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Expression of marker genes during early ear development in medaka

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Abstract

Induction of the otic placode involves a number of regulatory interactions. Early studies revealed that the induction of this program is initiated by instructive signals from the mesendoderm as well as from the adjacent hindbrain. Further investigations on the molecular level identified in zebrafish *Fgf3*, *Fgf8*, *Foxi1*, *Pax8*, *Dlx3b* and *Dlx4b* genes as key players during the induction phase. Thereafter an increasing number of genes participates in the regulatory interactions finally resulting in a highly structured sensory organ. Based on data from zebrafish we selected medaka genes with presumptive functions during early ear development for an expression analysis. In addition we isolated *Foxi1* and *Dlx3b* gene fragments from embryonic cDNA. Altogether we screened the spatio-temporal distribution of more than 20 representative marker genes for otic development in medaka embryos, with special emphasis on the early phases. Whereas the spatial distribution of these genes is largely conserved between medaka and zebrafish, our comparative analysis revealed several differences, in particular for the timing of expression.

Keywords: Otic induction; Otic placode; Otic vesicle; Ear, Foxi1; Dlx3b; Medaka; Zebrafish

1. Results and discussion

Ear development is a complex process, controlled by a network of regulatory interactions. In particular the early steps of otic development are highly conserved among vertebrates. During ear formation, presumptive otic cells give rise to an ectodermal thickening called the otic placode, which subsequently develops into the otic vesicle (Barald and Kelley, 2004; Riley and Phillips, 2003; Whitfield et al., 2002). Early transplantation studies in chick embryos revealed that instructive signals are already present before the otic placode forms and are lost during mid-to-late somitogenesis (Groves and Bronner-Fraser, 2000; Kil et al., 2005).

Molecular analysis revealed a number of genes implicated in the induction of this sensory structure. Members of the Fibroblast Growth Factor (Fgf) family of peptide ligands play a key role in this process (Leger and Brand, 2002; Lombardo and Slack, 1998; Maroon et al., 2002; Phillips et al., 2001; Vendrell et al., 2000; Wright and Mansour, 2003). In zebrafish, *Fgf3* and *Fgf8* redundantly induce otic development as could be demonstrated by both gain-of-function as well as loss-of-function experiments (Leger and Brand, 2002; Phillips et al., 2004; Vendrell et al., 2000). Here the signals of the secreted Fgf proteins are mediated within the presumptive otic placodes by the transcription factors *Dlx3b* and *Dlx4b* and independently by *Foxi1* (Liu et al., 2003; Solomon et al., 2004). Another early marker for otic development is *Pax8*, which in zebrafish depends on *Foxi1* function (Solomon et al., 2003, 2004). After this early phase of otic induction, a network of regulatory interactions is initiated at early somitogenesis, which stepwise leads to the formation of substructures within the developing ear. During this process members of the Pax-Six-Eya-Dach regulatory network are thought to play an important role (Riley and Phillips, 2003; Whitfield et al., 2002).

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Much of our knowledge about the genetic hierarchy of otic development originates from studies of a few model organisms. The data known for fish have been obtained from a single model system and in some aspects differ from other vertebrate species. We therefore started to analyse otic induction in medaka fish, a model system distantly related to zebrafish (reviewed in Wittbrodt et al., 2002). The aim of this study was to analyse the expression pattern of otic marker genes during early phases of ear development in a time dependent manner and to compare the results with zebrafish data. In total we analysed more than 20 genes by whole mount *in situ* hybridization from gastrulation until inner ear structures start to form. Expression patterns for the selected marker genes during the induction phase of otic development are presented in Fig. 1. The temporal distribution of their expression from neurula (stage 17) to mid-somi-

