THE FASEB JOURNAL • RESEARCH • www.fasebj.org

Transcriptional and translational dynamics during maternal-to-zygotic transition in early chicken development

Young Sun Hwang,[*,1] Minseok Seo,[†,‡,1] Sohyun Bang,[†] Heebal Kim,[*,†,§] and Jae Yong Han[*,§,2]

*Department of Agricultural Biotechnology, Research Institute of Agriculture and Life Sciences, Seoul National University, Seoul, Korea; [†]C & K Genomics, SNU Research Park, Seoul National University, Seoul, Korea; [‡]Channing Division of Network Medicine, Harvard Medical School and Brigham and Women's Hospital, Boston, Massachusetts, USA; and [§]Institute for Biomedical Sciences, Shinshu University, Minamiminowa, Japan

ABSTRACT: Maternal-to-zygotic transition (MZT) is the critical process for the establishment of embryonic identity across vertebrates. During this period, the massive transcriptional activation, called zygotic genome activation (ZGA), is mediated by maternally stored factors, and maternal mRNA clearance by conserved zygotic microRNAs (miRNAs) occurs; however, the important transition in avian species was identified by morphologic perspectives only. In this study, we performed transcriptome analysis to examine the molecular transitions of intrauterine development in chickens. On the basis of coexpression analyses on RNA sequencing data, 2 waves of ZGA-mediated MZT were observed across the early embryonic stages and were associated with transcriptional and translational dynamics. Furthermore, definite transitions were observed according to the distinct developmental characteristics between cleavage and the area pellucida formation period in the functional analysis. Finally, epigenetic modification and the evolutionarily conserved miRNA expression suggest that certain MZT proceeds from Eyal-Giladi and Kochav stage VIII in early chicken development. We expect our study to provide an evolutionary link among vertebrates from the perspective of MZT regulation.—Hwang, Y. S., Seo, M., Bang, S., Kim, H., Han, J. Y. Transcriptional and translational dynamics during maternal-to-zygotic transition in early chicken development. FASEB J. 32, 2004–2011 (2018). www.fasebj.org

KEY WORDS: aves · early embryo · RNA-seq · intrauterine development

Embryogenesis is the process of well-coordinated development with predetermined genetic programs. Fundamental cellular processes, such as transcription and translation, govern the initial and critical events of early embryonic development after fertilization. During the first round of early development, a rapid and reductive cellularization occurs that is mostly handled by maternal mRNAs and proteins stored in the oocyte (1). Through the early mitotic divisions, transcriptional inactivation is sustained until zygotic genome activation (ZGA) that is accompanied by lengthening of the cell cycle (2).

The massive induction of zygotic transcription and the degradation of maternal mRNA happens in the period called maternal-to-zygotic transition (MZT)—a crucial process for establishing an individual organism (3). The midblastula transition (MBT), which was originally defined in amphibian development, is a morphologic embodiment of MZT and applies to ZGA in nonmammalian species (3). The timing of ZGA and MZT varies among vertebrate species (1). The major ZGA in humans and mice occur in the 2- and 8-cell stages, respectively (4, 5). In zebrafish and frog, the 128-cell stage has been reported as ZGA. It is well known that ZGA is triggered by maternal transcription factors, such as Nanog (nanog homeobox), Pou5f1 (POU domain, class 5, transcription factor 1), and SoxB1 family, that are deposited in the oocyte (6, 7). Along with zygotic transcription, the conserved zygotic microRNAs (miRNAs), such as miR-430 in zebrafish, miR-427 in frog, and miR-290 in mouse, are induced by these factors and play a role in maternal RNA degradation *via* post-transcriptional regulation in vertebrates (8–12). In addition, the variable expression of rRNA suggests that translational dynamics, such as limited translation and transition of maternal and zygotic ribosome, pool in MZT (13, 14).

ABBREVIATIONS: DNMT, DNA methyltransferase; EGK, Eyal-Giladi and Kochav; FZD, frizzled class receptor; GO, gene ontology; HDAC, histone deacetylase; MBD, methyl binding domain; MBT, midblastula transition; miRNA, microRNA; MZT, maternal-to-zygotic transition; p-PolII, phosphorylation of RNA polymerase II C-terminal domain; TET, tet methylcytosine dioxygenase; WNT, wingless-type MMTV integration site family; ZGA, zygotic genome activation

[1] These authors contributed equally to this work.

[2] Correspondence: Research Institute of Agriculture and Life Sciences, Seoul National University, 1 Gwanak-ro, Gwanak-gu, Seoul 08826, South Korea. E-mail: jaehan@snu.ac.kr

doi: 10.1096/fj.201700955R

This article includes supplemental data. Please visit *http://www.fasebj.org* to obtain this information.

Intrauterine development of avian species consists of 2 definite periods, including cleavage and area pellucida formation, according to Eyal-Giladi and Kochav (EGK) criteria (15, 16). The first developmental transition equivalent to MBT that indicates a slower rate of cell cycle and morphogenesis occurs during area pellucida formation (17); however, to date, the molecular basis for the induction of zygotic developmental program, clearance of maternal factors, and post-transcriptional regulators during MZT has not been studied in avian species.

In this study, we provide the developmental transition in chickens according to transcriptional- and translational-related aspects. On the basis of RNA sequencing data from early chicken embryos—from oocyte to EGK.X—coexpression analysis was performed to analyze transcriptomic changes during the early developmental process. In addition, we tried to identify epigenetic dynamics, including conserved miRNA related to the transitional point in chickens. Finally, combining the identified transition and other biologic knowledge, we attempted to establish the connection between well-described vertebrates and avian species in the evolutionarily intermediate position.

MATERIALS AND METHODS

RNA sequencing data availability and pipeline for obtaining gene expression levels

Our previously generated RNA sequencing data were analyzed differently and deposited in the Gene Expression Omnibus database (GSE86592). Experimental use of chickens was approved by the Institute of Laboratory Animal Resources, Seoul National University (SNU-150827-1). A total of 21 raw-sequencing data—biologically triplicated for each embryonic stage—were preprocessed by using Trimmomatic (v0.32) (18). The number of embryos used for the biologic triplicates of raw-sequencing data were as follows: 5 oocytes for each replication; 3 zygotes for each replication; 6, 5, and 6 EGK.I embryos for each replication; 6 EGK.III embryos for each replication; 6, 6, and 7 EGK.VI embryos for each replication; 5, 5, and 3 EGK.VIII embryos for each replication; and 10 EGK.X embryos for each replication. After that, clean reads were mapped on the chicken reference genome (Ensembl; galGal4 and galGal5) using HISAT2 (19). The Python script, HTSeq-count (20), was employed to quantify the mapped reads in each Ensembl transcript, and the multidimensional scaling method was used to determine the relationship among samples (Supplemental Fig. 1*A*).

