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The *osr1* and *osr2* genes act in the pronephric anlage downstream of retinoic acid signaling and upstream of *wnt2b* to maintain pectoral fin development

Ana Neto[1], Nadia Mercader[2] and José Luis Gómez-Skarmeta[1,*]

SUMMARY

Vertebrate odd-skipped related genes (Osr) have an essential function during the formation of the intermediate mesoderm (IM) and the kidney structures derived from it. Here, we show that these genes are also crucial for limb bud formation in the adjacent lateral plate mesoderm (LPM). Reduction of zebrafish Osr function impairs fin development by the failure of *tbx5a* maintenance in the developing pectoral fin bud. Osr morphant embryos show reduced *wnt2b* expression, and increasing Wnt signaling in Osr morphant embryos partially rescues *tbx5a* expression. Thus, Osr genes control limb bud development in a non-cell-autonomous manner, probably through the activation of Wnt2b. Finally, we demonstrate that Osr genes are downstream targets of retinoic acid (RA) signaling. Therefore, Osr genes act as a relay within the genetic cascade of fin bud formation: by controlling the expression of the signaling molecule Wnt2ba in the IM they play an essential function transmitting the RA signaling originated in the somites to the LPM.

KEY WORDS: Osr genes, *wnt2b*, *tbx5a*, Limb, Zebrafish, Retinoic acid

INTRODUCTION

The *odd-skipped* (*Odd/Osr*) family of genes comprises evolutionary conserved zinc-finger transcription factors that lie at the top of the genetic hierarchy required for renal development in vertebrates and probably also in *Drosophila* (James et al., 2006; Tena et al., 2007; Wang et al., 2005). Mammalian genomes contain two paralogs, *Osr1* and *Osr2* (Lan et al., 2001; So and Danielian, 1999). In the mouse, *Osr1* expression starts early (E7.5) in the intermediate mesoderm (IM), from where renal structures derive (Mugford et al., 2008; So and Danielian, 1999), and is maintained until kidney organogenesis occurs. *Osr2*, by contrast, is activated at stage E9.25 in the mesonephros, and later (stage E14.5) in the mesenchyme that surrounds the ducts of the mesonephros and metanephros (Lan et al., 2001). *Osr1* knockouts lack renal structures (James et al., 2006; Wang et al., 2005), whereas *Osr2* mutants have apparently normal kidney development (Lan et al., 2004). *Xenopus* and zebrafish genomes also contain two Osr genes, and, in contrast to the mouse genes, both of them seem to contribute to some extent to the formation of the kidney (Tena et al., 2007). Indeed, knock down of both genes generates stronger kidney defects than single depletions, indicating partial redundancy between both genes. A partial redundancy of these two genes is further observed in gain-of-function assays in *Xenopus* and zebrafish (Tena et al., 2007), and can also be observed in knock-in experiments in mice (Gao et al., 2009).

Beside the kidney, vertebrate Osr genes are expressed in many other tissues. Analysis of mutant lines has indicated that these genes are required for proper formation and/or patterning of the endoderm, the heart, the teeth, the palate, the bones and the synovial joints in the limbs (Gao et al., 2011; Kawai et al., 2007; Lan et al., 2004; Mudumana et al., 2008; Wang et al., 2005; Zhang et al., 2009). Expression studies in mice and chicken indicate that, in the limb, Osr genes are expressed from very early stages in a highly dynamic pattern (Lan et al., 2001; So and Danielian, 1999; Stricker et al., 2006). These genes start to be expressed at E11.5 in mouse or HH22 stage in chick limb buds in largely overlapping domains in the bud mesenchyme, the tissue that will form, among other cell types, the bones. Slightly later, the expression of *Osr1* and *Osr2* becomes largely complementary, whereby *Osr2* is expressed more proximally and *Osr1* more distally. Finally, at later stages during limb development, both genes are again co-expressed in the developing joints. Despite this complex and dynamic expression patterns covering most of the developing limb, tissue-specific ablation using a lateral plate mesoderm (LPM)-specific Cre line (Prx1::Cre) has recently indicated that *Osr1* and *Osr2* genes are only required for joint development (Gao et al., 2011). To obtain insight about other possible functions of these genes during limb formation, and the degree of conservation of Osr function during the development of vertebrate appendages, we have examined the requirement of both *osr1* and *osr2* genes during development of the zebrafish pectoral fins, the structures that are equivalent to forelimbs in tetrapods.