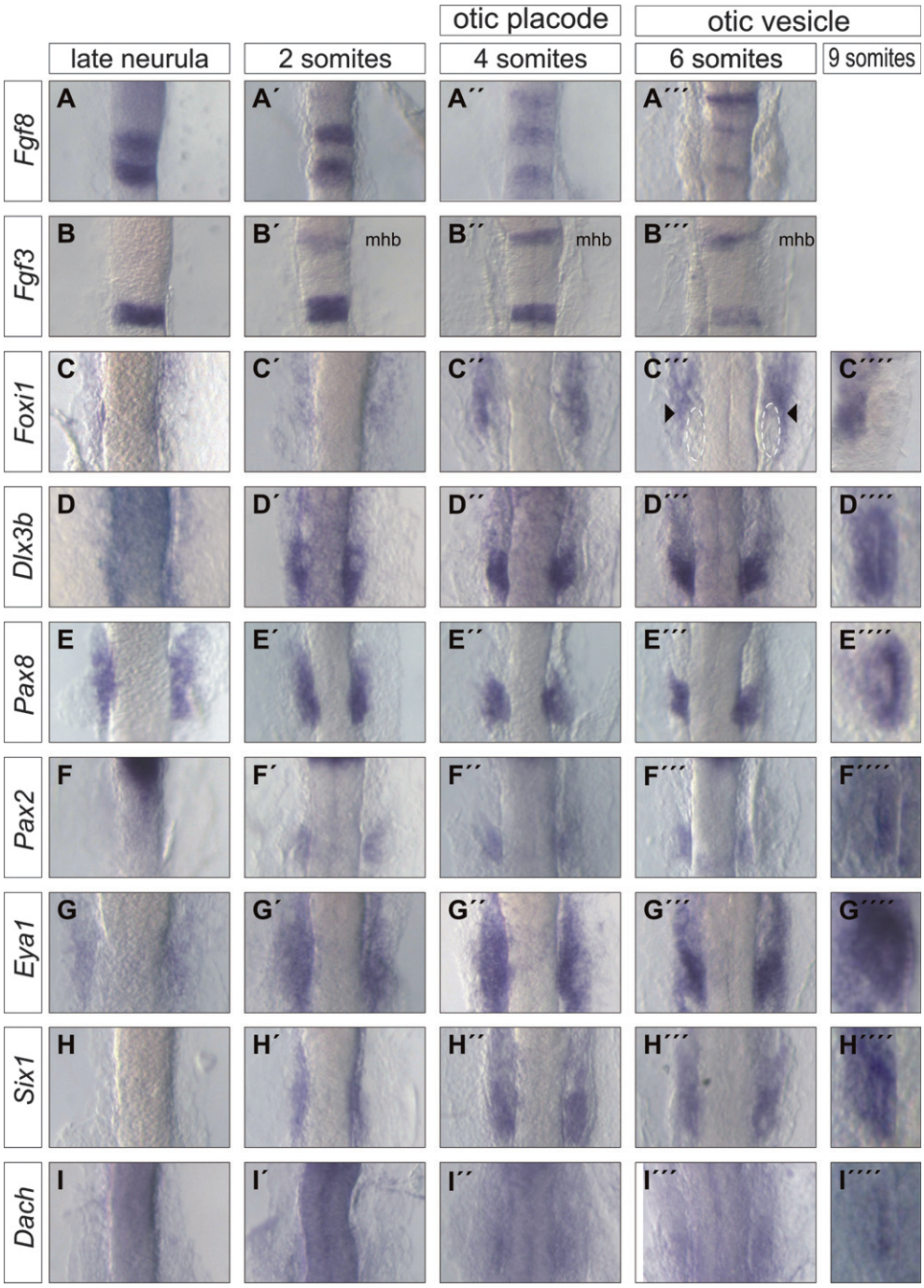

Fig. 1. Marker gene expression during early otic development in medaka. Summary of gene expression patterns involved in otic placode induction. The expression patterns of the indicated genes were analysed by whole mount in situ hybridization from stage 18 (late neurula) until 6 somites. Dorsal views of the otic vesicles (6 somites) at higher magnification are shown for selected genes. The arrowheads in (C‴) indicate the Foxi1 positive territory adjacent to the otic vesicle outlined by a dotted line. Dorsal views for all embryos, anterior to the top; mhb, midbrain–hindbrain boundary; r, rhombomere.

