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DNA Methylation and Regulatory Elements during Chicken Germline Stem Cell Differentiation

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SUMMARY

The production of germ cells *in vitro* would open important new avenues for stem biology and human medicine, but the mechanisms of germ cell differentiation are not well understood. The chicken, as a great model for embryology and development, was used in this study to help us explore its regulatory mechanisms. In this study, we reported a comprehensive genome-wide DNA methylation landscape in chicken germ cells, and transcriptomic dynamics was also presented. By uncovering DNA methylation patterns on individual genes, some genes accurately modulated by DNA methylation were found to be associated with cancers and virus infection, e.g., *AKT1* and *CTNNB1*. Chicken-unique markers were also discovered for identifying male germ cells. Importantly, integrated epigenetic mechanisms were explored during male germ cell differentiation, which provides deep insight into the epigenetic processes associated with male germ cell differentiation and possibly improves treatment options to male infertility in animals and humans.

INTRODUCTION

Germ cells are the only cell type capable of transmitting genetic information to the next generation. In many species, germ cells form at the fringe of the embryo proper and then traverse through several developing somatic tissues on their migration to the emerging gonads. Primordial germ cells (PGCs) are the only cells in developing embryos with the potential to transmit genetic information to the next generation (Nakamura et al., 2013). Chicken PGCs, unlike mammals, exhibit unique migration activity, appearing within the epiblast in the blastoderm and moving to the hypoblast of the area pellucida instead of moving into embryonic gonads through the hindgut (Petitte et al., 1997). During gastrulation, chicken PGCs move to the germinal crescent, then circulate through the blood vessels, finally settling in the gonadal ridge (Nakamura et al., 2007). In addition, chicken embryonic development occurs *in ovo* rather than *in utero* (Burt and Pourquie, 2003). These unique characteristics of chicken germ cells during early development make germ cell isolation easier and make it possible to gain a huge number of cells from chicken embryos to advance stem cell research (Li et al., 2004). Therefore, chicken models play a pivotal role in animal research as an alternative and outbreed experimental species to humans to compensate for ethical constraints and the accessibility of human germ cell studies, and understanding germ cell biology *in vivo* and *in vitro* in chicken models would be important for practical applications of avian reproductive biology and endogenous species conversation, especially for human medicine, including various birth defects, germ cell tumors, and drug target screening (Conti and Giudice, 2008).

DNA methylation is an essential epigenetic mechanism in developmental biology and plays important roles in sex chromosome dosage compensation, the maintenance of genome stability, and the coordinated expression of imprinted genes (Messerschmidt et al., 2014). PGCs, the precursors of sperm and eggs, are the route to totipotency and require the establishment of a unique epigenome in this lineage (Surani and Hajkova, 2010). In vertebrates, DNA methylation occurs almost exclusively on CpG islands (CGIs). Such methylation can be inherited through cell division and transmitted from one generation to the next via germ cells. CGI methylation plays a role in the maintenance of heterochromatin as well as the inhibition of promoter activity by inhibiting the interaction between transcriptional factors (TFs) and their promoters or by changing the chromatin structure (Jang et al., 2013). In general, TFs orchestrate the overall remodeling of the epigenome, including the priming of loci that

will change expression only at late stages of embryo development (Cantone and Fisher, 2013). Besides, TF binding sites are overlapping with regions of dynamic changes in DNA methylation and are linked to its targeted regulation (Stadler et al., 2011). It has also been shown that lineage-specific TFs and signaling pathways collaborate with the core regulators of pluripotency to exit the embryonic stem cell (ESC) state and activate the transcriptional networks governing cellular specification (Thomson et al., 2011).

Notably, long non-coding RNAs (lncRNAs) recently have emerged as an important class of gene expression regulators. lncRNAs exhibit several distinctive features that confer unique regulatory functions, including exquisite cell- and the tissue-specific expression and the capacity to transduce higher-order spatial information. Some lncRNAs were reported to be under control of pluripotency factors such as OCT4 and NANOG. Interestingly, these lncRNAs seemingly activate the transcription of pluripotent TFs in a regulatory positive feedback loop (Sheik Mohamed et al., 2010). In addition, the global DNA demethylation is associated with a cascade of chromatin-remodeling events, including the transient loss of linker histone H1, H3K27me3, and H3K9me3, and stable loss of H3K9ac and H2A/H4 R3me2, and, subsequently, reactivation of the X chromosome in females (Chuva de Sousa Lopes et al., 2008; Cantone and Fisher, 2013). During spermatogenesis, methylation of histone tails is achieved by H3-K4, and H3-K9 methyltransferases (Carrell et al., 2008). Although histone modification patterns during spermatogenesis and the interactions with DNA methylation have been reported to perform specific roles (Teng et al., 2010; Güneş and Kulaç, 2013), the orchestra among DNA methylation, TFs, lncRNAs, and histone modifications governing cellular specification during spermatogenesis is as yet poorly understood.

Recent evidence suggests that the DNA methylation pattern in the chicken is similar to that in mammals (Li et al., 2011), and DNA methylation and histone modifications are also involved in the pluripotency maintenance and differentiation process of chick embryonic germ cells (Jiao et al., 2013). Moreover, DNA methylation and histone modifications are expressed in time- and tissue-dependent manners in developing chick embryos, and epigenetic marks are relatively stable and kept at lower levels after birth (Gryzinska et al., 2013; Li et al., 2015). Also, differentially methylated signatures associated with gene expression were detected in PGCs during the early embryonic development of chickens (Jang et al., 2013). However, the precise and composited methylation regulation patterns, non-coding RNAs and TFs remain rarely studied in chick embryonic development. In this study, therefore, we aimed to explore precise DNA methylation regulation patterns during germline stem cell differentiation, especially differentiating into male germ cells, using methyl-CpG binding domain protein sequencing (MBD-seq) approach. The three kinds of chick germ cells—ESCs, PGCs, and spermatogonial stem cells (SSCs)—were collected to study epigenetic regulation mechanisms during spermatogenesis. Our results provided the comprehensive insight into epigenetic regulations during chicken spermatogenesis.

RESULTS

The Dynamics of DNA Methylation during Germ Cell Differentiation

To study DNA methylation dynamics in chick spermatogenesis, we performed DNA methylation sequencing on genome-wide by MBD-seq for ESCs, PGCs, and SSCs isolated from stage X blastoderm chick gonad at embryonic day 5 (E5), and chick testis at E19, respectively (Figures 1A and 1B). Our results showed that ESCs have the lowest methylation level (Figure 1C), which was consistent with genome-wide loss of DNA methylation during early mouse development, reaching a low point during the blastocyst stage (Santos et al., 2002). PGCs have a higher level of genome-wide methylation than ESCs and SSCs during chick germ cell differentiation. To investigate DNA methylation in different genomic regions across three cell types, we profiled DNA methylation plots covering upstream 20 kb, gene body region, and downstream 20 kb for all annotated chicken genes (Figure S1A). We observed that ESCs had a lower methylation level than PGCs and SSCs from outside of upstream 5 kb and downstream 5 kb, but a sharp increase occurred in gene body regions and around the transcriptional start and end sites (TSSs and TESs) for ESCs.

