[Frontiers in Bioscience E3, 158-165, January 1, 2011]

Repression of *Slc24a5* can reduce pigmentation in chicken

Xiao-fang Liu, Juan Luo, Xiao-xiang Hu, Hui Yang, Xiao-qing Lv, Chun-gang Feng, Jia Tong, Yan-qiang Wang, Shao-hua Wang, Xiao-juan Liu, Tong-hui Lin, Jing Fei, Ying Liu, Ning Li

State Key Laboratory for Agrobiotechnology, China Agricultural University, Beijing 100193, China

TABLE OF CONTENTS

1. ABSTRACT

Slc24a5 is a putative cation transporter, which is a member of the potassium-dependent sodium-calcium ion exchanger family. Association of the *Slc24a5* gene with pigmentation has been established in Zebrafish, mice and humans. Despite these findings, its function in chicken remains unknown. The intent of this study was to describe the association of *Slc24a5* with respect to melanin deposition in the chicken using RNAi. The objective was to detect the variety of melanin deposition caused by the down-regulation of *Slc24a5* in chicken retinal pigment epithelium (RPE) cells. Nine siRNAs that targeted against *Slc24a5* mRNA were found to be effective in suppressing *Slc24a5* gene expression in 293FT cells. The most effective target tested effectively inhibited *Slc24a5* expression in mRNA and subsequently reduced protein levels in RPE cells. These results show that down-regulation of *Slc24a5* results in a reduction of melanin content, as well as a decrease of melaeneous type β and type χ melanosomes simultaneously. Taken together, this work suggests that *Slc24a5* function is conserved in the chicken, and necessary for melanin synthesis.

2. INTRODUCTION

Pigmentation is a striking physical trait in chickens, and is due mainly to the deposition of melanin. Compared with more than 100 genes found related to melanogenesis in the mouse (1), only a few genes, including *MC1R* (melanocortin 1-receptor) (2, 3), *TYR* (tyrosinase) (4), *PMEL17* (silver homolog) (5), *Slc45a2* (solute carrier family 45, member 2) (6), and *MLPH* (melanophilin) (7), have been associated with pigmentation in the chicken. Moreover, the functions of pigmentation related genes in chickens are relatively unknown.

Slc24a5 (solute carrier family 24, member 5) is a pigmentation gene first identified in Zebrafish (8) which encodes a putative cation exchanger. Slc24a5 was predicted to be a member of the potassium-dependent sodium-calcium exchanger family, denoted NCKX5, by multiple sequence alignments (9). Slc24a5 is mainly located on the melanosomal membranes and the *trans*-Golgi network (TGN) as detected (9-11). The function of *Slc24a5* in melanin biogenesis, especially in the biogenesis of early melanosomes, has been identified to play very important

Table 1. siRNA sequence targeting on *Slc24a5* mRNA

siRNA name	sequence
siRNA-1	Sense 5′- CCA UCC UUG GAU CAG CCA UTT -3′ Anti-sense 5′- AUG GCU GAU CCA AGG AUG GTT -3′
siRNA-2	Sense 5′- GGU AUG AAA GUG CUU CCU UTT -3′ Anti-sense 5′- AAG GAA GCA CUU UCA UAC CTT -3′
siRNA-3	Sense 5′- GCU GGA CUA UUC UCA ACU UTT -3′ Anti-sense 5′- AAG UUG AGA AUA GUC CAG CTT -3′
siRNA-4	Sense 5′- CCA UGC CUG AAG AAG AUA UTT -3′ Anti-sense 5′- AUA UCU UCU UCA GGC AUG GTT -3′
siRNA-5	Sense 5′- GGU GUU AUC CCU UCC AAU UTT -3′ Anti-sense 5′- AAU UGG AAG GGA UAA CAC CTT -3′
siRNA-6	Sense 5′- GCA GCA UGG AUU UCU GCA ATT -3′ Anti-sense 5′- UUG CAG AAA UCC AUG CUG CTT -3′
siRNA-7	Sense 5′- GGG AUC CAA CGU AUU UGA UTT -3′ Anti-sense 5′- AUC AAA UAC GUU GGA UCC CTT -3′
siRNA-8	Sense 5′- CCG AAC CCA UAG AAG UAA ATT -3′ Anti-sense 5′- UUU ACU UCU AUG GGU UCG GTT -3′
siRNA-9	Sense 5′- GCC ACU UCU CUC AUC UCG UTT -3′ Anti-sense 5′- AAC AGA UGA GAG AAG UGG CTT -3′
Scrambled siRNA	Sense 5′- UUC UCC GAA CGU GUC ACG UTT -3′ Anti-sense 5′- ACG UGA CAC GUU CGG AGA ATT -3′

roles both in human and mouse melanocytes (9). SNPs of the *Slc24a5* gene were found to be associated with skin color in different human populations, which indicated positive selection in human populations (12-14). A *Slc24a5*$^{-/-}$ mouse model was generated with a hypopigmentation in the RPE (retinal pigmentation epithelium) phenotype, which indicates a melanogenesis function of *Slc24a5* in optic cup-derived pigmented neuroepithelium (15).

