

Spot pattern of *leopard* *Danio* is caused by mutation in the zebrafish *connexin41.8* gene

Masakatsu Watanabe¹, Motoko Iwashita^{1,2}, Masaru Ishii³, Yoshihisa Kurachi³, Atsushi Kawakami^{1,4}, Shigeru Kondo^{2,5} & Norihiro Okada^{1,6*}

¹Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology, Nagatsuta-cho, Midori-ku, Yokohama, Japan,

²Center of Developmental Biology, Riken, Chuo-ku, Kobe, Japan, ³Graduate School of Medicine, Osaka University, Suita, Osaka,

Japan, ⁴Graduate School of Science, The University of Tokyo, Bunkyo-ku, Tokyo, Japan, ⁵Graduate School of Science, Nagoya

University, Furo-cho, Chikusa-ku, Nagoya, Japan, and ⁶National Institute for Basic Biology, Nishigonaka, Myodaiji, Okazaki, Japan

***Leopard*, a well-known zebrafish mutant that has a spotted skin pattern instead of stripes, is a model for the study of pigment patterning. To understand the mechanisms underlying stripe formation, as well as the spot variation observed in *leopard*, we sought to identify the gene responsible for this phenotype. Using positional cloning, we identified the *leopard* gene as an orthologue of the mammalian connexin 40 gene. A variety of different *leopard* alleles, such as *leo^{tl}*, *leo^{iq270}* and *leo^{tw28}*, show different skin-pattern phenotypes. In this manuscript we show that the mutation in allele *leo^{tl}* is a nonsense mutation, whereas alleles *leo^{iq270}* and *leo^{tw28}* contain the missense mutations I202F and I31F, respectively. Patch-clamp experiments of connexin hemichannels demonstrated that the I202F substitution in allele *leo^{iq270}* disrupted the channel function of connexin41.8. These results demonstrate that mutations in this gene lead to a variety of *leopard* spot patterns.**

Keywords: connexin; *leopard*; pattern formation; positional cloning; zebrafish

EMBO reports (2006) 7, 893–897. doi:10.1038/sj.embor.7400757

INTRODUCTION

Colour patterns such as eye spots, dots, mottle and cross stripes are visually prominent features of many organisms and can be

involved in camouflage, threatening behaviour and mate choice. Among a large number of pattern types, stripes and dots are the most well known and attractive. Zebrafish (*Danio rerio*)—a model organism for genetic and developmental studies—is known, as its name indicates, for its stripes, comprising black (melanophore), orange (xanthophore) and silver (iridophore) pigment cells. Consequently the zebrafish is also a model organism for colour pattern studies (Johnson *et al*, 1995a; Hirata *et al*, 2003, 2005; Kelsh, 2004). Several zebrafish genes involved in the development of pigments and/or pigment cells have been identified. For example, *c-kit* (*sparse*), *ednrb1* (*rose*), *c-fms* (*panther*), *mitf* (*nacre*) and *slc24a5* (*golden*) have been isolated from mutant fish and investigated for their roles in pigment cell development (Lister *et al*, 1999; Parichy *et al*, 1999, 2000a,b; Rawls & Johnson, 2000; Lamason *et al*, 2005). Although these studies have contributed to our understanding of the development and migration of individual pigment cells, how these cells form a pattern in the skin remains largely unclear. Although the reaction–diffusion model, originally proposed by Turing (Turing, 1952; Kondo & Asai, 1995; Asai *et al*, 1999; Kondo, 2002), provides an explanation for how pigment cells make striped patterns, little is known about the molecular basis of this theoretical model. Thus, analysis of zebrafish mutants that do not show any defect in pigment cell development but have altered pigment cell localization would contribute to our understanding of pattern formation.

Leopard is one of the most well-known zebrafish mutants; it was introduced in the well-known textbook *Developmental Biology* as a mutant awaiting identification of its corresponding gene (Gilbert, 2003). In this study, we show that the zebrafish *connexin41.8* gene (*Cx41.8*) is responsible for the *leopard* phenotypes. Connexin is a component of gap junctions (Kumar & Gilula, 1996). In most eukaryotic tissues and organs, gap junction channels allow direct intercellular transfer of ions, secondary messengers (such as cyclic AMP, ATP, inositol 1,4,5-trisphosphate) and small metabolites of up to ~1,500 Da (Saez *et al*, 2005). Gap junctions are an aggregate of intercellular channels, each of which is composed of two connexons.