To refine gene body regions and explore DNA methylation changes in different functional elements, we divided chicken genome into the promoter, exon, intron, and intergenic region plus CGI. The results demonstrated that a large proportion of genomic methylation occurred on CGIs, which had five times methylation enrichment compared with the exon regions indicated in Figure 1D ($p < 0.01$, Fisher's exact test). Promoter regions were also enriched with abundant DNA methylation, which may be due to the fact that most annotated gene promoters overlap with a CGI (Deaton and Bird, 2011). It is noted that PGCs had significantly higher methylation than ESCs and SSCs across all these five functional elements, which was in agreement with Figure 1C, which shows that PGCs were experiencing *de novo* methylation and would last until male germ cells. In addition, some differentially methylated regions (DMRs) among three cell types were validated

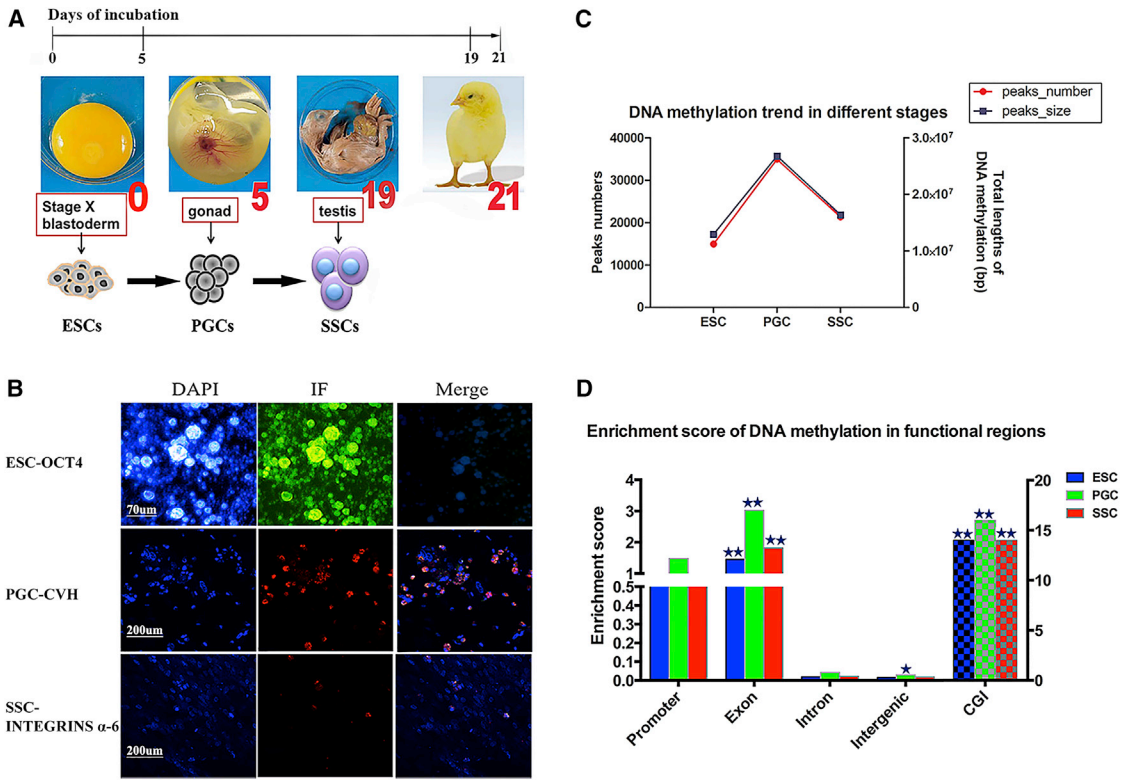

Figure 1. DNA Methylation Dynamics during Chick Germ Cell Differentiation

(A) ESCs were isolated from the blastoderm of fertile eggs at stage X, PGCs were isolated from chicken gonad at E5, and SSCs were isolated from chicken testis at E19.

(B) The immunocytochemical detection of chick ESCs, PGCs, and SSCs with three independent experiments. The ESC marker OCT4, PGC marker CVH, and SSC marker integrin α6 were DAPI staining and immunofluorescence (IF) staining.

(C) DNA methylation trend through different development stages of chick germ cells. The numbers of DNA methylation peaks on three cell types (the left y axis) and the total length of DNA methylation peaks for each cell types (bp, the right y axis) are shown. DNA-methylated fragment sequencing analyses were performed with two biological replicates per cell type.

(D) Enrichment score of DNA methylation in various annotated functional elements through three cell types. CGI is corresponding to the right y axis. The asterisks indicate statistically significant enrichment: *p < 0.05, **p < 0.01, Fisher's exact test) (see Figure S1).

by bisulfite cloning sequencing, and the results indicated that 89% putative DMRs identified by MBD-seq were confirmed (Figure S1B).

DNA Methylation and Gene Expression during Germ Cell Differentiation

To detect DNA methylation regulation of gene expression during germ cell differentiation, we examined differentially expressed genes between every two cell types and checked their DNA methylation levels in promoter regions. Interestingly, of 916 unique differentially expressed genes between ESCs and PGCs, 4.48% of genes are located on chromosome Z, and in which 65.85% of them were down-regulated from ESCs into PGCs; however, when PGCs were differentiated into SSCs, 7.02% of 726 unique differentially expressed genes are located on chromosome Z and 82.35% of them were upregulated (Figure 2A). These results demonstrated that most of the sex chromosome genes were activated at the second stage to drive sexual differentiation, which conformed to the biological characteristics of cell differentiation phenotype. The results of DNA methylation enrichment on promoter regions of unique differentially expressed genes showed that, in general, mRNA expression of genes and their DNA methylation of promoter regions had opposite expression directions, confirming that DNA methylation represses gene transcription. From PGCs to SSCs, their mRNA expression in ESCs was similar to that in PGCs or SSCs, which was with low mRNA expression in general (Figure 2B); thus, DNA methylation might switch certain genes to be on or off depending on cellular lineage and stage specificity.

To uncover methylation patterns associated with gene expression change and functions of these genes, we identified clusters of genes with similar methylation profiles and