A chicken *Slc24a5* gene was cloned from the eye of the White Leghorn. Expression levels of *Slc24a5* were examined in different chicken lines (White Leghorn, Beijing Fatty and Silky) with different skin colors and in different tissues with different pigmentation levels. It was found that *Slc24a5* expression was increased in tissues with higher melanin deposition (16), all of which indicates a potential role of the *Slc24a5* gene in melanogenesis in chicken.

In order to study the function of *Slc24a5* in chicken melanin synthesis, embryonic RPE (retinal pigment epithelium) cells were isolated from the White Leghorn chick embryos, and a primary RPE cell culture system was established. *Slc24a5* expression was proven to be required for melanin synthesis in RPE cells via siRNA-mediated knockdown. Additionally, down-regulation of *Slc24a5* results in a decrease of the type β and type χ melanosomes, as well as reduced expression levels of *TYR* (tyrosinase gene). These data suggest that the function of *Slc24a5* is conserved in the chicken, the down-regulation of *Slc24a5* results in a decrease of melanin, and that *Slc24a5* may play an important role in melanosomes.

3. MATERIAL AND METHODS

3.1. Chicken embryonic RPE cell preparation

Chicken embryonic RPEs were extracted from the eyes of 18-day old embryos of White Leghorn chickens (supplied by the paddock of China Agriculture University, China), and digested using trypsin to obtain RPE cells. Isolated RPE cells from 6 embryos were seeded into 12 wells of a 24-well plate. Cells were cultured in DF12 media (Hyclone, USA) supplemented with 2 mM L-Glutamine, 10% FBS (Gibco, USA) and Penicillin-Streptomycin (Invitrogen, USA) at 37 °C in 5% CO_2. Medium was replaced once daily. All the chicken embryo experiments were conducted in accordance with the International Guiding Principles for Biomedical Research Involving Animals as issued by the Council for the International Organizations of Medical Sciences (1985).

3.2. siRNA synthesis, transient transfection and psiCHECK-2-*Slc24a5* construction

siRNAs targeted on the *Slc24a5* mRNA sequence (GenBank accession no. DQ915178) were predicted by Invitrogen's RNAi Designer. Nine siRNAs (sequences are shown in Table 1) were chemically synthesized (Shanghai GenePharma Co., Ltd., China), and linked with FAM, which could indicate the transfection efficiency of RPE cell. Scrambled siRNA was synthesized and conjugated with FAM as a control, and displayed green fluorescence under a fluorescent microscope. All siRNAs were used at a concentration of 20 pmol/μl in DEPC water. 293FT cell transient transfection was mediated by Lipo-2K (Lipofectamine 2000™) (Invitrogen, USA) with 1μl in each well of a 24-well plate.

cDNA fragment of *Slc24a5* was amplified by RT-PCR and purified, then digested by *Xho* I and *Not* I (TaKaRa, Japan), finally was cloned into *Xho* I/*Not* I linearized psiCHECK-2 vector (Promega, USA), which included two report genes, *Renilla* and *Firefly* luciferase. The psiCHECK-2-*Slc24a5* vector could product a fusion mRNA containing the coding sequence of *Renilla* luciferase followed by the *Slc24a5* mRNA. siRNA and the psiCHECK-2-*Slc24a5* were transfected 293FT with together. Sixty hours post-transfection, the suppressed effect was verified via the double-luciferase detection system (Promega, USA) (17). The opti-MEM media (Invitrogen, USA) used for transfection was replaced with new complete medium 6-8 h after transfection.

3.3. Quantitative RT-PCR

Total RNA was extracted using an RNeasy Mini Kit (Qiagen, Germany) 48 hours after transfection. Genomic DNA was removed with DNase (Qiagen, Germany) following the manufacturer's recommended

Table 2. Sequence of PCR primers used in this study

Primer name	gene	Sequence(from 5' to 3')	Length of products
Slc24a5-F	Slc24a5	GGATGGAGGGAAGAGAGAGG	157 bp
Slc24a5-R	Slc24a5	CTTCAGGCATGGTGAAGACA	
β-actin-F	β-actin	GCCCATCTATGAAGGCTACG	153 bp
β-actin-R	β-actin	TCCTTGATGTCACGCACAAT	
TYR-2F	TYR	AAGAAAAGGAACTTCTCCAGAAATA	117 bp
TYR-2R	TYR	CACCTTAGAAACATCATTGAGATACTG	
ASIP-F	ASIP	CCCACACCTGCTGCTGACT	112 bp
ASIP-R	ASIP	TGAAAAATTCGGCATTTGCA	

protocol. 2μg of cDNA was reverse transcribed using M-MLV Reverse Transcriptase (Promega, USA) with oligo (dT) 18 primers and RNase inhibitor in a 20μl reaction system. Quantitative RT-PCR was performed on a Sequence Detection System (ABI Prism 7900HT, Applied Biosystems). The PCR protocol was as follows: 50°C for 2min; 95°C for 10min; 95°C for 20s, 60°C for 1min repeated 40 cycles; 95°C for 15s, 60 °C for 15s, 95°C for 15s. The specificity and quantity were validated via melting curve analysis. Relative expression levels of the *Slc24a5, TYR and ASIP* gene were normalized to chicken β-actin expression levels. All primer information is shown in Table 2.

