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Evolution of Developmental Control Mechanisms

Analyses of fugu *hoxa2* genes provide evidence for subfunctionalization of neural crest cell and rhombomere *cis*-regulatory modules during vertebrate evolution

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ABSTRACT

Hoxa2 gene is a primary player in regulation of craniofacial programs of head development in vertebrates. Here we investigate the evolution of a *Hoxa2* neural crest enhancer identified originally in mouse by comparing and contrasting the fugu *hoxa2a* and *hoxa2b* genes with their orthologous teleost and mammalian sequences. Using sequence analyses in combination with transgenic regulatory assays in zebrafish and mouse embryos we demonstrate subfunctionalization of regulatory activity for expression in hindbrain segments and neural crest cells between these two fugu co-orthologs. *hoxa2a* regulatory sequences have retained the ability to mediate expression in neural crest cells while those of *hoxa2b* include *cis*-elements that direct expression in rhombomeres. Functional dissection of the neural crest regulatory potential of the fugu *hoxa2a* and *hoxa2b* genes identify the previously unknown *cis*-element NC5, which is implicated in generating the differential activity of the enhancers from these genes. The NC5 region plays a similar role in the ability of this enhancer to mediate reporter expression in mice, suggesting it is a conserved component involved in control of neural crest expression of *Hoxa2* in vertebrate craniofacial development.

1. Introduction

In vertebrates, Hoxa2 is an important regulator of both hindbrain development and cranial neural crest patterning. Mouse loss-of-function studies have revealed multiple roles for *Hoxa2* during central nervous system (CNS) development, including regulation of properties of specific hindbrain segments or rhombomeres (r) (Davenne et al., 1999; Gavalas et al., 1997; Oury et al., 2006; Ren et al., 2002). In head development, *Hoxa2* expression is also spatially-restricted in cranial neural crest cells where convincing evidence implicate it as a master regulator of craniofacial programs and jaw formation (Couly et al., 2002, 1998; Hunt et al., 1991a). Mouse *Hoxa2*$^{-/-}$ mutants display duplications of lower jaw elements, such as Meckel's cartilage (Gendron-Maguire et al., 1993; Rijli et al., 1993; Santagati et al., 2005). Conversely, *Hoxa2* gain-of-expression experiments in a number of vertebrates, including mice, Xenopus and chickens have shown that ectopic *Hoxa2* represses jaw formation (Grammatopoulos et al., 2000; Kitazawa et al., 2015; Pasqualetti et al., 2000), in part through its ability to prevent chondrogenesis and inhibit bone formation (Kanzler et al., 1998). In compound mouse mutants where *Hoxa2* and *Hoxb2* are both lost, there appears to be very little difference in phenotypes, suggesting the *Hoxb2* paralog has a relatively minor input into cranial neural crest patterning (Santagati and Rijli, 2003). However, deletions of entire Hox clusters indicate that other *HoxA* genes and *HoxB* genes do contribute to craniofacial regulatory programs (Minoux et al., 2009; Vieux-Rochas et al., 2013). There is evidence in zebrafish (*Danio rerio*), based on morpholino experiments, that the functional roles for *hoxa2* are partially compensated for by its paralog *hoxb2*, as both genes must be knocked down to generate phenotypes analogous to those observed in mouse *Hoxa2* mutants alone (Hunter and Prince, 2002). In most vertebrates Hoxa2 is proposed to be a primary node of

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transcriptional regulation in controlling craniofacial programs to facilitate proper formation of the vertebrate jaw and pharyngeal arches (Minoux and Rijli, 2010; Santagati and Rijli, 2003).

Following their divergence from the tetrapods, the teleosts or ray-finned fish underwent genome duplication (Meyer and Malaga-Trillo, 1999; Meyer and Schartl, 1999; Meyer and Van de Peer, 2005; Ravi and Venkatesh, 2008; Smith and Keinath, 2015; Volff, 2005). This led to the duplication of a number of genes relative to mammals, including Hox genes. The mouse genome has a single *Hoxa2* gene that is expressed in the second branchial arch and in hindbrain rhombomeres, while there are two *hoxa2* co-orthologous genes present in striped bass (*Morone saxatilis*), fugu (*Takifugu rubripes)*, Nile tilapia (*Oreochromis niloticus*), cichlid (*Astatotilapia burtoni*), and medaka (*Oryzias latipes*). These duplicated *hoxa2* genes can display divergent patterns of expression in their respective teleost species. For example, in striped bass *hoxa2a* is expressed in r2-r7 and in the second pharyngeal arch while *hoxa2b* is expressed in r2-r5; whereas in fugu *hoxa2a* is expressed in thin stripes in r1-r2 while *hoxa2b* is expressed in r2-r5 (Scemama et al., 2002, 2006). This implies that there has been significant alteration in the *cis*-elements that govern hindbrain and pharyngeal arch expression leading to divergent patterns for *hoxa2* co-orthologs in several teleosts. Through mutation some teleosts have lost one of the duplicated *hoxa2* genes or one co-ortholog has become a non-functional pseudogene. Examples of this are medaka, with a single functional *hoxa2a* gene and a *hoxa2b* pseudogene, and zebrafish with a functional *hoxa2b* and a *hoxa2a* pseudogene (Davis et al., 2008; Hunter and Prince, 2002). In both these instances the single functional *hoxa2* ortholog has an expression pattern which matches that of mouse *Hoxa2,* in contrast to the variant expression patterns seen with the two functional co-orthologs in striped bass and fugu.

