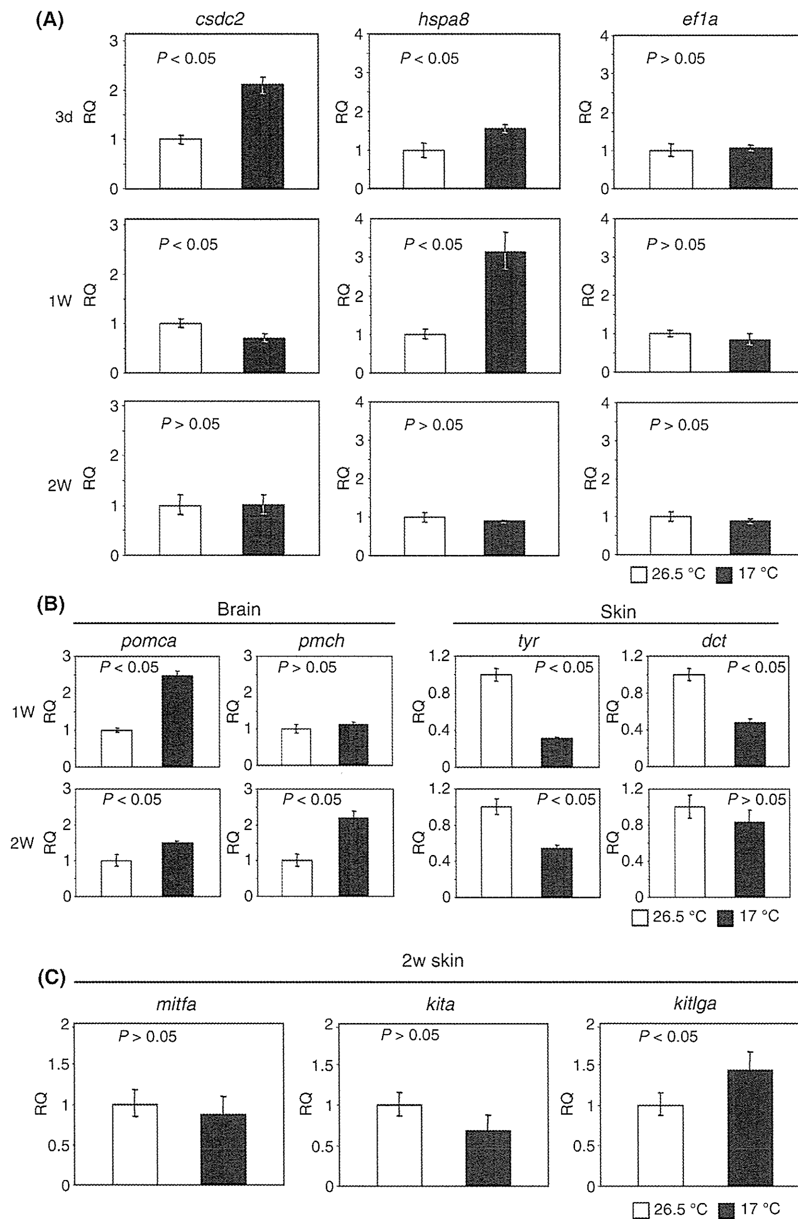

Figure 2 Expression levels of genes in normal and cold zebrafish (white and black bars, respectively). Levels of gene expression between normal and cold zebrafish were compared as relative quantities (RQ) on the *Y*-axis. (A) Expression levels of cold- and heat-shock protein genes, *csdc2* and *hspa8*, and a constitutively expressed gene, *ef1a*. Levels of *csdc2* and *hspa8* increased after 3 days (3 d) and 1 week (1 W) of cold exposure, whereas levels of *ef1a* were not changed. (B) Expression levels of genes involved in melanosome distribution, *pmoca* and *pmch*, and melanin synthesis, *tyr* and *dct*. In the brain of cold zebrafish, expression levels of *pmoca* increased at 1 W and slightly did at 2 W. Expression levels of *pmch* remained unchanged at 1 W, but increased at 2 W. In the skin, expression levels of *tyr* decreased both at 1 W and at 2 W, whereas *dct* decreased at 1 W and then recovered to normal levels. (C) Expression of genes involved in melanophore differentiation, *mitfa*, *kita* and *kitlga*, at the second week of cold exposure. Expression levels of *mitfa* and *kita* in cold zebrafish were not significantly different from normal zebrafish, whereas that of *kitlga* slightly was increased (~1.5-fold of normal zebrafish).

