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Original Article

The sonic hedgehog signaling pathway and the development of pharyngeal arch Derivatives in *Haplochromis piceatus*, a Lake Victoria cichlid

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ABSTRACT

Objectives: Pharyngeal arches develop in the head and neck regions, and give rise to teeth, oral jaws, the hyoid bone, operculum, gills, and pharyngeal jaws in teleosts. In this study, the expression patterns of genes in the sonic hedgehog (shh), wnt, ectodysplasin A (eda), and bone morphogenetic protein (bmp) pathways were investigated in the pharyngeal arches of *Haplochromis piceatus*, one of the Lake Victoria cichlids. Furthermore, the role of the shh pathway in pharyngeal arch development in *H. piceatus* larvae was investigated.

Methods: The expression patterns of lymphocyte enhancer binding factor 1 (*lef1*), ectodysplasin A receptor (*edar*), *shh*, patched 1 (*ptch1*), *bmp4*, sp5 transcription factor (*sp5*), sclerostin domain containing 1a (*sostdc1a*), and dickkopf 1 (*dkk1*) were investigated in *H. piceatus* larvae by *in situ* hybridization. The role of the shh pathway was investigated through morphological phenotypic characterization after its inhibition.

Results: We found that *lef1*, *edar*, *shh*, *ptch1*, *bmp4*, *dkk1*, *sostdc1a*, and *sp5* were expressed not only in the teeth, but also in the operculum and gill filaments of *H piceatus* larvae. After blocking the shh pathway using cyclopamine, we observed ectopic *shh* expression and the disappearance of *ptch1* expression. After six weeks of cyclopamine treatment, an absence of teeth in the oral upper jaws and a poor outgrowth of premaxilla, operculum, and gill filaments in juvenile *H. piceatus* were observed.

Conclusions: These results suggest that the shh pathway is important for the development of pharyngeal arch derivatives such as teeth, premaxilla, operculum, and gill filaments in *H. piceatus*.

1. Introduction

Pharyngeal arches develop on the ventrolateral side of the head in vertebrates. Teleost fish have seven pharyngeal arches, while amniotes have five [1]. The first and second pharyngeal arches form the oral jaws and operculum, respectively [1]. The remaining posterior arches of teleosts are involved in the formation of gills and gill-related skeletal structures [1]. An oral dentition is developed on the first pharyngeal arch and, thus, forms the functional jaws in teleosts, while a pharyngeal dentition is developed on the rostral margin of the seventh pharyngeal arch [2–4]. Most teleosts have multiple rows of teeth on both their oral and pharyngeal jaws, while zebrafish lost a set of oral dentitions [5]. The structural diversity of jaws and teeth provides clues into the adaptive radiations of cichlids in the East-African great lakes, including Lake Malawi [6,7] and Lake Victoria [2,8].

Genes in the sonic hedgehog (Shh), Wnt, ectodysplasin A (Eda), and bone morphogenetic protein (Bmp) pathways such as lymphocyte enhancer binding factor 1 (*Lef1*), ectodysplasin A receptor (*Edar*), Dickkopf 1 (*Dkk1*), sp5 transcription factor (*Sp5*), *Shh*, Patched 1 (*Ptch1*), sclerostin domain containing 1 (*Sostc1*), and *Bmp4* are known to be related to mouse tooth development [9–15]. Here, we investigated the expression pattern of *lef1, edar, shh, ptch1, bmp4, sostdc1, dkk1*, and *sp5* during the development of pharyngeal arch derivatives, specifically of teeth, operculum, and gill filaments, in the Lake Victoria cichlid *Haplochromis piceatus*, because this species is considered to be a morphologically and trophically generalized species [16].

Among the above pathways, a negative feedback loop mechanism between the Shh and Wnt pathways has been reported during

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mouse tooth development [14,15]. The Wnt pathway positively activates the Shh pathway, then, the Shh pathway induces the expression of *Sostdc1*, which inhibits the Wnt pathway [15]. In teleost, a negative feedback loop between the shh and wnt pathways has not been reported in developing pharyngeal arches. Here, we investigate the role of *shh* in the morphogenesis of pharyngeal arch derivatives in *H. piceatus*, and the relationship between the shh and other pathways by comparing morphological and molecular differences between cyclopamine-treated and control larvae. Cyclopamine is one of the chemical modulators used to experimentally manipulate the Shh signaling pathway.

2. Materials and methods

2.1. Animal care

Adult *H. piceatus* were kept in aquaria of $100 \times 50 \times 50$ cm^3 with a water temperature of 24.1 ± 0.5 °C. The wild-type stock was collected in 1984 in the Mwanza Gulf of Lake Victoria, East Africa. They were subsequently bred for 15–16 generations in our aquaria at Leiden University, Leiden, The Netherlands. They were kept under a day–night light cycle of 12:12 h and were fed daily with commercial flake fish food (Landman, Hoevelaken, The Netherlands) alternated with a range of frozen fish foods (Aquadistri BV, Klundert, The Netherlands). All procedures were carried out with the approval of the local ethics committee.

2.2. Collection and storage of larvae

Haplochromis piceatus is a mouthbrooding cichlid. Larval ages (in days post-fertilization, dpf) were determined using the first identification of mouthbrooding in a female as the starting point (0 dpf). Larvae were then obtained from the mouths of brooding females by manual compression, and maintained in separate culture tanks provided with flowing water and oxygenation for further development. The oxygen concentration in the water was 8 mg/L.

