Crucial Genes and Pathways in Chicken Germ Stem Cell Differentiation

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Background: Germ cells are critical for any species that multiplies through sexual reproduction.

Results: We found 173 candidate key genes and 18 key signaling pathways that are differentially activated.

Conclusion: Our results showed the crucial genes and pathways involved in the regulation of chicken male germ cell differentiation.

Significance: This study narrows the range of functional genes and pathways during ESC differentiation.

Male germ cell differentiation is a subtle and complex regulatory process. Currently, its regulatory mechanism is still not fully understood. In our experiment, we performed the first comprehensive genome and transcriptome-wide analyses of the crucial genes and signaling pathways in three kinds of crucial cells (embryonic stem cells, primordial germ cell, and spermatogonial stem cells) that are associated with the male germ cell differentiation. We identified thousands of differentially expressed genes in this process, and from these we chose 173 candidate genes, of which 98 genes were involved in cell differentiation, 19 were involved in the metabolic process, and 56 were involved in the differentiation and metabolic processes, like GAL9, AMH, PLK1, and PSMD7 and so on. In addition, we found that 18 key signaling pathways were involved mainly in cell proliferation, differentiation, and signal transduction processes like TGF-β, Notch, and Jak-STAT. Further exploration found that the candidate gene expression patterns were the same between in vitro induction experiments and transcriptome results. Our results yield clues to the mechanistic basis of male germ cell differentiation and provide an important reference for further studies.

The germ cell holds a singular fascination for cellular, reproductive, and developmental biologists because it is the only cell type that can penetrate from one generation to the next generation. Germ cells, without a doubt, are critical for any species that reproduce through sexual reproduction. It is important for fundamental research to understand the details of development and growth of the underlying germ line cells. The germ cell is an important cell type in which either gene expression and/or suppression were regulated temporally and spatially during embryonic development, according to gene expression switching triggered by interaction with the environment.

However, there were few reports about a transcriptome study of the germ stem cell in the chicken, especially in the early embryonic developmental stages because of technical difficulties for collecting early embryonic germ cells. Several previous studies have been reported finding some regulators (genes and/or pathways) that control the process of germ stem cell specification and differentiation. Saitou et al. (1) found that Ifitm3, Nanos2, Stella, Dppa4, Dnmt3l, and Piwi2 were involved in the early differentiation of germ cells. Blimp1/Prdm1 played an important role in the early stages of embryonic PGCs specialization. Genetic lineage tracing confirmed that almost all Blimp1 positive cells in early embryonic developmental stages would be eventually developed into Stella positive PGCs. BMP signal from the embryonic ectoderm can induce the two key regulatory genes (Blimp1 and Prdm14) that are responsible for the PGC specialization (2). Dazl (deleted in azoosperma) is a major controlling gene of mouse germ cell differentiation, and its expression promotes ESC differentiation to gametes in vitro (3). Dann et al. (4) used shRNA to inhibit Pou5f1, which resulted in the cloning reduction of the recipient mice SSCs after transplantation.

Ewen and Koopman (5) reported that Kit/KitL, FGFs pathways, and LIF cytokine factor have a positive regulatory role in proliferation and survival of PGCs, but TGFβ-activin/nodal signal has an inhibitory effect on PGCs proliferation. Saitou et al. (6) found that Wnt3a can affect BMP signaling pathways, also the ERK, MAPK, PI3K/AKT, Smad, and hedgehog signaling pathways were involved in the process of germ cell development. Rao (7) reported that basic FGF with tyrosine kinase receptor can activate multiple intracellular signaling pathways such as Ras/raf/mek, p38/MAPK, PKC, and PI3K pathways that are required for mammalian SSCs self-renewal and develop-
These findings suggested that there are some genes and pathways that may be responsible for investigation of germ cell development and differentiation, but its regulatory mechanism was not fully understood until now.

Here, we analyzed all the gene expression patterns of the three kinds of chicken stem cells throughout the whole genome. We identified thousands of differentially expressed genes (DEGs) in this process, and from these we chose 173 candidate genes, including 98 genes involved in cell differentiation, 19 involved in the metabolic process, 56 genes involved in the differentiation and metabolic processes, like GAL9, AMH, PLK1, PSMD7, and so on. In addition, we found that there were 18 key signaling pathways mainly involved in cell proliferation, differentiation, and signal transduction process like TGF-β, Notch, and Jak-STAT. Further exploration found that the candidate gene expression patterns were the same between in vitro induction experiments and transcriptome results. Our results yield clues to the mechanistic basis of male germ cell differentiation and provide an important reference for further studies.

Experimental Procedures

Samples—Procedures involving animals and their care were confirmed according to the U.S. National Institute of Health guidelines (publication no. 85-23, revised 1996) and approved by the laboratory animal management and experimental animal ethics committee of Yangzhou University.

This experiment was done using 18,340 freshly fertilized eggs of Suqin yellow chicken (Gallus gallus domesticus) that were obtained from Poultry Institute, Chinese Academy of Agricultural Sciences (Yangzhou, China). There were 10,540 (4,845 male and 4,854 female; lost 841) eggs at stage X used for the isolation of ESCs. PGCs were isolated from gonads of 3,400 eggs (1,594 male and 1,556 female; lost 250) that incubated for 72 h (stage 27) at 37 °C with 60% relative humidity, while 4,400 eggs were incubated for 18 days to isolate SSCs from the testis. The sex of the cells was determined using PCR, and then the cells with the same sex in each stage were collected for further experiments. Each experiment was repeated three times.