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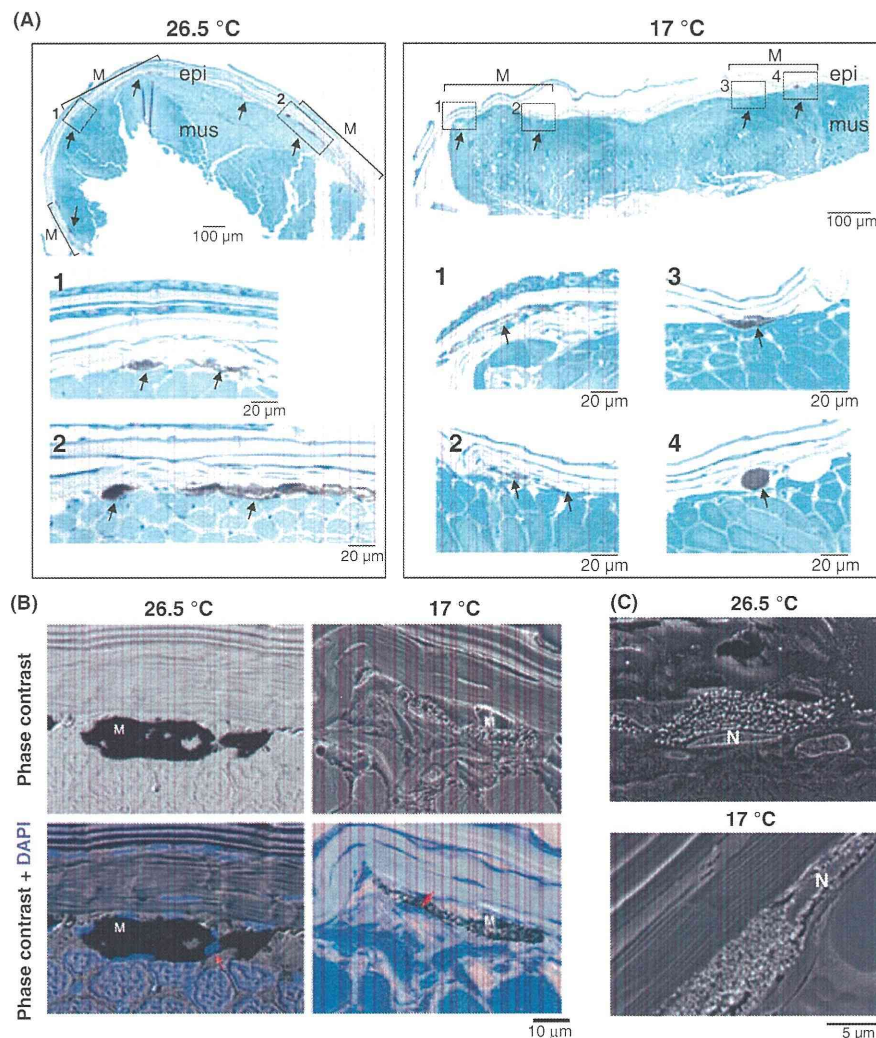

Figure 3 Histology of zebrafish skin. (A) Toluidine Blue O-stained zebrafish skin. Representative cross-sections of normal (26.5 °C) and cold (17 °C) zebrafish are shown. At lower magnification (upper panels), the number of melanophores (arrows) decreased in cold zebrafish. A series of melanophores (M) was localized between epidermis (epi) and muscle (mus) layers and named as melanophore band. The length of melanophore band in cold zebrafish (17 °C) was shorter than that of normal zebrafish (26.5 °C). At higher magnification (lower panels), the number of melanophores (arrows) decreased in melanophore bands of cold zebrafish compared with normal zebrafish. Lower panels are magnified view of boxes in upper panels. (B) Distribution of melanosomes in melanophores (M). Melanosomes were equally distributed in the cytoplasm of both normal and cold zebrafish. Nucleuses of melanophores were stained with DAPI and are located at the edge of cells (red arrows). There was no aggregation of melanosomes close to nucleus. (C) Images of scanning electron microscopy showed size, structure and distribution of melanosomes surrounding nucleus (N). There was no significant difference between normal and cold zebrafish.

