Construction of MC1R and ASIP Eukaryotic Expression Vector and its Regulation of Plumage Color in Japanese Quail (*Coturnix japonica*)

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The Japanese quail expresses polymorphism in plumage colors, including black, yellow, white, wild-type (maroon), and various intermediate colors through hybridization of quail with different plumage colors. The expression levels of MC1R and ASIP play important roles in the regulation of plumage colors in birds. In this study, the eukaryotic expression vector of pcDNA 3.1 + was used to analyze the effects of forced expression of MC1R and ASIP on the plumage colors of Japanese quail embryos. The constructed eukaryotic expression vectors of pcDNA 3.1 (+)-MC1R and pcDNA 3.1(+)-ASIP were transfected into wild-type Japanese quail embryos by Lipofectamine^{TM} 2000 liposome at 6 days of incubation. After 3 days, the embryos were collected to analyze the plumage colors and the expression levels of *MC1R*, *ASIP*, and *DCT* genes in skin tissue. Forced expression of the *MC1R* gene by transfection of the pcDNA 3.1(+)-MC1R vector led to hyperpigmentation (similar to black plumage), whereas forced expression of the *ASIP* gene by transfection of the pcDNA 3.1(+)-ASIP vector led to hypopigmentation (similar to white plumage) in wild-type quail embryos. Two kinds of ASIP alternative splicing (ASIP1 and ASIP2) were found in Japanese quail, which did not have a significant effect on the plumage color or the main motifs of the ASIP protein. This study indicated that the black plumage color may be caused by increased production of MC1R and the white plumage color may be caused by increased production of ASIP in Japanese quail.

Key words: ASIP, Japanese quail, MC1R, pcDNA3.1+, plumage color

Avian plumage colors depend on a combination of structural and chemical colors. Chemical color relies on a balance between eumelanin (black/brown pigments) and pheomelanin (yellow/red pigments) (Simon *et al*., 2009). These two kinds of pigments are produced in melanocytes, which are specialized cells that reside mainly in the epidermis, eye, and hair follicles. Melanin is produced in specialized organelles, the melanosomes, and is then transported via dendritic processes to the growing hair or the keratinocytes (Barsh and Cotsarelis, 2007; Weiner *et al*., 2007). There are numerous genes of melanogenesis in birds and mammalian; however, the Melanocortin 1 Receptor (MC1R) plays a crucial role in controlling the type of melanin synthesized by melanocytes (Jackson, 1997). MC1R encodes a seven-transmembrane domain G-protein-coupled receptor expressed primarily in melanocytes of developing feathers and hair (Mundy, 2005), which can influence the type and amount of melanin produced in developing feathers (Robbins *et al*., 1993). High activity of MC1R leads to the synthesis of black/brown eumelanin, whereas low activity leads either to the synthesis of reddish phaeomelanin, or an absence of melanin synthesis (Ha *et al*., 2003). Multiple studies have linked amino acid variability of MC1R to different plumage colors in wild bird populations. For example, MC1R variation is correlated with the plumage polymorphism of the bananaquit (*Coereba flaveola*), snow goose (*Anser c. caerulescens*) (Theron *et al*., 2001), Arctic skua (*Stercorarius parasiticus*) (Mundy *et al*., 2004), red-footed booby (*Sula sula*) (Baião *et al*., 2007), gyrfalcon (*Falco rusticolus*) (Johnson *et al*., 2012; Zhan *et al*., 2012), and Eleonora's falcon (*Falco eleonorae*) (Gangoso *et al*., 2011).

MC1R is furthermore regulated by the Agouti Signaling Protein (ASIP), which works as an antagonist/inverse agonist to MC1R function. The basal activity of MC1R is usually high in hair follicles and ASIP can reduce MC1R basal activity by displacing melanocyte stimulating hormone (MSH), which is the main activator of MC1R (Haitina *et al*., 2007). ASIP is produced by dermal papillae cells in which it governs the switch between production of eumelanin or pheomelanin. ASIP encodes a 131 amino acid protein with structural characteristics of a secreted protein, which has a hydrophobic signal sequence and lacks a transmembrane domain. The interaction between a-MSH/ASIP and MC1R

is involved in the establishment of the pigment pattern, control of chromatoblast survival, differentiation and/or proliferation, as well as melanin synthesis. The mutations and abnormal expression levels of the *ASIP* gene were reported to cause the change of feather colors in birds (Hiragaki *et al*., 2008).

