Effect of polymorphisms in the 5'-flanking sequence of *MC1R* on feather color in Taihang chickens

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ABSTRACT *MC1R* plays an important role in the regulation of the formation, transfer, and deposition of melanin in animals and is important for determining coat color. Many studies have reported on single nucleotide polymorphisms (**SNP**s) in the coding sequence of *MC1R*. However, few studies have investigated the polymorphisms in the 5'-flanking sequence of *MC1R*. In this study, we sequenced 2000 bp of the 5'-flanking sequence of *MC1R* in 300 Taihang chickens with brown feathers (**MTH**) and 300 Taihang chickens with black feathers (**HTH**). The sequencing results showed that 4 SNPs (*MC1R* g.18838722 G > C, g.18838624 T > C, g.18838694 G > A, and g.18838624 C > T) were located in the 5'-flanking sequence of *MC1R* between the MTH and HTH groups. Association analysis showed that there was a significant correlation between the 4 SNPs and feather color in Taihang chickens. The correlation between *MC1R* g.18838624 T >C and feather color of Taihang chicken was 100%, of which the CC (E1) genotype is MTH and the TT (E2) genotype is HTH. Furthermore, there was a significant correlation between *MC1R* g.18838624 T > C and egg production at 302 d. E1 (184.14 ± 0.674) was significantly higher than that in E2 (181.75 ± 0.577) ($P < 0.05$). Luciferase reporter assays were used to detect the transcriptional activity of MC1R with different SNP genotypes. The results showed that the luciferase activity of E2 was significantly higher than that of E1 ($P < 0.05$). In addition, transcription factor-binding site predictions showed that E2 creates a new binding site for *ZEB1*. RT−qPCR results revealed that the expression of *MC1R* in E2 was significantly lower than that in E1 ($P < 0.05$), and the expression of *ZEB1* in E2 was significantly higher than that in E1 ($P < 0.05$). Overexpression and shRNA experiments demonstrated that *ZEB1* regulates the expression of *MC1R* in DF1 cells. *ZEB1* has a negative regulatory effect on the transcriptional activity of *MC1R*; it inhibits the expression of *MC1R* and affects the feather color of Taihang chickens. This study provides new insight into the molecular mechanism of feather color formation and the transcriptional regulation of *MC1R* in Taihang chickens.

Key words: Taihang chicken, *MC1R*, 5'-flanking sequence, SNPs, *ZEB1*

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INTRODUCTION

The feather color of poultry is an important feature of different breeds, and has always been valued by breeders and poultry producers. Feather color is a genetic marker that can be used for determining hybrid combinations, variety and purity of genetic relationships, and evaluating product quality. In-depth studies of the genetic mechanisms that determine bird feather color show that feather color is mainly determined by pigmentation and the levels of melanin and melanocyte synthesis (Robbins et al., 1993; Jackson et al., 1994; Kerje et al., 2003). The appearance of the nerve crest, the generation, migration, proliferation and differentiation of melanin cells, and the regulation of melanin production after melanocyte migration to hair follicles are all important processes in melanin production (Li et al., 2015; Yang et al., 2017). Any mutation in the factors involved in the above process (including structural proteins, enzymes, cellular transcription factor receptors and growth factors) may lead to variation in feather color (Huang et al., 2020).

MC1R (melanocortin 1 receptor) is a G protein-coupled receptor located on the plasma membrane of melanocytes and has seven transmembrane domains. In chickens, the E locus encodes the MC1R protein consisting of 314 amino acids. The gene is located on chromosome 11 and has a long 945 bp CDS region (Kabir et al.,

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2020; Zanna et al., 2021). Previous studies have shown that *MC1R* plays a key role in the regulation of eumelanin (black/brown) and phaeomelanin (red/yellow) feather pigments (Robbins et al., 1993; Jackson 1997). The main regulatory mechanism of bird feather color is the binding of *MC1R* with α-melanocyte stimulating hormone (**αMSH**), which increases eumelanin, whereas binding with the inverse agonist Agouti protein increases pheomelanin and reduces eumelanin (Yeo et al., 2014). In terms of coat color variation, great progress has been made in understanding the effects of polymorphisms in *MC1R*. It has been found that *MC1R* mutations are related to coat color variation in mice, goats, pigs, dogs, horses and other animals (Marklund et al., 1996; Newton et al., 2000; Deng et al., 2009; Reissmann and Ludwig 2013; Wu et al., 2017; Dürig et al., 2018). In a study of chicken *MC1R* polymorphisms, several polymorphic sites, such as exon positions 69, 12, 274, 376, 398, 427, 636, and 834, have been shown to affect variation in chicken feather color (Hoque et al., 2013; Yeo et al., 2014; Yang et al., 2019). In a study of cream-colored Australian cattle dogs, Dürig found that coat color could not be explained by the common variant *MC1R* c.916C > T. Through genome-wide sequencing, a single nucleotide variant in the microphthalmia-associated transcription factor (**MITF**) binding site of the cream-colored Australian cattle dog *MC1R* promoter was identified that significantly decreased transcription of *MC1R* (Dürig et al. 2018). The decreased expression of *MC1R* led to a reduction in melanin synthesis and the appearance of cream-colored coats. That is, in cream-colored Australian cattle dogs, Chr5:63695679 was changed from G to C, while Chr5:63695679 of the normal coat color remained G. Although this SNP in the promoter region does not directly lead to changes in the MC1R protein, it changes the transcriptional activity and has effects on downstream genes to promote or inhibit transcription, thus changing biological traits. Yung found that the *MC1R* coding region was conserved in black-bellied voles and domestic mice and has homology with G protein-coupled receptors. At least 11 different *MC1R* transcription initiation sites were identified (Lai et al., 2016). However, polymorphisms in the promoter region of the chicken *MC1R* gene have not been reported. The purpose of this study was to examine the molecular structure of the promoter region of Taihang chickens.