ples using Toluidine Blue O staining and electron microscopy. At lower magnification (Fig. 3A, upper panels), we could observe a series of melanophores (arrows in upper panels of Fig. 3A) as if melanophores composed a band. The length of melanophore band in cold zebrafish (17 °C) was shorter than that of normal zebrafish (26.5 °C). When we examined a series of sections (results not shown), this observation was reproducible. At higher magnification, the number of melanophores decreased in the melanophore bands of cold zebrafish compared with normal zebrafish (Fig. 3A, lower panels). Taken together, it is likely that

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the number of melanophores decreased in cold zebrafish. To investigate the distribution of melanosome in melanophores, we stained skin with DAPI that visualized nucleus. Melanosomes were equally distributed in the cytoplasm of both normal and cold zebrafish (Fig. 3B). There was no aggregation of melanosome close to nucleus (Red arrows in Fig. 3B). To quantify melanosome further, we performed scanning electron microscopy (SEM). Size and structure of melanosome in cold zebrafish were similar to normal zebrafish (Fig. 3C and Fig. S3 in Supporting Information). Taken together, cold exposure down-regulated adult zebrafish pigmentation through decreasing the number of pigmented melanophores.

Discussion

We have developed a novel model for pigmentation research. We show that adult zebrafish maintained at 17 °C water undergo depigmentation by decrease in pigmented melanophores. Overall melanin content in skin decreased without alteration in melanosome distribution in cold zebrafish. Pigment of adult zebrafish is composed of xanthophores, reflective iridophores and melanophores or melanocytes. Melanophore differentiation has been extensively studied during fin regeneration, and markers have been well described: *mitfa* for melanoblasts, *kita* for melanocytes and *tyr* and *dct* for pigmented melanophores (Rawls & Johnson 2000). In combination with these marker genes, we could evaluate melanophore differentiation and pigmentation. Expression levels of *mitfa* and *kita* genes remained unchanged, whereas that of *kitlga* was increased. It implies that melanophore differentiation was not altered and the increased expression of *kitlga* in cold zebrafish was because of a compensation mechanism. Although depigmentation in cold zebrafish was associated with *tyr* and *dct* down-regulation, decrease in the melanin-synthesizing enzymes does not certify the decrease in melanin because the activity of degradation enzyme might be down-regulated at low temperature. Gene expression of *try* and *dct* is identified in melanophores (Rawls & Johnson 2000; White & Zon 2008). The decrease in the number of melanophores might cause *tyr* and *dct* down-regulation in cold zebrafish.

Zebrafish have adaptation mechanisms that allow them to change the color of their pigment stripes within 24 h after background exposure (Logan *et al.* 2006); their pigmentation becomes lighter when exposed to white backgrounds and darker when exposed to black backgrounds. However, it is unlikely that the reduced pigmentation seen in cold zebrafish was because of background adaptation, because (i) we observed slight difference in pigmentation in zebrafish that were maintained inside or outside of the incubator and (ii) depigmentation occurred after 3 days of cold exposure, and not within 24 h (results not shown). This adaptation is caused by complex mechanisms existing in the endocrine and/or nervous systems (Fujii 2000). Melanosome aggregation in melanophores is also controlled by sympathetic neurons (Iwashita *et al.* 2006). Although gene expression of *pomca* and *pmch* encoding pro-hormones of melanocyte-stimulating hormone (MSC) and melanin-concentrating hormone (MCH), respectively, was up-regulated in cold zebrafish, there was no alteration in melanosome distribution in cold zebrafish, implying that sympathetic nervous system adjusted the melanosome distribution in cold zebrafish. It will be further necessary to investigate the involvement of nervous system in the future. Low temperatures down-regulate many physiologic processes (Sonna *et al.* 2002), but fish have compensatory mechanisms that allow adaptive survival in low temperatures (Cossins *et al.* 2002; Schwarzbaum *et al.* 1992a,b). In Arctic fish, it has been reported that the activity levels of ion pumps and Na^+/K^+-ATPases in the kidney are increased to balance the altered ion concentrations and body fluid pH caused by temperature acclimation (Schwarzbaum *et al.* 1992a,b). The abnormal renal tubules and melanophores in the KM of cold zebrafish might be due to similar adaptation mechanisms.