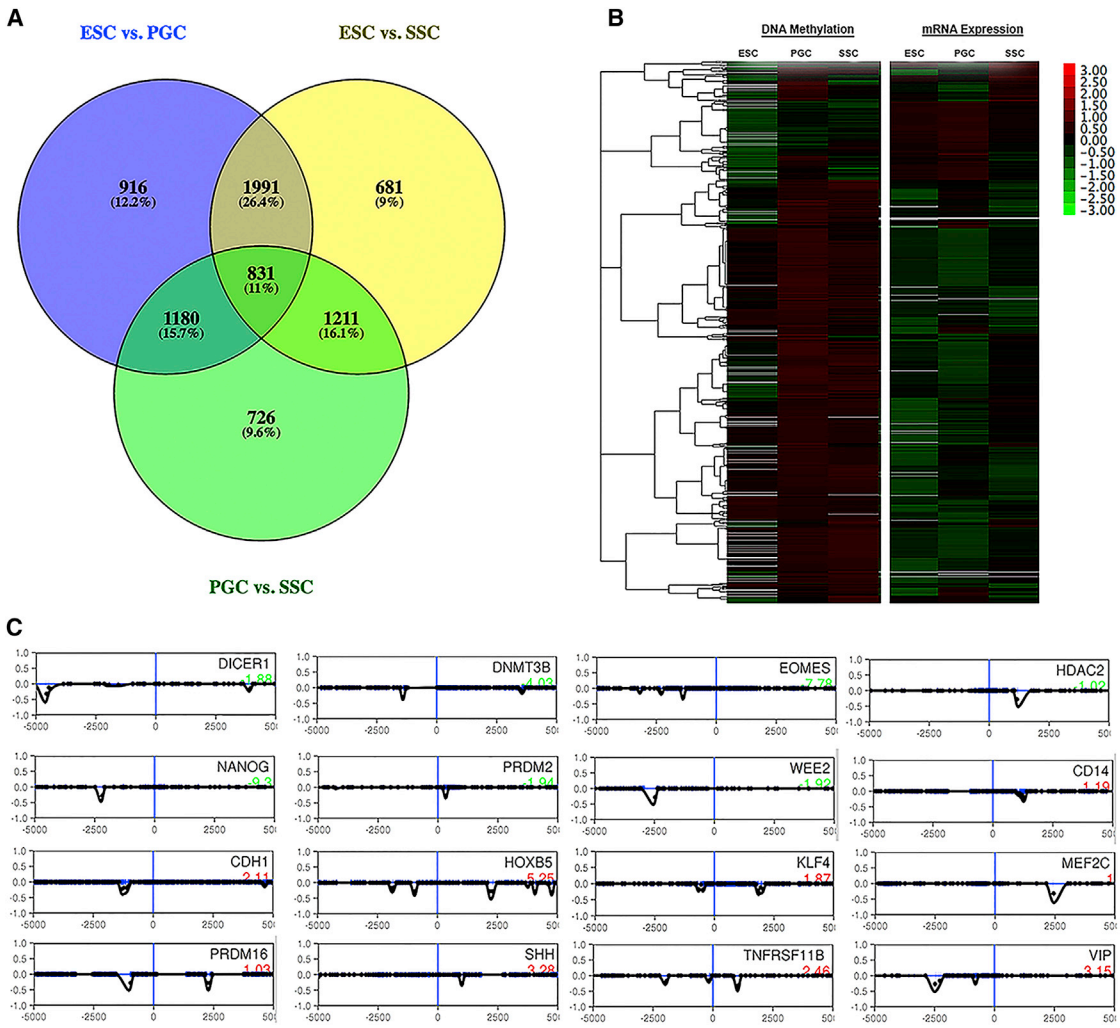

Figure 2. Regulation of DNA Methylation in Stage-Specific Differentially Expressed Genes

(A) At least three biological replicates for each cell type were used to run RNA sequencing (RNA-seq) experiments. Differentially expressed genes among three cell types with the criteria of fold change $\geq$ 2 and false discovery rate $\leq$ 0.01.

(B) DNA methylation and gene expression profiles in 726 unique differentially expressed genes between PGCs and SSCs.

(C) DNA methylation signatures in differentially expressed genes between ESCs and PGCs. Clustering was performed on 10 kb regions relative to the TSS. The y axis represents normalized methylation level and the x axis represents genome position relative to the TSS (0). The number at the lower right corner denotes log2 (gene expression fold change); green indicates downregulation, red indicates upregulation (see Figure S2, Tables 1 and S1–S3).

corresponding expression changes by combining MBD-seq and RNA sequencing (RNA-seq) data (Vanderkraats et al., 2013). Only one significant cluster ($p = 0.017$) showing a pattern of DNA methylation proximal to the TSS was discovered including 661 genes that were differentially expressed between ESCs and PGCs (Figure 2C). The investigation in terms of functions and annotations of these genes also further confirmed that they were enriched in cell growth, cell division, and cell migration processes, as well as cell cycle (Table 1). All the above are related to PGC function as a kind of "transgenerational stem cell" develops from a small population of cells that are specifically set aside in the extra-embryonic compartment very early during embryogenesis. Therefore, a lot of genes participated in cell division and cell migration when ESCs were differentiated into PGCs, and 5′ methylation change of these genes might play crucial roles to regulate their mRNA transcription. Likewise, we uncovered 7 significant clusters of 1,560 genes ($6.03 \times 10^{-13} < p < 0.024$) with same DNA methylation shape for each cluster and mRNA expression change. However, similar DNA methylation signatures were observed from clusters 1, 2, 3, and 4, with loss

Table 1. The Clustering of 661 Genes with 5′ Methylation Change Correlated with Expression Change between ESCs and PGCs with Literature Profiles in Humans, Related to Figure 2 and Table S1

Keyword	Hit	Total	p Value	q Value
Cell growth	162	3,912	2.97×10^{-8}	0.0001
Cluster 1 enrichment score: 5.22				
S phase	61	1,198	1.02×10^{-6}	0.0008
Cell division	57	1,203	3.54×10^{-5}	0.0084
RNAi	91	2,124	1.72×10^{-5}	0.0052
Cluster 2 enrichment score: 4.27				
Cell migration	88	2,072	3.60×10^{-5}	0.0081
Cell adhesion	101	2,496	8.07×10^{-5}	0.014
Protein complex	64	1,431	9.29×10^{-5}	0.0155

Enrichment score: the overall enrichment score for the group based on the p value of each term members. Hit, genes involved in the keywords link to the related abstract; Total, all genes involved in the keywords. p value, chi-squared test p value; q value, corrected p value.

methylation through TSS and CGI shores, while clusters 5, 6, and 7 have other similar methylation signatures with distal loss methylation of TSSs (Figure S2). Pathway analysis of genes from clusters 1, 2, 3, and 4 showed that they participated in the pathways related to the maintenance of cell and tissue structure and function, dorsoventral axis formation, and some cancers (Table S1). However, the genes from clusters 5, 6, and 7 with distal loss methylation involve metabolism pathways and axon growth guidance (Table S2). Therefore, DNA methylation change on TSS and CGI shores (TSS ± 3 kb) might more tend to regulate their gene expression than distal methylation change of genes during germ cell differentiation. Collectively, the results were also in agreement with reports that testicular DNA has eight times the hypomethylated loci, and most of them are generally away from the 5′ regions of genes compared with somatic tissues (Oakes et al., 2007).

DNA Methylation Regulation of Characteristic Genes

As reported that PGC formation depends on the bone morphogenetic protein (BMP) pathway, we found that the expressions of *ALK2* receptor and *SMAD1/5* signaling are activated and committed to developing into PGCs. Shortly thereafter, PGC fate and pluripotency are maintained by some genes, such as *BLIMP1*, *POUV (OCT4)*, *SOX2*, and *NANOG* (Pelosi et al., 2011). In our study, we found that these genes were also differentially expressed among ESCs, PGCs, and SSCs, suggesting that the *BMP* pathway, *SMAD* signaling, the *SOX* family, and *POUV*, as well as *NANOG*, could also be involved in chicken germline stem cell differentiation as they are in humans and mice. To validate and explore the function of the transforming growth factor β (TGF-β)/BMP signaling pathway in the regulation of male germ cell formation in the chicken, we used inhibitors, LY2109761 and LDN193189, to repress the expression of SMAD2 and SMAD5 *in vitro* and *in vivo*. The results indicated that the mRNA expression of *SMAD2* and *SMAD5* in inhibition groups was significantly suppressed compared with the control group during chick germ cell differentiation *in vitro* and *in vivo*; furthermore, their protein expression was consistent with mRNA expression before and after inhibition, while DNA methylation showed a decreasing trend during germ cell differentiation, implying that the TGF-β/BMP4 signaling pathway could promote male germ cell formation and that DNA methylation may regulate this process (see Figure 3).

