

# An *EAV-HP* insertion in the 5' flanking region of *SLCO1B3* is associated with its tissue-expression profile in blue-eggshell Yimeng chickens (*Gallus gallus*)

Jianfei Chen,<sup>\*,1</sup> Seyed Benyamin Dalirsefat,<sup>†,1</sup> Deping Han,<sup>‡</sup> Xianggui Dong,<sup>\*</sup> Guoying Hua,<sup>\*</sup> Xiaotong Zheng,<sup>\*</sup> Tianlan Xia,<sup>\*</sup> Tianqi Shao,<sup>\*</sup> Xuemei Deng,<sup>\*,2</sup> and Changxin Wu<sup>\*</sup>

<sup>\*</sup>Key Laboratory of Animal Genetics, Breeding and Reproduction of the Ministry of Agriculture & Beijing Key Laboratory of Animal Genetic Improvement, China Agricultural University, Beijing 100193, China; <sup>†</sup>Department of Animal Science, Faculty of Agricultural Sciences, University of Guilan, Rasht, Guilan, Iran; and <sup>‡</sup>College of Veterinary Medicine, China Agricultural University, Beijing 100193, China

**ABSTRACT** We previously reported that blue eggshell color in chickens is associated with a partial endogenous retroviral (*EAV-HP*) insertion in the promoter region of the solute carrier organic anion transporter family member 1B3 (*SLCO1B3*) gene. The *EAV-HP* sequence includes numerous regulatory elements, which may modulate the expression of adjacent genes. To determine whether this insertion influences the expression of neighboring genes, we screened the expression of solute carrier organic anion transporter family members 1C1, 1B1 (*SLCO1C1*, *SLCO1B1*), and *SLCO1B3* in 13 and 10 tissues from female and male Yimeng chickens, respectively. We observed that the insertion only significantly modulated the expression of *SLCO1B3* and did not majorly affect that of *SLCO1C1* and *SLCO1B1*. High expression of *SLCO1B3* was detected in the shell gland, magnum, isthmus, and vagina of the oviduct in female blue-eggshell chickens. We also observed ectopic

expression of *SLCO1B3* in the testes of male chickens. *SLCO1B3* is typically highly expressed in the liver; however, the *EAV-HP* insertion significantly reduces *SLCO1B3* expression. As a liver-specific transporter, a reduction in the expression of *SLCO1B3* may affect liver metabolism, particularly that of bile acids. We also detected higher ectopic expression of *SLCO1B3* in the lungs of birds heterozygous for the *EAV-HP* insertion than in homozygous genotypes. In conclusion, we confirmed that the *EAV-HP* insertion modifies *SLCO1B3* expression, and showed, for the first time, similar expression profile of this gene in all parts of the oviduct in females and testis in males. We also observed different levels of *SLCO1B3* expression in the liver, which were associated with the *EAV-HP* insertion, and significantly higher expression in the lungs of birds with heterozygous genotype. The effects of these changes in the *SLCO1B3* expression pattern on the function of the tissues warrant further investigation.

**Key words:** chicken, blue-eggshell, *EAV-HP*, *SLCO1B3*, expression profile

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## INTRODUCTION

Avian eggshell color diversity is known to contribute to crypsis, solar radiation filtering, enhanced eggshell strength, and sexual selection (Kilner, 2006; Maurer et al., 2012). The color of the eggshell is determined by the proportion of protoporphyrin IX, biliverdin, and

biliverdin zinc chelate (Gorchein et al., 2010). Blue eggshells contain higher levels of biliverdin, whereas brown and pink eggshells contain protoporphyrin IX, and white eggshells contain extremely low amounts or are completely devoid of any pigment (Kennedy and Vevers, 1976; Wang et al., 2007). Blue eggshell color in chickens has been reported as an autosomal dominant trait controlled by the oocyan (*O*) allele (Punnett, 1933), and some mapping regions and genes associated with the blue-eggshell phenotype have been identified (Bitgood et al., 1980; Wang et al., 2010, 2011; Wragg et al., 2012).

We previously demonstrated that the solute carrier organic anion transporter family member 1B3 (*SLCO1B3*) gene containing a partial endogenous

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<sup>1</sup>These authors contributed equally to the work.

<sup>2</sup>Corresponding author: [deng@cau.edu.cn](mailto:deng@cau.edu.cn)

retroviral (*EAV-HP*) insertion in the 5' flanking region is strongly associated with blue eggshells in chickens (Wang et al., 2013). Furthermore, the sequence of the causal mutation is similar to that of the *env* gene of avian leukosis virus subgroup J, which is a type of retrovirus (Smith et al., 1999; Sacco et al., 2000). The occurrence of retroviruses in plant and animal genomes has been extensively studied, and it has been found that partial retroviral fragments can result in high ectopic expression of adjacent genes or abnormal transcription (Matsumine et al., 1991; Chang et al., 2006; Butelli et al., 2012; Zhang et al., 2019). In addition to *SLCO1B3*, there are 2 other adjacent genes, solute carrier organic anion transporter family members 1C1 (*SLCO1C1*) and 1B1 (*SLCO1B1*) (reported as *LOC418189*), which are located 9 kb upstream and ~26 kb downstream of the insertion site, respectively. These 3 genes are members of the organic anion transporting polypeptide (OATP, *SLCO*) family, which are known to transport many endogenous and exogenous substances (Hagenbuch and Meier, 2003; Hagenbuch and Gui, 2008). Solute carrier organic anion transporter family members 1C1, *SLCO1B1*, and *SLCO1B3* are expressed in multiple chicken tissues (Barbosa-Morais et al., 2012; Merkin et al., 2012). Furthermore, *SLCO1C1* and *SLCO1B1* have been suggested as candidate genes affecting the brownness of eggshells (Chuanwei et al., 2014). Therefore, it is necessary to determine whether the expression of these 2 genes is also regulated by the *EAV-HP* insertion.

