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The first see-through frog created by breeding: description, inheritance patterns, and dermal chromatophore structure

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Masayuki Sumida¹, Mohammed Mafizul Islam¹, Takeshi Igawa^{1,2}, Atsushi Kurabayashi¹, Yukari Furukawa³, Naomi Sano³, Tamotsu Fujii³ & Norio Yoshizaki^{4,*}

We have succeeded in creating see-through frogs from natural color mutants of the Japanese brown frog *Rana japonica*, which usually possesses an ochre or brown back; this coloration enables the organs, blood vessels, and eggs to be observed through the skin without performing dissection. We crossed two kinds of recessive color mutant (black-eyed and gray-eyed) frogs through artificial insemination, and F2 offspring produced frogs whose skin is translucent throughout the life cycle. Three kinds of dermal chromatophores—xanthophores, iridophores, and melanophores—are observed in a layered arrangement in the skin of wild-type frogs, but few chromatophores were present in the skin of the see-through frogs. The translucent skin enables observation of organ growth and cancer formation and progression in the animal, which can be monitored over its entire life without the need for dissection. See-through frogs thus provide a useful animal model for environmental, medical, and biological research.

Dissection, particularly when performed in schools, has become increasingly controversial in much of the world. However, if the internal organs of an animal were clearly visible through the skin over the animal's entire life history, then there would be a greatly reduced need for dissection. In frogs, the skin is generally covered with dermal chromatophore units consisting of xanthophores, iridophores, and melanophores¹, which block the view of the internal organs through the skin. Although some small transparent fish species appropriate for experimental use have been previously reported^{2,3}, see-through tetrapods have not yet been developed. Several species of see-through glassfrogs belonging to the family Centrolenidae have recently been found in tropical wet forests and premontane rainforests in Central and South America; these frogs possess transparent parietal and cardinal peritonea^{4,5}. However, the creation of a see-through frog that is translucent over the entire body—to allow observation of organs, blood vessels, and eggs through the skin—remains a goal, with the primary purpose of providing an experimental model animal where organs can be observed over the entire life cycle without dissection.

The black-eyed and gray-eyed recessive color mutants seen in several frog species lack the normal iridophores and melanophores, respectively, resulting in pale or albino frogs^{6–13}. To create translucent frogs from these two color mutants of the Japanese brown frog *Rana japonica*^{12,14–16}, and to clarify the mode of inheritance in the resulting see-through frog, we crossed two mutant frogs through artificial insemination. F2 offspring produced frogs with skin that remains translucent throughout life. We also observed the dermal chromatophores in the skin of wild-type, black-eyed, gray-eyed, and see-through frogs with an electron microscope to examine the microstructure of the dermal chromatophores in the dorsal skin of see-through frogs. The present paper reports our experiments in detail, although a brief note on this topic has been previously published as a News-in-Brief (*Nature*)¹⁷.

¹Institute for Amphibian Biology, Graduate School of Science, Hiroshima University, Higashihiroshima 739-8526, Japan. ²Graduate School for International Development and Cooperation, Hiroshima University, Higashihiroshima 739-8529, Japan. ³Faculty of Human Culture & Science, Prefectural University of Hiroshima, Hiroshima 734-8558, Japan. ⁴Faculty of Applied Biological Science, Gifu University, Yanagido, Gifu 501-1193, Japan. *Deceased. Correspondence and requests for materials should be addressed to M.S. (email: msumida@hiroshima-u.ac.jp)

