Acquisition of glial cells missing 2 Enhancers Contributes to a Diversity of Ionocytes in Zebrafish

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Abstract

Glial cells missing 2 (gcm2) encoding a GCM-motif transcription factor is expressed in the parathyroid in amniotes. In contrast, gcm2 is expressed in pharyngeal pouches (a homologous site of the parathyroid), gills, and H⁺-ATPase-rich cells (HRCs), a subset of ionocytes on the skin surface of the teleost fish zebrafish. Ionocytes are specialized cells that are involved in osmotic homeostasis in aquatic vertebrates. Here, we showed that gcm2 is essential for the development of HRCs and Na⁺-Cl⁻ co-transporter–rich cells (NCCCs), another subset of ionocytes in zebrafish. We also identified gcm2 enhancer regions that control gcm2 expression in ionocytes of zebrafish. Comparisons of the gcm2 locus with its neighboring regions revealed no conserved elements between zebrafish and tetrapods. Furthermore, we observed gcm2 expression patterns in embryos of the teleost fishes Medaka (Oryzias latipes) and fugu (Fugu niphobles), the extant primitive ray-finned fishes Polypterus (Polypterus senegalus) and sturgeon (a hybrid of Huso huso × Acipenser ruhenus), and the amphibian Xenopus (Xenopus laevis). Although gcm2-expressing cells were observed on the skin surface of Medaka and fugu, they were not found in Polypterus, sturgeon, or Xenopus. Our results suggest that an acquisition of enhancers for the expression of gcm2 contributes to a diversity of ionocytes in zebrafish during evolution.

Introduction

Developmental evolutionary studies have played an important role in elucidating the molecular mechanisms that give rise to morphological divergence and acquisition of new morphological structures and/or functions. These studies have proposed a model for the evolution of a novel morphological structure by modification of temporal and spatial patterns of gene expression; that is, changes in gene regulatory networks by adaptive mutations of transcription factors, including cis-regulatory elements [1–3]. However, only a few actual examples have been reported. In our current study, we focused on the glial cells missing (gcm) family and investigated the evolution of ion-regulating organs in vertebrates that may have evolved by changing the molecular developmental mechanisms of gene regulation of the gcm family.

gcm encodes a GCM-motif transcription factor in Drosophila and functions as a determinant in glia/neuron and plasmacyte/macrophage cell fates [4–9]. Homologues of Drosophila gcm, gcm1 and gcm2, have been isolated from vertebrates [10–12], and both genes are expressed in non-neuronal organs with diverse functions [12–20]. Mouse gcm1 is expressed in the thymus, kidney, and placenta, where it is required for chorio-allantois morphogenesis [17,20]. In contrast, mouse gcm2 is expressed in the third pharyngeal pouch and the parathyroid gland that develops from the third pouch and is an essential gene for their development [21].

gcm2 is expressed in pharyngeal pouches of the teleost fish zebrafish and the small-spotted catshark Scyliorhinus canicula. Its expression patterns suggest that the evolutionary origin of the parathyroid organ may be traced back to the pharyngeal pouches of fishes [22–24]. Furthermore, it has been reported that gcm2 is expressed in H⁺-ATPase–rich cells (HRCs), a subset of ionocytes that are rich in H⁺-ATPase and located in the skin of zebrafish [25,26]. Ionocytes are ion transporters with abundant mitochondria and extensive basolateral membrane infoldings that form a tubular system [27]. They are located in gills and scattered over the skin surface of aquatic vertebrates but are not found in amniotes. They play a central role in maintaining body fluid homeostasis by actively taking up and excreting ions [28–30].

In our current study, we showed that gcm2 is essential for the development of HRCs and Na⁺-Cl⁻ co-transporter–rich cells (NCCCs), another subset of ionocytes in the skin of zebrafish. We also identified gcm2 enhancers specific for the skin of ionocytes in the developmental stages of the teleost fishes Medaka (Oryzias latipes) and fugu (Fugu niphobles), the primitive ray-finned fishes Polypterus (Polypterus senegalus) and sturgeon (a hybrid of Huso huso × Acipenser ruhenus), and the amphibian Xenopus (Xenopus laevis). gcm2-expressing cells were observed in the skin of Medaka and fugu but not in Polypterus, sturgeon, or Xenopus embryos. We will discuss an importance of our finding of cis-elements for ionocytes in the skin of zebrafish.