Taihang chickens are a local chicken breed located near the aihang Mountains with the advantage of producing high quality eggs. Our previous studies found that there was a polymorphism in *MC1R* c.637C > T in Taihang chickens that correlated with feather color (Fan et al., 2021). In this study, a 2000 bp fragment of the *MC1R* 5'-flanking sequence in Taihang chickens was sequenced, the SNPs in this region were screened, and a correlation between SNPs, feather color, and egg production in Taihang chickens was identified. The effects of polymorphic sites on *MC1R* transcription and the potential molecular mechanism of *MC1R* are discussed. These results are of great significance for elucidating the molecular characteristics of *MC1R*, understanding its function in egg production, and comprehensively exploring the molecular mechanism of feather color formation in Taihang chickens.

MATERIALS AND METHODS

Experimental Birds and Management

All animal experiments were conducted according to the guidelines of the Declaration of Helsinki and approved by the Institutional Animal Care and Use Committee at Hebei University of Engineering (AEEI-16015) (Hebei, China).

Sampling and DNA Extraction

A total of 600 Taihang chickens [300 black feather Taihang chickens (HTH group) and 300 brown feather Taihang chickens (MTH group)] were used in this study. All chickens were obtained from Hebei Jinai company and maintained in a controlled experimental environment (light cycles and feed nutrition were constant with unrestricted eating and drinking) and had a record of laying eggs for 500 d. From each chicken, 2 mL of blood was collected from the wing vein (using 20% EDTA as an anticoagulant) and stored at −20°C for later DNA extraction. In the 2 feather color groups, we selected 10 chickens according to egg laying (5 chickens in each group), collected the hair follicles in a 2 mL cryotube, stored them in liquid nitrogen, and transferred them to the laboratory for storage at −80°C. A DNA and RNA extraction kit (Tiangen, Beijing, China) was used to obtain total RNA and DNA.

Cloning, Sequencing, and Genotyping

Primers were designed according to the chicken *MC1R* 5'-flanking sequence (GenBank accession number: NM_001031462.1) and synthesized by Tsingke Company (Tianjin, China). *GAPDH* (accession number: NM_204305.2) was used as an internal reference gene. The primer sequences are shown in Table 1. Sanger sequencing was performed on the MC1R 5'-flanking sequences of 10 MTH and HTH chickens to identify polymorphisms (Tsingke, Tianjin, China). The sequencing results were aligned and compared with DNAMAN (LynnonBiosoft, San Ramon, CA). Based on the sequencing results, the candidate SNPs in the 5'-flanking sequence of the *MC1R* gene (*MC1R* g.18838624 T > C, *MC1R* g.18838694 A > G, *MC1R* g.18838624 C > T and *MC1R* g.18838721 G > A, *MC1R* g. 18838722 C > G) were genotyped by the Mass ARRAY technique (Zhou et al., 2018).

Calculation of Genetic Parameters of Polymorphic Loci

Online tools (http://www.msrcall.com/Gdicall.aspx) were used to calculate genetic parameters. A chi-square

Table 1. Primers used in this study.

Primers	Primer sequence (5'-3')	Tm (°C)	Size(bp)	Usage
S1	F:5' CCCAACCTTACGTGATCTCAC 3'	56.9	866	Primers for sequencing the first segment of the 5' extension of *MC1R*
	R:5' GCACACACGGTGAATTTCAG 3'	56.8		
S2	F:5' CTGCTGTGTAACGGTGTATGAG 3'	56.0	1230	Primers for sequencing the second segment of the 5' extension of *MC1R*
	R:5' AGCGGGTCACTGTGTACCTT 3'	56.9		
S3	F:5' CGCACTCAATCACTTCTTTGAC 3'	57.6	1212	Primers for sequencing the third segment of the 5' extension of *MC1R*
	R:5' CATGATGCTGTGGTAGCGC 3'	57.9		
S4	F:5' GAACAGGAATCTGCACTCGC 3'	57.9	1214	Primers for sequencing the fourth segment of the 5' extension of *MC1R*
	R:5' GCCGCCGTAGAACAAAGAC 3'	58.2		
S5	F:5' GTCAAGGCTGAGAACGGGA 3'	58.0	567	Primers for sequencing of *GAPDH*
	R:5' ACGGCAGGTCAGGTCAACA 3'	59.0		
S6	F:5' TGCACTCGCCCATGTACTACTT 3'	60.4	257	Primers of *MC1R* for real-time PCR
	R: 5' TCGATGACATTGTCCATGTGG 3'	60.1		
S7	F:5' AGATCAGAGACATGTGACGCAGT 3'	58.7	120	Primers of *ZEB1* for real-time PCR
	R:5' TTCTCTCCACTGTGGATTCGTAG 3'	58.7		
S9	F:5' CCTCTCTGGCAAAGTCCAAGTG 3'	60.1	193	Primers of *GAPDH* for real-time PCR
	R:5' CCATTTGATGTTGCTGGGGTC 3'	60.4		

test was used to explore the correlation between candidate SNP genotypes and feather color and 302-d egg production in Taihang chickens. The effects of genotypes were analyzed by single factor analysis of variance, multiple comparisons were performed by the LSD method, and all results are reported as the mean values ± SEMs (Bo et al., 2021). Statistical analysis was performed with independent sample t-tests using SPSS 20.0 statistical software (IBM Corp., Armonk, N.Y.)

Bioinformatic Analysis

The 5'-flanking sequence region of *MC1R* of different species was analyzed by MEGA 11 (Tamura et al., 2021), the promoter region of *MC1R* was analyzed using DNASTAR (DNASTAR Inc, Madison, WI), and the transcription binding sites of the promoter were predicted using JASPAR (http://jaspar.binf.ku.dk/).

Construction of Vectors

Chicken *MC1R* g.18838624 T > C TT genotype (E1) and CC genotype (E2) were cloned into the pGL3-Basic vector (Sangon, Shanghai, China), and the luciferase expression vectors pGL3-C and pGL3-T were constructed. The *ZEB1* CDS region of Taihang chicken was cloned into the pIRES2-EGFP (Sangon, Shanghai, China) vector to construct the *ZEB1* overexpression vector pIRES2-EGFP-*ZEB1*. Vectors were purified with a vector mini kit without endonuclease treatment (Tiangen, Beijing, China).