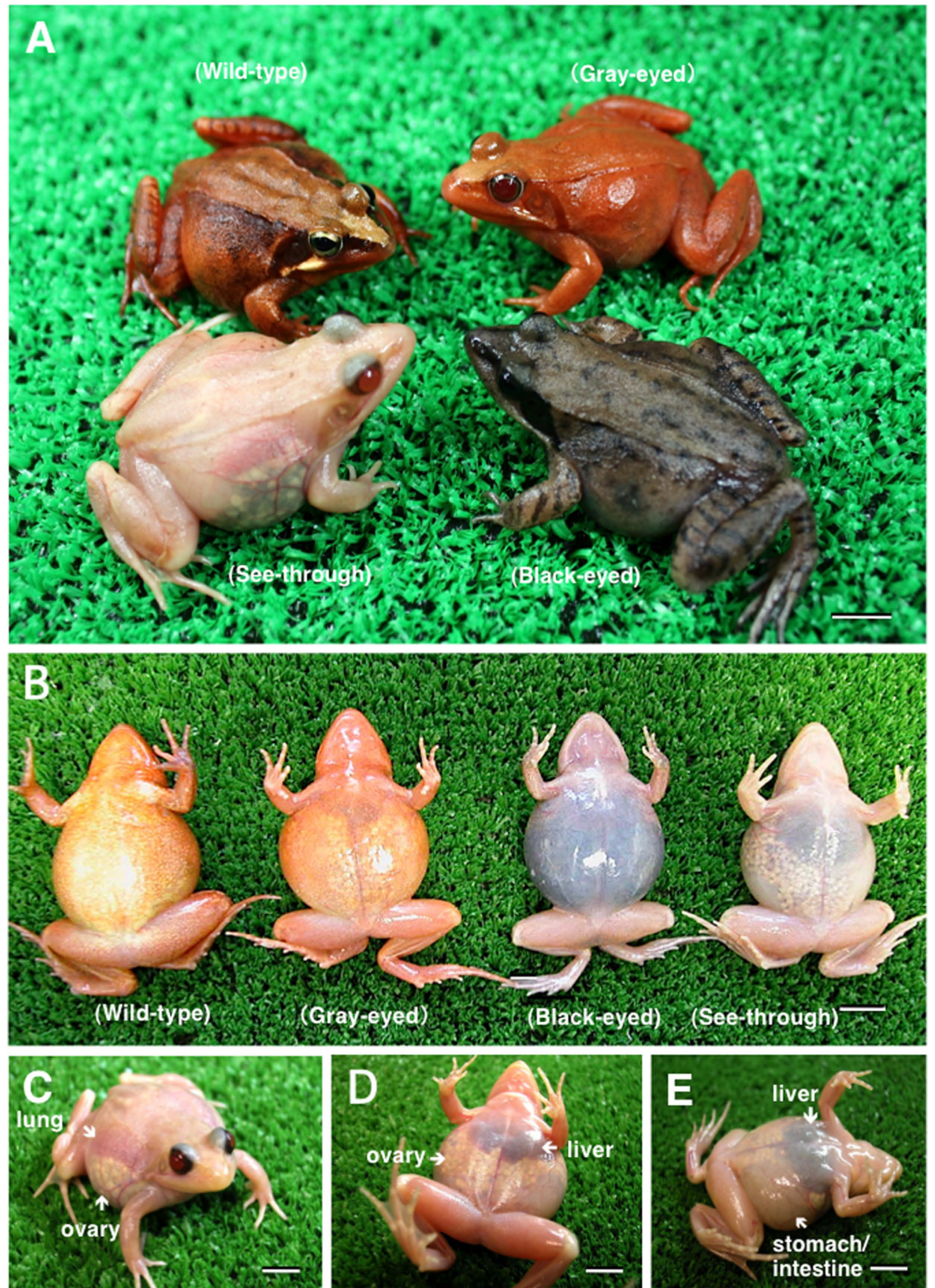


Figure 1. See-through, two color mutant, and wild-type adult *Rana japonica* frogs. (A) Dorsolateral view of four types. (B) Ventral view of four types. (C) See-through frog (frontal view). (D) See-through frog (ventral view). (E) See-through frog (ventrolateral view). (Scale bar 1 cm)

Results

Creation of see-through frogs. Upon crossing two color mutant (gray-eyed and black-eyed) frogs with recessive genes through artificial insemination, all of the offspring appeared normal (wild-type) due to the presence of dominant genes (Fig. 1A,B, Table 1). However, brother-sister mating among the wild-type offspring led to frogs with translucent skin from the tadpole stage, thereby successfully generating see-through frogs with visible viscera. The see-through frog is the first transparent four-legged animal to be developed artificially. Organs such as the lungs, liver, heart, ovaries, stomach, intestines, oviduct, and fat bodies are visible through the translucent

