods 25, 402–408.

Luo, X.G., Li, S.F., Lu, L., Liu, B., Kuang, X., Shao, G.Z., Yu, S.X., 2007. Gene expression of manganese-containing superoxide dismutase as a biomarker of manganese bioavailability for manganese sources in broilers. Poultry Sci. 86, 888–894.

Marsit, C.J., Eddy, K., Kelsey, K.T., 2006. MicroRNA responses to cellular stress. Cancer Res. 66, 10843–10848.

Qin, S.Z., Liao, X.D., Lu, L., Zhang, L.Y., Xi, L., Guo, Y.L., Luo, X.G., 2017. Manganese enhances the expression of the manganese superoxide dismutase in cultured primary chick embryonic myocardial cells. J. Integr. Agri. 16, 2038–2046.

Raver-Shapira, N., Marciano, E., Meiri, E., Spector, Y., Rosenfeld, N., Moskovits, N., Bentwich, Z., Oren, M., 2007. Transcriptional activation of miR-34a contributes to p53-mediated apoptosis. Mol. Cell 26, 731–743.

Riley, M.F., Bochter, M.S., Wahi, K., Nuovo, G.J., Cole, S.E., 2013. Mir-125a-5p-mediated regulation of Lfng is essential for the avian segmentation clock. Dev. Cell 24, 554–561.

Rodgers, A.B., Morgan, C.P., Bronson, S.L., Revello, S., Bale, T.L., 2013. Paternal stress exposure alters sperm microRNA content and reprograms offspring HPA stress axis regulation. J. Neurosci. 33, 9003–9012.

Smythies, J., Edelstein, L., Ramachandran, V., 2014. Molecular mechanisms for the inheritance of acquired characteristics-exosomes, microRNA shuttling, fear and stress: Lamarck resurrected? Front. Genet. 5, 133.

Suzuki, H.I., Yamagata, K., Sugimoto, K., Iwamoto, T., Kato, S., Miyazono, K., 2009. Modulation of microRNA processing by p53. Nature 460, 529–533.

Tang, Y., Zheng, J., Sun, Y., Wu, Z., Liu, Z., Huang, G., 2009. MicroRNA-1 regulates cardiomyocyte apoptosis by targeting Bcl-2. Int. Heart J. 50, 377–387.

Uno, M., Saitoh, Y., Mochida, K., Tsuruyama, E., Kiyono, T., Imoto, I., Inazawa, J., Yuasa, Y., Kubota, T., Yamaoka, S., 2014. NF-kappaB inducing kinase, a central signaling component of the non-canonical pathway of NF-kappaB, contributes to ovarian cancer progression. PLoS One 9, e88347.

Valinezhad, O.A., Safaralizadeh, R., Kazemzadeh-Bavili, M., 2014. Mechanisms of miRNA-mediated gene regulation from common downregulation to mRNA-specific upregulation. Int. J. Genomics 970607 2014.

Wang, H., Li, J., Chi, H., Zhang, F., Zhu, X., Cai, J., Yang, X., 2015. MicroRNA-181c targets Bcl-2 and regulates mitochondrial morphology in myocardial cells. J. Cell Mol. Med. 19, 2084–2097.

Wu, G.Y., Bazer, F.W., Cudd, T.A., Meininger, C.J., Spencer, T.E., 2004. Maternal nutrition and fetal development. J. Nutr. 134, 2169–2172.

Yamagishi, M., Nakano, K., Miyake, A., Yamochi, T., Kagami, Y., Tsutsumi, A., Matsuda, Y., Sato-Otsubo, A., Muto, S., Utsunomiya, A., Yamaguchi, K., Uchimaru, K., Ogawa, S., Watanabe, T., 2012. Polycomb-mediated loss of miR-31 activates NIK-dependent NF-kappaB pathway in adult T cell leukemia and other cancers. Cancer Cell 21, 121–135.

Yu, T., Zuo, Q.F., Gong, L., Wang, L.N., Zou, Q.M., Xiao, B., 2016. MicroRNA-491 regulates the proliferation and apoptosis of CD8(+) T cells. Sci. Rep. 6, 30923.

Zhu, Y., Lu, L., Liao, X., Li, W., Zhang, L., Ji, C., Lin, X., Liu, H.C., Odle, J., Luo, X., 2017. Maternal dietary manganese protects chick embryos against maternal heat stress via epigenetic-activated antioxidant and anti-apoptotic abilities. Oncotarget 8, 89665–89680.

Zhu, Y.W., Xie, J.J., Li, W.X., Lu, L., Zhang, L.Y., Ji, C., Lin, X., Liu, H.C., Odle, J., Luo, X.G., 2015. Effects of environmental temperature and dietary manganese on egg production performance, egg quality, and some plasma biochemical traits of broiler breeders. J. Anim. Sci. 93, 3431–3440.