Polyamine sensitivity of gap junctions is required for skin pattern formation in zebrafish

Masakatsu Watanabe, Daisuke Watanabe & Shigeru Kondo

Graduate School of Frontier Biosciences, Osaka University 1–3 Yamadaoka, Suita, Osaka 565-0871, Japan.

Gap junctions allow the direct and bidirectional transfer of small molecules between cells. Polyamine sensitivity, which has been observed for a certain gap junction in vitro, confers rectification property to gap junctions. Here we report that the polyamine sensitivity of gap junctions in vivo is crucial for skin pattern formation in zebrafish. Transgenic experiments have revealed that several connexin genes were able to rescue the spot phenotype of mutant zebrafish. Mutational analyses of the N-terminal region of connexins revealed that the ExxxE motif, a hypothetical polyamine-binding site, was important for connexin's role in pattern formation. Ectopic expression of spermidine/spermine N1-acetyltransferase (SSAT), a polyamine metabolic enzyme, also caused stripe pattern changes, which further indicates that the polyamine sensitivity of gap junctions is crucial. This is the first report to show that polyamine sensitivity has a physiologically relevant function and is related to skin pattern formation in animals.
cx41.8\textsuperscript{20–29} have missense mutations in the transmembrane regions of cx41.8. cx41.8 is expressed in both melanophores and xanthophores and functions to positively regulate the number of melanophores and negatively regulate the number of xanthophores\textsuperscript{20,27,29}.

In this study, we generated a series of transgenic zebrafish to identify the common features of various connexins that are required for zebrafish skin pattern formation. We found that polyamine sensitivity of gap junctions, which might induce rectification properties in gap junctions, is crucial for pattern formation in zebrafish.

**Results**

**Effect of connexin genes on pattern formation.** Using the Tol2 transposon system\textsuperscript{30,31}, a connexin gene was integrated into leopard zebrafish as described\textsuperscript{29}; the connexin gene was expressed under the control of the cx41.8 promoter (cx41.8pro) or the microphthalmia-associated transcription factor a (mitfa) promoter (mitfapro) (Fig. 1a). cx41.8pro regulates gene expression in both melanophores and xanthophores, whereas mitfapro controls expression only in melanophores of adult fish\textsuperscript{29}. We used the cx41.8\textsuperscript{t1} allele for the transgenic experiments here because this allele is a functional null allele; thus, endogenous cx41.8 would not influence the function of the transgenes. The phenotypes of the transgenic zebrafish were examined in the resulting F1 generations. Tg(cx41.8pro:cx41.8) fish were almost completely rescued by the transgene (Fig. 1d). When the promoter was changed to mitfapro, the resulting pattern was changed slightly with narrower stripes and wider inter-stripe distances (Fig. 1f). The difference in phenotypes between Tg(cx41.8pro:cx41.8) and Tg(mitfapro:cx41.8) fish was most likely caused by differences in gene expression in xanthophores; presumably, expression of cx41.8 in xanthophores decreased the number of xanthophores (see ref. 29 and the Discussion).

We assessed the effect of other connexin genes on pattern formation based on the phenotypic change from spots (Fig. 1c) to stripes (Fig. 1d and f). The promoter sequence used in each transgenic experiment was chosen based on the available restriction enzyme sites in each gene. Based on these experiments, we concluded that cx41.8 (Fig. 1d and f), cx45.6 (Fig. 1e), cx44.1 (Fig. 1g) and cx48.5 (Fig. 1h) rescued the leopard phenotype, whereas cx43 (Fig. 1i) failed to do so. We also generated transgenic zebrafish using cx27.5, cx32.2 and cx33.8 and found that these genes did not rescue the leopard phenotype (Fig. 2b right). Fig. 2a illustrates the structure of connexin, and the phylogenetic tree in Fig. 2b represents relationships among connexin genes in zebrafish\textsuperscript{32}. The connexin genes that were able to rescue the leopard phenotype were clustered (Fig. 2b; Cx48.5–Cx50.5). Here we have referred to this cluster as the "Cx41.8 cluster".

**Domain replacement and modification experiments.** Because cx43 did not rescue the leopard phenotype in the transgenic experiment, we next examined the differences between cx41.8, which was capable...
of rescue, and cx43. The N-terminal domain and C-terminal domain in Cx41.8 were each replaced with those of Cx43 (Fig. 2c). In parallel, we modified the cytoplasmic loop domain of Cx41.8 by inserting an extra 20 amino acid residues consisting of a 23Myc tag sequence (Cx41.8IMM). We then examined the effect of each construct on pattern formation. We found that Cx41.8C43 and Cx41.8IMM rescued the leopard phenotype, but N43Cx41.8 did not, indicating that the N-terminal domain of Cx41.8 is important for skin pattern formation in zebrafish.