FACS Sorting of ESCs, PGCs, and SSCs and RNA Extraction—Different cell surface markers were used to isolate different types of cells by the FACS. SSEA-1 and SOX2 were used for...
Genes/Pathways in Chicken Germ Stem Cell Differentiation

TABLE 1
DEGs related to |logFC| > 10 in ESCs versus PGCs of microarray
LHRH, luteinizing hormone-releasing hormone; PRL, prolactin.

| Probe name | Log FC (ESCs vs. PGCs) | Regulation | Gene symbol | Function |
|------------|------------------------|------------|-------------|----------|
| A_87_P054786 | 16.293575 | Up | GAL10 | A hypothalamic-hypophysiotropic hormone and a neuromodulator of LHRH secretion; action via inhibition of dopaminergic tone |
| A_87_P078616 | 15.038605 | Up | NPHS2 | Essential for the integrity of the glomerular filter and the associations of podocin with specialized lipid raft microdomains of the plasma membrane |
| A_87_P054986 | 13.116768 | Up | HBZ | Activates erythroblast-specific, globin gene expression |
| A_87_P274543 | 12.136964 | Up | HBG2 | Gamma chains make up the fetal hemoglobin F, in combination with alpha chains |
| A_87_P009555 | 12.067309 | Up | HBA4A | A liver-specific protein that is thought to be linked to hepatocyte growth |
| A_87_P009368 | 11.491803 | Up | LECT2 | A hypothalamic-hypophysiotropic hormone and a neuromodulator of LHRH secretion and action |
| A_87_P035104 | 11.244352 | Up | GAL9 | Controls lateral line cell migration |
| A_87_P132478 | 10.859593 | Down | OTX2 | A key regulatory gene in photoreceptor cell development |
| A_87_P037684 | 10.3792095 | Down | LUM | Interacts with collagen and limits growth of fibrils in diameter |
| A_87_P108903 | 10.637115 | Up | COL8A1 | A major component of the hexagonal lattice in the Descemet membrane |
| A_87_P146353 | 10.3792095 | Up | LUM | A major component of the hexagonal lattice in the Descemet membrane |
| A_87_P014692 | 10.170226 | Up | ACTA2 | Important regulators of smooth muscle cell differentiation |
| A_87_P132478 | 10.170226 | Up | ACTA2 | Important regulators of smooth muscle cell differentiation |
| A_87_P11002 | 10.933807 | Up | SLCO1A2 | Prevents the development of the Müllerian ducts into the uterus and other Müllerian structures, regulate production of sex hormones |
| A_87_P152088 | 10.653027 | Up | GADD153 | The association of podocin with specialized lipid raft microdomains of the plasma membrane |
| A_87_P054986 | 10.585939 | Up | HBZ | The zeta-globin polypeptide is synthesized in the yolk sac of the early embryo, while alpha-globin was a prerequisite for recruitment of nephron into rafts |
| A_87_P008801 | 10.369993 | Up | AMH | Important regulators of smooth muscle cell differentiation |
| A_87_P260443 | 10.933807 | Up | SLCO1A2 | Prevents the development of the Müllerian ducts into the uterus and other Müllerian structures, regulate production of sex hormones |
| A_87_P150703 | 10.657406 | Down | ATP5A1 | It mediates transport of estosterone-sulfate and, more weakly, prostaglandin E2. |
| A_87_P078616 | 10.859593 | Down | NPHS2 | The association of podocin with specialized lipid raft microdomains of the plasma membrane |
| A_87_P054986 | 10.585939 | Up | HBZ | The zeta-globin polypeptide is synthesized in the yolk sac of the early embryo, while alpha-globin was a prerequisite for recruitment of nephron into rafts |
| A_87_P058761 | 10.45761 | Down | HINTW | Prevents the development of the Müllerian ducts into the uterus and other Müllerian structures, regulate production of sex hormones |

TABLE 2
DEGs related to |logFC| > 10 in PGCs versus SSCs of microarray
LHRH, luteinizing hormone-releasing hormone.

| Probe name | Log FC (PGCs vs. SSCs) | Regulation | Gene symbol | Function |
|------------|------------------------|------------|-------------|----------|
| A_87_P008801 | 13.369993 | Up | AMH | Prevents the development of the Müllerian ducts into the uterus and other Müllerian structures, regulate production of sex hormones |
| A_87_P260443 | 10.933807 | Up | SLCO1A2 | It mediates transport of estosterone-sulfate and, more weakly, prostaglandin E2. |
| A_87_P150703 | 10.657406 | Down | ATP5A1 | The association of podocin with specialized lipid raft microdomains of the plasma membrane |
| A_87_P078616 | 10.859593 | Down | NPHS2 | The association of podocin with specialized lipid raft microdomains of the plasma membrane |
| A_87_P054986 | 10.585939 | Up | HBZ | The zeta-globin polypeptide is synthesized in the yolk sac of the early embryo, while alpha-globin was a prerequisite for recruitment of nephron into rafts |
| A_87_P058761 | 10.45761 | Down | HINTW | Prevents the development of the Müllerian ducts into the uterus and other Müllerian structures, regulate production of sex hormones |

ESCs, SSEA-1 and c-KIT were used for PGCs, and Integrinα6 and Integrinβ1 were used for SSCs isolation. Total RNA was extracted by TRIzol (Invitrogen), and its quality was evaluated with Nanodrop 2000 before the microarray and Illumina RNA sequencing assays.