3.4. Western blot

Total cell proteins were extracted using a Total Protein Extraction Kit (Applygen Technologies Inc., China) according to the manufacturer's instructions at 72 hours following transfection and protein was quantified using a BCA Assay Kit (Beyotime, China). 23 μg of protein extract was mixed with 3 μl sample buffer (producing a five-fold dilution). Protein samples were denatured at 98°C for 10 minutes, then separated with a 12% SDS–PAGE gel for about 3 hours. Protein was transferred from the polyacrylamide gel to PVDF membranes (GE Healthcare, UK) for about 1 hour under 350 mA current, then the PVDF membranes were blocked in a 5% solution of powdered milk (in TBST) overnight at room temperature. The membranes were hybridized with a polyclonal goat anti-mouse Slc24a5 antibody (Abcam, USA) for 2 hours at room temperature, followed by washing five times for 20 minutes using TBST, then hybridized with HRP-conjugated monoclonal rabbit anti-goat IgG antibody (Zhongshan Goldenbridge Biotechnology Co., Ltd., China) for 1 hour and washed as previously described. Membrane signal detection was conducted using Supersignal West Dura Extended Duration Substrate (Thermo Scientific). β-actin was used as an internal positive control, and hybridized anti-chicken β-actin antibody (Zhongshan Goldenbridge Biotechnology Co., Ltd., China) was used after clearing all antibodies and processing as previously described.

3.5. Measurement of melanin production

Five days after transfection, RPE cells were washed twice with DPBS, lysed in IP lysis buffer (Applygen Technologies Inc., China), and centrifuged for 20 minutes at 12,000 rpm at 4°C. Protein was quantified using a BCA Assay Kit (Beyotime, China). Melanin deposits were washed in 1:1 ethanol/ether, then dissolved using 1 M NaOH:10% DMSO (in PBS) for 30 minutes at 60°C. Melanin concentration was measured at 465nm, and the quantity was validated against a standard curve of melanin. Relative quantitative amounts of melanin were normalized against the total protein in every well.

3.6. Electron microscopic analysis using photoelectron emission microscopy

RPE cells were washed with DPBS twice, digested for 5 minutes in trypsin (0.25% m/v), centrifuged for 4 minutes at 1000 rpm, and resuspended in FBS, followed by another centrifugation for 10 minutes at 5000g. Pellets were collected and fixed in 2.5% glutaraldehyde overnight at 4°C. The pellets were post-fixed in 2% osmic acid for 1 hour at 4°C, and were subjected to successive dehydrations in 30%, 50%, 75% and 100% alcohol for 30 minutes each, followed by dehydration in 100% alcohol overnight. After dehydration, samples were treated with increasing gradients of epon. RPE cells were then embedded in 10% gelatin (11). Ultrathin sections were created and images were captured using a NP100 (Nikon, Japan).

4. RESULTS

4.1. Identification of siRNA that cause knockdown of *Slc24a5* mRNA

A 1.2 kb section of *Slc24a5* cDNA was inserted into a psiCHECK-2 vector, named psiCHECK-2-*Slc24a5*, which produced a fusion mRNA containing the coding sequence of *Renilla* luciferase followed by the *Slc24a5* mRNA, as well as *Firefly* luciferase for normalization. Nine siRNA against the *Slc24a5* mRNA sequence were co-transfected into 293FT cells containing the psiCHECK-2-*Slc24a5 construct.* The reduction of *Slc24a5* mRNA was indicated by the ratio of *Renilla/Firefly* luciferase activity. For every interferential group, mRNA expression was less 40% compared with the chotrols (Figure 1). The results demonstrate that all siRNAs could effectively knock down *Slc24a5* expression in 293FT cells.

4.2. siRNA-5 as an effective suppressor of *Slc24a5* expression

In this study, to verify the function of *Slc24a5* in melanogenesis in chicken, a chicken RPE cell culture system was established to knockdown *Slc24a5* by siRNA-mediated RNA interference.

The RPE cells tended to be contaminated by chick embryonic fibroblasts (CEF), mainly from the choroid membrane when using a direct trypsin digest. Success was achieved when separating RPEs from the eyes and subsequently digesting the RPE to obtain cells. RPE cells grew as a monolayer and were stably maintained in

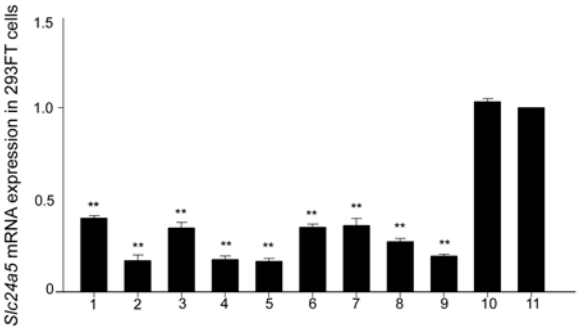

Figure 1. Detection of siRNAs interferential efficiency by the double-luciferase detection system in 293FT cells. The suppression level of different siRNA targeting to *Slc24a5* was indicated by the *Renilla* luciferase activity normalized to *Firefly* luciferase activity. All data are presented as the suppressive percentage of Rluc/Fluc compared with the blank. The knockdown of actual *Slc24a5* mRNA is as follow, 1: 60%, 2: 82%, 3: 65%, 4: 82.%, 5: 83%, 6: 64%, 7: 64%, 8: 72%, 9 :80%, 10: -3%, 11: blank well. Bar from 1 to 9 represents siRNA-1 to siRNA-9 respectively, Bar 10 represents scrambled siRNA, Bar 11 represents blank. * $p<0.05$, ** $p<0.01$.