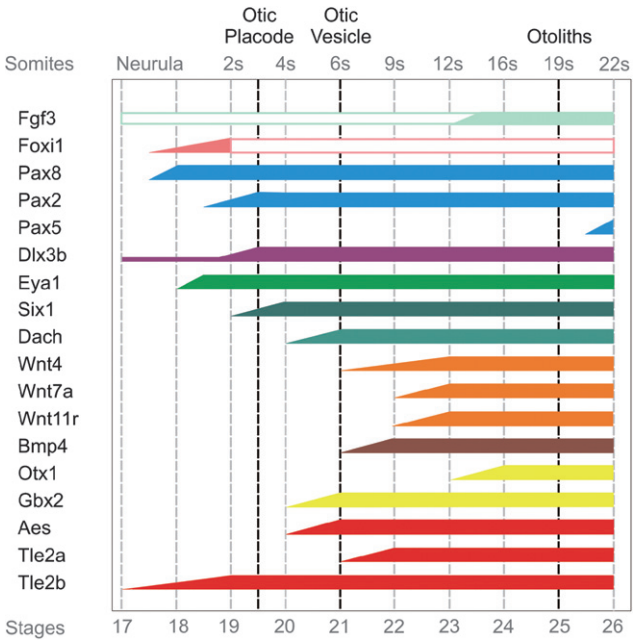

Fig. 2. Temporal analysis of marker gene expression during early otic development in medaka. Overview of the temporal distribution of gene expression during early otic development in wild-type medaka embryos from late gastrulation to mid-somitogenesis. The bars represent the expression of the indicated genes within the developing ears, empty bars indicate expression in adjacent tissues. The thin bar for *Dlx3b* represents the broad expression of the gene before it is upregulated specifically in the otic anlagen.

togenesis (stage 26; 22 somites) is summarised in Fig. 2. The spatial distribution of marker genes during later stages of inner ear development is presented in Fig. 3. In the following we discuss the results separately for each group of genes.

1.1. Fgf3 and Fgf8

The secreted factors Fgf3 and Fgf8 regulate otic development from a distance. Misexpression of both genes zebrafish as well as medaka embryos induces ectopic otic vesicles (Bajoghli et al., 2004; Phillips et al., 2004; Vendrell et al., 2000). Conversely, impairing functions of both genes in zebrafish causes strong reduction of otic tissue (Leger and Brand, 2002; Maroon et al., 2002; Phillips et al., 2001). In zebrafish, *Fgf3* is coexpressed with *Fgf8* at the end of gastrulation in the presumptive rhombomere 4, adjacent to the developing otic tissue (Leger and Brand, 2002; Maves et al., 2002; Phillips et al., 2001). We found similar expression patterns for the two genes in medaka during neurulation and early somitogenesis in the neural tube (Fig. 1). Transcripts of both genes first appear in the presumptive hindbrain at the end of gastrulation (Fig. 1A and B and data not shown) and with some delay in the mid-hindbrain boundary (starting at 4 somites; Fig. 1A′, A″, and B′). Within otic tissue they are not expressed up to stage 23 (12 somites), when *Fgf3* first shows an asymmetric expression pattern in the otic vesicle (restricted to the anterior part; Fig. 3A and data not shown).

1.2. Foxi1 andDlx3b

The first marker gene expressed during otic development in zebrafish is *Foxi1*, a forkhead-domain containing transcription factor (Weigel and Jackie, 1990). The role of the gene in ear development was revealed by the discovery of several zebrafish mutants, in which the *Foxi1* gene is affected (Lee et al., 2003; Nissen et al., 2003; Solomon et al., 2003).

