Divergent Wnt8a Gene Expression in Teleosts

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Abstract

The analysis of genes in evolutionarily distant but morphologically similar species is of major importance to unravel the changes in genomes over millions of years, which led to gene silencing and functional diversification. We report the analysis of Wnt8a gene expression in the medakafish and provide a detailed comparison to other vertebrates. In all teleosts analyzed there are two paralogous Wnt8a copies. These show largely overlapping expression in the early developing zebrafish embryo, an evolutionarily distant relative of medaka. In contrast to zebrafish, we find that both maternal and zygotic expression of particularly one Wnt8a paralog has diverged in medaka. While Wnt8a1 expression is mostly conserved at early embryonic stages, the expression of Wnt8a2 differs markedly. In addition, both genes are distinctly expressed during organogenesis unlike the zebrafish homologs, which may hint at the emergence of functional diversification of Wnt8a ligands during evolution.

Introduction

Teleosts are particularly suitable for comparative studies to elucidate evolutionary events resulting in genomic diversity. With as many as 25,000 species arising within the last 250 million years, it is the most diverse group of vertebrates and with the increasing number of genome sequences available an invaluable resource for gene/genome evolution species to gain insights into the functional complexity of a given gene and to address questions regarding gene/genome evolution related and morphologically similar but evolutionarily distant mammalian importance to study gene expression and function in duplicated mammalian homologs [11,12]. Thus, it is of fundamental value to study gene expression and function in related and morphologically similar but evolutionarily distant species to gain insights into the functional complexity of a given gene and to address questions regarding gene/genome evolution compared to mammalian systems. The Wnt signaling pathway is highly conserved and amongst the most intriguing signaling cascades studied to date. Its exceptionally wide range of functional properties ranging from cell proliferation and tissue homeostasis to cell differentiation and cellular diversity in development and disease has inspired many researchers investigating Wnt signaling [13,14]. However, by today, 28 different Wnt ligands have been identified in zebrafish [15] and only a very small subset of ligands has been analyzed in medaka [16]. Wnt8 genes are amongst the most prominent ligands in Wnt signaling. In vertebrates, two Wnt8 are present in the genome, Wnt8a and Wnt8b. In teleosts, the genome duplication resulted in the generation of an additional Wnt8a paralog in close genomic proximity and in tandem to the first [17]. This arrangement appears very conserved amongst teleosts [17–19]. Interestingly, besides a transcript for the second Wnt8a paralog, a bicistronic Wnt8a transcript encoding both Wnt8a proteins has been identified in zebrafish [17].

The two Wnt8a paralogs are largely overlappingly expressed during early zebrafish development and appear to exert similar but distinct functions [17,18]. However, it is not known whether these features are conserved within the teleost lineage. Moreover, an expression or functional analysis at later stages has not been carried out.

Here we report the expression analysis of the two medaka Wnt8a paralogs during embryonic development. Like for other teleosts, the two genes are arranged in close proximity to each other in the genome. However, unlike in zebrafish, both maternal and zygotic expression of the medaka Wnt8a genes differs in various developing tissues. Our data indicate that Wnt8a gene expression has diverged between distantly related teleost species and implies that they may have acquired different functions during evolution. Moreover, we find sites of medaka Wnt8a expression during organogenesis stages, which we did not detect in zebrafish but are found also in mammals with the exception of the caudal hematopoietic system and gall bladder.
Materials and Methods

Ethics statement
The approval for all animal work carried out was obtained from the Regierungspräsidium Karlsruhe (35-9185.64).

Fish maintenance
Wildtype *Oryzias latipes* from a closed stock at the University Heidelberg and the *Danio rerio* AB x TL line were kept as described [20–22].

Whole mount in situ labeling
Whole mount in situ hybridization using digoxigenin labeled RNA riboprobes for medaka *Wnt8a1* and *Wnt8a2* and zebrafish *Wnt8a ORF1* and *Wnt8a ORF2* were performed as described [23,24].