The ExxxE motif is the determinant of the Cx41.8 cluster. We then compared the amino acid sequences of the N-terminal domains of the zebrafish connexins (Fig. 2b). We found that the ExxxE motif in this region was highly conserved in the Cx41.8 cluster. In addition, we also examined the rescuing activity of the rat connexins rat-CX40 and rat-CX43, which are orthologs for zebrafish Cx41.8 and Cx43, respectively, and found that rat-CX40 also rescued the leopard phenotype, although rat-CX43 failed to do so (Fig. 2d, e). These results indicate that the functional similarities of connexins are well conserved between rat and zebrafish. We note that rat-CX40 has polyamine sensitivity, and the E9 and E13 residues in the N-terminal domain of rat-CX40 are predicted to be the residues that are sensitive to polyamine 9,10. We thus substituted K9 and K13 for E9 and E13, respectively, in rat-CX40, which destroys the spermine

Figure 2 | Summary of rescue experiments. (a) Connexin structure (see also the Introduction). The insertion site in Cx41.8IMM is indicated. (b) Phylogenetic tree of zebrafish connexins and comparison of their N-terminal domains. (c) Schematic diagrams of connexin chimeras and modified connexin. Cx41.8, wild-type zebrafish Cx41.8; N43Cx41.8, N-terminal domain of Cx41.8 was replaced with that of Cx43; Cx41.8C43, C-terminal domain of Cx41.8 was replaced with that of Cx43; Cx41.8IMM, 20 amino acid (aa) residues (23Myc tag sequences) were inserted into the cytoplasmic loop domain of Cx41.8 (black box). Red and pink boxes indicate amino acid sequences from Cx41.8. Pink boxes indicate the transmembrane regions. Blue boxes indicate amino acid sequences from Cx43. (d) N-terminal sequences of rat and zebrafish connexins from the Cx41.8 cluster that were analysed for rescue of the leopard phenotype. (e) A comparison of N-terminal sequences from mutant connexins and wild-type connexins. N43Cx41.8 is the Cx41.8 mutant with the N-terminal region of Cx43; Cx43SEEH is the Cx43 mutant with 4 aa substitutions; rat-CX40* is the rat-CX40 mutant in which the conserved E9 and E13 residues have been substituted with lysine residues. The results from rescue experiments are shown to the right of each panel (b–e, +, able to rescue the leopard phenotype; –, unable to rescue the leopard phenotype). Rat connexins are underlined (d, e). Red characters indicate acidic residues, and blue characters indicate basic residues (b, d and e).
sensitivity of rat-CX40 but barely affects channel function. As expected, our results clearly showed that the rat-CX40E9,13K mutant did not rescue the leopard phenotype (Fig. 2e; ratCX40*), indicating that the spermine sensitivity of this gap junction is important for pattern formation in zebrafish. Furthermore, we made a mutant Cx43, Cx43SEEHH, which mimics the N-terminal sequence of Cx41.8 by introducing the amino acid substitutions R9S, K13E, A16E and Y17H into Cx43 (Fig. 2e). We found that these amino acid substitutions were not sufficient to produce Cx41.8-like function in this version of Cx43 because the Cx43SEEH mutant did not rescue the leopard phenotype (Fig. 2e). This result is consistent with a previous report that rat-CX43 with K9,13E substitutions does not exhibit rectification property in vitro and that the E9 and E13 pair is not the only determinant of the rectification property of gap junctions⁴⁰.

We also compared the N-terminal sequences of connexins in the Cx41.8 cluster from various organisms and found that the consensus motif ExxxxE was also well conserved (Fig. 2d and see Supplementary Fig. S1 online). This degree of conservation suggests the importance of the Exxxx motif for the function of these connexins. We again used several of these rat connexins for rescue experiments. Rat-CX46, which is an ortholog to zebrafish Cx48.5, and rat-CX50, which is an ortholog to zebrafish Cx44.1, rescued the leopard phenotype, as did rat-CX40. These results indicated that the function of members of the Cx41.8 cluster might be well conserved between zebrafish and rat.

