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Osterix/Sp7 regulates biomineralization of otoliths and bone in medaka (*Oryzias latipes*)

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ABSTRACT

Osterix/Sp7 is a zinc finger transcription factor and critical regulator of osteoblast differentiation, maturation and activity. *Osterix* expression has also been described in non-skeletal tissues but functional analyses are lacking. In the present study, we show that in the teleost model medaka, *osterix* is present as two alternatively spliced transcripts, *osx_tv1* and *osx_tv2*. Knock-down of *osx_tv1* and/or *osx_tv2* results in mineralization loss in early intramembranous bones while cartilage formation is mostly unaffected. Formation of the parasphenoid, the earliest mineralized bone in the medaka skeleton, is impaired and fails to recover at later stages. Ossification of later bones, such as the operculum and cleithrum, is delayed but recovers during further development. In the axial skeleton, formation of the neural arches and centra is strongly delayed. *In vivo* analyses using *osterix*:nlGFP and *osteocalcin*:GFP transgenic medaka and whole mount *in situ* hybridization suggest that bone defects observed after knock-down of *osterix* are caused by a delay of osteoblast maturation and activity. Furthermore, we analyzed expression profile and function of *osterix* during ear and otolith formation. We show that *osterix* is expressed in otic placodes at the otic vesicle stage and that its knock-down results in a loss of otoliths. Taken together, we show that *osterix* is required for bone formation in a teleost fish and that its important regulatory functions are conserved between teleosts and mammals. Furthermore, we provide the first functional evidence for a role of Osterix in a non-skeletal tissue, i.e. the otoliths.

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1. Introduction

The zinc finger transcription factor Osterix/Sp7 belongs to the Sp (specificity protein) family and regulates osteoblast differentiation and maturation during embryonic bone formation and maintenance of adult bone in mammals (Nakashima et al., 2002; Baek et al., 2010; Zhou et al., 2010). On the other hand, a chondrocyte specific Osterix knock-out in mice leads to impairment of endochondral ossification indicating a role of Osterix also for chondrocyte differentiation and maturation after precursor cells have been committed to the chondrocyte lineage (Oh et al., 2012). Osterix expression is induced directly e.g. by Msx2 and indirectly by BMP2 (Matsubara et al., 2008). BMP2 stimulates Osterix expression via Runx2 or independent from Runx2 via Dlx5 (Lee et al., 2003). Osterix drives the expression of osteoblast-specific genes, such as *collagen type I* (*col1a1*), *osteonectin*, *osteopontin*, *bone sialoprotein* and *osteocalcin* (Nakashima et al., 2002; Koga et al., 2005). Furthermore, Osterix negatively controls osteoblast proliferation by inhibition of Wnt signaling (Zhang et al., 2008; Yang et al., 2010). More recently, a case study reported a mutation in the *Osterix* gene that leads to Osteogenesis imperfecta revealing an important function of Osterix for human bone development (Lapunzina et al., 2010). Interestingly, *Osterix* expression, formerly considered to be restricted to bone tissue, has also been shown in non-skeletal tissues such as the olfactory bulb and brain in mice and the otic placode in zebrafish (DeLaurier et al., 2010; Park et al., 2011). The function of Osterix in these non-skeletal tissues, however, remains unknown.

Teleost fish, such as the medaka, develop bones by chondral and intramembranous ossification. Genes involved in bone formation show a high degree of conservation at amino-acid (aa) and expression levels between teleosts and mammals. Previously, we analyzed the expression of *osterix* (*osx*) in medaka and found that *osx* promoter activity correlates temporally and spatially with bone ossification

Abbreviations: Bmp, bone morphogenetic protein; Col, collagen; Dlx, distal-less gene; Elf, elongation factor; Eya, eyes absent gene; MO, morpholino oligonucleotide; Msx, muscle segment homeobox gene; NFATc1, nuclear factor of activated T cells; nlGFP, nuclear green fluorescent protein; Ntr, nitroreductase; Osc, osteocalcin; Osx, osterix; Otx, orthodenticle gene; Rankl, receptor activator of nuclear factor-κB ligand; Pth, parathyroid hormone; RT, reverse transcriptase; Runx, runt-related transcription factor; Six, sine oculis gene; Sox, sex-determining region sry-related high-mobility-group box; Sp, specificity protein; s, somite stage; tv, transcript variant.