Microarray and RNA-seq Assays—RNA Libraries pools of the three kinds of cells were established following the protocols of the Agilent microarray and Illumina mRNA-seq with 50 ng of RNA, and the experiments were performed in the Oebiotech Company.

Data Analysis—Filtering and quality control checks of the raw reads from RNA-seq had been done by FastQC. The clean reads were mapped to reference sequences using SOAP2 aligner. The gene expression levels were calculated using RPKM method (reads per kb transcriptome per million reads). GO and pathway analyses of DEGs based on DAVID, FunNet, and WEGO databases were performed to analyze the regulating network of the candidate key genes.

Quantitative Real Time PCR (qRT-PCR)—Microarray and RNA-seq results were validated by quantitative real time PCR. One microgram of RNA was reverse transcribed to cDNA using the Takara reverse transcriptase Moloney murine leukemia virus (RNase H-) (Takara, Dalian, China). Quantitative real time PCR was performed on ABI PRISM 7500 HT sequence detection system (Applied Biosystems, Carlsbad, CA). Cycle number values were normalized against two housekeeping genes, β-Actin and GAPDH.
**In Vitro Induced Differentiation**—For further confirmation of the chosen key genes involved in the regulation of the male germ cell differentiation, several molecules including retinoic acid (RA), BMP4 (bone morphogenic protein 4), testosterone, and follicle-stimulating hormone were used to induce the differentiation of chicken ESCs toward male germ cells in vitro. The third generation of ESCs were seeded into the 24-well plate with supporting feeder cells with the density of $10^5$ cells/well. RA was added to the medium at a final concentration of $10^{-5}$ mol/liter, BMP4 with a final concentration of 40 ng/ml, testosterone with a final concentration of 15 ng/ml, and follicle-stimulating hormone with a final concentration of 25 ng/mg. The cells were collected every 2 days after incubation, and qRT-PCR was used to identify ESCs, PGCs, and SSCs by detecting the specific candidate gene markers and also to detect the key genes expression levels.

**Data Access**—Sequencing reads are available in the NCBI Sequence GEO accession numbers under accession number GSE57213.

**Results**

**Cell Sorting and Culture**—The purity of FAC-sorted ESCs, PGCs, and SSCs was demonstrated as shown in Fig. 1a as according to the results, 0.88% ESCs were SSEA1 and SOX2.
positive, 0.71% PGCs were SSEA1- and C-kit-positive, 2.43% SSCs were integrin α6- and integrin β1-positive, respectively. After FACS enrichment, the morphology of the three types of cells were shown in Fig. 1b. The ESCs were small and became bird nest-like clones after culture by 5–7 days, the PGCs were bigger and became mulberry-like clones after culture for 2–4 days, and the SSCs also were bigger than ESCs and became grape-like clones after culture for 5–6 days.

Analysis of Differentially Expressed Genes in Microarray Assay—Through microarray data difference analysis using $|\log_{10} FC| \geq 1$ as a standard for differential gene screening, there were 20,087 DEGs in the ESC versus the PGC group: 20,020 DEGs and 17,090 DEGs in the ESC versus the SSC group and PGC versus the SSC groups, respectively (Fig. 2). Concerning the up-regulated DEGs, there were 17 genes with more than 10-fold expression change in the ESC versus the PGC group, 33 genes in the ESC versus the SSC group, and 4 genes in the PGC versus the SSC group. In the down-regulated DEGs, there were 3, 13, and 11 genes detected in ESCs versus PGCs, ESCs versus SSCs, and PGCs versus SSCs, respectively. Most of DEGs belonged to the $|\log_{10} FC| \leq 4$ and $|\log_{10} FC| \leq 2$ groups, and only a few DEGs were related to the $|\log_{10} FC| > 10$ group, including AMH, HOXD8, GAL10, GAL9, and so on (Tables 1–3).

Gene Ontology (GO) analysis of these DEGs showed that more than 30% of DEGs related to the regulation of transcription according to the biological processes classification and more than 30% of DEGs belonged to cell nucleus in the cellular component classification. The molecular function assessment of these DEGs revealed that more than 40% of DEGs associated with ATP binding, nucleotide binding, and metal ion binding.

KEGG pathway assay for DEGs indicated that 11 signaling pathways were significantly enriched in the ESC versus the PGC group with enrichment in the MAPK signaling pathway (28.5%) and the focal adhesion pathway (27.2%) (Fig. 3A), although in the ESC versus the SSC group, 11 signaling pathways were significantly enriched with enrichment in the focal adhesion pathway (30%) and cell adhesion attached molecule pathway (16.1%) (Fig. 3B). In PGCs versus SSCs, in the other hand, 12 signaling pathways were enriched, and the most significantly enriched pathways were the focal adhesion (18.2%) and the cytoskeleton regulation (17.8%) (Fig. 3C).