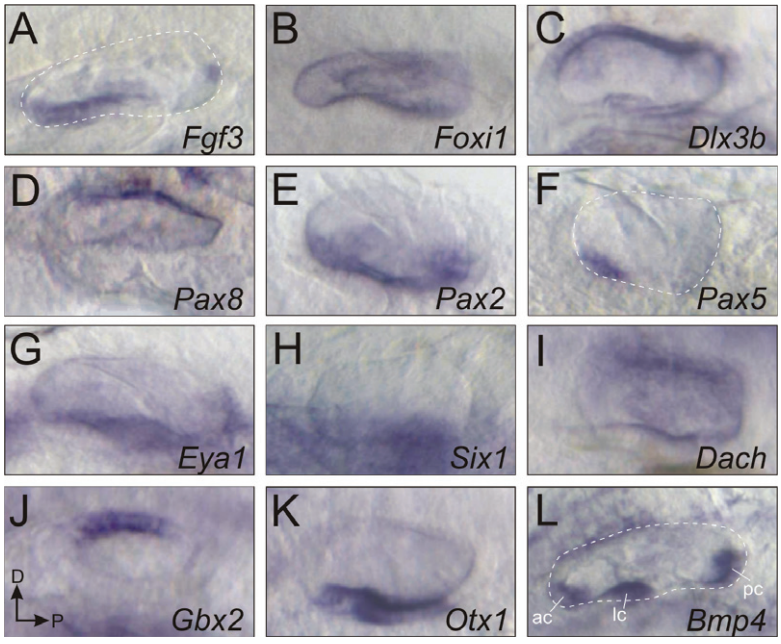

Fig. 3. Marker gene expression during later stages of otic vesicles. Late expression patterns of 12 selected otic marker genes in otic vesicles at stage 29 (34 somites), except for (J,K) stage 26 (22 somites). Lateral views for all otic vesicles; anterior to the left, dorsal to the top. ac, anterior cristae; lc, lateral cristae; pc, posterior cristae.

These mutants display a variable phenotype resulting in severe reduction or loss of the otic placode. Targeted inactivation of *Foxi1* in mice causes severely malformed inner ears, but does not affect otic induction (Hulander et al., 1998). In zebrafish *Foxi1* transcripts are first detected in presumptive epidermal regions, followed by expression in the otic anlagen at the end of gastrulation (Solomon et al., 2003). In order to isolate the medaka orthologue of *Foxi1* we performed a BLAST search of the medaka draft genome sequence (http://medaka.utgenome.org, revision 200406) using the zebrafish *Foxi1* amino acid sequence. The identified *Foxi* homologues were then compared with the known *Foxi* genes of zebrafish, in addition sequences of Fugu homologues were included in this analysis. The deduced phylogenetic tree and a sequence alignment within the forkhead domain are presented in Supplementary Figure S1. We thus unequivocally identified the medaka *Foxi1* gene on scaffold 8824 and isolated partial coding sequence by PCR from embryonic cDNA. Using this fragment as a probe for *in situ* hybridization experiments, we detected *Foxi1* expression in two domains positioned laterally to the neural plate (Fig. 1C and C′). This expression domain appears at the end of neurulation and includes the otic precursor cells, but then continuously moves both anteriorly and ventrally. Slightly later at four somites, *Foxi1* is not expressed anymore within otic tissue (Fig. 1C‴, arrowhead), as we could confirm by double staining experiments with *Foxi1* and *Dlx3b* probes (data not shown). Subsequently *Foxi1* is not detectable in otic tissue until stage 29 (34 somites) when expression reappears (Fig. 3B) in agreement with data from the mouse (Hulander et al., 1998).

The distal-less genes in vertebrates constitute a family of homeobox transcription factors, typically arranged in tandem on the chromosome. Thus *Dlx3b* and *Dlx4b* in zebrafish form a linked pair and share a highly similar expression pattern during embryonic development (Akimenko et al., 1994; Ekker et al., 1992; Ellies et al., 1997). Combined inactivation of both genes results in severely impaired otic and olfactory placodes (Solomon and Fritz, 2002). In zebrafish expression of both genes can first be detected at 75–80% epiboly in a continuous stripe of presumptive placodal ectoderm around the lateral edge of the neural plate (Akimenko et al., 1994; Ekker et al., 1992; Ellies et al., 1997). During early somitogenesis this expression concentrates in the prospective otic and olfactory primordia. Similar to *Foxi1* we first identified the medaka *Dlx3b* homologue *in silico* (scaffold 12; for sequence alignment and phylogenetic tree see Supplementary Fig. S2) and then isolated a fragment from embryonic cDNA. In medaka we found broad expression of *Dlx3b* during early gastrulation (Fig. 4A), which then starts to coalesce into a horseshoe-shaped stripe within the ectoderm around the lateral edge of the neural plate (Fig. 4B). During neurulation this stripe reaches strong intensity and includes the prospective otic and olfactory placodes (Fig. 4C). Eventually, the transcripts of *Dlx3b* accumulate in the placode regions (Fig. 1D′ to D‴ and Fig. 4D). The expression in the developing sensory organs then becomes more intense and starting from nine somites is complemented by expression in the visceral arches (Fig. 4E–H). At later stages of otic development *Dlx3b* transcripts become restricted to the dorsal side of the vesicles (stage 29, 34 somites; Fig. 3C). This pattern of *Dlx3b* expression in medaka closely resembles that of its zebrafish homologue.