High ectopic expression of *SLCO1B3* has been reported in the shell gland of blue-eggshell chickens (Wang et al., 2013). Wragg et al. (2013) noted that the *EAV-HP* insertion is associated with a high expression of *SLCO1B3* in the shell gland and oviduct of the Mapuche fowl, a native blue-eggshell chicken breed; however, the part of the oviduct to which these observations pertain remains unclear. OATP1B3 (encoded by *SLCO1B3*) is an important transporter involved in the specific transport of various endogenous and exogenous substances in the liver of humans, including CCK-8, bile acids, and some drug molecules (Ismair et al., 2001; Hagenbuch and Gui, 2008; Meyer and Kim, 2009). Considering that *SLCO1B3* is expressed primarily in the liver (König et al., 2000), a systematic analysis of the expression profile of *SLCO1B3* would be of interest, as this could reveal the systemic function of the *EAV-HP* insertion in blue-eggshell chickens. Moreover, as male and female chickens harbor the same insertional mutation, it would be of interest to examine the expression profile in both hens and roosters.

In this study, we examined the expression of *SLCO1C1*, *SLCO1B1*, and *SLCO1B3* in different *EAV-HP*-insertion genotypes, that is, homozygous dominant insertion (*EAV-HP*<sup>+/+</sup>), heterozygous insertion (*EAV-HP*<sup>+/-</sup>), and homozygous recessive insertion (*EAV-HP*<sup>-/-</sup>), in 13 hen and 10 rooster tissues. The findings of this study are expected to improve our understanding of the effects of the *EAV-HP* insertional mutation on *SLCO1B3* function and the associated changes in blue-eggshell chickens.

## MATERIALS AND METHODS

### Study Animals

Yimeng chickens, obtained from the Shandong Longsheng Agriculture and Animal Husbandry Group Co., Ltd. (Linyi City, Shandong Province, China) were used in this study. This is a native Chinese chicken breed in which the blue-eggshell trait is not fixed. All chickens were raised in cages under the same environment and *ad libitum* conditions. All experimental procedures were approved by the Animal Care and Use Committee of the China Agricultural University (Approval no. XK257).

### Diagnostic Genotyping Test for the EAV-HP Insertion

The chickens included in the experiments were approximately 300 d old. Blood was collected from a wing vein, which was then used for total genomic DNA extraction using the TIANamp Blood DNA Kit (Tiangen, Dalian, China). The concentration and purity of the extracted DNA were determined by spectrophotometry and agarose gel electrophoresis, respectively. All chickens were genotyped based on the retroviral *EAV-HP* insertion in *SLCO1B3* using a previously reported multiplex PCR method (Wang et al., 2013).

### Tissue Sample Collection

Yimeng chickens with different genotypes (*EAV-HP*<sup>+/+</sup>, *EAV-HP*<sup>+/-</sup>, and *EAV-HP*<sup>-/-</sup>) were randomly selected from population 1 (3 males and 3 females for each genotype, i.e., 9 males and 9 females in total) and population 2 (4 *EAV-HP*<sup>+/-</sup> hens and 4 *EAV-HP*<sup>-/-</sup> hens). The chickens were euthanized by severing the jugular vein after anesthesia. Tissue samples were collected from the heart, liver, spleen, lung, kidney, duodenum, jejunum, ileum, colon, ovary, oviduct (magnum, shell gland, isthmus, and vagina), and testis of birds from one or both populations. Samples of hens were collected ~3 to 5 h before the next expected oviposition.

### Real-Time Quantitative PCR

The total RNA was extracted using the RNAprep Pure Kit (Tiangen). The cDNA was synthesized from 1.5 µg of the extracted total RNA using the FastKing RT Kit (Tiangen). The expression of *SLCO1C1*, *SLCO1B1*, and *SLCO1B3* genes was quantified by real-time quantitative PCR using SuperReal PreMix Plus (SYBR Green) (Tiangen). Primers for quantitative PCR (Table 1) were designed using Primer Premier 5.0 software (Premier Biosoft International, Palo Alto, CA) and then synthesized (Sangon Biotech, Beijing, China). Data were collected from 3 biological replicates, and each biological replicate further had 3 technical replicates. The cycling conditions were as follows: 95°C for