Analysis of Differentially Expressed Genes in RNA-seq Assay—The RNA-seq results indicated that there were 7,697 DEGs in the ESC versus the PGC group, 7,868 DEGs in the ESC versus the SSC group, and 123 DEGs in PGCs versus SSCs.
TABLE 4
DEGs related to [log2] > 10 in ESCs versus PGCs of RNA-seq
FDR, false discovery rate; HPC, hematopoietic progenitor cell; MSCs, mesenchymal stem cells.

| Gene identification | Description | log2 (ESCs vs. PGCs) | Regulation | p value | FDR | Function | Reference |
|---------------------|-------------|----------------------|------------|---------|-----|----------|-----------|
| NM_205335.2         | TTR         | 11.8334779           | Up         | 1.94E-211 | 1.13E-210 | Initiates myoblast differentiation | Ref. 52 |
| NM_206989.1         | LIT2B       | 10.86825931          | Up         | 5.91E-170 | 3.76E-169 | Induces phenotypic differentiation, migration, and collagen synthesis | Ref. 53 |
| XM_001236998.1      | LOC777548   | 10.76811631          | Up         | 5.96E-17  | 1.29E-16  | Restricted to the early phases of HPC differentiation with down-modulation at intermediate/late stages of maturation | Ref. 57 |
| XM_426327.2         | ENPEP       | 10.7588003           | Up         | 0        | 0   | Contribute to the development of renal and hypertensive disorders | Ref. 54 |
| NM_001001611.2      | GAL9        | 10.45797343          | Up         | 9.20E-85  | 3.75E-84  | Induction of differentiation of MSCs into chondrocytes | Ref. 55 |
| XM_414212.2         | LOC15852    | 10.08034791          | Up         | 5.30E-50  | 1.67E-49  | Regulating the responsiveness of cells to adrenal androgens | Ref. 56 |
| XM_414795.2         | LOC98026    | 10.0212089           | Down       | 0        | 0   | Required for normal fertility and fecundity | Ref. 57 |
| XM_430154.2         | LOC24460    | 10.12099411          | Down       | 0        | 0   | Plays an essential role in host defense | Ref. 58 |
| XM_416906.2         | HISTII2AH   | 10.42067022          | Down       | 5.27E-77  | 2.03E-76  | Responsible for the nucleosome structure of the chromosomal fiber in eukaryotes | Ref. 59 |
| XM_001232474.2      | BPIF2       | 10.52057903          | Down       | 0        | 0   | Plays a role during avian skin and feather development | Ref. 60 |
| XM_001231344.1      | LOC768589   | 10.34284209          | Down       | 3.90E-115 | 1.91E-114 | Prevents apoptotic cell death | Ref. 61 |
| NM_204675.1         | WNT3A       | 11.68307778          | Down       | 0        | 0   | Facilitates clonal plating of hESCs exhibiting functional hepatic differentiation | Ref. 62 |
| XM_426984.2         | PRDM14      | 12.82024346          | Down       | 1.61E-66  | 5.77E-66  | Involved in the maintenance of the self-renewal of human ESCs by suppression of gene expression | Ref. 63 |

TABLE 5
DEGs related to [log2] > 10 in PGCs versus SSCs of RNA-seq

| Gene identification | Description | log2 (PGCs vs. SSCs) | Regulation | p value | FDR | Function | Reference |
|---------------------|-------------|----------------------|------------|---------|-----|----------|-----------|
| XM_429858.1         | LOCA21502   | 11.21551             | Up         | 2.7E-286 | 3.9E-285 | Required for chromatin cohesion and DNA recombination during meiosis and mitosis | Ref. 57 |
| XM_416467.2         | SCMB1       | 10.63431             | Up         | 0        | 0   | Reported as a positive regulator of the Notch pathway | Ref. 58 |
| XM_427005.2         | AAK1        | 10.14017             | Up         | 4.61E-20 | 1.14E-19 | Prevents development of the Müllerian ducts into the uterus and other Müllerian structures; regulates production of sex hormones | Ref. 59 |
| NM_205030.1         | AMH         | 10.05392             | Up         | 0        | 0   | Associated with intranuclear foci of condensed chromatin | Ref. 60 |
| XM_428866.1         | S-KER       | 10.74340             | Down       | 4.09E-54 | 1.61E-53 | Regulates the function of the alternative complement pathway in fluid phase and on cellular surfaces | Ref. 61 |

