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The N-terminal domain of gastrulation brain homeobox 2 (Gbx2) is required for iridophore specification in zebrafish

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ABSTRACT

Although body color pattern formation by pigment cells plays critical roles in animals, pigment cell specification has not yet been fully elucidated. In zebrafish, there are three chromatophores: melanophore, iridophore, and xanthophore, that are derived from neural crest cells (NCCs). A recent study has reported the differentially expressed genes between melanophores and iridophores. Based on transcriptome data, we identified that Gbx2 is required for iridophore specification during development. In support of this, iridophore formation is suppressed by *gbx2* knockdown by morpholino antisense oligonucleotide, at 72 h post fertilization (hpf) in zebrafish. Moreover, *gbx2* is expressed in *sox10*-expressing NCCs and guanine crystal plates-containing iridophores during development at 24 and 48 hpf, respectively. In *gbx2* knockdown zebrafish embryos, apoptosis of *sox10*-expressing NCCs was detected at 24 hpf without any effect on the formation of melanophores and xanthophores at 48 hpf. We further observed that the N-terminal domain of Gbx2 is able to rescue the iridophore formation defect caused by *gbx2* knockdown. Our study provides insights into the requirement of N-terminal domain of Gbx2 for iridophore specification in zebrafish.

1. Introduction

Characteristic animal body patterns provide animals with protection from irradiation and facilitate their communication with other animals. Zebrafish, one of the most useful model animals, have a conspicuous striped pattern, which is composed of three pigment cells; melanophores (black cells containing melanin), iridophores (silvery and/or white cells containing reflective guanine platelets), and xanthophores (yellow to orange cells containing pteridine and carotenoid) [1,2]. These pigment cells are derived from neural crest cells (NCCs), and key factors required for specification from the multipotent NCCs to pigment cell fate have been identified by genetical and molecular biological analyses in zebrafish. A previous report proposed that melanophores (terminal differentiation marker, *dopachrome tautomerase* (*dct*) [3]) and iridophores (terminal differentiation marker, *purine nucleoside phosphorylase 4a* (*pnp4a*)) are specified from *foxd3*-expressing NCCs via bi-potent precursors cells [4]. More specifically, the *microphthalmia-associated transcription factor a* (*mitfa*) is required for melanophore specification, and repression of *mitfa* by Foxd3 promotes iridophore fate [4]. In addition, a zebrafish mutation has indicated that the leukocyte tyrosine kinase (Ltk) as a promoting factor from *sox10*-expressing NCCs towards the iridophore lineage [5]. To facilitate the understanding of the fate specification mechanisms between melanophores and iridophores, transcriptome analysis was performed using zebrafish eyes [6]. A large number of differentially expressed genes in zebrafish pigment cells were identified [6]. However, the exact genes and molecular pathways leading to the pigment cell specification in zebrafish are still largely unknown.

Gastrulation brain homeobox proteins (Gbx), including Gbx1 and Gbx2, are members of the homeodomain transcription factors and have been identified in some vertebrates, including zebrafish, *Xenopus*, chicken, and mice [7]. It is well known that Gbx proteins are involved in the morphogenesis of the midbrain-hindbrain boundary (MHB), which is a center responsible for midbrain and rostral hindbrain patterning, as well as cerebellum formation [8]. Specification of the prospective MHB is determined by mutual

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repressive interaction between anterior Otx2/Wnt1 and posterior Gbx/Fgf8 signaling in the neural plate, during the development of zebrafish, chick, and mice [8]. Gain- and loss-of-function experiments have revealed that Gbx2 functions as a transcriptional repressor and both the N- and C-terminal regions of Gbx2 contribute to its suppressive activity [9]. In addition to MHB formation, *Gbx2* deficient mouse embryos show defects in NCC patterning and pharyngeal arch artery [10] while, in the *Xenopus*, Gbx2 promotes NCC induction downstream of Wnt signaling [11]. Although these results indicate the involvement of Gbx2 in NCC formation during development, the precise function of Gbx2 in NCCs is not fully elucidated.

A previous study reported results from a transcriptome analysis in zebrafish melanocytes, iridophores, and retinal pigmented epithelium [6]. Numerous differentially expressed genes among the three cell types were observed during development [6]. Among these genes that are enriched in iridophores and by using morpholino-mediated knockdown experiments, we have identified *gbx2* as a gene specific for iridophore formation. In the present study, we explored the function of Gbx2 in pigment cell specification during zebrafish development. We hereby show that the N-terminus domain of Gbx2 is required for iridophore specification in zebrafish.

2. Materials and methods

2.1. Fish husbandry

All zebrafish experiments were approved by the Hiroshima University Animal Research Committee (Permit Number: F17-6-2). Zebrafish embryos were incubated in 1/3 Ringer's solution at 28.5 °C [12] and staged according to a previous report [13]. *gbx2*^{fh253} mutant fish were obtained from the Zebrafish International Resource Center (Oregon), and were genotyped previously described [14]. The transgenic line Tg (*-4.9sox10:EGFP*) [15] was generous gift of M. Nikaido (University of Hyogo) with permission of R.N. Kelsh.