Cloning of the full length medaka *Wnt8a1* and *Wnt8a2* genes
Total RNA was isolated from 1, 2 and 3 dpf medaka embryos using Trizol® (Invitrogen, Darmstadt, Germany). First strand cDNA was prepared according to the manufacturer's protocol (Invitrogen SuperScript®III First-Strand kit, Darmstadt, Germany). To clone both medaka full-length *Wnt8a* genes from the synthesized cDNA, PCR was performed using the Taq DNA polymerase kit (QIAGEN, Hilden, Germany) with the following primers.

| Primer            | Sequence                  |
|-------------------|---------------------------|
| Wnt8a1 forward    | 5’-AGCGTGAGGGAGGCTGCAT-3’ |
| Wnt8a1 reverse    | 5’-CACGGTCCCTGCGCTTCGTT-3’|
| Wnt8a2 forward    | 5’-AGGAAAATTGAAGAAGCGAACCAGGA-3’ |
| Wnt8a2 reverse    | 5’-AGCCGTAATCTTTCATCTGGGGC-3’ |

PCR program: Denaturation for 30 sec at 95°C, annealing for 30 sec at 70°C for *Wnt8a1* and 62°C for *Wnt8a2* followed by extension for 1 min at 72°C for a total of 35 cycles. The first cycle was preceded with initial denaturation for 3 min at 95°C and the last cycle was followed by additional extension for 3 min at 72°C and cooling at 25°C for 30 sec. The purified full-length *Wnt8a* cDNAs (*Wnt8a1*: 1349 bp and *Wnt8a2*: 1110 bp) were cloned into the pCRII-TOPO vector (Invitrogen, Darmstadt, Germany) according to the manufacturer's instructions and sequenced.

RT-PCR detection of *Wnt8a* transcripts in medaka and zebrafish
To detect maternal and zygotic expression of medaka *Wnt8a* genes, we isolated total RNA from medaka embryos at 2-cells, 4-cells, 16-cells and 40% epiboly. The synthesized first strand cDNAs were used as templates for PCR carried out as above to obtain the *Wnt8a2* cDNA. To obtain a 530 bp long *Wnt8a1* fragment, we used the same PCR conditions as above with an annealing temperature of 60°C and for *Wnt8* the following primers: *Wnt8a1* forward 5’-AGCGTGAGGGAGGCTGCAT-3’, *Wnt8a1* reverse 5’-TGAGTGC CCCGTGTTCTGT-3’, *Wnt8a2* forward, 5’-AGGAAAATTGAAGAAGCGAACCAGGA-3’ and *Wnt8a2* reverse, 5’-AGCCGTAATCTTTCATCTGGGGC-3’.

Figure 1. Phylogenetic analysis of *Wnt8a* genes. The medaka genome contains two *Wnt8a* genes (bold). The *Wnt8a* cDNA sequence alignment of various species shows that the medaka paralogous copies cluster well with those of evolutionarily related teleosts. acul., aculeatus; nigro., nigroviridis.

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expression. (E,F) RT-PCR for Wnt8a. Sense RNA probes were used to validate the maternal transcript. Wnt8a1

Figure 2. Differential early gene expression. (A-D) 8-cell stage embryos are shown with the animal pole to the top. Sense RNA probes were used to validate the maternal Wnt8a1 expression. (E,F) RT-PCR for Wnt8a1 and Wnt8a2 transcripts on extracted RNA at stages indicated to the left. (G,H) Pictures focused on the dorsal margin of the dissected blastoderm of embryos at 40% epiboly with animal pole to the top. While Wnt8a1 (G) is expressed in cells of the blastoderm margin but not in cells of the shield (framed by black lines), Wnt8a2 (H) is weakly expressed in cells of the shield and the margin. Open rectangle indicates the broad Wnt8a2 expression domain at the margin. (I,J) Embryos at 80% epiboly with anterior to the left; (I) dotted lines frame the developing embryonic axis; inset and (J) show embryos dissected from the yolk for clarity. (J) Dotted line marks the blastoderm margin. 40%, 40% epiboly; c, cells; ma, margin; ea, embryonic axis; n.c., negative control; pam, paraxial mesoderm; st., stage; vma, ventral margin. doi:10.1371/journal.pone.0085303.g002