Clustering analysis was used to identify coexpressed genes across all developmental stages

A weighted correlation network analysis (21) was used to categorize gene expression patterns. As a result, 8 representative modules were detected in the analysis (Supplemental Fig. 1*B*). Of these, 7 representative patterns (cluster 1–7) were finally observed after repetitive patterns were removed (Supplemental Fig. 1C); removal was based on the patterns observed in the weighted correlation network analysis and those expected from our biologic assumption. In addition to 7 representative patterns, negatively correlated patterns (cluster −1 to −7) with cluster 1–7 were included to develop additional clusters. To detect genes that corresponded to the 7 representative patterns, supervised learning was performed recursively to detect coexpressed genes conservatively. First, to classify genes into the 7 patterns (Supplemental Fig. 1C), class labels were defined as follows: cluster 1 = (1, 0, 0, 0, 0, 1, 1); cluster 2 = (0, 1, 1, 1, 1, 1, 1); cluster 3 = (1, 2, 2, 2, 2, 1, 0); cluster 4 = (0, 0, 0, 0, 0, 1, 1, 1); cluster 5 = (0, 0, 0, 0, 1, 2, 2); cluster 6 = (0, 0, 0, 0, 0, 1, 1); and cluster 7 = (0, 1, 1, 1, 2, 2, 2).

The seven 0-based vectors were defined to express the degree of fluctuation across developmental stages, from oocyte to EGK.X. On the basis of these class labels, we performed a decision tree–based classification analysis. Log_2 trimmed mean of M value normalized values were used for a gene expression matrix to consider library size in each sample, calculated by using edgeR (22). Spearman's correlation coefficients were used for a distance matrix to characterize the linear relationship between class label and gene expression patterns:

$$r_{gc} = 1 - \frac{6\sum \left(\mathrm{ran}(\mathrm{TMM}_g), \mathrm{ran}(\mathrm{Cluster}_c)\right)}{n(n^2-1)} \quad (1)$$

where ran(X) represents the rank of observed values, g represents trimmed mean of M value normalized values for a specific gene, c represents the class label, and n represents the number of observations [7 (stages) × 3 (biologic replications) = 21; Eq. 1]. To test their significance, Spearman's correction test was used, as follows:

$$t = r_{gc}\sqrt{\frac{n-2}{1-r_{gc}^2}} \sim t(n-2) \quad (2)$$

Under the null hypothesis, H_0 = no linear relationship between c and g, (Eq. 2) approximately follows a Student's t distribution. On the basis of this statistic, a permutation test was performed (23), with a false discovery rate–adjusted $P < 0.1$ considered the threshold for a significant relationship. From these class labels ($Class_c$), the correlation-based distance matrix (C_{gc}), and decision matrix of significance test (S_{gc} 0 and 1 represent significant and not significant, respectively), decision tree–based classification was performed as follows:

Classification matrix denotes D_g for each gene g:
For i in 1 to g
  If ($\sum_{c=1}^{7} S_{ic} = 0$)
    D_g = "Undetermined"
  Else if ($\sum_{c=1}^{7} S_{ic} = 1$)
    Let $\boldsymbol{c}^*$ be a significantly detected category
    If ($C_{i,c^*} > 0$) #
      D_g = classfying category as $\boldsymbol{c}^*(+)$
    Else
      D_g = classfying category as $\boldsymbol{c}^*(-)$
  Else
    c^* = Max(| C_i |)
    If ($C_{i,c^*} > 0$)
      D_g = classfying category as $\boldsymbol{c}^*(+)$
    Else
      D_g = classfying category as $\boldsymbol{c}^*(-)$

From this decision rule, all genes were classified into 14 categories [7 training patterns (cluster 1–7) and 7 negative correlation patterns (cluster −1 to −7)]. The list of genes in each cluster and expressed values are shown in Supplemental Data 1.

Statistical test for 2-group comparison and functional enrichment analysis

For the gene-set enrichment analysis, the Database for Annotation, Visualization and Integrated Discovery (DAVID; *https://david.ncifcrf.gov/*) was employed with 2 biologic databases—gene ontology (GO; *http://www.geneontology.org*) and Kyoto Encyclopedia of Genes and Genomes (KEGG; *http://www.genome.jp*) (24)—using chicken gene IDs. In DAVID, 5% significance level was considered a biologically significant term.

Quantitative real-time PCR analysis of miRNA

First-strand cDNA of miRNAs was synthesized from total RNA (1 μg) by using an miRNA first-strand cDNA synthesis kit (Stratagene, La Jolla, CA, USA). To elongate miRNAs, total RNA was treated with *Escherichia coli* poly(A) polymerase to generate a poly(A) tail at the 3′-end of each RNA molecule. After polyadenylation, cDNAs were synthesized by using a real-time adaptor primer. Real-time PCR analysis of complete miRNA first-strand cDNAs was performed using the High-Specificity miRNA QPCR Core Reagent Kit (Stratagene). PCR mixture was prepared by adding 2.5 μl 10× core PCR buffer, 2.75 μl 50 mM $MgCl_2$, 10 μl 20 mM deoxynucleotide triphosphate, 1.25 μl 20× EvaGreen, 1.0 μl 3.125 μM universal reverse primer, 1.0 μl 3.125 μM miRNA-specific forward primers, and 0.5 μl high-specificity polymerase to prepared miRNA cDNA in a 25-μl reaction volume. Each miRNA forward primer and chicken small nucleolar RNA primer (Supplemental Table 1) was designed following the guidelines from Agilent Technologies (Santa Clara, CA, USA). miRNA expression was normalized to that of a chicken small nucleolar RNA, which has been validated as an internal control (25, 26), and was calculated by using the $2^{-\Delta\Delta Ct}$ method (27).