During early stages of development, the pectoral fin/forelimb field is induced with the specification of a group of LPM cells on either side of the embryo's trunk at precise positions along the anterior/posterior (A/P) axis (Capdevila and Izpisua Belmonte, 2001; Johnson and Tabin, 1997; Mercader, 2007; Tickle, 1999; Duboc and Logan, 2011). Interactions between the mesenchyme and the overlying ectoderm trigger the outgrowth of the fin/limb bud. The earliest molecular marker described as an initiator of limb bud formation is the T-box transcription factor Tbx5, which is expressed in the limb mesenchymal precursors of all tetrapods as well as all fish species analyzed so far (Agarwal et al., 2003; Begemann and Ingham, 2000; Gibson-Brown et al., 1996; Isaac et

[1]Centro Andaluz de Biología del Desarrollo, Consejo Superior de Investigaciones Científicas/Universidad Pablo de Olavide, Carretera de Utrera Km1, 41013 Sevilla, Spain. [2]CNIC, Fundación Centro Nacional de Investigaciones Cardiovasculares Carlos III, Melchor Fernandez Almagro 3, 28029 Madrid, Spain.

*Author for correspondence (jlgomska@upo.es)

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al., 1998; Logan et al., 1998; Ruvinsky et al., 2000; Saito et al., 2006; Tamura et al., 1999). Indeed, *Tbx5* is both necessary and sufficient for forelimb formation (Agarwal et al., 2003; Garrity et al., 2002; Minguillon et al., 2005; Ng et al., 2002; Rallis et al., 2003; Takeuchi et al., 2003; Ahn et al., 2002). In zebrafish, *tbx5a* mutant *heartstrings* (*hst*) embryos, as well as *tbx5a* morphants, show a complete loss of pectoral fins (Garrity et al., 2002; Ng et al., 2002; Ahn et al., 2002). The cells of the LPM expressing *tbx5a* fail to aggregate to form the compact circular structure of the wild-type fin bud. An upstream regulator of *Tbx5* in the establishment of the limb field is the retinoic acid (RA) pathway (Begemann et al., 2001; Gibert et al., 2006; Grandel and Brand, 2011; Grandel et al., 2002; Mercader et al., 2006; Mic et al., 2004; Zhao et al., 2009). During development, the limiting step in the synthesis of RA is catalyzed mainly by aldehyde dehydrogenase 1a2. The medaka, zebrafish and mouse mutants for *Raldh2*/*aldh1a2* display absence of *Tbx5* expression in the limb mesenchyme, and fail to form fins/limbs (Begemann et al., 2001; Grandel et al., 2002; Mic et al., 2004; Niederreither et al., 1999; Negishi et al., 2010). RA signaling has been proposed to regulate fin outgrowth in a two-step process: during gastrulation it promotes initiation of *tbx5* expression, whereas during early somitogenesis it is necessary to expand and maintain the *tbx5* expression domain (Begemann et al., 2001; Grandel et al., 2002; Grandel and Brand, 2011). These phenotypes can be rescued by the exogenous application of RA or by transplantation of wild-type cells (Gibert et al., 2006; Linville et al., 2004; Mercader et al., 2006; Mic et al., 2004; Niederreither et al., 1999; Zhao et al., 2009; Negishi et al., 2010).

The Wnt signaling pathway has also been proposed to regulate fin/limb outgrowth. In the chick, *Wnt2b* is detected in the IM as well as in the LPM of the wing bud field (Kawakami et al., 2001). In the zebrafish, two *wnt2b* orthologs have been described, *wnt2ba* and *wnt2bb*. *wnt2ba* expression is restricted to the IM, whereas *wnt2bb* expression has been described in the LPM at the time of fin formation (Ng et al., 2002; Ober et al., 2006). *Wnt2b* gain-of-function in the chick leads to ectopic limb formation (Kawakami et al., 2001), whereas morpholino-mediated *wnt2ba* gene silencing in the zebrafish downregulates *tbx5* expression and impedes proper fin bud outgrowth (Ng et al., 2002; Wakahara et al., 2007). *wnt2ba* expression is regulated by RA signaling (Mercader et al., 2006; Negishi et al., 2010), suggesting that RA might control limb development by controlling *wnt2b* expression.

The role of the IM, a kidney precursor tissue, in controlling limb outgrowth is still controversial. Classical experiments involving surgical ablation of the mesonephros in the chick led to impaired limb outgrowth (Geduspan and Solursh, 1992; Stephens and McNulty, 1981). On the contrary, physical block of IM and LMP did not interfere with limb development (Fernandez-Teran et al., 1997). In addition, genetic ablation of the kidney anlage in mouse appears to be compatible with the development of a normal limb (Bouchard et al., 2002). In opposition to this finding, a recently reported *Xenopus* mutant of the nephronectin gene, a small diffusible integrin ligand necessary for metanephros formation, display a complete lack of forelimbs (Abu-Daya et al., 2011).

