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The role of *gastrulation brain homeobox 2* (*gbx2*) in the development of the ventral telencephalon in zebrafish embryos

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ABSTRACT

During vertebrate brain development, the *gastrulation brain homeobox 2* gene (*gbx2*) is expressed in the forebrain, but its precise roles are still unknown. In this study, we addressed this issue in zebrafish (*Danio rerio*) first by carefully examining *gbx2* expression in the developing forebrain. We showed that *gbx2* was expressed in the telencephalon during late somitogenesis, from 18 h post-fertilization (hpf) to 24 hpf, and in the thalamic primordium after 26 hpf. In contrast, another *gbx* gene, *gbx1*, was expressed in the anterior-most ventral telencephalon after 36 hpf. Thus, the expression patterns of these two *gbx* genes did not overlap, arguing against their redundant function in the forebrain. Two-color fluorescence in situ hybridization (FISH) showed close relationships between the telencephalic expression of *gbx2* and other forebrain-forming genes, suggesting that their interactions contribute to the regionalization of the telencephalon. FISH further revealed that *gbx2* is expressed in the ventricular region of the telencephalon. By using transgenic fish in which *gbx2* can be induced by heat shock, we found that *gbx2* induction at 16 hpf repressed the expression of *emx3*, *dlx2a*, and *six3b* in the ventral telencephalon. Among secreted factor genes, *bmp2b* and *wnt1* were repressed in the vicinity of the *gbx2* domain in the telencephalon. The expression of forebrain-forming genes was examined in mutant embryos lacking *gbx2*, showing *emx3* and *dlx2a* to be upregulated in the subpallium at 24 hpf. Taken together, these findings indicate that *gbx2* contributes to the development of the subpallium through its repressive activities against other telencephalon-forming genes. We further showed that inhibiting FGF signaling and activating Wnt signaling repressed *gbx2* and affected the regionalization of the telencephalon, supporting a functional link between *gbx2*, intracellular signaling, and telencephalon development.

1. Introduction

The gastrulation brain homeobox (Gbx) group of transcription factor genes, composed of two genes, *gbx1* and *gbx2,* in vertebrates, is also present in invertebrates (Chiang et al., 1995), and can be regarded as widely conserved among animals. In particular, roles in various aspects of vertebrate development have been reported for *gbx2* in a variety of vertebrate species. At the primitive streak stage in mouse and chick embryos (Niss and Leutz, 1998; Wassarman et al., 1997), *gbx2* is expressed in the posterior neural plate and negatively interacts with *otx2*, which is expressed in the anterior neural plate. Mutual repression between these two genes contributes to the positioning of the midbrain-hindbrain boundary (MHB) (Simeone, 2000), which works as the local organizer patterning the midbrain and cerebellum (Nakamura et al., 2005; Rhinn and Brand, 2001) and thus called the isthmic organizer. Importantly, mouse embryos lacking *Gbx2* in rhombomere 1 (r1) after embryonic stage 9 (E9) still developed the cerebellum, although the expression of the isthmic organizer genes remained dependent on *Gbx2*, suggesting a distinct requirement for this gene in cerebellum formation (Li et al., 2002).

Along with another research group, we have shown that *gbx2* is expressed in zebrafish (*Danio rerio*) embryos only after the late gastrula stage in the anterior hindbrain, and that *gbx1* is instead expressed in the posterior neuroectoderm from the early gastrula to the early somitogenesis stages (Kikuta et al., 2003; Rhinn et al., 2003). Thus, *gbx1* determines the MHB position, whereas *gbx2* is involved in the formation and maintenance of the isthmus structure at the MHB (Kikuta et al., 2003). It is likely that the roles performed by *gbx2*