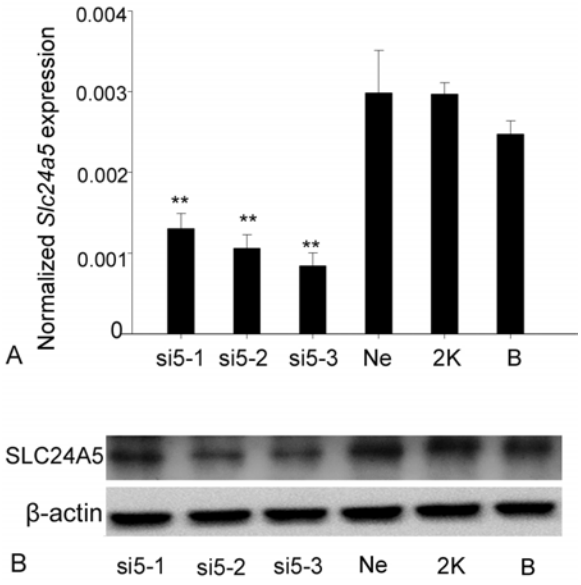

Figure 2. RNA interference using 21-nt siRNA (siRNA-5) chemically synthesized *in vitro* in RPE cells. (A) *Slc24a5* mRNA expressional levels were effectively down-regulated by transfection of siRNA-5 at different doses by quantitative RT-PCR. Relative reduction of *Slc24a5* mRNA was 47.3%, 57.2% or 65.9% respectively, corresponding to a dose of 5 pmol, 20 pmol, or 35 pmol. (B) Western blot results show that the Slc24a5 protein was knocked-down by transfection of siRNA-5 at different doses. si5-1, si5-2 or si5-3 represent the siRNA dose of 5 pmol, 20 pmol or 35 pmol, respectively, which was transfected to RPE cells; Ne: negative control, which was transfected with scrambled siRNA; 2K: negative control, which was transfected with no siRNA but only Lipofectamine 2000; B: negative control with no transfection. * $p<0.05$, ** $p<0.01$

culture for one week, with a rounded shape and obvious pigmentation Conjunct FAM with siRNA indicated the transfection efficiency of RPE cells was approximately 50%.

Different dosages of siRNA-5 targeted to chicken *Slc24a5* could down-regulate *Slc24a5* mRNA expression levels (Figure 2A). Normalized reduction of *Slc24a5* mRNA was 47.3%, 57.2% and 65.9% respectively, corresponding to 5 pmol (si5-1), 20 pmol (si5-2), and 35 pmol (si5-3). Western blot results show that Slc24a5 protein expression was also effectively suppressed in RPE cells (Figure 2B). Therefore, siRNA-5 could effectively suppress *Slc24a5* expression in RNA and protein levels.

4.3. Melanin production reduced by down-regulation of *Slc24a5* using RNAi in chicken RPE cells

Melanin deposits were consistent between control groups, however the melanin content in RNAi groups was significantly reduced (Fig 3A) compared with the controls. The reduced percentages of melanin content were 18.9% and 20.4% compared with the blank control, corresponding to transfected groups with siRNA-5 at doses of 20 pmol (si5-2) and 35 pmol (si5-3), respectively. These data demonstrate that down-regulation of *Slc24a5* decreased melanin deposition, and that *Slc24a5* plays an important role during melanin synthesis in chicken RPE cells.

4.4. Knock-down of the *Slc24a5* gene reduced *TYR*

The results of quantitative RT-PCR indicate that *TYR* gene expression is obviously reduced after down-regulation of *Slc24a5* in RPE cells (Figure 3B). These reduced percentages were 16.0% (si5-2) and 40.1% (si5-3). *TYR* is a rate-limited enzyme in the melanogenesis pathway, which was affected by the down-regulation of *Slc24a5*. Therefore, melanin reduction may be a result of reduction of *TYR* expression in this study. It can be concluded that down-regulation of *Slc24a5* may decrease tyrosinase activity either directly or indirectly.

4.5. Melanosomes were affected by a *Slc24a5* knock-down in chicken RPE cells

As melanin content was reduced in RPE cells after *Slc24a5* knock-down, it is important to understand the mechanism of the down-regulation of melanogenesis via down-regulation of *Slc24a5* expression. Five days after transfection with siRNA-5, RPE cells were collected and fixed in order to perform electron microscopic analysis using photoelectron emission microscopy. In the control cells transfected with scrambled siRNA (Negative control) or without transfection (Blank), large numbers of melanosomes of type βand type χ, which were filled with melanin, were observed (Figure 4). The number of type β and type χ melanosomes were reduced significantly in RPE cells transfected with siRNA-5 at both 20 pmol (si5-2) and 35 pmol (si5-3) concentrations. In addition, many type – and type α melanosomes with diluted pigmentation appeared in the RNAi treated groups of cells (si5-2 and si5-3). Despite these results, the shape of the melanosomes was not changed by siRNA-5 knock-down of *Slc24a5* gene expression. In conclusion, *Slc24a5* knockdown reduced