Japanese quail is the main strain for egg production in China. The plumage color of wild-type Japanese quail is maroon, whereas the white and yellow plumage color strains have also been bred in China. Recently, individuals with black plumage color were found amongst wild-type Japanese quail. The black plumage quail has black feathers covering the whole body, except for a few yellow feathers around the eyes during incubation. The beak and claws of the black plumage quail are more hyperchromic, and they do not have brown stripes on the back and head like the wild-type Japanese quail. The polymorphism and expression levels of *MC1R* and *ASIP* genes in black, white, and yellow quail have been analyzed in prior study and the results indicated the expression level of MC1R was significantly higher in the black plumage quail than that in the wild-type plumage quail, whereas the expression level of ASIP was significantly higher in the wild-type plumage quails than that in the black plumage quail (Zhang *et al*., 2013). Thus, in this study, the eukaryotic expression vectors of pcDNA 3.1(+)-MC1R and pcDNA 3.1(+)-ASIP were constructed and transfected into quail embryos to analyze the effects of forced expression levels of MCIR and ASIP on the plumage colors in Japanese quail.

Materials and Methods

Animals and Tissue Sample Selection

The wild-type plumage Japanese quail was bred and raised in a laboratory animal room in Henan University of Science and Technology. Ten fertile eggs of the wild-type Japanese quail were incubated and wing samples of embryos were collected after 12 days of incubation to clone MC1R and ASIP cDNA. All experimental and surgical procedures were approved by the Biological Studies Animal Care and Use Committee, Henan Province, P. R. China, and the ethics committee of Henan University of Science and Technology, P. R. China. All tissue samples were collected aseptically and immediately placed in liquid nitrogen.

Total RNA Extraction and cDNA Synthesis

Total RNA was isolated from wing tissue samples using RNAiso plus kits (Takara). The RNA quality was analyzed by 1.2% agarose gel electrophoresis and spectrophotometric absorption at 260 nm. After DNAse I (Takara) treatment, total RNA was reverse transcribed to cDNA using the Prime Script RT reagent Kit (Takara, Dalian, China) following the manufacturer's instructions.

Construction of MC1R and ASIP Eukaryotic Expression Vector

Two pairs of specific primers (Table 1), containing *Bam*HI and *Xba*I restriction enzyme cutting sites, were designed to clone the full-length coding sequence of MC1R (MR1 primers) and ASIP (AP1 primers) cDNA by RT-PCR. The RT-PCR products were separated with 1.5% agarose gel electrophoresis, and the target fragments were retrieved and purified by the Agarose Gel DNA Purification Kit v.2.0 (Takara). The target fragments were then ligated into the pMD19-T Simple Vector with the DNA Ligation Kit v.2.0 (TA Clone). The recombinant vectors of pMD19-T-MC1R and pMD19-T-ASIP were transformed into *E. coli* DH5α competent cells for amplification. Recombinant vectors were isolated from *E. coli* DH5α cells using the TaKaRa Mini BEST Plasmid Purification Kit v.2.0, and the vectors pMD19-T-MC1R and pMD19-T-ASIP were sequenced by an ABI 377 DNA sequencer (Applied Biosystems, USA). Table 1 contains the information on the primers

The vectors of pMD19-T-MC1R and pMD19-T-ASIP were digested by *Bam*HI and *Xba*I restriction enzymes (Takara), and the target fragments (full-length MC1R and ASIP cDNAs) were isolated and purified. The pcDNA 3.1 (+) eukaryotic expression vector was also digested by *Bam*HI and *Xba*I and then ligated with digested MC1R and ASIP

Table 1. **Information on primers**

Primer name	Primer sequence (5′–3′)	Tm (℃)	Product length (bp)
MR1	CCGCGGATCCACCATGGCGACGCTGGCCC TGTGCTCTAGACTACCAGGAGCACAGCACC	65	969
MR2	CAGAAGCAGCCCACCATC GAAGAAGACTCCCAGCAGG	55	78
AP1	GACCGGATCCACCATGGCAGTGGGATTTTTTCTC CCTCATCTAGATTAACACTTTGGGTTTAACAG	65	444
AP2	CACCCATCTCCATCGTAG GGGTGTCTTCAGTTCAGC	51	104
DCT	AAGAAGCCACCAGTTGTC TTTGCACGGTCTAGAGC	50	86
GAPDH	TGCCGTCTGGAGAAACC CAGCACCCGCATCAAAG	55	160

