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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 *Piwil2* 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 azoospermia) 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-

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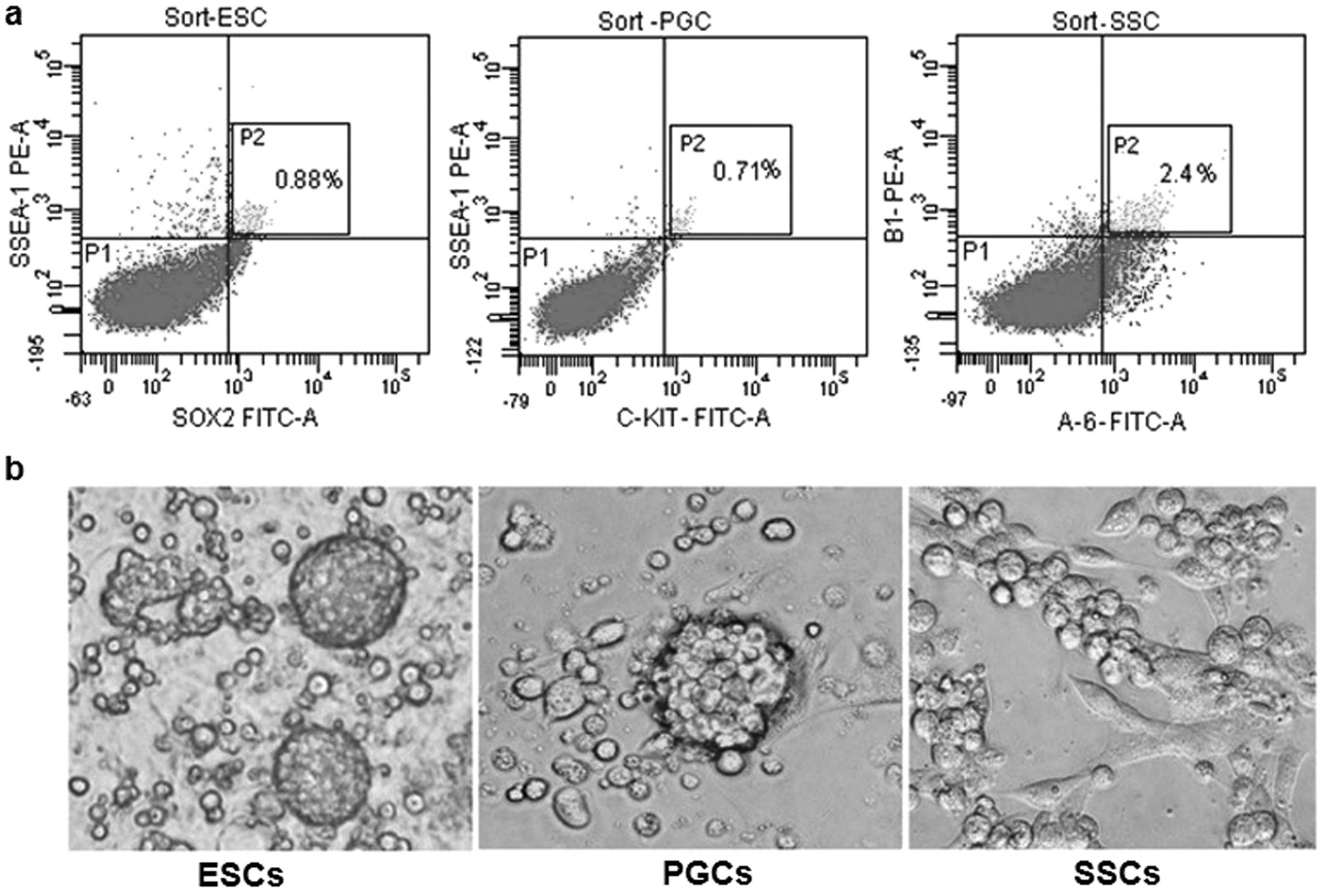

FIGURE 1. **Cell sorting and culture.** *a*, the purity of FAC-sorted ESCs, PGCs, and SSCs. *b*, the morphological characters of the three types of cells.

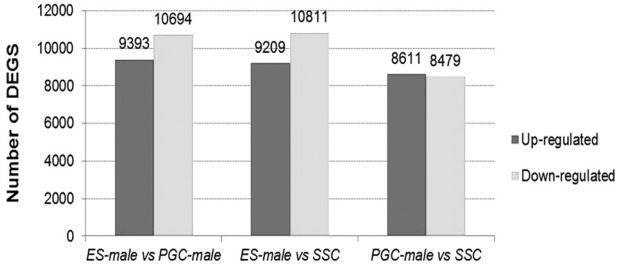

FIGURE 2. **DEGs in microarry.**

ment. 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)[4] 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

[4] The abbreviations used are: DEG, differentially expressed gene; ESC, embryonic stem cell; PGC, primordial germ cell; SSC, spermatogonial stem cell; RNA-seq, RNA sequencing; qRT-PCR, quantitative real time PCR; RA, retinoic acid; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

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	References
A_87_P054786	16.293575	Up	*GAL10*	A hypothalamic-hypophysiotropic hormone and a neuromodulator of LHRH secretion; action galanin is unlikely to influence PRL secretion via inhibition of dopaminergic tone	Refs. 21 and 22
A_87_P078616	15.038605	Up	*NPHS2*	Essential for the integrity of the glomerular filter	Ref. 23
A_87_P054986	13.116768	Up	*HBZ*	Activates erythroid-specific, globin gene expression	Ref. 24
A_87_P274543	12.136964	Up	*HBG2*	Gamma chains make up the fetal hemoglobin F, in combination with alpha chains	Ref. 25
A_87_P009555	12.067309	Up	*HBAA*		
A_87_P009368	11.491803	Up	*LECT2*	A liver-specific protein that is thought to be linked to hepatocyte growth	Ref. 26
A_87_P035104	11.244352	Up	*GAL9*	A hypothalamic-hypophysiotropic hormone and is a neuromodulator of LHRH secretion and action	Refs. 21 and 22
A_87_P057441	10.826411	Up	*HOXD8*	Controls lateral line cell migration	Ref. 27
A_87_P152088	10.653027	Up	*TSPAN8*	Encodes a cell surface glycoprotein defined by the monoclonal antibody CO-029, a 27–34-kDa membrane protein expressed in gastric, colon, rectal, and pancreatic carcinomas but not in most normal tissues	Ref. 28
A_87_P108903	10.637115	Up	*COL8A1*	A major component of the hexagonal lattice in the Descemet membrane	Ref. 29
A_87_P146353	10.3792095	Up	*LUM*	Interacts with collagen and limits growth of fibrils in diameter	Ref. 30
A_87_P014692	10.170226	Up	*ACTA2*	Important regulators of smooth muscle cell differentiation	Ref. 31
A_87_P132478	−10.0271225	Down	*OTX2*	A key regulatory gene in photoreceptor cell development	Ref. 32
A_87_P037684	−10.859593	Down	*ENS-3*	Nucleic acid binding; RNA-DNA hybrid ribonuclease activity; DNA integration	Ref. 33

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	References
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	Refs. 34 and 35
A_87_P260443	10.933807	Up	*SLCO1A2*	It mediate transport of estrone-3-sulfate and, more weakly, prostaglandin E2.	Refs. 36 and 37
A_87_P150703	−10.657406	Down	*ATP5A1W*	Cessation of recombination between avian sex chromosomes	Ref. 38
A_87_P078616	−11.816776	Down	*NPHS2*	The association of podocin with specialized lipid raft microdomains of the plasma membrane was a prerequisite for recruitment of nephrin into rafts	Refs. 39 and 40
A_87_P054986	−12.876568	Down	*HBZ*	The zeta-globin polypeptide is synthesized in the yolk sac of the early embryo, while alpha-globin is produced throughout fetal and adult life	Ref. 41
A_87_P058761	−14.45761	Down	*HINTW*	Is a W-chromosome HINT gene in chick, is expressed ubiquitously and is a robust female cell marker applicable in intraspecific chimera studies	Refs. 42 and 43

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.

