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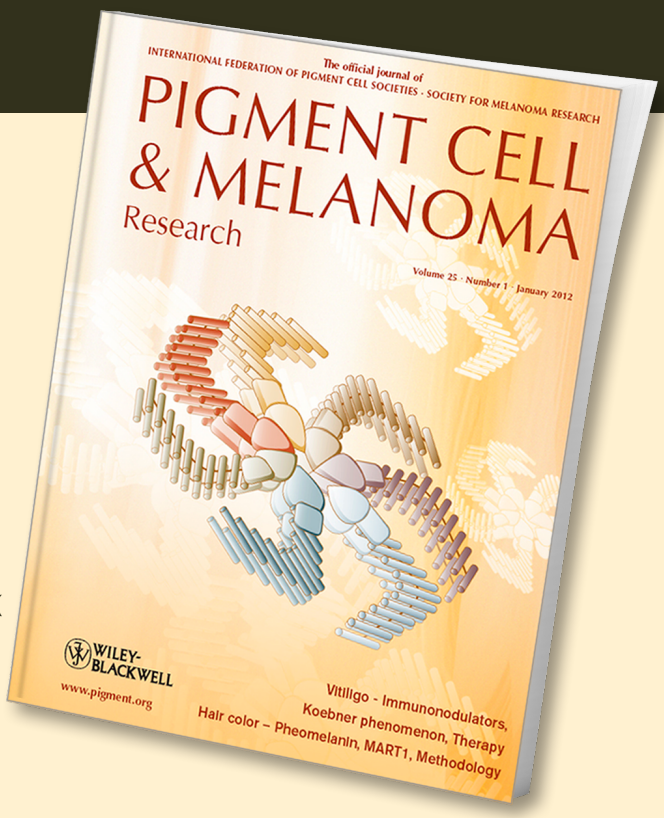

Changing clothes easily: *connexin41.8* regulates skin pattern variation

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Summary

The skin patterns of animals are very important for their survival, yet the mechanisms involved in skin pattern formation remain unresolved. Turing's reaction–diffusion model presents a well-known mathematical explanation of how animal skin patterns are formed, and this model can predict various animal patterns that are observed in nature. In this study, we used transgenic zebrafish to generate various artificial skin patterns including a narrow stripe with a wide interstripe, a narrow stripe with a narrow interstripe, a labyrinth, and a 'leopard' pattern (or donut-like ring pattern). In this process, *connexin41.8* (or its mutant form) was ectopically expressed using the *mitfa* promoter. Specifically, the leopard pattern was generated as predicted by Turing's model. Our results demonstrate that the pigment cells in animal skin have the potential and plasticity to establish various patterns and that the reaction–diffusion principle can predict skin patterns of animals.

Skin pattern is an important feature when measuring an animal's likelihood for survival and mating, and it is also an important marker for the evolutionary adaptation by a species to its environment. The fact that even closely related animals show extremely divergent patterns suggests that animal skin has a plasticity that can lead to great variability. Such variability can be described mathematically by Turing's reaction–diffusion (RD) model, which has been frequently cited to explain the diversity and robustness of naturally occurring patterns (Kondo and Asai, 1995; Kondo and Miura, 2010; Meinhardt, 1982; Turing, 1952). This diversity is achieved by changing the parameters in a series of partial differential equations, as demonstrated by the re-creation of naturally arising endophenotypic patterns (Miyazawa et al., 2010).