Mating Date	Parents (Genotype)		No. of eggs	No. of normally cleaved eggs (%)	No. of normal tail-bud embryos (%)	No. of normally hatched tadpoles (%)	No. of normally feeding tadpoles (%)	No. of metamorphosed frogs (%)	No. of offspring (Genotype) (%)			
	Female no.	Male no.							Wild-type	Gray-eyed	Black-eyed	See-through
2000.3.21	Gray-eyed (BBgg)1	Black-eyed(bbGG)1	62	55	50	33	30	25	25(BbGg)	-	-	-
	Gray-eyed (BBgg)2	Black-eyed(bbGG)1	58	50	47	38	35	32	32(BbGg)	-	-	-
	Gray-eyed (BBgg)3	Black-eyed(bbGG)1	92	85	80	68	65	63	63(BbGg)	-	-	-
	Gray-eyed (BBgg)4	Black-eyed(bbGG)1	38	32	30	20	18	16	16(BbGg)	-	-	-
	Total		250	222 (88.8)	207 (82.8)	159 (63.6)	148 (59.2)	136 (54.4)	136 (100)	0	0	0
2002.2.22	Wild-type (BbGg)1	Wild-type (BbGg)1	265	210	207	206	198	198	112 (B/G/)	40 (B/gg)	33 (bbG/)	13 (bbgg)
	Wild-type (BbGg)2	Wild-type (BbGg)1	50	42	38	37	32	32	18 (B/G/)	8 (B/gg)	4 (bbG/)	2 (bbgg)
	Wild-type (BbGg)3	Wild-type (BbGg)1	45	32	20	20	16	16	8 (B/G/)	4 (B/gg)	4 (bbG/)	0 (bbgg)
	Wild-type (BbGg)1	Wild-type (BbGg)2	240	215	211	204	190	190	97 (B/G/)	40 (B/gg)	41 (bbG/)	12 (bbgg)
	Wild-type (BbGg)2	Wild-type (BbGg)2	38	30	27	27	24	24	12 (B/G/)	5 (B/gg)	6 (bbG/)	1 (bbgg)
	Wild-type (BbGg)3	Wild-type (BbGg)2	34	28	24	24	16	16	7 (B/G/)	5 (B/gg)	4 (bbG/)	0 (bbgg)
	Total		672	557 (82.9)	527 (78.4)	518 (77.1)	476 (70.8)	476 (70.8)	254 (53.4) 267.8*	102 (21.4) 89.2*	92 (19.3) 89.2*	28 (5.9) 29.8*
(Segregation ratio 9:3:3:1, $\chi^2 = 2.7445$, $P = 0.433$)												
2004.3.7	Gray-eyed (Bbgg)1	Wild-type (BbGg)3	137	110	109	109	86	68	32 (B/G/)	27 (B/gg)	5 (bbG/)	4 (bbgg)
	Gray-eyed (Bbgg)1	Wild-type (BbGg)3	156	127	125	123	87	65	32 (B/G/)	18 (B/gg)	10 (bbG/)	5 (bbgg)
	Gray-eyed (Bbgg)2	Wild-type (BbGg)4	162	123	121	118	87	79	21 (B/G/)	38 (B/gg)	11 (bbG/)	9 (bbgg)
	Gray-eyed (Bbgg)2	Wild-type (BbGg)4	135	112	110	110	82	69	24 (B/G/)	27 (B/gg)	10 (bbG/)	8 (bbgg)
	Gray-eyed (Bbgg)3	Wild-type (BbGg)5	131	114	112	108	89	76	39 (B/G/)	29 (B/gg)	3 (bbG/)	5 (bbgg)
	Gray-eyed (Bbgg)3	Wild-type (BbGg)5	129	112	110	107	69	64	23 (B/G/)	24 (B/gg)	8 (bbG/)	9 (bbgg)
	Total		850	698 (82.1)	687 (80.8)	675 (79.4)	500 (58.8)	421 (49.5)	171 (40.6) 157.9*	163 (38.7) 157.9*	47 (11.2) 52.6*	40 (9.5) 52.6*
(Segregation ratio 3:3:1:1, $\chi^2 = 4.8660$, $P = 0.182$)												
2004.3.7	See-through (bbgg)1	See-through (bbgg)1	249	162	151	149	131	88	-	-	-	88 (bbgg)
	See-through (bbgg)2	See-through (bbgg)1	378	203	191	188	144	98	-	-	-	98 (bbgg)
	See-through (bbgg)1	See-through (bbgg)2	230	140	129	128	100	84	-	-	-	84 (bbgg)
	See-through (bbgg)2	See-through (bbgg)2	296	218	207	188	89	78	-	-	-	78 (bbgg)
	Total		1153	723 (62.7)	678 (58.8)	653 (56.6)	464 (40.2)	348 (30.2)	0	0	0	348 (100)