Note: The underline of MR1 and AP1 primers indicate the restriction enzyme sites of *Bam*HI and *Xba*I, respectively.

cDNA using the DNA Ligation Kit v.2.0. The recombinant plasmids of pcDNA 3.1(+)-MC1R and pcDNA 3.1(+)-ASIP were amplified in *E. coli* DH5α competent cells and isolated with the TaKaRa MiniBEST Plasmid Purification Kit v.2.0. The correct pcDNA 3.1(+)-MC1R and pcDNA 3.1(+)-ASIP plasmid sequence was verified by DNA sequencing.

Transfection of the Recombinant Eukaryotic Expression Vector in Quail Embryos

Eighty fertile eggs of wild-type Japanese quail were incubated for transfection of the recombinant eukaryotic expression vector. The normal quail embryos were selected by egg candling at 6 days of incubation and then divided into four groups: Lipofectamine™ 2000 (control), Lipofectamine™ 2000 + pcDNA 3.1(+)-MC1R, and two groups for Lipofectamine™ 2000 + pcDNA 3.1(+)-ASIP (alternative splicing ASIP1 and ASIP2). The preparation of transfection reagents mix was carried out according to Lipofectamine™ 2000 instructions and the procedure of microinjection was previously described (Song *et al*., 2015). Eight quail embryos were collected to conduct subsequent assays after 3 and 6 days of transfection.

Expression of Recombinant Eukaryotic Expression Vector in Quail Embryos

The wing samples from embryos of each group were collected to analyze the expression of the recombinant eukaryotic expression vector by qRT-PCR. qRT-PCR was performed on an iQ Real-Time PCR Detection System (Bio-Rad) using SYBR Premix Ex TaqII (Takara). Thermal cycling consisted of an initial step at 95℃ for 4 min, followed by 42 cycles at 95℃ for 30 s, annealing for 30 s, and extension/fluorescence acquisition at 72℃ for 30 s. *GAPDH* was chosen as the reference gene for normalization of all data because it was expressed more stably in tissues. Each qRT-PCR reaction (in 20 μl) contained 10 μl SYBR Premix Ex Taq™ II, 0.7 μl of each primer (MR2 primers for *MC1R* gene, AP2 primers for *ASIP* gene, and DCT primers for *DCT* gene, Table 1), 1 μl normalized template cDNA, and 7.6 μl ultrapure water. The qRT-PCR measurements were performed in triplicate on each cDNA sample.

Western Blot Analysis of MC1R and ASIP Proteins

The frozen wing samples were ground and transferred to centrifuge tubes with RIPA/PMSF (Solarbio, China). The tubes with comminuted samples were incubated in ice for 2 h. The whole divided protein was collected by centrifuging at 4℃ for 10 min (12000×g) and adjusted to almost the same protein level. Soluble protein with 4× sample loading buffer was separated on 8−12% SDS-PAGE gel and electrophoresed at 4℃. The protein was transferred onto NC membrane (Millipore Corporation, Billerica, MA, USA) by wet transfer after electrophoresis, then the NC membrane was blocked with 5% fat-free milk in TBST. The MC1R antibody (ab 180776, Abcam, UK), ASIP antibody (ab151033, Abcam, UK), and β-actin antibody (ab8227, Abcam) were incubated at 4℃ overnight. The membrane was washed 3 times using TBST buffer, then hybridized with HRP-conjugated goat anti-rabbit IgG for 2 h at room temperature. After washing 5 times with 1× TBST, the proteins of MCIR, ASIP, and β-

actin were detected on the membrane with the ultra-sensitive horseradish from the catalase DAB color kit (Sangon Biotech, China). The bands on the membrane were scanned by a densitometric analysis system (Bio-Rad).

Effects of Recombinant Eukaryotic Expression Vector on Plumage Colors in Quail Embryos

The quail embryos of each group were collected after 3 days and 6 days of transfection. All the embryos were photographed under the same conditions and the change in plumage color was analyzed by comparison with the control group.

Bioinformatics Analysis

The potential open reading frames (ORFs) were searched using the getorf program (version: 4.1.0) and the multiple sequence alignment was proceeded by the clustalW program. The signal peptide was predicted by the online SignalP program (http://www.cbs.dtu.dk/services/SignalP/) and the motifs were analyzed by online the ScanProsite program (http://www.expasy.org/tools/scanprosite).

