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RESEARCH ARTICLE

DMRT1 gene disruption alone induces incomplete gonad feminization in chicken

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Abstract

Compared with the well-described XY sex determination system in mammals, the avian ZW sex determination system is poorly understood. Knockdown and overexpression studies identified doublesex and mab-3-related transcription factor 1 (*DMRT1*) as the testis-determining gene in chicken. However, the detailed effects of *DMRT1* gene disruption from embryonic to adult development are not clear. Herein, we have generated *DMRT1*-disrupted chickens using the clustered regularly interspaced short palindromic repeats-associated protein 9 system, followed by an analysis of physiological, hormonal, and molecular changes in the genome-modified chickens. In the early stages of male chicken development, disruption of *DMRT1* induced gonad feminization with extensive physiological and molecular changes; however, functional feminine reproductivity could not be implemented with disturbed hormone synthesis. Subsequent RNA-sequencing analysis of the *DMRT1*-disrupted chicken gonads revealed gene networks, including several novel genes linearly and non-linearly associated with *DMRT1*, which are involved in gonad feminization. By comparing the gonads of wild type with the genome-modified chickens, a set of genes were identified that is involved in the ZW sex determination system independent of *DMRT1*. Our results extend beyond the Z-dosage hypothesis to provide further information about the avian ZW sex determination system and epigenetic effects of gonad feminization.

Abbreviations: AR, androgen receptor; αSMA, anti-α smooth muscle actin antibody; CASI, cell autonomous sex identity; CMV, cytomegalovirus; CRISPR/Cas9, clustered regularly interspaced short palindromic repeats-associated protein 9; CVH, chicken vasa homolog; CYP19A1, cytochrome P450 family 19 subfamily A member 1; DMRT1, doublesex and mab-3-related transcription factor 1; ESR1, estrogen receptor 1; ESR2, estrogen receptor 2; FOXL2, forkhead box L2; FSHR, follicle-stimulating hormone receptor; GFP, green fluorescence protein; GSD, genetic sex determination; HEMGN, hemogen; HH, Hamburger and Hamilton stage; LHCGR, luteinizing hormone/choriogonadotropin receptor; lncRNA, long non-coding RNA; MEF, mouse embryonic fibroblasts; MHM, male hypermethylated region; MSL, male-specific lethal; MSY, male-specific region of the Y chromosome; NHEJ, non-homologous end joining; PGC, primordial germ cells; PGR, progesterone receptor; RSPO1, R-spondin 1; SERPINB11, serpin family B member 11; SOX9, SRY-box transcription factor 9; SRY, sex determining region Y; TMM, trimmed mean of M-values; TOM, topological overlap matrix; WGCNA, weighted correlation network analysis; WL, White Leghorn; WNT4, Wnt family member 4; WT, wild-type.

Hong Jo Lee and Minseok Seo contributed equally to this work.

KEYWORDS
chicken, DMRT1, genome editing, sex determination

1 | INTRODUCTION

Genetic sex determination is accomplished by the master sex determination factor located on the sex chromosome. In mammals, a male-specific region of the Y chromosome (MSY) consists of genes responsible for male sex determination, as well as testicular development and functions.[1] The sex determining region Y (*SRY*) is an MSY-linked transcription factor gene that determines male sex in mammals.[2] *SRY* associates with or activates a number of genes, particularly SRY-box transcription factor 9 (*SOX9*), which is closely linked to male-specific functions, including regression of Mullerian duct and development of Wolffian duct, Sertoli cells, and male germ cells.[3,4] Mutations in the *SRY* gene cause male-to-female sex reversal and subsequent gonadal dysgenesis, probably due to insufficiency of its downstream testis determining factors, and activation of the default ovary determining factors such as forkhead box L2 (*FOXL2*), R-spondin 1, Wnt family member 4, and cytochrome P450 family 19 subfamily A member 1 (*CYP19A1*; also known as aromatase).[5,6]

In avian species, the male sex is homogametic (ZZ) and the female sex is heterogametic (ZW), and sex identity is largely cell autonomous and with less effects of sex steroid.[7,8] To date, there is no evidence for the presence of *SRY* gene in avian species; however, doublesex and mab-3-related transcription factor 1 (*DMRT1*), which is linked to the Z chromosome, was identified as the most reliable testis determining factor due to its Z-dosage effect.[9] DMRT1, a transcription factor containing a zinc finger-like DNA-binding motif (DM domain), is conserved in most mammalian, avian, reptile, amphibian, and fish species.[10–17] *DMRT1* regulates gonadal differentiation, gametogenesis, and sex determination in several species.[18] *DMY*, an orthologues of *DMRT1*, is the master sex determination factor in Medaka fish (*Orzias latipes*),[19] while another report suggests *DMRT1* is necessary for testis development in zebrafish (*Danio rerio*).[20] Studies in frog, turtle, mouse, and human revealed that *DMRT1* and its orthologues play critical roles in testis development.[21–26]

In chicken, *DMRT1* is expressed in the genital ridge of both sexes; then, it is expressed exclusively in the male embryonic gonad from E4.5 to E6.5 (the time of gonadal development).[10] RNA interference-mediated knockdown of *DMRT1* in chicken leads to feminization of the embryonic gonads in male embryos, whereby the left gonad shows female-like histology, decreased level of *SOX9*, and increased expression of *CYP19A1*.[9] Targeted mutations in the *DMRT1* gene in chicken supported that *DMRT1* is one of the most critical factors for testis development.[27] By contrast, overexpression of *DMRT1* promotes expression of male sex determination pathway-related genes, including *AMH*, *SOX9*, and hemogen, and represses expression of female sex determination pathway-related genes, including *FOXL2* and *CYP19A1*, resulting in testis development in female embryos.[17] Moreover, *DMRT1* is expressed in the paired Mullerian ducts of the chicken embryo. When *DMRT1* is knocked-down, the mesenchymal layer of developing Mullerian ducts is diminished, indicating that the *DMRT1* is also required for Mullerian duct development during early embryogenesis.[28]

Despite the notable discovery that *DMRT1* plays a role in chicken testis development during early embryogenesis, there is still no comprehensive analysis of the transcriptional network for *DMRT1*. Furthermore, the effects of *DMRT1* disruption on the physiological, hormonal, and molecular changes in both sexes of chickens are still not clear. Therefore, in this study, *DMRT1*-disrupted chickens were produced using clustered regularly interspaced short palindromic repeats (CRISPR)-associated protein 9 (CRISPR/Cas9) and non-homologous end-joining (CRISPR/Cas9-NHEJ)-mediated genome modification in chicken primordial germ cells (PGCs). Next, the *DMRT1*-disrupted chickens were analyzed for physiological, hormonal, and molecular changes in the gonads from embryonic to adult stages of development. Subsequently, global transcriptome changes in *DMRT1*-disrupted chicken gonads were analyzed by RNA sequencing.

2 | MATERIALS AND METHODS

2.1 | Experimental animals and animal care

The management and experimental use of chickens were approved by the Institute of Laboratory Animal Resources, Seoul National University (SNU-190401-1-1). The experimental animals were cared according to a standard management program at the University Animal Farm, Seoul National University, Korea. The procedures for animal management, reproduction, and embryo manipulation adhered to the standard operating protocols of our laboratory.

2.2 | Construction of CRISPR/Cas9 expression plasmids and donor plasmids

We constructed all-in-one CRISPR/Cas9 plasmids targeting the translation start site (ATG) of *DMRT1* gene. The CRISPR kit used for constructing multiplex CRISPR/Cas9 plasmids was provided by Takashi Yamamoto (Addgene Kit #1000000054).[29] For insertion of guide RNA (gRNA) sequences into the CRISPR/Cas9 plasmids, we designed sense and antisense oligonucleotides (Table S1) and synthesized by Bionics (Seoul, Korea). The annealing of sense and antisense oligonucleotides was carried out under the following thermocycling conditions: 30 s at 95°C, 2 min at 72°C, 2 min at 37°C, and 2 min at 25°C. For targeted gene insertion into *DMRT1* gene, the neomycin resistance gene with thymidine kinase promoter and a green fluorescence protein (GFP) gene with a cytomegalovirus (CMV) promoter from the *piggyBac* CMV-GFP-FRT plasmid[30] were cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA) using forward and reverse primers (Table S1) containing gRNA recognition sequences.

2.3 | Transfection and G418 selection of DF-1 and PGCs for targeted gene insertion

Chicken DF-1 fibroblast cells (CRL-12203; ATCC, Manassas, VA, USA) were maintained and sub-passaged in Dulbecco's minimum essential medium (DMEM; Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone) and 1× antibiotic–antimycotic (Thermo Fisher–Invitrogen, Carlsbad, CA, USA). DF-1 cells were cultured in an incubator at 37°C with 5% CO_2 and 60%–70% relative humidity. For genome editing in chicken DF-1, CRISPR/Cas9 plasmids (3 μg) and donor plasmids (3 μg) were mixed with 6 μl of Lipofectamine 2000 reagent (Thermo Fisher–Invitrogen) in 1 ml Opti-MEM (Thermo Fisher–Invitrogen), and this mixture was applied to 5×10^5 DF-1. About 6 h after transfection, the transfection mixture was replaced with DF-1 culture medium. Geneticin Selective Antibiotic (G418; GIBCO Invitrogen, Grand Island, NY, USA) (300 μg/ml) was added to the culture medium 1 day after transfection. The complete selection period was up to 7 days.