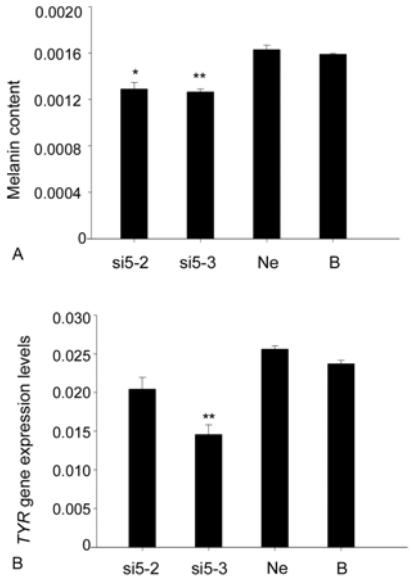

Figure 3. Variation in melanin content and *TYR* expression in RPE cells after the down-regulation of *Slc24a5*. There is a significant difference between the si5-2 and the control groups (Ne, B), and this change is much more obvious for si5-3 (A). Melanin content decreased significantly after knockdown of *Slc24a5* expression. (B) Quantitative RT-PCR analysis of the *TYR* gene expression in chicken RPE cells. *TYR* expression was affected significantly by down-regulation of *Slc24a5* (si5-3) (P<0.01), but indistinctively for si5-2 (p=0.094). * p<0.05, ** p<0.01

melanin deposition in melanosomes, but did not affect the overall shape of the melanosomes.

5. DISCUSSION

The finding of *Slc24a5* in Zebrafish as a pigmentation gene followed by the identification of important SNPs of *Slc24a5* in human populations suggests that *Slc24a5* is a very important gene in studying the evolution of pigmentation and melanogenesis. All prior studies have indicated that *Slc24a5* was conserved in Zebrafis*h*, mice and humans. Despite these findings, little is known of the association of *Slc24a5* with pigmentation in chicken.

In this study, a primary RPE cell culture system was established to investigate pigmentation following *Slc24a5* knockdown. The change of melanin content after down-regulation of the *Slc24a5* gene was consistent with previous reports (11, 15) and the reduction of *Slc24a5* results in a decrease in melanogenesis. The reduction in the levels of melanin content for RNAi groups is not dramatic (18.9% and 20.4%) (Figure 3) when compared with the reduction of melanosomal density in RPE cells (Figure 4). This reduction may be caused partially by the low transfection efficiency of primary RPE cells. Down-regulated *Slc24a5* resulted in a reduction in melanosomal density and melanin content, however this change in melanin content was in response to the global changes of melanin in the RPE cells, whether *Slc24a5* was down-regulation or not. Otherwise, the reduction of melanin may be caused partially by the limited number of new RPE cells generated during cell culture.

Slc24a5 is an ion exchanger, located mainly on Golgi apparatuses and melanosomes Rebecca S. Ginger proposed a model in which NCKX regulated melanogenesis occurred through the sorting of protein membranes correctly into the corresponding endosome in the membrane of Golgi apparatuses (9). If this theory is correct, *Slc24a5* knockdown would repress melanosome formation and result in a decrease in the total number of melanosomes. As a result, the proportion of the 4 melanosomal types would be similar to that of the control. Conversly, in this study it was found that type and type α melanosomes increased, while type β and type χ decreased as evidenced by electron microscope analysis. While the phenotype of the melanosome was normal, therefore it can be suggested that the Slc24a5 protein is a transporter, which may play a role in early stage of melanosome development, and that a reduction of *Slc24a5* expression results in a decrease of melanogenesis, affecting melanosome maturation. Furthermore, there is an absence of studies showing that abnormally high or abnormally low *Slc24a5* expression leads to abnormal melanosomal shapes. Lamason reported that the mouse melanoma (B16) showed 100-fold greater expression compared with normal skin or eyes (8,. Knockout of *Slc24a5* lead to hypopigmentaion in mouse RPE and IPE (iridal pigmented epithelium) cells (15).

While there are hundreds of genes related to melanogenesis, but few have been examined in detail, including Tyrosinase (*Tyr*), *MC1R*, *MAPT, P* (OCA2), *ASIP*, *Slc45a2* and *Slc24a5* (10, 18-20). Tyrosinase, whose catalytic activity is influenced by the abundance of tyrosine (21), is the key rate-limited enzyme of melanogenesis. Tyrosine takes a negative charge under physiological conditions or in the cytoplasm, and a positive charge inside of the melanosome according to its inherent pH (5.66). Authough tyrosine content is higher in cytoplasm than inside early stage melanosomes, tyrosine transport produces a voltage to block tyrosine facilitating diffusion from the cytoplasm to the inside of the melanosome, of which PH was about 5.0 (21, 22). Therefore, we think that *Slc24a5* proteins may maintain or produce opposing voltage by pumping bivalent cations out to facilitate tyrosine transport or perhaps to neutralize the voltage differential produced by tyrosine transportation. Following this theory, tyrosinase catalytic activity appears to be affected by Slc24a5. Quantitative RT-PCR results indicate that *TYR* expression decreased in the knockdown *Slc24a5* group. So it can be concluded that the reduction of melanin deposition may be caused by the decrease of tyrosinase catalytic activity. In other words, down-regulation of *Slc24a5* decreases melanogenesis indirectly by affecting tyrosine transportation.