1.3. Pax2/5/8

Expression of *Pax8*, a paired domain transcription factor and member of the *Pax2/5/8* family, can first be detected in the primordium of the otic placode during late gastrula-

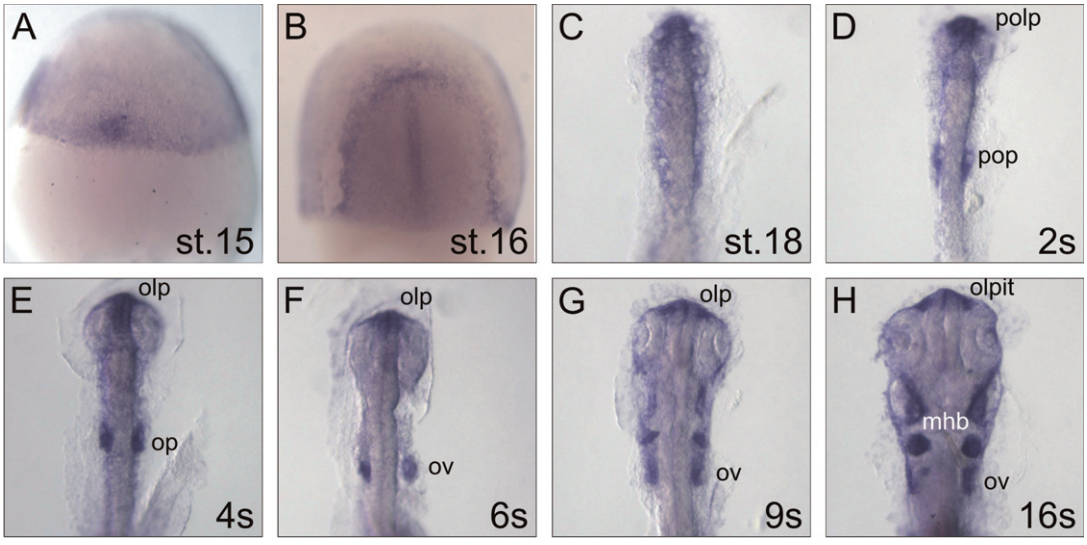

Fig. 4. Expression of the medaka Dlx3b gene during embryogenesis. *In situ* hybridization experiments for *Dlx3b* with medaka embryos of the indicated stages are shown. Dorsal views for all embryos, anterior to the top; mhb, midbrain-hindbrain boundary; polp, prospective olfactory placode; olp, olfactory placode; olpit, olfactory pit; pop, prospective otic placode; op, otic placode; ov, otic vesicle; s, somites.