Statistical Analysis

The relative expression levels of *MC1R*, *ASIP*, and *DCT* genes were determined by the comparative cycle threshold (CT) method. The Δ^{CT} value was calculated by subtracting the target gene CT value of each sample from its reference gene CT value (Livak and Schmittgen, 2001). The gene expression at different stages was analyzed by one-way analysis of variance (ANOVA) followed by Bonferroni testing for pair-wise comparisons.

Results and Discussion

The Recombinant of the Eukaryotic Expression Vector and its Expression in Quail Embryos

The RT-PCR products were loaded on 1.5% agarose gels, and the bands for the full-length coding sequence of MC1R, ASIP1, and ASIP2 cDNA were located at 969 bp, 444 bp, and 381 bp, respectively (Fig. 1). After insertion of MC1R, ASIP1, and ASIP2 cDNA fragments into the pcDNA 3.1(+) plasmid (5428 bp), the inserted plasmids were sequenced, and the results showed the correct inserted fragments of MC1R, ASIP1, and ASIP2 cDNA by blasting analysis.

After transfection, the expression of MC1R and ASIP was analyzed by qRT-PCR. The results showed that the MC1R and ASIP in transfected groups were overexpressed relative to that of the control group (Fig. 2). The concentration of MC1R protein and ASIP protein showed similar changes according to the western blot analysis (Fig. 3). The expression of DCT showed a decreasing trend from the pcDNA 3.1 (+)-MC1R transfected group, control group, and pcDNA 3.1 (+)-ASIP transfected groups (Fig. 4).

Notwithstanding the relationship between MC1R polymorphism and feather colors have been certified in many studies, whereas opposite results have often been observed. The mutation of MC1R often resulted in a change in its expression level, which affected the cAMP accumulation and led to decreased receptor function and reduced cell surface expression of the mutant protein in vitro (Guernsey *et al*., 2013). The expression of MC1R and ASIP could be tested in

chicken embryos as early as 2.5 day of incubation, which was associated with the proliferation and differentiation of melanoblasts. The expression level of MC1R was significantly higher in Silky Fowl than that in White Leghorn, whereas the expression level of ASIP in Silky Fowl was higher than that in White Leghorn during the early embryonic stage (Li *et al*., 2011). In Japanese quail, the expression of ASIP was significantly lower in black quail than that in wild-type quail (Hiragaki *et al*., 2008). In our prior experiment, the expression of MC1R was higher in black quail than that in wild-type quail and white quail, whereas the expression of ASIP was higher in wild-type quail than that in black quail and white quail (Zhang *et al*., 2013). The same phenomena have been found in many mammals (Norris and Whan, 2008; Kingsley *et al*., 2009; Linnen *et al*., 2009), including humans (Hunt *et al*., 1995).

Effects of Transfection on the Plumage Colors in Quail Embryos

After 3 days of transfection (9 days of incubation), half of the quail embryos were collected to analyze the effects of the eukaryotic expression vector on the plumage colors (Fig. 5). The plumage color in the pcDNA 3.1(+)-MC1R transfected

group was more melanic than that of the control group, whereas the plumage color in the pcDNA 3.1(+)-ASIP transfected groups was more hypochromic than that of the control group. This phenomenon was more conspicuous in the dorsal stripe region.

After 6 days of transfection (the 12 days of incubation), another half of the quail embryos were collected to analyze the effects of the eukaryotic expression vector on the plumage colors (Fig. 5). The same phenomenon appeared in this stage, except that the quail embryos were more fledged.

In our present study, the transient overexpression of MC1R in the wild-type Japanese quail embryos resulted in increasing melanogenesis in skin, which was similar to the black quail in phenotype, whereas the transient overexpression of ASIP in wild-type Japanese quail embryos resulted in
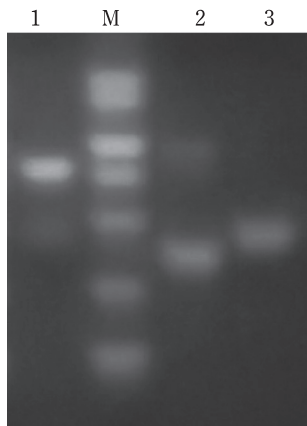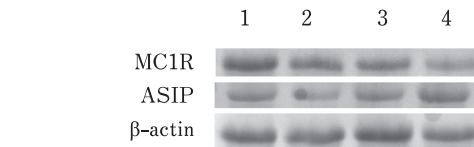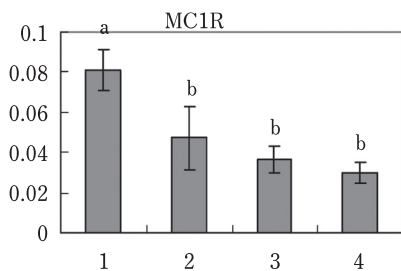

