Characterization of Chicken MMP13 Expression and Genetic Effect on Egg Production Traits of Its Promoter Polymorphisms

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ABSTRACT Extracellular matrix undergoes constant remodeling, cell–cell, and cell–matrix interactions during chicken ovarian follicle growth, which is coordinated by matrix metalloproteinases (MMPs), and their associated endogenous inhibitors (TIMPs). Transcriptome analysis revealed upregulation of MMP13 in sexually mature chicken ovaries. In this study, we found that the expression of MMP13 in chicken ovary was stably elevated from 60 d to 159 d, and was significantly higher at 159 d than at the other three developmental stages (P < 0.05). The expression of MMP13 mRNA increased from SW (small white follicles) to F5 (fifth largest follicles), then decreased to F1 (first largest follicles), and dramatically increased again in POF1 (newly postovulatory follicles) follicles (P < 0.05). The MMP13 protein was localized in stroma cells and primordial follicles of sexually immature chicken ovaries, in the theca cell layers of all sized follicles of sexually mature chicken ovaries. Furthermore, we identified a positive element (positions –1863 to –1036) controlling chicken MMP13 transcription, and, in this region, six single nucleotide polymorphisms were found and genotyped in chicken populations. In the White Recessive Rock population, hens with A–1356-C–1079/A–1356-C–1079 genotype had earlier “age at first laying” than those with G–1356-T–1079/G–1356-T–1079 genotype (P < 0.05), and exhibited significantly lower transcriptional activity (P < 0.01). Collectively, chicken MMP13 plays an important role in ovarian follicle growth and regression, and polymorphisms in its promoter region could be used as molecular markers for improving the trait “age at first laying” in chicken breeding.

The ovary represents a truly dynamic organ system characterized by structuring and restructuring events during the development of the ovarian follicle for ovulation. Follicle development is characterized as continued growth from the initial primordial follicle to a mature follicle that is approximately 400-fold larger. During this process, the extracellular matrix (ECM) requires constant remodeling, and cell–cell and cell–matrix interactions to control the wide range of cellular functions, which is coordinated by matrix metalloproteinases (MMPs) and their associated endogenous inhibitors (TIMPs) (Curry and Osteen 2003; Rodgers et al. 2003; Ohnishi et al. 2005; Curry and Smith 2006). The exquisite control of the MMP system has been postulated to regulate many of the cyclic changes, such as the follicular development, ovulation, the formation or destruction of vascularized ovarian structure (Fata et al. 2000; Curry and Osteen 2001; Ny et al. 2002; Smith et al. 2002; Page-McCaw et al. 2007), and therefore is essential for the proper follicular function in the ovary while simultaneously preserving ovarian integrity.

Unlike mammals, the domestic fowl has only a single left ovary, containing follicles of various sizes and developmental stages, including primordial follicles, primary follicles, prehierarchal follicles, such as small white follicles (SW) and small yellow follicles (SY), hierarchal follicles of F5 to F1, and postovulatory follicles (POFs); the POF in birds fails to form a functional corpus luteum (CL) and rapidly regresses. To maintain this hierarchy and daily ovulation, a follicle is selected from the pool of 6–8 mm SY follicles, and grows into a 40 mm preovulatory follicle in just 5–9 d (Gilbert et al. 1983). Follicular maturation also requires yolk deposition, which involves the process of transport, transformation, and...
deposition of yolk prerequisite material that is conveyed to the oocyte via the vascular system. In addition, the POFs regress shortly after ovulation, and are almost entirely regressed by cellular apoptosis accompanied by proteolysis and dissolution of the ECM within 4–6 d after ovulation (Sudaresan et al. 2008). These extensive cyclic changes in the follicular ECM throughout each reproductive cycle are postulated to occur via the action of a cascade of proteolytic events involving matrix metalloproteinases (MMPs) activity (Zhu et al. 2014).

