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Supplementary Information for

Instructive role of melanocytes during pigment pattern formation of the avian skin

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SI Materials and Methods.

Bird eggs and rearing.

Specific pathogen-free White Leghorn chicken eggs were purchased from Charles River (CT, USA); Japanese quail eggs were form AA laboratories (CA, USA). Bobwhite quail eggs were from Murray McMurray Hatchery (IA, USA). Barred Plymouth Rock chicken eggs and Black Australorp chicken eggs were from California Hatchery (CA, USA). Sicilian buttercup chicken eggs were provided by (Janicki Buttercups, WA, USA). All eggs were incubated at 38.5°C until the desired stage. Animal care and experiments were conducted according to the guidelines established by the USC Institutional Animal Care and Use Committee.

Cloning of Japanese quail genes.

Total mRNA was collected from E8 Japanese quail (JQ) skin by using Trizol reagent (Invitrogen). cDNA was synthesized from the RNA by using SuperScript III First-Strand Synthesis system (Invitrogen). *JQ-ASIP* (XM_015881357.1) was amplified with forward primer 5'-gctgGGATCCgccaccatgacagtgggatttttct-3' (qASIP_BamHI_F) and reverse primer 5'-AGTCaccggtttaacactttgggtttaaca -3' (qASIP_AgeI_R). *JQ-GJA5* (XM_015879861.1) was amplified with forward primer 5'-gctgACCGGTgccaccatgggggactggagtttcct-3' (qgja5_AgeI_kozak_F) and reverse primer 5'-AGTCgaattctcacacagacaaatcgtctg-3' (qgja5_EcoRI_R).

Plasmid construction.

Tol2 plasmids: pT2AL200R175-CAGGS-EGFP and pCAGGS-T2TP were provided by Dr. Kawakami (National Institute of Genetics, Japan). All Tol2 plasmids used here were generated by modifying pT2AL200R175-CAGGS-EGFP. pT2AL200R175-CAGGS-LynEGFP: First, LynEGFP sequence was generated by adding 48 bp 5' end of Lyn gene to the 5' end of EGFP with PCR. pT2AL200R175-CAGGS-EGFP was digested with NotI to remove EGFP, then LynEGFP fragment was inserted into the NotI site. pT2AL200R175-CAGGS-LynEGFP-IRES-Neo^r: IRES-Neo^r sequence was PCR-amplified from pEF1a-IRES-Neo. This fragment was inserted into the ClaI site of pT2AL200R175-CAGGS-LynEGFP. pEF1a-IRES-Neo was from Thomas Zwaka (Addgene plasmid # 28019). pT2AL200R175-CAGGS-H2BEGFP-IRES-Neo^r: pT2AL200R175-CAGGS-LynEGFP-IRES-Neo^r was digested with NotI to remove LynEGFP, then H2BEGFP fragment was inserted into the NotI site. pT2AL200R175-CAGG-mCherry-IRES-Neo^r: pT2AL200R175-CAGGS-LynEGFP-IRES-Neor was digested with NotI to remove LynEGFP, then mCherry fragment was inserted into the NotI site. pT2AL200R175-CAGGS-cx40-T2A-mCherry-IRES-Neo^r and pT2AL200R175-CAGGS-DNcx40-T2A-mCherry-IRES-Neo^r: cx40-T2A-mCherry or DNcx40-T2A-mCherry fragments were inserted into the EcoRI site of pT2AL200R175-CAGG-mCherry-IRES-Neo^r.

Lentivirus plasmids: FUGW was from David Baltimore (Addgene plasmid # 14883). pWPI was from Didier Trono (Addgene plasmid # 12254). FU-IRES-EGFP-W was constructed by replacing of EGFP of FUGW with IRES-EGFP of pWPI. FU-JQ ASIP-IRES-EGFP was constructed by inserting JQ ASIP into the BamHI and AgeI site of FU-IRES-EGFP-W.

Whole-mount in situ hybridization.

The promoter sequence for T7 RNA polymerase was added to the 5' end of the antisense primer. PCR products were amplified from E8 JQ skin cDNAs. Digoxigenin-labeled probes were synthesized with T7 RNA polymerase (Roche). *JQ-ASIP* probe (405bp): forward primer 5'-CTAATACGACTCACTATAGGGAGAgaaaaattcggcatttgcac-3' (JQ-ASIP-AS-T7) and reverse primer 5'-tgaggttttgactgaccttgg-3' (JQ-ASIP-S1). *JQ-GJA5* probe (685bp): forward primer 5'-CTAATACGACTCACTATAGGGAGAaaagtctggaggggggagtgt-3' (JQ-GJA5-AS-T7) and reverse primer 5'-gcagtccgatttcatgtgtg-3' (JQ-GJA5-S). Whole-mount in situ hybridization was done as described before (33). Briefly, embryos were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) over night at 4°C, and then serially dehydrated with methanol. Hybridization with probes were performed over night at 65°C. Color development was done with NBT/BCIP substrate (Promega).

Whole-mount and section immunostaining.