RESULTS

Global expression clustering to understand transcriptional transition among early developmental stage of chicken embryos

Coexpression analysis was performed to identify the transcriptional transition across all developmental stages, especially during MZT. According to EGK morphologic criteria in early chicken development, 7 representative stages, including oocyte, zygote, and intrauterine embryos (from EGK.I to EGK.X), were employed (**Fig. 1*A***). On the basis of the quantification of annotated genes, transcriptional relationships among samples and stages were examined (Supplemental Fig. 1*A*). In a multidimensional scaling plot, biologically replicated samples were clearly clustered into each developmental stage, and distinct dynamic changes in transcriptional features during intrauterine development were found. We next identified 8 coexpressed modules in coexpression analysis using WGCNA (Supplemental Fig. 1*B*). Of 8 coexpression

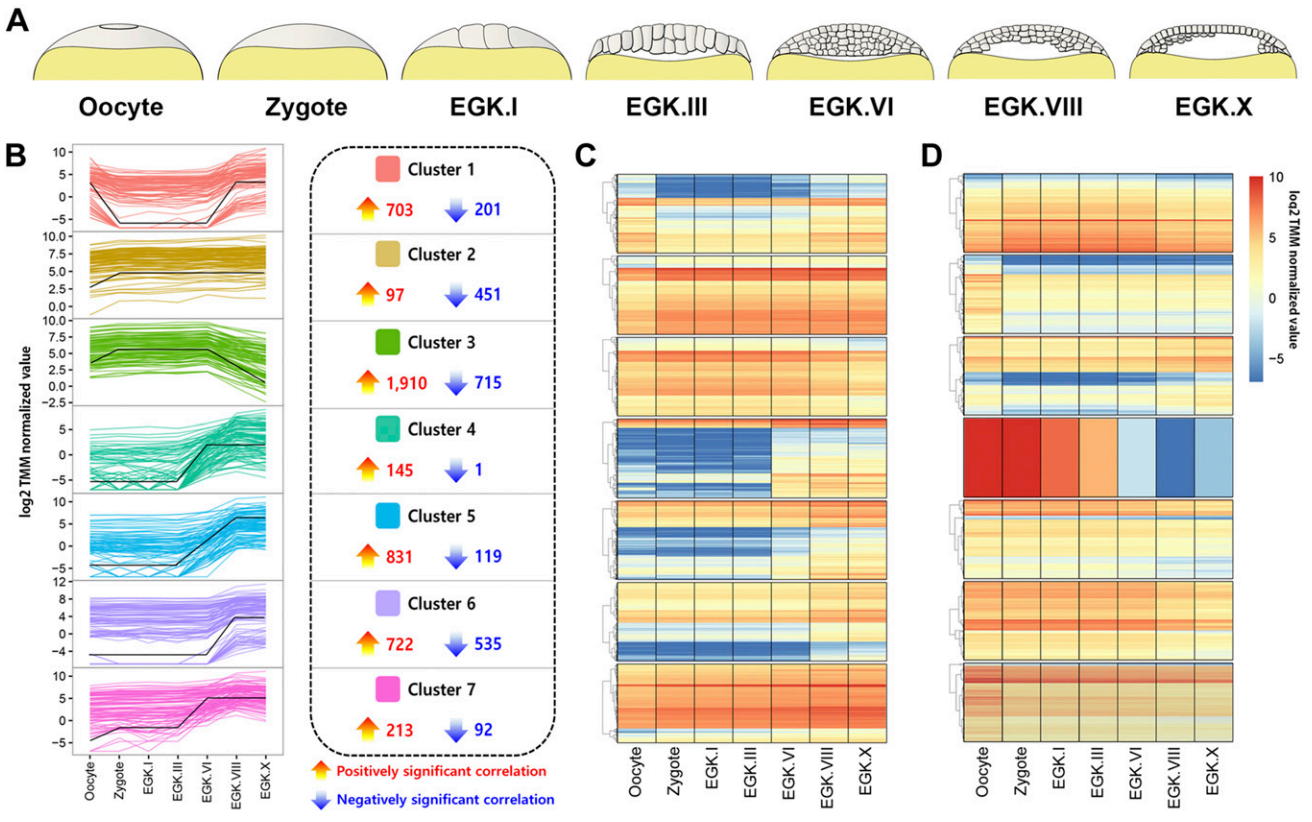

Figure 1. Expression clustering of RNA sequencing using early chicken embryos. *A*) Schematic of the morphology of early chicken embryos from oocyte to EGK.X. *B*) Line plots visualizing gene expression patterns across the developmental stages identified in our coexpression analysis. The *y* axis represents $\log_2$ trimmed mean of *M* value (TMM) normalized values, and the bold black line indicates representative patterns in each cluster. All significantly detected positive and negative genes in each cluster are summarized in the right dotted box. *C*, *D*) Heatmap of 7 positively (*C*) and negatively (*D*) clustered modules from coexpression analysis. From top to bottom, the heatmap shows the significantly enriched genes in clusters.

patterns, 7 representative patterns were determined, excluding repetitive patterns (Supplemental Fig. 1C), and supervised analysis was performed to detect statistically significant coexpressed genes according to such patterns. As a result, diverse gene expression patterns were observed and categorized into 7 representative patterns according to the properties of correlation measures—completely opposite correlation patterns were considered as being in the same category (Fig. 1*B–D*; false discover rate–adjusted $P < 0.1$). In the cluster, 1703 and 201 positive and negative correlated genes, respectively, were observed, and the expression of these genes was changed 2 times, between oocyte and zygote and between EGK.VI and EGK.VIII. Clusters 2, 3, and 7 demonstrate the first transition between oocyte and zygote stages. In cluster 4, only 1 negative correlated gene was observed, whereas 145 genes were positively correlated with the pattern of cluster 4, which suggests only an increased expression in EGK.VI. As with cluster 4, cluster 5 also demonstrated a transition pattern between EGK.III and EGK.VI, but the genes in cluster 5 displayed a steady increase or decrease in gene expression patterns up to EGK.VIII. In the case of the cluster, 6772 and 535 genes were significantly correlated, which demonstrated the transition of gene expression between EGK.VI and EGK.VIII. Coexpression analysis results suggest that there are three times the number of large transcriptional transitions at certain developmental stages, such as zygote, EGK.VI, and EGK.VIII, during intrauterine development. In particular, expression levels of a large number of genes were up-regulated at zygote and EGK.VI, which suggests that there may be 2 waves of ZGAs in chickens compared with other species. In addition, we found both increased and decreased gene expression in EGK.VIII.