MMPs, also known as collagenase-3, initiates the breakdown of the fibrilar collagens that form a key structural element of membranes. In chicken, MMP13 is involved in embryonic membrane remodeling, and corneal development related biochemistry and molecular changes (Lei et al. 1999; Huh et al. 2007). In the angiogenesis system of the chorio-allantoic membrane, MMP13 is the only enzyme associated with collagen remodeling (Zijlstra et al. 2004). Periodic changes of collagenase-3 have been detected in the rat ovary during the ovulatory process (Balbin et al. 1996); however, the role of MMP13 in chicken follicle growth, ovulation, and ovary function is unclear. In this study, we investigated the expression pattern and cellular localization of MMP13 during the reproductive cycle of hens. We also analyzed the regulatory elements of the chicken MMP13 promoter, and identified six single nucleotide polymorphisms (SNPs) that are associated with egg production traits in a White Recessive Rock population.

### Table 1

| Primer Name | Sequence (5′-3′) | Annealing Temperature (°C) | Size (bp) |
|-------------|-----------------|----------------------------|-----------|
| MMP13-F     | TTTGGATTAGGGTTGACGG | 56                        | 253       |
| MMP13-R     | CCACCTCGTATCTGGTA | 56                        | 251       |
| β-actin-F   | TGGATAGTATATTTGGC | 56                        | 251       |
| β-actin-R   | ATCTTCTCAATTTGATCC | 56                        | 251       |
| VEGFA-F     | GGAAGCCCACGAGATTA | 55                        | 231       |
| VEGFA-R     | GGCTATGTGACTCTGAT | 55                        | 231       |
| snpMMP13-F  | ACAATCCCAGTTCCCTCAG | 58                      | 807       |
| snpMMP13-R  | GGCACGGCTGTATCATC | 58                      | 807       |
| pGL3-MMP13-1F | GGACGCCGTGCGATGCTGTA | 58              | 807       |
| pGL3-MMP13-2F | CGACGGCTTCACTCTGTCGAG | 58           | 807       |
| pGL3-MMP13-3F | CGACGGCTACATCGTCTCAG | 58         | 807       |
| pGL3-MMP13-4F | CGACGGCTTGTCATGCTGTA | 58       | 807       |
| pGL3-MMP13-5F | CGACGGCTATGCATGCTGTA | 58     | 807       |
| pGL3-MMP13-R | GGACGCTGTATCATC | 58          | 807       |

All forward primers contained an MfeI site at their 5′-ends, and the reverse primers had an added BglII site at their 5′-ends (underlined).

### MATERIALS AND METHODS

#### Birds, sample collection, and sample preparation

Hy-line Brown commercial hens at the ages of 60 d, 90 d, 123 d (sexually immature), and 159 d (sexually mature) were used for analysis. Birds, sample collection, and sample preparation

#### Materials and Methods

**Total RNA extraction and real-time quantitative PCR**

Total RNA was extracted from all tissues using an RNA extraction kit (DNPV9, Tiangen). The amount and integrity of isolated total RNA were measured using a spectrophotometer (Eppendorf, Hamburg, Germany), and checked by loading total RNA onto a 1% agarose gel that was stained with ethidium bromide. The cDNA was synthesized using a PrimeScript RT reagent kit with gDNA Eraser (TaKaRa, Dalian, China) from 1 μg of total RNA mixed with 1 μl of oligo(dT)18 primer, 1 μl of PrimeScript RT Enzyme Mix I, 4 μl of 5× Prime Script Buffer, and RNase-free dtt419 (Tiangen, Beijing, China), and stored in TE (pH 8.0) until delivered to the laboratory. Genomic DNA was extracted from the blood sample using a DNA Extraction mini kit (Tiangen, Beijing, China), and stored in TE (pH 8.0) at −20°C. The birds were handled and treated according to the Animal Care and Use Committee of Shandong Agricultural University.