SSAT changes the skin pattern in zebrafish. To confirm that a polyamine such as spermidine/spermine was involved in zebrafish pattern formation, we introduced zebrafish-ssat⁴⁴, a metabolic enzyme of spermine and spermidine, into the zebrafish genome. Ectopic expression of sat in melanophores of fish with a wild-type background generated a spotted pattern (Fig. 1j), whereas scattered melanophores (i.e., the absence of spots or stripes) were observed in leopard background fish (Fig. 1k). These results indicated that polyamine is involved in the skin pattern formation of zebrafish and that the polyamine sensitivity of gap junctions may be crucial for pattern formation in zebrafish.

Discussion

Here, we have shown that polyamine binding of Cx41.8 is required for skin pattern formation of zebrafish. Based on the link between polyamine binding and the rectification property of rat-CX40 gap junction⁴⁰,⁴¹, we suggest that zebrafish Cx41.8 may also have a rectification property. To confirm its presence, we tried to perform an electrophysiological assay with Cx41.8 using mammalian cultured cells. We could not, however, detect gap junctional intercellular communication (GJIC) between transfected cells in culture and thus could not examine the rectification property of Cx41.8 (data not shown). We also examined the protein localisation of Cx41.8 in cultured cells, but we could not detect the membrane localisation of a Cx41.8:EGFP fusion protein or even of unmodified Cx41.8 (data not shown). We believe that the amount of Cx41.8 that is localised to the cell membrane is too small in mammalian cultured cells, but we could not detect the membrane localisation of Cx41.8:EGFP fusion protein or even of unmodified Cx41.8 (data not shown). Because we could not examine the rectification property of Cx41.8 directly, we used the ortholog rat-CX40 to suggest that the rectification property of gap junction may be required for skin pattern formation in zebrafish.

At present, it remains unclear what type of function the rectification of gap junctions leads to and when and where this property is required in zebrafish. Indeed, the role of gap junctions in pattern formation in zebrafish has not been fully elucidated⁴⁴. The expression of cx41.8 is detected in melanophores and xanthophores based on RT-PCR analysis⁴⁵; this localisation is also supported by transgenic experiments⁴⁶. The role of Cx41.8 in both melanophores and xanthophores was predicted based on observations of mutant fish and transgenic fish⁴⁷–⁴⁹. In fish expression the leopard mutant alleles cx41.8* and cx41.8tq⁵⁰, the number of melanophores is smaller than that in wild-type fish, indicating that the loss of function of Cx41.8 causes a reduction in the number of melanophores. When Cx41.8 was ectopically expressed in melanophores, the number of melanophores increased, although the area that was covered by the melanophores (i.e., the black stripes) was still smaller in the transgenic fish (Fig. 1d) than that in wild-type fish (Fig. 1b). Cx41.8 expression in xanthophores is likely required to make normal black stripes because the number of xanthophores and the area of xanthophores in Tg(mitfapox:cx41.8) fish are both larger than those in the wild type⁵⁰. Taken together, Cx41.8 functions to control the number of pigment cells, both melanophores and xanthophores, on the skin surface, and the rectification property of gap junction might be important for this function.

Concerning the existence of polyamines in pigment cells, this small molecule should be localised to and function at the intracellular membrane of melanophores to bind to gap junctions. Our previous work revealed that kir7.1, a member of the inwardly rectifying potassium channel, is responsible for one of the zebrafish skin pattern mutants, jaguar, which shows a broader stripe pattern than that of wild-type fish⁵¹. For Kir7.1 channels, polyamine is required to induce rectification property by binding to the pore domain of the channel⁵², where polyamine blocks the flow of ions from the inside to the outside of the cell. Kir7.1 is found in both melanophores and xanthophores based on RT-PCR⁵³, but its expression in melanophores is sufficient for skin pattern formation in zebrafish⁵⁴. These findings support the idea that a polyamine such as spermine could be distributed around the cell membrane of melanophores and that a polyamine may function to induce the rectification properties of both Kir7.1 and gap junction.

The results of transgenic experiments using sat, which encodes a metabolic enzyme of spermidine/spermine, also supported the existence and functional effects of a polyamine in melanophores. We note that zebrafish carrying the mitfassat transgene in the WT background showed an intermediate phenotype between those of leopard (spots) and jaguar (broad stripes) fish, namely, larger spots with larger inter-spot distances. Furthermore, the phenotype of mitfassat transgenic fish in the leopard background resembled that of leopard/jaguar double-homozygous mutant fish⁵⁵. Although the significance of polyamine sensitivity for gap junction communication relying on the function of Cx41.8 N-terminus remains unclear in vivo, these observations also support the function of spermine in pigment cells.