Investigating coexpression patterns of genes in GO terms related to transcription and translation

We expected to find an avian MZT along with the functional terms of transcription and translation because of coupled ZGA and MZT processes and the low 28s rRNA content of pre-MZT embryo that have reported in other well-known organisms (3, 13). In coexpression analysis, we observed transcriptional changes at zygote and EGK.VI stages. We presumed that, at the time point estimated as ZGA, there would be many genes involved in transcription- and translation-related GO terms. In this regard, we attempted to investigate the gene expression patterns of annotated genes in transcription- and translation-related GO terms (**Fig. 2*A***and Supplemental Table 2). As a result, all gene expression levels were coexpressed in their annotated transcription- and translation-related GO terms. In transcription-related terms, 5 terms showed significant expression during 2 stages: zygote (up-regulation of RNA processing and down-regulation of transcription repressor activity) and EGK.VI (up-regulation of transcription factor activity, regulation of transcription, and transcription). In the case of translation-related terms, 4 of the 6 terms—endoplasmic reticulum, ribosome, structural constituent of ribosome, and translation—demonstrated highly down-regulated patterns after fertilization, but all translation-related terms were up-regulated in EGK.VIII.

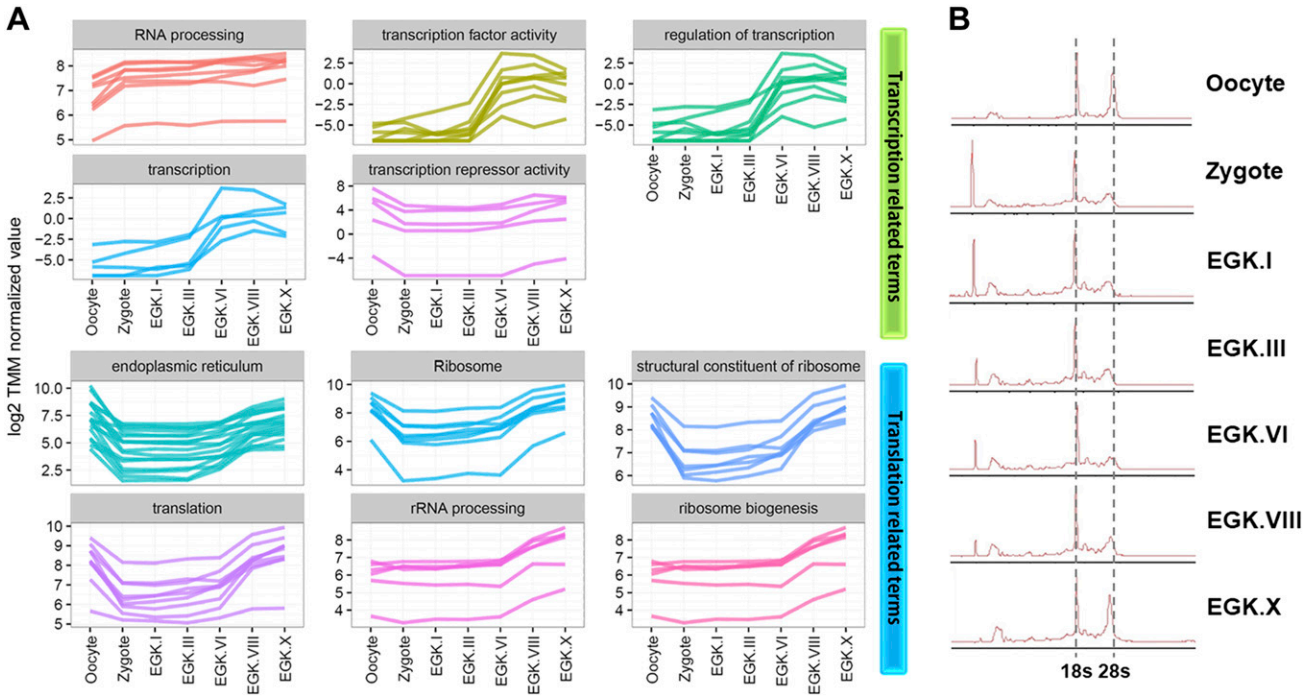

Figure 2. Transcriptional and translational regulation during ZGA and MZT in chickens. *A*) Expression patterns of clustered genes in relation to biologic terms of transcription and translation. *B*) Electrophoresis profile of the size distribution of total RNA content during early chicken development. Two dotted lines represent the peak of 18s and 28s rRNA. TMM, trimmed mean of *M* value.

Next, in accordance with translational terms, the relative amount of 28s rRNA was reduced markedly after formation of the zygote and recovered gradually after EGK.VIII, as indicated in electrophoresis profiles (Fig. 2*B*). This evidence supports the concept that MZT occurs in EGK.VIII in chicken.

Transition of molecular functions and signaling pathway during early embryonic development in chickens

To identify the functional transition of coexpressed genes in specific patterns, an enrichment test was performed on the basis of the GO and Kyoto Encyclopedia of Genes and Genomes databases (Supplemental Tables 3–15; enrichment test, $P < 0.05$) and the representative terms were summarized (**Fig. 3*A***). As a result, ion and cation transport were down-regulated from the oocyte stage to the zygote stage; however, they were up-regulated continuously after EGK.VI. In addition, 3 transcription-related terms, including RNA processing, regulation of transcription, positive regulation of transcription, and basal transcription factors, were indicated to be up-regulated after either or both zygote and EGK.VI stages (Fig. 3*A*). Functional terms, including ribosome and translation, were down-regulated at the zygote stage and up-regulated at the EGK.VIII stage (Fig. 3*A*). In addition, the regulation of small GTPase-mediated signal transduction, the MAPK signaling pathway, and the Notch signaling pathway showed similar transitions at two stages: up-regulation at the zygote stage and down-regulation after the EGK.VIII stage. Similarly, the regulation of small GTPase-mediated signal transduction and the MAPK signaling pathway by cluster 3 and the Notch signaling pathway by cluster 6 (negative correlated) were significantly observed (Fig. 3*B* and Supplemental Tables 5 and 14). Functional analysis on the basis of the galGal5 chicken genome build also demonstrated that the enriched functional terms that are associated with transcription, translation, and signaling pathways were categorized in the same clusters of galGal4 with minor differences (Supplemental Tables 16–28). These indicated that transcriptional and translational dynamics and transition in signaling pathways during MZT occurred, regardless of chicken genome build. Wnt signaling was involved in both transitions, but the Wnt ligand and receptor-related genes were differentially expressed in the 2 developmental stages (Fig. 3C). As shown in the heatmap, we observed distinct regulation between the cleavage and area pellucida formation periods. First, *WNT4* (wingless-type MMTV integration site family), *WNT6*, *FZD1* (frizzled class receptor), *RYK* (receptor-like tyrosine kinase), and *CCNYL1* (cyclin Y–like 1) were up-regulated during the cleavage period, from zygote to EGK.VI, and down-regulated after EGK.VI. In contrast, expression levels of *WNT5B*, *WNT8C*, *DKK1* (dickkopf WNT signaling pathway inhibitor 1), *FZD7*, *FZD10*, and *CTNNB1* (catenin-β1) were up-regulated during the area pellucida formation period, from EGK.VI to EGK.VIII.