For whole mount staining, skin explants incubated for 2-3 hours on culture insert were fixed with 4% paraformaldehyde in PBS over night at 4°C, and washed with PBS. For cryostat sections, the fixed samples were washed with PBS and embedded in OCT compound. Cryosections were prepared as 8μm thickness. Immunostaining was described before (33). Immunostaining antibodies: Mitf (Abcam, ab12039, 1:200 dilution); Mitf (Abcam, ab122982, 1:200 dilution); MelEM (MelEM was deposited to the DSHB by Le Douarin, N., 1:10 dilution); Hoechst 33342 (Invitrogen, 1:1000 dilution). Secondary

antibody was Alexa Fluor 488 (Invitrogen, 1:200 dilution), Alexa Fluor 594 (Invitrogen, 1:200 dilution) or Biotinylated horse anti-mouse IgG (Vector Laboratories, 1:200 dilution). For detection of Biotinylated horse anti-mouse IgG, Streptavidin Alexa Fluor 488 (Invitrogen, 1:300 dilution) was used.

Imaging.

Imaging of whole-mount embryos and skin explants was performed using a Nikon SMZ1500 or Zeiss Axio Zoom.V16. Images of in vitro melanocyte and sections were captured by a Keyence BZ-X700 or Zeiss LSM 5 Pascal upright laser scanning confocal microscope.

Skin transplantation.

E5 JQ skin was removed and kept in Hanks's buffered saline solution (HBSS) at 4°C. A part of the skin was removed from E6 WL in ovo, then the JQ skin was transplanted into the WL. The grafted skin was pinned with thinner glass needles to avoid detachment. Embryos were harvested at E11.

Skin explant culture, inhibitor treatment, and epidermis-dermis (E-M) recombination.

Removed embryonic skin was placed on a culture insert in 6-well culture plate (Falcon) containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 2% fetal bovine serum (FBS, GEMINI#100-106), 10% chicken serum (CS, Sigma C5405) and Penicillin-Streptomycin (Gibco) (1:1000). The explants were incubated at

38°C at an atmosphere of 5% carbon dioxide and 95% air. Chemical modulators: Nifedipine, carbenoxolone, 5-(N-Ethyl-N-isopropyl) Amiloride, silver nitrate and ω-Conotoxin were purchased from Sigma. For E-M recombination, detail of the method was described previously (33). Briefly, dissected E6 JQ skin was incubated 2x calcium-magnesium free saline with 0.25% EDTA on ice for 10 minutes. Epidermis and dermis were carefully separated by forceps, then rotated and recombined. The recombinants were incubated on the culture insert.

Neural tube transplantation.

Neural tube transplantation was performed as described previously (18). Briefly, 3 somite-length neural tube was removed from WL at HH 14 stage by using sharp tungsten needle. Identical developmental stage and position of neural tube was dissected from JQ and transplanted into WL embryo. The chimera was harvested at the desired stage.

In ovo electroporation.

A DNA solution (pT2AL200R175-CAGGS-LynEGFP-IRES-Neo^r: pCAGGS-T2TP = 7 μg/μl: 2μg/μl) was injected into the neural tube of E2 stage. Z-shape tungsten electrodes (diameter: 0.5 mm) were placed on both sides of the neural tube. Electric pulses of 50 V, 20 ms, were applied four times with 980 ms intervals by using CUY21EDIT (BEX).

Melanocyte incubation and transplantation.

An original method was described previously (20). Briefly, E8 skin electroporated with Tol2 plasmids was dissociated with 0.25% trypsin-EDTA (Gibco) at 37°C for 20 min. Dissociated tissue was filtrated with Sterile Cell Strainers (pore size, 70 μm, Corning). The filtrate was added to 10 ml PBS and centrifuged at 200 x g for 10 min. The pellet was suspended with DMEM (10% FBS and 2%CS) and incubated in 6 cm culture dish at 38°C at an atmosphere of 5% carbon dioxide and 95% air. After one day culture, the medium was replaced with Medium 254 (Gibco) containing with HMGS (Gibco) and 500 μg/ml G418 (Gibco). Incubation was kept until EGFP negative cells were killed (7-10 days). Melanocytes in the dish were washed with PBS and incubated with 1 ml 0.05% trypsin-EDTA at 38°C for 3 min. The reaction was stopped by adding 6 ml DMEM (10% FBS) and centrifuged at 200 x g for 5 min. The pellet was suspended with Medium 254 to make melanocyte-containing medium. This was moved to a hanging drop. 10-20 microlitres of the medium was placed on the inside of lid of a 6 cm dish and the bottom of dish was filled with 4 ml DMEM to maintain humidity. Melanocyte aggregates were formed after 1-2 days incubation, and transplanted between the E2 neural tube and presomitic mesoderm. The chimera were harvested at E11 stage to analyze the pigment pattern.

Neural crest cell incubation.

First, E2 neural tubes were labeled with the Tol2 plasmid by electroporation. 3 hours later, the neural tubes were dissected and placed on the 6 cm fibronectin coated (50 μg/ml) culture dish containing DMEM (10% FBS and 2% CS) at 38°C overnight. Replaced the DMEM with Medium 254 (Gibco) containing with HMGS (Gibco) and 500

μg/ml G418 (Gibco). For several days of culture, neural tubes were picked up and emigrated neural crest cells (NCCs) are incubated in a different dish coated with fibronectin. By incubating for 10 days, most cells showed LynEGFP positive signal. Then, those cells are moved to the hanging drop culture to form aggregates. The aggregates of NCCs were grafted in between neural tube and presomitic mesoderm of E2 WL embryos.