For PGC line establishment, the White Leghorn (WL) PGCs were maintained and sub-passaged on knockout DMEM (Invitrogen) supplemented with 20% FBS (Invitrogen), 2% chicken serum (Sigma-Aldrich, St. Louis, MO, USA), 1× nucleosides (Millipore, Temecula, CA, USA), 2 mM L-glutamine, 1× nonessential amino acids, β-mercaptoethanol, 10 mM sodium pyruvate, 1× antibiotic–antimycotic (Invitrogen), and human basic fibroblast growth factor (10 ng/ml; Sigma-Aldrich). Chicken PGCs were cultured in an incubator at 37°C under an atmosphere of 5% CO_2 and 60%–70% relative humidity. The PGCs were sub-cultured onto mitomycin-inactivated mouse embryonic fibroblasts at 5- to 6-day intervals via gentle pipetting. For genome editing in chicken PGCs, CRISPR/Cas9 plasmids (2 μg) and donor plasmids (2 μg) were co-introduced into 1×10^5 cultured PGCs with 4 μl of Lipofectamine 2000 reagent suspended in 1 ml Opti-MEM. Then, 4 h after transfection, the transfection mixture was replaced with PGC culture medium. Geneticin Selective Antibiotic (G418; 300 μg/ml) was added to the culture medium 1 or 2 days after transfection. The selection period was up to 3 weeks.

2.4 | Production of *DMRT1*-disrupted chickens

To produce genome-modified chickens, a window was cut at the sharp end of the Korean Ogye recipient egg, and more than 3000 genome-modified WL PGCs were transplanted into the dorsal aorta of Hamburger and Hamilton stages 14–17 recipient embryos. The egg window was sealed with paraffin film, and the eggs were incubated with the pointed end down until hatching. The hatched chicks were raised in the institutional animal farm. After sexual maturation, sperms from the recipient chickens were evaluated by breed-specific PCR conditions, and the chickens that have WL sperms were mated with WT female chickens. Genome-modified chickens were identified based on offspring feather color and subsequent genomic DNA analysis, and the sex of the chickens was evaluated by Z and W chromosome-specific PCR.[31]

2.5 | Detection of targeted gene insertion

To identify modified alleles in chicken DF-1, PGCs, and genome-modified chickens, genomic DNA was analyzed using knock-in PCR analysis. All reactions were performed under the same conditions, with a total PCR volume of 20 μl containing 100 ng of genomic DNA, 10× PCR buffer, 0.4 μl dNTP (10 mM each), 10 pmol of each primer (Table S1), and 0.5 U *Taq* polymerase (Bionics) under the following thermocycling conditions: 2 min at 95°C followed by 35 cycles of 20 s at 95°C, 40 s at 60°C, and 30 s at 72°C, and finally, 5 min at 72°C. For sequencing analysis, the amplicons were cloned into the pGEM-T-easy vector and sequenced using an ABI Prism 3730 XL DNA Analyzer (Thermo Fisher-Applied Biosystems, Foster City, CA, USA). The sequences were compared against assembled genomes using BLAST (http://blast.ncbi.nlm.nih.gov).

2.6 | Off-target analysis based on whole-genome sequencing

To identify off-target effects in *DMRT1*-disrupted chickens, genomic DNA from three *DMRT1*$^{-/Z}$ and three WT male chickens was extracted using the DNeasy Blood & Tissue Kit (QIAGEN, Germantown, MD, USA) and then sequenced using the Illumina NovaSeq 6000 System (Illumina, San Diego, CA, USA). Raw-read sequences generated from the six samples were cleaned-up using Trimmomatic (ver 0.39).[32] The clean reads were mapped to the GRCg6a genome using Bowtie2 (ver. 2.4.2). Single nucleotide polymorphisms and indels (insertions/deletions) were identified using bcftools (ver. 1.10.2). Identified variants with QUAL < 20 and DP < 30 were filtered out. Finally, variants that consistently showed different genotypes in three biological replications between *DMRT1*$^{-/Z}$ and *DMRT1*$^{Z/Z}$ groups were selected.

The potential off-target sites of the gRNA were estimated using Cas-OFFinder.[33] Less than two nucleotide mismatches and two DNA or RNA bulges compared to *DMRT1* gene targeting gRNA strand were regarded as potential off-sites. Variants at the nearest distance around these potential off-sites were searched. Based on the variants identified by whole-genome sequencing, the closest variants to these potential sites were investigated. No off-site mutation was found at the expected site, and the variant found at the nearest distance was 43 bp away from the potential off-site (Table S2).

2.7 | Histological analysis and immunohistochemical staining of tissues

Gonadal tissues, testis, and ovaries from the *DMRT1*$^{-/Z}$ and *DMRT1*$^{-/W}$ chickens ($n > 3$ at E6, E8, E10, E14, 1 W, 4–6 W, and 11–14 W. $n = 1$ at 30 W) along with WT male and female chickens were fixed in 4% paraformaldehyde (PFA) at 4°C overnight. Tissues were dehydrated in gradient ethanol, made transparent in xylene, and embedded in paraffine. Embedded tissues were cut into 10 μm sections, which were deparaffinized and rehydrated for histological analysis and immunohistochemistry. The sections on the slides were immediately stained with hematoxylin and eosin and dehydrated through graded ethanol solutions followed by a xylene-based mountant. For immunohistochemistry, tissue sections (after deparaffinization for paraffine-embedded tissues; thickness 10 μm) were washed three times with 1× phosphate-buffered saline (PBS) and blocked with blocking buffer (PBS containing 5% goat serum and 1% bovine serum albumin) for 1 h at room temperature. Samples were then incubated at 4°C overnight with rabbit anti-chicken vasa homolog (CVH) antibody,[34] DMRT1 rabbit anti-human polyclonal (aa98-128) antibody (LS-408137, LSBio, Seattle, WA, USA), anti-SOX9 antibody (AB5535, Sigma-Aldrich),[35] FOXL2-specific polyclonal antibody (#19672-1-AP, Proteintech, IL, USA),[36] or anti-α smooth muscle actin antibody (αSMA, ab5694, Abcam).[37] After washing three times with 1× PBS, samples were incubated with secondary antibodies labeled with phycoerythrin (PE) for 2 h at room temperature. Samples were then mounted with 4′,6-diamidino-2-phenylindole (DAPI, Vector Laboratories, CA, USA) and visualized using confocal microscopy (Carl Zeiss Microscopy LLC, NY, USA).

2.8 | In situ hybridization analysis

Localization of serpin family B member 11 (*SERPINB11*) mRNA in sections of chicken oviduct and vas deferens was determined by in situ hybridization. Briefly, for hybridization probes, total RNA from adult magnum was reverse transcribed, and the resulting cDNA was amplified using *SERPINB11*-specific primers (F: 5′-AGA TCC TGG AGC TCC CAT TT-3′ and R: 5′-AAT GGT GTT GGT TGG GTT GT-3′; product size: 452 bp) and ENSGAL00000051419-specific primers (F: 5′-CAA GAC AAC TCA GTG GCA GC-3′ and R: 5′-CTG CTC CGA AGA ACC AGA CC-3′; product size: 448 bp). PCR products of the correct size were cloned into the pGEM-T-easy vector (Promega). After verification of the sequences, plasmids containing gene sequences were amplified with T7- and SP6-specific primers (T7:5′-TGT AAT ACG ACT CAC TAT AGG G-3′; SP6:5′-CTA TTT AGG TGA CAC TAT AGA AT-3′). Then digoxigenin (DIG)-labeled RNA probes were transcribed using a DIG RNA labeling kit (Roche Applied Science, Indianapolis, IN). Tissues were collected, fixed in 4% PFA, embedded in paraffin, sectioned at 10 μm, and placed on APES-treated (silanized) slides. The tissue sections were then deparaffinized in xylene and rehydrated to diethylpyrocarbonate (DEPC)-treated water through a graded series of ethyl alcohol. The sections were treated with 1% Triton X-100 in PBS for 20 min and washed twice in DEPC-treated PBS. A standard protocol for in situ hybridization in chickens was followed.[38]

2.9 | Hormone assay

Blood was collected from the *DMRT1*$^{-/Z}$ and *DMRT1*$^{-/W}$ adult chickens ($n = 1$ each) along with WT male and female chickens ($n = 3$ each). The blood samples were allowed to clot for 2 h at room temperature or overnight at 4°C before centrifugation for 15 min at 1000× *g*. The collected serum was used for hormone assay immediately or aliquoted and stored at −20°C for later use. Then, the

serum levels of testosterone (T) and estradiol (E2) were quantified by ELISA method according to the hormone kit procedure. The hormone kits for chicken testosterone (LS-F10117) and chicken estradiol (LS-F10110) were purchased from the LifeSpan BioSciences, Inc (LSBio, Seattle, WA, USA).

2.10 | qRT-PCR analysis

Total RNA of the gonadal tissues from the *DMRT1*$^{-/Z}$ and *DMRT1*$^{-/W}$ chickens along with WT male and female chickens were isolated using the Trizol Reagent (Thermo Fisher-Invitrogen) according to the manufacturer's instructions, and the RNA quantity was determined using NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). cDNA was synthesized from the RNA (1 μg) using the Superscript III First-Strand Synthesis System (Thermo Fisher-Invitrogen). The cDNA was serially diluted fivefolds and was quantitatively equalized for PCR amplification. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed to examine the expression levels of chicken follicle stimulating hormone receptor (*FSHR*), luteinizing hormone/choriogonadotropin receptor (*LHCGR*), androgen receptor (*AR*), estrogen receptor 1 (*ESR1*), estrogen receptor 2 (*ESR2*), and progesterone receptor (*PGR*). All reactions were performed under the same conditions, with a total PCR volume of 20 μl containing 2 μl of cDNA, 10× PCR buffer, 0.4 μl dNTP (10 mM each), 10 pmol of each primer (Table S1), 20× EVA green, and 0.5 U *Taq* polymerase (Bionics) under the following thermocycling conditions: 3 min at 95°C followed by 40 cycles of 30 s at 95°C, 30 s at 60°C, and 30 s at 72°C, and finally, at the melting temperatures. Each test sample was run in triplicate. The relative quantification of gene expression was normalized with the *GAPDH*, and analyzed by the $2^{-\Delta Ct}$ method.

2.11 | cDNA library construction and RNA sequencing

Total RNA was isolated from the gonadal tissues of *DMRT1*$^{-/Z}$ and *DMRT1*$^{-/W}$ chickens along with WT male and female chickens using Maxwell (Promega)-based method. Total RNA integrity was checked using an Agilent Technologies 2100 Bioanalyzer and RiboGreen dye (Invitrogen), and quantified using a Trinean DropSense96 spectrophotometer. One milligram of total RNA was processed for preparing mRNA sequencing library using MGIEasy RNA Directional Library Prep Kit (MGI) according to the manufacturer's instruction.