¹Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology, 4259-B21, Nagatsuta-cho, Midori-ku, Yokohama 226-8501, Japan

²Center of Developmental Biology, Riken, 2-2-3 Minatojima-minamimachi, Chuo-ku, Kobe 650-0077, Japan

³Graduate School of Medicine, Osaka University, 2-2 Yamada-oka, Suita, Osaka 565-0871, Japan

⁴Graduate School of Science, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan

⁵Graduate School of Science, Nagoya University, Furo-cho, Chikusa-ku, Nagoya 464-8602, Japan

⁶National Institute for Basic Biology, Nishigonaka, Myodaiji, Okazaki 444-8585, Japan

*Corresponding author. Tel: +81 45 924 5742; Fax: +81 45 924 5835;

E-mail: nokada@bio.titech.ac.jp

Received 4 November 2005; revised 20 June 2006; accepted 20 June 2006; published online 14 July 2006

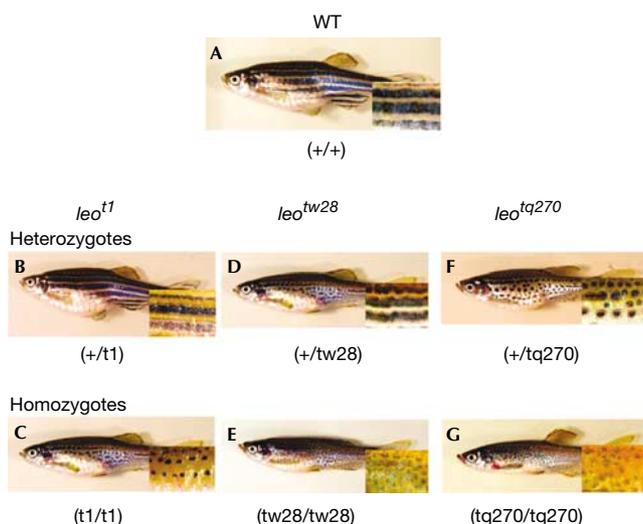


Fig 1 | Stripe patterns of zebrafish. (A) Wild-type (WT) zebrafish with normal horizontal stripes. (B,D,F) Zebrafish heterozygous for *leo*^{t1}, *leo*^{tw28} or *leo*^{tq270}. (C,E,G) Zebrafish homozygous for *leo*^{t1}, *leo*^{tw28} or *leo*^{tq270}. The inset boxes in each image show magnified images of stripes or spots on the skin of the fish.

Connexons are hexamers of connexin subunits, which are encoded by a multigene family, and more than 20 connexin genes have been identified in humans (Gerido & White, 2004). In zebrafish, almost 40 connexin genes were found through database searches (Eastman et al, 2006).

Here, we report that a variety of *leopard* allele phenotypes are generated with a change in connexin function, reflecting the effects of different mutations in *Cx41.8*.

RESULTS AND DISCUSSION

Zebrafish *leopard* phenotype

We examined three *leopard* alleles: *leo*^{t1}, *leo*^{tw28} and *leo*^{tq270}. Fig 1 illustrates the zebrafish phenotypes used in this study. Wild-type (WT) zebrafish (strain Tu) have several well-organized stripes consisting of melanophores and xanthophores (Fig 1A). The *leo*^{t1} heterozygote has a similar phenotype to the WT (Fig 1B), whereas the *leo*^{t1} homozygote shows a spot pattern consisting of well-organized melanophores (Fig 1C). The *leo*^{tw28} heterozygote shows a pattern of undulating stripes (Fig 1D), and the *leo*^{tw28} homozygote shows an interrupted stripe pattern (Fig 1E). In addition, there are fewer melanophores in the *leo*^{tw28} homozygote than in the *leo*^{tw28} heterozygote. The *leo*^{tq270} heterozygote shows a spot pattern similar to the *leo*^{t1} homozygote, whereas the *leo*^{tq270} homozygote has smaller spots (Fig 1F,G). These observations demonstrate that the *leo*^{tq270} mutant has a more pronounced phenotype than does the *leo*^{tw28} mutant.