TABLE 3

DEGs related to |logFC| > 10 in ESCs *versus* SSCs of microarray

LHRH, luteinizing hormone-releasing hormone; PRL, prolactin.

Probe name	Log FC (ESCs *vs.* SSCs)	Regulation	Gene symbol	Function	References
A_87_P009368	15.350238	Up	*LECT2*	Is a liver-specific protein that is thought to be linked to hepatocyte growth	Ref. 26
A_87_P274543	13.708621	Up	*HBG2*	The gamma globin genes (HBG1 and HBG2) are normally expressed in the fetal liver, spleen, and bone marrow	Ref. 41
A_87_P054786	13.304759	Up	*GAL10*	A hypothalamic-hypophysiotropic hormone and a neuromodulator of LHRH secretion; action galanin is unlikely to influence PRL secretion via inhibition of dopaminergic tone	Refs. 21 and 22
A_87_P129033	12.866734	Up	*GAL6*	Has bactericidal activity	Ref. 44
A_87_P100606	12.3010845	Up	*GAL7*	Has bactericidal activity	Ref. 44
A_87_P017854	11.8544855	Up	*POSTN*	Supports adhesion and migration of epithelial cells	Ref. 45
A_87_P009700	11.636215	Up	*CATHL2*	Has hemolytic activity and may play a role in the innate immune response	Ref. 46
A_87_P007819	11.55645	Up	*RGS5*	Involved in the regulation of heterotrimeric G proteins by acting as GTPase activators	Ref. 47
A_87_P037929	11.287436	Up	*KCNMB1*	Modulates the calcium sensitivity and gating kinetics	
A_87_P008896	11.176561	Up	*LYG2*	Lysozyme activity	Ref. 48
A_87_P059356	11.127887	Up	*GAL2*	Potent antibacterial activity against	Ref. 49
A_87_P146353	11.08161	Up	*LUM*	Regulate collagen fibril organization and circumferential growth, corneal transparency, and epithelial cell migration and tissue repair	
A_87_P191933	10.969956	Up	*CCDC80*	Promotes cell adhesion and matrix assembly (by similarity); may play a role in eye formation	Ref. 50
A_87_P014692	10.948489	Up	*ACTA2*	Involved in various types of cell motility and	
A_87_P263263	10.806091	Up	*RSFR*	ubiquitously expressed in all eukaryotic cells	
A_87_P023107	10.69445	Up	*CTSG*	May participate in the killing and digestion of engulfed pathogens and in connective tissue remodeling at sites of inflammation	
A_87_P008907	10.515071	Up	*ALDH1A1*	Involved in the regulation of the metabolic responses	
A_87_P282153	10.302805	Up	*COL1A2*	Matrix integrity	
A_87_P035106	10.2099	Up	*GAL4*		
A_87_P214693	10.184647	Up	*DCN*	Plays a role in matrix assembly; capable of suppressing the growth of various tumor cell lines	
A_87_P008852	−10.54513	Down	*GSC*	May play a role in spatial programing within	
A_87_P004087	−10.636513	Down	*LOC770611*	discrete embryonic fields or lineage compartments during organogenesis	
A_87_P151998	−10.693583	Down	*LOC430910*		
A_87_P037684	−10.859767	Down	*ENS-3*	Pol-like protein ENS-3	
A_87_P102041	−10.96241	Down	*EOMES*	Playing a crucial role during development. Functions in trophoblast differentiation and later in gastrulation, regulating both mesoderm delamination and endoderm specification. Plays a role in brain development being required for the specification and the proliferation of the intermediate progenitor cells	
A_87_P009011	−11.044117	Down	*CDX2*	Important in broad range of functions from early differentiation to maintenance of the intestinal epithelial lining of both the small and large intestine	
A_87_P150703	−11.242624	Down	*ATP5A1W*	Cessation of recombination between avian sex chromosomes	Ref. 38
A_87_P053051	−11.601723	Down	*CNOT2*	Involved in the maintenance of embryonic stem (ES) cell identity	Ref. 51
A_87_P058761	−14.244124	Down	*HINTW*	Strongly expressed in the gonads and other tissues of female chicken embryos	

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. 1*a* as according to the results, 0.88% ESCs were SSEA1 and SOX2