To understand the roles that development and genetics play in skin pattern formation, zebrafish is the preferred model organism. Previous studies have revealed the genetic factors related to the development and differentiation of pigment cells (Kelsh et al., 2009; Parichy et al., 1999; Rawls and Johnson, 2001), and interactions among these pigment cells have been proposed to be sufficient for skin pattern formation (Nakamasu et al., 2009; Takahashi and Kondo, 2008). Indeed, laser ablation experiments successfully reconstructed the RD phenomenon on zebrafish skin (Yamaguchi et al., 2007). However, the molecular factors that regulate these interactions remain largely unknown. One candidate is Connexin41.8 encoded by *cx41.8*. This gene is responsible for the skin pattern of *leopard* zebrafish, which has

Significance

In nature, we see various animal skin patterns such as stripes, spots, waves, donut rings, and tiles. Researchers are studying how the spatial patterning is determined. Theoretical explanations for how such patterns arise have been proposed, but the molecular mechanisms remain unsolved. In this report, we generated various skin patterns artificially on zebrafish skin using the *connexin41.8* gene and showed that *connexin41.8* functions to impart variation to animal skin patterns.

a spot pattern distinct from the stripe pattern typically seen in wild-type (WT) zebrafish (Figure S1) (Madersp-acher and Nusslein-Volhard, 2003; Watanabe et al., 2006). In fact, different *cx41.8* mutants show a variety of stripe–spot patterns (Figure S1). Furthermore the importance of Cx41.8 for pattern formation is supported by its function as one of the components of gap junctions, which connect neighboring cells and mediate cell–cell interactions (Kumar and Gilula, 1996). The interactions between two hypothetical factors are consistent with both the RD model and experimental observations (Kondo and Miura, 2010). In this study, we applied transgenic techniques to test whether the RD model could predict how *cx41.8* is related to skin patterning.

The 4.5-kb fragment upstream of exon 1 in *cx41.8* was cloned into a pTol2 plasmid (Figure 1A) (Kawakami, 2005), resulting in a pTol2-cx41.8 promoter vector. Transgenic experiments using the pTol2-cx41.8pro:cx41.8 plasmid revealed that the 4.5-kb fragment can act as a promoter for *cx41.8* and results in a stripe pattern in *leopard* zebrafish (Figure 1A–D), confirming that this system can be applied to pigment cells in adult zebrafish. Next, we replaced the *cx41.8* fragment in the pTol2-cx41.8pro:cx41.8 plasmid with an EGFP cassette and used EGFP expression as a reporter for *cx41.8*, finding EGFP signals in melanophores and xanthophores (Figure 1E–L; see legend). We next compared the EGFP expression driven by the *cx41.8* promoter (Figure 1M) with that of DsRed driven by the *mitfa* promoter (Figure 1N) (Lister et al., 1999) in young zebrafish (11 mmSL; Parichy et al., 2009). *mitfa* is a master control gene for the development and pigmentation of melanophores and is expressed in melanoblasts and melanophores. We named the *cx41.8* promoter as cx41.8pro and *mitfa* promoter as mitfapro here. We found that EGFP signals were expressed in the same cells as DsRed signals, suggesting that Cx41.8 may have a role in melanophore development and/or differ-