Results and Discussion

Wnt8a ligands are conserved amongst vertebrates

Like in other teleosts [17–19], the two medaka Wnt8a paralogs are organized in a tandem arrangement in the genome. We isolated the full length coding sequences of medaka Wnt8a (formerly named Wnt8-like) [16] and Wnt8a2. Phylogenetic analysis shows that the medaka Wnt8a paralogs cluster well with the respective Wnt8a paralogs of the evolutionarily close Fugu and Tetraodon (Figure 1). In contrast, the zebrafish Wnt8a genes cluster together on the Wnt8a1 branch of the tree. Consistent with its evolutionary distance, the medaka Wnt8a1 protein shares higher homology with its Fugu homolog (78% identical amino acids (aa)) rather than with zebrafish Wnt8a ORF1 (also named Wnt8.1, Wnt8a or Wnt8a.1) (69% identical aa). However, the second Wnt8a protein, Wnt8a2, appears similarly conserved (Fugu Wnt8.2 shares 71% identical aa with medaka Wnt8a2 and 72% with zebrafish Wnt8a ORF2; also named Wnt8a [17]).

Like zebrafish Wnt8a ORF1 (63% identical aa compared to 62% of Wnt8a ORF2), the medaka Wnt8a1 protein is most similar to the mammalian single Wnt8a protein (60% identical aa compared to 56% of Wnt8a2).

This indicates that the medaka genome contains two paralogous Wnt8a copies, which are well conserved within closely related teleosts and that Wnt8a1 is the gene more similar to the single mammalian Wnt8a.

Differential initiation of medaka Wnt8a gene paralog expression

Zebrafish Wnt8a ORF1 is expressed maternally [27] and the earliest Wnt8a expression in other animal models has been reported in the posterior epiblast prior to gastrulation in mice and chick [28,29]. Early zygotic zebrafish Wnt8a expression is found in lateral and ventral marginal cells of the blastoderm
**Table 1. Sites of Wnt8a gene expression in medaka, zebrafish, Xenopus and mouse.**

| Gene        | Medaka Wnt8a1 | Zebrafish Wnt8.1 | Xenopus Wnt8a | Mouse Wnt8a |
|-------------|---------------|------------------|---------------|-------------|
| maternal    | +             | + n.r.           | n.r.          | –           |
| blastoderm margin/embryonic ectoderm | + +*           | + +             | +             | +           |
| paraxial mesoderm | – +           | + n.r.           | +             | +           |
| tailbud     | +             | + +             | +             | +           |
| dht         | –             | – –             | n.r.          | n.r.        |
| gut/oesophagus | –           | – –             | +**           | +**         |
| gall bladder | –             | – –             | n.r.          | n.r.        |
| heart       | –             | – –             | n.r.          | +           |
| swim bladder | –             | – –             | n.a.          | n.a.        |
| otic vesicles | –             | – –             | n.r.          | +           |
| brain       | –             | – –             | +             | +           |
| fins/limbs, branchial arches, eye | –             | – –             | –             | +           |
| main references | (Yokoi et al., 2003) | (Kelly et al., 1995) | (Smith and Harland, 1991) (Christian et al., 1991) | (Bouillet et al., 1996) |

Wnt8.1 and Wnt8.2 correspond to zebrafish Wnt8a ORF1 and Wnt8a ORF2, respectively.

*, very transient; **, hindgut; +, expressed by in situ hybridisation; –, not expressed by in situ hybridisation. Abbreviations: dht, dorsal haematopoietic tissue; n.a., not applicable; n.r., not reported.

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[17,27] (Figure S1A and B). Its expression in the embryonic shield remains controversial. This gene was initially described to be expressed in the shield and downregulated subsequently [27]. A later study mentioned that Wnt8a ORF1 (and Wnt8a ORF2) is not expressed in the shield until about 75% epiboly [17]. At subsequent stages, Wnt8a ORF1 is expressed in the paraxial mesoderm [27] and Wnt8a ORF2 remains expressed in marginal cells similar to its paralog [17].