Cold- and heat-shock proteins are thought to play important roles in adaptation to cold exposure in vertebrates (Ali *et al.* 2003; Al-Fageeh & Smales 2006). Although their functions are not fully understood, it has been suggested that cold-shock proteins bind mRNA to regulate transcription and translation. We analyzed the expression levels of *csdc2* and *hspa8* genes in the skin of zebrafish to investigate the molecular relationships between cold exposure and pigmentation. Csdc2 contains an S1-like CSD, which is conserved in the cold-shock protein family proteins, and Hspa8 is one of the heat-shock protein 70 family proteins. It was reported that, under cold shock, Hspa8 is up-regulated in carp (Ali *et al.* 2003). In this study, gene expression of both *csdc2* and *hspa8* was up-regulated in the skin of cold zebrafish compared with normal zebrafish. This result implies that Csdc2 and Hspa8 directly or indirectly have a role in regulating pigmentation. Further investigation into this relationship is warranted.

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In humans, abnormal melanin synthesis results in diseases such as hypopigmentation in albinism and hyperpigmentation in melasma, postinflammatory melanoderma and solar lentigines (Ni-Komatsu & Orlow 2007). Novel genes involved in the regulation of melanin synthesis can be potentially identified with this cold zebrafish model. Moreover, *dct* is associated with cisplatin-resistant malignant melanoma (Miller & Mihm 2006; Pak *et al.* 2004). However, the relationship between *dct* and melanoma biology still remains to be elucidated (White & Zon 2008). Further investigations to understand better the molecular mechanisms involved in the formation of malignant melanomas are warranted. Our cold zebrafish model may provide important insights into melanoma biology in the future.

Experimental procedures

Maintenance of zebrafish

Zebrafish (*Danio rerio*, India strain) born from the same parent were housed in tanks with recirculating dechlorinated tap water at 26.5 °C (room temperature) for 2 months. Two-month-old zebrafish were then separated into two groups: the first group was grown in 17 °C water inside an incubator for 3 days, 1, 2 weeks and 7 months and the second group was continuously maintained at 26.5 °C. Food, water and light cycles for both groups were controlled under the same conditions. To assess the presence of background adaptation in the incubators, one set of zebrafish was maintained inside and one set outside of the incubators in 26.5 °C water for 2 weeks, under the same conditions as mentioned earlier. The inside of incubator was covered by metal sheet.

Measurement of melanin content in zebrafish skin by spectrophotometer

Melanin content was measured following the method of Hultman & Johnson (2010) with a little modification. Zebrafish skin at body and tail was separately dissected out and weighted. Soluene®-350 (PerkinElmer, MA, USA) was added at a ratio 250 μL/50 mg skin and was heated at 95 °C for 1 h. After cooling, samples were centrifuged. Melanin in supernatant was assayed by absorption at 500 nm on a Nanodrop spectrophotometer (Thermo Fisher Scientific Inc.).

Growth measurement

Body weight and length were measured before killing the adult zebrafish to observe the effects of cold exposure on growth. Phenotypes were observed under a back-lit microscope (Olympus SZX16, Tokyo, Japan), and high-resolution

Table 1 Primer sets for zebrafish genes that are involved in pigmentation. Primers were designed using PRIMER EXPRESS® software version 3.0

Genes		Sequence (5′ → 3′)	Size (bp)	Reference sequences
bactin2	F	TGCTCCCCGAGCTGTCTT	64	NM_181601.3
	R	ACCAACCATGACACCCTGATG		
csdc2	F	CACACACCTCTCTCGCTTTCAT	100	NM_001002690.1
	R	TTGGCAGTGGACTCAGAGGTT		
dct	F	TTCACTCTCTGAGTCCGGAAGAG	100	NM_131555.1
	R	CAGTGCTGTGTGGCGATCAT		
ef1a	F	TCTACAAATGCGGTGGAATCG	100	NM_131263.1
	R	TCCAACACCCAGGCGTACTT		
hspa8	F	GATCGGCAGGAGGTTCGA	100	NM_001110403
	R	TTCCACTGCAACTTTTGGCTTT		
kita	F	ATCCTGCTCCACCTCAAATG	112	NM_131053.1
	R	GAGTAATGGGCTCCGTCAGA		
kitlga	F	GCCTGATGACCCCGAAAAA	100	XM_001922513.1
	R	CAAAACCACCACTGCGATTG		
mitfa	F	ATTCTTGGGTTCATGGATGCA	100	NM_130923.1
	R	CAGCTGGAGGAAGAGCATGAT		
pmch	F	TGGATGAGCAACGTAACGTAGAA	100	FJ392644.1
	R	TGCCAGCAGGGCCTGTATAC		
pomca	F	GAAGAGGAATCCGCCGAAA	98	NM_181438.3
	R	CCAGTGGGTTTAAAGGCATCTC		
tyr	F	GGTGCCCTTCATCCCTCTCT	100	NM_131013.1
	R	AAACCGCTGACCTGGATCCT		