Results

Zebrafish gcm2 is essential for morphogenesis of ionocytes on the skin surface

To confirm that gcm2 is necessary for ionocyte development, zebrafish morphants injected with gcm2 antisense morpholinos

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(MOs) were examined using scanning electron microscopy. The observations focused on yolk sac areas because expression of gcm2 was localized in the gills and yolk sac (Figure 1A and B). In control embryos at 72 hours post-fertilization (hpf), ionocytes on the skin surface protruded between epithelial cells (Figure 2A), whereas in gcm2 morphants at 72 hpf, the number of ionocytes was reduced. We observed a reduction of approximately 40% in the number of ionocytes on the skin surface per 0.04 mm² when compared to the number of ionocytes in control embryos (Figure 2B and C). The reduction in the number of ionocytes in gcm2 morphants suggested that gcm2 MO treatment resulted in inhibition of ionocyte morphogenesis.

Zebrafish gcm2 is essential for the development of two of the three types of ionocytes

Although gcm2 morphants showed a reduction in ionocyte numbers on the skin surface, it was not clear which types of ionocytes were reduced during morphogenesis. A recently proposed model suggests that there are at least three subtypes of ionocytes on the zebrafish skin surface: H⁺-ATPase–rich cells (HRCs); Na⁺-K⁺-ATPase–rich cells (NaRCs); and Na⁺-Cl⁻ co-transporter–rich cells (NCCCs) [28]. It has been reported that gcm2 morphants are depleted of HRCs but not NaRCs [25,26], although the status of NCCCs is unknown. To determine which types of ionocytes require gcm2 for their development, we analyzed the expression patterns of ionocyte marker genes in gcm2 morphants. In control embryos at 48 hpf, we detected with whole-mount in situ hybridization skin surface expression of atp6v1a (H⁺-ATPase subunit A) as a HRC marker gene [31], atp1b1b (Na⁺-K⁺-ATPase transporting beta 1b subunit) as a NaRC marker gene [31], and slc12a10.2 (thiazide-sensitive Na⁺-Cl⁻ co-transporter, a member of the SLC12 family) as a NCCC marker gene [32] (Figure 3A, C, and E). In contrast, gcm2 morphants showed a strong reduction in the number of HRCs, no significant change in the number of NaRCs, and an increase in the number of NCCCs (Figure 3B, D, F, and G). These results suggested that gcm2 was essential for the development of HRCs, was not essential for the development of NaRCs, and suppressed the development of NCCCs.

zgcm2-expressing cells on the skin surface are present only in teleosts

To investigate if the induction of gcm2 expression in ionocytes on the skin surface is unique to teleosts, we examined gcm2 expression patterns in embryos of Medaka (13 days old), fugu (7 days old), Polypterus (48 hpf), sturgeon (stage 32), and Xenopus (stage 37). All embryos expressed gcm2 in the pharyngeal pouches and gills (Figure 4A, C, E, G, and J). The Medaka and fugu embryos also expressed gcm2 in a scattered pattern on the skin surface (Figure 4B and D). On the other hand, Polypterus, sturgeon, and Xenopus embryos did not express gcm2 on the skin surface (Figure 4E, G, and J), although Polypterus and sturgeon embryos showed atp1b1-positive

Enhancers Contributed to a Diversity of Ionocytes

Figure 1. Expression of gcm2 in the gills and the cells on the skin surface of zebrafish. Whole-mount in situ hybridization of 5-day-old zebrafish with gcm2 probes. (A) gcm2 is expressed in the gills. (B) Magnification of a yolk sac area. Some cells in the yolk sac express gcm2 (arrowheads).