In mammals, the overlapping expression of connexins occurs in many tissues such as the heart, retina, inner ear, skin and reproductive organs⁵⁶–⁵⁸, and complementation experiments have been carried out to determine the functional differences between connexin genes⁵⁹. Based on our greater understanding of the relevance of polyamine sensitivity to gap junction function, it would be of interest to carry out a functional comparison among the Cx41.8 cluster members CX40, CX46 and CX50 for further understanding of gap junction function.

Our study provides new insight into gap junction research. In particular, the rectification property of gap junction should be considered when the function of gap junction is examined. Further analysis will reveal the role of the rectification property of gap junction in pattern formation and in other biological phenomena.

Methods

Fish. Zebrafish (Danio rerio) were bred and maintained under standard laboratory conditions⁶⁰. Images were acquired using a Leica MZ16FA microscope after the fish were anaesthetised with MS222 (Sigma). All experiments were approved by the Animal Experiments Committee of Osaka University, Japan.
1. Kumar, N. M., Gillula, N. B. The gap junction communication channel. Cell 84, 381–388 (1996).
2. Simon, A. M. & Goodenough, D. A. Diverse functions of vertebrate gap junctions. Trends Cell Biol. 8, 477–483 (1998).
3. Saez, J. C., Berthoud, V. M., Branes, M. C., Martinez, A. D. & Beyer, E. C. Plasma membrane channels formed by connexins: their regulation and functions. Physiol. Rev. 83, 1359–1400 (2003).
4. Purnick, P. E., Benjamin, D. C., Verselis, V. K., Bargiello, T. A. & Dowd, T. L. Structure of the amino terminus of a gap junction protein. Arch. Biochem. Biophys. 381, 181–190 (2000).
5. Ohshima, A., Tani, K., Hiroski, Y., Fujiyoshi, Y. & Somasundar, G. E. Three-dimensional structure of a human connexin26 gap junction channel reveals a plug in the vestibule. Proc. Natl. Acad. Sci. USA 104, 10034–10039 (2007).
6. Maeda, S. et al. Structure of the connexin 26 gap junction channel at 3.5 Å resolution. Nature 458, 597–602 (2009).
7. Nakagawa, S., Maeda, S. & Tsukihara, T. Structural and functional studies of gap junction channels. Curr. Opin. Struct. Biol. 20, 423–430 (2010).
8. Oh, S., Rubin, J. B., Bennett, M. V., Verselis, V. K. & Bargiello, T. A. Molecular determinants of electrical rectification of single channel conductance in gap junctions formed by connexins 26 and 32. J. Gen. Physiol. 114, 339–364 (1999).
9. Musa, H. & Veenstra, R. D. Voltage-dependent blockade of connexin40 gap junctions by spermine. Biophys. J. 84, 205–219 (2003).
10. Musa, H. et al. Amino terminal glutamate residues confer spermine sensitivity and affect voltage gating and channel conductance of rat connexin40 gap junctions. J. Physiol. 557, 863–878 (2004).
11. Gemel, J., Lin, X., Veenstra, R. D. & Beyer, E. C. N-terminal residues in Cx43 and Cx40 determine physiological properties of gap junction channels, but do not influence heteromeric assembly with each other or with Cx26. J. Cell Biol. 119, 2258–2268 (2006).
12. Hibino, H. et al. Inwardly rectifying potassium channels: their structure, function, and physiological roles. Physiol. Rev. 90, 291–366 (2010).
13. Perrais, D., Veran, J. & Mulle, C. Gating and permeation of kainate receptors: determinants of electrical rectification of single channel conductance in gap junctions formed by connexins 26 and 32. J. Gen. Physiol. 114, 339–364 (1999).
14. Parichy, D. M. Mutational analysis of endothelin receptor b1 (rose) during neural crest development and pigment pattern formation in the zebrafish Danio rerio. Dev. Biol. 227, 294–306 (2000).
15. Perias, J. A., Robertson, C. P., Lepage, T., Johnson, S. L. & Raible, D. W. nacre signaling and photoreceptor development in the zebrafish retina. Dev. Biol. 227, 3715–3724 (2000).
16. Lister, J. A., Robertson, C. P., Lepage, T., Johnson, S. L. & Raible, D. W. nacre signaling and photoreceptor development in the zebrafish retina. Dev. Biol. 227, 3715–3724 (2000).