#### Western blotting

Total protein was extracted from homogenized ovaries and different developing follicles using the Cell Lysis Reagent (Fermentas). Protein concentration was determined by the bicinchoninic acid assay (BCA Protein Array kit, Tiangen). The mouse anti-rat MMP13 monoclonal antibody was produced by Chemicon (Temecula, CA). An equal amount of protein was separated by 10% SDS gel electrophoresis under denaturing and nonreducing conditions, after which the proteins were
and 159-d-old (sexually mature) hens. Tissues were collected from 45-d-old (sexually immature) hens and 159-d-old hens. (A) Messenger RNA expression of MMP13 was analyzed by real-time PCR. (B) Expression of MMP13 protein was analyzed by Western blot analysis. A band of approximately 48 kDa corresponding to the molecular mass of MMP13 was detected. β-actin (41 kDa) was used as the loading control. Data are presented as mean ± SEM from at least four independent experiments. Bars with different superscript letters are significantly different (P < 0.05).

Transferred to a polyvinylidene fluoride membrane, and then incubated (1 hr, 37°C) with the MMP13 (1:250) antibodies in a 5% bovine serum albumin/PBS solution. After washing in PBST (1000 ml PBS:500 µl Tween-20), the blots were incubated with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G antibody (1:1000; Abcam) in a 5% bovine serum albumin/PBS solution (1 hr, 37°C). Specific binding was visualized using diaminobenzidine (Tiangen).

**Immunohistochemistry**

Ovaries and follicles were collected from 45-d-old (sexually immature) and 159-d-old (sexually mature) hens. Tissues were fixed in 10% buffered formalin and paraffin-embedded, and then cut into 5 µm tissue sections. All the sections were deparaffinized, rehydrated through a graded ethanol series, boiled in 10 mM sodium citrate buffer, quenched in 3% hydrogen peroxide, and blocked with 10% goat serum for 20 min. Next, the slides were incubated with mouse anti-rat MMP13 monoclonal antibody (1:50) for 2 hr at 37°C. Then, the sections were incubated with the biotinylated secondary antibody and avidin-biotin-peroxidase complex for 0.5 hr according to the Histostain-plus kit instructions (Zhongshan Golden Bridge Biotechnology, China). Finally, immunoprecipitates were visualized by incubation with a diaminobenzidine kit (Zhongshan Golden Bridge Biotechnology). The sections were counterstained with hematoxylin after immunostaining, dehydrated, and covered. Negative control staining was performed using PBS instead of primary antibody. No specific staining was found on the control slides.

**Plasmid construction**

The 5′-regulatory region of chicken MMP13 gene was inserted upstream of the firefly luciferase gene of the pGL3-Basic vector (Promega, Madison, WI) to generate reporter plasmids. To construct serial deletion promoter reporters, five forward primers (from pGL3-MMP13-1F to pGL3-MMP13-5F, Table 1), and one reverse primer (pGL3-MMP13-R, Table 1) located downstream of the transcription start site of chicken MMP13 gene were synthesized. All forward primers contained an MluI site at their 5′-ends, and the reverse primers had an added BglII site at their 5′-ends. The PCR amplification of the chicken MMP13 promoter was carried out with high-fidelity Taq DNA polymerase (TaKaRa, Dalian, China) using the chicken genome as template, and all PCR fragments were inserted into pGL3-Basic between the MluI and BglII restriction sites to generate 5′-serially deleted promoter constructs. The PCR products and pGL3-Basic vector were separately digested with the same restriction enzyme for 0.5 hr at 37°C, and then the two purified products were ligated for 30 min at 22°C. To evaluate the effect of the SNPs (g.−1356 G > A, g.−1128 A > G, g.−1094 C > A, g.−1079 T > C) on MMP13 promoter activity, two reporter plasmids (wt-MMP13 and mut-MMP13 constructs), which encompassed the core promoter region, were constructed with individuals of G−1356A−1128C−1094T−1079G−1356GA−1128CT−1094GA−1079G genotype and A−1356G−1128A−1094C−1079A−1356GC−1128AG−1094CG−1079G genotype as a template, respectively. PCR amplification was performed in 20 µl volumes containing 4 µl of 5× primer STAR HS Buffer, 1.6 µl (2.5 mM) of dNTPs (TaKaRa, Dalian, China), 0.2 µl of prime STAR HS polymerase (5 U/µl) (TaKaRa, Dalian, China), 0.5 µl of each forward (pGL3-MMP13-3F) and reverse (pGL3-MMP13-R, Table 1) primers (10 µM), 1 µl genomic DNA (50–100 ng), and 12.7 µl of nuclease dH2O, and run on a Mastercycler gradient (Eppendorf, Germany) according to the following program: 98°C for 10 min, 35 cycles of 98°C for 10 sec, annealing at 58°C for 30 sec, and 72°C for 1.5 min, and final extension at 72°C for 5 min. All constructs were sequenced in both directions to confirm the authenticity of the sequences. Plasmids were reproduced in Escherichia coli DH5α, and purified using the Endo-Free Plasmid Purification Kit (Tiangen).