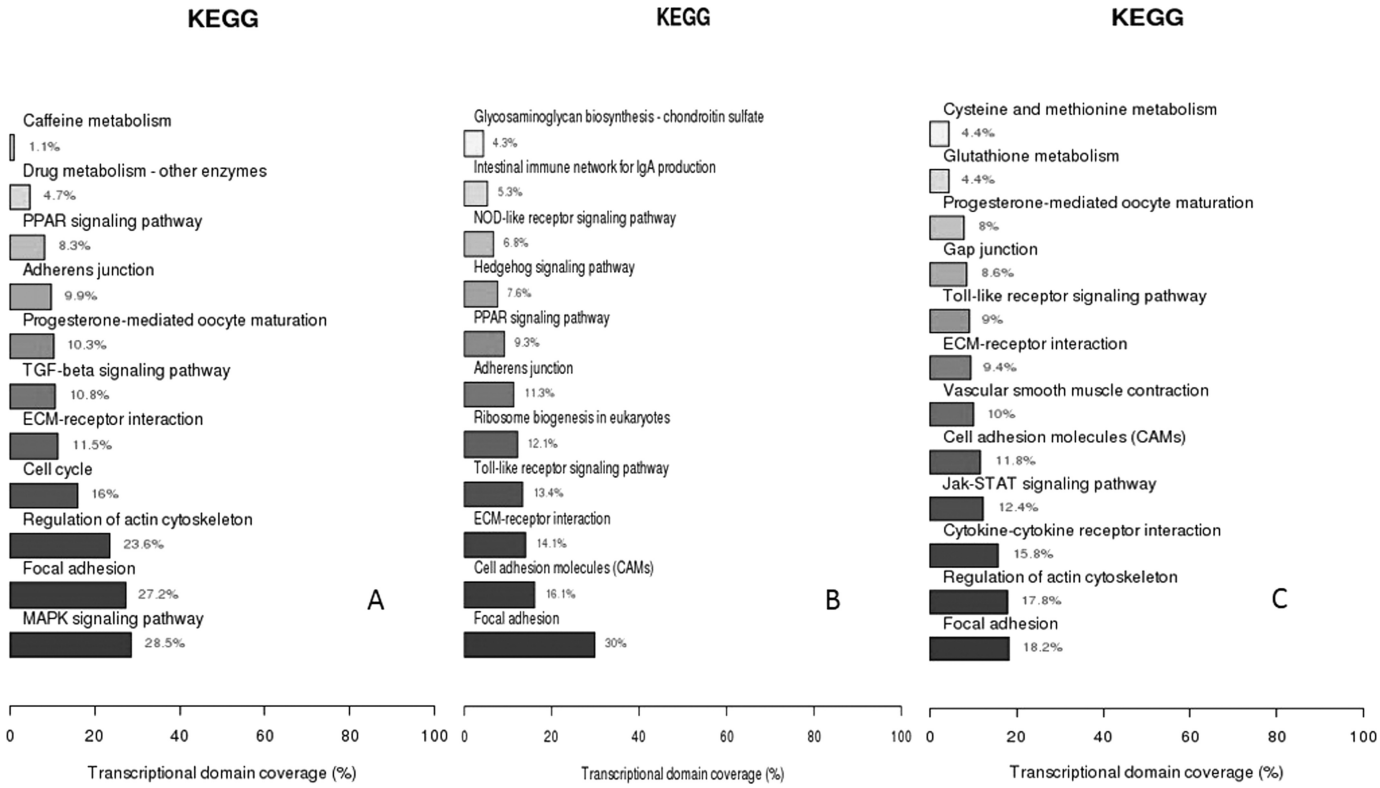

FIGURE 3. **KEGG classification of DEGs in microarray.** *A*, ESCs *versus* PGCs. *B*, ESCs *versus* SSCs. *C*, PGCs *versus* SSCs.

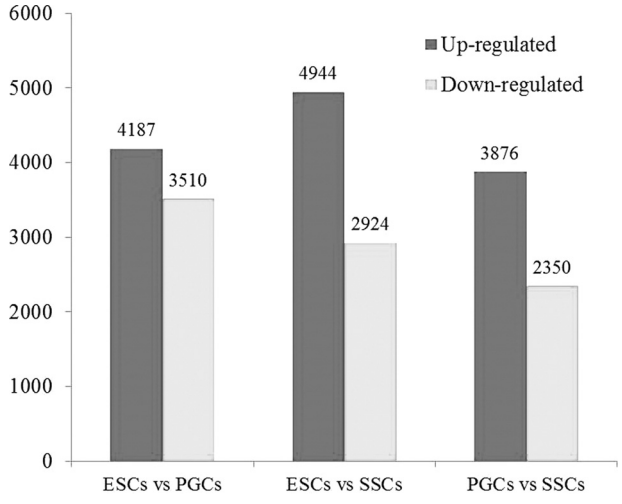

FIGURE 4. **DEGs in RNA-seq.**

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. 1*b*. 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 $|\log FC| \geq 1$ 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 $2 < |\log FC| \leq 4$ and $\log FC \leq 2$ groups, and only a few DEGs were related to the $|\log FC| > 10$ 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. 3*A*), 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. 3*B*). 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. 3*C*).

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

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*	11.83347797	Up	1.49E-211	1.13E-210	Initiates myoblast differentiation	Ref. 52
NM_206989.1	*UTS2B*	10.86825931	Up	5.91E-170	3.76E-169	Induces phenotypic differentiation, migration, and collagen synthesis	Ref. 53
XM_001236989.1	*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	
XM_426327.2	*ENPEP*	10.7588003	Up	0	0	Contribute to the development of renal and hypertensive disorders	
NM_001001611.2	*GAL9*	10.4579734	Up	9.20E-85	3.75E-84	Induction of differentiation of MSCs into chondrocytes	Ref. 54
XM_414212.2	*LOC415852*	10.08034791	Up	5.30E-50	1.67E-49	Regulating the responsiveness of cells to adrenal androgens	
XM_414795.2	*LOC398026*	−10.0212089	Down	0	0	Required for normal fertility and fecundity	Ref. 16
XM_430154.2	*LOC424460*	−10.12099411	Down	0	0		
XM_416906.2	*HIST1H2AH*	−10.42067022	Down	5.27E-77	2.03E-76	Responsible for the nucleosome structure of the chromosomal fiber in eukaryotes	
XM_001234742.1	*BPIL2*	−10.52057903	Down	0	0	Plays an essential role in host defense	Ref. 55
XM_001231344.1	*LOC768589*	−10.54284209	Down	3.90E-115	1.91E-114	Prevents apoptotic cell death	
NM_204675.1	*WNT3A*	−11.68307778	Down	0	0	Facilitates clonal plating of hESCs exhibiting functional hepatic differentiation	Ref. 56
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. 14

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	*LOC421502*	11.21551	Up	2.7E-286	3.9E-285		
XM_416467.2	*SMC1B*	10.64341	Up	0	0	Required for chromatid cohesion and DNA recombination during meiosis and mitosis	Ref. 57
XM_427005.2	*AAK1*	10.14017	Up	4.61E-20	1.14E-19	Reported as a positive regulator of the Notch pathway	Ref. 58
NM_205030.1	*AMH*	10.04345	Up	0	0	Prevents the development of the Müllerian ducts into the uterus and other Müllerian structures; regulates production of sex hormones	Refs. 34 and 35
XM_428866.1	*S-KER*	−10.7443	Down	4.09E-54	1.61E-53		

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	References
XM_422203.2	*LOC424360*	11.05914	Up	9.10E-245	7.40E-244		
NM_001039453.1	*AQP1*	11.01037	Up	1.20E-141	6.30E-141		
XM_421648.2	*PBIP*	10.53929	Up	0	0	Induces endochondral bone formation in adult animals	Ref. 59
NM_213579.1	*GEM*	10.53732	Up	0	0	Regulation of Ca^{2+} channel expression at the cell surface	Ref. 60
XM_419553.2	*LOC421508*	10.52041	Up	0	0	Defective regulation in failing hearts	Ref. 61
XM_001232973.1	*LCAT*	10.51207	Up	4.39E-22	1.00E-21		
NM_204897.1	*MENT-1*	10.43276	Up	0	0	Associated with intranuclear foci of condensed chromatin	Ref. 62
NM_204679.1	*CDERMO-1*	10.21531	Up	1.60E-103	7.00E-103	A role during avian skin and feather development	Ref. 63
XM_427005.2	*AAK1*	10.14017	Up	4.94E-19	1.08E-18	Reported as a positive regulator of the Notch pathway	Ref. 58
NM_205259.2	*LOC396194*	10.11269	Up	1.24E-91	5.13E-91		
XM_426613.2	*LOC429057*	10.1068	Up	0	0	Regulates the function of the alternative complement pathway in fluid phase and on cellular surfaces	Ref. 64
NM_001030541.1	*POSTN*	10.09206	Up	0	0	Periostin as a mediator of matrix remodeling by cushion mesenchyme towards a mature valve structure	Ref. 65
NM_204311.1	*CDX2*	−12.264	Down	0	0	Critical for establishing the trophoectoderm, the precursor of the placenta	Ref. 66
XM_415985.2	*LOC417741*	−10.6646	Down	3.10E-160	1.80E-159	Encodes a secreted antagonist of Wnt signaling likely involved in inhibiting Xwnt8 and XmyoD ventrally	Ref. 67
XM_001231344.1	*LOC768589*	−10.5428	Down	1.20E-107	5.30E-107	Prevents apoptotic cell death	
XM_001234907.1	*LOC771651*	−10.3586	Down	4.05E-11	7.60E-11		