Cx41.8 is the *leopard* gene

To understand the role of the *leopard* gene in stripe pattern development in zebrafish skin, we used positional cloning to identify the corresponding gene (supplementary information online). Meiotic mapping with chromosomal walking identified the critical region for *leopard* (Fig 2A). This region yielded two

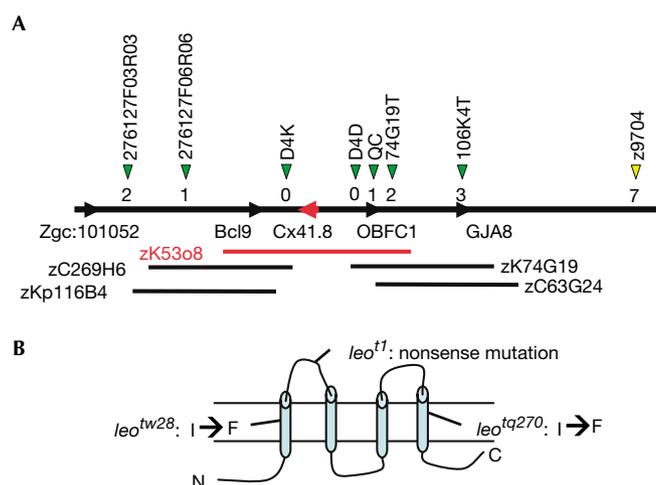


Fig 2 | Zebrafish *connexin41.8* as a *leopard* gene. (A) Map of the *leopard* region. The yellow arrowhead indicates the microsatellite marker z9704. The red bar indicates the bacterial artificial chromosome (BAC) clone zK53o8, which includes the critical region for *leopard*. The black bars show the BAC clones located around this region. Green arrowheads indicate single nucleotide polymorphism marker sites. Each site was named as shown above the arrowhead. The numbers below the green arrowheads refer to the number of recombination events (supplementary information online). This region includes two genes, *bcl9* and *Cx41.8*. *Cx41.8* is indicated by a red arrowhead. (B) Schematic of zebrafish *connexin41.8* showing predicted structural motifs and mutations detected in *leopard* alleles.

candidate genes, *bcl9*, the B-cell chronic lymphocytic leukaemia/lymphoma 9 gene, and *Cx41.8*, the *connexin41.8* gene. We sequenced the coding regions of both genes from two WT strains, AB and Tu, and the three *leopard* alleles. No mutation was detected in the *bcl9* gene when the deduced amino-acid sequences of the WT and *leopard* alleles were compared. However, mutations were identified in the *Cx41.8* sequence of each of the *leopard* mutant alleles *leo*^{t1}, *leo*^{tw28} and *leo*^{tq270}, as shown in Fig 2B and supplementary Fig S1 online. *leo*^{t1} carries a nonsense mutation in the first extracellular loop domain, which reduces the predicted molecular size of *Cx41.8* encoded by this allele from 42 to 8 kDa. Because the *leo*^{t1} heterozygote had the same phenotype as the WT fish, *leo*^{t1} represents a recessive allele caused by a null mutation of *Cx41.8*. A missense mutation, I31F, was identified in the allele *leo*^{tw28}, and another missense mutation, I202F, was identified in the allele *leo*^{tq270} (supplementary Fig S1 online). Because the I31F mutation lies in the first transmembrane domain of *Cx41.8*, and I202F lies in the fourth transmembrane domain, these substitutions were presumed to be critical mutations for connexin function. *leo*^{tw28} and *leo*^{tq270} heterozygotes have phenotypes different from that of WT; thus, they represent dominant-negative alleles.

To confirm that this critical region includes the *leopard* gene, we performed transgenic experiments. We injected the bacterial artificial chromosome (BAC) clone, zK53o8, into 500 fertilized eggs of homozygous *leo*^{t1} mutants, and observed that some transgenic mosaic fish showed melanophore spots that are larger than those of *leo*^{t1} homozygote fish (supplementary Fig S2 online).