2.3. Whole-mount in situ hybridization

In situ hybridization was carried out as described in Vonk et al. [17]. For probe synthesis, we isolated total RNA from *H. piceatus* larvae with Trizol (Invitrogen, Breda, The Netherlands) and synthesized cDNA from the RNA by a reverse transcriptase (SuperScript III, Invitrogen or RevertAid, Fermentas, Leon-Roth, Germany). Primers for the targeted genes were designed using alignments of fugu (*Takifugu rubripes*), medaka (*Oryzias latipes*), stickleback (*Gasterosteus aculeatus*), tetraodon (*Tetraodon nigroviridus*), Nile tilapia (*Oreochromis niloticus*), and zebrafish (*Danio rerio*) nucleotide sequences. The resulting PCR products from the targeted genes were sequenced and the sequences were deposited in GenBank (accession numbers KJ129615–KJ129622 and KJ705283, Table 1). We investigated the expression patterns of *lef1, edar, shh, ptch1, bmp4, sp5*, and *dkk1* in larvae at 5, 6, 6.5, and 7 dpf. At least three larvae of each stage were hybridized with each probe.

2.4. Cyclopamine treatment

Cichlid larvae were treated with 10 μM cyclopamine (Toronto Research Chemicals, ON, Canada). The cyclopamine stock solution, dissolved in pure dimethyl sulfoxide (DMSO), had a concentration of 1 mM. This stock was diluted to 1:100 in the aquarium water to a final concentration of 10 μM cyclopamine and 1% DMSO. As a control, we used 1% DMSO (vehicle only) under the same conditions. Treatment and control experiments were performed in Petri dishes kept at 26 °C on an oscillating platform in a culture incubator (KS 130 basic, IKA Works GmbH & Co., Germany). Following DMSO or cyclopamine treatment for 24 h, cichlid larvae were fixed with 4% paraformaldehyde in phosphate buffered saline for *in situ* hybridization analysis or kept for one day in aquaria containing approximately 3 L of fresh water and then transferred to an aquarium containing 10 L of fresh water for six weeks for morphological analysis.

3. Results

3.1. lef1, edar, shh, ptch1, sostdc1, bmp4, sp5, and dkk1 are expressed in the oral jaws of the H. piceatus at 6 dpf

The expression patterns of *lef1, edar*, *shh*, *ptch1, sostdc1, bmp4, sp5*, and *dkk1* were investigated in *H. piceatus* larvae at 5, 6, 6.5, and 7 dpf. The developing premaxillae and mandibles of *H. piceatus* expressed *lef1, edar, dkk1, sp5, shh, ptch1,* and *bmp4* at 6 dpf. All but *ptch1* and *shh* showed a punctate expression pattern in the oral jaws (Fig. 1A–D, H). In contrast, *shh* was expressed as a continuous band (Fig. 1E) and *ptch1* was widely expressed (Fig. 1F), which indicates the strong activity of the shh signaling pathway in the oral jaws.

Two copies of *sostdc1*, *sostdc1a* and *sostdc1b*, have been found in *H. piceatus*. Here, we show only the expression pattern of *sostdc1a. sostdc1a* was found to be expressed in the oral jaws, as well as in the lacrimal and nasal bones during development (Fig. 1G). These results indicate that these genes are involved in tooth development.

3.2. lef1, edar, shh, ptch1, sostdc1, bmp4, sp5, and dkk1 are expressed in the operculum and gills of H. piceatus at 6 dpf

Genes expressed during tooth development were also exprressed in the developing operculum and gill filaments. The developing operculum of *H. piceatus* at 6 dpf showed striped expression of *lef1*, *edar*, *dkk1*, *shh*, and *bmp4* (Fig. 2A–C, E, H). The operculum margin expressed *sp5* and *ptch1* (Fig. 2D, F). The tips of developing gill filaments showed expression of *lef1*, *edar*, *dkk1*, *sp5*, *shh*, *ptch1*, and *bmp4*. These results indicate that these genes are involved not only in tooth development but also in the development of the operculum and gill filaments. Interestingly, *sostdc1a* was expressed in developing gill filaments at 5 dpf (Fig. 2G′) but not at 6 dpf (Fig. 2G).

3.3. Disruption of the shh signaling pathway alters shh and ptch1 expression patterns in developing teeth, operculum, and gill filaments

In normal cichlid larvae at 6 dpf, *shh* was expressed as multiple odontogenic bands in the premaxilla, vomer, and palate (Fig. 3A). At 6.5 dpf, *shh* expression disappeared in the vomer, but was maintained in the premaxilla and palate (Fig. 3B). At 7 dpf, *shh* was expressed as multiple dots on the rostral end of the premaxillary odontogenic band (Fig. 3C). A caudal margin of *shh* expression in the premaxillary odontogenic band was evident; however, *shh* expression was not observed in the regions between the odontogenic bands of the premaxilla and palate (Fig. 3A–C). This band-like *shh* expression pattern was conserved in control larvae but changed in cyclopamine-treated larvae (Fig. 3D–F). While *shh* expression in the odontogenic bands of the premaxilla and palate was discrete in control larvae (Fig. 3D), its expression expanded to the regions between the odontogenic bands of the premaxilla and palate after cyclopamine treatment (Fig. 3E, F). Although *shh* expression pattern was altered after cyclopamine treatment, its