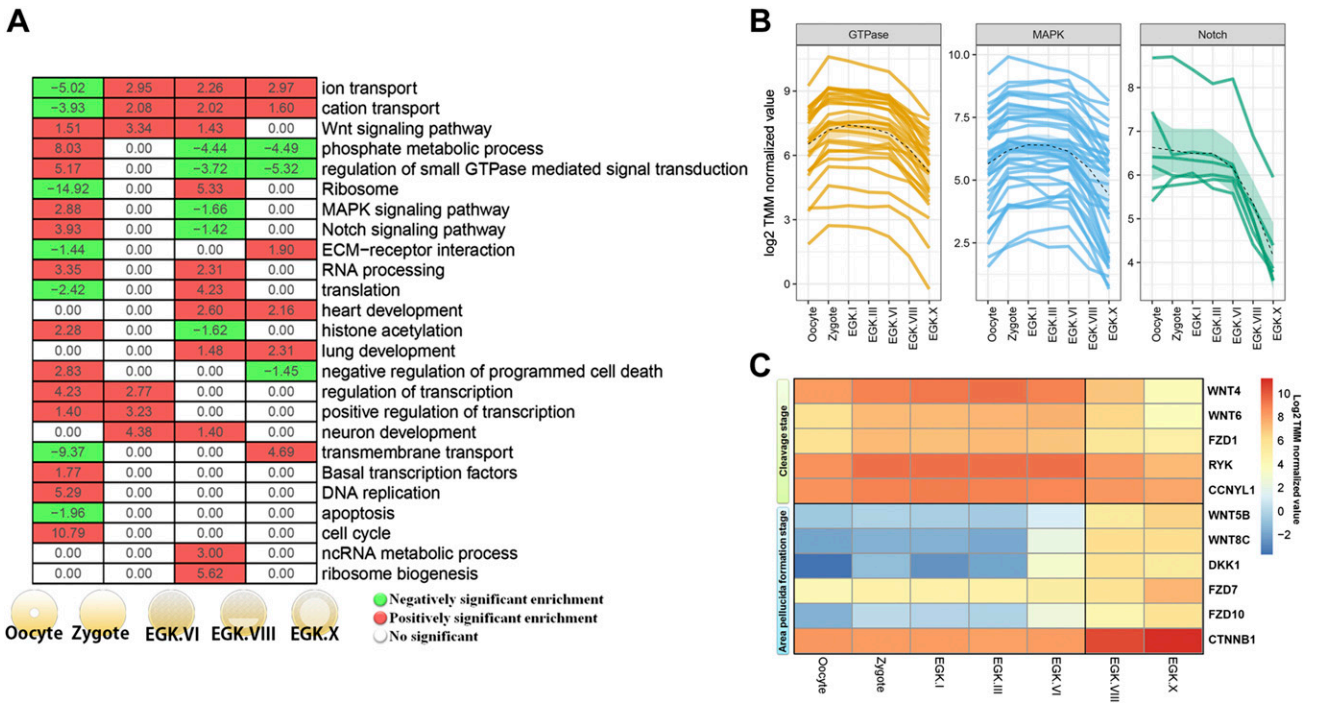

Figure 3. Embryonic developmental transition at the EGK.VIII stage in chickens. *A*) Summary of the significant functional terms across the 4 transitions. Average $\log_2$ fold-change values of genes that belong to the functional terms are represented in each transition. Positively and negatively enriched terms are filled in green and red, respectively. Nonsignificantly detected terms during a specific transition are filled in white. *B*) Degradation of up-regulated signaling pathways after fertilization from the EGK.VIII stage. *C*) Detailed gene expression related to Wnt signaling *via* early embryogenesis. ECM, extracellular matrix; ncRNA, noncoding RNA; TMM, trimmed mean of *M* value.

Epigenetic dynamics and post-transcriptional regulators in chicken MZT

On the basis of the results of the functional analysis, we could speculate global epigenetic status and histone modifications. Genes that are related to histone H3 acetylation (GO: 0043966) were induced after the zygote stage and decreased after the EGK.VIII stage, which indicates that dynamic modulation occurs in chromatin structures from paternal and maternal pronucleus or zygotic nucleus after fertilization for ZGA (**Fig. 4*A***). In addition, gene expression of epigenetic enzymes, such as DNA methyltransferases (DNMTs), histone deacetylases (HDACs), methyl binding domains (MBDs), tet methyl-cytosine dioxygenases (TETs), and histone acetyltransferases, was investigated during the various developmental stages (Fig. 4*A*). In this heatmap, expression levels of *HDAC4*, *HDAC11*, *MBD3*, and *TET2* were down-regulated >2-fold after the zygote, but these enzymes, with the exception of *TET2*, were up-regulated again after EGK.VIII. Other epigenetic enzymes—*DNMT3A*, *DNMT3B*, and *TET1*—were up-regulated in the transition between EGK.VIII and EGK.X, whereas *DNMT1* and *HDAC9* were down-regulated. These expression patterns seem to be associated with epigenetic reprogramming during MZT in chickens.

To additionally investigate the roles in maternal RNA clearance of miRNA in chicken, miRNA machinery genes, such as *DROSHA* (drosha ribonuclease III), *DICER1* (dicer 1 ribonuclease type III), *DGCR8* (DGCR8 microprocessor complex subunit), *XPO5* (exportin 5), and *AGO2* (argonaute RISC catalytic subunit 2), were investigated primarily (Fig. 4*B*). Of the 5 representative miRNA machinery examples, *DROSHA* and *DGCR8* were significantly up-regulated during EGK.VIII. These results indicate that miRNAs could be actively maturated during EGK.VIII to degrade maternal transcripts *via* post-translational regulation as zygote-expressed miRNAs are essential for MZT and maternal mRNA clearance in various species (11). Generally, strong evidence for MZT can be found in miRNAs that share the same proximal AAGUGC motif, which has been widely studied in other species, such as *Danio rerio*, *Xenopus laevis*, and *Mus musculus* (11, 12). On the basis of this knowledge, a motif search was performed in whole-chicken mature miRNAs to detect orthologous miRNAs with the conserved 5′-motif. As a result, the miR-302 family was identified as orthologous to well-known MZT markers, such as dre-miR-430s, xla-miR-427, and mmu-miR-290s (Fig. 4C). To measure the expression of mature miR-302, quantitative real-time PCR was performed. Results demonstrated