Haplotype Quantification

Total RNA was extracted from Taihang chickens with E1 and E2 genotypes using an RNA Extraction Kit (Tiangen, Beijing, China). The expression levels of *ZEB1* and *MC1R* in E1 and E2 genotype Taihang chickens were detected by RT–qPCR with *GAPDH* as the internal reference. The RT–qPCR primer sequence information is shown in Table 1.

Cell Culture

The chicken DF-1 cells and HEK293T cells (obtained from laboratory preservation) were grown in DMEM (GIBCO, Waltham, MA) supplemented with 10% fetal bovine serum (GIBCO, Waltham, MA) in an incubator at 37°C and 5% CO_2. According to the instructions, the luciferase reporter vector was transfected into the HEK293T cell line using Lipofectamine 2000 (Thermo Fisher, Waltham, MA). The steps of transfection were as follows: HEK293T cells were grown to a density of 70% in a 24-well plate. Lipofectamine 2000 reagent and pGL3-T, pGL3-C, and pGL3-Basic were diluted in OPTI-MEM(GIBCO, Waltham, MA). Diluted pGL3-T, pGL3-C and pGL3-Basic were added to each tube of diluted Lipofectamine 2000 reagent (1:1) and incubated for 20 min. Finally, the pGL3-T-lipid complex, pGL3-C-lipid complex and pGL3-Basic-lipid complex were added to the cells. After transfection for 48 h, HEK293T cells were collected and treated to measure luciferase activity.

The overexpression vector of *ZEB1* pIRES2-EGFP-*ZEB1* and the control vector pIRES2-EGFP-basic were transfected into DF-1 cells with Lipofectamine 2000 (Thermo Fisher, Waltham, MA). After transfection for 48 h, DF-1 cells were collected, and RNA was extracted with a cell RNA extraction kit (Tiangen, Beijing, China). The expression levels of *ZEB1* and *MC1R* in pIRES2-EGFP-*ZEB1* and pIRES2-EGFP transfected cells were detected by RT–qPCR.

Transcription Factor Interference

The shRNA vector of *ZEB1*, shRNA-pSGU6/GFP/Neo-*ZEB1*, was constructed (Sangon, Shanghai, China). Lipofectamine 2000 was used to transfect pSGU6/GFP/Neo-*ZEB1* and a corresponding negative control NC (Sangon, Shanghai, China) into DF-1 cells. After transfection for 48 h, the cells were collected, and RNA was extracted using a cell RNA extraction kit (Tiangen, Beijing, China). The expression levels of *ZEB1* and *MC1R*

in pSGU6/GFP/Neo-*ZEB1*- and NC-transfected cells were detected by RT–qPCR.

Statistical Analyses

All statistical analyses were performed with independent sample t-tests using SPSS 20.0 statistical software (IBM Corp., Armonk, N.Y.), and the mean of three replicates was evaluated and is displayed as the mean ± standard error (SE).

RESULTS

Identification of SNPs in the Promoter Region of MC1R and Correlation Analysis with Production Performance in Taihang Chicken

The sequencing results showed that there were four SNPs in the 5'-flanking sequence of *MC1R* in MTH and HTH Taihang chicken populations (g.18838722 G > C, g.18838624 T > C, g.18838694 G > A and g.18838624 C > T) (Figure 1). The genotyping results showed that all 4 loci were polymorphic. Population genetic analysis showed that all 4 polymorphic loci were moderately polymorphic ($0.25 < PIC < 0.5$) in the Taihang chicken population (Table 2), and there were significant differences in the gene frequency and genotype frequency of the 4 polymorphic loci between MTH and HTH (Table 3). The egg production of the *MC1R* g.18838624T > C CC genotype was significantly higher than that of the TT genotype (181.75 ± 0.577 and 184.14 ± 0.674, respectively; $P < 0.01$), and there was no significant correlation between other loci and egg production (Table 4). These results showed that the 4 polymorphic sites in the core promoter region of *MC1R* in Taihang chickens were significantly correlated with feather color ($P < 0.01$), and the g.18838624 T > C was significantly correlated with egg production at 302 d in Taihang chickens ($P < 0.01$). Sequence analysis of the promoter region of the *MC1R* gene in different species showed that chickens share high homology with Japanese quail, followed by duck, and poor homology with zebrafish (Figure 2), and that MC1R g.18838712 T > C is not highly conserved across species (Figure 3).

Effect of MC1R g.18838712 T > C on the Activity of the MC1R Promoter

To study the effect of the g.18838624 T > C polymorphism on the promoter activity of *MC1R*, the constructed pGL3-T, pGL3-C and pGL3-Basic vectors were transfected into HEK293T cells. The results showed that the luciferase activity of pGL3-C in HEK293T cells was significantly higher than that of pGL3-T (Figure 4).

The MC1R g.18838712 T > C Polymorphism Adds a Novel Transcription Factor-Binding Site

To further identify the function of *MC1R* g.18838624 T > C, an online tool was used to predict the binding of

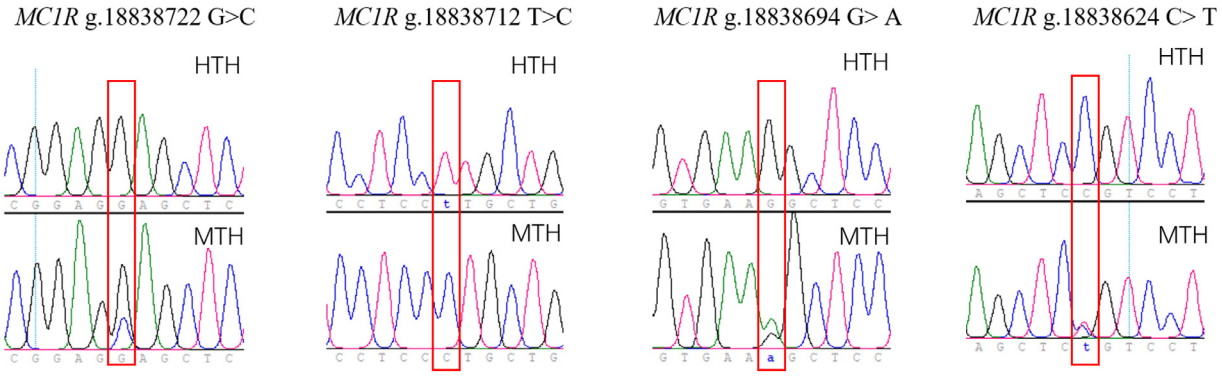

Figure 1. The sequencing results of the 5'-flanking regions of *MC1R* in MTH and HTH populations (MTH represents the brown feather Taihang chicken population, HTH represents the black feather Taihang chicken population).