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throughout MHB development in mice and chicks are played sequentially by *gbx1* and *gbx2* in zebrafish, and this functional division of labor may be due to alterations in the *cis*-regulatory elements of the *gbx* genes during vertebrate evolution (Islam et al., 2006). Meanwhile, studies using zebrafish *gbx1* and/or *gbx2* mutants showed that the isthmus was severely disrupted only when both *gbx1* and *gbx2* functions were affected, raising the possibility that the two zebrafish *gbx* genes function redundantly in MHB formation (Su et al., 2014). In fact, as *gbx1* and *gbx2* suppress the development of the anterior brain in very similar manners (Kikuta et al., 2003), it is likely that they possess similar biochemical properties. However, gene disruption in zebrafish often gives rise to silent mutations due to genetic compensations by paralogous genes (Rossi et al., 2015), making further studies necessary to reveal the exact functions of respective *gbx* genes. Recently, we re-examined the roles of *gbx2* in MHB formation using transgenic (Tg) zebrafish (*Tg(hsp70l:gbx2)*), harboring heat-inducible *gbx2* (*hsp-gbx2*) (Nakayama et al., 2013). By inducing *gbx2* in embryos of this Tg line, we found that the sensitivity of the neural plate to *gbx2* activity is highest at the bud stage, suggesting that *gbx2* plays a crucial role in MHB formation at this particular stage. The analyses using this Tg line further showed that *gbx2* upregulates or downregulates many developmental regulatory genes, and that *gbx2* drives neurogenesis apart from regulating MHB development (Nakayama et al., 2017).

Additionally, *gbx2* functions in a variety of developmental processes after MHB establishment. This gene was shown to be required for neural crest (NC) formation in mice (Li et al., 2009; Roeseler et al., 2012). It was further shown in *Xenopus* that *gbx2* is the earliest factor for specifying NC cells, and that *gbx2* is directly regulated by NC-inducing signaling pathways, such as Wnt/β-catenin signaling (Li et al., 2009). In the spinal cord, cell-tracking experiments showed that mouse *Gbx2* is expressed in motor neurons and interneurons. In fact, interneuron precursor cells and spinal cord patterning are abnormal in *Gbx2* mutant embryos at E12.5 (Luu et al., 2011).

In the forebrain, murine *Gbx2* is expressed in the medial ganglionic eminence (MGE) of the basal ganglia, which is the primordium of the pallidum, and in the thalamus at E12.5 (Bulfone et al., 1993; Waters et al., 2003). In the MGE, tangentially migrating *Gbx2* lineage-derived cells exclusively give rise to striatal cholinergic interneurons, whereas radially migrating *Gbx2* lineage cells mainly give rise to GABAergic and other non-cholinergic neurons in the basal forebrain. *Gbx2* deletion leads to an abnormal distribution and significant reduction of cholinergic neurons in the striatum (Chen et al., 2010). In the thalamus primordium, mouse *Gbx2* is activated in neural progenitors of different thalamic nuclei at particular stages, contributing to their differentiation (Li et al., 2012; Mallika et al., 2015). In addition, conditional disruption showed that *Gbx2* is also involved in thalamocortical projections from the thalamus to the cortex (Li et al., 2012).

In contrast to murine *Gbx2* and zebrafish *gbx1/gbx2*, murine *Gbx1* is not expressed in the anterior-most hindbrain, but is expressed in r3 and r5 of the hindbrain, optic vesicles, and MGE (Waters et al., 2003). In the basal telencephalon, *Lhx7* and *Gbx1* play roles in the development of the cholinergic system (Asbreuk et al., 2002). In the dorsal horn of the spinal cord, *Gbx1* controls the differentiation of a specific subset of GABAergic neurons (John et al., 2005). Furthermore, *Gbx1*-null mice showed gross locomotive defects, disruption of the proprioceptive sensorimotor circuit within the spinal cord, and a reduction in *Isl1*-positive ventral motor neurons (Buckley et al., 2013). Therefore, *Gbx1* and *Gbx2* apparently function in different aspects of mouse brain formation except for the MGE, where both genes are reportedly expressed at the same stage (Waters et al., 2003).