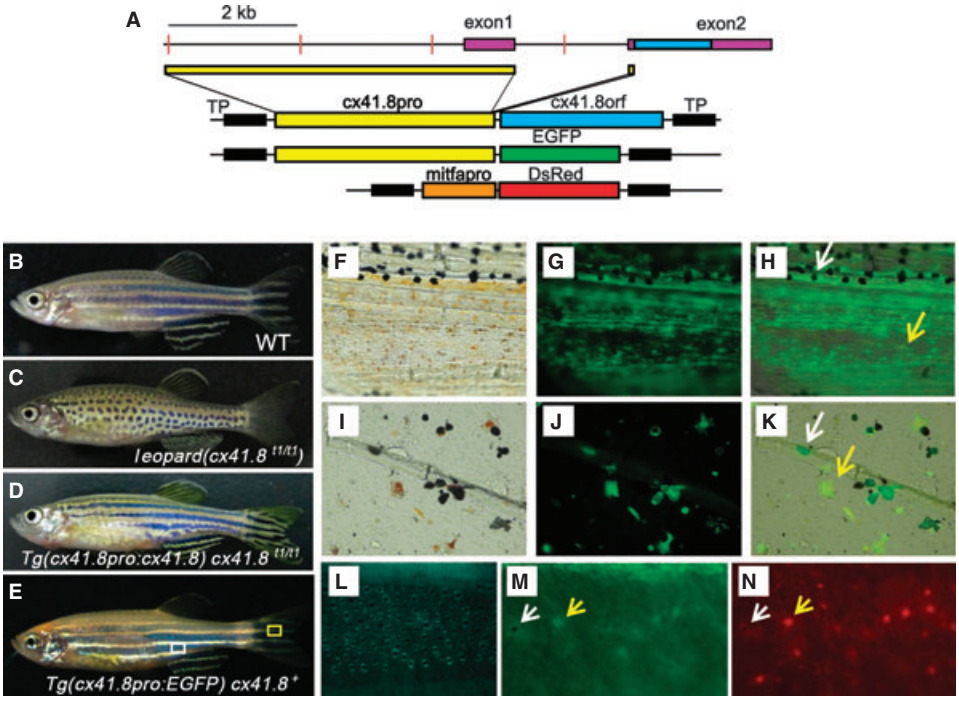

Figure 1. Expression of *cx41.8*. Design of plasmids for the transgenic experiments. (A) Upper line indicates the genome structure around the zebrafish *cx41.8* locus. Lower lines indicate the pTol2 regions of the plasmid constructs: (top to bottom) pTol2-cx41.8pro:cx41.8, pTol2-cx41.8pro:EGFP, and pTol2-mitfapro:DsRed. Purple boxes indicate the *cx41.8* exons; blue boxes, open reading frame; yellow boxes, the upstream region of *cx41.8*, which includes the *cx41.8* 5′ untranslated region; black boxes, transposon sequences (TP); and green and red boxes, the EGFP and DsRed cassettes, respectively. 'cx41.8pro' indicates the *cx41.8* promoter, and 'mitfapro' indicates the *mitfa* promoter. Panels B–E show skin patterns of WT zebrafish (B), $cx41.8^{t1}$ allele (C), *leopard* zebrafish rescued with pTol2-cx41.8pro:cx41.8 (D; $cx41.8^{t1/t1}$ background, F1 generation), and transgenic fish with pTol2-cx41.8pro:EGFP (E; WT background, F1 generation). EGFP signals were detected in melanophores and xanthophores of untreated caudal fin (F–H; magnified image of the yellow boxed region in E) and trypsin-treated caudal fin (I–K). F, I: color images of the melanophores and xanthophores; G, J: EGFP signals derived from the *cx41.8* promoter; H, K: overlaid images of F and G and of I and J, respectively. White arrows and yellow arrows in H and K indicate EGFP signals in melanophores and xanthophores, respectively. EGFP signals were also detected in the trunk region of adult zebrafish (L), a region that is indicated by the white box in E. (M and N). *EGFP* expression (M) and *DsRed* expression (N) driven by the cx41.8pro promoter and mitfapro promoter, respectively, were compared at the trunk region in young zebrafish (11 mmSL).

entiation and that the *mitfa* promoter can be used to ectopically express *cx41.8* in melanophores of adult zebrafish. Recent studies have revealed that *mitfa* expression also can be detected in precursor cells for iridophores and xanthophores in zebrafish embryos (Curran et al., 2011), indicating the possibility that *cx41.8* activated by the *mitfa* promoter was also expressed in these precursor cells (see below). On the other hand, the zebrafish skin pattern is changeable even after the adult phenotype is fixed (Yamaguchi et al., 2007). Furthermore, DsRed expression driven by the *mitfa* promoter was detected only in melanophores (not in xanthophores) in adult fin tissue (Figure S2). These observations prompted us to consider the *mitfa* promoter as a melanophore-specific promoter for our study of adult phenotypes, although further analysis in embryos might be required to determine the Cx41.8 function in the precursor cells.