Table 2. Population genetic analysis of four polymorphic loci of *MC1R*.

Locus	Genotype			Gene frequency		*PIC*	*He*	*Ne*
MC1R g.18838722 G > C	GG (n = 328)	GC (n = 156)	CC (n = 47)	G 0.76	C 0.24	0.30	0.36	1.57
MC1R g.18838712 T > C	TT (n = 259)	CT (n = 0)	CC (n = 285)	T 0.48	C 0.52	0.37	0.50	2.00
MC1R g.18838694 G > A	GG (n = 323)	GA (n = 158)	AA (n = 48)	G 0.76	A 0.24	0.30	0.36	1.57
MC1R g.18838624 C > T	CC (n = 326)	CT (n = 160)	TT (n = 47)	C 0.76	T 0.24	0.30	0.36	1.57

He, heterozygosity; Ne, effective number of alleles; PIC, polymorphic information content

Table 3. Genotype and allele frequencies of four polymorphic loci of *MC1R* in MTH and HTH.

Locus	Genotype	Genotype frequencies		Sample size		*P*	Allele gene	Gene frequency	
		HTH	MTH	HTH	MTH			HTH	MTH
MC1R g.18838722 G > C	GG	1.00	0.25	262	66	3.12148E−70	G	1.00	0.54
	GC	0.00	0.58	0	156		C	0.00	0.46
	CC	0.00	0.17	0	47				
MC1R g.18838712 T > C	TT	1.00	0.00	259	0	2.5424E−120	T	1.00	0.00
	TC	0.00	0.00	0	0		C	0.00	1.00
	CC	0.00	1.00	0	285				
MC1R g.18838694 G > A	GG	1.00	0.25	256	67	1.99871E−69	G	1.00	0.53
	GA	0.00	0.58	0	158		A	0.00	0.47
	AA	0.00	0.17	0	48				
MC1R g.18838624 C > T	CC	1.00	0.23	263	63	2.59599E−72	C	1.00	0.53
	CT	0.00	0.59	0	160		T	0.00	0.47
	TT	0.00	0.17	0	47				

Table 4. Effects of four polymorphic loci in *MC1R* on egg production in Taihang chickens (mean ± SEM)[c].

Locus	Genotype	Egg production/MS
MC1R g.18838722 G > C	CC	182.47 ± 0.516[a](n = 328)
	CC	183.52 ± 0.870[a](n=156)
	CC	185.26 ± 2.026[a](n = 47)
MC1R g.18838712 T > C	TT	181.75 ± 0.577[a](n = 259)
	CC	184.14 ± 0.674[b](n = 285)
MC1R g.18838694 G > A	GG	182.38 ± 0.519[a](n = 323)
	GA	183.83 ± 0.905[a](n = 158)
	AA	185.48 ± 1.996[a](n = 48)
MC1R g.18838624 C > T	CC	182.41 ± 0.516[a](n = 326)
	CT	183.89 ± 0.895[a](n = 160)
	TT	185.30 ± 2.031[a](n = 47)

Note: Data in the same column should be shoulder marked with the same letter or no letter to indicate that differences are not significant, and different letters indicate that differences are significant.

[a]indicate no significant difference, while superscripts.

[b]indicate significant difference.

[c]Superscripts

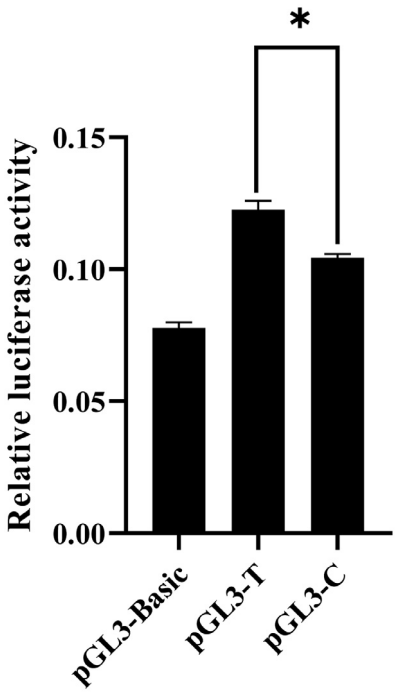

Figure 2. The *MC1R* g.18838712 T>C mutation regulates *MC1R* promoter activity. The constructed pGL3-T, pGL3-C and pGL3-Basic constructs were transfected into HEK293T cells, and the luciferase activity was detected by a dual-luciferase reporter analysis system. The results are expressed as the mean ± SEM (n = 3). $^{*}P < 0.05$; $^{**}P < 0.01$.

transcription factors to this site. The results showed that the *MC1R* g.18838624 T > C polymorphism creates a new binding site for the transcription factor *ZEB1* (Figure 5).

Haplotype Quantification

The RT–qPCR results showed that the expression of *MC1R* in the E1 genotype was significantly higher than that in the E2 type, while the expression of *ZEB1* in E2 was significantly lower than that in E1 (Figure 6).

ZEB1 Regulated the Expression of MC1R

To determine whether *ZEB1* is involved in regulating the expression of *MC1R*, the overexpression vector pIRES2-EGFP-ZEB1 was transfected into DF1 cells. The RT–qPCR results showed that the expression level of *MC1R* in DF-1 cells transfected with the pIRES2-EGFP-ZEB1 vector was significantly lower than that in the control group ($P < 0.01$), and the expression level of *ZEB1* increased significantly after pIRES2-EGFP-ZEB1 transfection ($P < 0.01$) (Figure 7). The pSGU6/GFP/Neo-ZEB1 vector was transfected into DF-1 cells. RT–qPCR results showed that the expression level of *MC1R* in DF-1 cells transfected with the pSGU6/GFP/Neo-ZEB1 vector was significantly higher than that in the negative control group ($P < 0.01$) (Figure 6), and the expression level of *ZEB1* decreased significantly after pSGU6/GFP/Neo-ZEB1 transfection. The results showed that *ZEB1* overexpression significantly decreased the expression of *MC1R*, while interference with *ZEB1* significantly increased the expression of *MC1R*. These results suggested that *ZEB1* inhibited the expression of *MC1R* in DF-1 cells by binding to the promoter region of *MC1R*.