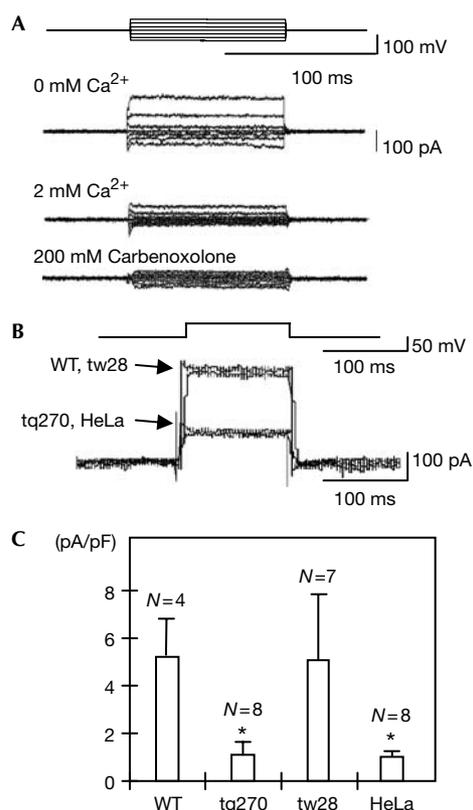


Fig 3 | Patch-clamp analysis of *Cx41.8* hemichannels expressed in HeLa cells. (A) Establishment of conditions for patch-clamp analysis with HeLa cells transfected with the wild-type (WT) *Cx41.8* cDNA. I_m responses recorded in solution with no added Ca^{2+} (upper), with 2 mM Ca^{2+} (middle) and with 200 μ M carbenoxolone (lower) in the bath solution. (B) Superimposed I_m traces from HeLa cells expressing WT, *leo^{tw28}* or *leo^{tq270}* connexin41.8, and untransfected HeLa cells (negative control). (C) Bar graph of WT ($N=4$), mutant *leo^{tw28}* ($N=7$) and mutant *leo^{tq270}* ($N=8$), compared with untransfected HeLa cells ($N=8$, negative control). Data represent the means \pm s.e.m. Each value was compared with that of WT. * $P<0.005$.

This observation is consistent with a transplantation experiment in which transplanted mosaic fish show a larger or irregular form of melanophore spots (Maderspacher & Nusslein-Volhard, 2003). Accordingly, this result indicates that this BAC clone actually contains a factor for melanophore development and/or stripe pattern formation (supplementary Fig S2 online).

Functional analysis of *Cx41.8*

To determine whether the mutations identified in the *Cx41.8* gene from the *leopard* mutants affect the ability of connexin to form functional channels, we performed patch-clamp analysis to record hemichannel currents. We first prepared HeLa cells transfected with either WT or mutant *Cx41.8* complementary DNA. After establishing the conditions for whole-cell recording, the membrane potential was clamped to +20 mV. To measure the current flowing through the connexin hemichannel, bipolar voltage pulses of 100 ms were delivered to alter the V_m in 20 mV increments from -80 to +40 mV (Fig 3A). Hemichannels composed of connexins

are known to open on removal of extracellular Ca^{2+} . Concordantly, the presence of 2 mM extracellular Ca^{2+} elicited a very small current, whereas depolarization produced activating currents in the absence of Ca^{2+} . The hemichannel blocker carbenoxolone also blocked the hemichannel ability (Fig 3A). Fig 3B represents a superimposed I_m trace of HeLa cells transfected with the WT, *leo^{tw28}* or *leo^{tq270}* connexin41.8 gene, or of untransfected HeLa cells (negative control). After establishing conditions for whole-cell recording, V_m was clamped to -40 mV. Voltage pulses of 100 ms were then delivered to depolarize V_m up to +15 mV. Currents were recorded in the absence of Ca^{2+} . Fig 3C summarizes the patch-clamp data for the WT, *leo^{tw28}* and *leo^{tq270}* connexin hemichannels compared with untransfected HeLa cells. Interestingly, *connexin41.8* from *leo^{tq270}* had the same low-level basal current as untransfected HeLa cells, indicating that connexin hemichannel function was disrupted by the *leo^{tq270}* mutation. This result led us to conclude that the I202F substitution has a crucial effect on the ability of connexin to conduct ions and that the phenotypes of zebrafish skin pattern might depend on connexin channel function. Conversely, the trace from zebrafish allele *leo^{tw28}* could not be distinguished from that of WT, indicating that the I31F substitution does not have a substantial effect on ion channel function. Small differences in current might not be detected using this assay and/or this mutation might only manifest its effect after the formation of intercellular channels (Castro et al, 1999; Bao et al, 2004). Further analysis might demonstrate whether the mutation I31F actually affects the function of connexin gene. Therefore, we concluded that the *leopard* allele with the most severe spot phenotype, *leo^{tq270}*, showed the most pronounced disruption in channel function.