**Table 1.** Primers used for real-time quantitative PCR.

Primer names	Primer sequence (5' → 3')	GenBank number	Size (bp)	Anneal temperature (°C)
<i>SLCO1B1</i> -F	TCACATAACTGGAATGGCAA	CGNC:50698	158	58
<i>SLCO1B1</i> -R	AACTGTTTTTAGGGGAGGAA			
<i>SLCO1B3</i> -F	TGGTGATTGCATTTGTAAGCTA	CGNC:50697	197	58
<i>SLCO1B3</i> -R	TGGAGAGCAGGGATTTATGC			
<i>SLCO1C1</i> -F	TGCTGTTGCTCTCATTGTTT	CGNC:9932	184	58
<i>SLCO1C1</i> -R	TAGGTGATGCCATTCTCTCC			
<i>GAPDH</i> -F	CTCTGTTGTTGACCTGACCT	CGNC:49077	125	58~60
<i>GAPDH</i> -R	CAACCTGGTCCTCTGTGTAT			

15 min, followed by 40 cycles of 95°C for 10 s, 58°C (an optimized annealing temperature for each primer pair, Table 1) for 15 s, and 72°C for 24 s. In addition, a mixed sample containing the cDNAs of all sampled tissues from an *EAV-HP*<sup>+/-</sup> chicken was analyzed in each cycle for correcting of system errors. The PCR was performed using CFX96 Real-Time System (Bio-Rad, Hercules, CA). Glyceraldehyde-3-phosphate dehydrogenase was selected as the housekeeping gene for normalization of the expression of target genes.

### Statistical Analysis

Data were collected and analyzed using Bio-Rad CFX Manager. The cycle threshold (CT) values of the genes were exported to Microsoft Excel. The relative gene expression was determined by the  $2^{-\Delta\Delta CT}$  method described by Schmittgen and Livak (2008). For example, the  $\Delta\Delta CT$  for *SLCO1B3* was calculated using the following formula:

$$\Delta\Delta CT = [(CT_{SLCO1B3} - CT_{GAPDH})_{\text{sample A}} - (CT_{SLCO1B3} - CT_{GAPDH})_{\text{sample mix}}]$$

The sample mix allowed for comparison of the gene expression level of all samples. The relative expression of the other 2 genes was calculated using the same formula. Differences in gene expression among the 3 genotypes were analyzed using Kruskal–Wallis test in R, version 4.0.1, software (<https://www.r-project.org>) and considered significant at  $P \leq 0.05$ .

## RESULTS

### Tissue Expression Profile of *SLCO1C1* and *SLCO1B1* in Female and Male Chickens

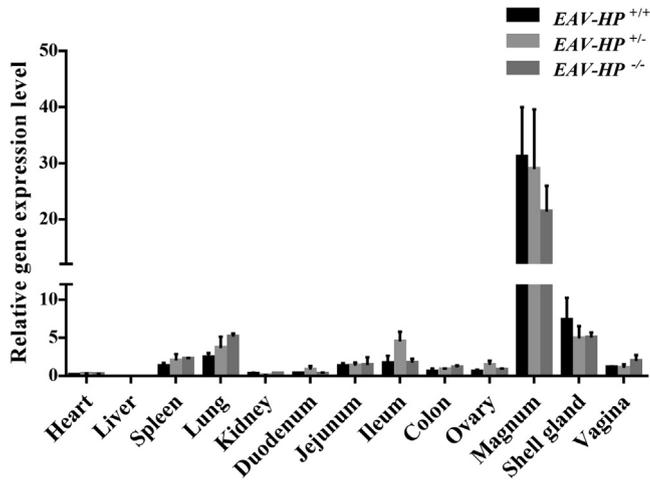
Analysis of *SLCO1C1* and *SLCO1B1* expression patterns in the 13 and 10 tested tissues from female and male chickens, respectively, revealed low expression levels in most of the selected tissues. The expression level of *SLCO1C1* was very low, almost undetectable, in the heart, liver, kidney, and duodenum tissues, whereas that of *SLCO1B1* was extremely low in most tissues, except the lung, kidney, magnum, shell gland, and vagina. Significantly ( $P \leq 0.05$ ) higher expression levels

of these 2 genes were observed in the magnum than in other tissues. Although differences were detected in the expression levels of *SLCO1B1* between some tissues, including the colon and testis in male chickens, because of the extremely low expression levels, such differences were deemed irrelevant; moreover, this gene exhibited different expression patterns in hens. No significant differences were detected in the expression of *SLCO1C1* and *SLCO1B1* among the 3 genotypes in any tissue in both male and female chickens (Figures 1 and 2, Supplementary Figures 1 and 2).

### *SLCO1B3* Exhibits Higher Ectopic Expression in Reproductive Tissues

Analysis of the relative expression levels of *SLCO1B3* in 13 and 10 tissues from female and male chickens, respectively, revealed significant differences in several tissues among the *EAV-HP*<sup>+/+</sup>, *EAV-HP*<sup>+/-</sup>, and *EAV-HP*<sup>-/-</sup> genotypes (Figures 3 and 4). Notably, high

