Altered retinoic acid signalling underpins dentition evolution

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Small variations in signalling pathways have been linked to phenotypic diversity and speciation. In vertebrates, teeth represent a reservoir of adaptive morphological structures that are prone to evolutionary change. Cyprinid fish display an impressive diversity in tooth number, but the signals that generate such diversity are unknown. Here, we show that retinoic acid (RA) availability influences tooth number size in Cyprinids. Heterozygous adult zebrafish heterozygous for the cyp26b1 mutant that encodes an enzyme able to degrade RA possess an extra tooth in the ventral row. Expression analysis of pharyngeal mesenchyme markers such as dlx2a and lhx6 shows lateral, anterior and dorsal expansion of these markers in RA-treated embryos, whereas the expression of the dental epithelium markers dlx2b and dlx3b is unchanged. Our analysis suggests that changes in RA signalling play an important role in the diversification of teeth in Cyprinids. Our work illustrates that through subtle changes in the expression of rate-limiting enzymes, the RA pathway is an active player of tooth evolution in fish.

1. Introduction

Small variations in signalling molecules linked to species variation were first demonstrated in Darwin’s finches with bmp4 demonstrated to be a major actor in beak shape variation [1]. To identify additional signalling molecules that play a role during evolution, a number of criteria are likely to be important: (i) that a particular tissue or organ displays sufficient variation in closely related species and (ii) that the developmental biology of the organ or tissue development is sufficiently known and can be manipulated. Teeth represent an ideal candidate tissue for such studies as they display an impressive diversity in terms of size, shape, number and localization [2]. With more than 3000 species, Cypriniformes is the most diverse order of freshwater fishes with an impressive diversity in pharyngeal dentition [3]. Retinoic acid (RA) is a morphogen molecule with pleiotropic roles during embryonic development in vertebrates [4] and has long been proposed to play an important role during evolution [5,6]. RA levels are tightly controlled in the developing embryo, mainly by the expression of specific synthetizing (aldh1a) and degrading (cyp26) enzymes. These provide the ability to precisely govern the local levels of RA during development, alteration in which could impact developmental processes. We have previously established that RA is required for tooth induction in the Cypriniform zebrafish [7]. Here, we investigate how local levels of RA signalling in the tooth-forming region influence tooth number, providing a mechanism to underpin dentition evolution in Cyprinids.

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2. Results

(a) Cyprinid’s dentition is highly diverse

We previously characterized the diversity of tooth row organization and tooth shape in Cyprinids [8,9]. Whereas in most species, the main tooth row contains five teeth, we found at least four independent cases in which six teeth are present on the fifth ceratobranchial bone (red circles, figure 1): in Cultrinae, in which at least three genera form a monophyletic group [10]; in Leuciscinae, in which *Rutilus* and *Chondrostoma* represents two independent lineages [11] harbouring six teeth; and in *Raiamas*, a Danioninae distantly related to the zebrafish. The case of *Rutilus* is particularly interesting as it displays an asymmetric organization with the left ceratobranchial harbouring six teeth, whereas the right one contains only five (see the electronic supplementary material, figure S1 for more details).

This suggests that there were convergent gains of one tooth during evolution in these various clades.