Abundant evidence from mammalian studies indicates that cortical interneurons comprise distinct neuronal subpopulations by their anatomical, neurochemical, and physiological properties (Markram et al., 2004). In the late 1990s, it was discovered that GABAergic interneurons arise not in the pallium, but in distinct subpallial regions (Wonders and Anderson, 2005). Specifically, fate-mapping and loss-of-function analyses showed that many cortical interneurons arise from the MGE and caudal ganglionic eminences (CGE). Many studies have suggested that the different subpopulations of interneurons are derived from regional differences in the ventral telencephalon, or subpallium, which depend on the expression of particular combinations of transcription factors (Flames et al., 2007; Wonders and Anderson, 2006; Wonders et al., 2008). However, the factors governing the generation of interneuron diversity in the cerebral cortex and the functional involvement of mouse *Gbx1* and *Gbx2* in this process still remains unclear.

In zebrafish embryos, *gbx2* is expressed in the forebrain after MHB establishment, as in mice. At 24 h post-fertilization (hpf), *gbx2* expression was noted in the telencephalon (Su and Meng, 2002). Consistent with this observation, we found that the upstream DNA of *gbx2* drives transcription in the telencephalon besides the MHB region (Islam et al., 2006). The expression of *gbx2* in the thalamus, which is well established in mice, was also observed in zebrafish (Kikuta et al., 2003). However, the details of the forebrain expression of zebrafish *gbx2* and its significance have not been examined. It is also unknown whether *gbx1* is involved in forebrain development, although its expression has been noted in the ventral telencephalon (Rhinn et al., 2003).

Studies in zebrafish brains have also demonstrated that the basic organization of the telencephalon has been conserved during vertebrate evolution (Mueller and Wullimann, 2009; Wullimann, 2009), and that the subpallium is the origin of GABAergic interneurons, as in mammals (Mione et al., 2008). Since this animal model provides an excellent platform for analyzing the molecular mechanisms of vertebrate brain development, elucidating the role of zebrafish *gbx* in subpallial development will greatly contribute to the overall understanding of the generation of various neurons in the telencephalon.

In the present study, we investigated whether zebrafish *gbx* genes are directly involved in the development of the telencephalon, as has been shown in mice. We carefully examined the expression of the two *gbx* genes, especially *gbx2*, in the anterior brain after somitogenesis. We also analyzed the effects of the overexpression and disruption of *gbx2* on telencephalon development. Our results show that *gbx2* is expressed in the ventricular zone of the telencephalon and involved mainly in the development of the ventral telencephalon.

2. Materials and methods

2.1. Fish husbandry and maintenance

Adult zebrafish were maintained at 27 °C under a 14-h light:10-h dark cycle. Embryos were raised at 25 °C or 28.5 °C until they reached appropriate stages. Morphological features and hpf were used to stage embryos (Kimmel et al., 1995). When necessary, 0.2 mM 1-phenyl-2-thiourea (Nacalai Tesque) was added to cultures to prevent pigment formation. The Tg fish line harboring heat shock-inducible *gbx2*, *Tg(hsp70l:gbx2)*, was established previously (Nakayama et al., 2013). The *gbx2*-null mutant fish line (*gbx2*^{fh253}) used in this work was established by the Targeting Induced Local Lesions in Genomes (TILLING) method (Su et al., 2014). All experiments using live fish were conducted in accordance with the protocols approved by the Committee for Animal Care and Use of Saitama University.

2.2. Whole-mount in situ hybridization

Digoxigenin (DIG)- or fluorescein (FLU)-labeled RNA probes were synthesized using T3 or T7 RNA polymerases (Agilent Technologies) with the DIG RNA Labeling Mix (Roche Diagnostics) or the FLU RNA Labeling Mix (Roche Diagnostics), respectively, according to the manufacturers' protocols. Whole-mount in situ hybridization (WISH) was performed essentially as described previously (Kikuta et al., 2003; Nakayama et al., 2013).

Images of WISH-stained embryos were captured using a Leica