DISCUSSION

The difference in feather color in chickens is mainly affected by the production and transformation of melanin, and *MC1R* plays an important role in this process (Zhang et al., 2017; Khumpeerawat et al., 2021; Schwochow et al., 2021). *MC1R* is a highly polymorphic

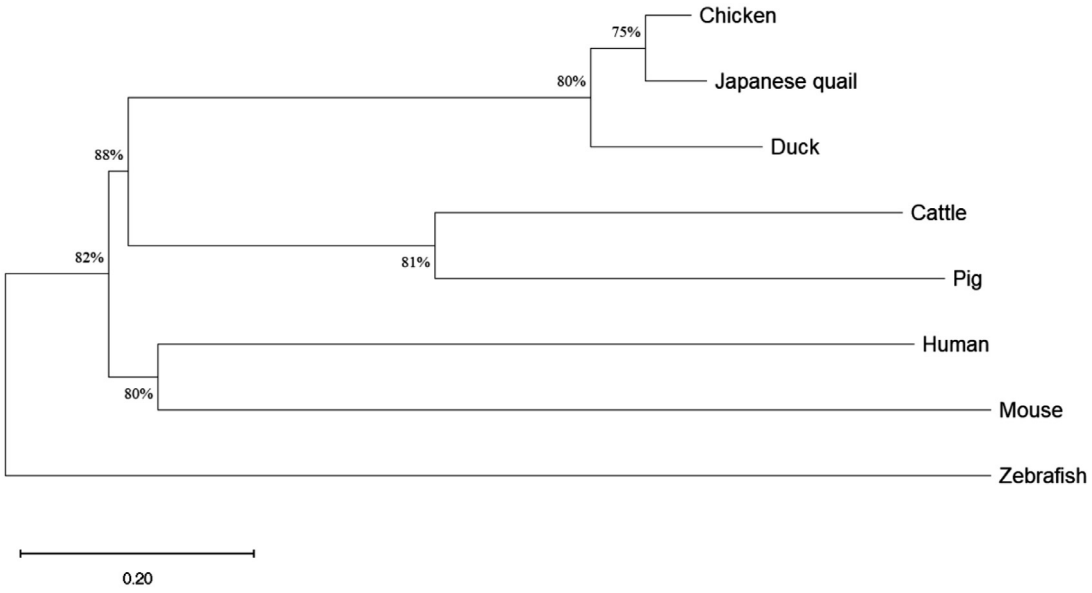

Figure 3. Phylogenetic tree of 2000 bp 5'-flanking region of *MC1R* in chicken and other species.

gene, and more than 200 variants have been found (Zanna et al., 2021). Some studies have shown that *MC1R* mutations or base substitutions can affect various phenotypes of feather color in chickens (Liu et al., 2010; Oh et al., 2010; Kim and Production 2020). When Zhang (Zhang et al., 2020) studied the formation of Yunnan Piao chickens' tile gray feathers, through sequencing and alignment of the whole coding region of *MC1R*, a total of 10 SNP sites were detected, of which 8 were nonsynonymous mutations that caused amino acid changes. Among these, C69T, T212C, and A274G were significantly correlated with tile gray feathers. At the same time, SNPs of the *MC1R* promoter region can also affect the formation of hair color. When studying the relationship between *MC1R* polymorphisms and coat color in Chinese yak, Dongmei Xi (Xi et al., 2012) found that there were 13 SNPs in Chinese yak compared with other bovine *MC1R* sequences. These included 4 SNPs (T-129C, A-127C, C-106T, G-1A) in the 5' flanking sequence and 9 SNPs (C201T, T206C, C340A, C375T, T663C, G714C, C870T, G871A, and T890C) in the coding sequence. This study revealed the high genetic variability of *MC1R* but did not experimentally verify the effects of these SNPs on coat color. In this study, we genotyped the 5'-flanking sequence of *MC1R* in MTH and HTH. The genotyping results showed that all 4 polymorphic sites in the promoter region of *MC1R* were significantly correlated with feather color in Taihang chickens ($P < 0.05$). Interestingly, the Taihang chicken population corresponding to the CC genotype in *MC1R* g.18838624 T > C is MTH, while the Taihang chicken population corresponding to the TT genotype is HTH,