Lentivirus infection.

Lentivirus packaging was performed in HEK293T cells (which was a gift from Erin Weber) co-transfected with FUGW, pCMV-VSVG (which was a gift from Bob Weinberg, Addgene plasmid #8454) and pCMV-dR8.2 (which was a gift from Didier Trono, Addgene plasmid # 12263). Lentivirus were concentrated by ultracentrifuging at 20,000 rpm for 2 hours. The virus was suspended in 100 μl HBSS and stored at -80°C. The virus was injected into E2 somites of JQ.

RNA seq. analysis.

Black and yellow stripes were dissected from E7 JQ skin and total mRNA was extracted by Trizol reagent (Invitrogen). The quality of samples was analyzed by bioanalyzer. 2 μg RNA was used for library construction with TruSeq RNA Sample Prep Kit Version2 (Illumina). Two biological replicates were sent for sequencing with Illumina NextSeq 500 in the USC Epigenome Center. The expression analysis was performed by TopHat and Cufflinks software (34). 12,420 genes have been annotated by IPA software (Qiagen). Differential expression genes ($p<0.01$) were analyzed and

visualized with Excel according to the website of HOMER (http://homer.ucsd.edu/homer/basicTutorial/excelTips.html).

Statistics.

The analysis was performed with R software. In Fig. 5*C*, the statistical significance was analyzed by Mann-Whitney U test. In Fig. 5*G*, values are sum of the number of feather from six (mC), four (DNcx40) and four (cx40) individuals. The statistical significance of among each group was analyzed by the chi-squared test followed by the Ryan test. The Ryan test was performed using the code written by Dr. Shigenobu Aoki ("http://aoki2.si.gunma-u.ac.jp/R/src/p_multi_comp.R", encoding="euc-jp").

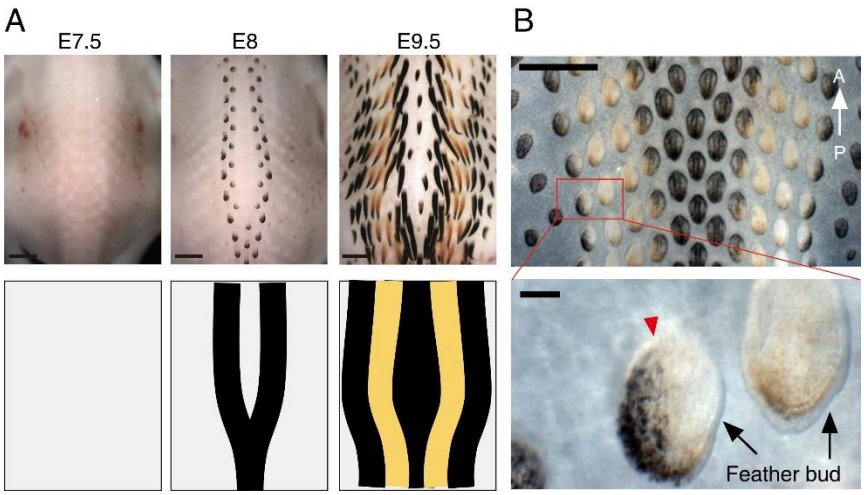


Fig. S1. Characterization of the pigment pattern of Japanese quail (JQ). (*A*) Dorsal view of the pigment pattern of JQ skin during development. (*B*) E8 JQ skin explant incubated for 2 days. A: anterior; P: posterior. Red arrowhead indicates the color boundary (enlarged view). Scale bars, 2 mm in (*A*) and 1 mm in (*B*, whole mount view), 100 μm in (*B*, enlarged view).

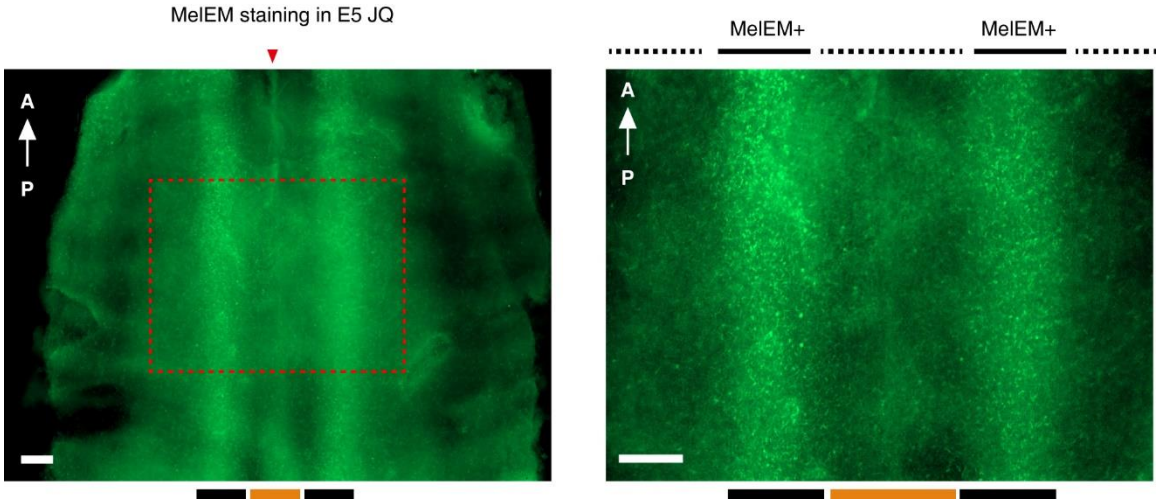


Fig. S2. Immunofluorescent image of MelEM in E5 JQ skin. MelEM+ melanocytes form two stripes along midline (red arrowhead). The box in the left panel is enlarged in the right panel. Solid lines: MelEM+ regions; dotted lines: MelEM- regions. A: anterior; P: posterior. Scale bars, 200 μm.