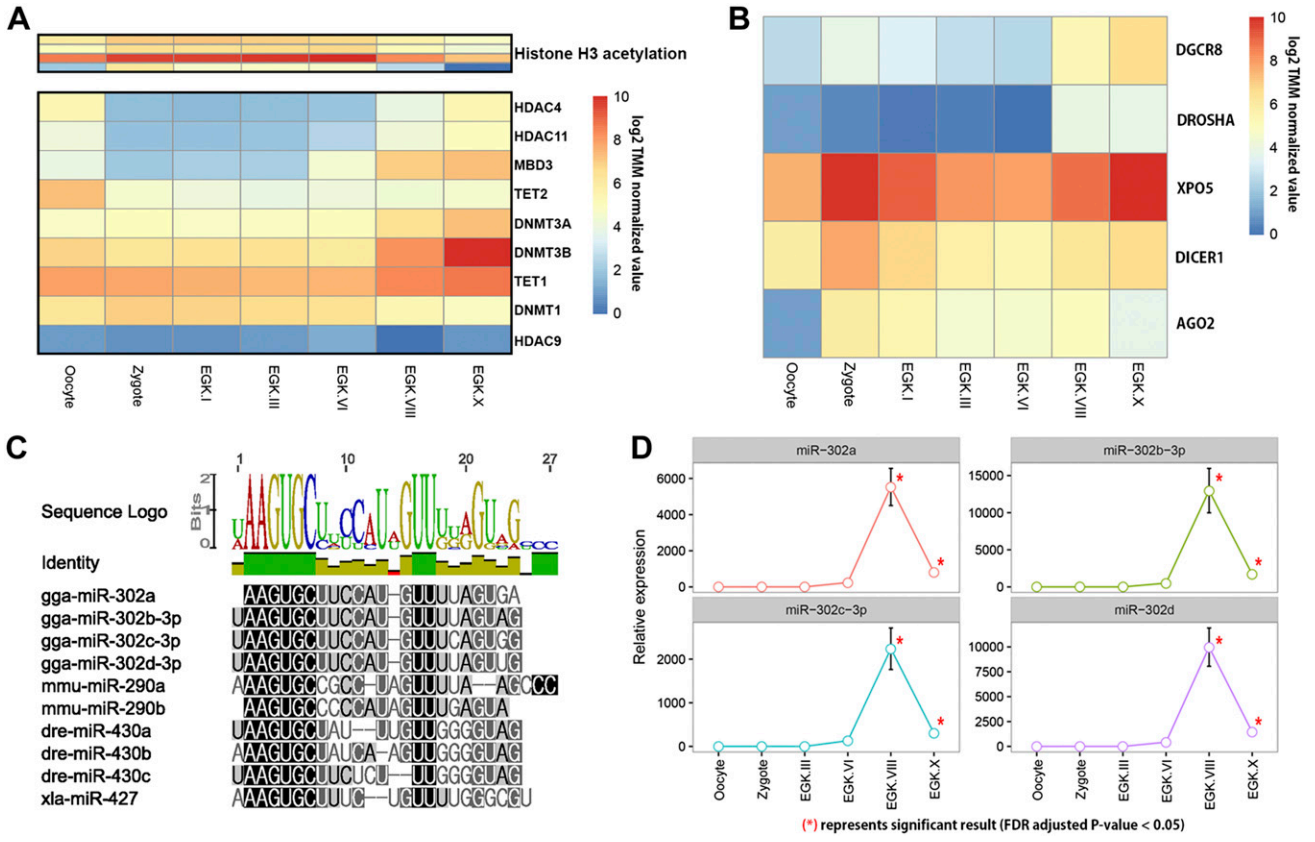

Figure 4. Epigenetic dynamics in early chicken development. *A*) Expression patterns of histone H3 acetylation and epigenetic enzymes. *B*) Expression profiling of miRNA machinery genes, including *DGCR8*, *DROSHA*, *XPO5*, *DICER1*, and *AGO2*, *via* early embryogenesis. *C*) Multiple sequence alignment of several mature miRNAs. From 2 to 7 bp, the 5′-proximal AAGUGC motif was conserved. *D*) Mature gga-miR-302 family expression patterns in early chicken development. Quantitative RT-PCR was conducted to quantify gene expression, and relative gene expression was measured by comparison with the control gene that encodes a small nucleolar RNA (snoRNA). Error bars indicate SE among 3 biologic replicates. FDR, false discovery rate; TMM, trimmed mean of *M* value.

significant up-regulation during EGK.VIII (Fig. 4*D* and Supplemental Table 29). These results provide strong evidence that MZT occurs in the EGK.VIII stage during early chicken development in the functional aspects.

DISCUSSION

During MZT, the maternal program is cleared and the zygotic developmental program is induced in vertebrates; thus, this period is the crucial point at which an organism becomes independent with respect to morphologic and genetic changes. In birds, MBT seems to occur at the beginning of second half of intrauterine development, the area pellucida formation around EGK.VI to EGK.VIII stage (Fig. 1*A*) (15–17). In addition to the morphologic concept, we examined the systematic transition of transcriptional and translational dynamics on the basis of RNA sequencing in chickens, a representative model of avian species.

Our transcriptomic and coexpression patterned analysis indicated 2× transcriptional transition between oocyte and zygote stages and EGK.III and EGK.VI stages. In mammals, the first transcriptional activation, called minor ZGA, occurs during the pronucelus stage as a result of the reprogramming of the chromatin state and histone modifications (28–32). Similarly, after fertilization in chickens, certain gene sets were affected, including the up-regulation of histone H3 acetylation and down-regulation of several epigenetic modulators that are involved in transcriptional repression—*HDAC4*, *HDAC11*, and *MBD3*.

After the zygote upon fertilization, no dynamic changes in transcription were observed until EGK.VI on the basis of gene expression clusters. During these cleavage stages, a rapid cellularization takes place, and MBT-like events, such as cell cycle lengthening, occur around EGK.VI on the basis of cell counting analysis (17, 33). Coincidently, the second transcriptional activation at a later point between EGK.III and EGK.VI was observed, although the phosphorylation of the RNA polymerase II C-terminal domain became apparent during the late EGK.II to early EGK.III stage (33).