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TIFF images were acquired using an Olympus DP71 camera (Tokyo, Japan) attached to the microscope.

Dissection of zebrafish

Dissections were performed as previously described (Ivanovski *et al.* 2009). Briefly, adult zebrafish were anesthetized with 0.02% tricaine. Under the Olympus SZX16 (Tokyo, Japan), the superficial layers (outer epidermis and the underlying dermis) were carefully dissected out using two microforceps with 0.3-mm tips (World Precision Instruments, Sarasota, FL) and placed in RNA*later* (Life Technologies, Carlsbad, CA, USA). Muscles were dissected along the body. KMs, which are dark red–colored tissue with black dots and flanked by vertebral bones (Ivanovski *et al.* 2009), and brains were also dissected out and placed in RNA*later* (Life Technologies).

Real-time PCR

RNA extractions from skin, brain and KM were carried out using RiboPure™ kits (Life Technology), and mRNA was reverse-transcribed using High-Capacity RNA-to-cDNA kits (Life Technology). cDNA quality was evaluated by amplifying *bactin2*. Thirty thermal cycles were performed as follows: first denaturation at 95 °C for 10 s, annealing at 60 °C for 20 s and extension at 72 °C for 20 s. Gene expression levels were measured by StepOnePlus™ real-time PCR (Life Technology) using Fast SYBR® Green Master Mix. Primers were designed using PRIMER EXPRESS® software version 3.0 (Life Technology) and are shown in Table 1.

Histology of KM

Kidney marrows were fixed in 4% paraformaldehyde and 0.25% glutaraldehyde overnight. After three washes in PBS and once in 20% sucrose in PBS, the zebrafish were embedded into O.C.T. compound (SAKURA Tissue-Tek®; Torrance, CA, USA) and snap-frozen in liquid nitrogen before storage in −80 °C. For cryosections, the embedded zebrafish were cut cross-section into 10-μm slices and stained with Toluidine Blue O (Wako Pure Chemical Industries Ltd., Osaka, Japan). Stained cryosections were magnified at ×10, and the images were captured on an Olympus CKX41 microscope (Tokyo, Japan).

Histology of skin

Zebrafish were fixed in 0.1% paraformaldehyde and 4% glutaraldehyde overnight. After three washes in PBS, zebrafish were fixed in 2% OsO_4 at 4 °C for 2 h. Samples were dehydrated in 50%, 75%, 85% and 100% acetone for 10 min each. Dehydrated zebrafish were embedded in Technovit 8100 (Heraeus Kulzer GmbH, Hanau, German). The embedded zebrafish were cut cross-section into 3–4 μm by ultramicrotome (semi-thin) and stained with Toluidine Blue O (Wako Pure Chemical Industries Ltd.) or DAPI.

Electron microscopy

Scanning electron microscopy and TEM were performed as described by previously (Yahiro & Nagato 2005) with modification. For SEM, the embedded zebrafish were cut cross-section as semi-thin on Si-Chips. Ion etching was performed by hard mode 3 min × 2 times. Then, ion bombarder was performed by soft mode 3 min × 1 time. Tissues were coated with osmium (2.5 nm) and observed. For TEM, KMs were embedded in Epon 812 (Shell Chemical, San Francisco, CA, USA) and cut cross-section into 90 nm (ultrathin). Tissues were stained with uranyl acetate and lead and observed.

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Supporting Information/Supplementary material

The following Supporting Information can be found in the online version of the article:

Figure S1 Gross appearance of cold zebrafish before and after cold exposure.

Figure S2 Electron microscopic observation of zebrafish kidney marrow.

Figure S3 Electron microscopic observation of melanophore in skin of cold zebrafish.

Additional Supporting Information may be found in the online version of this article.

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