ectopic expression of *SLCO1B3* was detected along the entire oviduct in blue-eggshell chickens. In *EAV-HP*<sup>+/+</sup> and *EAV-HP*<sup>+/-</sup> hens, *SLCO1B3* was significantly overexpressed in the magnum, shell gland, and vagina of the oviduct, exhibiting 800- to 6,000-fold higher expression than that in *EAV-HP*<sup>-/-</sup> hens. Furthermore, in *EAV-HP*<sup>+/+</sup> hens, the expression levels of *SLCO1B3* were similar in different parts of the oviduct, whereas in *EAV-HP*<sup>+/-</sup> hens, the expression levels of *SLCO1B3* in the magnum were approximately 1/5-fold compared with those in the other 2 parts of the oviduct. In addition, the expression levels of *SLCO1B3* were 2-fold higher in the shell gland and vagina of *EAV-HP*<sup>+/+</sup> hens than in those of the *EAV-HP*<sup>+/-</sup> hens. Analysis of the expression levels of *SLCO1B3* in male chickens of different genotypes revealed higher expression in the testes of *EAV-HP*<sup>+/+</sup> and *EAV-HP*<sup>+/-</sup> roosters than in *EAV-HP*<sup>-/-</sup> roosters. The expression levels of *SLCO1B3* in *EAV-HP*<sup>+/+</sup> roosters were approximately 2-fold higher than those in *EAV-HP*<sup>+/-</sup> roosters (Figure 4). Moreover, the expression levels of *SLCO1B3* in testicular tissues from the different genotypes were similar to those in the entire oviduct of hens, indicating that this gene exhibits higher

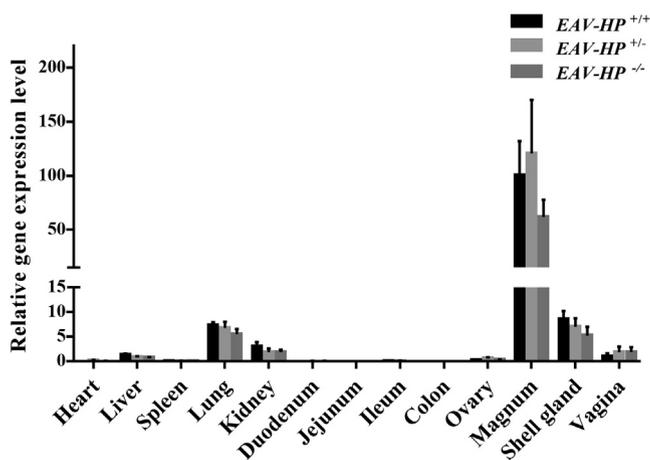


**Figure 1.** Relative expression of *SLCO1C1* in the 13 tested tissues of Yimeng hens with different *EAV-HP*-insertion genotypes. Error bars indicate the SE (n = 3). Abbreviation: *SLCO1C1*, solute carrier organic anion transporter family members 1C1.

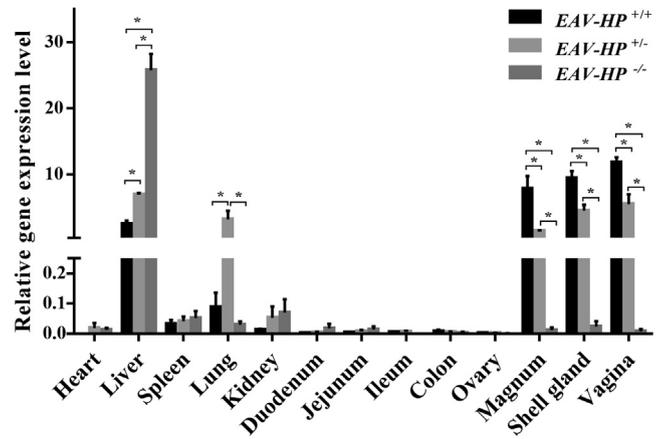
ectopic expression in the reproductive tissues of both male and female blue-eggshell chickens.

### ***SLCO1B3* Expression Profile in the Liver and Other Tissues**

In addition to the high ectopic expression of *SLCO1B3* in the reproductive tissues of blue-eggshell chickens, we observed that *SLCO1B3* was specifically expressed in the liver of non-blue-eggshell hens, whereas the expression level was low in other tissues, which is similar to the tissue-expression profile of human *SLCO1B3*. Moreover, the expression of *SLCO1B3* in the liver was inversely related to that in the oviduct and testis in all 3 genotypes, with *EAV-HP*<sup>-/-</sup> chickens characterized by a lower expression in the oviduct or testis and a higher expression in the liver than those in *EAV-HP*<sup>+/+</sup> and *EAV-HP*<sup>+/-</sup> chickens. Furthermore, although the expression levels of *SLCO1B3* were low in the liver of



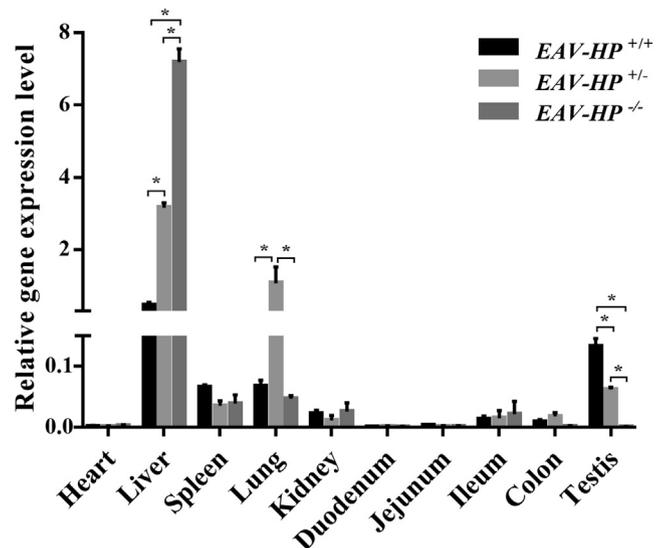
**Figure 2.** Relative expression of *SLCO1B1* in the 13 tested tissues of Yimeng hens with different *EAV-HP*-insertion genotypes. Error bars indicate the SE (n = 3). Abbreviation: *SLCO1B1*, solute carrier organic anion transporter family members 1B1.



