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Comparison of the expression of medaka (*Oryzias latipes*) *pitx* genes with other vertebrates shows high conservation and a case of functional shuffling in the pituitary

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Abstract

With the availability of an increasing number of whole genome sequences in chordates, exhaustive comparisons of multigene families become feasible. Relationships of orthology/paralogy can not only be inferred from sequence similarity but also by comparing synteny conservation on chromosomes. More accurate scenarios for gene and expression domain gain or loss can now be proposed. Here, we take benefit from the recent release of the medaka (*Oryzias latipes*) genome to analyse the orthology relationships and expression patterns of the three different sub-families of the *pitx* homeobox genes belonging to the *paired* class. They are involved in a wide variety of developmental processes and have pleiotropic expression patterns, especially in the case of the *pitx2* sub-family. The emerging picture is a strong conservation of expression domains, suggesting that most functions have been present in the common ancestor of actinopterygians and sarcopterygians. Almost all *pitx* genes are expressed in anterior placodes in all species studied so far, including medaka. It has previously been shown that in mammals, *pitx1* and *2* are expressed in the pituitary. Interestingly we demonstrate here that only *pitx3* is expressed in medaka pituitary. It will be interesting to analyze what are the corresponding changes in the regulatory elements of *pitx* genes.

Keywords: Zebrafish, Placode, Stomodaeum, Hypophysis, Evolution, Development

1. Introduction

Gene duplications are known to have occurred early in the vertebrate lineage, prior to gnathostome radiation (Abi-Rached et al., 2002; Robinson-Rechavi et al., 2004). It is important to determine the relationships of paralogy or orthology between the genes created by these processes, as well as the gain or loss of expression domains of pleiotropically expressed paralogues, and finally the chronology of fixation of their actual functions. These data constitute a mandatory prerequisite for further studies aimed at documenting, for instance, the changes that have occurred at the level of regulatory *cis*-acting sequences, which are often considered to be the driving force in the evolution of genetic networks (Carroll et al., 2001).

To date however, such precise results on phylogenetic relationships and expression patterns have been obtained only for a limited number of gene families (Ekker et al., 1997; Avaron et al., 2003; Coolen et al., 2005). Homeobox gene families are especially attractive for that kind of analysis, because i) they are well known to play major roles in the body patterning and differentiation events taking place during animal development, ii) in the gnathostomes, they constitute well-defined multigene families with a limited number of members, and iii) very often, single orthologues have been characterised, in urochordates or in cephalochordates.

Their classification is based on sequence similarities in the homeodomain motif (Duboule, 1994), or on the presence of specific peptides, as is the case for the *bicoid*-type homeoboxes

Abbreviations: ANB, anterior neural boundary; WMISH, whole-mount *in situ* hybridization.

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of the *paired-like* class, which possess a characteristic lysine residue in position 50. Within this family, the whole *Otx* gene family has been submitted to complete analysis of expression patterns, which led to the conclusion that specific functions of each *Otx* orthology class were fixed prior to the gnathostome radiation. These studies have underlined the prominent role played by regulatory changes in the functional diversification of this gene family (Plouhinec et al., 2005).

Here we analyse another homeobox gene family, the pituitary homeobox (*pitx*) family, which contains three members in gnathostomes. Given the above-mentioned occurrence of gene duplications in this animal group (and notably in the teleostean lineage), this small and stable number of *pitx* paralogues implies that many (presumably independent) gene losses have occurred in the course of gnathostome evolution. Indeed, the most common fate of one of the duplicates is to "degenerate" into a pseudogene (Li et al., 1981; Lynch and Conery, 2000).