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

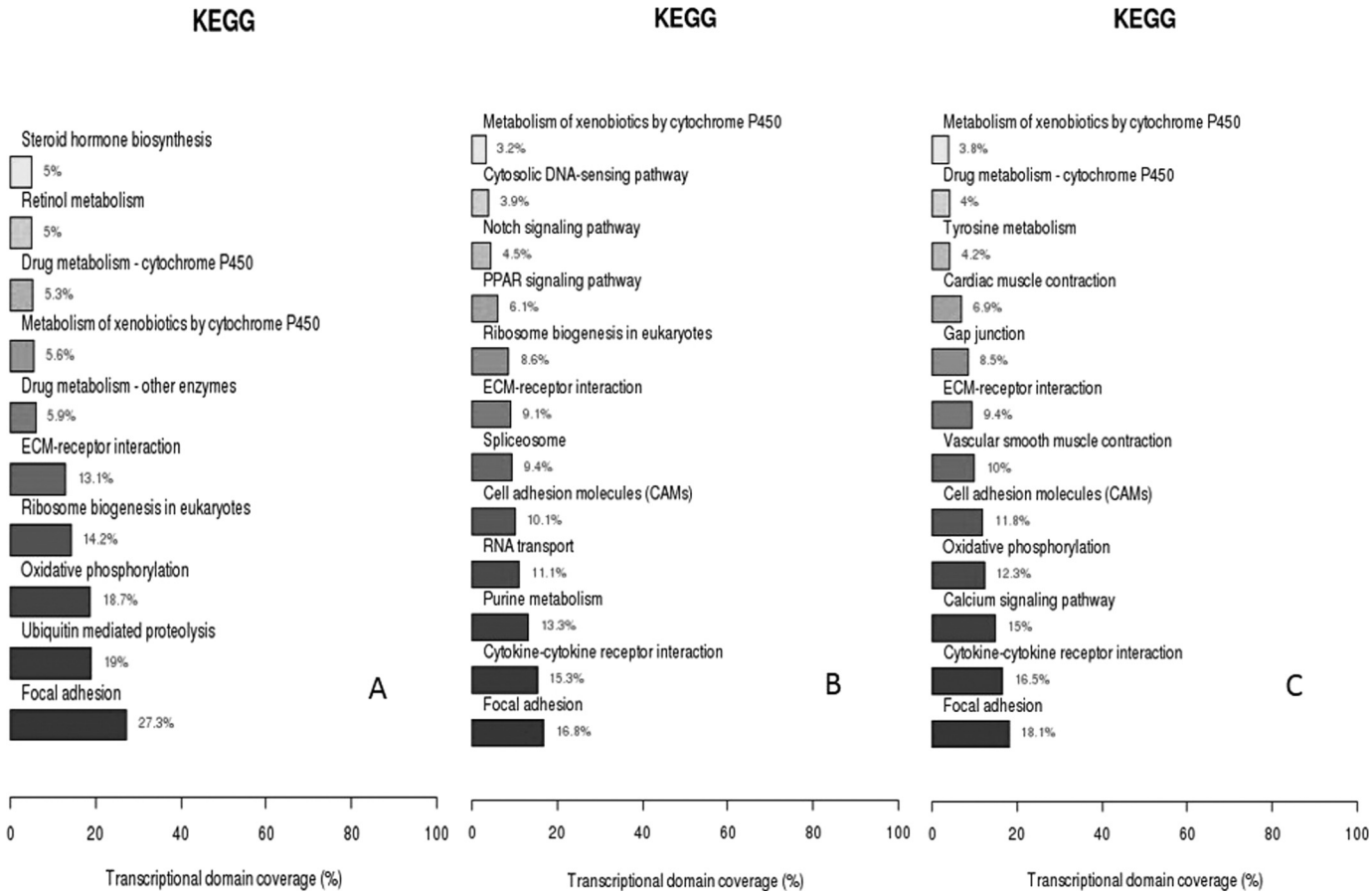

FIGURE 5. **KEGG classification of DEGs in RNA-seq.** *A*, ESCs *versus* PGCs. *B*, ESCs *versus* SSCs. *C*, PGCs *versus* SSCs.

TABLE 7

DEGs related to |log2| > 10 in ESCs *versus* PGCs of microarray and RNA-seq

LHRH, luteinizing hormone-releasing hormone.

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 protein 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* Gal 9 (*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	PREDICTED: *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		
XM_414795.2	PREDICTED: *Gallus gallus* similar to LOC398026 protein (*LOC416488*), mRNA	Required for normal fertility and fecundity	Ref. 16

versus SSCs, and 2 genes in PGCs *versus* SSCs. Most of the DEG fold change distributed in $2 < \log2 \leq 4$ and $\log2 \leq 2$, 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

TABLE 8
DEGs related to |log2| > 10 in ESCs *versus* SSCs of microarray and RNA-seq

Gene identification	Description	Function	References
XM_422203.2	PREDICTED: *Gallus gallus* hypothetical LOC424360 (*LOC424360*), mRNA		
NM_001039453.1	*Gallus gallus* aquaporin 1 (*AQP1*), mRNA		
XM_421648.2	PREDICTED: *Gallus gallus* similar to prepro bone inducing protein (*LOC423776*), mRNA	Has a key role in physiological and pathological angiogenesis	Ref. 68
NM_213579.1	*Gallus gallus* GTP-binding protein overexpressed in skeletal muscle (*GEM*), mRNA	Participates in receptor-mediated signal transduction at the plasma membrane	Ref. 69
XM_419553.2	PREDICTED: *Gallus gallus* similar to cardiac muscle ryanodine receptor (*LOC421508*), mRNA		
XM_001232973.1	PREDICTED: *Gallus gallus* similar to lecithin-cholesterol acyltransferase (*LOC769683*), mRNA		
NM_204897.1	*Gallus gallus* heterochromatin-associated protein MENT (*MENT-1*), mRNA	Associated with intranuclear foci of condensed chromatin	Ref. 62
NM_204679.1	*Gallus gallus* Dermo protein (*CDERMO-1*), mRNA	A role during avian skin and feather development	Ref. 63
XM_427005.2	PREDICTED: *Gallus gallus* similar to AP2-associated protein kinase 1 (adaptor-associated kinase 1) (*LOC429449*), partial mRNA	Reported as a positive regulator of the Notch pathway	Ref. 58
NM_205259.2	*Gallus gallus* leukocyte ribonuclease A-1 (*LOC396194*), mRNA		
XM_426613.2	PREDICTED: *Gallus gallus* similar to complement regulator factor H (*LOC429057*), mRNA	Regulates the function of the alternative complement pathway in fluid phase and on cellular surfaces	Ref. 64
XM_001235325.1	PREDICTED: *Gallus gallus* similar to AP2-associated protein kinase 1 (adaptor-associa1ted kinase 1) (*LOC772149*), partial mRNA		
NM_001030541.1	*Gallus gallus* periostin, osteoblast specific factor (*POSTN*), mRNA	Mediator of matrix remodeling by cushion mesenchyme towards a mature valve structure	Ref. 65
NM_204311.1	*Gallus gallus* caudal type homeobox transcription factor 2 (*CDX2*), mRNA	Critical for establishing the trophoectoderm, the precursor of the placenta	Ref. 66
XM_415985.2	PREDICTED: *Gallus gallus* similar to secreted Xwnt8 inhibitor sizzled (*LOC417741*), mRNA	Encodes a secreted antagonist of Wnt signaling likely involved in inhibiting Xwnt8 and XmyoD ventrally	Ref. 67
XM_001231344.1	PREDICTED: *Gallus gallus* hypothetical protein LOC768589 (*LOC768589*), mRNA	Prevents apoptotic cell death	
XM_001234907.1	PREDICTED: *Gallus gallus* hypothetical protein LOC771651 (*LOC771651*), partial mRNA		