From the transitional point of EGK.VI, the definite morphologic and genetic changes, including the establishment of the naive pluripotent state, progressed (15, 16, 34). In addition to transcriptional dynamics, functional and translational components clearly suggest that the EGK.VIII stage is a critical point for MZT in chickens. Of the significant functional terms, gene sets, such as the regulation of small GTPase-mediated signal transduction, the MAPK signaling pathway, and the Notch signaling pathway, were first down-regulated between EGK.VI and EGK.VIII stages, which suggests the clearance of maternal mRNA. Of interest, the Wnt signaling pathway is up-regulated in both transitions, and the associated genes, including ligands and receptors, were converted after MZT. In a previous report, pre-MZT bovine embryos displayed atypically low levels of 28s rRNA (13). This was also observed in our study, from the zygote to the EGK.VI stage, in chicken. We observed that the 28s rRNA level recovered after MZT, which follows EGK.VIII. These findings were consistent with translation-related genes being significantly down-regulated during the zygote stage and up-regulated during the EGK.VIII stage.

Indeed, during these stages, epigenetic modulators, such as *DNMT3A*, *DNMT3B*, and *TET1*, which regulate DNA methylation and demethylation, were up-regulated again during the EGK.VIII stage. These findings are also coincident with the global reduction in transcriptional activity during the EGK.VIII stage (35), which seems to represent epigenetic reprogramming along with MZT at the cellular level, as in the case of *Xenopus* and zebrafish (36, 37). Moreover, zygotic expression of miRNAs is essential for MZT and maternal mRNA degradation. On the basis of our expression analysis of miRNA machinery, along with previous reports (11), we confirmed that the expression of mature miR-302 members that contain the proximal AAGUGC motif increased during the EGK.VIII stage. Previous reports have shown that the miR-302 family was abundantly expressed in chicken EGK.X blastoderm (25), as was the miR-290 family in the mouse embryo after ZGA (11). Thus, the miR-302 family may regulate MZT and maternal mRNA degradation.

In summary, we demonstrated transcriptomic transitions of the early developmental stages and the first establishment of embryonic identity during MZT in chicken, which has previously been limited to morphologic studies. All evidence taken together, we conclude that MZT proceeds from the EGK.VIII stage after transcriptional activation in the EGK.VI stage during early chicken development. Our results provide insights into the issues of MZT mechanism among vertebrates. FJ

ACKNOWLEDGMENTS

This work was supported by a National Research Foundation of Korea grant funded by the South Korean government (Grant NRF-2015R1A3A2033826). The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

Y. S. Hwang and J. Y. Han designed the experiments; Y. S. Hwang generated whole-transcriptome RNA sequencing reads; Y. S. Hwang, M. Seo, S. Bang, H. Kim, and J. Y. Han analyzed the data; and Y. S. Hwang, M. Seo, H. Kim, and J. Y. Han wrote and edited the manuscript.

REFERENCES

1. Tadros, W., and Lipshitz, H. D. (2009) The maternal-to-zygotic transition: a play in two acts. *Development* **136**, 3033–3042
2. Newport, J., and Kirschner, M. (1982) A major developmental transition in early *Xenopus* embryos: II. Control of the onset of transcription. *Cell* **30**, 687–696
3. Lee, M. T., Bonneau, A. R., and Giraldez, A. J. (2014) Zygotic genome activation during the maternal-to-zygotic transition. *Annu. Rev. Cell Dev. Biol.* **30**, 581–613
4. Xue, Z., Huang, K., Cai, C., Cai, L., Jiang, C. Y., Feng, Y., Liu, Z., Zeng, Q., Cheng, L., Sun, Y. E., Liu, J. Y., Horvath, S., and Fan, G. (2013) Genetic programs in human and mouse early embryos revealed by single-cell RNA sequencing. *Nature* **500**, 593–597