tion (Pfeffer et al., 1998). In zebrafish, *Pax2a*, a second member of this gene family, is expressed in this region slightly later (Pfeffer et al., 1998). *Pax8* and *Pax2a* in zebrafish are activated by different pathways depending on *Foxi1* and *Dlx3b/4b*, respectively (Hans et al., 2004). The two *Pax* genes exhibit a high degree of redundancy, but combined inactivation results in complete loss of otic structures (Hans et al., 2004; Mackereth et al., 2005). Later during zebrafish otic development *Pax2b* and Pax5, two other members of this gene family are activated in otic tissue (Pfeffer et al., 1998). In medaka *Pax5* and *Pax8* genes are known, but so far only a single *Pax2* gene has been isolated (Koster et al., 1997). We performed extensive BLAST searches of the medaka genome but could identify only one *Pax2* gene, contrary to *Pax2a* and *Pax2b* genes present in zebrafish. When we analysed the three *Pax2/5/8* genes in medaka during otic development, we found exactly the same chronological order of expression. *Pax8* expression is initiated in the prospective otic placode at late neurula together with *Foxi1* (stage 18; Fig. 1E). Thereafter, a progressive restriction of *Pax8* transcripts to the otic placode and vesicle region is seen (Fig. 1E′ to E″). In zebrafish expression of *Pax8* in the otic region is reported to disappear shortly after the otic vesicle has formed (Pfeffer et al., 1998). In medaka *Pax8* expression persists in otic vesicles and later becomes restricted to the dorso-medial part of the vesicle at stage 29 (34 somites; Fig. 3D and data not shown). *Pax2* mRNA in the otic anlage could first be detected at two somites (stage 19). *Pax2* is also active in otic vesicles during later stages, but contrary to its paralogue, *Pax2* expression becomes restricted to the ventro-medial wall (Fig. 3E). Transcripts of *Pax5*, the third member of this *Pax* gene subfamily, appear in the ventro-anterior part of the otic vesicle after the otoliths have formed (Fig. 3F), which is consistent with data from zebrafish (Pfeffer et al., 1998). Therefore all three *Pax2/5/8* paralogues obtain distinct expression domains during later ear development. Taken together, the overall expression pattern of these genes during otic development is conserved, but in some aspects of their expression the *Pax2/5/8* gene family differs between zebrafish and medaka.

1.4. Pax-Six-Eya-Dach network

The Pax-Six-Eya-Dach network consists of transcriptional feedback loops and protein–protein interactions and was discovered during *Drosophila* eye development (Pignoni et al., 1997). Similar interactions were later found in vertebrate eye and muscle development (Relaix and Buckingham, 1999; Treisman, 1999). Homologues of all four gene families are active during inner ear development, but their regulatory relationship is poorly understood so far (reviewed in Riley and Phillips, 2003; Whitfield et al., 2002). Targeted disruption of mouse *Eya1*, causes severe phenotypes in otic vesicle differentiation (Xu et al., 1999). In mouse, *Xenopus* and zebrafish, *Eya1* is expressed in the preplacodal ear region before the onset of *Pax2* expression (David et al., 2001; Heanue et al., 2002; Sahly et al., 1999). In accordance we show here that medaka *Eya1* is expressed after *Pax8* mRNA can be detected, but shortly before *Pax2* expression starts (Fig. 1G–G‴′). The initially uniform expression of *Eya1* in the otic vesicles becomes later restricted to the ventral side (stage 29, 34 somites; Fig. 3G).

In medaka *Eya1* expression in the preplacodal region precedes that of *Six1*, being activated shortly before the otic vesicles appear (Fig. 1H′ and H″). Both genes remain coexpressed in the otic region as well as in the adjacent tissues (Fig. 3G and H). Starting with otic placode formation, *Dach* is expressed in otic tissue (Figs. 1I and 3I and Loosli et al., 2002). These results are in accordance with data from zebrafish, where *DachA* transcripts are first detected in the otic placodes (Hammond et al., 2002).

1.5. Wnt, TGF-β and Groucho/Tle genes

Members of the highly conserved Wnt family of secreted signalling molecules play important roles in many developmental processes (Nusse, 2005). In mice, functions of this signal transduction pathway have been demonstrated during later stages of ear development (Riccomagno et al., 2005), but its role during the induction phase is controversial (Ladher et al., 2000; Ohyama et al., 2006; Phillips et al., 2001). We analysed the expression of selected *Wnt* genes within otic tissue and could detect transcripts of *Wnt4* in otic vesicles starting with six somites, consistent with published data (Yokoi et al., 2003). Expression for *Wnt7a* and *Wnt11r* appeared in otic vesicles at nine somites (data not shown and Fig. 2). We could not detect transcripts of *Wnt1* and *Wnt5a* in otic tissue (data not shown), *Wnt5b* and *Wnt8b* were previously described to be inactive during otic development (Yokoi et al., 2003).