Pitx genes have been first isolated in mammals as transcriptional activators of endocrine genes (*pitx1*, (Szeto et al., 1996)), by positional cloning for the Axenfeld–Rieger syndrome (*pitx2*, (Semina et al., 1996)), or by homology cloning (*pitx3*, (Semina et al., 1997)). Later, several isoforms have been identified in various chordates (Arakawa et al., 1998; Essner et al., 2000; Schweickert et al., 2000; Tremblay et al., 2000; Boorman and Shimeld, 2002a,b; Christiaen et al., 2005). In addition to the homeodomain, Pitx proteins exhibit a 14 amino acid protein–protein interaction domain called Aristaless (Meijlink et al., 1999) and a C-terminal CQY motif. They participate in many developmental processes: all three play important roles in the development of the pituitary gland (Lamonerie et al., 1996; Szeto et al.,1996; Tremblay et al., 1998); *pitx1* and *pitx2* are involved in the development of the hindlimbs (Lanctot et al., 1999b; Logan and Tabin, 1999; Szeto et al.,1999), teeth (Fraser et al., 2006) as well as in the determination of left–right asymmetry of the embryonic limbs, heart, lungs, and digestive tract (Yoshioka et al., 1998; Campione et al., 1999; Logan and Tabin,1999; Fraser et al., 2006). Indeed, an isoform-specific asymmetric expression in the lateral plate mesoderm and heart is a distinctive feature of the *pitx2* sub-family. *Pitx3* has a more restricted expression in tetrapods, but mutations in this paralogue are known to lead to dominant cataracts and malformations of the eye mesenchyme (Semina et al., 1997). Also, mammalian *pitx* genes are expressed in the midbrain where they regulate the fate of dopaminergic neurons (Messmer et al., 2007).

Here, we re-examine the orthology relationships and genomic contexts in the *pitx* family through a survey of teleosts and human genomes and we study the expression patterns of the three paralogues in the medaka *Oryzias latipes*, a small freshwater teleost possessing many of the biological properties which makes the zebrafish an attractive model (Wittbrodt et al., 2002). Medaka are also quite easy to breed and amenable to transgenesis (Thermes et al., 2002). More importantly in the context of this study, they are distantly related to zebrafish, a position making them especially valuable for comparative functional genomic studies.

Our results clearly point to a conservation of the expression domains of the three *pitx* orthologues within teleosts, as well as between teleosts and tetrapods, a fact in agreement with the idea that most developmental functions of *pitx* genes were acquired early during, or even before, the gnathostome radiation (Boorman and Shimeld, 2002a). However, a careful examination of *pitx* expression patterns in the anterior placodes and developing pituitary and lens provides a clear example of function shuffling between paralogous genes. These results will be of interest in a future analysis of *pitx cis*-regulatory sequences evolution. In this context, it is noteworthy that conserved non-coding sequences are especially numerous in the gene desert found upstream from the *pitx2* genes, the sub-family having the most diversified expression patterns in vertebrates.

2. Materials and methods

2.1. Cloning of Ol-pitx genes and probe synthesis

BLAST searches of *pitx* genes in the genome of the medaka (medaka genome sequencing project: (http://dolphin.lab.nig.ac.jp/medaka/)) allowed the identification of three scaffolds containing at least the third exon of *pitx* genes. Sequences can now be found at http://www.ensembl.org/Oryzias_latipes/familyview?family=ENSF00000001786.

A phylogenetic analysis confirmed that the three *Oryzias latipes* sequences identified belong to the three well known subfamilies of vertebrate *pitx* genes (*pitx1*, *pitx2*, *pitx3*) (data not shown). In order to synthesize antisense RNA probes, exonic regions were amplified from genomic DNA with oligos located at the C-terminal extremity (pitx1/3-F : 5′-GTGTGGTTYAAAAACMGSCG; pitx1-R: 5′-TCAGCTGTTGTACTGGCAGGC; pitx3-R : 5′-TCATACCGGCCGGTCYACAGC; pitx2-F : 5′-GTTTGGTTCAAGAACAGGCG; pitx2-R : 5′-SACCGGYCTRTCCACKGCGTA). We thus ensured that probes exhibit a high level of identity only in the very short 3′ part of the homeobox and have no risk to cross-hybridize during whole-mount *in situ* hybridization (WMISH). The length of the sequence of each RNA probe used for *in situ* hybridizations are 549, 555 and 569 bp, for the *pitx1, 2* and *3* genes, respectively. PCR products were cloned in pGEM-T. Antisense RNA digoxigenin-UTP probes were prepared according to Joly et al. (1997) using T7 polymerase.

2.2. Analysis of pitx loci

To confirm the hypotheses of orthology suggested by phylogenetic analysis, we looked at human, mouse, *Xenopus*, zebrafish and medaka *pitx* paralogs in respect to their flanking genes with the Ensembl Database Version 42. We used ENSEMBL Compara (Hubbard et al., 2007) to extract orthologous genes. Gene models were then redrawn (Fig. 1) as boxes to show the relative distances between genes based on ENSEMBL Multicontigview Release 42.

2.3. Whole-mount in situ hybridization (WMISH)

Medaka embryos of a Carbio strain were used in all experiments. Embryos were collected daily and incubated in