2.12 | Expression quantification of RNA sequencing

Fundamental quality control was performed by excluding the adapter sequence from the raw paired-end sequence generated from 24 subjects using Trimmomatic (ver 0.39)[32] with TruSeq3-PE-2.fa:2:30:10 MINLEN:75 options. The generated clean reads were aligned using HISAT2 (ver 2-2.1.0)[39] on the Ensembl Chicken reference genome (*Gallus gallus* 6.0a). Subread (ver 2.0.0) with strand-specific options were employed to quantify the mapped read.[40] There were no quality control issues in all 24 samples.

2.13 | Co-expression network analysis

Based on the gene expression level of all quantified 23 650 genes, weighted correlation network analysis[41] was performed to identify linear co-expression patterns in 24 subjects. We used the trimmed mean of M-values (TMM) normalized expression in co-expression analysis. We used the soft threshold method,[41] which uses a point where the power is 0.9. The topological overlap matrix-based dissimilarity was used as the distance metric, and an unsigned network was constructed to include both positive and negative correlations among genes. The option was decided so that at least 30 co-expressed genes should be included to be determined by the co-expression module.

2.14 | Statistical analysis

Statistical tests were performed using edgeR[42] based on quantified raw counts from the RNA-sequencing pipeline. We used a three-way ANODEV model to consider all three key experimental factors: sex (testis and ovary), *DMRT1* gene disruption (case and control), and time point (E6 and 1 W). Since there are three biological replications for all conditions, the interaction term with *DMRT1* gene disruption was considered to investigate the non-linear effect of *DMRT1*. We considered significant results in all statistical tests at 5% significance level after multiple testing adjustments.[43] We used the log2-fold change (logFC) calculated from edgeR.

2.15 | Functional enrichment analysis

The PANTHER software[44] was used to explore functional characteristics of the identified genes. We performed a statistical overrepresentation test with Ensembl gene IDs from each gene expression analysis at 5% significance

level. The list of reference genes used is Gallus gallus annotation. The observed functional terms and genes were visualized in a network format using qgraph R package. The relationship to the functional term network was determined according to the similarity of genes corresponding to each functional term. In the gene network, relationship was represented based on Pearson's correlation.

2.16 | Investigation of DMRT1's DM-domain binding site

Among the mechanisms known for the regulation of gene expression performed by DMRT1 so far, the regulation mechanism through binding of the DM domain revealed in the mouse model is representative. In the existing mouse model study, 1477 candidate genes with DMRT1 binding sites were identified.[45] We identified 1:1 orthologue gene between these 1477 DMRT1 binding genes and the annotated genes of galGal 6.0a reference genome. It was performed based on the gene model of Ensembl Biomart version 100, and only results with "high orthology confidence" and "1:1 homolog relationship" were considered. As a result, we found 743 genes that are supposed to have DMRT1's DM binding sites in chicken.

2.17 | Data availability

The whole-genome sequencing data used in the off-site analysis were uploaded to the SRA database (PRJNA694352). All RNA-sequencing data generated in this study have been submitted to the GEO database under accession number GSE150339.

3 | RESULTS

3.1 | CRISPR/Cas9–NHEJ-mediated disruption of *DMRT1* in chickens

We applied the CRISPR/Cas9–NHEJ-mediated genome editing method to generate *DMRT1*-disrupted chickens, as previously reported.[46] By introducing the CRISPR/Cas9 and donor plasmids into chicken cells, the expression of *DMRT1* can be disturbed by targeted insertion of the donor plasmid (Figure 1A). To validate the targeted insertion in chicken cells, we applied the strategy to a chicken somatic cell line (DF-1) and PGCs. By introducing the CRISPR/Cas9 and donor plasmids, the transfected cells expressed GFP. Therefore, GFP-expressing DF-1 cells (Figure 1B) and PGCs (Figure 1D) could be selected from the population by drug selection. Subsequent sequencing analysis of the 5′ junction between the endogenous gene and donor plasmid demonstrated successful integration into the *DMRT1* locus of chicken DF-1 cells (Figure 1C) and PGCs (Figure 1E), as well as the presence of indel mutations that will disrupt the gene. To generate genome-modified chickens, *DMRT1*-modified WL PGCs were transplanted into recipient Korean Ogye males and then subsequently crossed with wild-type (WT) WL females. The germline transmission efficiency varied from 3.8% to 76.9% in each germline chimeric chicken, while the genome modification efficiency, which is distinguishable by GFP expression (Figure 1F), was 12.5% to 50.0% (Table S3). Sequencing analysis of genomic DNA from three representative genome-modified chickens (one female and two males) showed that the donor plasmid was integrated into the target loci with identical indel mutations in *DMRT1* (Figure 1G). The off-target analysis based on whole-genome sequencing demonstrated that *DMRT1* editing was specific (Table S2).

3.2 | *DMRT1* gene disruption induces morphological and molecular changes in the chicken

To identify the effects of *DMRT1* disruption, we first traced the development of reproductive organs, including the testis, ovary, and oviduct of *DMRT1*-disrupted chickens, during embryogenesis. At embryonic day 6 (E6), there were no morphological differences between the gonads of WT and genome-modified embryos in either sex (Figure 2A). However, from E8, morphological differences were observed. The size of the left gonads of heterozygous knockout male ($DMRT1^{-/Z}$) embryos was similar to those of WT female embryos from E8 onwards (Figure S1A), suggesting feminization of the gonads of $DMRT1^{-/Z}$ chickens, as previously described.[9]

Histological and immunohistochemical analyses showed no significant difference in terms of morphology at E6; however, the expression of sex-specific markers was altered distinctively. The Sertoli cell markers, DMRT1 and SOX9, were expressed at low levels, while there was a slight increase in FOXL2 expression in the $DMRT1^{-/Z}$ chicken embryonic gonads. DMRT1 and SOX9 expression were absent from the gonads of *DMRT1* gene-modified female chickens ($DMRT1^{-/W}$), which also showed reduced expression of the granulosa cell and theca cell markers FOXL2 and αSMA (Figure 2B). Subsequent transcriptional analysis confirmed that *DMRT1* disruption decreases expression of *DMRT1* in the embryonic gonads of both sexes. *DMRT1* disruption also increases the expression of *FOXL2*, and decreased that of *SOX9* in embryonic gonads (Figure 2C), which indicates that *DMRT1* is indispensable