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Figure 2. Scanning electron microscopy of the yolk sac membrane in 3-day-old zebrafish. (A, B) Magnification of the yolk sac membrane. (A) An embryo injected with a control morpholino (MO). (B) An embryo injected with a gcm2 MO. (C) Quantitative comparison of the ionocyte number per 0.04 mm² between embryos injected with control MO and gcm2 MO. The ionocyte number on the yolk sac membrane area in gcm2 morphants is less than that in control MO. Arrowheads indicate ionocytes. Because it is likely that cells were still alive or had differentiated without expression of a marker gene by loss of function, we observed the external morphology of the skin surface in morphants using scanning electron microscopy.

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enhancers are located 8 kb upstream and 41 kb downstream of gcm2

To characterize gcm2 enhancers specific for ionocytes on the skin surface, several fragments derived from the zebrafish genomic region containing the gcm2, elovl2, and mak loci were PCR amplified, cloned into the Tol2 transposon-based Venus expression vector (pTol2Ⅷ), and injected into zebrafish eggs. Transient transgenic embryos injected with the pTol2Ⅷ vector containing either the 8 kb upstream region or the 41 kb downstream region of the gcm2 translation site showed Venus expression specifically in ionocytes on the skin surface (Figure 5F). Moreover, all reporter constructs with the 8 kb upstream region between −8249 and −8004 bp (−8 kb enhancer) or the 41 kb downstream region between +41277 and +41497 bp (+41 kb enhancer) displayed Venus positivity, indicating that each region contained enhancers that specifically regulated expression of gcm2 in ionocytes on the skin surface.

Venus-expressing cells on the skin surface express a HRC marker gene

To examine whether Venus-expressing cells on the skin surface are HRCs, zebrafish embryos were injected with a construct containing the −8 kb or +41 kb enhancer and examined with situ hybridization using an atp6v1al probe in combination with immunohistochemistry using anti-GFP. All Venus-expressing cells on the skin surface expressed atp6v1al mRNA (Figure 6C, D, E, and F), which demonstrated that the gcm2 enhancers we identified were enhancers specific for HRCs in the skin.

gcm2 enhancer regions specific for HRCs in zebrafish are not found in tetrapods

We used VISTA software [33] to examine whether the two gcm2 enhancers are conserved among different species of vertebrates. Analysis of the gcm2 locus and neighboring sequences in zebrafish and some tetrapods, including Xenopus, chick, mouse, and human, showed no conserved sequences in these vertebrate species (Figure 7).

Discussion

The genes that regulate the specification of NaRCs, HRCs, and NCCCs from their common precursor cells in zebrafish remain to be determined. Previous studies [23,26] and our current study suggest that regulation of gcm2 plays a key role in the specification and differentiation of common precursor cells into each type of ionocyte. Ionocytes are derived from common precursor cells of keratinocytes [31,34]. Furthermore, a positive regulatory loop involving foxi3a and foxi3b [31] regulates the specification and differentiation of HRCs and NaRCs; however, the genes that regulate the specification and differentiation of NCCCs remain unknown. Our current study using gcm2 morphants showed that
cells on the skin surface (Figure 4F and H). These results suggested that the cells expressing gcm2 on the skin surface were unique to teleosts.

Zebrafish 160 kb BAC includes gcm2 enhancers specific for ionocytes on the skin surface