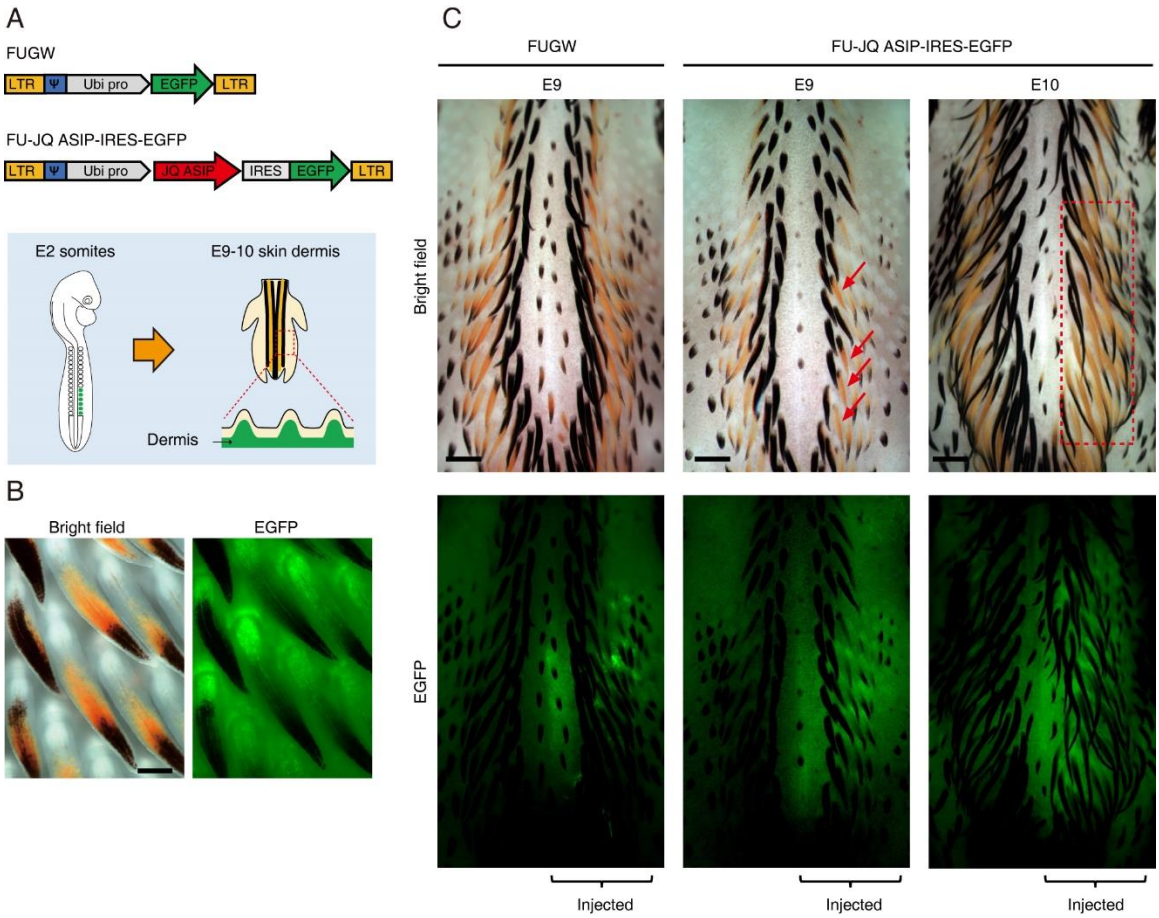


Fig. S3. Lentivirus-mediated over-expression of ASIP. (*A*) Lentivirus construct expressing EGFP (FUGW), Japanese quail ASIP (FU-JQ ASIP-IRES-EGFP) under the control of the human ubiquitin promoter (Ubi pro) and a diagram depicting the infection strategy. Virus was injected into somites on the right side of the body at E2, then the phenotype was observed at E9-10. (*B*) E9 JQ skin infected by lentivirus with EGFP. Signals were detected in the dermis of feather and skin. (*C*) E9-10 JQ embryos infected with lentivirus harboring either EGFP (n=3) or ASIP-IRES-EGFP (n=10). The region expressing ASIP (EGFP+) showed the ectopic yellow pigmentation (red arrows and dotted rectangle). Scale bars, 200 μm in (*B*) and 1 mm in (*C*).