5. Yan, L., Yang, M., Guo, H., Yang, L., Wu, J., Li, R., Liu, P., Lian, Y., Zheng, X., Yan, J., Huang, J., Li, M., Wu, X., Wen, L., Lao, K., Li, R., Qiao, J., and Tang, F. (2013) Single-cell RNA-Seq profiling of human preimplantation embryos and embryonic stem cells. *Nat. Struct. Mol. Biol.* **20**, 1131–1139
6. Lee, M. T., Bonneau, A. R., Takacs, C. M., Bazzini, A. A., DiVito, K. R., Fleming, E. S., and Giraldez, A. J. (2013) *Nanog, Pou5f1* and *SoxB1* activate zygotic gene expression during the maternal-to-zygotic transition. *Nature* **503**, 360–364
7. Leichsenring, M., Maes, J., Mössner, R., Driever, W., and Onichtchouk, D. (2013) *Pou5f1* transcription factor controls zygotic gene activation in vertebrates. *Science* **341**, 1005–1009
8. Giraldez, A. J., Mishima, Y., Rihel, J., Grocock, R. J., Van Dongen, S., Inoue, K., Enright, A. J., and Schier, A. F. (2006) Zebrafish miR-430 promotes deadenylation and clearance of maternal mRNAs. *Science* **312**, 75–79
9. Tang, F., Kaneda, M., O'Carroll, D., Hajkova, P., Barton, S. C., Sun, Y. A., Lee, C., Tarakhovsky, A., Lao, K., and Surani, M. A. (2007) Maternal microRNAs are essential for mouse zygotic development. *Genes Dev.* **21**, 644–648
10. Lund, E., Liu, M., Hartley, R. S., Sheets, M. D., and Dahlberg, J. E. (2009) Deadenylation of maternal mRNAs mediated by miR-427 in *Xenopus laevis* embryos. *RNA* **15**, 2351–2363
11. Svoboda, P., and Flemr, M. (2010) The role of miRNAs and endogenous siRNAs in maternal-to-zygotic reprogramming and the establishment of pluripotency. *EMBO Rep.* **11**, 590–597
12. Yartseva, V., and Giraldez, A. J. (2015) The maternal-to-zygotic transition during vertebrate development: a model for reprogramming. *Curr. Top. Dev. Biol.* **113**, 191–232
13. Gilbert, I., Scantland, S., Sylvestre, E. L., Gravel, C., Laflamme, I., Sirard, M. A., and Robert, C. (2009) The dynamics of gene products fluctuation during bovine pre-hatching development. *Mol. Reprod. Dev.* **76**, 762–772
14. Ihara, M., Tseng, H., and Schultz, R. M. (2011) Expression of variant ribosomal RNA genes in mouse oocytes and preimplantation embryos. *Biol. Reprod.* **84**, 944–946
15. Eyal-Giladi, H., and Kochav, S. (1976) From cleavage to primitive streak formation: a complementary normal table and a new look at the first stages of the development of the chick. I. General morphology. *Dev. Biol.* **49**, 321–337
16. Kochav, S., Ginsburg, M., and Eyal-Giladi, H. (1980) From cleavage to primitive streak formation: a complementary normal table and a new look at the first stages of the development of the chick. II. Microscopic anatomy and cell population dynamics. *Dev. Biol.* **79**, 296–308
17. Park, H. J., Park, T. S., Kim, T. M., Kim, J. N., Shin, S. S., Lim, J. M., and Han, J. Y. (2006) Establishment of an *in vitro* culture system for chicken preblastodermal cells. *Mol. Reprod. Dev.* **73**, 452–461
18. Bolger, A. M., Lohse, M., and Usadel, B. (2014) Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* **30**, 2114–2120
19. Kim, D., Langmead, B., and Salzberg, S. L. (2015) HISAT: a fast spliced aligner with low memory requirements. *Nat. Methods* **12**, 357–360
20. Anders, S., Pyl, P. T., and Huber, W. (2015) HTSeq–a Python framework to work with high-throughput sequencing data. *Bioinformatics* **31**, 166–169
21. Langfelder, P., and Horvath, S. (2008) WGCNA: an R package for weighted correlation network analysis. *BMC Bioinformatics* **9**, 559
22. Robinson, M. D., McCarthy, D. J., and Smyth, G. K. (2010) edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* **26**, 139–140
23. Kendall, M. G., Stuart, A., and Ord, J. K. (1968) *The Advanced Theory of Statistics*, Vol. 3, Charles Griffin, London
24. Huang, D. W., Sherman, B. T., Tan, Q., Kir, J., Liu, D., Bryant, D., Guo, Y., Stephens, R., Baseler, M. W., Lane, H. C., and Lempicki, R. A. (2007) DAVID bioinformatics resources: expanded annotation database and novel algorithms to better extract biology from large gene lists. *Nucleic Acids Res.* **35** (suppl 2), W169–W175
25. Lee, S. I., Lee, B. R., Hwang, Y. S., Lee, H. C., Rengaraj, D., Song, G., Park, T. S., and Han, J. Y. (2011) MicroRNA-mediated post-transcriptional regulation is required for maintaining undifferentiated properties of blastoderm and primordial germ cells in chickens. *Proc. Natl. Acad. Sci. USA* **108**, 10426–10431
26. Rengaraj, D., Lee, S. I., Park, T. S., Lee, H. J., Kim, Y. M., Sohn, Y. A., Jung, M., Noh, S. J., Jung, H., and Han, J. Y. (2014) Small non-coding RNA profiling and the role of piRNA pathway genes in the protection of chicken primordial germ cells. *BMC Genomics* **15**, 757
27. Livak, K. J., and Schmittgen, T. D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-delta delta C(T)) method. *Methods* **25**, 402–408
28. Bouniol, C., Nguyen, E., and Debey, P. (1995) Endogenous transcription occurs at the 1-cell stage in the mouse embryo. *Exp. Cell Res.* **218**, 57–62
29. Aoki, F., Worrad, D. M., and Schultz, R. M. (1997) Regulation of transcriptional activity during the first and second cell cycles in the preimplantation mouse embryo. *Dev. Biol.* **181**, 296–307
30. Aoshima, K., Inoue, E., Sawa, H., and Okada, Y. (2015) Paternal H3K4 methylation is required for minor zygotic gene activation and early mouse embryonic development. *EMBO Rep.* **16**, 803–812
31. Wu, J., Huang, B., Chen, H., Yin, Q., Liu, Y., Xiang, Y., Zhang, B., Liu, B., Wang, Q., Xia, W., Li, W., Li, Y., Ma, J., Peng, X., Zheng, H., Ming, J., Zhang, W., Zhang, J., Tian, G., Xu, F., Chang, Z., Na, J., Yang, X., and Xie, W. (2016) The landscape of accessible chromatin in mammalian preimplantation embryos. *Nature* **534**, 652–657
32. Dahl, J. A., Jung, I., Aanes, H., Greggains, G. D., Manaf, A., Lerdrup, M., Li, G., Kuan, S., Li, B., Lee, A. Y., Preissl, S., Jermstad, I., Haugen, M. H., Suganthan, R., Bjørås, M., Hansen, K., Dalen, K. T., Fedorcsak, P., Ren, B., and Klungland, A. (2016) Broad histone H3K4me3 domains in mouse oocytes modulate maternal-to-zygotic transition. *Nature* **537**, 548–552
33. Nagai, H., Sezaki, M., Kakiguchi, K., Nakaya, Y., Lee, H. C., Ladher, R., Sasanami, T., Han, J. Y., Yonemura, S., and Sheng, G. (2015) Cellular analysis of cleavage-stage chick embryos reveals hidden conservation in vertebrate early development. *Development* **142**, 1279–1286
34. Mak, S. S., Alev, C., Nagai, H., Wrabel, A., Matsuoka, Y., Honda, A., Sheng, G., and Ladher, R. K. (2015) Characterization of the finch embryo supports evolutionary conservation of the naive stage of development in amniotes. *Elife* **4**, e07178
35. Lee, H. C., Choi, H. J., Lee, H. G., Lim, J. M., Ono, T., and Han, J. Y. (2016) DAZL expression explains origin and central formation of primordial germ cells in chickens. *Stem Cells Dev.* **25**, 68–79
36. Akkers, R. C., van Heeringen, S. J., Jacobi, U. G., Janssen-Megens, E. M., Françoijs, K. J., Stunnenberg, H. G., and Veenstra, G. J. (2009) A hierarchy of H3K4me3 and H3K27me3 acquisition in spatial gene regulation in *Xenopus* embryos. *Dev. Cell* **17**, 425–434
37. Lindeman, L. C., Andersen, I. S., Reiner, A. H., Li, N., Aanes, H., Østrup, O., Winata, C., Mathavan, S., Müller, F., Aleström, P., and Collas, P. (2011) Prepatterning of developmental gene expression by modified histones before zygotic genome activation. *Dev. Cell* **21**, 993–1004

Received for publication September 8, 2017.
Accepted for publication November 13, 2017.