TABLE 6
DEGs related to [log2] > 10 in ESCs versus SSCs of RNA-seq

| Gene identification | Description | log2 (ESCs/SSCs) | Regulation | p value | FDR | Function | Reference |
|---------------------|-------------|------------------|------------|---------|-----|----------|-----------|
| XM_42203.2          | LOCA24360   | 11.05914          | Up         | 9.10E-245 | 7.40E-244 | Induces endochondral bone formation in adult animals | Ref. 59 |
| NM_001039453.1      | AQP1        | 10.01037          | Up         | 1.20E-141 | 6.30E-141 | Regulates the Ca^{2+} channel expression at the cell surface | Ref. 60 |
| XM_421648.2         | PBIP        | 10.53929          | Up         | 0        | 0   | Defective regulation in failing hearts | Ref. 61 |
| NM_213579.1         | GEM         | 10.53732          | Up         | 0        | 0   | Associated with intranuclear foci of condensed chromatin | Ref. 62 |
| XM_419553.2         | LOCA21508   | 10.52041          | Up         | 0        | 0   | A role during avian skin and feather development | Ref. 63 |
| XM_001232973.1      | LCAT        | 10.51207          | Up         | 4.39E-22 | 1.00E-21 | Associated with intranuclear foci of condensed chromatin | Ref. 64 |
| NM_204897.1         | MENT-1      | 10.43276          | Up         | 0        | 0   | Associated with intranuclear foci of condensed chromatin | Ref. 65 |
| NM_204679.1         | CDERMO-1    | 10.21531          | Up         | 1.60E-103 | 7.00E-103 | Regulates the function of the alternative complement pathway in fluid phase and on cellular surfaces | Ref. 66 |
| XM_427005.2         | AAK1        | 10.14017          | Up         | 4.94E-19 | 1.08E-18 | Periostin as a mediator of matrix remodeling by cushion mesenchyme towards a mature valve structure | Ref. 67 |
| NM_205259.2         | LOC396194   | 10.11269          | Up         | 1.24E-91 | 5.13E-91 | Critical for establishing the trophoectoderm, the precursor of the placenta | Ref. 68 |
| XM_426613.2         | LOCA249057  | 10.1068           | Up         | 0        | 0   | Prevents apoptotic cell death | Ref. 69 |
| NM_001030551.1      | POSTN       | 10.09206          | Up         | 0        | 0   | Prevents apoptotic cell death | Ref. 70 |
| XM_204311.1         | CDX2        | 10.1264           | Down       | 0        | 0   | Prevents apoptosis signal invasion by nested Wnt signaling involved in inhibiting Xwnt8 and XmyoD ventrally | Ref. 71 |
| XM_415985.2         | LCC477741   | 10.6646           | Down       | 3.10E-160 | 1.80E-159 | Prevents apoptotic cell death | Ref. 72 |

versus the SSC group, and 6,226 in the PGC versus the SSC group (Fig. 4). In the up-regulated DEGs, there were six genes with a significant difference more than 10-fold in the ESC versus the PGC set, 13 genes in the ESC versus the SSC pair, and 4 genes in the PGC versus the SSC pair, whereas there were 7 genes down-regulated in ESCs versus PGCs, 4 genes in ESCs versus the SSC pair, and 4 genes in the PGC versus the SSC pair, whereas there were 7 genes down-regulated in ESCs versus PGCs.
versus SSCs, and 2 genes in PGCs versus SSCs. Most of the DEG fold change distributed in $2 < \log_2 \leq 4$ and $\log_2 \leq 2$, and there were a few DEGs more than 10-fold, including PRDM14, KPNA7, HOXB6, GAL9, and TWIST2, etc. (Tables 4–6). In these DEGs, more than 15% related to the multicellular development within the biological processes classification and more

| Gene identification | Description | Function | References |
|---------------------|-------------|----------|------------|
| NM_205335.2         | Gallus gallus transthyretin (TTR), mRNA | Transports thyroxine from the bloodstream to the brain | |
| NM_206989.1         | Gallus gallus prepro-urotensin II-related peptide (LOC404534), mRNA | Induces phenotypic differentiation, migration, and collagen synthesis | Ref. 53 |
| XM_001236989.1      | PREDICTED: Gallus gallus hypothetical LOC773980 (LOC777548), partial mRNA | Required for the correct speed and extent of migration | Ref. 27 |
| XM_426327.2         | PREDICTED: Gallus gallus similar to aminopeptidase A (LOC428771), mRNA | Probably plays a role in regulating growth and differentiation of early B-lineage cells | |
| NM_001001611.2      | Gallus gallus Gal9 (GAL9), mRNA | GAL is a hypothalamic-hypophysiotropic hormone and is a neuromodulator of LHRH secretion and action | Ref. 21 and 22 |
| XM_414212.2         | PREDICTED: Gallus gallus hypothetical LOC415852 (LOC415852), mRNA | Regulating the responsiveness of cells to adrenal androgens | |
| XM_426984.2         | PREDICTED: Gallus gallus hypothetical LOC429428 (LOC429428), partial mRNA | Involved in the maintenance of the self-renewal of human ES cells by suppression of gene expression | Ref. 14 |
| NM_204675.1         | Gallus gallus wingless-type MMTV integration site family, member 3A (WNT3A), mRNA | Facilitates clonal plating of hESCs exhibiting functional hepatic differentiation | Ref. 56 |
| XM_001231344.1      | Gallus gallus hypothetical protein LOC768589 (LOC768589), mRNA | Prevents apoptotic cell death | |
| XM_001234742.1      | PREDICTED: Gallus gallus similar to bactericidal/permeability-increasing protein-like 2 (LOC771461), mRNA | Plays an essential role in host defense | Ref. 55 |
| XM_416906.2         | PREDICTED: Gallus gallus hypothetical LOC418708 (LOC418708), mRNA | Responsible for the nucleosome structure of the chromosomal fiber in eukaryotes | |
| XM_430154.2         | PREDICTED: Gallus gallus hypothetical LOC424460 (LOC424460), mRNA | Required for normal fertility and fecundity | Ref. 16 |
| XM_414795.2         | PREDICTED: Gallus gallus similar to LOC398026 protein (LOC416488), mRNA | Function | References |

**TABLE 7**

DEGs related to $|\log_2| > 10$ in ESCs versus PGCs of microarray and RNA-seq

LHRH, luteinizing hormone-releasing hormone.
than 30% belonged to cell membrane within the cellular component classification, but the molecular functional assessment of these DEGs revealed that more than 30% associated with ATP binding. KEGG pathway assay indicated that 10 signaling pathways were significantly enriched in ESCs versus PGCs with the most enrichments in the focal adhesion signaling pathway (27.3%) and the ubiquitin-mediated proteolysis (19%) (Fig. 5A), whereas in the ESCs versus SSCs, 12 signaling pathways were significantly enriched in the focal adhesion pathway (16.8%) and cytokine-cytokine receptor interaction pathway (15.3%) (Fig. 5B). On the other hand, there were 12 enriched signaling pathways in the PGCs versus SSCs within the focal adhesion (18.1%) and the cytokine-cytokine receptor interaction pathway (16.5%) (Fig. 5C).