**Cell culture and luciferase assay**

The F1, F2, and F3 follicles of the ovaries of egg-laying hens were separated from the ovaries and placed in PBS (HyClone). Theca cells were isolated according to the protocol described in Gilbert et al. (1977). The theca cells were dispersed by treatment with 0.1% collagenase II at 37°C for 25 min with gentle agitation in a flask. After centrifugation, the cells were suspended in culture medium (M199 with 10% fetal bovine serum, and 1% penicillin/streptomycin), and subsequently seeded in 24-well culture plates at a density of 1 × 10^6/well. The number of viable cells (90%) was estimated using Trypan blue. Cells were cultured at 38.5°C in a water-saturated atmosphere of 95% air and 5% CO2 for 24 hr.

**Figure 1** Expression of MMP13 in ovaries from 60-, 90-, 123-, and 159-d-old hens. (A) Messenger RNA expression of MMP13 was analyzed by real-time PCR. (B) Expression of MMP13 protein was analyzed by Western blot analysis. A band of approximately 48 kDa corresponding to the molecular mass of MMP13 was detected. β-actin (41 kDa) was used as the loading control. Data are presented as mean ± SEM from at least four independent experiments. Bars with different superscript letters are significantly different (P < 0.05).

**Figure 2** Expression of MMP13 in different follicles from 159-d-old hen ovaries. (A) Expression of MMP13 mRNA in small white follicles (SW), fifth largest follicles (F5), third largest follicles (F3), first largest follicles (F1), and newly postovulatory follicles (POF1). (B) Expression of chicken MMP13 protein was analyzed by Western blot analysis. β-actin (41 kDa) was used as the loading control. Data are presented as mean ± SEM from at least four independent experiments. Bars with different superscript letters are significantly different (P < 0.05).
Polymorphism analysis

PCR amplification was performed in 20 μl volumes containing 2 μl of 10× Ex-buffer, 1.6 μl (2.5 mM) of dNTPs (TaKaRa, Dalian, China), 0.1 μl (5 U/μl) of Ex-Taq DNA polymerase (TaKaRa, Dalian, China), 0.4 μl of each forward and reverse primers (10 μM, snpMMP13-F/R in Table 1) designed according to the chicken MMP13 gene (GenBank accession No. NC_006088.3), 1 μl genomic DNA pools (50–100 ng), and 14.5 μl of nuclease ddH2O, and run on a Mastercycler gradient (Eppendorf, Germany) according to the following program: 94°C for 4 min, 35 cycles of 94°C for 30 sec, annealing at 58°C for 30 sec, and 72°C for 45 sec, and final extension at 72°C for 5 min. Fragments of 807 bp were resolved by electrophoresis on a 1% agarose gel, purified with AxyPrep DNA Gel Extraction Kit (Axygen, Union City, CA), and sequenced using forward primer. Sequences were aligned with the reference sequence (GenBank accession No. NC_006088.3) to identify nucleotide changes using the DNAMAN program.