The findings in this study prove that *Slc24a5* gene function is conserved in the chicken for the first time. Previous studies have proven this for Zebrafish, mice and

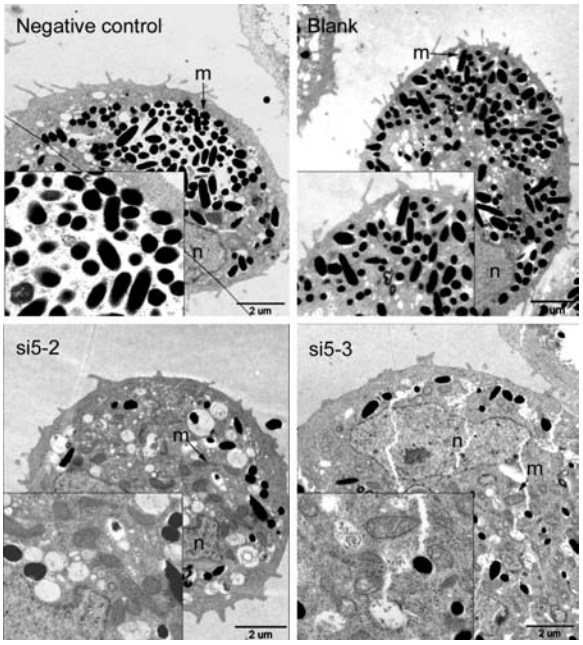

Figure 4. Melanosomal density was affected by down-regulation of *Slc24a5* gene by ultrastructure of chicken embryonic RPE cells. RPE cells transfected with scrambled siRNA (Negative), or without transfection (Blank) contained numerous type β and type χ melanosomes (m) around the nucleus (n). RPE cells transfected with siRNA-5 at 20 pmol (si5-2) or 35 pmol (si5-3) contained mainly type – and type α melanosomes, type β and type χ melanosomes were reduced in those RPE cells compared with control cells (Negative and Blank). A larger magnification of melanosomes is shown in the left frame. The scale bar is 2 μm.

humans. Down-regulation of Slc24a5 results in melanin depositing abnormally, which indicate that the Slc24a5 gene is necessary in chicken RPE cells. More importantly, it is illustrated that the Slc24a5 gene may play a key role in the membrane of melanosomes, and not Golgi apparatuses, and also plays a role in maintaining or producing voltage opposite to that produced via tyrosine transport. This voltage regulation is necessary to facilitate tyrosine transport or to neutralize the voltage resulting from tyrosine transport. Interestingly, ASIP (agouti signaling protein), which could bind with MC1R to stimulate a switch from eumelanin to phaeomelanin synthesis (23), exhibited erratic changes in this study (the results not shown). More studies are required to determine if there is any relationship between Slc24a5 and TYR in the melanogenesis pathway.

6. ACKNOWLEDGMENTS

This work is supported by the State Major Basic Development Program of China (2006CB102100), the National Hi-Tech Research and Development Program of China (2006AA12A120).

7. REFERENCES

1. Bennett, D. C. and M. L. Lamoreux: The color loci of mice--a genetic century. *Pigment Cell and melanoma Res* 16 (4), 333-44 (2003)

2. Kerje, S., J. Lind, K. Schutz, P. Jensen and L. Andersson: Melanocortin 1-receptor (MC1R) mutations are associated with plumage colour in chicken. *Anim Genet* 34 (4), 241-8 (2003)

3. Ling, M. K., M. C. Lagerstrom, R. Fredriksson, R. Okimoto, N. I. Mundy, S. Takeuchi and H. B. Schioth: Association of feather colour with constitutively active melanocortin 1 receptors in chicken. *Eur J Biochem* 270 (7), 1441-9 (2003)

4. Sato, S., T. Otake, C. Suzuki, J. Saburi and E. Kobayashi: Mapping of the recessive white locus and analysis of the tyrosinase gene in chickens. *Poult Sci* 86 (10), 2126-33 (2007)

5. Kerje, S., P. Sharma, U. Gunnarsson, H. Kim, S. Bagchi, R. Fredriksson, K. Schutz, P. Jensen, G. von Heijne, R. Okimoto and L. Andersson: The Dominant white, Dun and Smoky color variants in chicken are associated with insertion/deletion polymorphisms in the PMEL17 gene. *Genetics* 168 (3), 1507-18 (2004)

6. Gunnarsson, U., A. R. Hellstrom, M. Tixier-Boichard, F. Minvielle, B. Bed'hom, S. Ito, P. Jensen, A. Rattink, A. Vereijken and L. Andersson: Mutations in SLC45A2 cause plumage color variation in chicken and Japanese quail. *Genetics* 175 (2), 867-77 (2007)

7. Vaez, M., S. A. Follett, B. Bed'hom, D. Gourichon, M. Tixier-Boichard and T. Burke: A single point-mutation within the melanophilin gene causes the lavender plumage colour dilution phenotype in the chicken. *BMC Genet* 9, 7 (2008)