**Figure 3.** Relative expression of *SLCO1B3* in the 13 tested tissues of Yimeng hens with different *EAV-HP*-insertion genotypes. Error bars indicate the SE (n = 3). \* indicates significant differences ( $P \leq 0.05$ ). Abbreviation: *SLCO1B3*, solute carrier organic anion transporter family member 1B3.

*EAV-HP*<sup>+/+</sup> chickens, they were comparable with those in the oviduct (magnum, shell gland, and vagina) of *EAV-HP*<sup>+/-</sup> hens (Figure 3). These observations indicate that the *EAV-HP* insertion significantly reduces *SLCO1B3* expression in the chicken liver.

No differences were detected in the expression of *SLCO1B3* in the lung tissues between *EAV-HP*<sup>+/+</sup> and *EAV-HP*<sup>-/-</sup> hens; however, *SLCO1B3* was significantly overexpressed in male and female *EAV-HP*<sup>+/-</sup> chickens (Figures 3 and 4). In addition, the expression of *SLCO1B3* in the colon tissues of male chickens was similar to that in the lung tissues for all 3 genotypes, although the expression levels in both these tissues tended to be low. In contrast, no such trend could be detected in the colon tissues of hens.



**Figure 4.** Relative expression of *SLCO1B3* in the 10 tested tissues of Yimeng roosters with different *EAV-HP*-insertion genotypes. Error bars indicate the SE (n = 3). \* indicates significant differences ( $P \leq 0.05$ ). Abbreviation: *SLCO1B3*, solute carrier organic anion transporter family member 1B3.

To confirm the different patterns in the expression of *SLCO1B3* in the liver, lung, magnum, and shell gland, samples from population 2 were analyzed. Similar expression patterns of this gene were detected in the liver, lung, magnum, isthmus, and shell gland (Supplementary Figure 3), and the *EAV-HP* insertion promoted the ectopic expression of *SLCO1B3* in the lung, magnum, isthmus, and shell gland in all individuals from population 2.

## DISCUSSION

In blue-shelled chickens, genes near the *EAV-HP*-insertion region include *SLCO1C1*, *SLCO1B3*, and *SLCO1B1*, which are all members of the OATP family of membrane transporters. Of these, *SLCO1C1* is known to play a prominent role in the transport of molecules, including thyroid hormones, which is a well-documented function of this gene in humans (Pizzagalli et al., 2002; Sugiyama et al., 2003). To date, no functional analyses of *SLCO1B1* have been conducted in poultry. Wang et al. (2013) did not report *SLCO1C1* expression in the shell glands of either blue- or brown-shelled hens. In contrast, Wragg et al. (2013) reported the expression of this gene in the liver; however, no such observation could be made in the present study, which may be attributed to the differences in the chicken breeds and primers used. We further detected low expression levels of *SLCO1C1* and *SLCO1B1* in most of the tissues examined, which was consistent with previous results of transcriptome analysis of chicken tissues (Barbosa-Morais et al., 2012; Merkin et al., 2012). However, higher expression levels were observed in the magnum of the oviduct than in other tissues. As OATPs, *SLCO1C1* and *SLCO1B1* are expected to play certain roles in the oviduct tissues; however, apart from their lower expression levels in the male colon and testes, no significant differences could be detected among the different genotypes, indicating that the *EAV-HP* insertion does not significantly affect their expression.

We focused on the expression profile of *SLCO1B3*, as it determines the blue-eggshell phenotype in chickens and is also an important liver transport gene. In comparison with *EAV-HP*<sup>-/-</sup> hens, *SLCO1B3* was significantly overexpressed in the shell gland, which is the site for pigment incorporation into the eggshell during egg formation, of *EAV-HP*<sup>+/+</sup> and *EAV-HP*<sup>+/-</sup> hens. Furthermore, the expression levels of *SLCO1B3* in the shell gland were approximately 2-fold higher in homozygous individuals than in heterozygous individuals. These observations are consistent with the findings of Wang et al. (2013), who showed that *SLCO1B3* is expressed exclusively in the shell gland of blue-eggshell chickens.