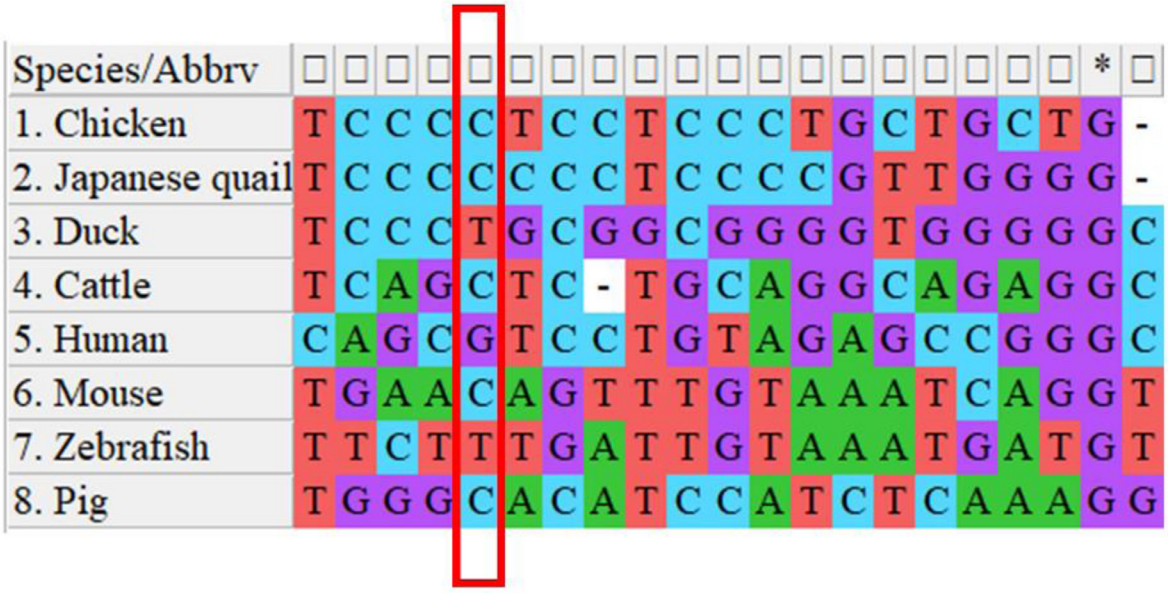

Figure 4. Comparison of *MC1R* g.18838712 T > C homology between chicken and other species.

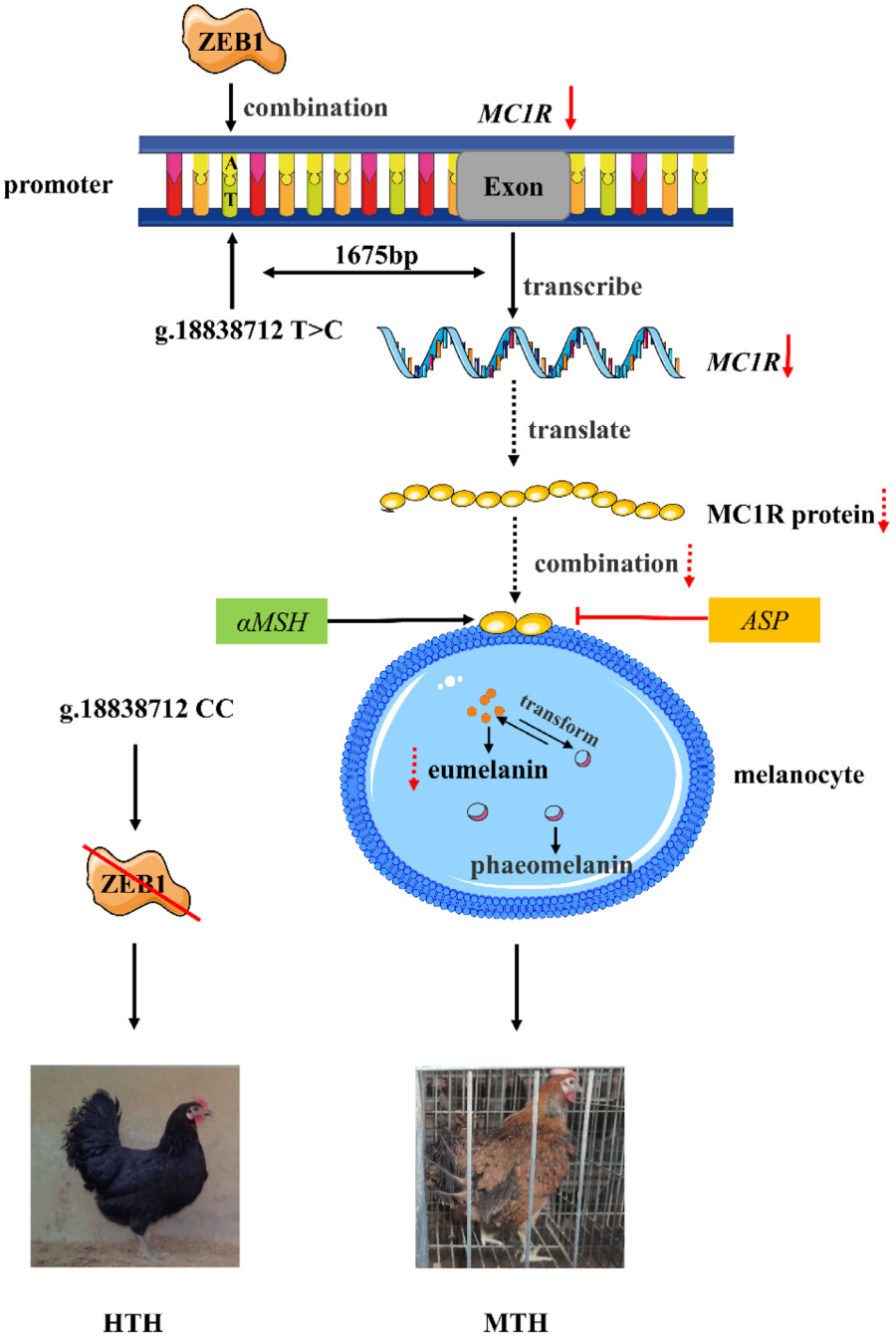

Figure 5. Flow diagram of *MC1R* g.18838712 T>C CC mutation regulates feather color in Taihang chickens through *ZEB1*.

and the correlation between genotype and feather color is 100%. The haplotype quantitative expression results showed that transcription of *MC1R* in the TT genotype was significantly higher than that in the CC genotype, and the expression of *MC1R* in HTH was significantly higher than that in MTH. After associating SNPs of the *MC1R* promoter region with egg production in Taihang chickens, we found that egg production of the CC genotype in *MC1R* g.18838624 T > C was significantly higher than that of the TT type. These results indicate that the egg production of MTH was significantly higher than that of HTH, which was consistent with the egg production data recorded in actual production. We found that this polymorphic site was in linkage disequilibrium, which may be a spurious association. More experiments are needed to verify its role in functional studies exploring this polymorphic locus, such as recalculating correlations after increasing the amount sample information, controlling for other variables such as day and weight of laying chickens when collecting data on egg production as these factors may impact subsequent data analysis.