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during embryogenesis (Renn and Winkler, 2009). Furthermore, transgenic expression in adult animals suggested a possible role of Osx in *de novo* osteoblast differentiation/maturation and activity in bone remodeling. In osteoblast ablation and *rankl* overexpression studies, we demonstrated that *osx* expressing cells are crucial for bone formation as well as activity and survival of osteoclasts in medaka (To et al., 2012; Willems et al., 2012). Taken together these data suggest a conserved function of *osx* for bone formation in teleost fish. Importantly, despite its essential role in mammals, a functional analysis of *osx* in teleost fish is lacking. In the present study, we identified and characterized two full-length medaka *osx* mRNAs that originate from alternative splicing. We used morpholino knock-down approaches to show the importance of Osx for osteoblast maturation and activity during embryonic bone development in medaka. Additionally, we analyzed *osx* expression at early stages of medaka embryogenesis before the onset of bone formation and analyzed its function during induction of the otic placode and otolith formation. We provide first evidence that Osx plays a role during otolith formation in medaka revealing a novel function of Osx in a non-skeletal tissue.

2. Results

2.1. Expression of two osterix mRNA splice isoforms in wild-type embryos

A cDNA and deduced protein sequence of medaka *osx* is annotated in the Ensemble genome database (Transcript ID: ENSORLT00000006574). Compared to the data base entry, we isolated a cDNA with extended exon1 containing 15 additional nucleotides resulting in an open reading frame of 460 amino-acids (aa) from embryos at 1.5–2 dpf (Fig. 1A, C). Exon1 contains the 5′UTR and sequence coding for the first twelve aa, while exon2 encodes most of the protein and contains the 3′UTR. Additionally, we identified a second *osx* splice form (*osx_tv2*) encoding for a deduced protein of 346 aa that lacks aa 8–121 of *osx_tv1* at the N-terminus (Fig. 1A, C, and D). For *osx_tv2*, alternative splice donor and acceptor sites lead to a shorter mRNA lacking the last 15 nucleotides in exon1 and the first 327 nucleotides in exon2 (Fig. 1D). To examine the expression profiles of *osx_tv1* and *osx_tv2* during medaka embryogenesis, we performed RT-PCR on cDNA derived from different stages using a primer pair that detects both isoforms. As shown in Fig. 1B, *osx_tv1* is the predominantly expressed isoform during embryogenesis of medaka.

Both deduced protein isoforms contain all functional domains that have been described for mammalian Osx. Medaka Osx is highly conserved at the aa level when compared to mammals (human OSX to medaka Osx_tv1: overall identity 57%, similarity 66%; Fig. S1). The zinc finger domains of medaka and human Osx for example differ in only two residues. The residues, which have been proposed to establish contact with target DNA (Gao et al., 2004), are 100% conserved (arrowheads in Fig. S1). Other functional domains, such as the binding region for nuclear factor of activated T cells (NFATc1) (Koga et al., 2005) and the region with strongest transactivation activity (Hatta et al., 2006), are also highly conserved between fish and mammals. In contrast, the C-terminal region directly following the zinc finger domain is less conserved. For human and mouse, a shorter *Osx* transcript containing an alternative translation start site has been reported that encodes a truncated protein (arrow in Fig. S1; Gao et al., 2004; Nishio et al., 2006). This second start codon is conserved in other vertebrates. However, medaka *osx_tv2* lacks this particular ATG (Fig. 1C, D).

2.2. Osterix is expressed in sensory placodes during early embryogenesis

We previously described the expression of *osx* during osteogenesis in medaka from 4 dpf onwards (Renn and Winkler, 2009, Fig. 2F). We extended our analysis and performed whole mount *in situ* hybridization on embyros before 3 dpf. Surprisingly, we found that already at late gastrula stages, *osx* is expressed at the rostral edge of the neural plate (Fig. 2A). During preplacodal stages (neurula until 3 somite stage, s) and otic placode stages (4–6 s) *osx* is broadly expressed in the prospective otic and olfactory placodes (Fig. 2B, C). At otic vesicle stages (7 to 10 s), *osx* expression ceases in the olfactory placodes. Its expression in the otic vesicle is reduced at 8 ss and no longer detectable at 16 ss. Instead, expression appears laterally to the otic vesicle (Fig. 2D, E). Hence, *osx* is expressed during the induction of the sensory placodes and disappears shortly after their formation indicating a very narrow temporal window for its function in these tissues.