Furthermore, we found that the *EAV-HP* insertion altered the expression of *SLCO1B3* not only in the shell gland but also in the liver, lung, testis, and the entire oviduct, including the magnum, isthmus, and vagina. In hens, the different parts of the oviduct play distinct roles during egg formation: the magnum is primarily associated with the formation of egg white, and the isthmus and shell gland are associated with the

formation of the eggshell membrane and eggshell itself, respectively. Although the production of blue-shelled eggs has been confirmed to be associated with high ectopic expression of *SLCO1B3* in the shell gland, the roles played by the other parts of the oviduct (the magnum, isthmus, and vagina) remain to be determined. As no evidence indicating the origin of the biliverdin content of blue eggshells is available, it can be conjectured that this compound may be derived from the shell gland, either directly or indirectly via accumulation from other sites of biliverdin production, such as the blood or other parts of the oviduct. Furthermore, *SLCO1B3* exhibited higher expression in the testes of blue-eggshell chickens. The high expression of this gene in both male and female reproductive organs indicated that there may be specific hormone-related signals in the *EAV-HP* sequence. This inference is consistent with the function of the U3 region transcription factor predicted by Wragg et al. (2013). It would thus be informative to determine whether the ectopic expression of *SLCO1B3* in the testis has any prominent effect on male reproduction.

Considering the limited number of studies on chicken OATP1B3, we attempted to interpret its function based on its role in humans. OATP1B3 is an important transport protein in the basolateral hepatocyte membrane (König et al., 2000), transporting various substrates to the liver for metabolism, thereby eliminating xenobiotics, such as bile acids. In our literature search, we found multiple studies showing that jaundice and hyperbilirubinemia in humans are associated with mutations in *SLCO1B3* (Serena et al., 2009; Keppler, 2014). Moreover, certain forms of cancer have been shown to be linked to an abnormal transcript of this gene, which results in the deletion of the first 28 amino acids in the encoded protein (Serena et al., 2009; Keppler, 2014). In the present study, we observed that the *EAV-HP* insertion is associated with a significant reduction in the expression of *SLCO1B3* in the liver. In this context, human *SLCO1B3* is activated by the farnesoid X receptor (FXR), hepatocyte nuclear factor 1-alpha (HNF1α), and hepatocyte nuclear factor 3-beta, in the promoter region located within 120 bp of the 5' flanking region (Jung et al., 2001, 2002; Ohtsuka et al., 2006). Consistently, we predicted the potential binding elements in chicken *SLCO1B3* for FXR, HNF1α, and hepatocyte nuclear factor 3-beta (Supplementary Figure 4), namely, an inverted hexanucleotide repeat motif element that can be activated by the bile acid and an HNF1α site near the inverted hexanucleotide repeat motif element. We believe that insertion of the large *EAV-HP* fragment separates the original transcriptional elements of *SLCO1B3*, such that activation of *SLCO1B3* expression is disrupted, to a certain extent, by the large distance between the promoter and transcription start site. However, *SLCO1B3* was found to have retained a moderate expression level in the liver tissues, which may be attributed to the function of the long terminal repeat region. However, the possibility that the original transcription start site remains functionally active, albeit with reduced transcriptional efficiency owing to the *EAV-*

*HP* insertion, should not be excluded. A marked decrease in the expression levels of *SLCO1B3* in the liver may predictably perturb the transport and metabolism of certain endogenous and exogenous substances, some of which, including bile acids, serve as ligands for nuclear receptors, such as FXR and retinoid X receptor, of which the former plays a key role in the metabolism of bile acids and fatty acids (Makishima et al., 1999; Shen et al., 2011). We speculate that a significant decrease in the expression of *SLCO1B3* in chicken liver may adversely affect lipid metabolism, which requires further investigation.

Interestingly, we detected unexpectedly high expression levels of *SLCO1B3* in the lung tissues of *EAV-HP*<sup>+/-</sup> chickens, whereas such high expression levels were not detected in male and female *EAV-HP*<sup>+/+</sup> chickens. To the best of our knowledge, this is the first study demonstrating the expression of *SLCO1B3* in the lungs of chickens, further suggesting a heterozygous expression advantage. In this context, we initially suspected that the *EAV-HP* insertion may stimulate the expression of *SLCO1B3* in the lung tissues; however, considering that the expression of this gene in *EAV-HP*<sup>+/+</sup> individuals was virtually the same as that in *EAV-HP*<sup>-/-</sup> individuals, a more thorough study of the chicken *EAV-HP* activation region, including the identification of transcription factors that bind to the *EAV-HP* sequence, is required.

In conclusion, we observed an alteration in the expression patterns of *SLCO1B3*, an important membrane transport protein-coding gene, due to the retroviral *EAV-HP* insertion in different genotypes and sexes of blue-eggshell Yimeng chickens. The high ectopic expression of *SLCO1B3* detected in the entire oviduct of hens and testes of roosters reflects similar regulation of gene expression in both these reproductive tissues. However, we observed that the expression of *SLCO1B3* in the liver was inversely related to that in the reproductive tissues in both male and female chickens. The effects of these contrasting patterns of the expression of *SLCO1B3* in the liver and reproductive tissues of blue-eggshell Yimeng chickens require further study. Nevertheless, our findings contribute significantly to our current knowledge on the role of *EAV-HP* transcriptional regulatory elements in the regulation of gene expression. The expression profile of *SLCO1B3* will provide a valuable basis for further investigating the associated transcription factors.

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Conflict of Interest Statement: We declare no competing financial interests.

## SUPPLEMENTARY DATA

Supplementary data associated with this article can be found in the online version at <https://doi.org/10.1016/j.psj.2020.09.002>.