Table 1. Crossing experiments for creating see-through frogs. Asterisks show the expected values from the segregation ratios.

skin when the animal is viewed ventrally or laterally (Fig. 1C–E, Fig. 2A,B). Dramatic changes during metamorphosis such as gill disappearance, lung development, intestinal shortening, and emergence of forelimbs can be easily viewed in the developmental process of metamorphosis (Fig. 2C,D). See-through frogs also allow observation of ovulation (Fig. 2F,G). Ovulation was induced in mature female see-through frogs by injecting a suspension of bullfrog pituitary extract into the abdominal cavity. Deposited see-through frog eggs were white in color, as opposed to the normal black color of wild-type eggs (Fig. 2E). All of these processes are visible through the skin, without dissecting the tadpole or the frog. Videos of the see-through frogs and tadpoles produced in this study are available online (adult frog, <https://www.youtube.com/watch?v=3TumwZ40LQM>; tadpole <https://www.youtube.com/watch?v=tYRjZtaxOgg>).

As shown in Table 1, the production rates of see-through frogs depended on the combination of parental genotypes following the law of segregation (9:3:3:1, $\chi^2 = 2.7445$, $P = 0.433$; 3:3:1:1, $\chi^2 = 4.8660$, $P = 0.182$). The most effective crossing combinations were between Bbgg female and BbGg male parents. See-through frogs produced by the F2 generation had low viability, probably due to the presence of two recessive genes.

Microstructure of dermal chromatophores in the dorsal skin. Three kinds of dermal chromatophores—xanthophores, iridophores, and melanophores—were observed in a layered arrangement in the skin of wild-type frogs (Fig. 3A). Pterinosomes and carotenoid vesicles were found in the xanthophores (Fig. 3B), and reflecting platelets and melanosomes were observed in the iridophores and melanophores, respectively (Fig. 3C). The skin of gray-eyed frogs contained no melanophores, and had only xanthophores and iridophores (Fig. 3D). Normal pterinosomes and many abnormal round reflecting platelets were observed in the xanthophores and iridophores, respectively (Fig. 3E,F). The skin of black-eyed frogs contained xanthophores and melanophores, but not iridophores (Fig. 3G). Normal melanosomes were found in the melanophores, whereas immature pterinosomes or carotenoid vesicles were observed in the xanthophores (Fig. 3H). In the skin of see-through frogs, there was a low number of chromatophores (Fig. 3I), but immature xanthophore-like cells containing melanosome-like structures were observed (Fig. 3J).

Discussion

The see-through frogs produced in this study allowed us to observe changes in the internal tissues and organs in detail, externally, in both early development and senescence, to evaluate the effects of chemicals on the viscera and bones in a simple and inexpensive manner, to view the development and progression of cancer, and to assess the effects of toxins over time. See-through frogs do not need to be dissected to perform such viewing and therefore enable the repeated ongoing observation of the viscera over the entire life course in a single frog. Furthermore, the dramatic changes in organs that occur during the metamorphosis of tadpoles into adult frogs can be easily seen.

Genetic engineering of see-through frogs is an approach that could be used to produce see-through, fluorescent frogs. In the near future, we plan to produce frogs that are both see-through and fluorescent by injecting fluorescent GFP-tagged genes into see-through frogs, causing the frogs to fluoresce to indicate expression of the tagged gene¹⁸. This could be used for many purposes, for example, showing when cancer starts. See-through frogs can also be used as experimental model animals in various fields, such as medicine, veterinary medicine, biology, and education. Commercial production and marketing applications for see-through frogs as ornamental pets is another possibility, and the frogs have been nicknamed “*sukeru-pyon*”, combining the Japanese words for “see-through” and “frog”.

The see-through frogs produced in the present study appear pale when viewed dorsally, as xanthophores remain in the dorsal skin. However, these frogs are almost transparent when viewed ventrally, because there are little to no xanthophores in the ventral skin. If we could obtain color mutants that lack xanthophores, we would be able to produce completely see-through frogs by using these mutants to produce triple mutants lacking genes for all three chromatophores. In fact, variant blue ranid frogs have been found in several frog species^{19–22}, but we have not yet found this kind of color mutant in the Japanese brown frog *Rana japonica*. We hope to create completely see-through frogs in the near future. It is worth noting that it is unrealistic to apply the present method to mammals such as mice, given the differences in skin structure, although the development of whole-body imaging at single-cell resolution has recently enabled system-level approaches to studying cellular circuits in mice by using tissue decolorization²³. See-through frogs are not stable enough to produce offspring, probably due to inbreeding depression caused by two recessive genes. This is another problem that must be addressed in the future by using outbreeding, via artificial insemination, to avoid inbreeding depression.