The Combination Analysis of Microarray and RNA-seq—The combined analysis of both microarray and RNA-seq results of the total DEGs revealed that there were 19 genes with expression differences more than 8-fold in the ESCs versus PGCs including GAL10, HBB, HBZ, and HBA1 genes and GAL9, which showed the highest fold change with successively increased expression in the three kinds of cells. In the ESCs versus SSCs, there were 31 genes with expression changes more than 8-fold including SRY, GAL6, GAL7, and GAL9 genes and the CDX2 that had the highest fold change in the three types of cells. In the PGCs versus SSCs, six genes were found to have more than 8-fold changes in expression, and AMH, HBZ, and HINTW showed highly specific expression in SSCs (Tables 7–9).

**Screening of Candidate Genes Involved in Male Germ Cell Development**—Venny analysis was used to find the specifically expressed genes in these three types of cells. The results showed that there were 1,023 DEGs in ESCs versus PGCs, 957 in ESCs versus SSCs, and 688 in the PGC versus the SSC group. GO analysis found that DEGs in ESCs versus PGCs were mainly enriched in 326 GO terms, of which 32 were associated with development and differentiation. In the ESC versus the SSC group, The DEGs were mainly enriched in 370 GO terms,
of which 24 were associated with development and differentiation, whereas in PGCs versus SCCs, DEGs were mainly enriched in 107 GO terms, of which 13 genes were associated with development and differentiation. All DEGs in three groups were mainly enriched in 117 GO terms, of which 32 were associated with development and differentiation (Fig. 6).

FIGURE 6. Venn diagram comparing DEGs among the three analyses.

FIGURE 7. Heat map representation (left) and GO classification (right) of differentiation-related genes among ESCs, PGCs, and SSCs.
Further analyzes revealed that there are 173 genes are related to development, differentiation, and metabolism, according to their GO classification as shown in the heat map in Fig. 7. Of these genes, 25 were successively up-regulated, and 14 were down-regulated; 18 genes were specifically expressed in ESCs, 58 genes were specifically expressed in PGCs, 16 genes were specifically expressed in SSCs, and 46 genes were specifically expressed in both types of cells. Ontological analysis indicated that 98 DEGs (57%) associated with cell differentiation, 19 genes (11%) were accompanied to metabolic process, and 56 (32%) genes were related to both processes. When we paid attention to the highly expressed 33 genes as candidate players involved in male germ cell development (Table 10), we found that there were 11 DEGs in the three types of cells (9 were up-regulated, and 2 were down-regulated), although 11 genes were specifically expressed only in PGCs, and 2 genes were only specifically expressed in SSCs. We also identified that IARS, TARS1L2, EPRS, and THRSP4 genes were specifically expressed in ESCs, but SARS, SLC13A3, TLL2, and SDF2 were specifically expressed in PGCs, whereas other genes were expressed in both two types of cells. Network analysis of 173 candidate genes associated with differentiation and development revealed the regulatory network and the interaction of DEGs. The FunNet analysis found that DEGs were mainly clustered in the three groups (Fig. 8), in which PLK1 and PSMD7 were two key nodes of regulatory networks. The results showed that PLK1 and PSMD7 expressions were decreased from ESCs to SSCs and that GAL9, AMH, PLK1, PSMD7, SDF2, DANA7, MYH13, PRDM14, KPN7, HOX6B, TWIST2, SHISA2, SIX1, USP2, MH13, and PR3 might be candidate genes related to chicken male germ cell development, differentiation, and cell metabolism processes.

**Screening of the Candidate Pathways Involved in Male Germ Cell Development**—Based on the above KEGG pathway enrichment analysis of the DEGs, further functional classification detected that most of the enriched pathways were related to the metabolism regulation processes (32.94–33.72%), diseases (21.71–21.96%), environmental information regulatory process (8.53–8.63%), and cellular process regulation (5.04–5.10%). While analyzing the KEGG pathways in cell growth and apoptosis, one pathway involved in cell apoptosis and in signal transduction pathways (Fig. 9). Among 13 pathways regulating the cellular processes, there were four pathways involved in cell communication, four pathways in cell growth and apoptosis, one pathway involved in cell motility, and four pathways in the transport and catabolism (Fig. 10a). The participating 19 pathways in the environmental information regulatory process were classified as follows: 1 pathway related to membrane transport, 13 involved in signal transduction, 4 related to signaling molecules interaction, and 1 pathway involved in the transport and catabolism (Fig. 10b). In these pathways, the DEGs were significantly expressed in TGF-β signaling pathway, Notch signaling pathway, Jak-STAT signaling pathway, ErB signaling pathway, ABC transporter,
extracellular matrix receptor interaction, cytokines, and their receptor interaction and cell adhesion molecule pathways. Among the 76 pathways related to the regulation of metabolism, 14 pathways regulate the carbohydrate metabolism, 2 pathways control the energy metabolism, 15 pathways are for lipid metabolism, 2 pathways are for nucleotide metabolism, 20 pathways are for amino acid metabolism, 6 pathways are for polysaccharides biosynthesis and metabolism, 12 pathways are for co-enzyme factor and vitamin metabolism, 2 pathways are for terpenoids and polyketide metabolism, 2 pathways are for biosynthesis of other secondary metabolites, and 3 pathways are related to xenobiotics metabolism and biodegradation (Fig. 10c). Most of the DEGs were significantly expressed in the following 17 metabolic pathways: arginine and proline metabolism, steroid biosynthesis, glutamic acid, serine, threonine metabolism, alanine, aspartic acid, glutamate metabolism, primary bile acid production, purine metabolism, sphingolipid metabolism, mucopolysaccharide metabolism, chondroitin sulfate, tyrosine metabolism, carbohydrate digestion and absorption, retinol metabolism, steroid hormone production, and oxidative phosphorylation pathways.