## REFERENCES

- Barbosa-Morais, N. L., M. Irimia, Q. Pan, H. Y. Xiong, S. Gueroussov, L. J. Lee, V. Slobodeniuc, C. Kutter, S. Watt, R. Colak, T. Kim, C. M. Misquitta-Ali, M. D. Wilson, P. M. Kim, D. T. Odom, B. J. Frey, and B. J. Blencowe. 2012. The evolutionary landscape of alternative splicing in vertebrate species. *Science* 338:1587–1593.
- Bitgood, J. J., R. N. Shoffner, J. S. Otis, and W. E. Briles. 1980. Mapping of the genes for pea comb, blue egg, barring, silver, and blood groups A, E, H, and P in the domestic fowl. *Poult. Sci.* 59:1686–1693.
- Butelli, E., C. Licciardello, Y. Zhang, J. Liu, S. Mackay, P. Bailey, G. Reforgiato-Recupero, and C. Martin. 2012. Retrotransposons control fruit-specific, cold-dependent accumulation of anthocyanins in blood oranges. *Plant Cell* 24:1242–1255.
- Chang, C., J. Coville, G. Coquerelle, D. Gourichon, A. Oulmouden, and M. Tixier-Boichard. 2006. Complete association between a retroviral insertion in the tyrosinase gene and the recessive white mutation in chickens. *BMC Genomics* 7:19.
- Chuanwei, Z., L. Zesheng, Y. Ning, and N. Zhonghua. 2014. Quantitative expression of candidate genes affecting eggshell color. *Anim. Sci. J.* 85:506–510.
- Gorchein, A., C. K. Lim, and P. Cassey. 2010. Extraction and analysis of colourful eggshell pigments using HPLC and HPLC/electrospray ionization tandem mass spectrometry. *Biomed. Chromatogr.* 23:602–606.
- Hagenbuch, B., and C. Gui. 2008. Xenobiotic transporters of the human organic anion transporting polypeptides (OATP) family. *Xenobiotica* 38:778–801.
- Hagenbuch, B., and P. J. Meier. 2003. The superfamily of organic anion transporting polypeptides. *Biochim. Biophys. Acta* 1609:1–18.
- Ismair, M. G., B. Stieger, V. Cattori, B. Hagenbuch, M. Fried, P. J. Meier, and G. A. Kullak-Ublick. 2001. Hepatic uptake of cholecystokinin octapeptide by organic anion-transporting polypeptides OATP4 and OATP8 of rat and human liver. *Gastroenterology* 121:1185–1190.
- Jung, D., B. Hagenbuch, L. Gresh, M. Pontoglio, P. J. Meier, and G. A. Kullak-Ublick. 2001. Characterization of the human OATP-C (SLC21A6) gene promoter and regulation of liver-specific OATP genes by hepatocyte nuclear factor 1 alpha. *J. Biol. Chem.* 276:37206–37214.
- Jung, D., M. Podvinec, U. A. Meyer, D. J. Mangelsdorf, M. Fried, P. J. Meier, and G. A. Kullak-Ublick. 2002. Human organic anion transporting polypeptide 8 promoter is transactivated by the farnesoid X receptor/bile acid receptor. *Gastroenterology* 122:1954–1966.
- Kennedy, G. Y., and H. G. Vevers. 1976. A survey of avian eggshell pigments. *Comp. Biochem. Physiol. B* 55:117–123.
- Keppler, D. 2014. The roles of MRP2, MRP3, OATP1B1, and OATP1B3 in conjugated hyperbilirubinemia. *Drug Metab. Dispos.* 42:561–565.
- Kilner, R. M. 2006. The evolution of egg colour and patterning in birds. *Biol. Rev. Camb. Philos. Soc.* 81:383–406.
- König, J., Y. Cui, A. T. Nies, and D. Keppler. 2000. A novel human organic anion transporting polypeptide localized to the basolateral hepatocyte membrane. *Am. J. Physiol. Gastrointest. Liver Physiol.* 278:G156–G164.