Here, we report that in zebrafish Osr genes are required within the kidney anlage to maintain proper levels of *tbx5a* expression in the LPM during early stages of limb development. This dependence is mediated, at least in part, by Wnt2b signaling, which originates at the pronephros and requires Osr function. We also show that Osr genes are controlled by RA signaling. Therefore, our studies allow connecting the RA and the Wnt pathways during early limb formation through Osr function.

MATERIALS AND METHODS

Zebrafish in situ hybridization

Antisense RNA probes were prepared from cDNAs using digoxigenin or fluorescein (Boehringer Mannheim) as labels. Zebrafish specimens were prepared, hybridized and stained as described (Harland, 1991; Jowett and Lettice, 1994). For *wnt2ba* in situ hybridization, the blocking solution used was 2% blocking powder (Roche) in maleic acid solution (0.1 M, pH 7.5) and the antibody was diluted in the same blocking solution. For in situ hybridization on sections, embryos were cut using a Leica VT100S vibratome at 30 μm after staining and examined under the microscope.

In vitro RNA synthesis, microinjection of mRNA and morpholinos and RT-PCR from injected embryos

All DNAs were linearized and transcribed as described previously (Harland and Weintraub, 1985) with a GTP cap analog (New England Biolabs), using SP6, T3 or T7 RNA polymerases. After DNAse treatment, RNA was extracted with phenol-chloroform, column purified and precipitated with ethanol. mRNAs for overexpression studies were resuspended in water and injected at the desired concentration in the yolk at the one-to two-cell stage. For knockdown experiments, zebrafish embryos were injected in the yolk at the one-to two-cell stage with 7.5-20 ng of morpholinos. The translation specific *MOosr1* and *MOosr2* morpholinos and the Osr overexpression constructs have been described previously (Tena et al., 2007). The *MOosr1sp* morpholino was described by Mudumana et al. (Mudumana et al., 2008). The *MOosr2sp* morpholino used in this study targets the acceptor splicing site from exon 3 (5′-ATATATCTGAGGAACAGGCGAGAGG-3′). To evaluate its efficiency on blocking *osr2* mRNA splicing, we designed primers in exons 2 and 4 (5′-GGGAGACACTTCACCAAATCC-3′ and 5′-CGTGATCGTGAGTGCTATGG-3′). For RT-PCR, total RNA was extracted at 48 hpf from 25 morphants and control embryos and amplification was

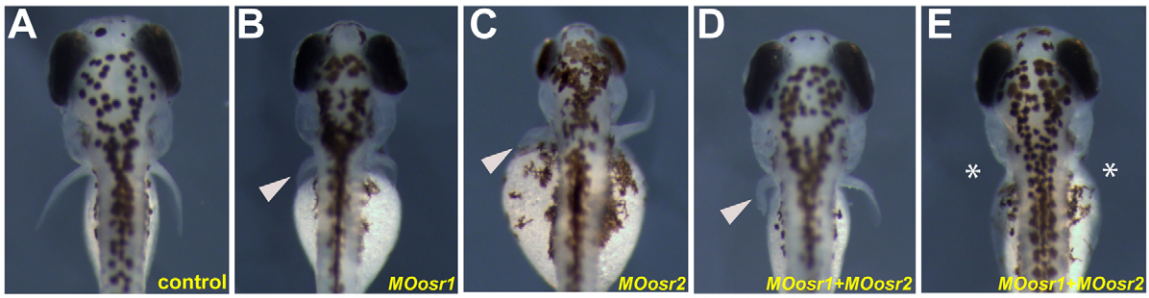

Fig. 1. Zebrafish Osr genes are necessary for pectoral fin formation. All panels show the dorsal view of 5 dpf zebrafish larvae with anterior towards the top. (**A**) Control uninjected embryos. (**B**,**C**) Embryos injected with 20 ng of *MOosr1* (B) or *MOosr2* (C). (**D**,**E**) Embryos injected with 10 ng of each morpholino. Note that the reduction of the pectoral fin is stronger in double-injected embryos (D,E) than in embryos morphant for each individual gene (B,C). Arrowheads indicate reduced fins and the asterisks indicate the complete elimination of the fins in a double morphant larvae.

carried out for 30 cycles. A band of 297 bp is produced only if the *MOosr2sp* cause the skipping of exon 2. The elimination of this exon of the *osr2* mRNA introduces several precocious stop codons. The MO*wnt2ba* morpholino used targets acceptor splicing site from exon 2 (5′-CTGCAGAAACAA-ACAGACAATTAAG-3′) and was injected at 6 to 10 ng per embryo. Primers used for RT-PCR to test efficiency of gene silencing were wnt2ba_ex1-F (5′-GCCAGAGTGTGATGGAGTTG-3′) and wnt2ba_ex5-R (5′-CATGCCTCTGGAGGACTTGT-3′). A band of 982 bp spanning exon2 to exon5 disappears in MO*wnt2ba*-injected embryos.