Fig. 3. **MC1R and ASIP protein expression in skin tissues.** 1: pcDNA 3.1(+)-MC1R; 2: control; 3: pcDNA 3.1(+)-ASIP1; 4: pcDNA3.1(+)-ASIP2.

Fig. 1. **Agarose gel electrophoresis analysis of MC1R and ASIP.** M: DL2000 DNA marker; 1: MC1R; 2: ASIP1; 3: ASIP2.
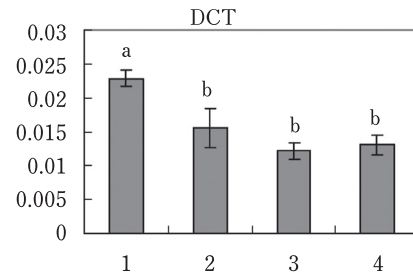

Fig. 4. **DCT expression in skin tissues after 3 days of transfection.** 1: pcDNA 3.1(+)-MC1R; 2: control; 3: pc DNA3.1(+)-ASIP1; 4: pcDNA 3.1(+)-ASIP2. Note: Treatments preceded by the same letter denotes no significant difference ($P>0.05$).
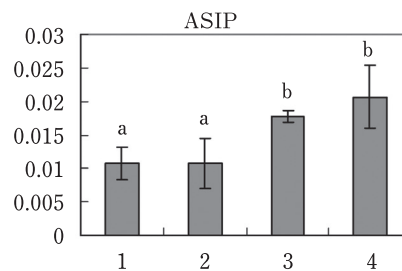

Fig. 2. **MC1R and ASIP expression in skin tissues after 3 days of transfection.** 1: pcDNA 3.1(+)-MC1R; 2: control; 3: pcDNA 3.1(+)-ASIP1; 4: pcDNA 3.1(+)-ASIP2. Note: Treatments preceded by the same letter denotes no significant difference ($P>0.05$).
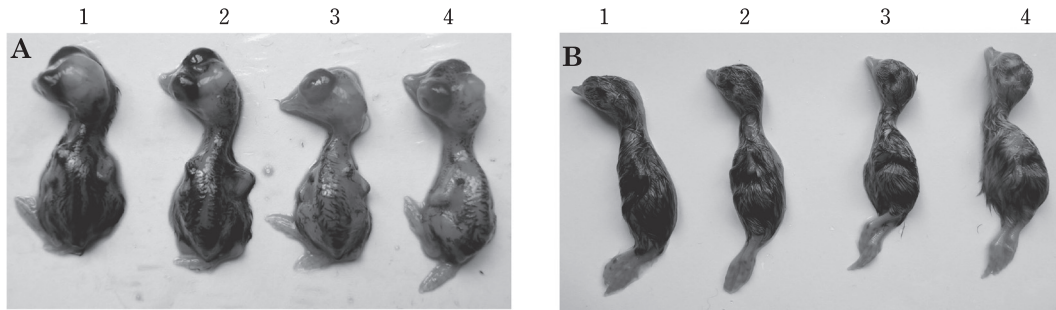
Fig. 5. **Effects of eukaryotic expression vector transfection on plumage colors of Japanese quail embryos after 3 and 6 days.** A: after 3 days of transfection; B: after 6 days of transfection. 1: pcDNA 3.1(+)-MC1R; 2: control; 3: pcDNA 3.1(+)-ASIP1; 4: pcDNA 3.1(+)-ASIP2
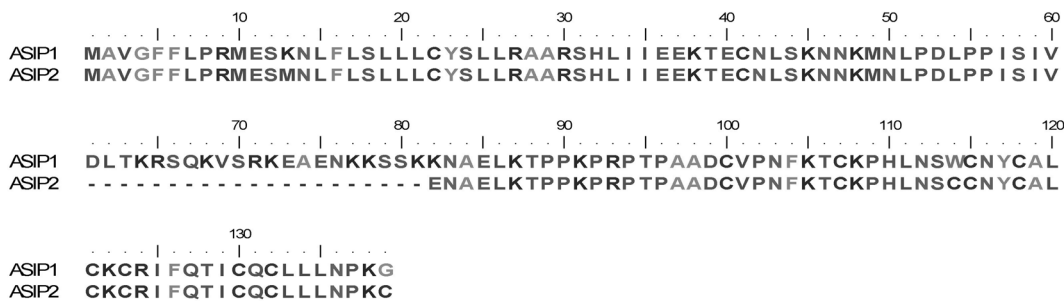