The dominant-negative phenotypes of *leo^{tw28}* and *leo^{tq270}* could be explained by the generation of heterohexamers of connexin to form the gap junctions, although we did not obtain functional data about *leo^{tw28}*. Homotypic heterohexamers can be generated from mutated and normal *Cx41.8*, and heterotypic heterohexamers can be formed from mutated *Cx41.8* and another zebrafish connexin, such as *Cx43* or *Cx45.6* (Christie et al, 2004). It is likely that the phenotype of the heterozygous *leo^{tw28}* mutant is due to formation of homotypic heterohexamers that consequently cause a wavy striped pattern in zebrafish. The *leo^{tq270}*-encoded protein showed disrupted channel function, predicting that the skin pattern phenotype of the *leo^{tq270}* heterozygote would be the same as that of the null mutant *leo^{t1}* homozygote. In fact, the dominant-negative *leo^{tq270}* homozygote has a more severe phenotype than the null mutant *leo^{t1}* homozygote. This more severe phenotype of *leo^{tq270}* homozygotes might be explained by the formation of heterotypic heterohexamers composed of *leo^{tq270}* connexin41.8 and other connexins, such as *Cx43* or *Cx45.6*, that form irregular connexons in the homozygous *leo^{tq270}* mutant. Another report supports this hypothesis: connexin 26 and connexin 32 can form a heterohexamer connexon that has different signalling molecule selectivity compared with connexin 26 or connexin 32 homohexamer connexons (Bevans et al, 1998). In addition, a mouse gap junction consisting of WT connexin 43 and a dominant-negative mutant connexin 43 has a smaller channel pore size than normal gap junctions, thereby allowing the transfer of relatively smaller molecules (Upham et al, 2003). This hypothesis of heterohexamer formation further suggests that other connexin genes contribute to stripe pattern formation.

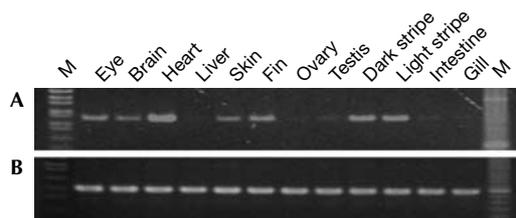


Fig 4 | *Cx41.8* expression in adult tissues. Reverse transcription-PCR was performed with RNA samples isolated from the indicated adult fish tissues. M indicates molecular size marker. Total RNAs were reverse-transcribed with oligo-d(T)₁₈ primer, and the resultant complementary DNAs were amplified by PCR using specific primers for (A) *Cx41.8* and (B) β -actin.

Expression of the *Cx41.8* gene

Because gene expression levels of connexins are generally too small to detect using *in situ* hybridization, we performed reverse transcription-PCR (RT-PCR) analysis to determine the tissue specificity of *Cx41.8* expression (supplementary information online). RT-PCR was carried out using cDNA generated from adult eye, brain, heart, liver, skin, fin, ovary, testis, intestine and gill. The fin was divided into the dark portion (melanophore) and light portion (light portion) of each stripe, and each fraction was subjected to RT-PCR. *Cx41.8* expression was strongly detected in the eye, brain, heart, skin and fin. The gene was also expressed in melanophore and xanthophore portions of the fin (Fig 4). To confirm these results further, and to exclude the possibility of cross contamination of the pigment cells in each fraction, an alternative approach was undertaken as described in the supplementary text and supplementary Fig S3 online. These data indicate that the gene is expressed in melanophores and that expression in xanthophores is also likely.