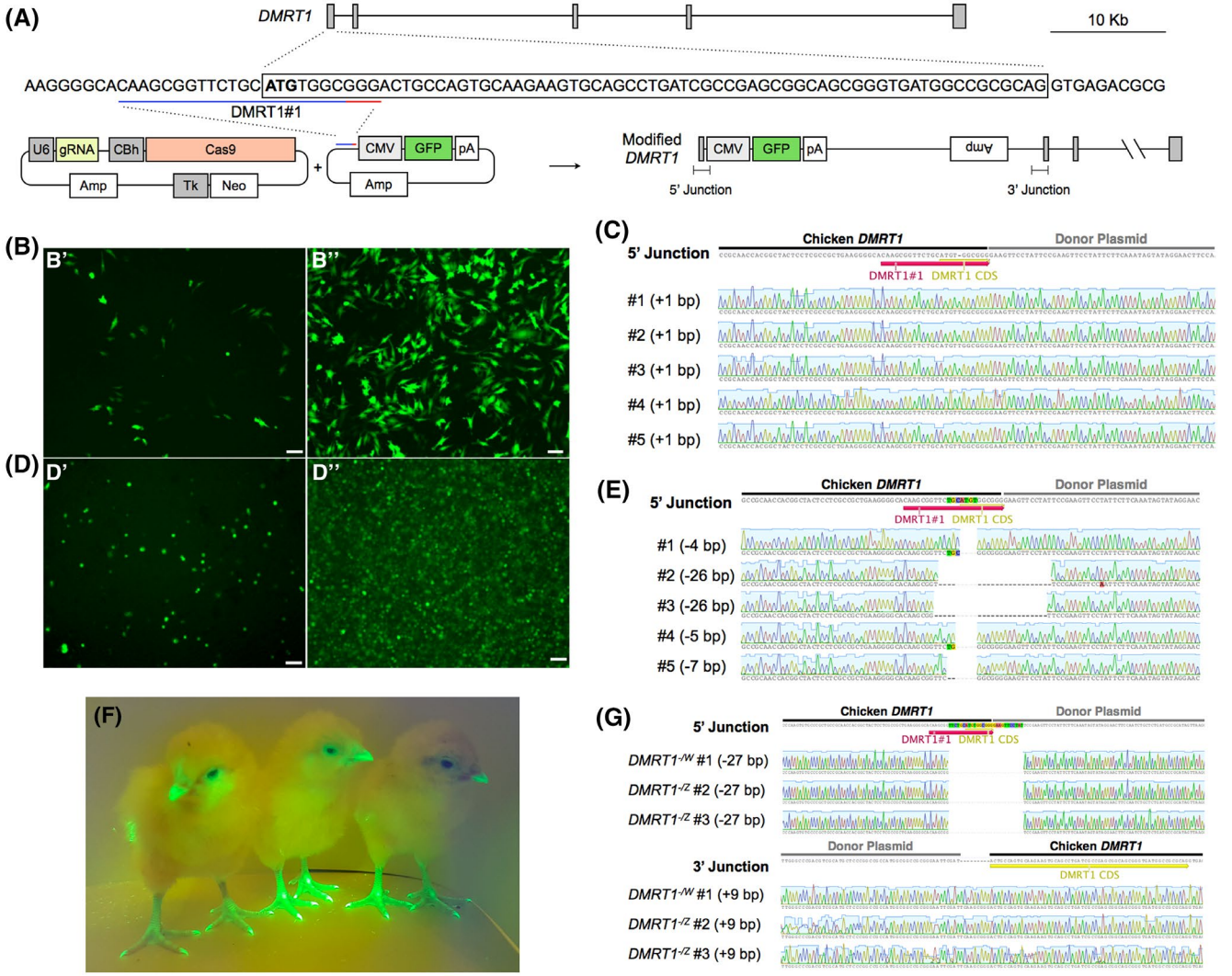

FIGURE 1 Targeted gene disruption of the *DMRT1* gene in chicken. (A) Schematic of the CRISPR/Cas9 vector containing a gRNA targeting *DMRT1* (DMRT1#1), and a donor plasmid containing the same gRNA recognition site and a green fluorescent expression cassette. In the modified allele, the *DMRT1* gene was disrupted by the targeted insertion of the donor plasmid. The targeted insertion was confirmed by sequencing the 5′ or 3′ junction between the endogenous *DMRT1* gene and the donor plasmid. Grey rectangles indicate exons. Scale bar = 1 Kb. Blue bar indicates the gRNA binding site, and red bar indicates the protospacer adjacent motif sequence. Targeted insertion into the chicken *DMRT1* gene in chicken DF-1 cells (B) and PGCs (D). GFP expression in the CRISPR/Cas9 and donor plasmid-introduced chicken DF-1 (B') and PGCs (D'). GFP-expressing DF-1 (B") and PGCs (D") were enriched by drug selection. Scale bar = 100 μm. Sequencing analysis of the GFP-expressing DF-1 (C) and PGCs (E). Targeted gene insertion was confirmed by sequencing analysis of the 5′ junction region between endogenous *DMRT1* and the integrated donor plasmid. Five TA clones were analyzed, and the patterns of indel mutation were identified. (F) GFP expression in the *DMRT1*-disrupted genome-modified chickens. (G) Sequencing analysis of the GFP-expressing *DMRT1* gene-disrupted chickens. Targeted gene insertion was confirmed by sequencing analysis of the 5′ and 3′ junction regions between endogenous *DMRT1* and donor plasmid. Three representative genome-modified chickens were analyzed by Sanger sequencing, and the patterns of indel mutation are shown. Red arrow indicates the *DMRT1* target site, yellow arrow indicates the DMRT1 coding sequence (CDS), and donor plasmid is presented. Amp, ampicillin-resistant gene; CBh, chicken beta-actin short promoter; CMV, cytomegalovirus promoter; GFP, green fluorescent protein; Neo, neomycin-resistant gene; pA, poly A signal; TK, thymidine kinase promoter; U6, human U6 promoter

for Sertoli, granulosa, and theca cell differentiation from gonadal mesenchymal precursors.[47]

In the *DMRT1*$^{-/Z}$ chickens at 1-week of age, the left gonad resembled the WT ovary with a morphological oviduct attached, whereas the right gonad degenerated. Seminiferous tubules were absent in the gonads of 1-week-old *DMRT1*$^{-/Z}$ chickens, which contained a distinct medulla and cortex region similar to the ovaries of WT females (Figure 3A). The gonads of E14 and 1-week-old *DMRT1*$^{-/Z}$ chickens weakly expressed FOXL2 and αSMA,

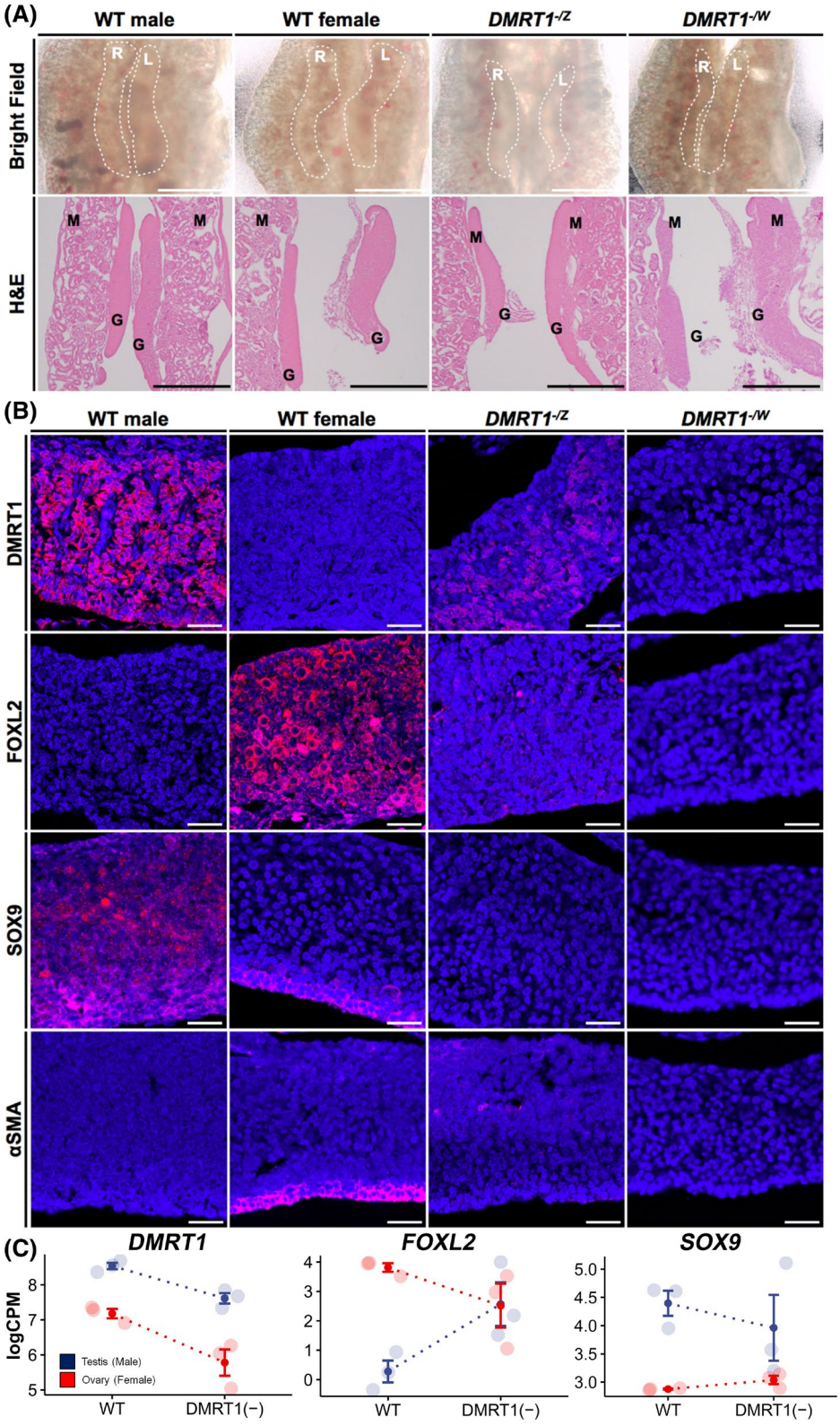

FIGURE 2 Morphological, histological, and immunohistochemical analysis of *DMRT1*-disrupted chicken gonads at embryonic day 6 (E6). (A) Morphology and histological image of wild-type (WT) and *DMRT1*-disrupted chicken gonads at embryonic day 6 (E6). G, gonads; L, left gonads; M, mesonephros; R, right gonads. Gonadal sections were stained with hemotoxylin and eosin (H&E). Scale bars = 1 mm (Bright field image) and 50 μm (H&E staining). (B) Gonadal sections were stained with DMRT1, FOXL2, SOX9, and αSMA primary antibodies, followed by secondary antibodies labeled with PE. Stained sections were then mounted with 4′,6-diamidino-2-phenylindole (DAPI) to visualize cell nuclei. Scale bars = 50 μm. (C) Expression patterns of *DMRT1*, *FOXL2*, and *SOX9* genes in the gonads, which were measured by RNA sequencing