Fig. 6. **Effect of alternative splicing on the ASIP protein structure.**

decreasing melanogenesis in skin, which was similar to white quail in phenotype. It is therefore suggested that melanic coat color in animals may be caused by either increased production of MC1R or decreased production of ASIP.

The transient overexpression of ASIP in the dorsal melanic side of flatfish by injection of homologous capped mRNA can induce skin paling in vivo (Guillot *et al*., 2012). Overexpression of ASIP induced a significant reduction of TYRP1 expression, which promoted final steps of eumelanin synthesis supporting that ASIP inhibited melanogenesis and/or melanophore differentiation (Le Pape *et al*., 2009).

The Bioinformatics Analysis of ASIP1 and ASIP2

Quail ASIP proteins retained the same structure exhibited by all agouti family of peptides. The putative ASIP precursors had the characteristics of secreted proteins, displaying a putative hydrophobic signal peptide of 31 amino acids residues. Processing of the potential signal peptide produced 108 and 87-amino acid mature proteins in quail ASIP1 and ASIP2, including an N-terminal region, a basic central domain with a high proportion of lysine residues, as well as a proline-rich region that immediately preceded the C-terminal polycysteine domain.

ASIP1 and ASIP2 were different alternative splicing of the *ASIP* gene and the predicted ASIP2 protein lacked 21 amino acid residues in the middle of the protein (Fig. 6). The alternative splicing in ASIP2 had no effect on the signal

peptide, the potential N-glycosylation site, or the disulfide bonds by bioinformatics analysis.

Quail ASIP protein exhibited one potential N-glycosylation site within the N-terminal region by bioinformatics analysis. In mice, glycosylation of ASIP was an important factor for protein functionality as disruption partially reduced peptide activity in transgenic mice (Perry *et al*., 1996). Similar to mammalian species, the basic domain of the quail ASIP peptides exhibited a high proportion of lysine (K) and arginine (R) residues. The integrity of this basic domain was also key for the full activity of the ASIP protein (Miltenberger *et al*., 2002). The N-terminal region of the mouse ASIP protein had been shown to down-regulate the melanocortin receptor signaling in *Xenopus* melanophores (Ollmann *et al*., 1999) and was also thought to mediate low-affinity interactions with the product of the mahogany locus (He *et al*., 2001).

In this study, the alternative splicing in ASIP2 had no effect on the formation of disulfide and the nuclear envelope localization domain (KASH domain), which was a highly hydrophobic domain of approximately 60 amino acids, comprising a 20 amino-acid transmembrane region and a 30−35 residue C-terminal region that lied between the inner and outer nuclear membranes (Morimoto *et al*., 2012). The KASH domain linked the dynein motor complex of the microtubules, through the outer nuclear membrane to the

Sad1 domain in the inner nuclear membrane, which then interacted with the bouquet proteins Bqt1 and Bqt2 that were combined with Bqt4, Rap1, and Taz1 and attached to the telomere (Fujita *et al*., 2012). This indicated that the lack of 21 amino acid residues in ASIP2 had no effect on the main structure and function, which was consistent with the results of no significant difference in transfection with pcDNA 3.1 (+) -ASIP1 and pcDNA 3.1(+)-ASIP2 in Japanese quail embryos.

Conclusion

The forced expression of the *MC1R* gene by transfection of the pcDNA 3.1(+)-MC1R plasmid resulted in increasing melanogenesis, which was similar to black quail in phenotype, whereas the forced expression of the *ASIP* gene by transfection of the pcDNA 3.1(+)-ASIP plasmid resulted in decreasing melanogenesis, which was similar to white quail in phenotype. This suggested that melanic coat color in animals may be caused by either increased production of MC1R or decreased production of ASIP.

Acknowledgments

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