8. Lamason, R. L., M. A. Mohideen, J. R. Mest, A. C. Wong, H. L. Norton, M. C. Aros, M. J. Jurynec, X. Mao, V. R. Humphreville, J. E. Humbert, S. Sinha, J. L. Moore, P. Jagadeeswaran, W. Zhao, G. Ning, I. Makalowska, P. M. McKeigue, D. O'Donnell, R. Kittles, E. J. Parra, N. J. Mangini, D. J. Grunwald, M. D. Shriver, V. A. Canfield and K. C. Cheng: SLC24A5, a putative cation exchanger, affects pigmentation in zebrafish and humans. *Science* 310 (5755), 1782-6 (2005)

9. Ginger, R. S., S. E. Askew, R. M. Ogborne, S. Wilson, D. Ferdinando, T. Dadd, A. M. Smith, S. Kazi, R. T. Szerencsei, R. J. Winkfein, P. P. Schnetkamp and M. R. Green: SLC24A5 encodes a trans-Golgi network protein with potassium-dependent sodium-calcium exchange activity that regulates human epidermal melanogenesis. *J Biol Chem* 283 (9), 5486-95 (2008)

10. Sturm, R. A: A golden age of human pigmentation genetics. *Trends Genet* 22 (9), 464-8 (2006)

11. Cheli, Y., F. Luciani, M. Khaled, L. Beuret, K. Bille, P. Gounon, J. P. Ortonne, C. Bertolotto and R. Ballotti: {alpha}MSH and Cyclic AMP elevating agents control melanosome pH through a protein kinase A-independent mechanism. *J Biol Chem* 284 (28), 18699-706 (2009)

12. Izagirre, N., I. Garcia, C. Junquera, C. de la Rua and S. Alonso : A scan for signatures of positive selection in candidate loci for skin pigmentation in humans. *Mol Biol Evol* 23 (9), 1697-706 (2006)

13. Sabeti, P. C., P. Varilly, B. Fry, J. Lohmueller, E. Hostetter, C. Cotsapas, X. Xie, E. H. Byrne, S. A. McCarroll, R. Gaudet, S. F. Schaffner, E. S. Lander, K. A. Frazer, D. G. Ballinger, D. R. Cox, D. A. Hinds, L. L. Stuve, R. A. Gibbs, J. W. Belmont, A. Boudreau, P. Hardenbol, S. M. Leal, S. Pasternak, D. A. Wheeler, T. D. Willis, F. Yu, H. Yang, C. Zeng, Y. Gao, H. Hu, W. Hu, C. Li, W. Lin, S. Liu, H. Pan, X. Tang, J. Wang, W. Wang, J. Yu, B. Zhang, Q. Zhang, H. Zhao, H. Zhao, J. Zhou, S. B. Gabriel, R. Barry, B. Blumenstiel, A. Camargo, M. Defelice, M. Faggart, M. Goyette, S. Gupta, J. Moore, H. Nguyen, R. C. Onofrio, M. Parkin, J. Roy, E. Stahl, E. Winchester, L. Ziaugra, D. Altshuler, Y. Shen, Z. Yao, W. Huang, X. Chu, Y. He, L. Jin, Y. Liu, Y. Shen, W. Sun, H. Wang, Y. Wang, Y. Wang, X. Xiong, L. Xu, M. M. Waye, S. K. Tsui, H. Xue, J. T. Wong, L. M. Galver, J. B. Fan, K. Gunderson, S. S. Murray, A. R. Oliphant, M. S. Chee, A. Montpetit, F. Chagnon, V. Ferretti, M. Leboeuf, J. F. Olivier, M. S. Phillips, S. Roumy, C. Sallee, A. Verner, T. J. Hudson, P. Y. Kwok, D. Cai, D. C. Koboldt, R. D. Miller, L. Pawlikowska, P. Taillon-Miller, M. Xiao, L. C. Tsui, W. Mak, Y. Q. Song, P. K. Tam, Y. Nakamura, T. Kawaguchi, T. Kitamoto, T. Morizono, A. Nagashima, Y. Ohnishi, A. Sekine, T. Tanaka, T. Tsunoda, P. Deloukas, C. P. Bird, M. Delgado, E. T. Dermitzakis, R. Gwilliam, S. Hunt, J. Morrison, D. Powell, B. E. Stranger, P. Whittaker, D. R. Bentley, M. J. Daly, P. I. de Bakker, J. Barrett, Y. R. Chretien, J. Maller, S. McCarroll, N. Patterson, I. Pe'er, A. Price, S. Purcell, D. J. Richter, P. Sabeti, R. Saxena, S. F. Schaffner, P. C. Sham, P. Varilly, D. Altshuler, L. D. Stein, L. Krishnan, A. V. Smith, M. K. Tello-Ruiz, G. A. Thorisson, A. Chakravarti, P. E. Chen, D. J. Cutler, C. S. Kashuk, S. Lin, G. R. Abecasis, W. Guan, Y. Li, H. M. Munro, Z. S. Qin, D. J. Thomas, G. McVean, A. Auton, L. Bottolo, N. Cardin, S. Eyheramendy, C. Freeman, J. Marchini, S. Myers, C. Spencer, M. Stephens, P. Donnelly, L. R. Cardon, G. Clarke, D. M. Evans, A. P. Morris, B. S. Weir, T. Tsunoda, T. A. Johnson, J. C. Mullikin, S. T. Sherry, M. Feolo, A. Skol, H. Zhang, C. Zeng, H. Zhao, I. Matsuda, Y. Fukushima, D. R. Macer, E. Suda, C. N. Rotimi, C. A. Adebamowo, I. Ajayi, T. Aniagwu, P. A. Marshall, C. Nkwodimmah, C. D. Royal, M. F. Leppert, M. Dixon, A. Peiffer, R. Qiu, A. Kent, K. Kato, N. Niikawa, I. F. Adewole, B. M. Knoppers, M. W. Foster, E. W. Clayton, J. Watkin, R. A. Gibbs, J. W. Belmont, D. Muzny, L. Nazareth, E. Sodergren, G. M. Weinstock, D. A. Wheeler, I. Yakub, S. B. Gabriel, R. C. Onofrio, D. J. Richter, L. Ziaugra, B. W. Birren, M. J. Daly, D. Altshuler, R. K. Wilson, L. L. Fulton, J. Rogers, J. Burton, N. P. Carter, C. M. Clee, M. Griffiths, M. C. Jones, K. McLay, R. W. Plumb, M. T. Ross, S. K. Sims, D. L. Willey, Z. Chen, H. Han, L. Kang, M. Godbout, J. C. Wallenburg, P. L'Archeveque, G. Bellemare, K. Saeki, H. Wang, D. An, H. Fu, Q. Li, Z. Wang, R. Wang, A. L. Holden, L. D. Brooks, J. E. McEwen, M. S. Guyer, V. O. Wang, J. L. Peterson, M. Shi, J. Spiegel, L. M. Sung, L. F. Zacharia, F. S. Collins, K. Kennedy, R. Jamieson and J. Stewart: Genome-wide detection and characterization of positive selection in human populations. *Nature* 449 (7164), 913-8 (2007)