Screening and filtration of the closely related pathways to cell proliferation, differentiation, and signal transduction revealed that TGF-β, focal adhesion signal pathways, and ABC transporter were the most enriched in the ESC versus the PGC group, playing an important role during differentiation of ESCs to PGCs, whereas the most enriched pathways that regulate PGCs to SSC differentiation were Jak-STAT signaling pathway, ErbB signaling pathway, cell adhesion molecules, cytokine receptors and their interactions, extracellular matrix receptor interaction, focal adhesion, tight junctions and gap junctions pathways. In a ESC versus SSC comparison, there were seven significantly enriched pathways that were suspected to be responsible for in vivo differentiation of ESCs to SSCs cells; they are cytoplasmic DNA sensing, Notch signaling, PPAR signaling, the Focal adhesion, extracellular matrix receptor interaction, cytokines and interaction with their receptors, and cell adhesion molecule pathways (Fig. 11).

Validation of Microarray and RNA-seq Results by qRT-PCR—qRT-PCR was used to validate gene expression levels by detecting 10 randomly selected DEGs. As shown in Fig. 12, most of the qRT-PCR results were significantly correlated with the microarray and RNA-seq results, indicating
the reliability and accuracy of microarray and RNA-seq expression analysis.

Verification of the Microarray and RNA-seq Results by in Vitro Induction Experiments—The RA and supporting Sertoli cell induction group showed the embryoid body formation, and PGCs cells appeared after 2 days of induction, accompanying the beginning of SHISA2 gene expression, which was significantly up-regulated from 2–4 days and then increased at a slower rate after 6 days. The BMP4 induction group showed the embryoid body formation and PGCs cell appearance and the SHISA2 gene activation at the sixth day of induction. SIX1 (SIX homeobox 1) gene expression was increased in a linear growth trend until 4–6 days followed by a declined expression at the eighth day of stimulation. BMP4 expression was gradually increased at 2–8 days postinduction, whereas USP2 (ubiquitin specific peptidase 2) expression was gradually increased at 2–4 days and then linearly up-regulated at 4–6 days but began to

FIGURE 10. Screening of the candidate pathways involved in male germ cell development. a, classification of the 13 enriched pathways related to cellular processes regulation. b, distribution of the participating 19 pathways in environmental information regulatory process. c, percentages of the related pathways to the regulation of metabolism.

FIGURE 11. Signaling regulate different stages of male germ cell.
when treated with BMP4, USP2 expression was gradually increased during 2–6 days with a linear upward trend at 6–8 days after induction. The specifically expressed genes in SSCs (MH13, PR3, and DNAH7) were expressed at 6–8 days postinduction, whereas it began to express at day 8 in the BMP4 induction group, consistent with the beginning time of SSCs cell formation. When the induction occurred using the sex hormones in combination with supporting cells and RA, it was observed that EPHA3 expression was continuously increased and reached its highest value at the time of SSC formation, and GAP43 expression was up-regulated during PGC formation, reaching its maximum level of expression at the eighth day postinduction. Then its expression level was declined with the disintegration of embryoid bodies. On the other hand, both LOC773389 and LOC773586 expression showed a continued up-regulation and reached the highest value at SSC formation (Fig. 13).

**Discussion**

We have examined, for the first time, the entire gene expression pattern in three kinds of stem cells in chicken through a systematic whole genome and whole transcriptome approaches. Through comparison of the results, we identified that the male germ cell differentiation is a complex regulatory process associated with a lot of genes and signaling pathways. Until now, however, the molecular mechanisms during this process were not fully announced; only some genes and pathways had been confirmed. As an attempt at a complete understanding, two high throughput methods (microarray and RNA-seq) were used to detect the gene expression pattern during male germ cell specification and differentiation.

Light had been shed on some crucial genes that were predicted to be the controller of this process. Our work confirmed the roles of some previously mentioned genes in the differentiation process as SHISA2, AMH, SOX9, ALDH1A1, and others. Boudreau and Jones (8) reported that SHISA2 gene blocks the expression of Wnt signaling pathway. It is necessary to ensure the normal development of mouse PGCs as it was observed by Miles (9). The same findings were obtained in our experiment because the SHISA2 gene was detected in a high expression level in PGCs cells during either the normal in vivo male germ cell differentiation or during its in vitro induction. Also, our results revealed that AMH and SOX9 genes were specifically expressed in SSCs with more than 10-fold difference of the expression.
expression changes. These results are consistent with other studies (10) showing that the anti-Mullerian hormone (AMH) was responsible for early male development in vertebrate as AMH, which is a major downstream of SOX9, which boosts the AMH expression through binding to its promoter, leading to stimulation of male germ cell differentiation.