The following primers were used to detect *lef1* mRNA levels in control and injected embryos by qRT-PCR assays: lef1F (5′-CATCCAGCCA-TTGTCAACC-3′), lef1R (5′-CAGCATGAAAGCGTTTAGAGG-3′). Amplification of the ef1a gene (McCurley and Callard, 2008) was used for normalization (primers ef1aF 5′-CTTCTCAGGCTGACTGTGC-3′ and ef1aR 5′-CCGCTAGCATTACCCTCC-3′).

Pharmacological treatments

Embryos were incubated in the dark at 28°C in 10^{-8} M all-trans retinoic acid (Sigma), diluted in E3 embryo medium, from a 10^{-5} M stock solution in DMSO. DEAB (4-diethylaminobenzaldehyde, Sigma Aldrich), a competitive reversible inhibitor of retinaldehyde dehydrogenases (Begemann et al., 2004), was applied at a concentration of 10^{-4} M diluted from a 0.1 M stock in DMSO in E3 media. As controls, wild-type embryos were treated with similar dilutions of DMSO without drugs. Embryos injected with inducible domain of β-catenin (Afouda et al., 2008), were incubated with dexamethasone (4 μg/ml, Sigma Aldrich) in E3 embryo medium (Kolm and Sive, 1995).

RESULTS

Osr genes are required for fin development in zebrafish

We have previously reported two zebrafish morpholinos (*MOosr1*and *MOosr2*) that effectively impair *osr1* and *osr2* mRNA translation in zebrafish (Tena et al., 2007) (supplementary material Fig. S1A). Embryos injected with 20 ng of any of the individual MOs show reduction of fin buds at 5 dpf (see Table 1 for phenotypes in embryos injected with different MOs), although the proportion of affected embryos and the reduction of fin size was higher in *osr1* morphant (Table 1, Fig. 1A-C). At these higher doses, we also observed craniofacial defects (Fig. 1C) similar to those described in mouse embryos deficient for *Osr2* (Lan et al., 2004). Co-injection of both MOs

Table 1. Effect of morpholino injections in different markers at different developmental stages

Reduction on comparison with wild-type siblings	MOzosr1 (20 ng/embryo)	MOzosr2 (20 ng/embryo)	MOzosr1 (10 ng/embryo) + MOzosr (10 ng/embryo)	MOzosr1sp (7.5 ng/embryo)	MOzosr2sp (10 ng/embryo)	MOzosr1sp (3.75 ng/embryo) + MOzosr2sp (5 ng/embryo)
Fins at 5 days	*n*=89; wild type, 23%; reduction, 47%; absent or reduced unilaterally, 25%; absent bilaterally, 5%	*n*=33; wild type, 52%; reduction, 39%; absent or reduced unilaterally, 9%; absent bilaterally, 0%	*n*=201; wild type 15%; reduction, 48%; absent or reduced unilaterally, 25%; absent bilaterally, 12%	*n*=146; wild type, 19%; reduction, 66%; absent or reduced unilaterally, 15%; absent bilaterally, 0%	*n*=36; wild type, 50%; reduction, 47%; absent or reduced unilaterally, 15%; absent bilaterally, 0%	*n*=88; wild type, 36%; reduction, 32%; absent or reduced unilaterally, 25%; absent bilaterally, 7%
tbx5a at 8 somites	ND	ND	*n*=25; wild type, 100%; reduction, 0%	*n*=58; wild type, 84%; reduction, 16%	*n*=60; wild type, 97%; reduction, 3%	*n*=62; wild type, 95%; reduction, 5%
tbx5a at 13 somites	ND	ND	*n*=60; wild type, 25%; reduction, 75%	*n*=32; wild type, 25%; reduction, 75%	*n*=109; wild type, 28%; reduction, 72%	*n*=79; wild type, 24%; reduction, 76%
tbx5a at 21 somites	*n*=113; wild type, 43%; reduction, 57%	*n*=203; wild type, 53%; reduction, 47%	*n*=147; wild type, 32%; reduction, 68%	*n*=32; wild type, 17%; reduction, 83%	*n*=32; wild type, 24%; reduction, 76%	*n*=32; wild type, 12%; reduction, 88%
tbx5a at 24 hpf	*n*=90; wild type, 40%; reduction, 60%	*n*=88; wild type, 44%; reduction, 56%	*n*=162; wild type, 33%; reduction, 67%	ND	ND	ND
pax2.1 at 21 somites	*n*=29; wild type, 34%; reduction, 66%	*n*=11; wild type, 73%; reduction, 27%	*n*=36; wild type, 33%; reduction, 67%	*n*=53; wild type, 36%; reduction, 64%	*n*=40; wild type, 80%; reduction, 20%	*n*=50; wild type, 42%; reduction, 58%
tbx5 and *pax2.1* at 24 hpf	*n*=33; wild type, 21%; reduction of both markers, 76%; reduction of *tbx*5 only 3%	*n*=27; wild type, 22%; reduction of both markers, 14%; reduction of *tbx*5 only, 64%	*n*=98; wild type, 25%; reduction of both markers, 67%; reduction of *tbx*5 only, 8%	ND	ND	ND
wnt2ba at 13 somites	*n*=32) ; wild type, 10%; reduction, 90%	*n*=18; wild type, 94%; reduction, 6%	*n*=20; wild type, 20%; reduction, 80%	ND	ND	ND
wnt2bb (*prt*) at 21 somites	*n*=36; wild type, 22%; unilateral reduction, 33%; bilateral reduction, 45%	*n*=16; wild type, 31%; unilateral reduction, 31%; bilateral reduction, 38%	*n*=34; wild type, 26%; unilateral reduction, 18%; bilateral reduction, 56%	ND	ND	ND