(b) Retinoic acid signalling in the tooth-forming region

To test if RA signalling plays a role in the diversification of the tooth row in Cyprinids, we first studied the expression of RA signalling pathway genes during the development of the first three teeth (4V1, which appeared first, and then 3V1 and 5V1), and the first replacement tooth 4V2 [12,13]. *aldh1a2*, the rate-limiting enzyme of RA formation required for tooth induction, is expressed in the posterior ventral pharynx from 24 to 72 hpf [7,14], but excluded from the tooth bud itself (figure 2a, f, g). We found that *cyp26b1*, an RA degrading enzyme [15,16], is expressed at 48 hpf, where the first tooth 4V1 will be formed, but also in the entire lateral length of
the ventral pharynx, where other teeth will subsequently emerge from 48 to 52 hpf (figure 2b,c). Later in development, cyp26b1 is expressed in the mesenchyme of the second forming tooth 3V1, whereas expression remains detected in a broader region that contains more than the tooth buds (figure 2d). At 96 hpf, expression is detected in the pharyngeal epithelium surrounding half of the first replacement tooth germ 4V2 (figure 2e; the same pictures without any annotation are available in the electronic supplementary material, figure S2). We also checked the expression pattern of the other cyp26 enzymes (cyp26a1, cyp26c1) and showed that they are not expressed in the tooth-forming region at these stages (see the electronic supplementary material, figure S3). In zebrafish, the RA receptors α (RARAα-A and α-B) are implicated in 4V1 induction [7,17]. We monitored their expression at the time of 4V1 (43 hpf) and 3/5V1 (48 hpf) induction [18], and found them expressed in the entire ventralmost part of the pharynx, not restricted to the region of 4V1 or 3/5V1 induction (figure 2f,i). These expression patterns suggest that RA is produced broadly in the posterior ventral pharynx, but its availability is tightly regulated by cyp26b1 expression, which prevents RA being present outside of its domain of action in both a spatial and temporal manner (figure 2f).

(c) Retinoic acid signalling controls tooth number

Based on this tight regulation of RA availability in the ventral posterior pharynx, we then studied the effect of exogenous RA on the development of the tooth row. Under RA exposure at 10⁻⁸ M from 56 hpf to 12 days post fertilization (dpf), embryos often (55% of observed cases, n = 18) display six rather than five teeth in each pharyngeal jaw at 14 dpf detected either by Alcian blue or by Alizarin red staining (figure 3a). The presence of this extra tooth is difficult to interpret as it could correspond to an extra tooth in the row or, alternatively, to an earlier appearance of the first replacing tooth 4V2 [19]. As it is very difficult to discriminate between these possibilities, we studied this effect in adult fishes in which the amount of RA varies within a more physiological range. For this, we used the stocksteif (sst) mutant in which the cyp26b1 gene is mutated, therefore compromising RA degradation and leading to more endogenous RA [16], especially in the

Figure 2. Retinoic acid signalling in the tooth-forming region. (a) Transverse sections of the posterior ventral pharynx of a 48 hpf Danio rerio embryo stained with haematoxylin and eosin. The 4V1 tooth germs are marked by dashed lines. (b–e) Transverse sections of the posterior ventral pharynx of embryos post in situ hybridization embryos stained with cyp26b1 at (b) 48 hpf, (c) 52 hpf, (d) 80 hpf and (e) 96 hpf. The 4V1 tooth bud is marked by dashed lines in (b,c). The 3V1 and 4V2 tooth buds are marked by dashed lines in (d) and (e), respectively. (f) Transverse and (g) longitudinal sections of the posterior ventral pharynx of an embryo post in situ hybridization embryos stained with aldh1a2 at 48 hpf. 4V1 is marked in (f), whereas expression in the ventral posterior pharynx in the vicinity of the forming tooth bud is indicated by an arrow in (g). Note that aldh1a2 expression is excluded from the developing tooth bud in (f). (h) rara and (i) rarab expression in transverse sections of the posterior ventral pharynx at 48 hpf. The position of the 4V1 tooth bud is indicated. Note that both rara and rarab are expressed within the tooth bud but also in the entire posterior ventral pharynx. (j) Summary of the temporal and regional expression pattern of RA signalling actors: at 36 hpf, aldh1a2 is expressed in the ventral pharynx until 48 hpf. cyp26b1 expression starts at 50 hpf in the tooth bud mesenchyme. rara and rarab are expressed through the entire ventral pharynx. The RA availability within the tooth-forming region is temporally sharpened by the expressions of both synthesizing (aldh1a2) and degrading (cyp26b1) enzymes. Scale bars, 25 μm.
ventral posterior pharynx where cyp26b1 is highly expressed (figure 2b). Using computed microtomography, we observed a high penetrance (51%, n = 45) of tooth rows harbouring six teeth in the adult sst heterozygous mutants, whereas five were observed in wild-type fishes (figure 3c,d). Among the heterozygous fish that had a sixth tooth, 65% had unilateral extra tooth, whereas 35% had bilateral formation of an extra tooth. This sixth tooth is present in an extra position in the normal row as it is present in wild species harbouring six teeth in the first row (e.g. *Rutilus* or *Pseudobrama*; figure 1; electronic supplementary material, figure S1). In many cases, we observed, as in *Rutilus*, asymmetric distribution with, respectively, five and six teeth on each side. However, in contrast to *Rutilus*, in about one-third of the sample, the affected side varies randomly, exhibiting six teeth on both sides. When we compare the six-tooth row with the five-tooth row in the same individuals, we observed that the six-tooth row is greater (113% the size of the five-tooth row) and that the teeth are slightly shifted medially. We observed the same increase in size (112%) when comparing the right five-tooth row with the left six-tooth row in *Rutilus* (see the electronic supplementary material, figure S4). Interestingly, we observed that RA also induces the formation of an extra tooth in another cyprinid species: the goldfish *Carassius auratus*. In goldfish, four teeth are present in a single row, but after RA treatment (5/10−8 M, 2–14 dpf or 3–16 dpf), we observed a fifth tooth in about 50% (n = 15) of the treated embryos (figure 3b). This result demonstrates that RA has the same effect of increasing number of teeth in distant cyprinid species. We noted, however, that the goldfish appears to develop its extra tooth laterally, whereas the zebrafish develops it mesial to the tooth closest to the midline. This may suggest that there is an interesting difference to explore between the way these two species form their extra teeth. We concluded that a tight regulation of RA levels restricts tooth number in Cyprinids and that small local variations in RA are sufficient for the formation of an extra tooth.