2.2. Whole-mount in situ hybridization, FISH, and TUNEL assay

Whole-mount *in situ* hybridization and FISH were performed as previously described [16], and riboprobes were prepared according to previously published methods. For FISH experiments, expression of *gbx2* and *sox10* was detected with DyLight633-tyramide and FAM-tyramide [17,18] To detect apoptotic cells, we performed TUNEL assay using an *In Situ* Cell Death Detection Kit (Sigma-Aldrich) according to the manufacturer's instructions.

2.3. Morpholino oligonucleotide (MO) and mRNA injections

The *gbx2-ΔNt* [9] and *gbx2-ΔNtΔCD* were amplified by PCR with pCS2^{+}-*gbx2* [19] as a template using pairs of primers (5′-CCGCCACCATGACTGCATTCAGCATTGACTCGC-3′ and 5′-GGGGCTCGAGTCAAGGTCGGGCCTGTTCTAAC-3′) and (5′-CCGCCACCATGACTGCATTCAGCATTGACTCGC-3′ and 5′-GGGGCTCGAGTTAATCTAAATCACTGTCCATGGAG-3′), followed by ligation into the pCS2^{+} vector, respectively. The pCS2^{+} vector carrying a cDNA fragment encoding *gbx2-ΔNt*, *gbx2-ΔNtΔCD*, or *egfp* was used in this study. Capped mRNA was synthesized using an SP6 mMESSAGE mMACHINE (Ambion). For the overexpression experiments, *gbx2-ΔNt*, *gbx2-ΔNtΔCD*, or *egfp* mRNA (80 pg each) was injected at the one-cell stage. The morpholino oligonucleotide (MO) (Gene-Tools, Inc.) that was targeted to the 5′-terminal sequence of the cording *gbx2* region (*gbx2* MO) [19] (4 pg) and the control *gbx2* MO (*gbx2* 5mis MO) (4 pg) was injected into one-cell stage eggs. The morpholino sequences were as follows: *gbx2* MO, 5′-ACGGTGTGCTGAAAGCTGCACTCAT- 3’; *gbx2* 5mis MO,5′-ACCGTCTGCTCAAAGCTCCACTGAT-3’.

2.4. Statistical analyses

Data are expressed as averages from repeated experiments with standard deviation. Statistical significance was determined by using the Student's *t*-test. *p* values of ≤ 0.05 were considered to be statistically significant.

3. Results and discussion

3.1. Morpholino-mediated gbx2 knockdown leads to iridophore reduction during development

In order to identify the genes involved in iridophore formation, we have examined morpholino (MO)-mediated knockdown experiments targeted at certain iridophore-enriched genes that were identified from a previously reported transcriptome analysis [6]. Among these genes, we found that the number of iridophores in *gbx2* MO-injected larvae was significantly reduced compared to control *gbx2* 5mis MO-injected larvae at 72 hpf (Fig. 1). Although

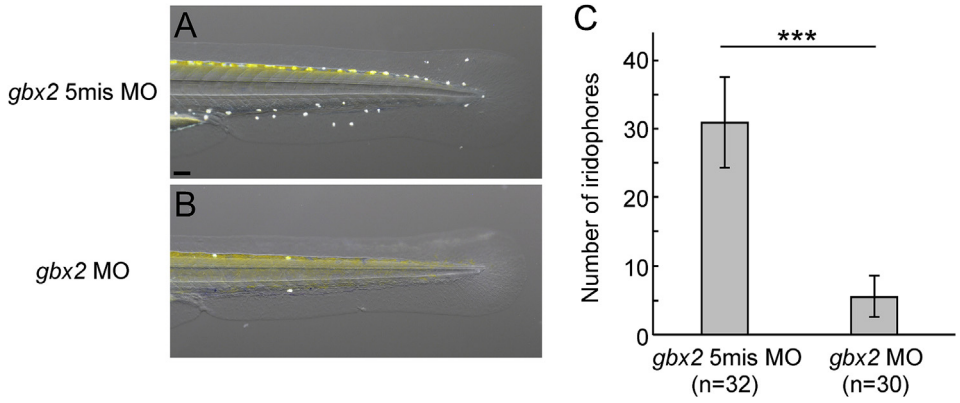

Fig. 1. Iridophore numbers are reduced in *gbx2* knockdown zebrafish larvae.
(A and B) Incident light images of 72 hpf zebrafish larvae indicate the presence of iridophores in the trunk and tail (A and B). Lateral views, anterior to the left. Scale bars: 100 μm. (C) The number of iridophores in *gbx2* MO injected-larvae is significantly reduced, when compared to that in *gbx2* 5mis MO-injected larvae. n = number of larvae examined. $^{***}p < 0.001$ by Student's *t*-test. Error bars represent the standard deviation.