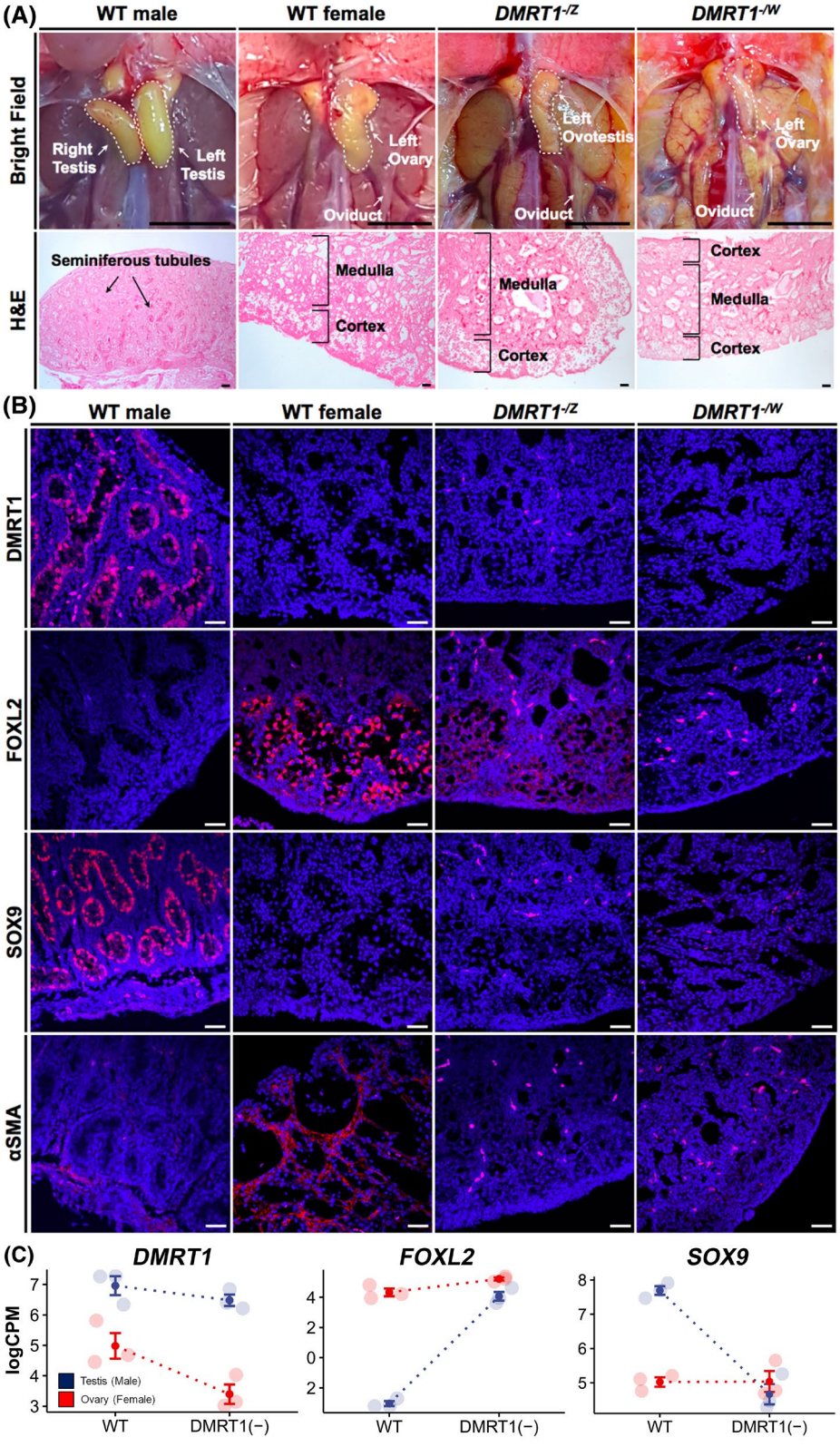

FIGURE 3 Morphological, histological, and immunohistochemical analysis of *DMRT1*-disrupted chicken gonads at 1 week of age (1 W). (A) Morphology and histological image of wild-type (WT) and *DMRT1*-disrupted chicken gonads at 1 week of age (1 W). Gonadal sections were stained with hemotoxylin and eosin (H&E). Scale bars = 1 mm (Bright field image) and 50 μm (H&E staining). (B) Gonadal sections were stained with DMRT1, FOXL2, SOX9, and αSMA primary antibodies, followed by secondary antibodies labeled with phycoerythrin. Stained sections were then mounted with 4′,6-diamidino-2-phenylindole (DAPI) to visualize cell nuclei. Scale bars = 50 μm. (C) Expression patterns of *DMRT1*, *FOXL2*, and *SOX9* genes in gonads, which were measured by RNA sequencing

with a marked reduction in expression of SOX9 and DMRT1 compared to WT male chickens. Transcriptional analysis confirmed that *DMRT1*$^{-/Z}$ chicken embryonic gonads showed increased expression of *FOXL2* and decreased expression of *SOX9* (Figure 3C). In *DMRT1*$^{-/W}$ embryos, the gonads were morphologically similar to those of WT female embryos. FOXL2 and αSMA were also down-regulated in *DMRT1*$^{-/W}$ chicken gonads compared with WT female chicken gonads (Figures 3B and S1B). Taken together, these results indicate that loss of one allele of *DMRT1* causes feminization of ZZ chicken gonads in terms of morphogenesis, but incompletely alters the gene expression profile; these findings are in contrast to those of a report from a mammalian study.[23] Furthermore, *DMRT1* disruption in the ZW animal altered the female-specific gene expression profile; however, it is dispensable for ovary morphogenesis.

3.3 | Immature development of reproductive organs in *DMRT1*$^{-/Z}$ and *DMRT1*$^{-/W}$ chickens

Morphologically, the ovary and oviduct were underdeveloped in the adult (>30 weeks old) *DMRT1*$^{-/Z}$ chickens, although the ovotestis and oviducts developed normally in juvenile chickens (Figure 4A). The external appearance of the *DMRT1*$^{-/Z}$ chicken was normal (Figure 4C); however, there was increased mortality. Histological analysis of the ovotestis of *DMRT1*$^{-/Z}$ chicken at 30 weeks revealed that it contains a cortex, medulla, and immature follicles compared with age-matched WT testis (Figure 4D,F). In *DMRT1*$^{-/W}$ chickens, the ovaries were similar to WT female chickens at early postnatal stages (up until 6 weeks). However, at the adult stage (>30 weeks), the ovary and oviduct were underdeveloped in the *DMRT1*$^{-/W}$ chicken (Figure 4B). The external appearance of the *DMRT1*$^{-/W}$ chickens was also normal (Figure 4C); however, there was also increased mortality as *DMRT1*$^{-/Z}$ chicken. *DMRT1*$^{-/W}$ chicken had only a few follicles in the immature ovary compared with age-matched WT ovary (Figure 4E,G).

The weight of *DMRT1*$^{-/Z}$ and *DMRT1*$^{-/W}$ chickens at 30 weeks was only 1100 and 960 g, respectively, compared with 1750 and 1780 g in age-matched WT male and female chickens, respectively. *DMRT1*$^{-/Z}$ chicken did not produce sperm, and *DMRT1*$^{-/W}$ chicken did not lay eggs. The serum testosterone level of adult *DMRT1*$^{-/Z}$ chicken was significantly lower than that of WT males (Figure S2A), and the serum estradiol level of adult *DMRT1*$^{-/W}$ chicken was significantly lower than that of WT females (Figure S2B). Histological and immunohistochemical analysis showed that *DMRT1*$^{-/Z}$ and *DMRT1*$^{-/W}$ chickens (30 weeks old) have immature oviducts and expressed low levels of the oviduct-specific marker, *SERPINB11*, which is expressed exclusively in the WT female oviduct but not in the WT male vas deferens (Figure S2C).

We further investigated the expression patterns of the hormone receptor genes, including *FSHR*, *LHCGR*, *AR*, *ESR1*, *ESR2*, and *PGR*, in WT and *DMRT1*-disrupted chicken gonads at 1 and 30 weeks old. The results showed that expression of most of the genes was altered in a time- or tissue-dependent manner in the *DMRT1*-disrupted chicken gonads. Specifically, *DMRT1* disruption induced significant differential expression of *PGR*, *ESR1*, *ESR2*, and *AR* in *DMRT1*$^{-/Z}$ chicken gonads at 1 week of age. Additionally, expression of *FSHR*, *LHCGR*, *ESR1*, and *AR* increased significantly in 30-week-old *DMRT1*$^{-/Z}$ chicken compared to WT male chickens. In 1-week-old *DMRT1*$^{-/W}$ chickens, *FSHR*, *PGR*, *ESR1*, and *AR* expression was increased by *DMRT1* disruption; more interestingly, *LHCGR*, *ESR1*, and *AR* expression levels decreased markedly in 30-week-old *DMRT1*$^{-/W}$ chickens (Figure S2D). Collectively, the results indicate that *DMRT1* disruption dysregulates the hormone receptor genes that are critical for female gonadal development.

3.4 | Germ cell depletion in the gonads of *DMRT1*$^{-/Z}$ and *DMRT1*$^{-/W}$ chickens

To examine the status of germ cells in the *DMRT1*-disrupted chickens, the WT and genome-modified male and female gonads in E6, E14, 1-week-old, 4- to 6-week-old, and >30-week-old chickens were subjected to immunohistochemical analysis for the germ cell marker, CVH. The results showed that CVH-positive germ cells were dispersed throughout the embryonic gonads in all samples at E6 (Figure S3A–D), while in the *DMRT1*$^{-/W}$ chickens, CVH-positive germ cells were localized mainly in the cortex region from E14 and 1 week, similar to the WT female and *DMRT1*$^{-/W}$ chickens (Figure S3E–L). Interestingly, the germ cells of *DMRT1*$^{-/Z}$ chickens followed the developmental patterns of female germ cells until 4 to 6 weeks, but the overall growth of the follicle was retarded (Figure S3M–O). However, CVH expression was no longer detected in the germ cells of *DMRT1*$^{-/W}$ chickens at 5 and 30 weeks, or in the *DMRT1*$^{-/Z}$ at 30 weeks (Figure S3P–T), suggesting that the DMRT1 is also critical for germ cells development in the chicken.

3.5 | Disruption of *DMRT1* induces incomplete feminization of the gonads

To explore the role of *DMRT1* in gonad feminization and regulation of sex-related genes, RNA sequencing analysis