This gene duplication, divergence, and differential expression in teleosts can be informative from a regulatory standpoint in helping to identify *cis*-elements required for tissue-specific gene expression. Functional domains within and around a gene have a slower rate of change compared to non-functional domains, presumably due to selective pressures. However, following gene duplication, functional regions of one gene can be free to mutate if its paralog preserves or fulfills the relevant functions. Through this process, paralogous genes can adopt new functions (neofunctionalization) or partition ancestral functions between the duplicate genes (subfunctionalization) (Jimenez-Delgado et al., 2009). An example of regulatory subfunctionalization can be seen when comparing mouse *Hoxb1* to the zebrafish *hoxb1* genes, *hoxb1a* and *hoxb1b*. Zebrafish *hoxb1b* is expressed in early gastrula stage embryos, like its mouse counter-part, through a conserved pair of retinoic acid response elements (RAREs) located downstream of both the mouse and zebrafish genes (Marshall et al., 1994; McClintock et al., 2001, 2002). In contrast, the zebrafish *hoxb1a* gene is the co-ortholog expressed in r4, like the mouse gene, through an equivalent highly conserved Hox auto- and cross-regulatory *cis*-element (McClintock et al., 2002; Pöpperl et al., 1995; Studer et al., 1998, 1994). Therefore, each individual zebrafish *hoxb1* co-ortholog has maintained separate functions of the ancestral *Hoxb1* gene, in part through conservation and divergence of *cis*-regulatory elements essential for modulating distinct aspects of the expression pattern. Therefore, non-coding sequences conserved only in teleost paralogs, whose gene expression matches specific aspects of their tetrapod counter-parts, have the potential to be associated with distinct regulatory activities.

Compared with hindbrain rhombomeres and segmentation, very little is known about the upstream regulatory network, signals and transcription factors that couple *Hox* expression to cranial neural crest patterning (Hunt et al., 1991a, 1991b). With respect to *Hoxa2*, an enhancer that mediates its spatially-restricted and temporally dynamic patterns of expression in cranial neural crest cells important for craniofacial patterning, has been identified upstream of the mouse gene (Maconochie et al., 1999). This neural crest enhancer contains four separate regions (NC1-NC4) which are all required for regulatory activity. The NC4 *cis*-element contains binding sites for the AP2 family of transcription factors, known to be important for neural crest development (Mitchell et al., 1991; Morriss-Kay, 1996; Schorle et al., 1996).

The *Hoxa2* neural crest enhancer is embedded within a highly conserved enhancer that mediates segmental expression of the gene in r3 and r5 (Maconochie et al., 1999, 2001; Nonchev et al., 1996a, 1996b; Parker et al., 2014). Hence, it has been challenging to characterize the evolution of this neural crest enhancer independent of constraints on the rhombomeric enhancer. In regulatory analyses of the two fugu *Hoxa2* co-orthologous genes, *hoxa2a* and *hoxa2b,* using reporter genes in transgenic chicken embryos, evolutionary divergence in key *cis*-elements of the enhancer regions that mediate segmental expression in hindbrain rhombomeres has previously been shown to account for their differential gene expression (Tümpel et al., 2006). However, no reproducible neural crest regulatory activity with either enhancer was detected in these assays in chicken embryos. It is surprising that the regulatory assays of the fugu *hoxa2a* and *hoxa2b* enhancer in chicken embryos failed to uncover any robust cranial neural crest regulatory potential in either enhancer. In light of the fact that in zebrafish *Hoxb2* partially compensates for the function of *Hoxa2* in neural crest cells (Hunter and Prince, 2002), it is possible that the fugu *Hoxa2* genes are not expressed in neural crest and their functional roles have been taken over by *Hoxb2*. Alternatively, *cis*-elements directing neural crest expression in fugu may not function effectively in the chicken embryo or there may be species-specific differences in their requirements.

In this study, since *Hoxa2* has such a key role in craniofacial patterning in many vertebrates, we addressed this important problem by investigating the evolution of the neural crest regulatory potential of the fugu *hoxa2a* and *hoxa2b* enhancers. Our findings reveal that there has been subfunctionalization of neural crest activity between the two co-paralogs, such that fugu *hoxa2a* appears to retain the ability to mediate expression in neural crest cells while *hoxa2b* possesses the *cis*-elements that direct expression in rhombomeres. Furthermore, our sequence comparisons and regulatory analyses have uncovered an additional *cis*-element (NC5) which plays a conserved role in potentiating neural crest regulatory activity of this enhancer.

2. Materials and methods

2.1. Transgenic zebrafish and mouse reporter assays

Slusarski AB (wild type) and *egr2b:KalTA4BI-1UASkCherry* (r3r5-mCherry) (Distel, Wulliman, Koster 2009) lines were maintained at 28 °C. Embryos were raised in Embryo Medium (Nüsslein-Volhard and Dahm) and staged according to hours post-fertilization (hpf). Enhancer regions to be tested were inserted, using Gateway cloning, into a Tol2 transposon based vector containing a cFos minimal promoter-EGFP reporter cassette (pGW-cfos-EGFP) (Fisher et al., 2006). Transient transgenic fish embryos (F_0) were injected at the 1-cell stage with an injection mix of phenol red (0.05%), Tol2 transposase (Fisher et al., 2006) and the expression vectors. Each embryo was injected with a bolus (visualized by the phenol red) one-fifth the size of the cell formed once the cytoplasm begins to separate from the yolk towards the animal pole. A minimum of 350 embryos were injected to account for mosaicism and position effects. Embryos expressing GFP were raised to adulthood and crossed to wild-type fish to create stable transgenic