Next, *cx41.8* was expressed in melanophores using the *mitfa* promoter in several genetic backgrounds. The transgene causes presumably mixed expression of the endogenous protein and transgenic protein in melanophores, whereas the xanthophores express only the endogenous protein. The observed differences in the patterns obtained imply that this transgenic expression results in heteromeric gap junctions having modified properties. Our transgenic approach yielded several skin patterns (Figure 2), which we compared with those generated in simulations (Figures 2, 3, and S3). For example, the skin pattern of WT zebrafish resembled the stripe pattern seen in Chapman's zebra (Figures 2A and S3A), whereas the spot pattern in *leopard* zebrafish resembled that seen in cheetah (Figs 2B and S3B). The transgene *mitfapro:cx41.8* expressed in a *cx41.8*$^{t1/t1}$ background generated a bongo pattern consisting of broad stripes interlaced with narrow ones (Figures 2C and S3C), whereas a labyrinth pattern was generated when the mutant Cx41.8M7, in which six amino acid residues were deleted at the N terminus of Cx41.8, was used in the WT background (Figures 2D, S3D, and S4, Cx41.8M7). Furthermore, an insertion of three residues at the N terminus of Cx41.8 (Figure S4, MKLCx41.8) changed the skin pattern in WT zebrafish, as the black stripes of the melanophores were gradually cleaved and replaced with xanthophores during growth, resulting in a narrow-stripe pattern (Figure 2E). This phenomenon is easily constructed in the two-stage Turing model described by Liu et al. (2006). Initially, parameters were set to produce the WT stripe pattern (Figure 3A, left column). D_u and D_v were then changed