Members of the Groucho/Tle family of corepressor proteins participate in the regulation of otic development (Bajoghli et al., 2005). We previously analysed the expression pattern of the seven family members in medaka embryos (Aghaallaei et al., 2005) and included these data in Fig. 2. Out of the full length Groucho genes, *Tle2b* was shown to be expressed in otic tissue from early neurula stage on, whereas *Tle2a* followed later (six somites). Transcripts of the truncated family member *Aes* first appeared at stage 20 (four somites). In addition we detected transcripts of the TGF-β family member *Bmp4* in otic vesicles starting at six somites (Fig. 2). The expression of *Bmp4* is finally retained to the anterior, posterior and lateral cristae (Fig. 3L).

1.6. Patterning of the medaka otic vesicle along the dorso-ventral axis

Undifferentiated otic vesicles persist relatively long in medaka (6–34 somites) before inner ear development proceeds. An important decision during this time is the determination of the otic axes. We analysed the marker genes according to their distribution along the dorso-ventral

axis. Asymmetry along this axis could first be detected at 12 somites, when transcripts of *Otxl* appear at the ventral side of the vesicle (Fig. 3K). *Gbx2* is first expressed in otic placodes (4 somites) in a uniform pattern (data not shown; Fig. 2). This expression becomes asymmetric at 12 somites, but contrary to *Otx2*, *Gbx2* activity is confined to the dorsal part of the vesicle (Fig. 3J). Therefore the homeobox genes *Otxl* and *Gbx2* are the first markers showing a dorso-ventral distribution in medaka otic vesicles at 12 somites. The majority of other otic marker genes still keep a uniform expression pattern along this axis, until they switch to an asymmetric distribution at 30–34 somites (e.g. *Pax8* and *Dlx3b* dorsal; *Pax2*, *Six1* and *Eya1* ventral; see Fig. 3).

1.7. Comparison of otic marker gene expression between zebrafish and medaka

The timing of otic development differs between zebrafish and medaka. Otic placodes and the otic vesicles in medaka form at three and six somites, respectively (Iwamatsu, 2004). Overall development in zebrafish proceeds much faster than in medaka, nevertheless, compared to the rapidly forming body axis, development of the ear seems delayed. Thus, otic placodes and vesicles appear at 14 and 20 somites, respectively (Kimmel et al., 1995). Taking common morphological landmarks of embryonic development as a reference, otic development in medaka therefore proceeds faster than in zebrafish. The overall expression pattern of marker genes is in good agreement between the two fish species, however we detected several important differences.

In zebrafish *Pax8* is the earliest known marker of otic fate. It is expressed in preotic cells during 80% epiboly. After placode formation its expression gradually diminishes until it cannot be detected any more in otic vesicles (Heller and Brandli, 1999; Pfeffer et al., 1998). In medaka *Pax8* expression appears considerably later (late neurulation; Fig. 1E); but then persists and is still detectable when the inner ear structures start to form (Fig. 3D). Interestingly the late expression domains of Pax8 (dorso-medial) and Pax2 (ventro-medial) differ, although initially crossregulation exists (Hans et al., 2004).

In zebrafish *Eya1* is expressed at the end of gastrulation in a horseshoe-shaped region around the anterior neural plate including otic tissues (Sahly et al., 1999). This expression pattern, closely resembling that of *Dlx3b* is however not seen in medaka, where we detected *Eya1* transcripts first in preotic cells during late neurulation.

In agreement with its zebrafish homologue medaka *Foxi1* is coexpressed together with *Dlx3b* and *Pax8* in otic precursor cells. In both species *Foxi1* is not detectable in otic placodes and vesicles during mid-somitogenesis (Fig. 1C, Solomon et al., 2003), however in medaka *Foxi1* expression in the otic vesicles reappears at 34 somites (Fig. 3B). This late activity *of Foxi1* is similarly seen for the mouse homologue (Hulander et al., 1998).

Taken together, zebrafish and medaka clearly differ in the timing of otic development; nevertheless, our comparative analysis of marker genes for otic development revealed a high overall similarity of the expression patterns suggesting analogous mechanisms leading to ear formation in the two fish species. These results should largely facilitate the application of medaka for the study of early ear development.