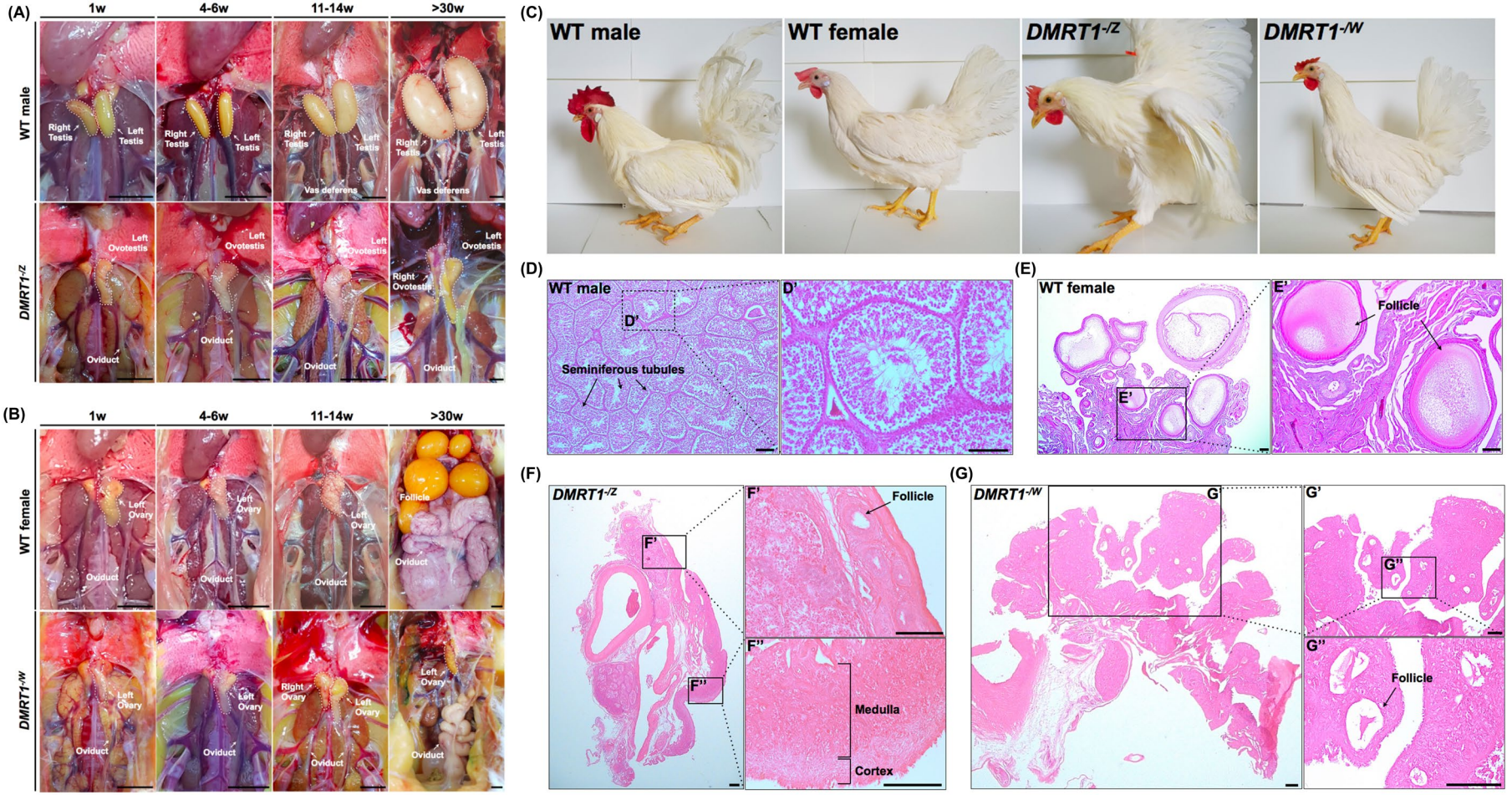

FIGURE 4 Reproductive organ development in *DMRT1*-disrupted chickens. (A) Reproductive organ development in the wild-type (WT) male and $DMRT1^{-/Z}$ chickens (1-week-old to >30-weeks-old). Dashed lines and/or arrows indicate testis, ovotestis, vas deferens, and oviduct of individuals. Scale bar = 1 cm. (B) Reproductive organ development in the WT female and $DMRT1^{-/W}$ chickens (1-week-old to >30-weeks-old). Dashed lines and/or arrows indicate ovaries, oviducts, and follicles of individuals. Scale bar = 1 cm. (C) External appearance of WT- and *DMRT1*-disrupted chickens at adult stage (>30-weeks-old). (D–G) Histological analysis of testis/ovotestis and ovary in WT and *DMRT1*-disrupted chickens at adult stage (>30-week-old). The ovotestis of $DMRT1^{-/Z}$ chicken clearly shows a few immature follicles with cortex and medulla structures. The ovary of $DMRT1^{-/W}$ chicken shows structural disruption. Scale bar = 200 μm. $DMRT1^{-/Z}$, heterozygous *DMRT1* knockout male chicken; $DMRT1^{-/W}$, *DMRT1* knockout female chicken

was conducted in the WT, *DMRT1*$^{-/Z}$, and *DMRT1*$^{-/W}$ chicken gonads at two morphologically distinguished time points (E6 and 1 week of age for morphologically undifferentiated and differentiated gonads, respectively). The overall expression pattern of *DMRT1* disruption demonstrated a feminized phenotype (Figure 5A). Co-expression network analysis identified a set of genes associated with the feminization phenomena. We found 62 modules with distinct co-expression patterns across all samples used, of which 6 were similar to the expression pattern of *DMRT1* (Figure S4). We found that the genes included in Module 16 did not change as a result of *DMRT1* disruption but were significantly different between the sexes (Supporting Information Data 1). A total of 213 genes were included in Module 16, of which 168 were located on the sex chromosomes.

We found two novel long non-coding RNAs (lncRNAs), *ENSGALG00000050012* (p_{adj}: 1.42e-49) (Figure 5B) and *ENSGALG00000051419* (p_{adj}: 1.42e-232) (Figure 5C), located on the Z chromosome (Figure 5D). These two lncRNAs were not affected by *DMRT1* disruption and were significantly differentially expressed according to the original sex of each subject (Figure 5B,C). Both *ENSGALG00000050012* and *ENSGALG00000051419* lncRNAs were located in the male hypermethylated region (MHM), which is localized adjacent to the *DMRT1* locus and associated with the *DMRT1* expression.[48,49] In the latest version of the Ensembl gene annotation for GRCg6a, these two lncRNAs are known to produce three different transcript isoforms, respectively. We further investigated the expression pattern at the exon level to clarify which of these transcripts shows such expression patterns. As a result, the same significant difference was observed in one exon in *ENSGALG00000050012* and four exons in *ENSGALG00000051419* (Figure S5). Based on these results, in situ hybridization was performed for additional biological confirmation, and a significant difference between sexes was observed in *ENSGALG00000051419* among the two lncRNAs. The *ENSGALG00000051419* lncRNA was expressed specifically in the cortex region of WT and *DMRT1*$^{-/W}$ chicken gonads, which indicates it may play a role in oocyte development (Figure 5E).

Meanwhile, we found that several lncRNAs were upregulated in the gonads of 1-week-old *DMRT1*$^{-/Z}$ chickens and WT females, indicating potential roles in male gonad feminization (Figure S6). Looking at the 62 co-expression patterns, most genes have varied expression levels depending on the interaction between the sex and *DMRT1* disruption experimental factors. A total of 3358 genes were found through statistical analysis of the interaction term between these two experimental factors, and it was once again confirmed that most of them are lncRNAs (Figure 5F).

3.6 | *DMRT1* linearly and non-linearly associated with the expression of sex determination genes

We performed co-expression network analysis to identify potential genes that could be linearly regulated depending on the expression level of *DMRT1*. We found that *DMRT1* is included in Module 41, and a total of 147 genes were within this cluster, of which 129 were sex chromosome genes (Supporting Information Data 2). Interestingly, the average correlation between *DMRT1* and Z-chromosomal genes was positive (0.803), whereas the average correlation with W-chromosomal genes was negative (−0.784). This indicates that genes belonging to Module 41 are linearly associated by *DMRT1*, and may promote Z chromosome gene regulation and inhibit W chromosome genes.

Meanwhile, we found several genes that were not co-expressed linearly with the expression level of the *DMRT1*, but were differentially expressed following *DMRT1* disruption. Although they do not exhibit a linear co-expression pattern with the *DMRT1* gene, these genes are also potential genes that are non-linearly regulated by *DMRT1*. At the E6 time point, 240 significant genes were identified with an FDR adjustment of $p < .05$, of which 15 were Z chromosome genes. Most genes, including the lncRNA *ENSGALG00000050161* (p_{adj}: 1.02e-07), were downregulated, similar to the expression pattern of WT males. Eight major directionalities were visualized by the interaction of the two experimental conditions, with several genes biased toward the female direction at the E6 time point (Figure 6A). We successfully classified and revealed novel lncRNAs, such as *ENSGALG00000044392* (p_{adj}: .0077), and genes known to be important for sexual development, such as *AMH* (p_{adj}: .0004), *CYP19A1* (p_{adj}: 1.77e-10), *FOXL2* (p_{adj}: .016), and *FSHR* (p_{adj}: 2.03e-07), which are non-linearly associated with the *DMRT1* disruption. Functional enrichment tests on the 240 genes that were non-linearly associated with the *DMRT1* at the E6 time point revealed 61 significant terms at a 5% significance level, including uterus development (p: 6.75e-05), female genitalia development (p: 8.95e-03), and female sex differentiation (p: 1.11e-02) (Figure 6B). We found that only one or two genes were associated with sex-related biological GO terms, such as female sex determination (p: 2.31e-02) and female somatic sex determination (p: 2.31e-02).

We found that 25 of the 240 genes were functionally uncharacterized lncRNAs, which could not be considered in the functional enrichment analysis (Figure S7). From the 61 functionally relevant terms, we found 37 belonged to protein-coding genes (Figure S8). Since these genes are non-linearly associated with *DMRT1*, we speculated that there could be a co-expression pattern between lncRNAs and protein-coding genes. Co-expression pattern