Results show reduction in comparison with wild-type siblings.

at half doses caused a fin reduction similar or slightly higher to that observed with *MOosr1* alone (Table 1, Fig. 1D,E). Accordingly, in the double knockdown there was a higher proportion of embryos with no fins, than in *osr1* single morphants (Table 1, Fig. 1E). Similar effects (Table 1) were observed with a different set of MOs that block the correct splicing of *osr1* and *osr2* mRNAs (Mudumana et al., 2008) (supplementary material Fig. S1B). These results indicate that both genes influence the genetic cascade that operates during fin development. We then examined the expression patterns of these genes during stages of early fin bud formation and compared them with that of *tbx5a*. The expression of *tbx5a* in the LPM starts at the 7-somite stage (Begemann and Ingham, 2000) (Fig. 2C). At this stage, *osr1* was expressed all along the IM, whereas *osr2* was present at very low levels only in the posterior IM (Fig. 2A,B). At the 13-somite stage, the expression of *tbx5a* in the LPM was stronger and both *osr1* and *osr2* localized adjacent to this domain at the anterior IM in the pronephric anlage (Fig. 2D-F). At this stage, in the posterior IM, *osr1* was found in a more lateral domain (Fig. 2D, asterisk), as previously shown (Mudumana et al., 2008). The same relative distribution of *tbx5a*, *osr1* and *osr2* was observed at 24 hours post fertilization (hpf), although at this stage *osr1* also showed expression in endodermal cells (Mudumana et al., 2008; Tena et al., 2007) (Fig. 2G-I). At 48 hpf, although *osr1* is very weakly expressed in the growing limb bud (Fig. 2K), *osr2* is observed in two patches, one in the anterior and another in the posterior edges of this territory (Fig. 2L). A stronger and broader expression of *osr2* was observed in the fins at 72 hpf, whereas *osr1* is only moderately expressed in the fin at this stage (Fig. 2M-O).

Osr genes are required within the pronephric anlage for proper fin bud formation

The rather late expression of Osr genes in the fin fields let us examine at what stage pectoral fin development was affected in the Osr morphant embryos. In embryos injected with single or combinations of *osr1* and *osr2* MOs, the expression of *tbx5a* was not clearly affected at the 7- to 8-somite stage (Table 1, Fig. 3A-D). However, from the 12-somite stage onwards, we observed a reduction of *tbx5a* (Table 1, Fig. 3E-P). In these experiments, we used as an internal control *tbx5a* expression in the eye (Fig. 3Q-T), which was not affected in injected embryos. Importantly, *tbx5a* rarely disappeared completely in the morphant embryos, as we frequently observed for *pax2a* in the anterior pronephros (Fig. 3U,X). This indicates that Osr genes influence the maintenance, but not the triggering, of *tbx5a* expression. By analyzing in the same embryos the expression of the pronephric marker *pax2a* and *tbx5a*, we observed that there is a high correlation between kidney and fin defects in the Osr morphant embryos (Fig. 3U-X). Nevertheless, a fraction of the morphant embryos, which is higher in *osr2*-deficient ones (see Table 1), showed reduced *tbx5a* expression but no apparently affected kidneys (Fig. 3W). This indicates that the fin phenotype can occur even at Osr levels that are enough for kidney formation. As both genes seem to participate in fin formation, we