**Figure 3.** Retinoic acid controls tooth number. (a) Alizarin red staining of 12 dpf untreated and treated zebrafish (*Danio rerio*) embryos with 10−8 M of RA from 56 hpf onwards. (b) Alizarin red staining of 14 dpf untreated and treated goldfish (*Carassius auratus*) embryos with 5 × 10−8 M of RA from 48 hpf onwards. A black arrowhead indicates the extra tooth induced by RA treatment. (c,d) Computed microtomography scans of (c) wild-type zebrafish adults (cyp26b1+/+) and of (d) stocksteif (sst) heterozygous mutants (cyp26b1+/−). Teeth on the ventral row are numbered and the sixth extra tooth observed in sst mutants is highlighted in red. Chromatograms of genotyping showing the A>T nonsense mutation in heterozygous is provided.
(d) Exogenous exposure expands the domain of expression of lateral pharyngeal mesenchyme markers

We investigated the expression of the dental and pharyngeal mesenchyme markers in RA-treated zebrafish embryos. Under RA exposure, *dlx2a* expression is detected in a larger and wider expression domain in the pharyngeal arch mesenchyme (figure 4a–f). This expansion of *dlx2a* expression is specific to the tooth region as its expression in the forebrain is unaffected. Similarly, *lhx6* expression, which is normally confined to the dental mesenchyme and the lateral pharyngeal mesenchyme at this developmental stage, is expressed throughout the entire pharyngeal mesenchyme and part of the epithelium under RA exposure (figure 4g–l). *pitx2a*, which is normally restricted to the dental epithelium, has its expression expanded to the pharyngeal epithelium under RA treatments (figure 4m–r). The dental epithelium-specific markers *dlx3b* and *dlx2b* do not display such a dramatic expansion of their respective domain of expression under RA treatments (figure 4n–r). At most, we note a slightly increase of expression in the dental epithelium of the 4V1 tooth bud (especially for *dlx3b*) under RA exposure. We therefore concluded from these experiments that exogenous RA exposure can expand the expression of the pharyngeal mesenchyme markers such as *dlx2a*, *lhx6* and dental epithelium *pitx2a*, providing a broader domain for tooth induction that cannot be compensated by *cyp26b1* expression owing to an excess of RA signalling in this region. However, it remains to be demonstrated that this expansion of pharyngeal markers may reflect an expansion of tooth-competent mesenchyme.