To comprehensively study DNA methylation regulation on stem cell differentiation, we concentrated on genes associated with human stem cell differentiation and further investigated their methylation status and gene expression in chicken germline stem cells. The results showed that some genes were unmethylated in ESCs with no change of their transcriptions, but these genes could be repressed by DNA methylation when ESCs were differentiated into PGCs. Interestingly, they were activated in SCCs, such as imprinting genes, and related TFs *IGF2*, *KLF4*, and *GDNF* (see Figure S3A, upper panel). Furthermore, some genes with low methylation levels showed high expression through all three cell types, and they participate in pathways in cancers, including colorectal cancer, endometrial cancer, and lung cancer, and also in hepatitis B, which suggested that these genes regulated by DNA methylation might be associated with carcinogenesis in early embryonic development, e.g., *AKT1*, *CCND1*, *MYC*, *CTNNB1*, and *PTEN* (see Figure S3A, lower panel and S3B). However, mRNA transcription of some genes seems not be affected by DNA methylation (see Figure S3A, middle panel). To refine the relationship between DNA methylation and gene expression, we extracted genes showing a correlation between gene expression and DNA methylation at promoter or gene body regions (CGI shores). The results showed an obvious linear correlation between DNA methylation and gene expression such as *Nanog* (Figure S3C). In addition, 31 of the genes (3.2%) related to human stem cell differentiation not only showed linear decrease dependence between their gene expression and DNA methylation but were also found to have significant methylation signatures (Table S3). Therefore, DNA methylation of these genes might directly control their mRNA transcriptions during chicken germline stem cell differentiation.

It is known that X chromosome inactivation (XCI) is a mechanism of dosage compensation that silences the

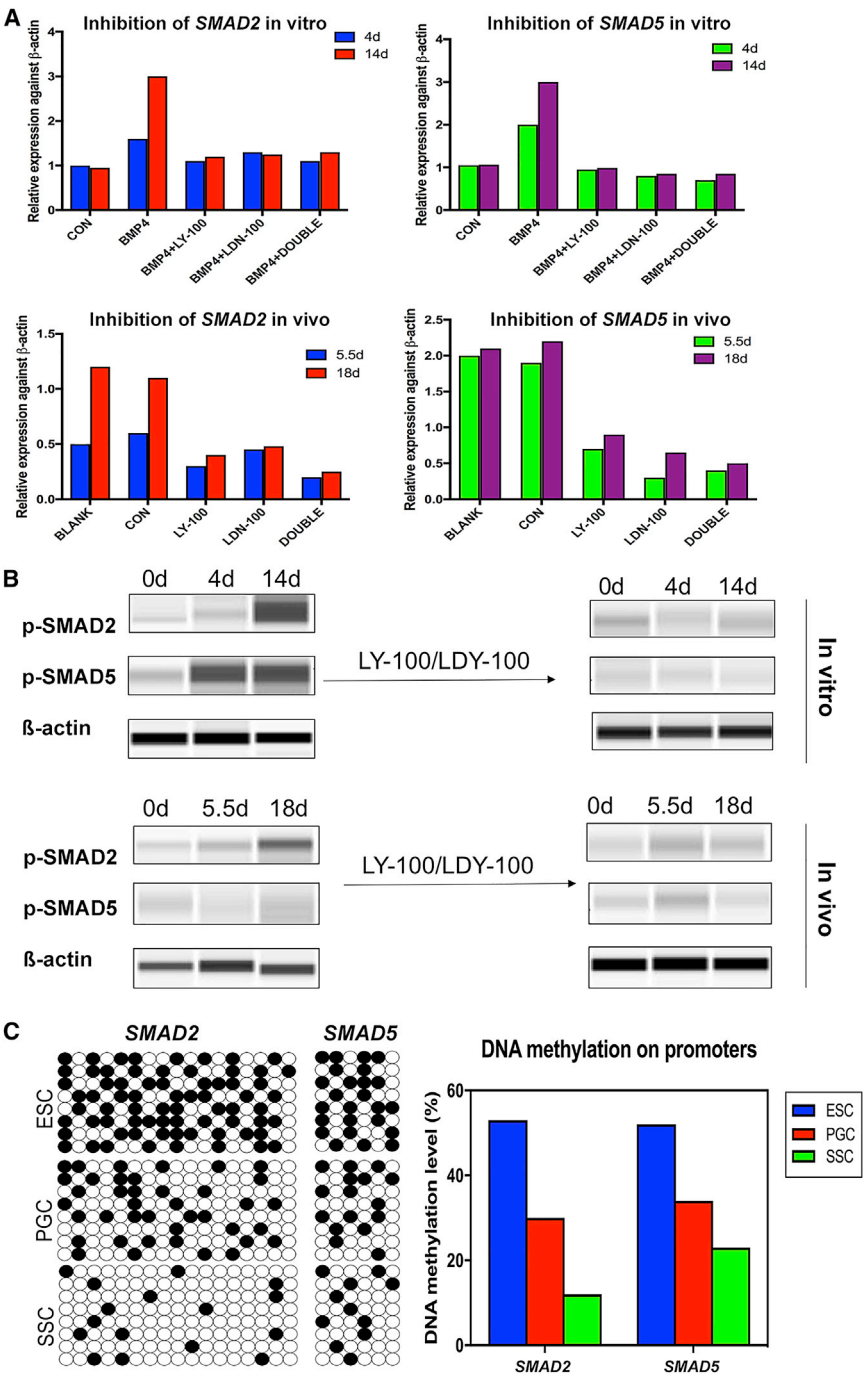

Figure 3. Inhibition of the TGF-β/BMP Signaling Pathway *In Vitro* and *In Vivo*

(A) *SMAD2* and *SMAD5* expression were measured in control and inhibition groups on the differentiation days 4 and 14 *in vitro* with three independent experiments (CON, control group; LY-100, 100 nM of LY2109761; LDN-100, 100 nM of LDN193189). The same procedure was also performed *in vivo* on embryo development days 5.5 and 18.

(B) Western blot was conducted in three cell types with phosphorylated SMAD2 and SMAD5 antibodies against β-actin before and after inhibition *in vitro* and *in vivo* with three independent experiments. p-SMAD2 against SMAD2 (58 kDa); p-SMAD5 against SMAD5 (52 kDa); and β-actin against β-actin (42 kDa).

(C) DNA methylation of *SMAD2* and *SMAD5* on promoter regions was measured by bisulfite cloning sequencing at three stages *in vivo* with three independent experiments. The left panel is DNA methylation status in each clone. White circle, unmethylated CpG; black circle, methylated CpG. The right panel is the statistic result for the left panel (see Figures S3 and S4).

majority of genes on one X chromosome in each female cell (Sharp et al., 2011). In chickens, males are the homogametic sex (ZZ), while females are the heterogametic sex (ZW). The Z chromosome is larger and has more genes, like the X chromosome in the XY system. To reveal whether this event accompanies chicken germ cell differentiation, we investigated DNA methylation distributions on chicken chromosomes and found that DNA methylation densities were higher on chromosome W than on chromosome Z, which was due to their huge difference in chromosome sizes and gene numbers (Figures S4A and S4B). To explore what genes on chromosome Z were methylated and involved in XCI, we profiled DNA methylation enrichment of promoter regions and mRNA expression for all genes on chromosome Z; the results demonstrated that DNA methylation inactivated their gene expression in

PGCs and SSCs, and more genes lost DNA methylation in ESCs at the blastocyst stage; but low mRNA expression in ESCs might be due to other factors (Figure S4C). Overall, mRNA transcription of most genes on chromosome Z was inactivated in chickens, which is consistent within mammals.