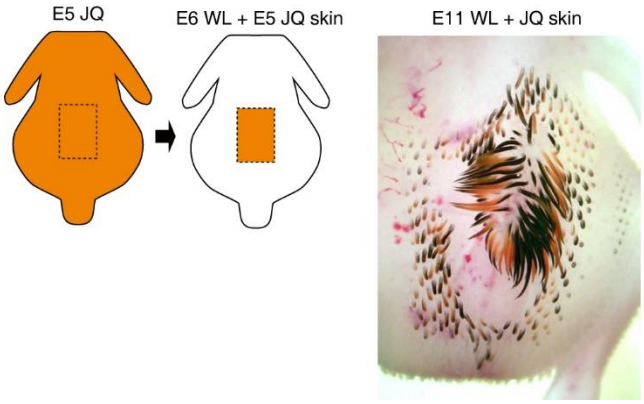


Fig. S4. JQ skin transplantation into WL. E5 JQ full thickness skin is grafted to E6 WL skin and evaluated at E11. Longer feather buds in the center region are derived from transplanted JQ skin. Pigment cells in shorter feather buds are derived from melanocytes in transplanted JQ skin.

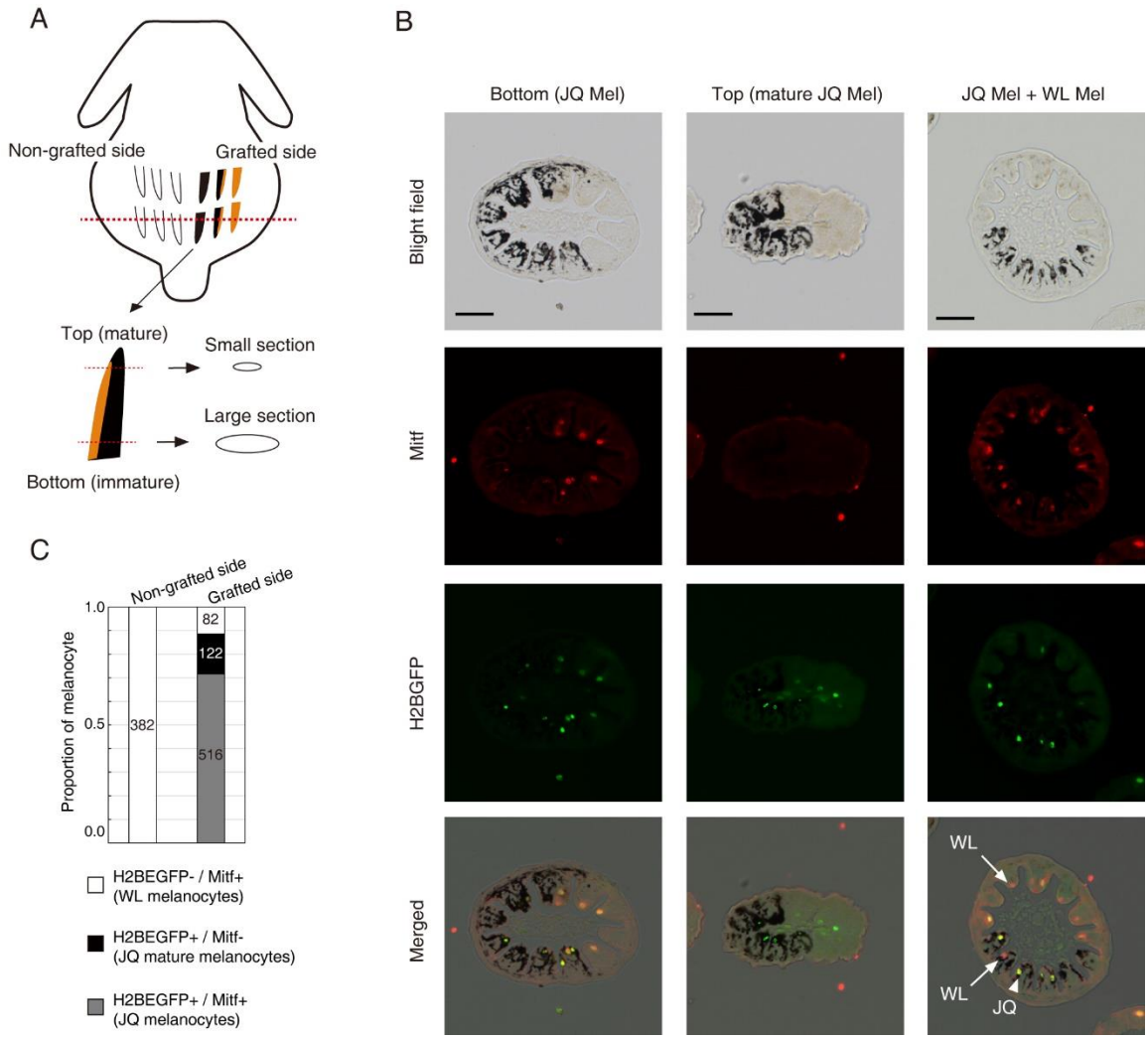


Fig. S5. Analysis of WL-derived melanocytes in E11 WL with JQ melanocytes expressing H2BGFP. (*A*) Position of transverse sections in E11 WL with JQ melanocytes. (*B*) Section images at different feather bud positions. JQ Mel are from near the bottom of feathers (left column). Mature JQ Mel are from near the top of feathers (middle column). Mitf signal in mature melanocytes tend to be negative. Arrowhead and arrows indicate JQ melanocytes (Mitf+; H2BGFP+) and WL melanocytes (Mitf+; H2BGFP-), respectively (right column). WL melanocytes may acquire pigment by interacting with JQ melanocytes. (*C*) Proportion of JQ melanocyte types in WL chickens transplanted with JQ melanocytes expressing H2BEGFP. Values are the sum of cell numbers from four individuals. Scale bars, 50 μm.