TABLE 9
DEGs related to |log2| > 10 in PGCs *versus* SSCs of microarray and RNA-seq

Gene identification	Description	Function	References
XM_429858.1	PREDICTED: *Gallus gallus* hypothetical LOC421502 (*LOC421502*), mRNA		
XM_416467.2	PREDICTED: *Gallus gallus* structural maintenance of chromosomes 1B (*SMC1B*), mRNA	Required for meiotic chromosome dynamics	Ref. 70
XM_427005.2	PREDICTED: *Gallus gallus* similar to AP2-associated protein kinase 1 (adaptor-associated kinase 1) (*LOC429449*), partial mRNA	Reported as a positive regulator of the Notch pathway	Ref. 58
NM_205030.1	*Gallus gallus* anti-Mullerian hormone (*AMH*), mRNA	Has a critical role in regression of the mullerian duct system during development	Ref. 71
XM_428866.1	PREDICTED: *Gallus gallus* similar to Scale keratin (*S-KER*) (*S-ker*) (*LOC431315*), mRNA		
XM_001232144.1	PREDICTED: *Gallus gallus* similar to Wpkci (*LOC771438*), mRNA	Involved in triggering the differentiation of ovary	Ref. 72

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. 5*A*), 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. 5*B*). 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. 5*C*).

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,

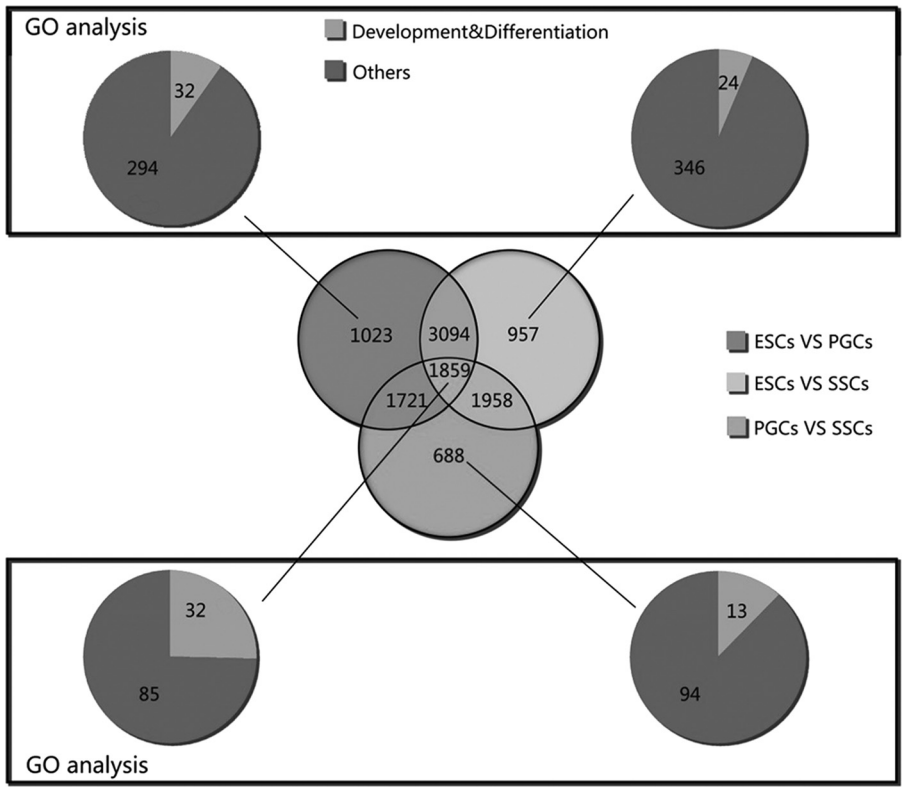

FIGURE 6. **Venn diagram comparing DEGs among the three analyses.**

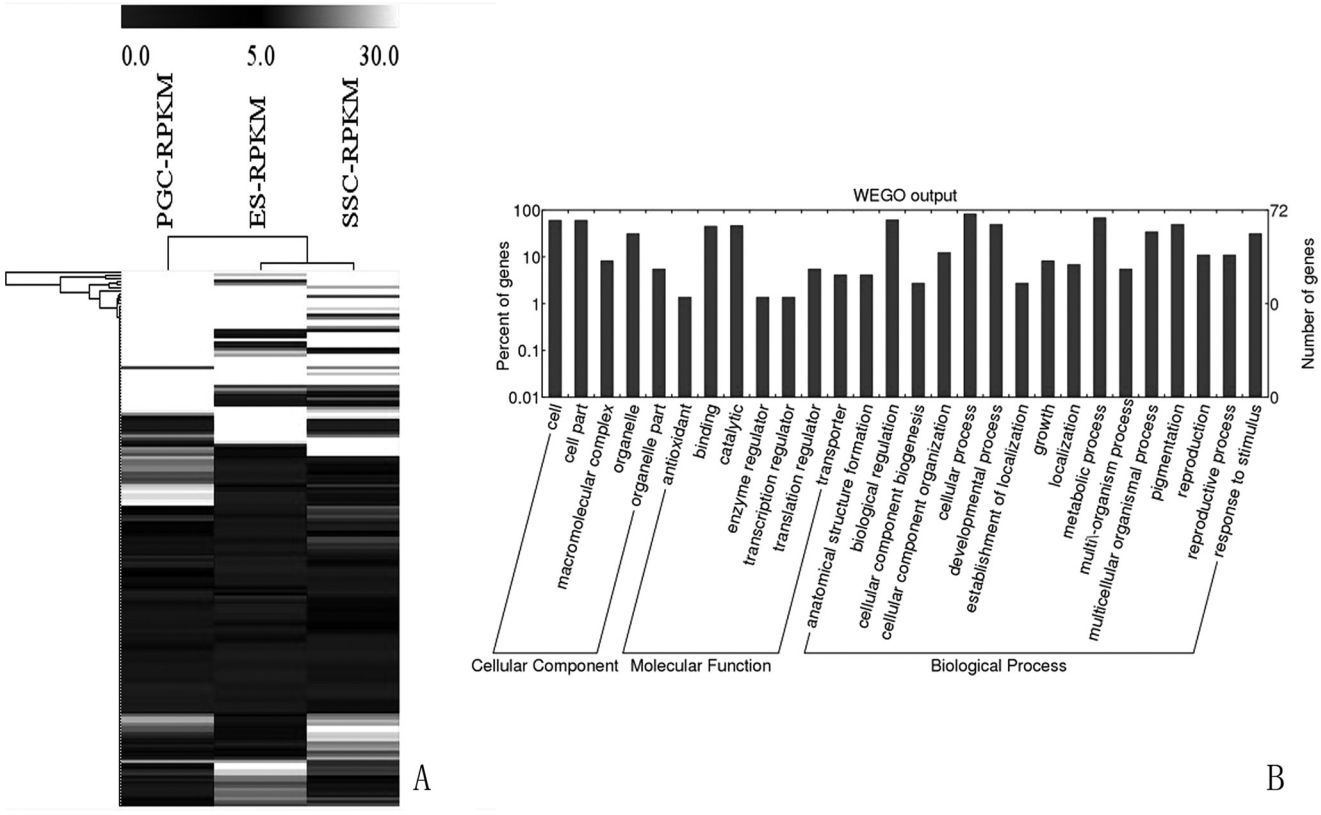

FIGURE 7. **Heat map representation (*left*) and GO classification (*right*) of differentiation-related genes among ESCs, PGCs, and SSCs.**

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).