Both ALDH1A1 and CYP26b1 are suspected to be key candidate genes because they are involved in the synthesis, degradation, and maintaining the in vivo homeostasis of RA, which plays an important role in mammalian spermatogenesis process through controlling the activation of the meiotic-related genes, such as STRA8 (stimulated by retinoic acid gene 8) (11–13). At the same time, we observed a continuous up-regulation of ALDH1A1 and CYP26b1 that explain the nonconstant level of RA during the differentiation process caused by the effect of these genes on the RA equilibrium. The maximum levels of ALDH1A1 and CYP26b1 expression were detected in SSCs, indicating its stimulatory role in the meiosis process. A recent study identified the PRDM14 expression by microarray assay, in particular, undifferentiated human ESCs (14), and Chia et al. (15) described it as an important transcription factor in human pluripotency maintenance. HU stated that PLK1, a polo-like kinase family member, was involved in cell mitosis and also expressed in a high significant manner in many of human malignancies so it is considered a carcinogenic gene (16).

Our study observed high expression levels of PRDM14 and PLK1 especially in chicken ESCs in contrast to its decreased levels in the other two types of cells (PGCs and SSCs). As a further confirmation of those previous observations, it also speculated that PRDM14 and PLK1 may play an important role in pluripotency maintenance and their down-regulation and so may be related to the ESC differentiation process. Analysis of candidate genes regulatory network found that PLK1 is located at a key node of the entire regulatory network, as a bridge connecting the entire cell differentiation process.

Studies have shown that ECM (extracellular matrix protein) can provide support for the cell adhesion and also help through the integrin receptor to deliver the extracellular signals that regulate stem cell proliferation, migration, and differentiation. ECM could activate the expression of Integrinα6 and Integrinβ1 that led to cell morphology and the function change (17). In our study, Integrinα6 and Integrinβ1 were expressed in a continuous upward manner in the three types of cells, indicating that the ECM signals may promote the expression of SSCs marker genes and induce the formation of the male germ cells. Cell adhesion molecules could activate FAK signal leading to the reorganization of the actin cytoskeleton and subsequently cause cell differentiation (18). TGF-β signaling could regulate testis formation and male germ cell development (9). Notch signaling was reported antagonistically to regulate germ line stem cell niche formation in Drosophila male embryonic gonads (19) and deletion of jagged1, a Notch ligand, could lead to the formation of multicystic follicles in the mouse ovary (20). These reports provide support that our selected candidate key signaling pathways play an important role in the differentiation of the male germ cells. p53 signaling pathway and MAPK signaling pathway have been reported to be involved in the cell differentiation.

Some genes were not involved in any signal pathway. GO assay results suggested that these genes have a role in cell differentiation regulation because they showed significant differences among the three types of cells. Therefore the regulatory
mechanism and function of these genes and its position in the signal pathways require further researches. The genes that we identified in this study will provide important candidates, which are potential markers identifying ESCs, PGCs, SSCs, and the potential regulators controlling germ cell differentiation.

In this study, we successfully induced differentiation of ESCs into the male germ cells in vitro using an induction system containing RA, supporting cells, BMP4, testosterone, and follicle-stimulating hormone. During these induction procedures, the expression level of the selected crucial genes was detected using quantitative real time PCR. The consistency of microarray and RNA-seq results with our confirmatory experimental observations using this established model indicated its successful ability for induction of in vitro differentiation of ESCs toward male germ cells, and so the candidate crucial genes can be validated based on this system. It was concluded that these candidate crucial genes have the ability of specific expression during male germ cell development either in the normal in vivo differentiation process or in vitro induction system. However, its specific functions remain under further investigation.

Differentiation of male germ cells is an intricate regulatory process that involved a large number of genes and signaling pathways with a lot of unclear mechanisms. Currently, there is not any system or complete comprehensive research report that reveals the variations of these genes, so it cannot be an accurate search for the key regulatory genes or signaling pathways responsible for this process. This study revealed the whole gene expression changes in the entire process of germ cell differentiation to explore the molecular mechanism of its variation and to obtain the candidate crucial genes and signaling pathways, and it was also observed that some of these genes and signaling pathways were first reported in poultry. This research will provide a more reliable reference for the researchers to study the mechanism of germ cell differentiation and narrow the selection of genes or signal pathways. Moreover, it will suggest more precise ideas to be focused on in the future, especially for the in vitro spermatogenesis process because it will support the exploration of promoting induction methods. Currently, we have obtained a partial screening of the critical candidate genes and signaling pathways to start further functional validation experiments for in-depth study and analysis. Using poultry as a research model to study the regulatory mechanism of ESC differentiation toward the male germ cells will contribute a better understanding of cell biology and developmental biology and will be useful for human health-applicable studies.

Our results showed the crucial genes and pathways involved in the regulation of chicken male germ cell differentiation. These results will be helpful for researchers to narrow the range of functional genes and pathways during the ESC differentiation to male germ cells providing an important reference for future research.

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