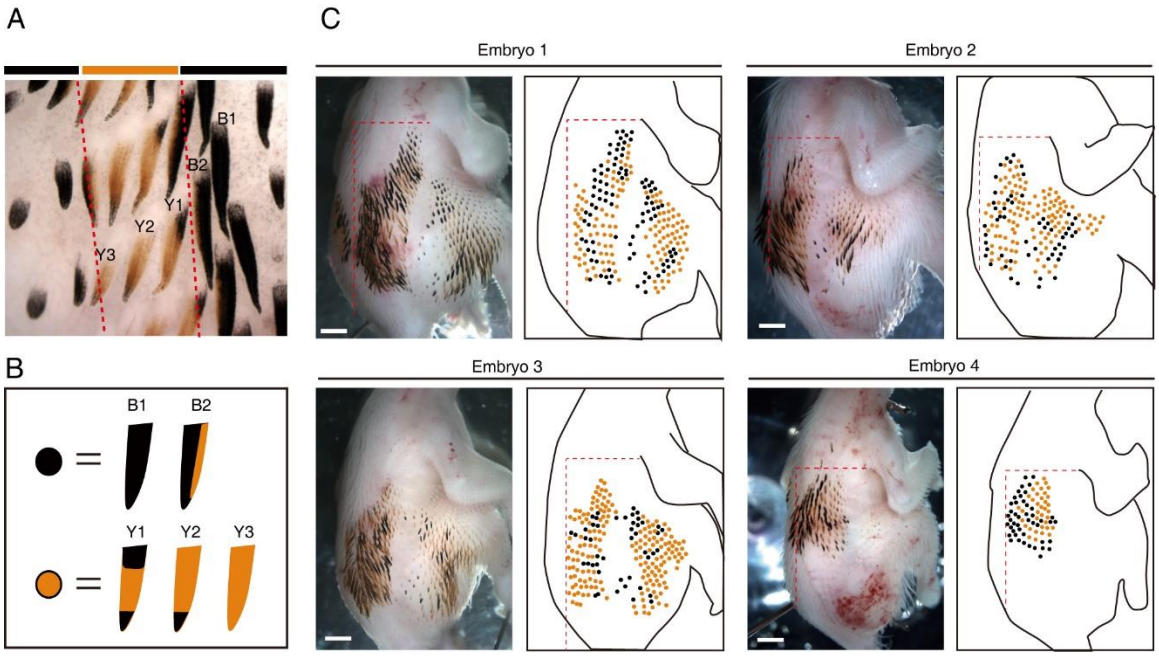


Fig. S6. Pigment variations in WL chickens transplanted with JQ melanocytes. (*A*) Feathers in E9.5 JQ skin. Two types of feather colors found in black regions. B1: whole black; B2: half black and half yellow. Three types of feather color are present in yellow regions. Y1: yellow with black tip and black bottom; Y2: yellow with black tip; Y3: whole yellow. (*B*) The criteria for feather color. (*C*) E11.5 WLs with JQ melanocytes. Right side of body (dotted lines) were analyzed and the pigment pattern was described by the criteria. Note that the pigment pattern is different among individuals. Scale bar, 2 mm.