Because gcm2 was uniquely expressed in ionocytes on the skin surface of teleosts, the zebrafish was used to identify potential cis-regulatory elements (i.e., enhancers of gcm2 that may regulate its expression on the skin surface). To examine whether putative enhancers are located near the gcm2 locus, we prepared a 160 kb BAC (Bacterial Artificial Chromosome) clone containing zebrafish gcm2 and its neighboring genes, elongation of very long chain fatty acids like 2 (elovl2), and male germ cell–associated kinase (mak). Venus was then inserted into the gcm2-coding region of the 160 kb BAC clone, and this construct was injected into zebrafish eggs (Figure 5A). In transient transgenic embryos, Venus was detected on the skin surface, especially over the yolk sac membrane after 24 hpf, but it was not observed in the gills at any of the observed stages of
development (Figure 5B). Because all Venus-expressing cells also were labeled with MitoTracker, an ionocyte marker that stains mitochondria (Figure 5C, D, and E), our results demonstrated that Venus-expressing cells in the transgenic embryos were ionocytes and that the 160 kb BAC-Venus clone contained gcm2 enhancers specific for ionocytes in the skin. In addition, when the 160 kb BAC-Venus construct was injected into fugu embryos, Venus-expressing cells were observed on the skin surface (Figure 5F). This result suggested that there were common trans factors that induced gcm2 on the skin surface in zebrafish, but whether the common factors are actually present in fugu remains to be investigated.
gcm2 is involved in the differentiation of both HRCs and NCCCs on the skin surface. gcm2 morphants showed not only extremely reduced numbers of HRCs but increased numbers of NCCCs (Figure 3). Therefore, we propose that gcm2 functions as a binary switch in determining HRC or NCCC fate on the skin surface. Because gcm in Drosophila functions as a binary switch in determining glial or neuronal cell fate [4,5], it is possible that vertebrate gcm2 has a similar function.

If gcm2 functions as a binary switch, then what trans factors directly regulate gcm2 in progenitor cells of HRCs? fox3a is a candidate gene because positive feedback of gcm2 and fox3a in the specification of HRCs may maintain the expression of gcm2 [26].

Figure 4. Expression of gcm2 in Medaka, fugu, Polypterus, sturgeon and Xenopus. (A, B) Whole-mount in situ hybridization for gcm2 in 13-day-old Medaka. (C, D) Whole-mount in situ hybridization for gcm2 in 7-day-old fugu. (B, D) Magnification of the skin surface. gcm2 is expressed in the gills (arrowheads) and the cells on the skin surface of the teleostean fishes, Medaka and fugu. (E, F) Whole-mount in situ hybridization for gcm2 and atp1b1 probes in 48-hpf Polypterus. (B, D) gcm2 is expressed in the gills (arrowhead). (F) atp1b1 is expressed in the kidney and the cells on the skin surface. (G, H) Whole-mount in situ hybridization for gcm2 and atp1b1 in sturgeon at stage 32. (G) gcm2 is expressed in the gills (arrowhead). (H) atp1b1 is expressed in the cells on the skin surface. (I) Scanning electron microscopy on the yolk sac membrane of sturgeon at stage 32. Ionocytes (arrowheads) protrude between the epithelial cells. (J) Whole-mount in situ hybridization with the gcm2 probe in Xenopus at stage 37. gcm2 is expressed in the gills (arrowhead) but not on the skin surface of Polypterus, sturgeon, and Xenopus embryos.
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This raises the question of whether foxi3a directly regulates gcm2 enhancers. Because the −8 kb enhancer region contains a consensus sequence for a Fox protein binding site [35] (Figure S1), this binding site may directly control the −8 kb enhancer upon its binding to foxi3a. However, a transient transgenic zebrafish that was injected with a pTolIF vector containing the −8 kb enhancer region with a deletion of the Fox protein binding site did not lose Venus activation (Table S2). In addition, the +41 kb enhancer region did not contain a sequence identical to the Fox protein binding site (data not shown). These results and the genetic data of Esaki et al. (2009) suggest that foxi3a indirectly regulates gcm2 enhancers [26].

It has been hypothesized that the acquisition of enhancers during the evolution of animals is an evolutionary opportunity for animals to obtain new cells and organs [1–3]. Because the enhancers we identified in our current study are required for the expression of gcm2 in progenitor cells of HRCs, gcm2 enhancers may have enabled zebrafish to acquire new HRC ionocytes. When the zebrafish 160 kb BAC-Venus construct that contained gcm2 enhancers was injected into fugu embryos, Venus-expressing cells were observed on the skin surface but not in the gills. (C) Several cells on the yolk sac expressed Venus. (D) Ionocytes stained with MitoTracker. (E) Merge of (C) and (D), showing overlap in staining with a subset of ionocytes. (F) A 160 kb BAC-Venus transient transgenic 7-day-old fugu. Venus-expressing cells are observed on the skin surface.