Statistical analysis

The SHEsis software (http://analysis.bio-x.cn) was used to analyze the pairwise linkage disequilibrium and haplotype frequency. The genotype and allelic frequencies, Hardy-Weinberg equilibrium χ² test, polymorphism information contents (PIC), heterozygosities (Hs), and the effective population of the allele (Ne), were calculated using Tools for Population Genetic Analyses software (http://www.marksgeneticsoftware.net/tfpga.htm). Haplotypes were constructed in chicken populations using the PHASE v2.0 program. The associations of MMP13 genotypes with egg production traits including age at first laying, and egg number at 32 wk, were analyzed in White Recessive Rock hen populations that were derived from the same chicken farm using the General Linear Model of SAS (version 9.2; Cary, NC). The linear model is represented as follows: Yij = μ + Gi + ei + eij, where Yij is the phenotypic value of traits, μ is the population mean, Gi is the fixed effect of genotype, and ei is the random error effect. For qRT-PCR analysis, western blotting analysis, and luciferase assay, differences between the experimental groups were evaluated by ANOVA, followed by Duncan’s multiple range test (P < 0.05) using the General Linear Model procedure of SAS (SAS version 9.2; Cary, NC). All the expressions were repeated at least three times, and all data were presented as the mean ± SEM.

Data availability

Supplementary file File S1 contains genotype and phenotype data of chicken individuals used for diplotype and egg production trait association analysis. Sequence data are available at GenBank and the accession number is 1971458461 for SNP g.-1356G>A.

RESULTS

Expression of MMP13 in chicken ovaries of different developmental stages

Illumina/solexa sequencing revealed that the expression of chicken MMP13 was significantly higher in the ovaries of laying hens than in prepubertal hens (Zhu et al. 2014). This expression dynamic was also validated in Jining Bairi and Hy-line hens (Supplemental Material, Figure S1). In this study, we further analyzed the expression of MMP13 mRNA in the ovary of Hyline-brown hens during development. A stably elevated mRNA expression of chicken MMP13 from 60 d to 159 d was observed, and, at 159 d, it was significantly higher
than at the other three developmental stages ($P < 0.05$) (Figure 1A). Similarly, the protein level of chicken MMP13 in the ovary was also increased during chicken development, reaching the highest level at 159 d ($P < 0.05$) (Figure 1B).

Expression of MMP13 mRNA and protein during chicken follicular development

The expression of MMP13 mRNA increased from SW to F5, then decreased to F1 and dramatically increased again in POF1 follicles ($P < 0.05$) (Figure 2A). The expression of MMP13 protein among SW, F5, F3, F1 to POF1 follicles was not significant ($P > 0.05$) (Figure 2B).

Spatiotemporal changes in the localization of chicken MMP13 protein

Cellular localization of MMP13 protein was examined in chicken sexually immature (45-d-old) and mature (159-d-old) ovaries. In sexually immature chicken ovaries, MMP13 protein staining was observed in stroma cells and primordial follicles (Figure 3). In sexually mature chicken ovaries, MMP13 protein is localized mainly in theca cell layers of all sized follicles, and in follicles of F5, F3, F1, and POF1, its expression is restricted mainly to blood vessels of theca cell layers (Figure 4). In chicken prevolutulatory follicles, MMP13 mRNA level was significantly higher in theca cells than in granulosa cells (Figure 5).