TABLE 10
Development and differentiation-related genes among three kinds stem cells of chicken

Gene identification	Gene name	Pattern	Function	References
XM_001231469.1	Hypothetical protein LOC768468	Up-regulate		
XM_001231338.1	Similar to pol; hypothetical protein LOC769775	Up-regulate		
NM_204283.1	ST8 α-*N*-acetyl-neuraminide α-2,8-sialyltransferase 4 (*ST8SIA4*)	Up-regulate	Critical gene for the formation of neural cell adhesion molecule	Ref. 73
NM_205254.1	Myosin IF (*MYO1F*)	Up-regulate	Have a role to play in cell motility	Ref. 74
NM_204894.1	ATP-binding cassette, subfamily B (MDR/TAP), member 1 (*ABCB1*)	Up-regulate	Involved in the cell type-specific transport or release of estrogen that is essential for avian follicular development	Ref. 75
NM_204735.1	Myosin VI (*MYOVI*)	Up-regulate	Plays a role in the maintenance of Golgi morphology and in exocytosis	Ref. 76
NM_001008476.1	Histone deacetylase 9 (*HDAC9*)	Up-regulate		
XM_001231534.1	CD80	Up-regulate		
NM_205430.1	EPH receptor A3 (*EPHA3*)	Up-regulate	Play an important role during development and in signal transduction pathways	Ref. 77
XM_418915.2	E2F transcription factor 3 (*E2F3*)	Down-regulate	Critical regulators of the genes responsible for cell cycle progression and growth	Ref. 78
XM_427876.2	Myosin VB (*MYOVB*)	Down-regulate		
XM_425527.2	Growth-associated protein 43 (*GAP43*)	PGCs specific		
XM_001231480.1	Similar to Wpkci; histidine triad nucleotide binding protein W	PGCs specific	Involved in triggering the differentiation of ovary	Ref. 72
NM_204926.1	Ubiquitin-specific peptidase 2 (*SUP2*)	PGCs specific		
NM_204501.1	Transmembrane protein 46; hypothetical protein LOC771239	PGCs specific		
NM_204769.1	Visual system homeobox 1 (*VSX1*)	PGCs specific	Retinal differentiation	Ref. 79
NM_204115.1	Rho-related BTB domain containing 2 (*RHOBTB2*)	PGCs specific		
NM_001044685.1	SIX homeobox 1 (*SIX1*)	PGCs specific	Controls craniofacial and brain development	Ref. 80
NM_204202.1	Claudin 3 (*CLDN3*)	PGCs specific	Promotes tubule formation and expansion of the ureteric bud epithelium	Ref. 81
XM_421631.2	Paired-like homeodomain 3	PGCs specific	Pituitary and lens formation	Ref. 82
XM_001234694.1	Histone deacetylase 9	PGCs specific		
XM_001231443.1	Hypothetical protein LOC768737	PGCs specific		
XR_027197.1	Dynein, axonemal, heavy chain 7 (*DNAH7*)	SSCs specific		
XM_001231455.1	Myosin, heavy chain 13, skeletal muscle (*MYH13*)	SSCs specific	Early specialization of the superfast myosin	Ref. 83
XM_416513.2	Ubiquitin-specific peptidase 5 (isopeptidase T)	cESCs and PGCs		
NM_204559.1	msh homeobox 2 (*MSX2*)	cESCs and PGCs	Crucial role in directing the growth and patterning of limb mesoderm	Ref. 84
XM_001231369.1	Leucine-rich repeat containing 28	cESCs and SSCs		
XM_001231533.1	Hypothetical protein LOC768631	cESCs and SSCs		
NM_001012929.1	RAS p21 protein activator 2	cESCs and SSCs		
NM_001012927.1	myb-like, SWIRM and MPN domains 1	cESCs and SSCs	Essential gene for the growth and the differentiation for various types of cells	Ref. 85
XM_422634.2	Nicotinamide nucleotide adenylyltransferase 3	cESCs 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*, *TARSL2*, *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*, *DNAH7*, *MYH13*, *PRDM14*, *KPNA7*, *HOXB6*, *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%), regulation of genetic information process (8.53–8.63%), environmental information regulatory process (7.36–7.45%), and cellular process regulation (5.04–5.10%) (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. 10*a*). 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. 10*b*). In these pathways, the DEGs were significantly expressed in TGF-β signaling pathway, Notch signaling pathway, Jak-STAT signaling pathway, ErbB signaling pathway, ABC transporter,