Cell-Type-Specific Regulators in Germ Cell Differentiation

In adult animals, spermatogenesis involves a continuous differentiation of the spermatogonial stem and progenitor cell population into mature sperm. A unique aspect of this developmental process is the intensive germ-cell-specific transcription of genes encoding many TFs, often from alternative promoters (Kolthur-Seetharam et al., 2008). However, the TFs for chicken germ cell differentiation remain uncharacterized. Here, we uncovered putative TFs by detecting enriched TF motifs and cell-type-specific regions of DNA methylation, and quantified their activity and specificity on nearby genes (Pinello et al., 2014). Twenty-nine ESC-specific TF motifs were found, and 9 PGC-specific and 25 SSC-specific TF motifs were identified (Table S4). In ESCs, the most significant TF, EWSR1-FLI1 ($q = 4.34 \times 10^{-11}$), which can affect *EGR2* expression, resulting in decreased cell proliferation and tumor growth when *EGR2* is silent (Gomez and Davis, 2015). KLF5 ($q = 9.81 \times 10^{-8}$) is involved in self-renewal of mouse ESCs (Parisi et al., 2008). TF TFAP2C has been reported to be essential for PGC maintenance (Schemmer et al., 2013). Moreover, the HOX family including HOXA5, HOXA9, and HOXC9, were identified in ESCs; unlike *HOX* genes, HOX TFs are usually activated in varying spatial and temporal patterns in the development of ESCs (Seifert et al., 2015). Of them, HOXA5 was studied with regard to involvement in embryo and organ development, and cell proliferation and methylation pathways (Wang et al., 2015). As shown in Figures 4A–4C, downregulation of HOXA5 implies that, during early embryonic development, it commits ESCs into different lineages. Chromatin immunoprecipitation assay (ChIP)-qPCR of HOXA5 on the *GFRA2* gene indicated that HOXA5 can bind the *GFRA2* gene and their affinity would decrease when ESCs were differentiated into PGCs and then SSCs (Figure 4E). Thus, low mRNA expression of the *GFRA2* gene in ESCs might be caused by HOXA5 repression, while decreased affinity or loss of HOXA5 on *GFRA2* gene may result in activation of *GFRA2* in PGCs, but a dramatic reduction of *GFRA2* mRNA expression in SSCs could be caused by high methylation on its promoter instead of HOXA5 suppression (Figures 4D and 4E). In PGCs, TP53 might be involved in the regulation of cell proliferation through DNA methylation. Similarly, tumor protein p63, one of the p53 homologs encoded by the *TP63* gene, was also found in PGCs (Petre-Lazar et al., 2007). Moreover, SREBF1 and SREBF2 in SREBF were identified in PGCs, appearing to have a unique function as determinants of germ-cell-specific gene expression (Wang et al., 2006). In addition, we found that some TFs were associated with somatic testicular cells, e.g., GABPA in SSCs (Chalmel et al., 2012). Furthermore, the SSCs expressed several TFs (Pou5f1, Sox2) required for reprogramming fibroblasts into a pluripotent state, suggesting that a single SSC can acquire pluripotentiality in chicken (Kanatsu-Shinohara et al., 2008). FOXP factors, e.g., FOXP1 and FOXP2 in chicken SSCs, act mainly as transcriptional repressors mediated through interaction with HDAC proteins (Herriges et al., 2012), implying that these TFs might regulate spermatogenesis by histone modification ways.

Long Non-coding RNA and Germ Cell Differentiation

As Figure S4A shows, most DNA methylation was enriched on chromosomes 16 and 25, while DNA methylation enrichment was not high on their genes, demonstrating that DNA methylation mainly occurred in intergenic regions of these two chromosomes. To disclose whether non-coding RNA participates in germ cell differentiation, long intergenic non-coding RNA (lincRNA), one type of non-coding RNA, was identified. In total, there were 5,925 lincRNAs recognized from three cell types. Differentially expressed lincRNAs between different cell types were analyzed, and some of them were also confirmed by qPCR (Figures S5A and S5B). Interestingly, a differentially expressed lincRNA, *MAPKAPK5*, a target gene of p38 mitogen-activated protein kinase (MAPK) signaling in the embryonic gonads in mice (Ewen et al., 2010), locates on the upstream of lincRNA5 (TCONS_00016108) and had an opposite expression profile compared with its neighboring lincRNA5 (Figures 5A and 5B). The Dual-Luciferase Reporter Assay of *MAPKAPK5* and lincRNA5 demonstrated that lincRNA5 can bind the promoter of the *MAPKAPK5* gene. Therefore, lincRNA5 could fractionally bind the promoter of *MAPKAPK5* to repress gene expression in SSCs compared with that in ESCs, while high methylation might block the binding in PGCs and suppress gene expression as an alternative way (Figures 5C and 5D). To explore the expression correlation of lincRNAs and their neighboring genes, 451 differentially expressed genes between two cell types and their neighboring lincRNA were applied for expression correlation analysis (Figure S5C). Our results demonstrated that expression distribution of lincRNAs was, in general, similar to their neighboring gene. For some lincRNAs, their expression directions at two stages were that same as their neighboring genes. However, most lincRNAs seemingly did not correlate with their

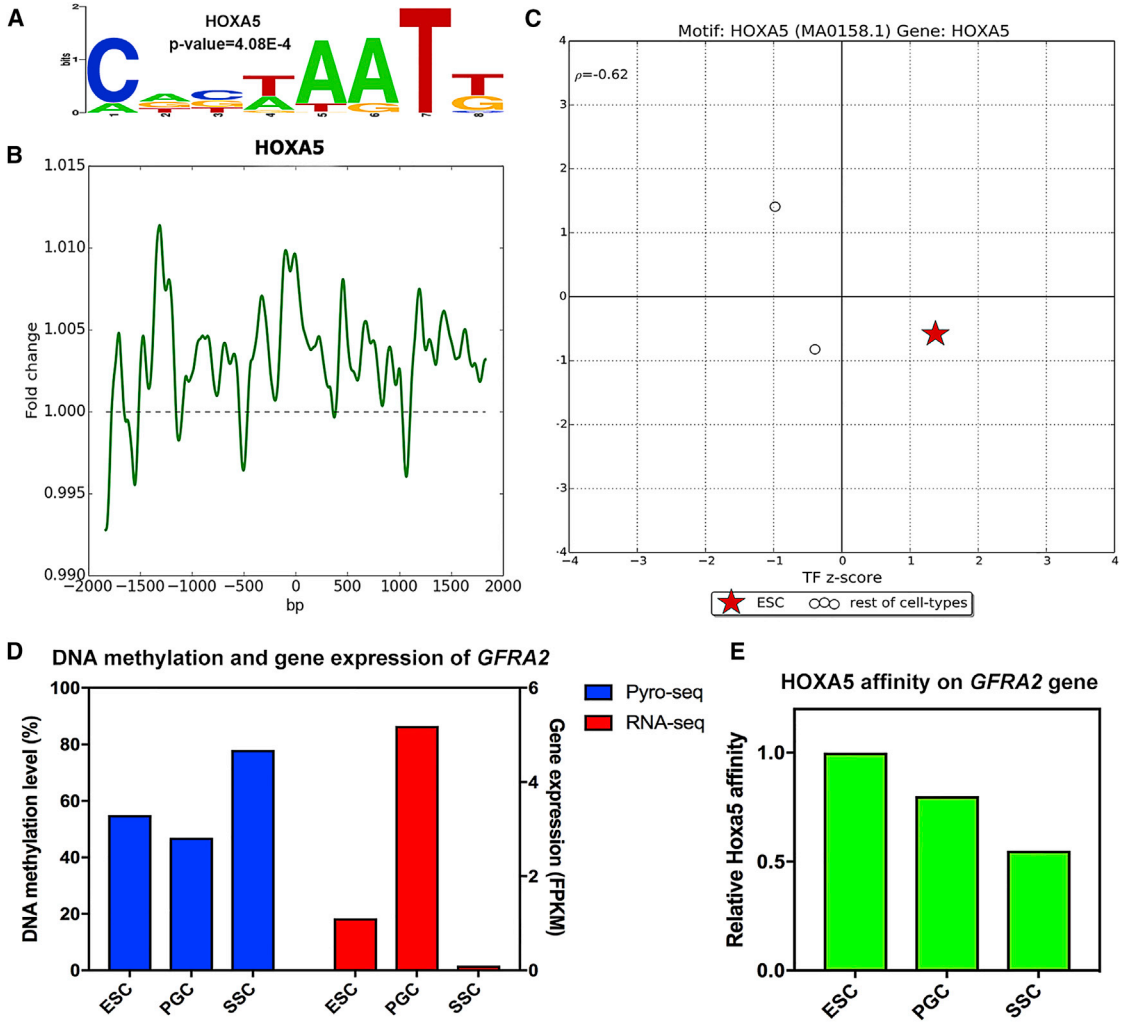

Figure 4. HOXA5 Motif and Its Biological Functions

(A) HOXA5 motif logo. HOXA5 is significantly identified in chicken ESCs ($q = 4.08 \times 10^{-4}$).