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are positive for ionocyte markers (Figure 4) [39,40]. In addition, gcm2 enhancer regions in zebrafish were not found in the synteny block that contained the gcm2 locus and its neighboring regions in the tetrapods that we examined (Figure 7). All together, these results suggest that an acquisition of enhancers for the expression of gcm2 contributes to a diversity of ionocytes in zebrafish. Whether the same enhancers are responsible for development of ionocytes in other teleosts including fugu remains to be investigated. Since gcm2-expressing cells were not observed in primitive actinopterygian fishes and because gcm2 enhancers are not found in tetrapods, the evolution of gcm2 enhancers is one of a few examples to provide a fish model for evolutionary developmental studies of a novel morphological structure through changes in gene regulatory networks after the teleost-specific whole genome duplication [41–43].

Materials and Methods

Animals

Zebrafish and Medaka were purchased at a local pet shop and maintained as described [44]. Fertilized eggs were incubated in E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl₂, and 0.33 mM MgSO₄) at 28.5°C. Fugu (F. niphobles) eggs were collected on Arai beach, Kanagawa prefecture, Japan, and incubated in artificial seawater, Tetra marine salt pro (Tetra), at 20°C. Polypterus (P. senegalus) was purchased at a local pet shop, and fertilized eggs were incubated in E3 medium. Sturgeon is a hybrid of H. huso × A. ruhenus. Their eggs were supplied by Fujikin Incorporated, and embryos were incubated in freshwater at 20°C. Embryos were staged according to Ballard and Needham (1964) [45]. Xenopus laevis was purchased at a Xenopus culture shop, and fertilized eggs were obtained in vitro using standard methods. Embryos were staged according to Nieuwkoop and Faber (1967) [46].

Scanning electron microscopy

Zebrafish and sturgeon embryos were fixed in 2% glutaraldehyde in 0.1 M PBS at 4°C overnight, washed with 5% sucrose in 0.1 M PBS for 15 minutes, and fixed in 1% osmium tetroxide in 0.1 M PBS for 60 minutes. Fixed embryos were washed with 5% sucrose in 0.1 M PBS for 15 minutes and dehydrated in a graded series of ethanol. The embryos were critical-point dried using
A GFP microscope, SteREO Lumar, V. 12 (Carl Zeiss).

Injection of zebrafish embryos

Supercoiled plasmid and antisense MOs were injected into zebrafish embryos with a glass micro needle using NARISHIGE IM-30 (NARISHIGE). Control zebrafish MOs and MOs against gcm2 were injected as described previously [24]. MOs (1 μM/embryo) were injected into cells at one- to two-cell-stage zebrafish embryos.

Molecular cloning and probe synthesis

Total RNA was extracted from whole bodies of zebrafish (5 days old), Medaka (3–13 days old), fugu (3–7 days old), Polypterus (1–3 days old), Medaka (3–13 days old), xenopus (3–7 days old), sturgeon (stage 32), and fugu (3–7 days old). The samples then were transferred to TRizol (Invitrogen) and treated with DNase (Invitrogen). Single-stranded cDNA was synthesized from total RNA using a PrimeScript 1st strand cDNA synthesis kit (TAKARA) according to the manufacturer’s instructions.

Zebrafish cDNA clones of gcm2 (NM_001005603), atp1b1b (NM_131671), atp6v1al (NM_201135), and slc12a10.2 (EF591989) were isolated by PCR using the following forward and reverse primers: gcm2, 5'-CAGGACACGAGGCAGTATCGCTTCTCC-3' and 5'-GTGAAGACATCGCTTCTCCAGGACTCTCC-3'; atp1b1b, 5'-CAGGACTGCTCTCCAGGCTCTC-3' and 5'-CGGAGGCTCCGCTCTCAGTATTAC-3'; atp6v1al, 5'-CCAGCTGCTAGTACATCGAG-3' and 5'-GAGGGGACAACTGGATCAGGA-3' and 5'-CCAGACGCCATGCTGCCATG-3'.