Immature xanthophore-like cells containing melanosome-like structures were observed in the skin of the see-through frogs. Some evidence suggesting the conversion of xanthophores into melanophores *in vitro* and *in vivo* has been reported^{24–27}, although it is difficult to follow the conversion of a single xanthophore precisely, and there has been no conclusive evidence demonstrating the conversion of a xanthophore into a melanophore *in vivo*. Evidence that melanosomes and pterinosomes are homologous organelles has been reported in several studies^{28–31}, and similarities exist in the morphological characteristics between the two. Ide³² demonstrated the conversion of amphibian xanthophores into melanophores *in vitro*. In the cloned xanthophore, pre-existing pterinosomes disappeared and melanosomes were formed instead during proliferation. Ide considered these melanosomes to originate *de novo* from melanosomes via premelanosomes, because of the presence of premelanosomes in the melanized xanthophores. The results of the present study also suggest that xanthophores may be converted into melanophores, demonstrating the *de novo* formation of melanosomes via premelanosomes.

Another possible explanation may be that xanthophores contain melanosome-like organelles, but not trans-differentiated chromatophores, as melanosome-like structures were also observed in xanthophores in the skin of gray-eyed frogs (Fig. 3D–F). According to Bagnara³³, pigment cells occasionally occur that contain not only their definitive organelle type, but also the pigment organelles of other pigment cell types. Chromatophore mosaicism,