(B) Average enrichment profile of HOXA5 motif in cell-type-specific regions with HOXA5 motif hits. The regions above the horizontal black line and with a low q value mean that this TF likely binds these sequences.

(C) TF activity for HOXA5 in ESCs (red star) compared with the other cell types (circles). The ρ value is a correlation value between the expression level of HOXA5 and the expression of the genes nearby. The x axis represents the specificity of the expression level of HOXA5. The TF *Z* score is above 0, which means that HOXA5 is more expressed in ESC cell types than in others. The y axis denotes effects on the gene nearby the regions containing the HOXA5 motif. *Z* score targets, marked with the red star, are below 0, which means that the target genes are downregulated by HOXA5 in ESCs.

(D) DNA methylation level of *GFRA2* promoter region measured by pyrosequencing and normalized mRNA expression of *GFRA2* detected by RNA-seq across three cell types.

(E) HOXA5 affinity on *GFRA2* promoter was measured by ChIP-qPCR with HOXA5 antibody in three cell types with three independent experiments (see Table S4).

neighboring gene expression. Hence, lincRNA as a regulator might coordinate gene expression during germline stem cell differentiation in a sophisticated way.

Because lincRNAs are spatially correlated with TFs, often acting as scaffolds that help localize chromatin-modifying complexes important for gene transcription in *cis* or in *trans* (Ulitsky and Bartel, 2013; Herriges et al., 2014), TF motifs binding to lincRNAs were screened for each cell type: 29 TF motifs, 9 TF motifs, and 14 TF motifs were found to be correlated with lincRNAs in ESC-, PGC-, and SSC-specific cells, respectively (Table S5). Therefore, all TFs previously predicted from ESCs and PGCs could act by lincRNAs, but partial TFs from SSCs could function through lincRNAs and epigenetic mechanisms.

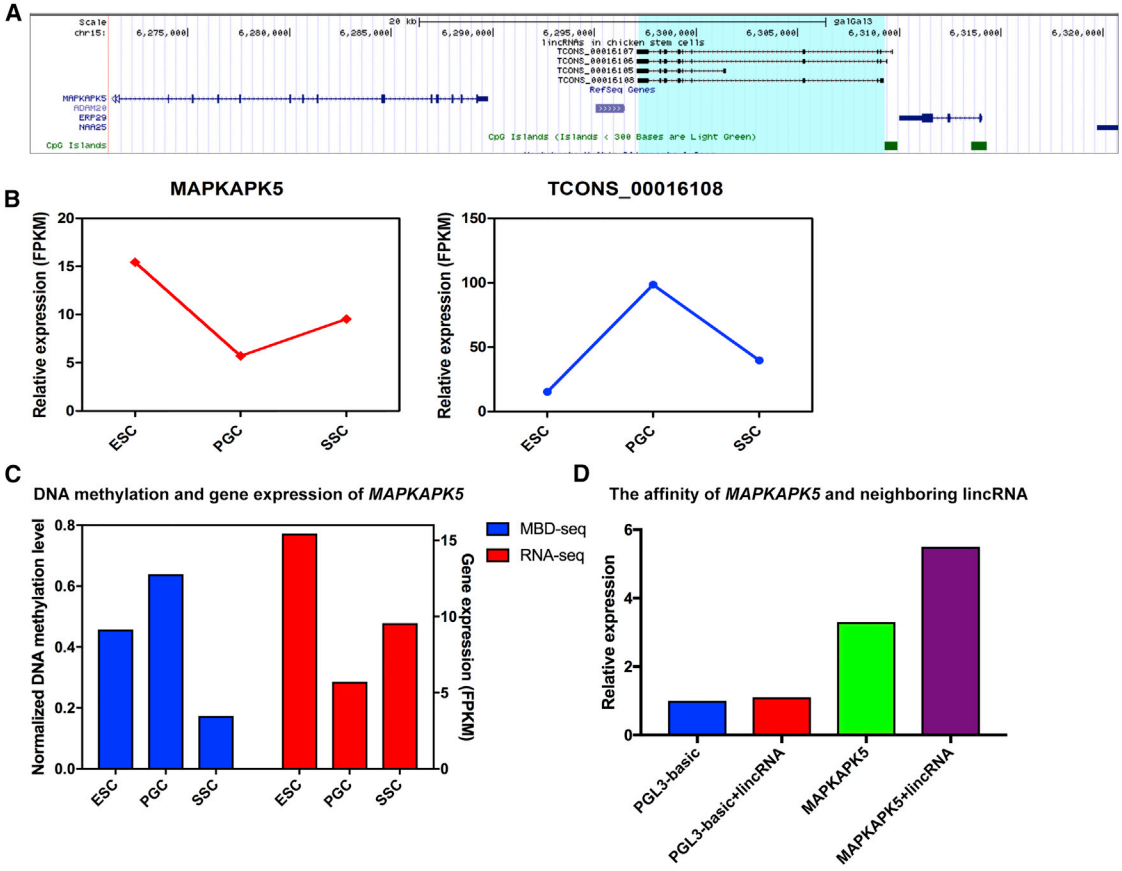

Figure 5. *MAPKAPK5* and Its Neighboring lincRNA *TCONS_00016108*

(A) Genomic location shown for lincRNA *TCONS_00016108* (highlighted) and its neighboring gene *MAPKAPK5*.

(B) Expression levels for *TCONS_00016108* (right) and *MAPKAPK5* (left) are shown across three cell types.

(C) DNA methylation of *MAPKAPK5* promoter measured by MBD-seq and its gene expression measured by RNA-seq.

(D) The affinity of *MAPKAPK5* with lincRNA *TCONS_00016108* was measured by the Dual-Luciferase Reporter Assay with three independent experiments (see Figure S5).