2.3. Knock-down of Osx_tv1 leads to an increase of osx_tv2 mRNA levels

For functional studies, we injected splice and translation blocking antisense morpholino oligonucleotides (MOs; Gene Tools, Corvallis). Atg1MO blocks the translation of *osx_tv1* and *osx_tv2*, while splice MOs (*splMO1* and *splMO2* co-injected; *splMO*) and *atg2MO* interfere exclusively with *osx_tv1* splicing and *osx_tv1* translation, respectively (Fig. 1C). We performed RT-PCR to validate and quantify the efficiency of gene knock-down and to determine levels of correctly spliced *osx* mRNA using a primer pair that binds in exon1 and exon2 (Figs. 2G, H and S2). We observed a reduction of correctly spliced *osx_tv1* RNA in splice MO injected embryos at 1 and 2 dpf (Fig. 2G, H). At 4 dpf, the levels of correctly spliced *osx_tv1* transcripts were not different from the controls (Fig. S2). In injected embryos at 1 and 2 dpf, we detected mRNAs of slightly smaller length containing pre-mature stop codons and most likely originating from the use of alternative splice acceptors (Fig. 2G, H). However, we also detected an additional band at 342 base pairs in length. Sequencing revealed that this fragment corresponds to *osx_tv2*. Although levels of correctly spliced *osx-tv1* were similar in morphants and controls at 4 dpf, the expression of *osx-tv2* was still increased in morphants (Figs. 2H and S2). No reduction of correctly spliced transcripts or presence of alternatively spliced mRNAs could be detected when we injected mismatch MO (mMOs) as control (Figs. 2H and S2). To examine whether an increase of *osx_tv2* was specific for knock-down of *osx_tv1* or an artefact of splice MO injection, we performed RT-PCR on *atg2MO* injected embryos. Injection of *atg2MO* also led to an increase of *osx_tv2* (Fig. 2G, H). This suggests that interference with splicing and translation of *osx_tv1* results in transcriptional upregulation of *osx_tv2* possibly indicating an autoregulatory feedback mechanism. Co-injection of *atg1MO* and *atg2MO* (*atg1 + 2MO*) also resulted in an increase in *osx_tv2* transcripts, while injection of *atg1MO* alone surprisingly did not (Figs. 2G, H and S2). *Osx_tv1* contains an alternative downstream start codon that results in a deduced protein shortened by 23 aa. It is possible that this alternative product might not be capable of increasing *osx_tv2* transcription. In contrast, *atg2MO* interferes with the second start codon most likely leading to reduced

Fig. 1. (A) Medaka expresses two *osx* transcript variants (tv) each encoding a full-length open reading frame. (B) Semi-quantitative RT-PCR shows that *osx_tv1* is the predominantly expressed variant in wild-type medaka at all embryogenic stages analyzed. Note that *osx_tv2* is not detectable at any stage under the experimental conditions used. *Elongation factor 1α* (*elf-1α*) was used as loading control. Synthesis of cDNA was performed with (+) or without (−) reverse transcriptase. (C) Nucleotide and amino acid sequences of medaka *osx_tv1* and *osx_tv2*. Gray shadowed letters indicate nucleotides and amino acids, which are not included in *osx_tv2*. Coding and non-coding regions are indicated by small and big letters, respectively. Positions of MOs are marked by bars. (D) Diagram showing possible splicing of *osx_tv1* and *osx_tv2* transcripts. Numbers designate nucleotides according to the first ATG. Black and gray boxes represent exons and UTR, respectively. Figure is not drawn to scale. The size of the intron is according to Ensembl annotation. Lengths of 5′ and 3′UTRs are unkown. Note that ATG2 is not included in *osx_tv2*.

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