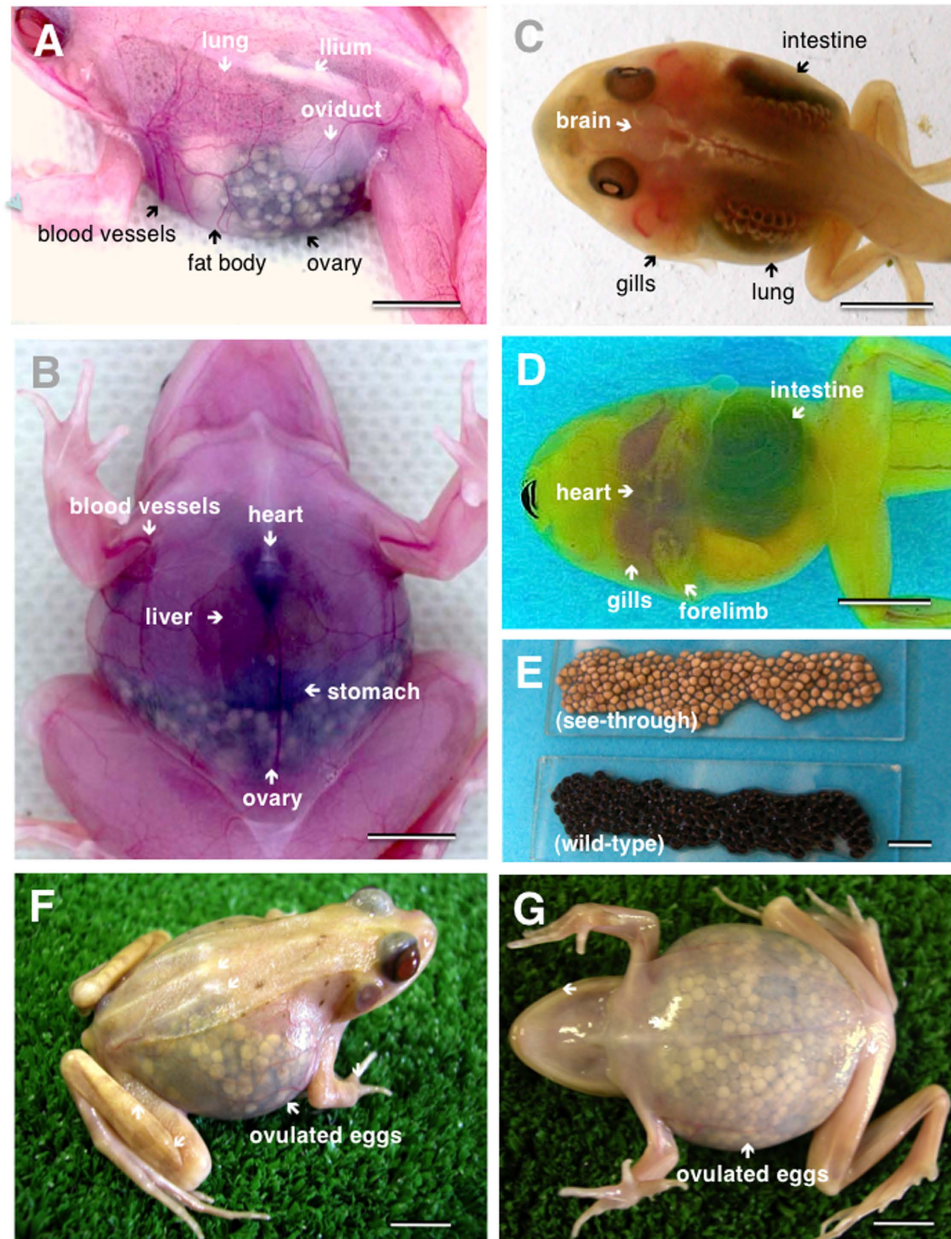


Figure 2. Internal organs of live see-through frogs and tadpoles visible through the translucent skin. (A) Breathing see-through frog (dorsolateral view). (B) Heart beating see-through frog (ventral view). (C) See-through tadpole (dorsal view). (D) See-through tadpole (ventral view). (E) Eggs of see-through and wild-type frogs (just after insemination). (F) Ovulated see-through frog (lateral view). (G) Ovulated see-through frog (ventral view). (Scale bar 1 cm)

where different types of pigment granules are contained within a single pigment cell, have been reported in several kinds of frogs^{13,33,34–36}. Some mosaic or polychromatic hybrid chromatophores appear to possess organelles characteristic of other chromatophores, for example, melanophores containing pterinosomes or xanthophores containing melanosomes³⁴. These mosaic cells are given compound names, such as “irido-melanophores” for chromatophores that reflect light and produce melanin^{35,36}. The pigment granules contained in a single pigment cell are variable, and mosaic chromatophores that contain three types of pigment granules have also been found^{13,35}. Melanophores, xanthophores, and iridophores are fundamentally distinct in their appearance, composition, and function, but all migrate from their site of origin at the neural crest to populate the integument. Their respective pigments—melanins, pteridines, and purines—are found in organelles designated as melanosomes, pterinosomes, and reflecting platelets, respectively. These organelles are all derived from endoplasmic reticular vesicles. This commonality is in keeping with the hypothesis of the common origin of pigment cells from a stem cell containing a primordial organelle with the potential to become any derived pigmentary organelle³⁷.