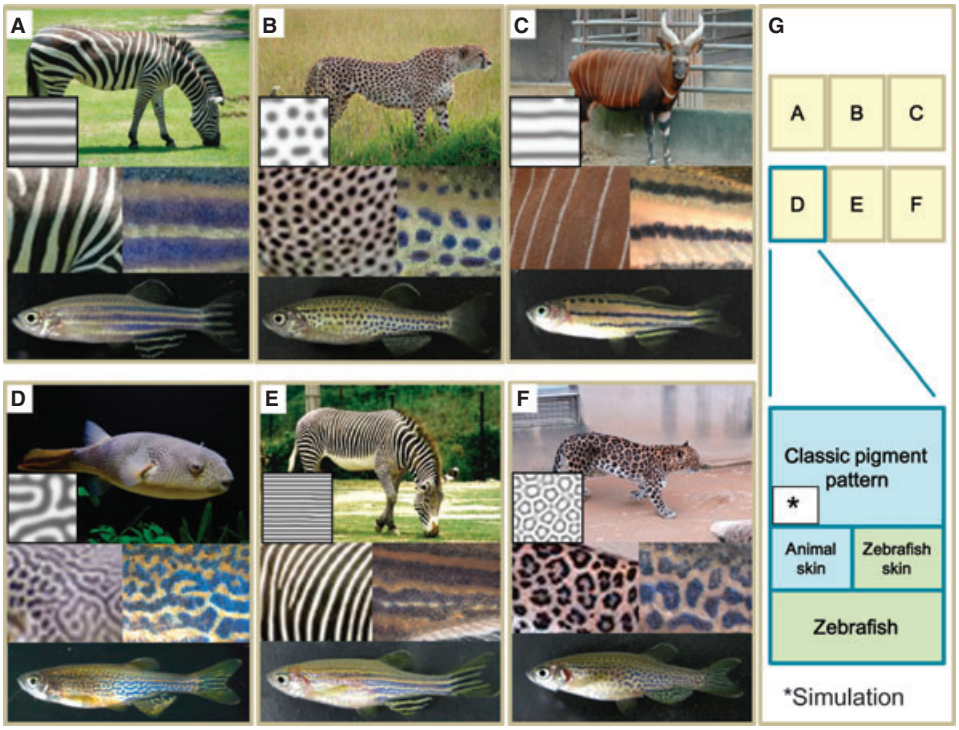

Figure 2. Natural skin patterns observed in zebrafish. Animals showing classic pigment patterns: (A) Chapman's zebra (*Equus quagga chapmani*), (B) cheetah (*Acinonyx jubatus*), (C) bongo (*Tragelaphus eurycerus eurycerus*), (D) puffer fish (*Tetraodon mbu*), (E) Grevy's zebra (*Equus grevyi*), and (F) leopard (*Panthera pardus*). Zebrafish: (A) WT (*cx41.8*$^{+}$), (B) *leopard* zebrafish (*cx41.8*$^{t1/t1}$), (C) *Tg(mitfapro:cx41.8)cx41.8*$^{t1/t1}$, (D) *Tg(mitfapro:M7cx41.8)cx41.8*$^{+}$, (E) *Tg(mitfapro:MKLcx41.8)cx41.8*$^{+}$, and (F) *Tg(mitfapro:MKLcx41.8)cx41.8*$^{tq270/+}$. Simulation conditions are described in Data S1 and Fig. S3.

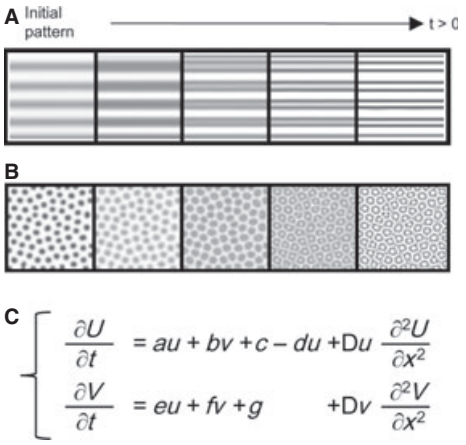

Figure 3. Skin patterns predicted by the RD model. (A) Cleaved-stripe and (B) ring patterns generated by the RD model, where u and v are concentrations of hypothetical factors, au + bv + c and eu + fv + g are the respective reaction kinetics, and D_u and D_v are the respective hypothetical diffusion coefficients for u and v (C). Parameters were as follows: a = 0.08, b = −0.08, d = −0.03, e = 0.1, f = −0.06, g = −0.12, D_u = 0.02, D_v = 0.5, 0 ≤ au + bv + x ≤ synUmax = 0.2, and 0 ≤ eu + fv + g ≤ synVmax = 0.5. Finally, c = 0.16 for (A) and 0.02 for (B). The starting pattern is one thin line (A) or random dots (B). After the initial patterns were fixed (left columns in A and B), D_u and D_v were changed to 0.002 and 0.05, respectively, in both simulations.

such that cleaved stripes appeared (Figure 3A). Similarly, a donut ring pattern could be theoretically generated if the initial conditions led to the spot pattern (Figure 3B). To confirm this donut phenomenon experimentally, we inserted the *MKLcx41.8* transgene into the *cx41.8*$^{tq270/+}$ background because this mutant fish had the largest spot pattern among the fish with *leopard* alleles (Figure S1). The resulting ring pattern resembled those seen on leopard (Figure 2F). We therefore named this pattern the 'leopard pattern'.