Staining with MitoTracker

MitoTracker Orange CMTMRos (Invitrogen) was used to illuminate ionocytes [51]. Zebrafish that were injected with the 160 kb BAC-Venus were stained with MitoTracker at a final concentration of 1 μM in E3 medium for 30 minutes. Observations were made with confocal microscopy using an LSM 510 microscope (Carl Zeiss).

Enhancer analysis in zebrafish

A BAC containing the gcm2 locus in zebrafish was identified by the gcm2 cDNA sequence using the BAC sequence from the Sanger Institute (http://www.sanger.ac.uk/cgi-bin/blast/submit?blast/d_rerio). The BAC clone 160 kb BAC (CH211-203G10), which was purchased from BACPAC Resources Center, CA, USA, had a ~160 kb insert, of which ~120 kb was upstream of gcm2. To generate gcm2 reporter constructs, we followed the method of Lee et al. (2001) [47] using the BAC homologous recombination system (a gift from N. Copeland, National Cancer Institute, Frederick, MD, USA). To make a plasmid construct of a targeting DNA template, we subcloned Venus [48], the SV40 polyA signal, and a kanamycin resistance gene (Kmr) in this order into pBluescript-SK. The gcm2-derived sequences were: 5’ arm, 5’-CTTCAAGCTGACACGGCTCGTCG-3’, which is located 300 bp downstream of the putative translation start site of gcm2, and 3’ arm, 5’-CTTTTGCCGAATGACTCCGCGCC-3’, which is located 354 bp downstream of the putative translation start site.

The gcm2 locus around sequence fragments in the zebrafish genome was subcloned with the homologous recombination system [47] or amplified by PCR and cloned into the Tol2 transposon-based vector, pTolV, which is converted to Tol2000 [49] with the c-fos promoter, Venus, and the SV40 polyA signal in the opposite direction of the Tol2 element. Each construct and the in vitro–synthesized capped RNA of Tol2 [49] were co-injected as described [50]. All injected embryos at 24 hpf were observed with a GFP microscope, SteREO Lumar, V. 12 (Carl Zeiss).

Staining with MitoTracker

MitoTracker Orange CMTMRos (Invitrogen) was used to illuminate ionocytes [51]. Zebrafish that were injected with the 160 kb BAC-Venus were stained with MitoTracker at a final concentration of 1 μM in E3 medium for 30 minutes. Observations were made with confocal microscopy using an LSM 510 microscope (Carl Zeiss).

Figure 7. Two gcm2 enhancers in zebrafish are not found in tetrapods. VISTA plots of zebrafish, Xenopus, chick, mouse, and human genomic sequences of the gcm2 locus with its neighboring genes, elovl2 and mak. doi:10.1371/journal.pone.0023746.g007
5’-GARTGGMCNGAYGGNTAYGT-3’ and 5’-ACYTTNGT-
NGTENGTNGTDAT-3’. The sturgeon cDNA clones for gcm2 were amplified or isolated by degenerate PCR using the following forward and reverse primers: 5’-GARTGGMCNGAYGGNTAYGT-3’ and 5’-TCTGACNCGCTTYNGCYTG-3’. The Xenopus cDNA clone of gcm2 (AB175676) was isolated as described previously [24]. The Polyphemus cdNA clone of atp1b1 (AB615441) was identified from a Polyphemus EST project (M. Okabe, unpublished results) and isolated by PCR using the following forward and reverse primers: 5’-CGAAATAGGGAGGAAGGAAAGAAG-3’ and 5’-CACATTAGGCCTGACTTGGCTCT-3’. The sturgeon cdNA clone of atp1b1 was isolated by degenerate PCR using the following forward and reverse primers: 5’-CARGAYMGW-GTYGCMCCCTCAGG-3’ and 5’-TTTCKCARTARTAKGGR-TARTA-3’.

The PCR clone in the pGEM-T-Easy Vector (Promega) was used as a template, and a digoxigenin (DIG)-labeled antisense RNA probe was synthesized by in vitro transcription with T7 RNA polymerase (Promega) and DIG RNA labeling mix (Roche).