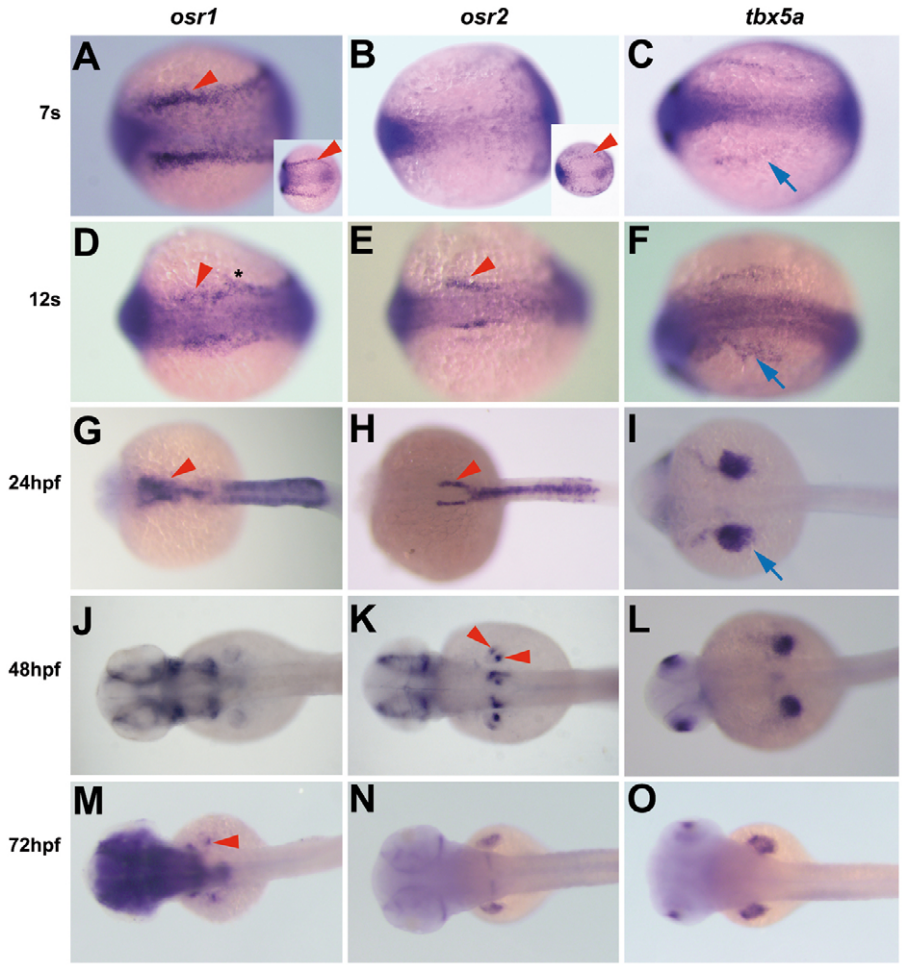

Fig. 2. Comparative expression pattern of Osr genes and *tbx5a* during pectoral fin development. All panels show the dorsal view of zebrafish embryos with the anterior towards the left after whole-mount in situ hybridization using riboprobes indicated. (**A-C**) At 7 somites, *osr1* is expressed in the IM (A, arrowhead) adjacent to the LPM domain expressing *tbx5a* (C, arrow). At this stage, *osr2* is observed only in the posterior IM (arrowheads in A and B insets). (**D-F**) At 12 somites, *osr1* and *osr2* are found in the kidney anlage (D,E, arrowheads) medial to the fin precursor territories showing *tbx5a* expression (F, arrow). Asterisk in D shows a lateral displacement of *osr1* expression in the posterior IM. (**G-I**) The same situation is observed at 24 hpf. (**J-L**) At 48 hpf, *osr1* expression is detected very weakly in the fin bud (J), whereas *osr2* is found in two patched domains within the *tbx5a*-expressing territory (K, arrowheads). (**M-O**) At 72 hpf, *osr2* is expressed in a broader domain (N) overlapping with most of the *tbx5a*-expressing territory (O), whereas *osr1* expression occurs only in small patches in the developing limbs (M, arrowheads). hpf, hours postfertilization; s, somites.