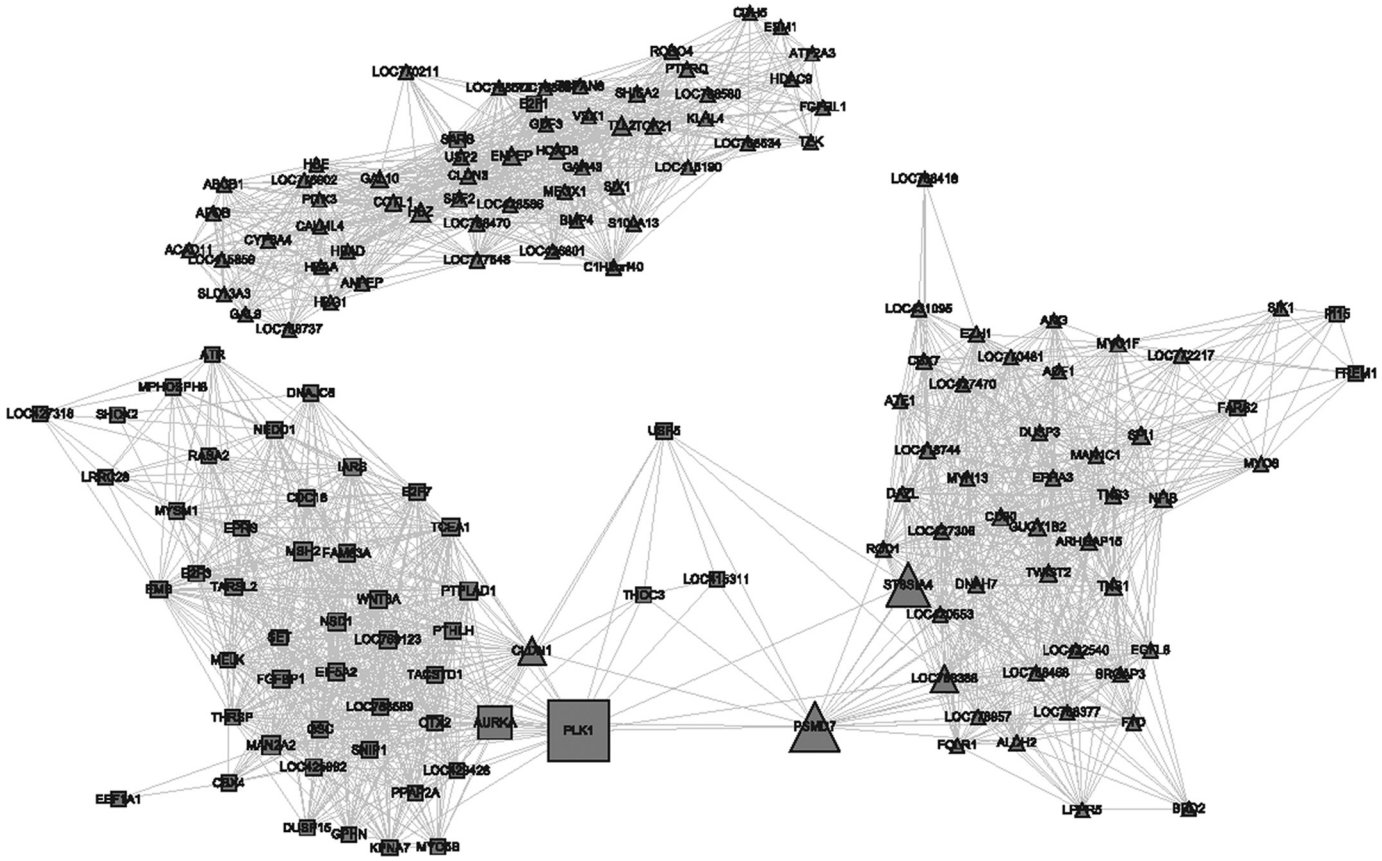

FIGURE 8. **Gene regulatory network analysis.**

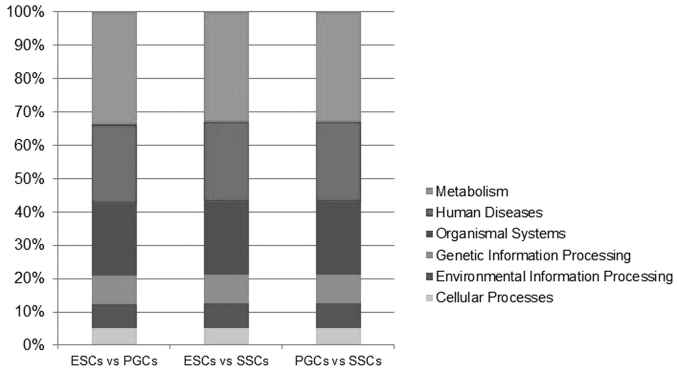

FIGURE 9. **KEGG pathway enrichment analysis of the DEGs.**

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. 10*c*). 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

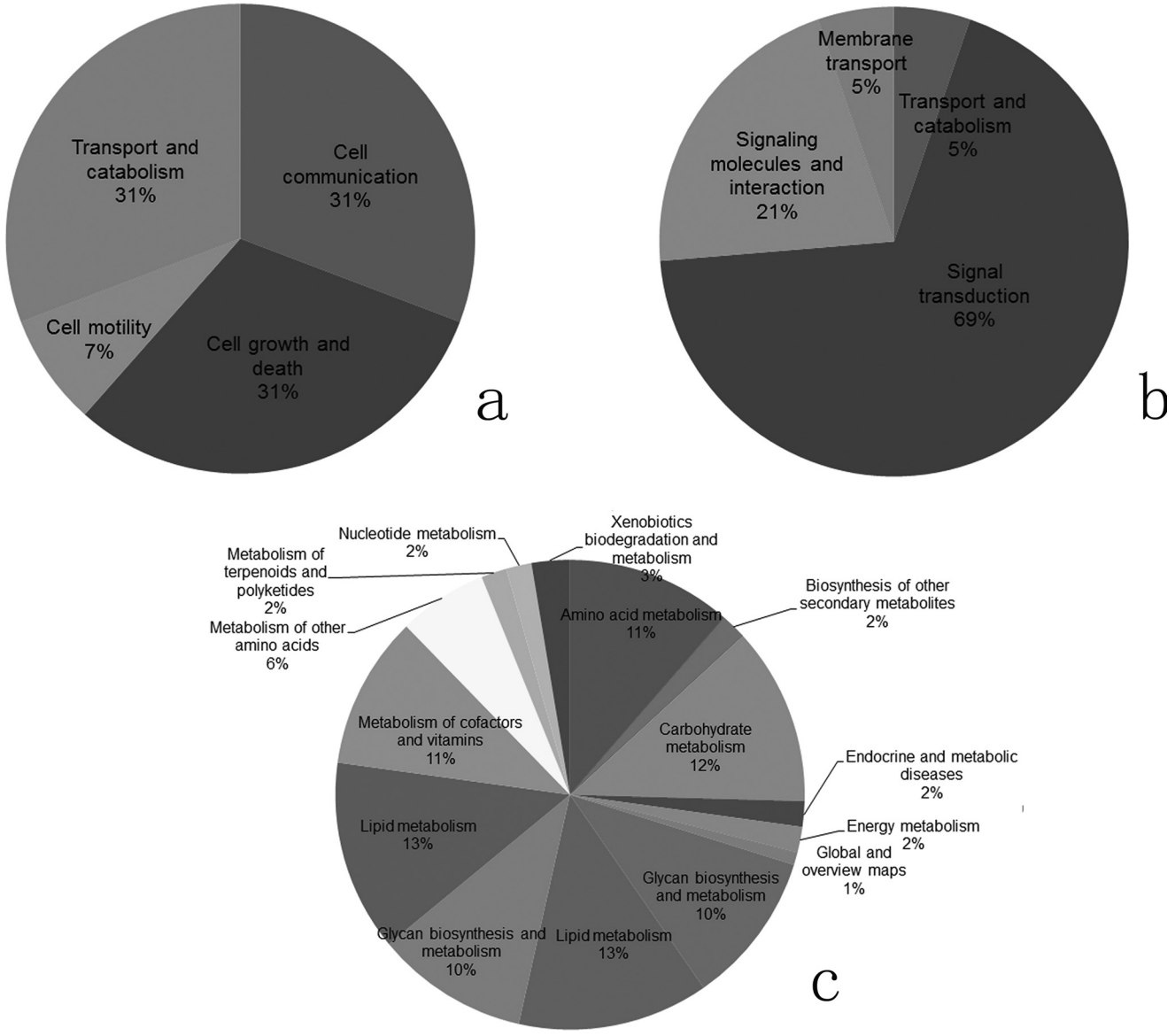

FIGURE 10. **Screening of the candidate pathways involved in male germ cell development.** *a*, classification of the 13 enriched pathways related to cellular processes regulation. *b*, distribution of the participating 19 pathways in environmental information regulatory process. *c*, percentages of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