DISCUSSION

While global DNA methylation analyses have been conducted in chickens (Li et al., 2011; Gryzinska et al., 2013), and the roles of DNA methylation in embryos explored (Rocamora and Mezquita, 1989; Jang et al., 2013; Jiao et al., 2013), we reported genome-wide DNA methylation patterns during chicken germline stem cell differentiation in this study. Our results showed that chicken ESCs isolated from blastoderm, the layer of cells forming the wall of the blastocyst, experienced demethylation, while chicken PGCs experienced *de novo* methylation, and SSCs had decreased methylation, which is similar to the patterns observed in the mouse and human (Morgan et al., 2005; Smith et al., 2012; Guo et al., 2014). Moreover, we found that DNA methylation profiles of chicken embryos spanning upstream, TSS, gene body, TES, and downstream of a gene are similar to those of human and mouse embryos, and that the overall DNA methylation level of the gene body was higher than that of neighboring intergenic regions (Lister et al., 2009), indicating that the dynamic changes of DNA methylation are in general universal throughout the entire genome among species. It is known that the process of methylation is catalyzed by three DNA methyltransferases (DNMT1, DNMT3A, and DNMT3B). In our study, *DNMT3A* and *DNMT3B* were expressed much higher in chick ESCs than in PGCs and SSCs, and they predominated in ESCs of female chicks compared with male chicks, which was consistent with previous reports that *DNMT3A* is maternally provided and that they are both expressed in early preimplantation embryos (Okano et al., 1998) (Figure S6A). In addition, *DNMT3B* was more active than *DNMT3A* in ESCs, implying that *DNMT3B* might predominate in earlier embryonic development, which was compatible with the conclusion that the deletion of *DNMT3B* causes embryonic lethality, but

that *DNMT3A* knockouts are partially viable (Okano et al., 1999). However, the expression of *DNMT1* that can sustain genomic methylation status after DNA replication (Arand et al., 2012) was undetected in all three cell types, which might be because *DNMT1* mainly contributes to the cell proliferation in early preimplantation embryos.

So far, it has been apparent that DNA methylation and histone modifications depend on each other. Certain histone methylations cause a readily reversible local formation of heterochromatin, whereas DNA methylation leads to stable long-term repression (Cedar and Bergman, 2009), especially in embryonic germ cells (Jiao et al., 2013). Therefore, a site-specific DNA methylation pattern or other epigenetic marks are likely to participate in the regulation of chick embryo development. In Figure S3C, we found that the transcription of *KDM5B*, which encodes a lysine-specific histone demethylase, was repressed from ESCs to PGCs, but DNA methylation during this process remained stable, suggesting that the transcription of histone demethylase KDM5B might be affected by a histone methylation strategy (Dey et al., 2008; Xie et al., 2011). To investigate the interaction between DNA methylation and histone methylation, we combined sequencing data of H3K4me3 and H3K27me3 (GEO: GSE65961) to check the enrichment of DNA methylation on histone methylation regions and lincRNA regions across three kinds of germ cells (Figure S6B). The results indicated that DNA methylation was more enriched on H3K27me3 regions than on H3K4me3 regions, which was in agreement with the conclusion that DNA methylation is a "repressed" switch and that H3K27me3 is a "repressed" mark to gene transcription. Moreover, there were more overlaps between DNA methylation and histone methylation in PGCs than in ESCs and SSCs, further confirming that DNA methylation and histone modifications are dependent on each other in individual development. It is noted that DNA methylation was enriched much less in lincRNA regions compared with histone methylation regions, demonstrating that DNA methylation has more interaction with histone methylation than with lincRNAs. To check the relationship between lincRNAs and histone modifications, lincRNA enrichment scores were calculated in two histone methylation marks, and the results showed that lincRNAs were more enriched in H3K27me3 regions than in H3K4me3, and that lincRNA enrichment on the H3K27me3 mark was about six times more than DNA methylation enrichment on the H3K27me3 mark (see Figures S6B and S6C), which implied that lincRNAs might prefer to interact with repressed histone marks to depress gene expression, and that lincRNAs are likely to be involved in more events of histone modifications compared with DNA methylation, which is consistent with the fact that some lincRNAs contain multiple binding sites for distinct protein complexes that direct specific combinations of histone modifications on target gene chromatin (Tsai et al., 2010). In our study, 36 SSC gene markers were detected in the chicken and they showed cell-type-specific expression during chick germ cell differentiation, which is similar within the human and mouse, suggesting that these markers could be universal gene makers to identify male germ cells (Table S6). Chicken-unique SSC gene markers (121) were uncovered, and their expression change could be controlled by DNA methylation (Table S7).

In terms of similar epigenetic discoveries of male germ cell differentiation in different organisms, we explored their shared mechanisms (Figure 6). Interestingly, we found that increased DNA methylation on promoter regions or CGI shores would repress gene expression or adjust their expression in a dose-dependent manner. For example, decreased DNA methylation for *BCL2* and *CSF3R* genes from ESCs to PGCs activated their mRNA transcription in PGCs, and continual decrease from PGCs to SSCs caused their expression to be higher in SSCs. However, for the *IGF2R* gene, tremendously increased methylation at the stage of ESCs to PGCs turned off its mRNA transcription in PGCs, but the subsequently decreased methylation caused this gene to be fractionally expressed in SSCs (Figure 6A). We also found that DNA methylation at gene promoters has a negative effect on certain TFs. In our study, HOXA5 was identified from chick ESCs and it downregulates its target genes. As Figures 4D and 4E show, *GFRA2* as a target gene of HOXA5 had a strong affinity in ESCs, suppressing *GFRA2* gene transcription in ESCs. However, its affinity with HOXA5 decreased in PGCs, accompanying a mild methylation change in the promoter region, which released *GFRA2* and caused it to reach a high expression in PGCs. In SSCs, increasing methylation of the *GFRA2* gene blocked HOXA5 binding to the promoter region and extremely repressed gene expression in SSCs in an alternative way (Figure 6B). *GFRA2* involves stem cell differentiation with stem markers (Garcia-Lavandeira et al., 2009; Santiago et al., 2014), implying that HOXA5 and its target genes play vital roles in chicken male germ cell differentiation. Most interestingly, lincRNAs may bind to gene promoters. For the *MAPKAPK5* gene, extremely increased methylation on its promoter region when ESCs were differentiated into PGCs made its expression very low, even silent, in PGC cells. When PGCs became SSCs, the methylation was dramatically removed for a neighboring lincRNA binding, which could repress *MAPKAPK5* transcription instead of DNA methylation (see Figures 5 and 6C). Here, we consider lincRNAs acting as scaffolds for RNA-binding proteins recruiting chromatin-modifying complexes. For *CDX2* gene, there was no DNA methylation change across all three chicken germ cell types, but we identified STAT1 and TFAP2c TFs in ESCs, and they can be assembled by a