The effect of gene promoter region polymorphisms on downstream genes has been studied in many species. For example, Li et al. found that the single base mutation located 25 kb upstream of chicken *TBX5* activates the expression of *TBX5* (Li et al., 2020). The change in TBX5 expression affects the color of chicken leg feathers. Experiments show that there is a significant correlation

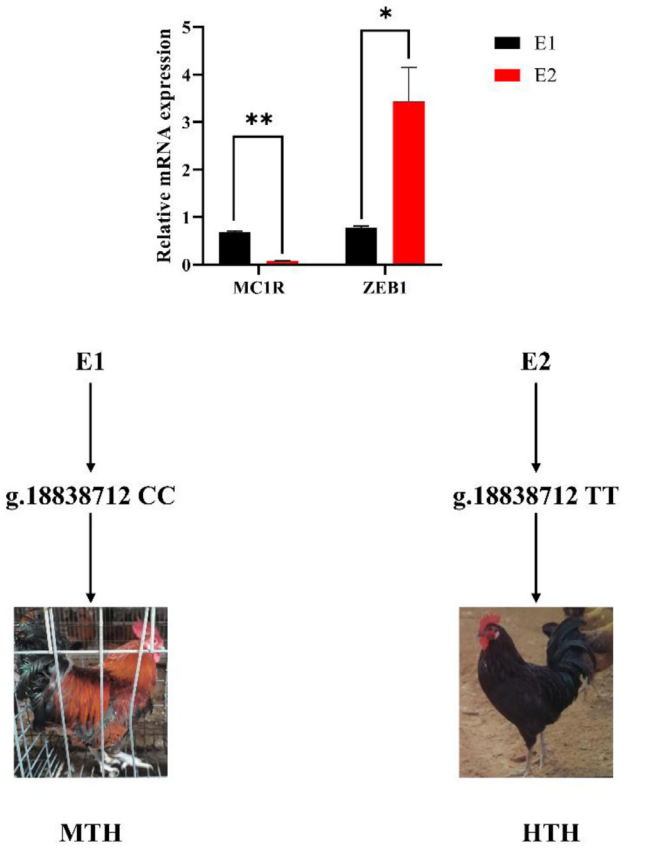

Figure 6. Expression of *MC1R* and *ZEB1* in E1 and E2 genotype Taihang chickens. The results are expressed as the mean ± SEM (n = 3). $^{*}P < 0.05$; $^{**}P < 0.01$. (E1: *MC1R* g.18838712 T>C TT; E2: *MC1R* g.18838712 T > C CC.

between the TBX5 promoter region polymorphism and the color change of chicken leg feathers. The T > G polymorphism in the promoter region of sheep *NR5A2* located at −700 nt significantly affected the average litter size of ewes and created a new binding site for the transcription factor *MTF-1*. The results showed that *MTF-1* positively regulated the transcriptional activity of *NR5A2*, which increased the expression of *NR5A2* and improved the fecundity of Hu sheep (Li et al., 2019). There is a transcription factor-binding site for *GATA2* in the 5'-flanking sequence of chicken *KLF7*. Overexpression and gene knockout experiments showed that *GATA2* increased the expression of *KLF7,* whereas *GATA3* inhibited expression. These results provide an important reference for the molecular mechanism of transcriptional regulation of *KLF7* in chicken adipose tissue (Lin et al., 2021). Promoter haplotypes of *ABCB1* encoding P-glycoprotein differentially affect its promoter activity by altering transcription factor binding (Speidel et al., 2018). By constructing pGL3-T, pGL3-C, and pGL3-Basic for dual luciferase reporting assays, we found that *MC1R* g.18838624 T > C polymorphism significantly regulated the activity of the *MC1R* promoter, and the promoter activity in the CC genotype was significantly higher than that in the TT genotype. These findings indicate that this polymorphism may be involved in regulating the expression of *MC1R*, thus affecting the feather color of Taihang chickens.

In this study, we show that *MC1R* g.18838712 T > C produced a new transcription factor-binding site for ZEB1, and that ZEB1 may be directly or indirectly involved in regulating the transcriptional activity of *MC1R*. ZEB1 is a transcriptional inhibitor originally

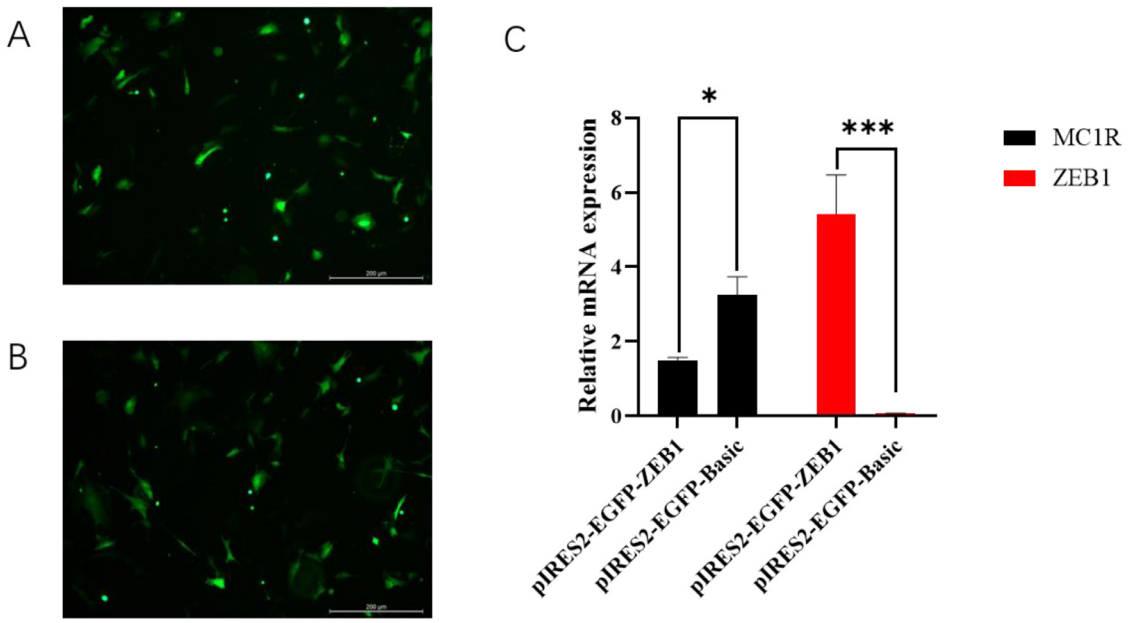

Figure 7. (A) Fluorescence after transfection of the overexpression vector pIRES2-EGFP-ZEB1 in DF-1 cells. (B) Fluorescence after transfection of the overexpression empty vector pIRES2-EGFP-Bacic in DF-1 cells. (C) After DF-1 cells were transfected with overexpression vector pIRES2-EGFP-ZEB1 and overexpression empty vector pIRES2-EGFP-Basic for 48 h, cells were collected, RNA was extracted, and *MC1R* and *ZEB1* expression in different transfection groups was analyzed. The results are expressed as the mean ± SEM (n = 3). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