Polymorphisms in the critical promoter region of the chicken MMP13 gene

We further identified a critical region controlling the transcription of chicken MMP13 gene, and found that deletion from $–1863$ bp to $–1036$ bp significantly decreased the promoter activity as assayed by luciferase activity ($P < 0.05$) (Figure 6). In this region, six single nucleotide polymorphisms (SNPs), i.e., g.$–1719$ T $> C$ (rs731299043), g.$–1661$ C $> A$ (rs314003171), g.$–1356$ G $> A$ (rs1971458461) identified by this study, g.$–1128$ A $> G$ (rs316193109), g.$–1094$ C $> G$ (rs315077856), and g.$–1079$ T $> C$ (rs312778897) were found, among which complete linkage disequilibrium exist between g.$–1719$ and g.$–1661$, g.$–1356$ and g.$–1128$, and g.$–1094$ and g.$–1079$ loci, respectively (Figure 7). At SNPs g.$–1719$ T $> C$, g.$–1661$ C $> A$, g.$–1356$ G $> A$, g.$–1128$ A $> G$, g.$–1094$ C $> G$, g.$–1079$ T $> C$, alleles T, C, G, A, C, and T were predominant in White Recessive Rock, Hy-line brown, Wenchang, Jining Bairi, and Wenshang Barred chicken populations, respectively (Table 2).

Associations of diplotypes of chicken MMP13 gene with egg production traits

To analyze the associations of SNPs in the chicken MMP13 promoter region with egg production traits, haplotypes were constructed using the two SNPs of g.$–1356$ G $> A$ and g.$–1079$ T $> C$. Three haplotypes of A (G$^{1356}$/C$^{1079}$), C (A$^{1356}$/T$^{1079}$), and D (A$^{1356}$/C$^{1079}$) were detected in the White Recessive Rock population. Due to the fact that the frequency of haplotype C was less than 0.01, association with egg production traits was analyzed only with diplotypes AA, AD, and DD. The results indicated that hens with diplotype DD had earlier age at laying than those with diplotype AA and AD ($P < 0.05$), but its effect on egg number at 32 wk was not significant ($P > 0.05$) (Table 3).

Genetic effect of the SNPs on chicken MMP13 expression

To further investigate the genetic effect of promoter SNPs on chicken MMP13 expression, the transcriptional activity of the 5’-flanking region of the chicken MMP13 gene, which contains the G$^{1356}$/A$^{1128}$/C$^{1094}$/T$^{1079}$ and A$^{1356}$/G$^{1128}$/G$^{1094}$/C$^{1079}$ (designated as wt-MMP13 and mut-MMP13), respectively, was compared. As shown in Figure 8, the wt-MMP13 promoter showed significantly higher transcriptional activity than mut-MMP13 ($P < 0.05$).

DISCUSSION

In chicken, during follicle growth and ovulation, MMPs and their associated endogenous inhibitors, TIMPs, play critical roles in ECM remodeling. Our previous study has characterized the expression and regulation mechanism of chicken MMP1, MMP3, and MMP9 in the ovary and ovarian follicles (Zhu et al. 2014), and identified an indel polymorphism in the promoter region of the chicken MMP9 that is associated with egg number at 28 wk (Zhu and Jiang 2014). From a transcriptome study, we also found that the MMP13 mRNA is elevated in sexually mature chicken ovary (Zhu et al. 2014); therefore, in this study, we further analyzed the expression dynamics and regulation of reporter plasmid was used as the internal control to correct for transfection efficiency. Data are presented as mean ± SEM from at least four replicates for each construct. Means with the different lowercase letters within the same column are significantly different ($P < 0.05$).
chicken MMP13 transcription, and identified a haplotype that is associated with the trait “age at first laying”. MMP13, also called collagenase-3, was first cloned from a cDNA library derived from a breast tumor (Freije et al. 1994). In this study, we first validated the expression dynamics of MMP13 mRNA in Jining Bairi and Hy-line hens by qRT-PCR (Figure S1), and found that, in both breeds, its mRNA expression level was significantly increased in sexually matured ovaries (140 d for Jining Bairi hens, 300 d for Hy-line hens) compared with sexually immature ovaries (90 d for Jining Bairi hens, 70 d for Hy-line hens), which is consistent with transcriptome results (Zhu et al. 2014). Second, we analyzed the expression dynamics of chicken MMP13 during chicken development from 60-d-old to 159-d-old hens, and found that MMP13 expression in the ovary of 159-d-old hens was significantly higher than other developmental stages, suggesting that MMP13 plays a role in the proper function of the ovary in laying hens. When expression of MMP13 was further analyzed in different sized follicles of sexually mature Hy-line hens, two expression peaks in F5 and POF1 follicles at mRNA level, and one in POF1 follicles at the protein level were revealed, suggesting MMP13 is an important enzyme in follicle selection and follicle regression. In rat ovary, the expression of collagenase-3 mRNA was upregulated by 32-fold at 48 h after eCG injection; MMP13 is likely