2. Experimental procedure

2.1. Isolation of medaka Foxi1 and Dlx3b

Total RNA was extracted from mixed stages of medaka embryos using the Roti-Quick-Kit (ROTH). Reverse transcription was done with 1 μg total RNA using Revert M-MuLV Reverse Transcriptase (Fermentas) and random primers. The primers used for the PCR were as follows: forward primer for Foxi1: 5′-CCAACCTTCTACCCAAGCAGAG-3′; reverse primer for Foxi1: 5′-AAGCAGTCATTCAGCGACAAG-3′; forward primer for Dlx3b: 5′-GGGATCCATGAGCGCCGGACAGACC-3′; reverse primer for Dlx3b: 5′-GCTCGAGATAAACAGCTC CCACGCTCT-3′. PCR conditions were as follows: denaturation at 95 °C for 10 min, then 35 cycles at 94 °C for 30 s, annealing (67 °C for *Foxi1* and 68 °C for *Dlx3b)* for 1 min and 72 °C for 1 min extension. The DNA fragments were isolated, subcloned into the pGEM-T easy vector (Promega, Madison, WI) and sequenced.

2.2. Sequence and genomic analysis

For identification of the Fugu (*Fugu rubripes*) *Foxi* gene sequences we used the BLAST search program available at the Ensembl Genome Browser (http://www.ensembl.org) in the version v4.0 June05. Gene-IDs for the identified genes in the Fugu genomic sequence database are given in brackets: *Foxi1* (NEWSINFRUG00000124804); *Foxi2* (NEWSINFRUG00000148104); *Foxi3* (NEWSINFRUG00000163934). Human and Zebrafish *Dlx* and *Foxi* sequences were obtained from the NCBI server under the following accession numbers: human *Dlx3*, NP_005211; *Dlx4*, NP_612138; *Dlx5*, NP_005212; zebrafish *Dlx3b*, NP_571397; *Dlx4b*, NP_571393; *Dlx4a*, NP_571375; *Dlx5a*, NP_571381; *Foxi1*, NP_859424; *Foxi2*, NP_944598; *Foxi3a*, NP_944599. Sequence predictions for medaka were improved using the Genewise program (www.ebi.ac.uk/Wise2/). BioEdit software (v7.0.5.2, http://www.mbio.ncsu.edu/BioEdit/) was used for multiple alignments and trees were plotted using Treeview software (vl.6.6; http://taxonomy.zoology.gla.ac.uk/rod/rod.html).

2.3. Whole mount in situ hybridization and probes

Medaka embryos of the Cab inbred strain were used for all experiments. Embryonic stages were determined according to Iwamatsu (2004). Embryos were fixed over night in 4% PFA/PTW (PBS/Tween) at 4 °C and subsequently dechorionated manually. Whole-mount *in situ* hybridizations were performed as described (Hauptmann and Gerster, 2000) using DIG- or FITC-labelled RNA probes. The colour reaction was carried out with NBT/BCIP for single stainings, followed by dehydration of embryos in 100% MeOH. Embryos were then, fixed in 4% paraformaldehyde/PTW over night and mounted in 87% glycerol through a graded series of glycerol/PTW.

For the *in situ* experiments the following probes were used: *Pax8*, *Pax5*, *Wnt7a* and *Fgf3*, MEPD database (Henrich et al., 2005; Quiring et al., 2004) (*Pax8*, 631-134-03-M; *Pax5*, McF0001MGR-lB01bdl; *Wnt1a*, McF0003I12-MGRbd1; *Fgf3*, 631-136-21-K); *Eya1*, *Six1*, *Pax2* (Koster et al., 1997); *Dach* (Loosli et al., 2002); *Bmp4*; *Aes*, (Lopez-Rios et al., 2003); *Tle2a*, *Tle2b* (Aghaallaei et al., 2005); *Gbx2* (Heimbucher et al., submitted); *Wnt4*, *Wnt5a*, *Wnt5b* and *Wnt8b* (Yokoi et al., 2003); *Wntl* (Carl and Wittbrodt, 1999); *Otxl* (F. Loosli and J. Wittbrodt, unpublished); *Wntllr* medaka EST database (Kimura et al., 2004)(MF01FSA036G14).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.modgep.2006.07.008.

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