17. Ohashi, A., Tani, K., Hiroaki, Y., Fujii, Y. & Sosin, G. E. Three-dimensional structure of a human connexin26 gap junction channel reveals a plug in the vestibule. Proc. Natl. Acad. Sci. USA 104, 10034–10039 (2007).
18. Maeda, S. et al. Structure of the connexin 26 gap junction channel at 3.5 Å resolution. Nature 458, 597–602 (2009).
19. Nakagawa, S., Maeda, S. & Tsukihara, T. Structural and functional studies of gap junction channels. Curr. Opin. Struct. Biol. 20, 423–430 (2010).
20. Oh, S., Rubin, J. B., Bennett, M. V., Verselis, V. K. & Bargiello, T. A. Molecular determinants of electrical rectification of single channel conductance in gap junctions formed by connexins 26 and 32. J. Gen. Physiol. 114, 339–364 (1999).
21. Musa, H. & Veenstra, R. D. Voltage-dependent blockade of connexin40 gap junctions by spermine. Biophys. J. 84, 205–219 (2003).
22. Musa, H. et al. Amino terminal glutamate residues confer spermine sensitivity and affect voltage gating and channel conductance of rat connexin40 gap junctions. J. Physiol. 557, 863–878 (2004).
23. Gemel, J., Lin, X., Veenstra, R. D. & Beyer, E. C. N-terminal residues in Cx43 and Cx40 determine physiological properties of gap junction channels, but do not influence heteromeric assembly with each other or with Cx26. J. Cell Biol. 119, 2258–2268 (2006).
24. Hibino, H. et al. Inwardly rectifying potassium channels: their structure, function, and physiological roles. Physiol. Rev. 90, 291–366 (2010).
25. Parichy, D. M. et al. Mutational analysis of endothelin receptor b1 (rose) during neural crest and pigment pattern development in the zebrafish Danio rerio. Dev. Biol. 227, 294–306 (2000).
26. Lister, J. A., Robertson, C. P., Lepage, T., Johnson, S. L. & Raible, D. W. nacre encodes a zebrafish microphthalmia-related protein that regulates neural crest-derived pigment cell fate. Development 126, 3757–3767 (1999).
27. Rawls, J. F., Johnson, S. L. & Raible, D. W. nacre signaling and photoreceptor development in the zebrafish retina. Dev. Biol. 227, 3715–3724 (2000).
28. Parichy, D. M., Ransom, D. G., Paw, B., Zon, L. I. & Johnson, S. L. An orthologue of the kit-related gene fms is required for development of neural crest-derived xanthophores and a subpopulation of adult melanocytes in the zebrafish, Danio rerio. Development 127, 3031–3040 (2000).
29. Kondo, S. & Muura, T. Reaction-diffusion model as a framework for understanding biological pattern formation. Science 329, 1616–1620 (2010).
30. Asai, R., Taguchi, E., Kume, T., Saito, M. & Kondo, S. Zebrafish leopard gene as a component of the putative reaction-diffusion system. Mech. Dev. 89, 87–92 (1999).
31. Yamaguchi, M., Yoshimoto, E. & Kondo, S. Pattern regulation in the stripe of zebrafish suggests an underlying dynamic and autonomous mechanism. Proc. Natl. Acad. Sci. USA 104, 4793–4798 (2007).
32. Nakamura, A., Takahashi, G., Kaneh, A. & Kondo, S. Interactions between zebrafish pigment cell genes responsible for the generation of Turing patterns. Proc. Natl. Acad. Sci. USA 106, 8429–8434 (2009).
33. Kondo, S., Iwashita, M. & Yamaguchi, M. How animals get their skin patterns: fish pigment pattern as a live Turing wave. Int. J. Dev. Biol. 53, 851–856 (2009).
34. Takahashi, G. & Kondo, S. Melanophores in the stripes of adult zebrafish do not have the nature to gather, but disperse when they have the space to move. Pigment Cell & Melanoma Res. 21, 677–686 (2008).

Additional information
Supplementary information accompanies this paper at http://www.nature.com/scientificreports/competition

M.W. and S.K. designed and M.W. and D.W. performed the research; M.W., D.W. and S.K. analysed data; M.W. wrote the paper.

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M.W. and S.K. designed and M.W. and D.W. performed the research; M.W., D.W. and S.K. analysed data; M.W. wrote the paper.

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