| Locus or Haplotype | Allele | White Recessive Rock (n = 510) | Hy-Line Brown (n = 45) | Wenchang (n = 53) | Jining Bairi (n = 37) | Wenshang Barred (n = 46) |
|--------------------|--------|-------------------------------|-----------------------|-------------------|---------------------|-------------------------|
| g.-1719 T>C        | T      | 0.917                         | 1.000                 | 0.774             | 0.838               | 0.935                   |
| g.-1661 C>A        | C      | 0.917                         | 1.000                 | 0.774             | 0.838               | 0.772                   |
| g.-1356 G>A        | G      | 0.819                         | 0.600                 | 0.953             | 0.622               | 0.989                   |
| g.-1128 A>G        | A      | 0.819                         | 0.600                 | 0.679             | 0.595               | 0.804                   |
| g.-1094 C>G        | C      | 0.825                         | 0.600                 | 0.755             | 0.676               | 0.989                   |
| g.-1079 T>C        | T      | 0.825                         | 0.600                 | 0.755             | 0.676               | 0.837                   |
being expressed only in the regressing CL, suggesting a speci-
expression of the rat CL, collagenase-3 had a separate expression pattern,
function. In ovarian follicles, the expression of
mature chicken ovary, suggest that MMP13 is involved in the action of a
sexually immature chicken ovary, and in the theca cells of sexually
consistent, a higher expression of MMP13 was also observed in POF1.
formation of a CL is not observed, follicle regression is a similar process;
follicular growth (Cooke
Means with the different lowercase letters within the same column are
significantly different (P < 0.05).

The distribution of chicken MMP13 protein in the stroma cells of
sexually immature chicken ovary, and in the theca cells of sexually
mature chicken ovary, suggest that MMP13 is involved in the action of a
cascade of proteolytic events for folliculogenesis, and proper follicle
function. In ovarian follicles, the expression of MMP13 is significantly higher in theca cells than in granulosa cells. These results suggest that
the MMP13 is secreted mainly by theca cells to regulate follicle growth
and ovulations. In accordance with the results of this study, MMP13
was also shown to be localized in rat ovary (Balbin et al. 1996), and in
the granulosal and thecal layers of bovine preovulatory follicles (Bakke
et al. 2004). In addition, the expression of MMP13 is stimulated by LH
in rat (Komar et al. 2001), PGF2α in sheep (Ricke et al. 2002), and
GnRH in cattle (Bakke et al. 2004). In broiler hens consuming feed ad
libitum compared to feed-restricted hens, granulosa cells from F1 fol-
licles have less collagenase-3-like gelatinolytic activity (Liu et al. 2014),
consistent with the fact that less ovulation of mature follicles occurs in
the former, emphasizing the importance of MMP13 in chicken follicle
maturation. Whether the expression of MMP13 in laying hens is regu-
lated by the aforementioned hormones requires further investigation.
Studies have indicated that MMP13 plays a role in the processes of
vascularization and ossification (Inada et al. 2004; Negev et al. 2008),
a nd induction and expression of MMP13 coincided with the onset of
angiogenesis and blood vessel formation in the choroida.ntic mem-
brane (Zijlstra et al. 2004). We also found that chicken 
MMP13 was
expressed in the veins of the theca cell layer; consistently, the expression of
VEGFA mRNA exhibits a similar pattern (Figure S2), which suggests
that MMP13 is likely involved in angiogenesis. Further study is re-
quired to clarify the relationship between MMP13 and VEGFA in
chicken follicle growth.