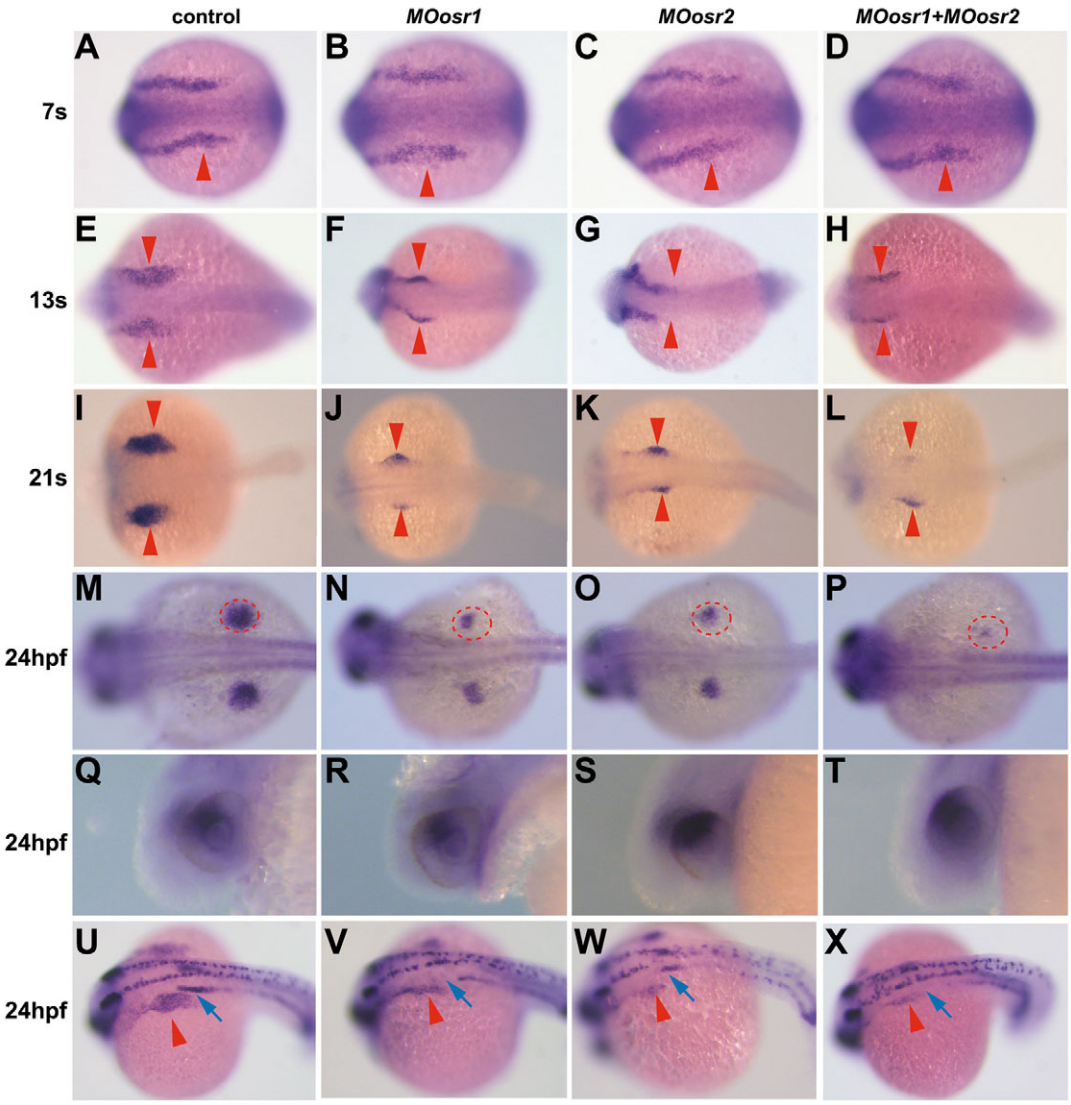

Fig. 3. Osr genes are required for *tbx5a* expression during fin bud formation. Dorsal (A-P,U-X) and lateral (Q-T) views of *tbx5a* whole-mount in situ hybridization on zebrafish embryos injected with MO*osr1*, MO*osr2* or a combination of both, as well as control embryos. Anterior is towards the left. (**A-P**) Osr MO-injected embryos reveal a smaller *tbx5a* expression domain in the pectoral fin field when compared with controls. Red arrowheads (A-L) or circles (M-P) mark *tbx5a* expression in the developing fins. (**Q-T**) At 24 hpf, the expression of *tbx5a* in the eye is not affected in morphant embryos. (**U-X**) *tbx5a* and *pax2a* double in situ hybridization. Red arrowheads and blue arrows indicate *tbx5a* and *pax2a* expression in the fin buds and anterior pronephros, respectively. The anterior *pax2a* expression domain disappears in MO*osr1* and MO*ors1*+MO*osr2*-injected embryos.