3. Discussion

Taken together, our results suggest that the RA pathway has a dramatic effect on tooth row formation in zebrafish and that modulation of RA level in zebrafish resembles what is observed in wild species: RA controls the number of teeth in the dental row and a decrease in *cyp26b1* level in *sst* heterozygous mutant shows that this effect can be observed for a relatively narrow range of concentrations. Interestingly, the variation of tooth number (from five to six teeth) has occurred several times during cyprinid evolution. Indeed, we previously characterized the diversity of tooth row organization and tooth shape in cyprinids [8]. Whereas in most species, the main tooth row contains five teeth, we found at least four independent cases in which six teeth are present on the fifth ceratobranchial bone (red circles, figure 1). This is confirmed by the analysis of tooth evolution in Leuciscinae that we are currently performing and that will be published separately (E.P.-V. and L.V. 2015, unpublished data). The case of *Rutilus* is particularly interesting as it displays an asymmetric organization with the left ceratobranchial harbouring six teeth, whereas the right one contains only five. The most parsimonious explanation of this pattern is effectively a convergent gain of one tooth during evolution in these various clades. Unfortunately, we cannot test directly whether RA was implicated in these convergent changes as we cannot have access to the embryos of these species.

We previously hypothesized that early RA exposure can initiate homeotic transformation of anterior and dorsal pharyngeal tissue in the vicinity of tooth bud formation to a more posterior and ventral identity. This could explain why early exogenous RA exposure is able to induce the formation of anterior ectopic teeth in zebrafish [20]. This was exemplified by the anterior expansion of *pitx2a* expression in the pharyngeal epithelium under RA treatments. Our novel results indicate that pharyngeal mesenchyme markers also experience an expansion of their domain of expression in the three directions: laterally, anteriorly and dorsally under RA exposure (figure 4j). We therefore speculate that the new availability of cells expressing pharyngeal mesenchymal markers in a broader domain of the pharynx in the proximodistal axis under RA treatments is sufficient to induce an extra tooth in the ventral most part of the fifth ceratobranchial arch. This change in cell identity, unlike what we proposed for ectopic anterior tooth induction, does not need to be linked to a homeotic transformation for the following reasons.

First, the timing of treatments does not fit anymore with a homeotic transformation: when we begin our RA treatments after 24 hpf, we no longer observed the induction of anterior ectopic teeth, as expression of the dental epithelium markers *dlx2b* and *dlx3b* remain restricted to the 4V1 tooth bud in the posterior pharynx. Second, the formation of an extra tooth in the ventralmost row of the fifth ceratobranchial arch does not require a dramatic change such as would be needed to induce anterior ectopic teeth. A simple lateral expansion of the competent domain of cells that can form a tooth is sufficient. Although in our RA treatment, we observed an expansion of pharyngeal markers in three directions, and we speculate that the lateral expansion of these markers is enough. The nature of signals and genes responsive to the higher number of cells expressing *dlx2a* and *lhx6* that are needed to induce the extra tooth remain to be determined.

In addition, we also consistently note that exogenous RA has an effect on the shape of individual teeth, and this may be linked to a stronger variation in RA level as this is not observed in adult *sst* heterozygous but only after high and/or early treatment with exogenous RA. We are currently analysing this effect in more detail to better understand its developmental basis and to see if it may corresponds to change in wild species. It will be particularly interesting to study if there is co-occurrence of changes in tooth number and tooth shape during cyprinid evolution, and if change in RA signalling is the mechanism that has been recruited to promote tooth elongation in such cases.

Our analysis reveals that in addition to its action on body plan evolution in vertebrates [5,6,21], RA signalling can also intervene in the fine-tuning of tooth number at a taxonomically restricted level. Our findings provide a link, at a microevolutionary scale, between a specific change in RA signalling and a novel morphology. This is reminiscent of the situation of Hox genes, which, after having been considered solely as regulators of major body plan transitions, were demonstrated to be micromanagers involved in morphological differences between Drosophila species [22,23]. Our observation offers a coherent framework to explore the implication of such a signalling pathway in evolutionary adaptations.

4. Experimental procedures

(a) Zebrafish handling

Zebrafish and their embryos were handled according to standard protocols [24] and in accordance with the animal
welfare committees of the Ecole Normale Supérieure de Lyon and Deakin University. The stocksteif zebrafish mutant was used as previously presented [16,25].