In situ hybridization combined with immunohistochemistry

All samples for whole-mount in situ hybridization in combination with immunohistochemistry were fixed in 4% paraformaldehyde in 0.1 M PBS at 4°C overnight and were breached with breaching solution (1% H2O2, 5% formamide, 0.5× SSC:75 mM NaCl, and 7.5 mM sodium citrate, pH 7). Fixed samples were incubated in hybridization buffer (50% formamide, 50 mM NaCl, 50 mM MgCl2, 100 mM Tris-HCl (pH 9.5), and 0.1% SSC, 500 μg/ml yeast tRNA, 50 μg/ml heparin, and 0.1% Tween-20, pH 6.0) containing DIG-labeled antisense RNA probe at 60°C overnight. The hybridized embryos were washed several times for 3 hours each with 2× SSC and 0.2× SSC and then for 10 minutes with MABT (100 mM maleic acid, 150 mM NaCl, and 0.1% Tween 20, pH 7.5). After incubation for 1 hour with blocking buffer (2% Blocking Reagent (Roche) in MABT), embryos were immunoreacted overnight at room temperature with an anti-DIG Fab fragment conjugated to alkaline phosphate (Roche, 1:500) and rabbit anti-GFP (MBL, 1:500). After several washes for 6 hours with MABT and three washes with NTMT (100 mM NaCl, 50 mM MgCl2, 100 mM Tris-HCl (pH 9.5), and 0.1% Tween-20), samples were treated with BM purple AP Substrate precipitating solution (Roche). When satisfactory coloration was achieved, samples were washed with PBST and then reacted with Alexa Fluor 485-conjugated goat anti-rabbit IgG (Invitrogen, 1:500 in PBST) overnight at room temperature in the dark. Finally, samples were washed with PBST and photographed.

Phylogenetic analysis of the conserved sequences

The VISTA program (http://www-gsd.lbl.gov/vista/index.shtml) was used for homology comparison. Draft genome sequence of zebrafish, Xenopus, chick, mouse, and human were obtained from the databases of UCSC Genome Bioinformatics (http://genome.ucsc.edu/) and the Wellcome Trust Sanger Institute, Genome Research Limited (http://www.sanger.ac.uk/).

In vivo deletion assay of gcm2 enhancer regions

A deletion assay for upstream region between −8249 and −8004 bp of gcm2 were carried out using Gene Tailor™ Site-Directed Mutagenesis System (Invitrogen). All the fragments were cloned into pTolfV and injected into zebrafish eggs. All injected embryos at 24 hpf were observed under a GFP microscope, SteREO Lumar, V. 12 (Carl Zeiss).

Supporting Information

Figure S1 The sequence of −8 kb enhancer regions.

The possible transcription factor binding sites in the sequences. The box was analyzed in search of transcription factor binding sites using TFSEARCH Data Base (http://www.cbrc.jp/research/db/TFSEARCHJ.html). A candidate for FOX protein binding site was previously described [52]. GATA: GATA-binding factor, SRY: sex-determining region Y gene product, FOX: Forkhead box protein, C/EBPβ: CCAAT/enhancer binding protein beta, HNF-3β: hepatic nuclear factor 3beta, OCT1: octamer-binding factor 1. (TIF)

Table S1 Transient transgenic analysis of the gcm2 enhancer regions.

| Fragment | Direction | Description |
|----------|-----------|-------------|
| upstream | (+)       | gcm2 loci |
| downstream | (+) | gcm2 loci |

(TIF)

Table S2 In vivo deletion assay of −8 kb enhancer regions.

Deletion sites, a-f, are shown in Figure S1. The sites were deleted and all the fragments were cloned into pTolfV. All reporter constructs displayed Venus positivity, indicating that the possible transcription factor binding sites of −8 kb enhancer regions in the analysis do not affect the enhancer activity. (TIF)

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Author Contributions

Conceived and designed the experiments: TS MO. Performed the experiments: TS DK TM. Analyzed the data: TS DK TM MO. Wrote the paper: TS TM MO.

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