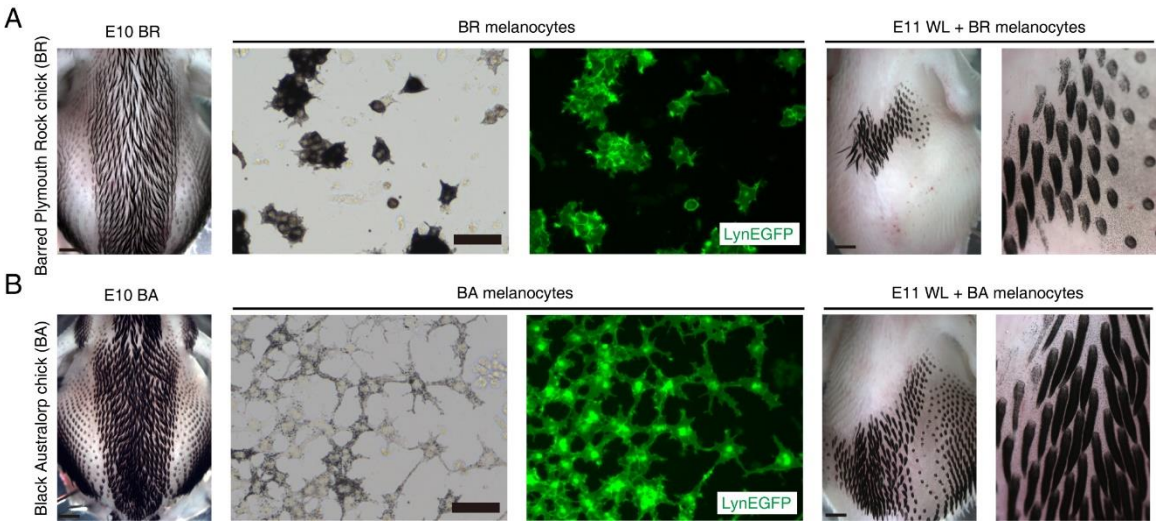
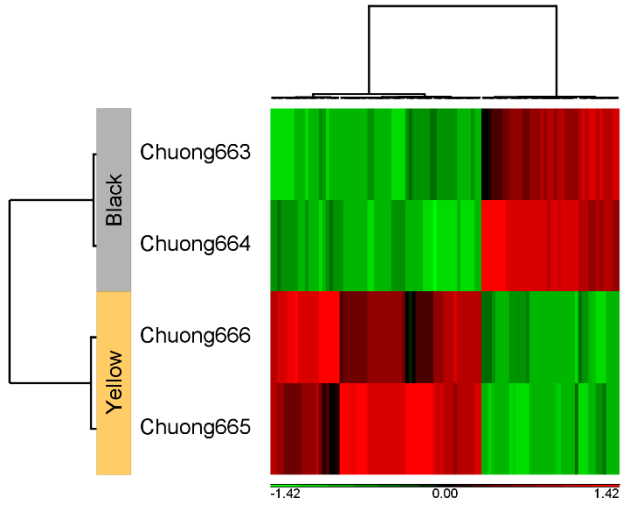
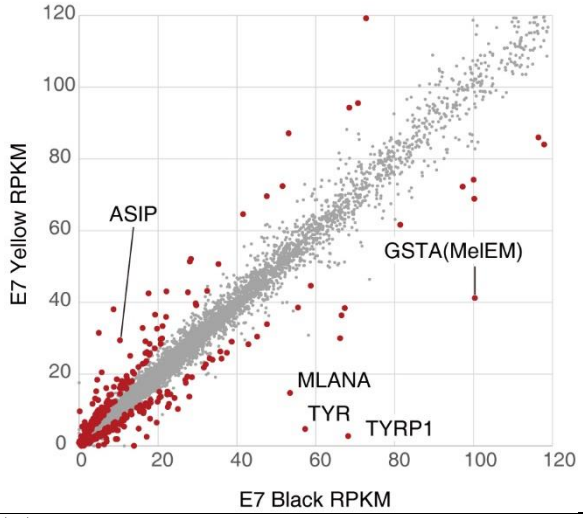


Fig. S7. Melanocytes of Barred Plymouth Rock (BR) and Black Australorp (BA) chicks and their transplantation into WL. (*A*) BR chick (n=4), (*B*), BA chick (n=7). The expression of LynEGFP is used to identify BR or BA melanocytes cultured for 9 days. Cultured melanocytes were transplanted to WL at E2 and analyzed at E11. Scale bars, 2 mm in whole view of the embryos and 100 μm in cell images.

A



B



(C) Pathway enriched analysis

Pathway	Gene
Eumelanin Biosynthesis ($P = 6.63 \times 10^{-5}$)	DCT, TYR
Melanocyte Development and Pigmentation Signaling ($P = 2.14 \times 10^{-3}$)	DCT, TYR, TYRP1
Glutathione-mediated Detoxification ($P = 2.95 \times 10^{-3}$)	Gsta1, HPGDS

Fig. S8. RNA-seq analysis between yellow and black JQ E7 skin. (*A*) Hierarchical clustering based on 101 DEGs. (*B*) Differential gene expression between black and yellow strips in E7 JQ ($p < 0.05$). **(***C***),** Pathway analysis by IPA with Fisher's Exact Test P-value and associated genes.