14. Stokowski, R. P., P. V. Pant, T. Dadd, A. Fereday, D. A. Hinds, C. Jarman, W. Filsell, R. S. Ginger, M. R. Green, F. J. van der Ouderaa and D. R. Cox: A genomewide association study of skin pigmentation in a South Asian population. *Am J Hum Genet* 81 (6), 1119-32 (2007)

15. Vogel, P., R. W. Read, R. B. Vance, K. A. Platt, K. Troughton and D. S. Rice: Ocular albinism and hypopigmentation defects in Slc24a5$^{-/-}$ mice. *Vet Pathol* 45 (2), 264-79 (2008)

16. Luo, J., W. Zhang, B. Yu, X. Hu and N. Li: cDNA cloning, expression analysis of Slc24a5 and its relationship with melanin deposition in chicken. *Progress in Biochemistry and Biophysics* (35), 69-76 (2008)

17. Wang, S., X. Lv, K. Zhang, T. Lin, X. Liu, J. Yuan, Y. Dai and N. Li: Knockdown of the prion gene expression by RNA interference in bovine fibroblast cells. *Mol Biol Rep (*2009)17. Schraermeyer, U. and K. Heimann. Current understanding on the role of retinal pigment epithelium and its pigmentation. *Pigment Cell and melanoma Res* 12 (4), 219-36 (1999)

18. Anno, S., T. Abe and T. Yamamoto: Interactions between SNP alleles at multiple loci contribute to skin color differences between caucasoid and mongoloid subjects. Int J Biol Sci 4 (2), 81-6 (2008)

19. Soejima, M. and Y. Koda: Population differences of two coding SNPs in pigmentation-related genes SLC24A5 and SLC45A2. *Int J Legal Med* 121 (1), 36-9 (2007)

20. Dimisianos, G., I. Stefanaki, V. Nicolaou, V. Sypsa, C. Antoniou, M. Poulou, O. Papadopoulos, H. Gogas, E. Kanavakis, E. Nicolaidou, A. D. Katsambas and A. J. Stratigos: A study of a single variant allele (rs1426654) of the pigmentation-related gene SLC24A5 in Greek subjects. *Exp Dermatol* 18 (2), 175-7 (2009)

21. Fuller, B. B., D. T. Spaulding and D. R. Smith: Regulation of the catalytic activity of preexisting tyrosinase in black and Caucasian human melanocyte cell cultures. *Exp Cell Res* 262 (2), 197-208 (2001)

22. Ancans, J. and A. J. Thody: Activation of melanogenesis by vacuolar type H $^{(+)}$-ATPase inhibitors in amelanotic, tyrosinase positive human and mouse melanoma cells. *FEBS Lett* 478 (1-2), 57-60 (2000)

23. Alaluf, S., D. Atkins, K. Barrett, M. Blount, N. Carter and A. Heath: The impact of epidermal melanin on objective measurements of human skin colour. *Pigment Cell and melanoma Res* 15 (2), 119-26 (2002)

Key Words: *Slc24a5*, Melanin Synthesis, Retinal Pigment Epithelium Cell, RNA Interference, Tyrosinase

Send correspondence to: Ning Li, State Key Laboratory for Agrobiotechnology, China Agricultural University,

Beijing 100193, China, Tel: 86-10-62733323, Fax: 86-10-62733904, E-mail: ninglcau@cau.edu.cn

http://www.bioscience.org/current/volE3.htm