As observed in transgenic zebrafish shown in Figures 1 and 2, during pattern formation gap junctions were predicted to activate melanophore production and repress xanthophore production because without *cx41.8*, the number of melanophores decreased and the number of xanthophores increased. In addition, the number of melanophores increased when the transgene *mitfapro:cx41.8* was expressed only in melanophores whereas the number of xanthophores did not change (Figure 2C). In this study, some of the simulations were carried out considering the known function of *cx41.8* in pigment cells. Namely, for the spot and bongo patterns, parameter c (concentration of a hypothetical component) was changed, which may affect the number or ratio of pigment cells (Figure S3). Furthermore, the simulated cleaved-stripe pattern (Figure 2E, F) was obtained by changing the diffusion constants D_u and D_v after the initial patterns were produced, which was consistent with the cleaved-stripe pattern observed during the growth period of transgenic fish. We previously reported that xanthophores enhance melanophore survival and that melanophores indeed cannot survive in the absence of xanthophores (Nakamasu et al., 2009). These observations led us to hypothesize that melanophores tend to maintain the stripe width because these cells cannot survive at a distance from xanthophores. Furthermore, a modified N-terminal domain in MKLCx41.8 might affect the diffusion rate of small molecules between pigment cells, which may be related to melanophore survival and cause stripe cleavage. On the other hand, it was unclear how and why the type of pattern changed when the N-terminal domain encoded by *cx41.8* was deleted (Figures 2D and S3D). To address this issue, we next should investigate the impact of the initial conditions in vivo, such as precursor cell arrangement.

Our study reveals that unique skin patterns can be generated and that animal skin patterning is indeed a plastic phenomenon. Interestingly, the skin patterns obtained were generated by manipulating the expression (and therefore function) of a single gene, namely *cx41.8*. Gap junctions constitute bidirectional channels that directly connect neighboring cells. Recent studies have revealed that many kinds of small molecules of less than 1000 Da can be transferred through gap junctions. However, it is still difficult to identify a molecule(s) that is specific to a particular cellular phenomenon because of the unselective properties of the gap-junction channel. Our study suggests that the precise regulation of the N-terminal domain of connexin, which works as a plug for the channel (Maeda et al., 2009; Oshima et al., 2007), is required for gap junction function. Interestingly, different types of mutant connexins yield different zebrafish phenotypes (Figures 2D, E), which suggests that gap junctions tune cell–cell interactions for pattern making and that slight functional changes in the junctions are related to pattern diversity. We recently found that not only *cx41.8* but also other connexins such *cx44.1*, *cx45.6*, and *cx48.5* also rescued the *leopard* phenotype whereas *cx43* did not. In addition, rat *cx40*, an ortholog of zebrafish *cx41.8*, also rescued the *leopard* phenotype (M. Watanabe, D. Watanabe and S. Kondo, unpublished data); this information may prove useful in future studies of *connexin* gene function in skin pattern formation.

Acknowledgements

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Supporting information

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

Figure S1. Leopard alleles. (A) WT fish, (B) *leopard* fish, (C) *cx41.8^{tw28}* heterozygous allele, (D) *cx41.8^{tq270}* heterozygous allele, (E) *cx41.8^{tw28}* homozygous allele, and (F) *cx41.8^{tq270}* homozygous allele. *cx41.8^{t1}* has a nonsense mutation in the first extracellular loop domain; *cx41.8^{tw28}*, an I31F mutation in transmembrane-1 (TM-1), and *cx41.8^{tq270}*, an I203F mutation in TM-4 of Cx41.8. The phenotype of *cx41.8$^{t1/+}$* is the same as that of WT fish.

Figure S2. DsRed signals driven by the *mitfa* promoter were detected only in melanophores of adult fish fins. DsRed expression driven by the *mitfa* promoter was examined in *Tg(mitfapro:DsRed)* in the F1 generation. (A) Intrinsic fluorescence of xanthophores (green); (B) melanophores expressed DsRed signals driven by the *mitfa* promoter; (C) bright field image; and (D) merged image of A–C.

Figure S3. RD simulations. (A) Stripe, (B) spot, (C) stripe with wide interstripe, and (D) labyrinth. Simulation conditions are given in the supplementary text.

Figure S4. Alignment of N-terminal region of Cx41.8. Cx41.8M7 has its initial codon at the seventh position of WT Cx41.8, which was generated by deleting the second through seventh amino acid residues (ADWSLL). MKLCx41.8 has an extra three residues at the N-terminal end.

Data S1. Materials and Methods.

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