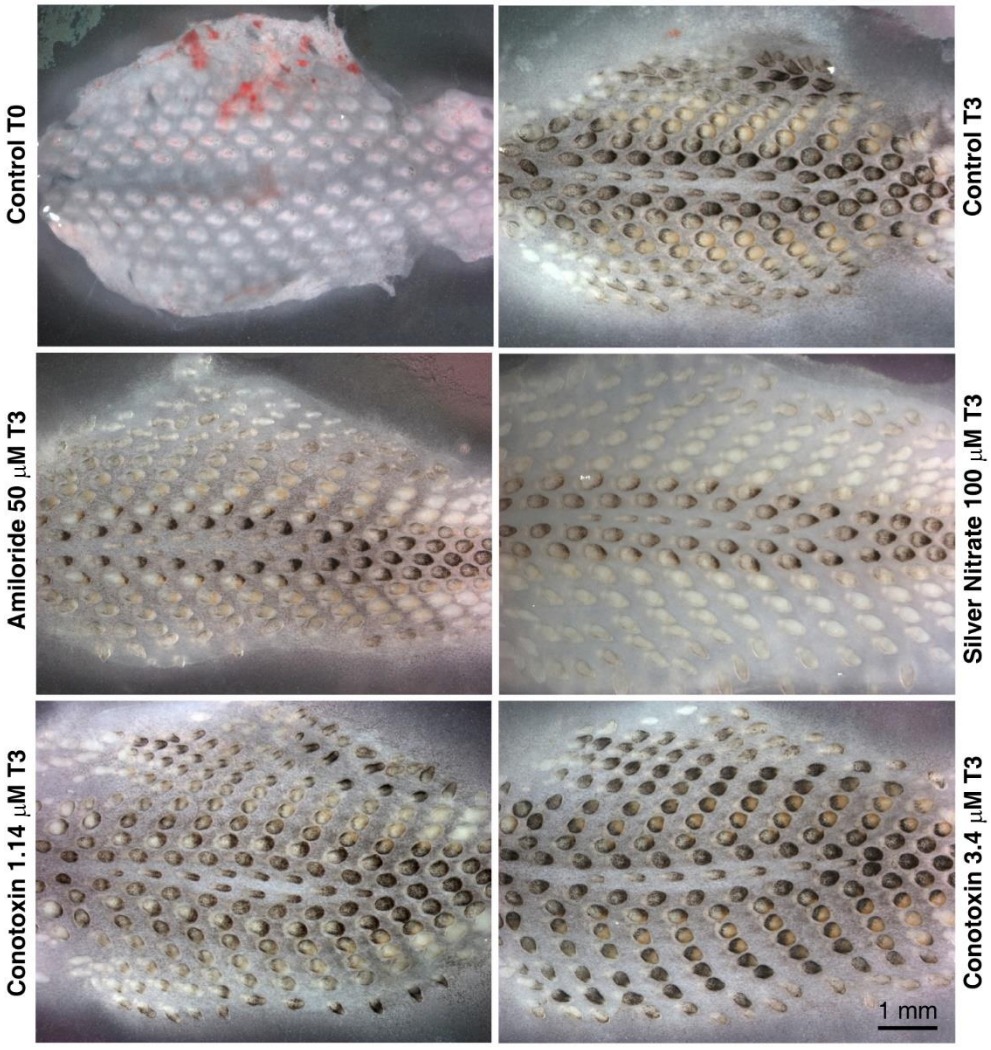


Fig. S9. Skin explant of Japanese quail (JQ) with inhibitors. E7 quail dorsal skin dissected and grown as explants on a culture insert dish in 10% FBS, 2% CS in DMEM for 3days. Control skin explants were grown for 0 days (T0). The addition of Amiloride (inhibitor of Na+ and Ca++ transport across epithelial membrane) and Silver nitrate (inhibitor of K+ homeostasis) for 3 days (T3) suppressed the pigmentation. Conotoxin (inhibitor of voltage gated Ca++ channel) increased the pigmentation.

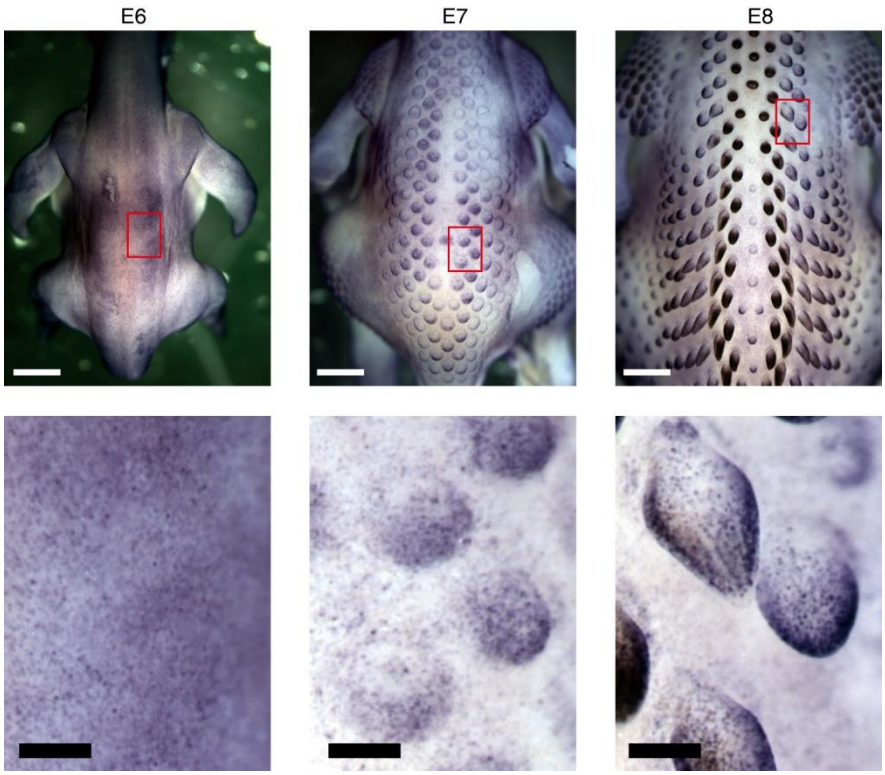


Fig. S10. Connexin40 expression pattern of JQ during development. Whole mount in situ hybridization of JQ connexin40 in developing JQ embryos. The area within the red box of the upper images are enlarged below. Scale bars, 1 mm in whole view and 200 μm in enlarged view.

References

33. Chuong C-M (1998) Molecular basis of epithelial appendage morphogenesis (R.G. Landes, Austin, TX).
34. Trapnell C, et al. (2012) Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. Nat Protoc 7(3):562–578.