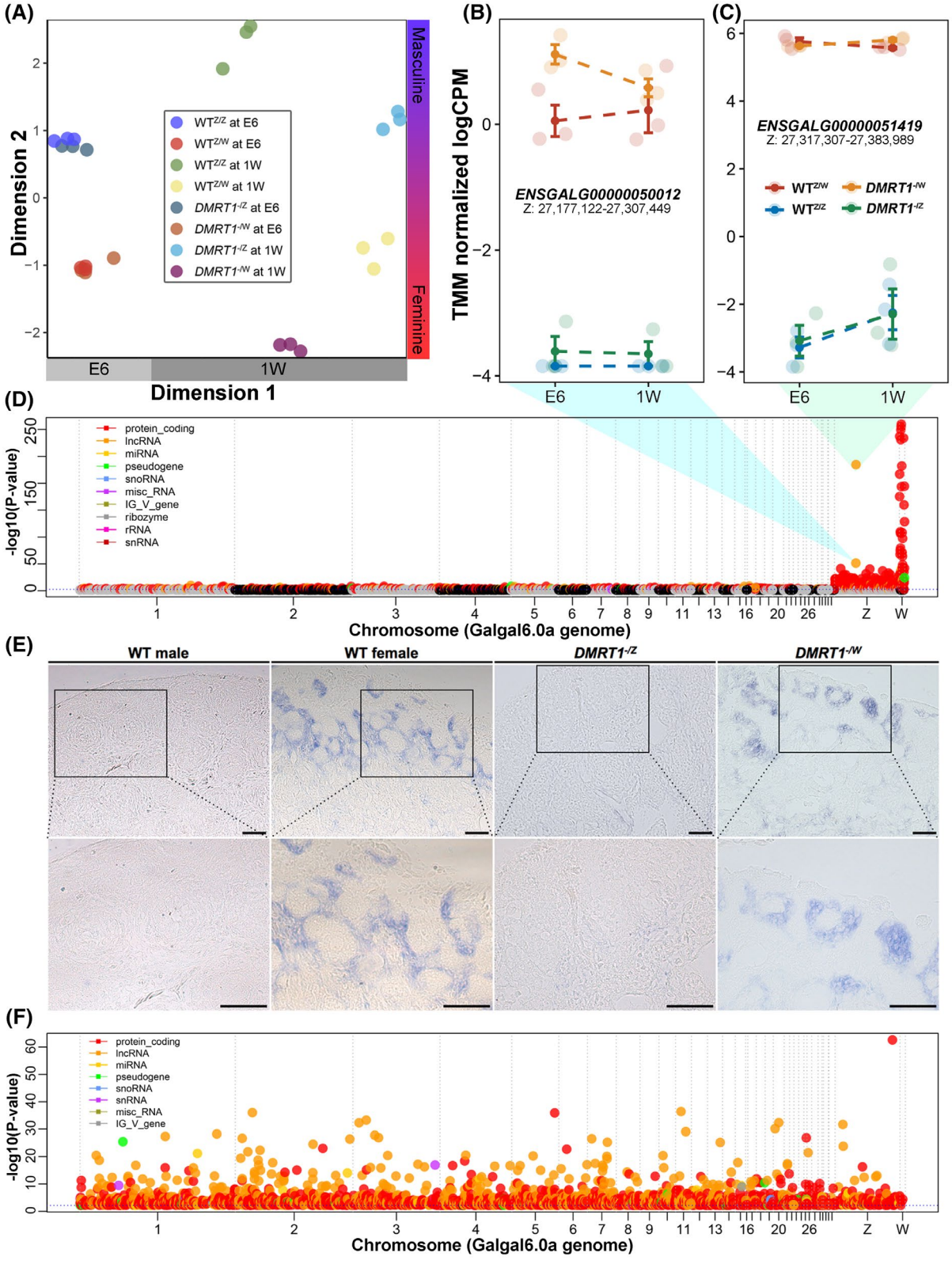

FIGURE 5 Expression profiling of RNA sequencing data from *DMRT1*-disrupted chicken gonads. (A) Multidimensional scaling plot of the global expression pattern of all 24 samples (including triplicates of $WT^{Z/Z}$, $WT^{Z/W}$, $DMRT1^{-/Z}$, and $DMRT1^{-/W}$ at E6 and 1 week time points). WT samples were designated as the control, and *DMRT1*-disrupted samples were designated as the case. The experimental conditions (*DMRT1* knockout status, time point, and tissue type) were simultaneously analyzed and grouped. (B and C) Identified lncRNAs showing strong female-biased expression patterns in the Z chromosome. (D) Manhattan plot of statistical hypothesis test results for sexual differences in a three-way ANODEV model. Sex-specific expression patterns of 1071 genes with FDR adjustment ($p < .05$) and their chromosomal location. (E) In situ hybridization for ENSGAL00000051419 lncRNA. Scale bars = 50 μm. (F) Manhattan plot of statistical test for interaction term sex**DMRT1* disruption

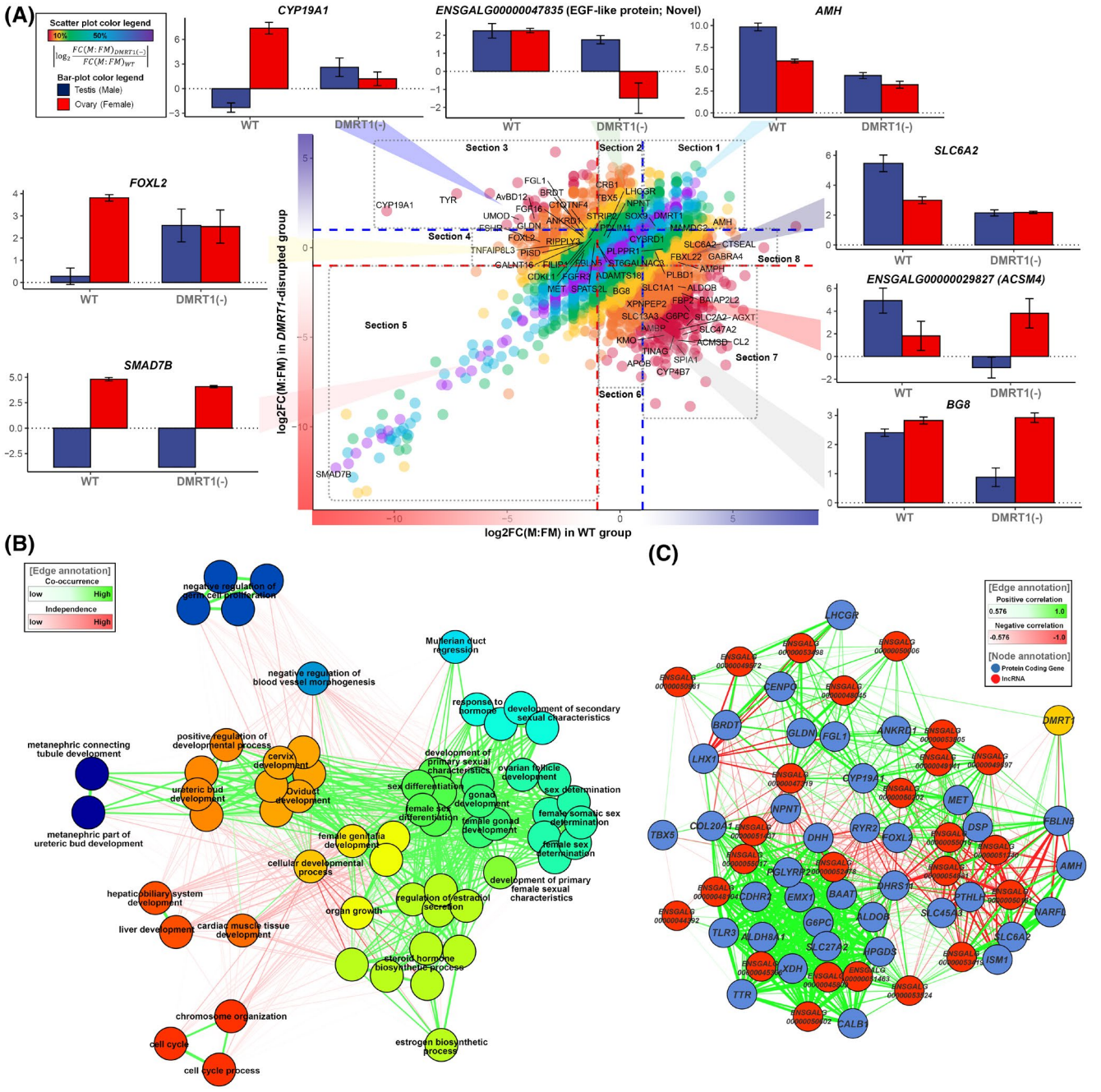

FIGURE 6 Interaction plot based on sex and *DMRT1* disruption at the E6 time point. (A) Based on the interaction between sex and *DMRT1* disruption variables, the directionality of expression patterns can be classified into a total of eight sections. The *x*-axis represents the log2FC value calculated from the sex of WT samples, and the *y*-axis represents the log2FC value calculated from *DMRT1* disruption. The color of the scatterplot is the log2 ratio of absolute values calculated from sex and *DMRT1* disruption groups. All measured 23 650 genes are displayed, with the top 1% and 5% differentially expressed genes shown in red and orange, respectively. Significant genes identified from the interaction of sex**DMRT1*(−) performed at E6 are annotated by known gene symbols. Representative bar-plot to visualize the expression patterns of each directionality pattern. The *y*-axis represents the TMM normalized logCPM value. Only known gene symbols from Ensembl are displayed. If the gene is unknown, the Ensembl gene ID and predicted symbol through the BLAST search are displayed. (B) Results of functional enrichment analysis for significant genes from the interaction between sex**DMRT1*(−) at the E6 time point and following FDR adjustment ($p < .05$). Significant biological process terms within a 5% significance interval from a gene ontology analysis are shown. Each node is arranged according to the similarity of the functional term, and the edge indicates the strength of co-expression or independence. (C) Co-expression network plot of genes identified by enrichment analysis from Figure 6B. The *DMRT1* was added to examine its relationship with the gene set. The blue nodes are the protein-coding genes, and the red nodes are the lncRNA with unknown functions. Edges are only displayed between genes with significant correlation and FDR adjustment of $p < .05$. The expression patterns of each gene are visualized in Figures S12 and S13

analysis demonstrated that genes with known functional roles in sex determination, such as *FOXL2* and *CYP19A1*, were co-expressed with genes of unknown function, including *GLDN*, *ANKRD1*, *RYR2*, *DHH*, *DHRS11*, and *MET* (Figure 6C). Furthermore, 25 lncRNAs that could be regulated indirectly by *DMRT1* were strongly co-expressed with these protein-coding genes. This provides evidence that genes linearly associated with *DMRT1* expression levels and genes with newly identified non-linear associations could play a role in gonad feminization.

4 | DISCUSSION

The sex of birds is determined by sex chromosomes Z and W; however, the molecular networks of sex determination in birds remain largely unclear. Recently, DMRT1 was recognized as the most promising candidate for male testis formation.[9,17] To identify whether disruption of *DMRT1* could induce male-to-female sex reversal in the chickens, and to identify the molecular mechanisms underlying sex determination, the CRISPR/Cas9-NHEJ method was used to modify the genome, followed by RNA sequencing analysis.