(b) Zebrafish assays
RA and DEAB treatments were performed as previously described [7]. Whole mount in situ hybridizations were

Figure 4. Expansion of pharyngeal mesenchyme markers in RA treated embryos. (a–f) expression of dlx2a in the tooth bud of 4V1 and lateral arch mesenchyme marked by an arrow in (a,c,e) control and (b,d,f) RA-treated embryos. Note the lateral, anterior and dorsal expansion of dlx2a expression in RA-treated embryos (arrowhead in (f)). (g–l) Expression of lhx6 in the lateral pharyngeal mesenchyme marked by an arrow in (g,i,k) control and (h,j,l) RA-treated embryos. As for dlx2a, note the lateral, anterior and dorsal expansion of lhx6 expression in RA-treated embryos (arrow in transverse section in (l)). (m–t) Expression of dlx2b and dlx3b in the dental epithelium marked by an arrow in (m,o,q,s) control and (n,p,r,t) RA-treated embryos. RA exposure has only a minor effect on the expression of these dental epithelium markers. (u–x) Expression of pitx2a in the pharyngeal and dental epithelium marked by an arrow in (u,w) control and (v,x) RA-treated embryos. Note the lateral, anterior and dorsal expansion of pitx2a expression in RA treated embryos. (y) Diagram of a longitudinal section of the posterior ventral pharyngeal epithelium and mesenchyme of the 4V1 tooth germ during early morphogenesis, dorsal up, anterior to the left of control embryo (top) and RA-treated embryo (bottom). In red, dlx2b and dlx3b are restricted to the folding dental epithelium, whereas dlx2a is expressed in both the developing tooth (red) germ and the lateral pharyngeal mesenchyme (purple); lhx6 is expressed in both the dental mesenchyme (red) and in lateral arch mesenchyme (purple); pitx2a is expressed in the dental epithelium (red) and the pharyngeal epithelium (purple). Under RA exposure, expression of dlx2a, lhx6 and pitx2a is expanded in three directions (laterally, dorsally and anteriorly), whereas expression of dlx2b and dlx3b remains confined in the dental epithelium, but their expression domains are slightly enlarged compared with control.
performed as described [26]. Pictures were taken on an Axiosimager (Zeiss) and processed using Adobe PHOTOSHOP. Alcian blue and Alizarin red stainings were performed as previously described [7].

(c) Phylogenetic analysis

The phylogenetic tree was built using three markers: cytochrome b (cytb), cytochrome oxidase 1 (cox) and recombination activating gene (rag1). Sequences were downloaded from GenBank (references available upon request). Sequences were aligned and concatenated with SEAVIEW. Phylogenetic reconstruction was performed using RAxML, with a partitioned GTR + G + I model. Bootstrap support was calculated with 1000 repetitions. Carassius auratus was taken as outgroup.

(d) Microtomography

Specimens all came from the collections from the Museum of Natural History in Paris (MNHN), except Danio rerio and Carassius auratus, which came from our breeding facilities. All specimens were scanned by conventional microtomography using a nanotom Phoenix X-ray (General Electric) with the following parameters: 70 kV tension, 100 mA tension, 3000 images, 500 ms exposure time per image and three images for average per position. After scanning, reconstruction was performed with the software attached to the machine (data rec). Three-dimensional volumes were then analysed with VGSTUDIO MAX in order to virtually extract the pharyngeal bones.

(e) Histology

Whole-mount hybridized embryos stored in glycerol were dehydrated through several baths of absolute ethanol, then cross-sections. Sections were mounted in eukitt, observed and photographed under bright field. H&E staining was performed as previously described [10].

(f) sst genotyping

We amplified the part of the cyp26b1 gene that is targeted in the sstAs2 mutant by genomic PCR using custom primers (F: CACTCTCCTAATTAGGTTACCC, R: GGAAAGG CACAAGGAATGC). The A–T mutation introduces a new NheI restriction site, so we checked the genotype by digestion of the amplicon (data not shown). We confirmed the result by sequencing the amplicon and looking at the raw chromatogram.

References