- Makishima, M., A. Y. Okamoto, J. J. Repa, H. Tu, R. M. Learned, A. Luk, M. V. Hull, K. D. Lustig, D. J. Mangelsdorf, and B. Shan. 1999. Identification of a nuclear receptor for bile acids. *Science* 284:1362–1365.
- Matsumine, H., M. A. Herbst, S. H. Ou, J. D. Wilson, and M. J. McPhaul. 1991. Aromatase mRNA in the extragonadal tissues of chickens with the henny-feathering trait is derived from a distinctive promoter structure that contains a segment of a retroviral long terminal repeat. Functional organization of the Sebright, Leghorn, and Campine aromatase genes. *J. Biol. Chem.* 266:19900–19907.
- Maurer, G., S. J. Portugal, and P. Cassey. 2012. Review: an embryo's eye view of avian eggshell pigmentation. *J. Avian Biol.* 42:494–504.
- Merkin, J., C. Russell, P. Chen, and C. B. Burge. 2012. Evolutionary dynamics of gene and isoform regulation in mammalian tissues. *Science* 338:1593–1599.
- Meyer, Z. S. H., and R. B. Kim. 2009. Hepatic OATP1B transporters and nuclear receptors PXR and CAR: interplay, regulation of drug disposition genes, and single nucleotide polymorphisms. *Mol. Pharm.* 6:1644–1661.
- Ohtsuka, H., T. Abe, T. Onogawa, N. Kondo, T. Sato, H. Oshio, H. Mizutamari, T. Mikkaichi, M. Oikawa, T. Rikiyama, Y. Katayose, and M. Unno. 2006. Farnesoid X receptor, hepatocyte nuclear factors 1alpha and 3beta are essential for transcriptional activation of the liver-specific organic anion transporter-2 gene. *J. Gastroenterol.* 41:369–377.
- Pizzagalli, F., B. Hagenbuch, B. Stieger, U. Klenk, G. Folkers, and P. J. Meier. 2002. Identification of a novel human organic anion transporting polypeptide as a high affinity thyroxine transporter. *Mol. Endocrinol.* 16:2283–2296.
- Punnett, R. C. 1933. Genetic studies in poultry. IX. The blue egg. *J. Genet.* 27:465–470.
- Sacco, M. A., M. J. F. D., K. Howes, and K. Venugopal. 2000. Avian endogenous retrovirus EAV-HP shares regions of identity with avian leukosis virus subgroup J and the avian retrotransposon ART-CH. *J. Virol.* 74:1296–1306.
- Schmittgen, T. D., and K. J. Livak. 2008. Analyzing real-time PCR data by the comparative C(T) method. *Nat. Protoc.* 3:1101–1108.
- Serena, S., B. Fabio, M. Andrea, P. F. Mcardle, U. Gianluca, D. Mariano, L. Sandra, M. Antonella, P. Maria Grazia, and P. Lucia. 2009. Common variants in the *SLCO1B3* locus are associated with bilirubin levels and unconjugated hyperbilirubinemia. *Hum. Mol. Genet.* 18:2711–2718.
- Shen, L., H. Liu, J. Peng, L. Gan, L. Lu, Q. Zhang, L. Li, F. He, and Y. Jiang. 2011. Effects of farnesoid X receptor on the expression of the fatty acid synthetase and hepatic lipase. *Mol. Biol. Rep.* 38:553–559.
- Smith, L. M., A. A. Toye, K. Howes, N. Bumstead, L. N. Payne, and K. Venugopal. 1999. Novel endogenous retroviral sequences in the chicken genome closely related to HPRS-103 (subgroup J) avian leukosis virus. *J. Gen. Virol.* 80:261–268.
- Sugiyama, D., H. Kusuhara, H. Taniguchi, S. Ishikawa, Y. Nozaki, H. Aburatani, and Y. Sugiyama. 2003. Functional characterization of rat brain-specific organic anion transporter (Oatp14) at the blood-brain barrier: high affinity transporter for thyroxine. *J. Biol. Chem.* 278:43489–43495.
- Wang, X. T., J. R. Bai, C. J. Zhao, H. Zhang, H. G. Bao, G. Y. Xu, J. Y. Li, L. S. Lian, C. X. Wu, and X. M. Deng. 2010. Localisation of the genomic sequence interval for the blue eggshell gene using an F2 resource population of Dongxiang chickens. *Br. Poult. Sci.* 51:507–509.
- Wang, X. T., X. M. Deng, C. J. Zhao, J. Y. Li, G. Y. Xu, L. S. Lian, and C. X. Wu. 2007. Study of the deposition process of eggshell pigments using an improved dissolution method. *Poult. Sci.* 86:2236–2238.
- Wang, Z. P., R. F. Liu, A. R. Wang, J. Y. Li, and X. M. Deng. 2011. Expression and activity analysis reveal that heme oxygenase (decycling) 1 is associated with blue egg formation. *Poult. Sci.* 90:836–841.
- Wang, Z., L. Qu, J. Yao, X. Yang, G. Li, Y. Zhang, J. Li, X. Wang, J. Bai, G. Xu, X. Deng, N. Yang, and C. Wu. 2013. An EAV-HP insertion in 5' flanking region of *SLCO1B3* causes blue eggshell in the chicken. *PLoS Genet.* 9:e1003183.
- Wragg, D., J. M. Mwacharo, J. A. Alcalde, C. Wang, J. L. Han, J. Gongora, D. Gourichon, M. Tixier-Boichard, and O. Hanotte. 2013. Endogenous retrovirus EAV-HP linked to blue egg phenotype in Mapuche Fowl. *PLoS One* 8:e71393.
- Wragg, D., J. M. Mwacharo, J. A. Alcalde, P. M. Hocking, and O. Hanotte. 2012. Analysis of genome-wide structure, diversity and fine mapping of Mendelian traits in traditional and village chickens. *Heredity* 109:6–18.
- Zhang, L., J. Hu, X. Han, J. Li, Y. Gao, C. M. Richards, C. Zhang, Y. Tian, G. Liu, H. Gul, D. Wang, Y. Tian, C. Yang, M. Meng, G. Yuan, G. Kang, Y. Wu, K. Wang, H. Zhang, D. Wang, and P. Cong. 2019. A high-quality apple genome assembly reveals the association of a retrotransposon and red fruit colour. *Nat. Commun.* 10:1494.