Speculation on connexin functions in pattern formation

It is possible that the change in skin pattern can be explained by the loss of cell-cell interactions owing to the gap-junction defect of *connexin41.8*. Maderspacher & Nusslein-Volhard (2003) provided an important observation by transplantation experiments that showed that the spots became larger in transplanted fish, suggesting that the *leopard* gene might have a crucial role in cell-cell interactions of melanophore-melanophore, xanthophore-xanthophore and melanophore-xanthophore during the process of stripe pattern formation. This observation is consistent with our results, because gap junction proteins are responsible for the formation of cell-cell interactions. Conversely, Hirata *et al* (2003, 2005) showed that there are layer structures of pigment cells, such as xanthophores/iridophores/melanophores, on zebrafish skin and this structure might prevent direct interaction between melanophores and xanthophores. These observations indicate that the *leopard* gene might also have an indirect effect on cell-to-cell interactions between melanophores and xanthophores. Indirect effects might be explained by the function of the connexin hemichannel. Pearson *et al* (2005) proposed a model in which ATP release and its diffusion from chick embryonic retinal pigment epithelium through connexin 43 hemichannels activates mitosis of the progenitor cells in the adjacent neural retina. In this model, connexin hemichannels are important for differentiation of

neighbouring cells. Although there is no evidence that a similar ATP signalling pathway through connexin hemichannels contributes to melanophore differentiation in zebrafish, defects in the function of mutant *Cx41.8* hemichannels could explain the observation that zebrafish *leopard* mutants have a reduced number of melanophores (Fig 1; Johnson *et al*, 1995a).

Recently, we identified a *jaguar/obelix* gene as a potassium channel (Iwashita M, Watanabe M, Ishii M, Chen T, Johnson SL, Kurachi Y, Okada N & Kondo S, unpublished data). Mutations in this gene alter the localization of pigment cells, causing broadened stripe patterns. Although the functions of the *leopard* and *jaguar/obelix* genes in pigment cell localization in zebrafish skin remain unknown, it is a novel and fascinating finding that channels that form gap junctions and ion channels contribute to pigment cell localization. This leads to further questions on how small molecules such as ATP and ions contribute to the positioning of pigment cells on the skin to yield a specific pattern.

METHODS

Positional cloning. *leo^{fl}* homozygous fish were crossed with WT strains Tu, AB, and India to produce a reference mapping cross. Heterozygous F₁ were intercrossed, and separate pools of F₂ homozygous *leo^{fl}* fish (1,738 individuals) were used for sequence length polymorphism and single nucleotide polymorphism analyses. Because the linkage number in which the *leopard* gene was mapped was previously predicted to be linkage group 1 (LG1; Johnson *et al*, 1995b), we started screening using LG1 polymorphism markers that were obtained from the Z-FIN database. (See the supplementary information online for the positional cloning strategy for the *leopard* gene.)

HeLa cell transfection. Patch-clamp experiments were performed with HeLa cell clones that were transfected with cDNA constructs containing the coding sequence of WT or mutant *Cx41.8*. HeLa cells were transfected with 5 μ g of recombinant *Cx41.8*-pcDNA plasmid using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The cells were seeded onto sterile glass coverslips placed in 12-well dishes and used 24–48 h after plating (see the supplementary information online for further details).

Hemichannel current measurement. Experiments were carried out on single cells attached to a pipette. The channel current was recorded using a patch-clamp amplifier, Axon200B (Axon Instrument, CA, USA), and analysed with a commercially available software, pCLAMP9 (Axon Instrument). Results are shown as mean values, and error bars represent \pm s.e.m. (see the supplementary information online for further details).

Supplementary information is available at *EMBO reports* online (<http://www.emboreports.org>).

ACKNOWLEDGEMENTS

This work was supported by a Grant-in-Aid for Scientific Research on Priority Areas ‘Molecular Mechanism of Speciation’ and ‘Comparative Genomics’ from the Ministry of Education, Culture, Sports, Science and Technology of Japan to N.O.

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