DMRT1 gene disruption was accomplished by targeted insertion of donor GFP using the CRISPR/Cas9-NHEJ method, as previously described.[46] The results suggest that the CRISPR/Cas9-NHEJ method is a precise and efficient tool for targeted gene disruption and insertion without significant nucleotide mutations. However, in this study, the *DMRT1*-disrupted chickens showed growth retardation and low survivability. In a recent study, *DMRT1* knockout chickens developed by ssODN-mediated gene replacement showed normal growth over a 28-wk period.[27] Compared to those *DMRT1* knockout chickens, the growth retardation and low survivability shown in this study may not be caused by *DMRT1* disruption. Instead, it could be caused by an off-target effect, as reported in previous genome editing studies.[50–53] In our whole-genome sequencing analysis, variants near predicted off-target sites were detected, and it may cause the difference, although no off-site mutation was found at predicted potential off-sites. For future studies, we will further validate the effect of identified variants and apply diverse genome editing methods for reducing variants.

Nevertheless, gonad development of *DMRT1*$^{-/Z}$ chickens is feminized postnatally, similar to early embryogenesis both in terms of morphological and molecular aspects.[9,27] The genome-modified chickens did not show any other significant differences in their appearance except in higher mortality and reproductive organ development compared to *DMRT1*-disrupted chicken reported from another research group,[27] supporting the cell autonomous sex identity theory, as proposed in gynandromorphic chickens.[7] However, disruption of *DMRT1* led to incomplete growth of the mature female reproductive organs as same as the another *DMRT1* knockout study,[27] despite the distinct morphological and molecular change in the chicken gonads. The results indicate that the establishment of female reproductive capacity requires additional factors with one copy of *DMRT1*. One possibility is the existence of co-factors that induce hormone-producing gene expression. In this study, adult *DMRT1*$^{-/Z}$ chicken have undetectable levels of serum estradiol. Furthermore, qRT-PCR analysis showed that expression of hormone receptors, including *FSHR*, *AR*, and *PGR*, was significantly lower in *DMRT1*$^{-/Z}$ chicken than in WT females (*DMRT1*$^{+/W}$) at 30 weeks, but not at 1 week. These results support the hypothesis that co-factors are required for development of the female reproductive organs, despite the mostly cell autonomous avian sex determination system.[7] This hypothesis could be supported by introducing estrogen, which plays essential roles in chicken ovarian development, or by disturbance to hormone production by either an aromatase inhibitor or gonadectomy, which can change the sex properties of the gonads.[54,55] Furthermore, this study revealed that ovary morphogenesis in female chicken (*DMRT1*$^{-/W}$) is not affected by *DMRT1* disruption, indicating that DMRT1 is dispensable for ovary morphogenesis, unlike estrogen, which has a dominant role in forming the ovarian cortex.[56]

The RNA-seq analysis results suggest that *DMRT1* could linearly and non-linearly regulate a set of genes involved in sex determination and other developmental processes. In particular, the two lncRNAs (*ENSGALG00000050012* and ENSGALG00000051419) may play roles in chicken ovary development, similar to *Sex-1* and *Ceh-39*, which are regulators of the master sex determination gene *Xol-1* in *Caenorhabditis elegans*.[57] Previous research shows that methylation and subsequent expression of lncRNAs were present only in individuals lacking a W chromosome, suggesting the importance of the W chromosome in early ovary development.[48] Furthermore, our results provide evidence that some of the lncRNAs may have a dosage effect mechanism, which has not been identified in chickens to date. LncRNAs may play roles in various developmental processes by regulating transcriptional and post-transcriptional pathways.[58] One of the most well-known lncRNAs is X-linked X-inactive specific transcript (*XIST*) in mammals, which plays a critical role in dosage compensation in the XY sex determination system via inactivation of one X chromosome in females.[59,60] *XIST* spreads to coat one of the X chromosomes in females (XX), and initiates the X chromosome inactivation process by recruiting heterochromatin-related complexes such as Polycomb repressive complex 2.[61] By contrast, dosage

compensation in drosophila is mediated mainly by male-specific lethal (MSL) complex, which activates male X chromosome genes.[62] The MSL complex contains several proteins, as well as X chromosome-specific lncRNAs *roX1* and *roX2*, which are critical for MSL complex assembly.[63] Mutations in *roX1* and *roX2* induce reduced expression of X chromosome genes, suggesting that these lncRNAs are indispensable for dosage compensation.[64] Considering our results and the importance of lncRNAs in dosage compensation, we speculate that these novel lncRNAs may play an important role in the dosage effect of the chicken ZW sex determination system.

We classified genes that showed a significant interaction between sex**DMRT1* disruption at E6 in eight directions (Figure 6A). The genes located in Sections 1 and 5 were genes that exhibit constant sex-specific biased expression without strong interaction effects. We found that relatively large numbers of female-biased genes with strong signals were skewed toward Section 7 (versus Section 3). This suggests that the tendency toward feminization observed after genome editing is also reflected in the gene expression pattern. There were already well-known male-bias genes found in Section 1, such as *AMH*, *DMRT1*, and *SOX9*, while the other genes present in the other Sections were relatively unknown. These genes, which have been well-studied in the chicken sex determination system, show a similar positive linear relationship to the *DMRT1* expression patterns; therefore, these genes could have been relatively easily detected in the past. In this study, the newest classification attempt provides evidence for novel sex determination genes. For example, genes located in Section 2 showed a male-biased expression pattern in *DMRT1*-disrupted chicken, but no distinct sex-specific differences in WT birds. This expression pattern was observed for the unclassified *ENSGALG00000047835* protein-coding gene (p_{adj}: .0135). In addition, genes belonging to Section 3 showed a female-biased expression pattern in WT chickens, which converted into a male-biased expression pattern in *DMRT1*-disrupted chickens. We found that genes involved in hormone synthesis, such as *CYP19A1* and *TYR* (p_{adj}: .0003), exhibit such interaction effects. Although these genes have no linear relationship with *DMRT1* expression, disruption of the gene identified novel non-linear patterns. Indeed, aromatase (*CYP19A1*) is responsible for synthesizing estrogen, a critical factor for ovarian cortex development.[56] Therefore, the male-specific increase in *CYP19A1* expression level validated by RNAseq could be one of the major factors for gonad feminization in $DMRT1^{-/Z}$ chicken, as suggested before.[27]

Given the nature of the sex-determining mechanisms that are very important in living organisms, there will inevitably be many genes involved. However, compared with mammalian studies, studies on the mechanisms of sex determination in chickens have focused on very few genes. One reason may be that very few genes relating to sex determination have been annotated in chickens. Thus, re-classification of novel non-linearly associated genes could reveal potential new candidates and identify previously unknown mechanisms involved in chicken sex determination.

Notably, the results from this study suggest that DMRT1 has critical roles in female secondary reproductive organ development, specifically in germ cell differentiation in both sexes. Previous researches show that DMRT1 is dispensable for ovarian development in zebrafish, mice, and humans. However, DMRT1 regulates transcriptional activation of *STRA8* for the proper entry of oogonia into meiosis.[20,65,66] In males, loss of *DMRT1* in early spermatogonia disrupts spermatogenesis by precocious and uncontrolled meiotic initiation, causing germ cell apoptosis.[67] Similar expression patterns of DMRT1 in human gonads suggest a possible role in the regulation of meiotic entry.[68] Furthermore, Dmrt1 depletion causes apoptosis of germline stem cells,[69] and DMRT1 mutation inhibits vasa-positive germ cell self-renewal and induces apoptosis in fish.[70] Here, we found that the disruption of *DMRT1* gene caused severe germ cells lose in the gonads, as well as immature growth of reproductive organs in both male and female chickens. Since *DMRT1* is critical for SSCs maintenance, germ cells maintenance, and development of germ cell-nursing cells (including Sertoli cells) in males,[71] *DMRT1* disruption may cause germ cells loss and impaired gonadal development. The recently published study also reported germ cell lose in *DMRT1* knockout female chickens, and suggest that the absence of oocyte/follicles in the gonads may be due to a delay or failure of meiosis.[27] To clarify the question, the germ cell-specific or Sertoli cell-specific *DMRT1* knockout should be conducted, as previous research.[23]

In this study, we demonstrated that *DMRT1* disruption was insufficient to induce complete testis feminization in genome-modified chickens. Furthermore, RNA-seq analysis identified potentially novel genes that could be either linear or non-linearly involved in gonad feminization. To date, the most comprehensive understanding of DMRT1 regulation is through transcription factor binding.[45] Unfortunately, there were few identified genes that could only be explained by the transcriptional regulation mechanism by transcription factor binding of DMRT1 (Supporting Information Data 3). Further investigation into the functions of the identified genes, along with genome-wide DNA binding analysis, could provide vital clues about sex determination in birds.

There are several practical limitations in this study. First, although genome editing using CRISPR/Cas9 is a cutting-edge method, the off-target effects are still

unresolved. Even though we investigated the off-target effects of CRISPR/Cas9 through the simultaneous in silico method and whole-genome sequencing analysis, there is no way to comprehensively investigate this, as many studies have revealed.[72] Second, functional validation of the two lncRNAs discovered through RNA-seq analysis in this study was not performed. Likewise, the novel genes linearly and non-linearly co-expressed with *DMRT1* identified by RNA-seq analysis need further research to better understand their exact functional roles in chickens. We believe that genome editing experiments on these genes will improve our understanding of the sex-determining mechanisms in chickens in the near future. Third, analysis of the DMRT1 protein, and its effects on downstream gene expression, has been limited due to the lack of available commercial antibodies for chicken. To confirm the hypothesis proposed in this study, an in-depth analysis of the protein, including quantitative measurement and identification of binding partners, should be investigated in the future. Despite these practical limitations, this study notably emphasized the critical roles of *DMRT1* plays in the development and maintenance of reproductive organs and germ cells of chickens from embryonic stages to the adult stage.

ACKNOWLEDGMENTS

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DISCLOSURES

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

Hong Jo Lee and Minseok Seo participated in the design of the study, carried out the experiments, statistical analysis, and wrote the first draft of the manuscript. Hee Jung Choi, Deivendran Rengaraj, Kyung Min Jung, Jin Se Park, Kyung Youn Lee, and Young Min Kim carried out the experiments and revised the first draft of the manuscript. Kyung Je Park, Soo Taek Han, and Kyu Hyuk Lee cared for experimental animals. Humphrey Hung-Chang Yao revised the first draft of the manuscript. Jae Yong Han participated in study design and coordination.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.

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