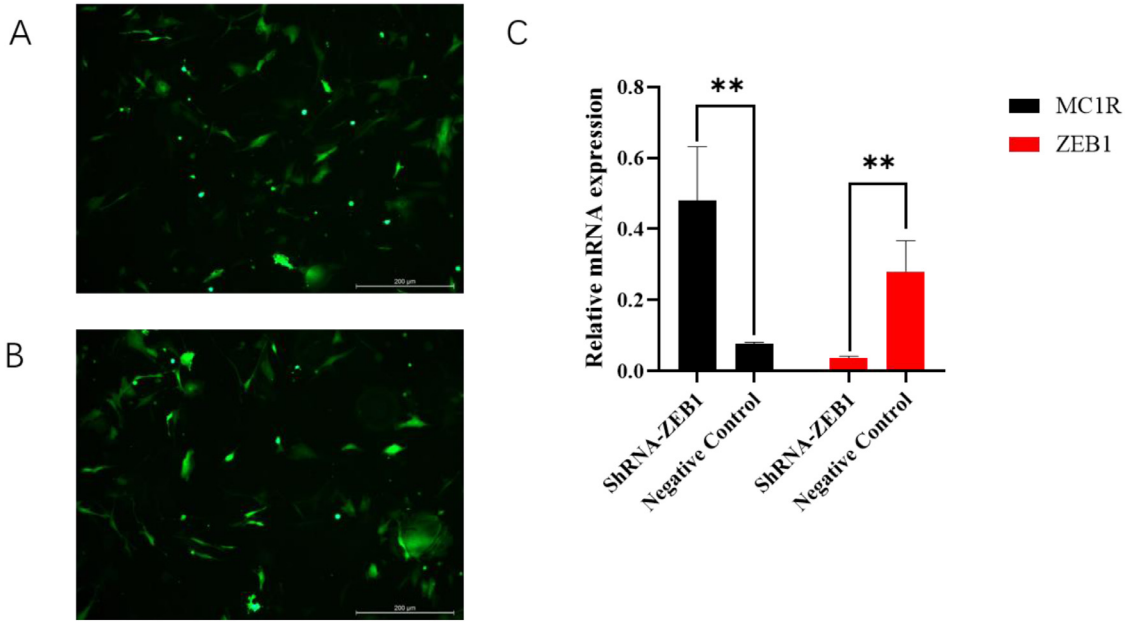

Figure 8. (A) Fluorescence after transfection of the vector-shRNA-ZEB1 in DF-1 cells; (B) Fluorescence after transfection of the vector-negative control in DF-1 cells; (C) After DF-1 cells were transfected with vector shRNA-ZEB1 and vector-negative control for 48 h, cells were collected, RNA was extracted, and *MC1R* and *ZEB1* expression in different transfection groups was analyzed. The results are expressed as the mean ± SEM (n = 3). $^{*}P < 0.05$; $^{**}P < 0.01$.

named δ EF-1. It has been reported for the first time that ZEB1 plays an important role in the development of the lens in chicken as a transcriptional inhibitor of δ 1-crystal enhancer (Birkhoff et al., 2021). ZEB1 belongs to the ZEB transcription factor family, which is composed of the ZEB1 and ZEB2 proteins. The ZEB transcription factor family is involved in the development and differentiation of many cell lines. For example, in vertebrates, the ZEB transcription factor family has been shown to play a key role in the formation of neurospinal cells (**NCC**) and their subsequent differentiation into various derived cells (Bruneel et al., 2020). In a study of the regulation of melanocytes by the family of ZEB transcription factors, Denecker et al. found that ZEB2 and ZEB1 seem to play opposite roles in regulating the proliferation and migration of melanocytes. ZEB2 promotes the proliferation of melanocytes, while ZEB1 is an inhibitor that indirectly regulates the proliferation and migration of melanocytes by regulating MITF. The deletion of ZEB2 in the melanocyte lineage led to the downregulation of MITF and melanocyte differentiation markers and the upregulation of ZEB1 (Denecker et al., 2014). MITF is the first identified melanocyte-specific marker, and it is strongly expressed in differentiated adult melanocytes. MITF is considered to be the main regulator of the melanocyte lineage because it regulates differentiation, cell growth, survival, and melanin synthesis. At the same time, MITF has also been shown to be involved in the regulation of *MC1R* transcription (Guo et al., 2021). This further indicates that ZEB1 may be involved in the transcriptional regulation of *MC1R*. Neurospinal cells are motor neurons and can produce a variety of cell types, including melanocytes, glial cells, adipose tissue, and cardiac smooth muscle cells (Plaschka et al., 2022). As a transcription factor involved in a variety of biological processes and playing an important role in the proliferation and migration of melanocytes, the function of ZEB1 requires further study. Here, the *ZEB1* overexpression vector pIRES2-EGFP-ZEB1 and the interference vector pSGU6/GFP/Neo-ZEB1 were constructed and transfected into DF1 cells. RT–qPCR results showed that *ZEB1* may be involved in negative regulation of *MC1R* expression in DF1 cells by binding to the promoter region of *MC1R* to inhibit the transcription of *MC1R*. We found that *ZEB1* is a transcriptional inhibitor that can bind to the *MC1R* g.18838624T > C CC promoter, reduce its promoter activity and expression levels in DF-1 cells, and regulate the production of melanin to affect feather color. These results provide insight into the molecular mechanism of feather color formation in Taihang chickens.

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DISCLOSURES

The authors declare that there is no conflict of interest.

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