As the expression dynamics of chicken MMP13 accompany fol-
lle growth and ovulation, we set out to analyze the regulatory
mechanism of MMP13 transcription and identified a critical region
stimulating its transcription. In this region, six SNPs were identified,
among which haplotype D caused by SNPs at –1356 and –1079 sites
has a positive effect on the age at first laying trait in the White
Recessive Rock population, and hens of diplotype DD are expected
to lay the first egg about 8 d earlier (Table 3). Moreover, luciferase
assay indicated that the promoter of a chicken MMP13 gene har-
boring 
G-1356/A-1128/C-1094/T-1079 has a higher transcriptional activity
than one harboring A-1356/G-1128/G-1094/C-1079. Using
Genomatix Online Software (http://www.genomatix.de/index.
html), transcription factors that can bind to these cis-elements were
predicted. The two polymorphic alleles may have different binding
affinity for MEL1 DNA-binding domains 2 (MELS), Kruppel-like
factor 7 (KLF7), and Grainyhead-like2 (Grhl2) at g.–1356 (G > A),
g.–1128 (A > G), and g.–1094 (C > G), respectively. MEL1 en-
codes a zinc finger protein, and overexpression of a MEL1 lacking
PR domain blocked granulocytic differentiation, acting as one of the
causative factors in the pathogenesis of myeloid leukemia (Nishikata
et al. 2003). Chicken KLF7 contains three C2H2-type zinc fingers
domain at the C-terminus (Zhang et al. 2013) that are important
regulators of cell proliferation and differentiation in several differ-
ent organ systems (Laub et al. 2005; Lei et al. 2005; Caiazzo et
al. 2010). Grhl2 belongs to the grainyhead-like transcription factor
family, plays an important role in growth and development, neural
tube closure, and epithelial cell differentiation (Werth et al. 2010;
Pyrgaki et al. 2011; Senga et al. 2012). Whether these SNPs affect
transcription of chicken MMP13 by interfering with the binding of
MELS, KLF7, and Grhl2 remains to be determined.

In conclusion, in laying hens, the expression of both chicken 
MMP13 mRNA and protein was increased significantly in the ovary of 159-d-
old hens and POF1 follicles, and chicken MMP13 protein was pre-
dominantly expressed in theca cells of sexually mature ovaries. Positive
cis-acting element controlling chicken 
MMP13 transcription was iden-
tified, and, in this region, six SNPs were found and genotyped in
chicken populations. In the White Recessive Rock population, hens
with A-1356/C-1079 haplotype had earlier age at first laying than those
with the G-1356/T-1079 haplotype, and exhibited lower transcriptional activity by luciferase assay. These results collectively suggest that
MMP13 plays an important role in chicken follicle growth and POF
regression, and that polymorphisms in its promoter region could be
used as molecular markers for improving the trait age at first laying in
chicken breeding.

Table 3 Effect of the six single nucleotide poly-
morphisms on chicken MMP13 transcriptional
activity in theca cells. Data are presented as
mean ± SEM from at least four replicates for each
construct.

| Diplootype | Number | Age at First Laying | Egg Number at 32 wk |
|------------|--------|---------------------|---------------------|
| AA         | 335    | 176.27 ± 0.48 a     | 31.65 ± 0.39        |
| AD         | 162    | 176.73 ± 0.69 a     | 31.35 ± 0.55        |
| DD         | 8      | 168.86 ± 3.33 b     | 35.57 ± 2.62        |

Means with the different lowercase letters within the same column are
significantly different (P < 0.05).
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LITERATURE CITED

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