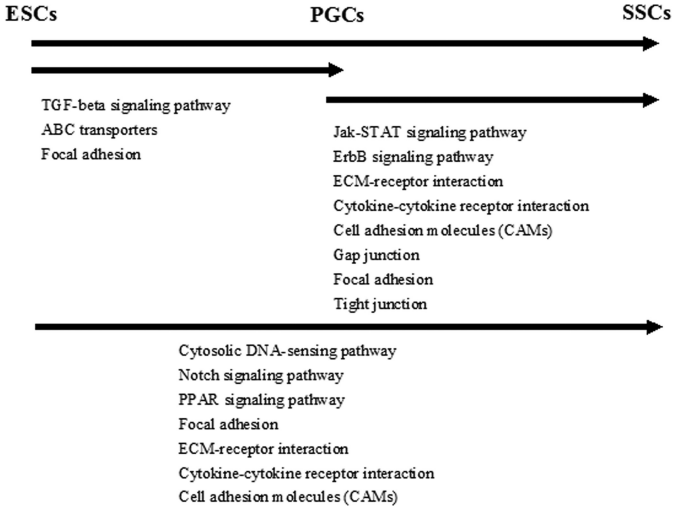

FIGURE 11. **Signaling regulate different stages of male germ cell.**

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

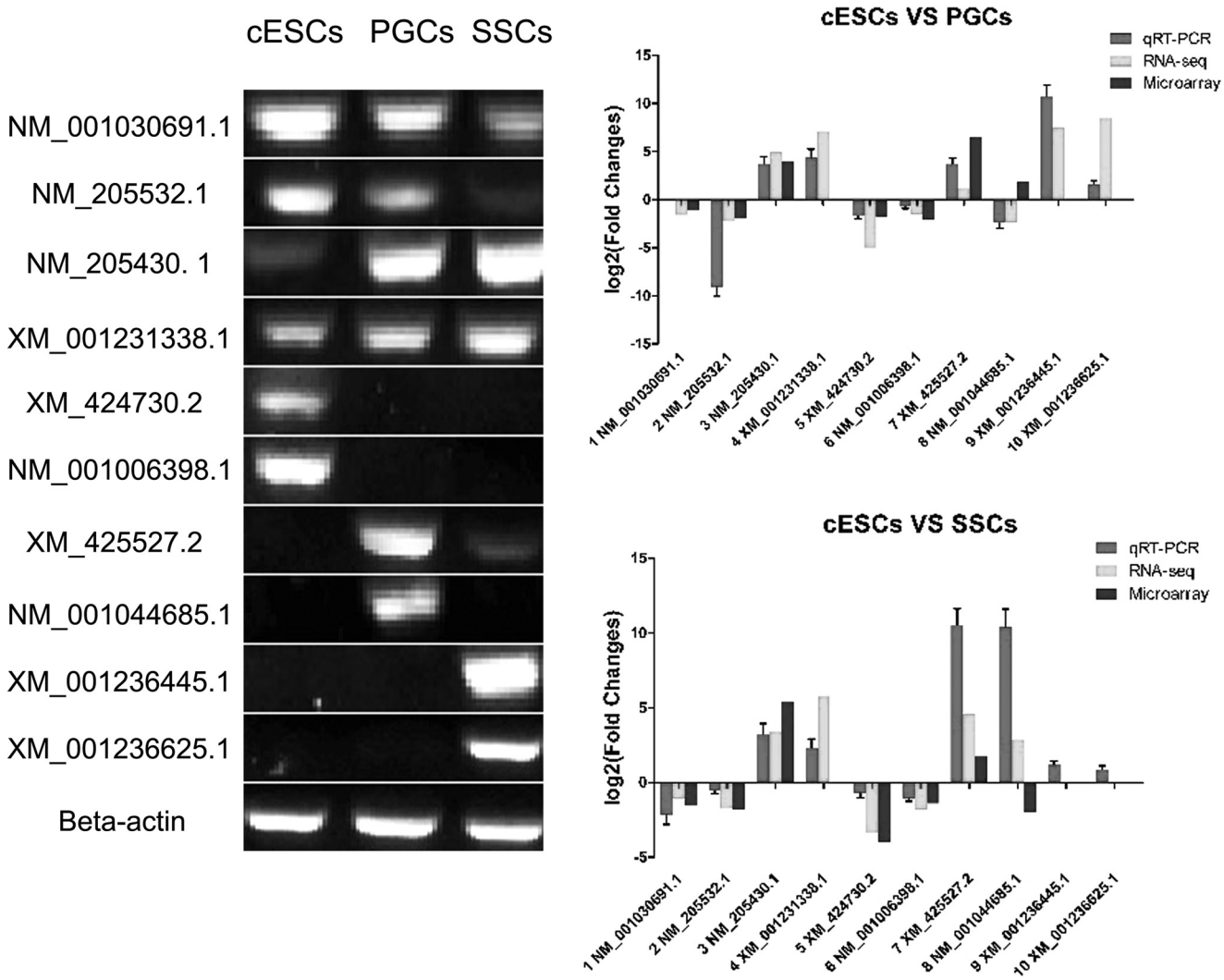

FIGURE 12. **Validation of RNA-seq results by quantitative PCR.**

decline at the day eight after induction. 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

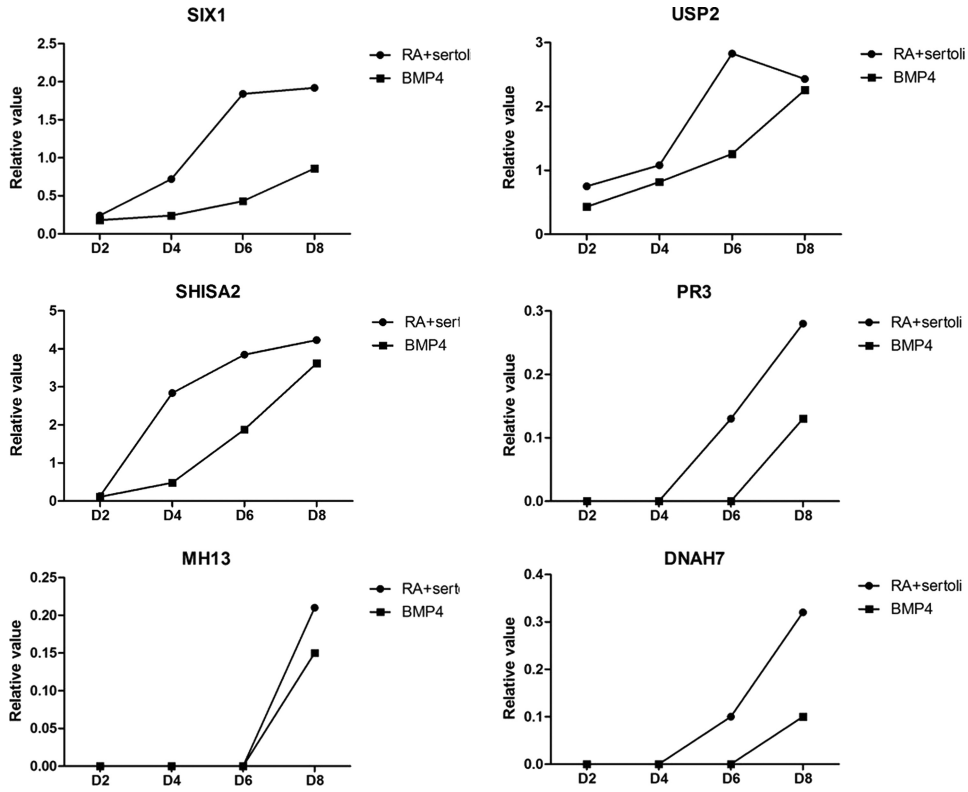

FIGURE 13. **Express trend of mark genes during differentiation.**

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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