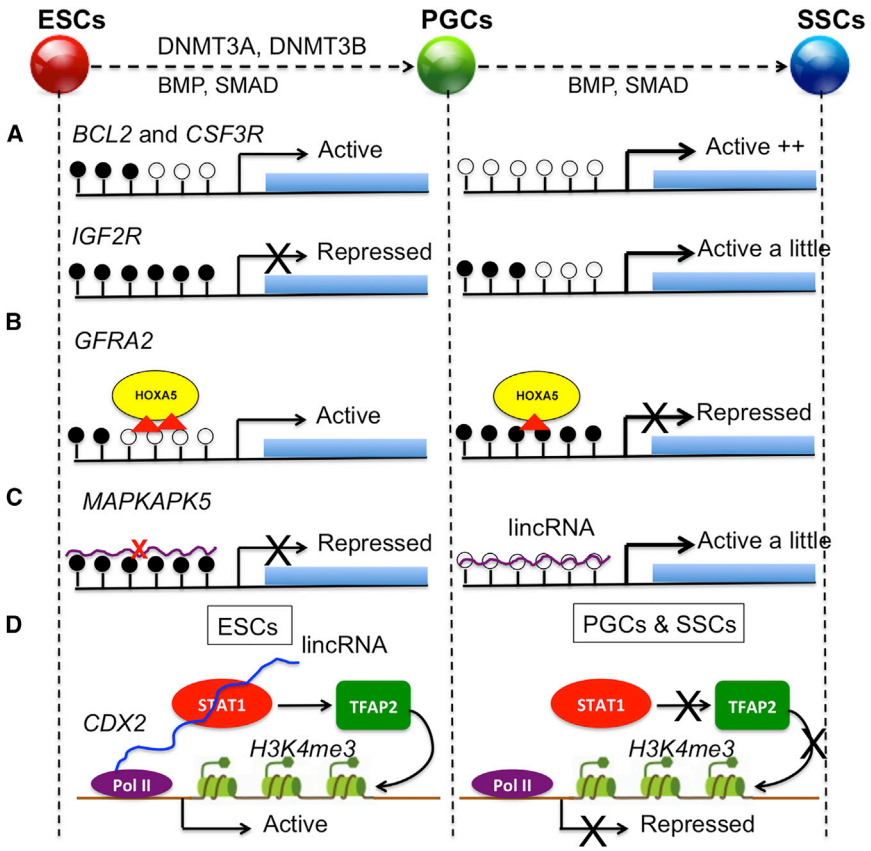

Figure 6. A Schematic Representation of the Epigenetic Mechanisms and a Summary of Major Changes that occur in Chick Germline Stem Cells

(A) Hypermethylation of DNA silences gene expression and hypomethylation activates gene expression.

(B) The interaction of TFs and DNA methylation changes gene expression.

(C) lncRNAs repress nearby gene expression in *cis*.

(D) lncRNAs act as scaffolds for assembling RNA-binding TFs to recruit chromatin-modifying complexes for regulating gene expression in specific cell lineage. White circle, unmethylated CpG; black circle, methylated CpG (see Figure S6 and Table S5).

lincRNA to form a complex with RNA polymerase II. The expression of TFAP2c was induced by STAT1 in ESCs, and then caused TFAP2c to act on active histone mark H3K4me3, consequently upregulating *CDX2* expression. However, there were no STAT1 and TFAP2c found in PGCs and SSCs, so the loss of functions of H3K4me3 caused the *CDX2* gene to be repressed (Figure 6D). In addition, methyltransferases DNMT3A and DNMT3B, BMP pathway, and SMAD signaling are also involved in chicken male germ cell differentiation (Figures 3 and 6). Taken together, multiple epigenetic events, including DNA methylation, histone modifications, and non-coding RNAs, may act synergistically instead of single regulation mode during embryonic development, and this kind of regulation mode owns typical cell lineage specification.

Conclusions

In summary, our study provides a comprehensive atlas at the genome-wide scale of the DNA methylation landscape in chicken germline stem cells; transcriptomic dynamics is also presented. Universal gene markers and unique chicken markers were discovered for identifying male germline stem cells. Moreover, the integrated epigenetic mechanisms were explored during chicken male germ cell differentiation, which will help us understand the epigenetic processes associated with male germ cell differentiation and possibly improve treatment options for male infertility in animals and humans.

EXPERIMENTAL PROCEDURES

Full experimental methods are provided within Supplemental Experimental Procedures.

Sample Collection

All eggs were immediately collected for isolation of three kinds of germline stem cells (ESCs, PGCs, and SSCs) after fertilization in the National Poultry Institute at the Chinese Academy of Agricultural Sciences. All procedures involving the care and use of animals conformed to U.S. National Institute of Health guidelines (NIH Publication No. 85-23, revised 1996), and were approved by the Laboratory Animal Management and Experimental Animal Ethics Committee of Yangzhou University.

MBD-Seq and RNA-Seq

Genomic DNA from ESCs, PGCs, and SSCs was extracted for performing MBD-seq with two biological replicates per cell type. Total RNA from three cell types was prepared with multiple biological replicates. All sequencing libraries were analyzed on the Illumina HiSeq 2000 Analyzer following manufacturer protocols.

Bioinformatics Analysis

All sequencing data were evaluated and trimmed off for high-quality assurance, and then aligned to the galGal4 reference genome by bowtie v.1.1.1 for MBD-seq data and TopHat v.2.0.9 for RNA-seq data. For data manipulation, filtration, and format conversion, a combination of procedures available in SAMtools and BEDtools was applied. Peaks of DNA methylation were called using MACS1.4.2, and the following DMRs were identified by DiffBind R package with an edgeR analysis. Mapped RNA-seq reads were assembled and analyzed by cufflinks v.2.1.1 series, and, finally, normalized gene expression was output as FPKM (fragments per kilobase of transcript per million mapped reads). The differentially expressed genes were filtered out by particular criteria. lincRNAs were identified from RNA-seq data with a robust pipeline developed by our lab (He et al., 2016). HAYSTACK pipeline was used to identify cell-type-specific TF motifs with DNA methylation data and quantify their activity on nearby genes. To uncover functional genes with differential methylation patterns associated with expression change of these genes, WIMSi was applied to identify groups of genes with similarly shaped methylation signatures and corresponding expression changes based on MBD-seq and RNA-seq data. Our previous sequencing data in terms of H3K4me3 and H3K27me3 (GEO: GSE65961) were also introduced for integration analysis.

Validation Experiments

A few DMRs were selected and validated by bisulfite cloning sequencing as well as DNA methylation of *SMAD2* and *SMAD5* on promoters. The results were analyzed by QUMA (http://quma.cdb.riken.jp), and DNA methylation levels for each region and group were obtained. DNA methylation of some genes involved in Figure 6 was confirmed using bisulfite pyrosequencing technology. Real-time PCR using iQ SYBR Green Supermix was utilized to validate differentially expressed lincRNAs between cell types.

To explore the function of the TGF-β/BMP signaling pathway in the regulation of male germ cell formation, TGF-β signaling pathway-specific inhibitors, LY-100 and LDN-100, were added to inhibit Smad2 and Smad5 expression *in vitro* and *in vivo*. qRT-PCR was performed to evaluate the inhibition efficiency of TGF-β signaling. The phosphorylated SMAD protein levels were identified by western blotting before and after inhibition *in vitro* and *in vivo* with triple biological replicates. ChIP was performed with HOXA5 antibody in three cell types, and a subsequent qPCR was applied for measuring HOXA5 affinity on *GFRA2* promoter through all cell types. To validate whether the *MAPKAPK5* gene is bound to its neighboring lincRNA, the Dual-Luciferase Reporter Assay was conducted in the T293 cell line to avoid the effects of chicken endogenous genes. The *MAPKAPK5* reporter gene vector and the lincRNA overexpression vector were constructed and transfected in 293T cells. Finally, luciferase expression indicated the binding of lincRNA to the cloned *MAPKAPK5* target sequence.

ACCESSION NUMBERS

Sequencing data are available in the NCBI SRA database. SRA: SRR3720923, SRR3720924, and SRR3720925 for transcriptome sequencing data of ESCs, PGCs, and SSCs. SRA: SRR6967724, SRR6967725, SRR6967726, SRR6927727, SRR6967728, and SRR6967729 for DNA methylation sequencing data of three kinds of stem cells.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and seven tables and can be found with this article online at https://doi.org/10.1016/j.stemcr.2018.03.018.

AUTHOR CONTRIBUTIONS

J.S. and Y.H. conceived and designed the experiments. Q.Z. and B.L. provided chickens and performed cell isolation and determination. Y.H. performed a series of MBD-seq experiments. Q.Z. and B.L. performed RNA-seq-related experiments. Y.H., J.E., J.L., Q.N., and W.C. conducted the data analysis. B.L., K.Z., and J.S. participated in the explanation of results. Y.H. and J.S. drafted the manuscript. All authors read and approved the final manuscript.

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