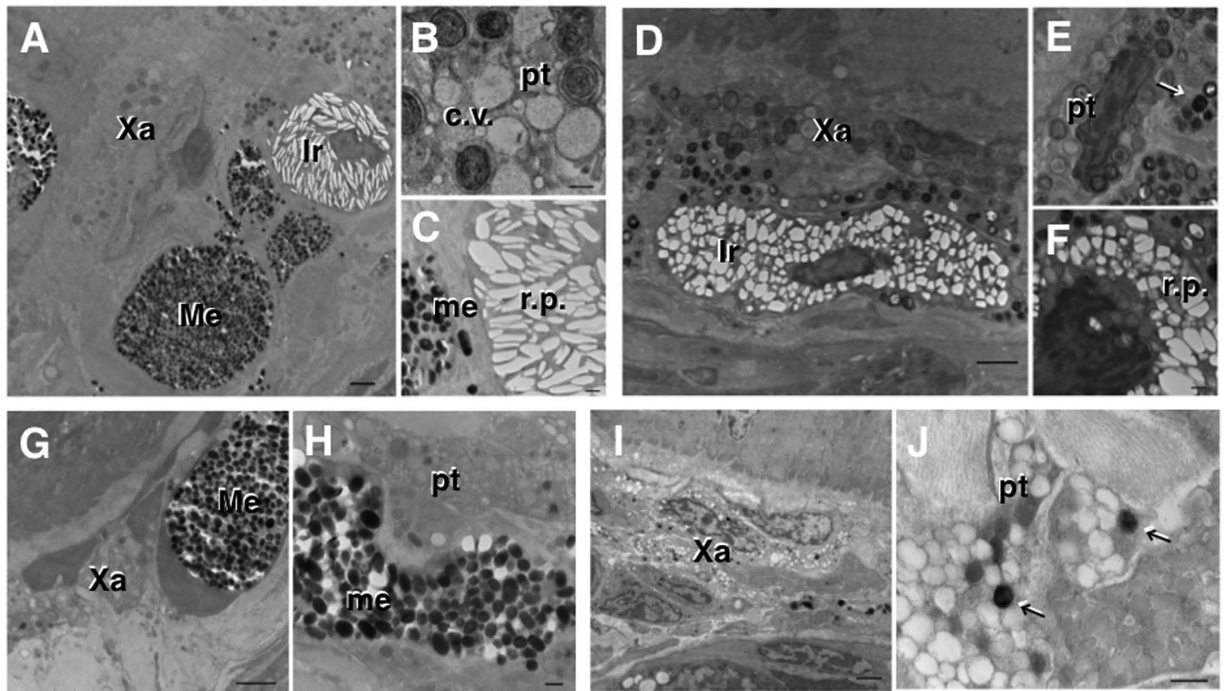


Figure 3. Electron microphotographs of dermal chromatophores in the dorsal skin. (A–C) Wild-type frog. (D–F) Gray-eyed frog. (G,H) Black-eyed frog. (I,J) See-through frog. Me: melanophore, Xa: xanthophore, Ir: iridophore, me: melanosome, pt: pterinosome, c.v.: carotenoide vesicle, r.p.: reflecting platelet. Arrows indicate melanosome-like structures. (Scale bar A,D,G,I: 2 μ m; B,C,E,F,H,J: 500 nm)

In light of these findings, further studies will be necessary to clarify the processes involved in chromatophore formation in more detail, and to examine the fine structure of pigment organelles by observing pigment cell development in see-through tadpoles and frogs using electron microscopy.

This study has received a patent under “Creation and Use of See-through Frogs”, application number (2006-203987), open number (2008-029223) on application date (2006.7.26), open date (2008.2.14), applicant (Hiroshima University), creator (Masayuki Sumida).

Methods

Animal ethics. All animal experiments were performed in accordance with the approved guidelines of Hiroshima University regarding protection and management of animals and standards for the breeding, safe-keeping, and minimization of suffering of experimental animals. All experimental protocols were approved by the Hiroshima University Animal Experiment Committee, and were in keeping with the basic guidelines of Hiroshima University regarding animal experiments (Approval numbers: 24-116, 26-209).

Creation of see-through frogs. We used two color mutants (black-eyed and gray-eyed) that were originally caught in the field, and then bred and maintained in the Institute for Amphibian Biology, Hiroshima University^{12,22}. These mutants were crossed by artificial insemination to produce frogs homozygous at both gene loci over several generations. Ovulation was accelerated by injecting a suspension of bullfrog pituitary into the abdominal cavity. Fertilization was always performed artificially. Tadpoles were fed boiled spinach and metamorphosed frogs were fed crickets^{38,39}.

Microstructure of dermal chromatophores in the skin. We observed the dermal chromatophores in the dorsal skin of wild-type, black-eyed, gray-eyed, and see-through adult frogs with an electron microscope to examine the microstructure of the skin of see-through frogs. Pieces of the dorsal skins removed from each of these frogs were cut into minute pieces in cold 0.1 M phosphate buffer (pH 7.4) containing 3% glutaraldehyde and kept in the same solution for 2 h after renewal of the fluid. The minute pieces were then postfixed in 0.1 M phosphate buffer (pH 7.4) containing 2% osmic acid for 2 h. These fixation procedures were performed at 2–4 °C. The fixed samples were dehydrated in an ethanol series and embedded in Epon 812. Sections were cut on a Porter-Blum MT-1 ultramicrotome with a glass knife and double stained with saturated uranyl acetate and alkaline lead citrate. Observations were made using a Hitachi Hs-8 electron microscope.

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Author Contributions

M.S. conceived the research, directed the project, and wrote the main text. M.M.I., T.I. and A.K. bred the strains and maintained them for subsequent research. T.I. also statistically analyzed the segregation ratios of F2 offspring in the genetic crosses. Y.F., N.S. and T.F. examined and analyzed the electron microscopic data. N.Y. prepared specimens for electron microscopy and acquired the electron microscope images. All the authors reviewed and approved the manuscript.

Additional Information

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