We find that medaka Wnt8a1 is maternally expressed (Figure 2A,C,E), while we did not detect any Wnt8a2 transcripts by whole mount in situ hybridization or RT-PCR (Figure 2B,D,F). The early zygotic medaka Wnt8a1 expression is well conserved amongst vertebrates and is seen in lateral and ventral marginal cells at 40% epiboly with the exception of the shield (Figure 2G) [16,17,27,30–32]. At this stage, Wnt8a2 is expressed weakly in a slightly broader region of the blastoderm margin including cells of the shield (Figure 2H). At late gastrulation, medaka Wnt8a1 is also found in cells of the dorsal blastoderm margin with lower expression in the ventral part similar to its homologs in other vertebrates [17] (Figure 2I and inset). However, no Wnt8a2 transcripts are found in any of the marginal cells. Therefore the temporal expression of medaka Wnt8a2 in the shield and the blastoderm margin markedly differs from medaka Wnt8a1 and zebrafish Wnt8a ORF2. This may imply functional differences of the medaka Wnt8a genes during early embryonic development, which may be more prominent than in zebrafish [17].

Shortly before the end of gastrulation, we find Wnt8a2 transcripts specifically in the paraxial mesoderm (Figure 2J). This transient Wnt8a2 expression is similar to the reported expression of zebrafish Wnt8a ORF1 and the single Wnt8a genes of Xenopus and mouse [17,28,33,34] (Table 1).

**Medaka Wnt8a expression during somitogenesis and organogenesis**

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addition, Wnt8a1 is expressed in the outflow tract of the heart, the swim bladder and the gall bladder (Figure 3G,I and inset). These observations represent evolutionary diversification of gene expression within the teleost lineage and amongst vertebrate species in general. Unlike other model systems analyzed, we were not able to detect expression of zebrafish Wnt8a genes at stages after somitogenesis by in situ hybridization. Conversely, Wnt8a gene expression in cells of the developing hindbrain rhombomeres seen in various species including zebrafish appears not to be conserved in medaka [17,28,29,33]. Moreover, Wnt8a expression in limbs and branchial arches of mouse and chick embryos is not seen in fish [36]. Intriguingly, expression in the gut, heart and otic vesicles appears to be conserved between medaka and mouse [36–38], while only medaka Wnt8a gene expression is found in the caudal hematopoietic tissue, gall- and swim bladder (Table 1).

In summary, the medaka Wnt8a paralogs have acquired very different sites of expression when compared to each other, but the sum resembles much of Wnt8a expression in animals with a single Wnt8a gene in their genome.

The early medaka Wnt8a1 gene expression is similar to Wnt8a expression of other vertebrate species. However, the temporal-spatial dynamics of Wnt8a2 expression appears to have diverged during teleost evolution. It is tempting to speculate that this may be due to differences on the transcriptional level such that for instance unlike zebrafish and Fugu Wnt8a, medaka may not have a bicistronic Wnt8a transcript [17,18].

Our phylogenetic analysis shows that the zebrafish Wnt8a genes do not separate into the two Wnt8a gene clusters of other teleosts, which may explain their similar expression and semi-redundant function described in zebrafish [17]. Conversely, the separation of medaka Wnt8a paralogous copies together with the divergence of their expression suggests that the two genes may have acquired different functions during evolution.

Supporting Information

Figure S1 Analysis of zebrafish Wnt8a gene expression. (A,B) Dorsal views and (C,D,G,H) lateral views of zebrafish embryos labeled for Wnt8a ORF1 and Wnt8a ORF2 expression at stages indicated to the left. (A–D) Embryos at 80% epiboly and 22 hpf exhibit the described Wnt8a gene expression in cells of the blastoderm margin and the tail tip respectively; no labeling is detected using the Wnt8a sense probes (insets in A and B). (E–H) Low levels of blue color is ubiquitously distributed in the brain of 2 dpf and 4 dpf old embryos, which is however also seen using the sense probe (insets in E and F). (I) RT-PCR analysis to detect Wnt8a gene transcripts reveals low levels of Wnt8a gene expression in both heads and tails of zebrafish embryos. Therefore the color visible in the head is likely to be background. 80%, 80% epiboly; n.c., negative control; st., standard; tb, tailbud.

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Author Contributions

Conceived and designed the experiments: MC. Performed the experiments: NM. Analyzed the data: NM MC. Contributed reagents/materials/analysis tools: MC. Wrote the paper: MC. Generation and analysis of the phylogenetic tree: CB.

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