also evaluated the ability of individual Osr genes to rescue *tbx5a* expression in double Osr-morphant embryos. For this experiment, we used the Myc-tagged Osr mRNAs (*MTosr1* or *MTosr2*), which are insensitive to the translation-blocking MOs (Tena et al., 2007). Both Osr genes were similarly capable of partially rescuing the expression of *tbx5a* in the pectoral fin territory, as well as the expression of *pax2a* in the pronephros (Fig. 4A-D). Thus, whereas 93% of the embryos morphant for both Osr genes showed reduced *tbx5a* and *pax2a* expression, this proportion was reduced to 83% (n=138) or 76% (n=250) in embryos co-injected with the Osr MOs and *MTosr1* or *MTosr2* mRNAs, respectively. As shown previously (Tena et al., 2007), the overexpression of the Osr mRNAs caused strong gastrulation defects, which is likely to preclude a higher proportion of rescued embryos. We conclude that both Osr genes influence pectoral fin development at the time when they are not expressed in this territory but are co-expressed in the kidney anlage (Tena et al., 2007).

Osr genes are necessary for Wnt2b expression in the intermediate mesoderm

Both in zebrafish and chick embryos, it has been reported that *wnt2ba,* which is expressed in the IM, is essential for limb formation by controlling *tbx5a* expression (Kawakami et al., 2001; Ng et al., 2002). In order to analyze the epistatic relationship of Wnt2b and Osr genes in controlling limb development, we first compared the temporal expression profile of the Osr and the *wnt2ba* gene. At the 7-somite stage, when the expression of *tbx5a* initiates (Fig. 2A), *wnt2ba* is still not expressed (Fig. 5A). The

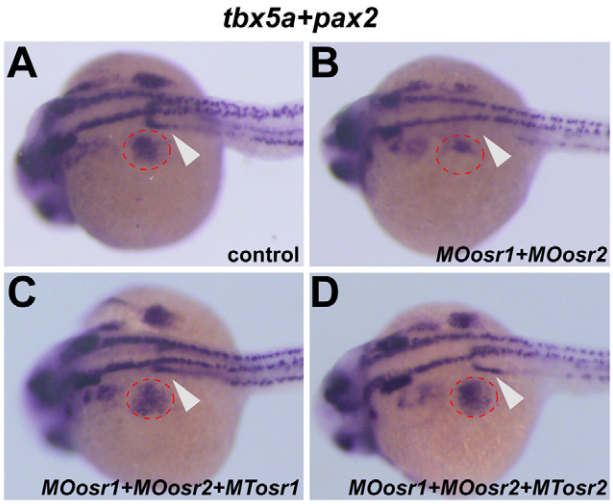

Fig. 4. Osr mRNAs rescue *tbx5a* and *pax2a* expression in double morphants embryos. Whole-mount in situ hybridization using *tbx5a* and *pax2a* riboprobes on zebrafish embryos injected with *osr1/osr2* morpholinos and osr1/osr1 splicing insensitive mRNA (*MTosr1*, *MTosr2*), as indicated. Panels show a dorsolateral view at 24 hpf. In each embryo, the limb bud is encircled in red and the pronephros is indicated with an arrowhead. (**A**) Control uninjected embryo. (**B**) Embryos co-injected with MO*ors1* and MO*osr2*. (**C**,**D**) Embryos co-injected with the two Osr morpholinos and the *MTosr1* (C) or the *MTosr2* (D) mRNA. Co-injection of either *MTosr1* or *MTosr2* mRNA rescues *tbx5a* and *pax2a* expression in limb buds and pronephros, respectively.

expression of this gene became only detectable in the IM from the 10- to 12-somite stage onwards, in a pattern reminiscent to that of the Osr genes (Fig. 5B,C). Indeed, sections through 22 hpf embryos showed that Osr genes and *wnt2ba* are both co-expressed in the pronephric area (Fig. 5D-I). A similar pronephric expression was found for the related *wnt2bb* gene (Ober et al., 2006), which we detected expressed in the kidney territory only from 21-somite stage onwards (Fig. 5F, inset).

As Osr depletion downregulated *tbx5a* expression precisely at the time *wnt2ba* became activated, we next examined the expression of the Wnt2b genes in Osr morphant embryos. In *osr1* morphant embryos, the expression of both *wnt2ba* and *wnt2bb* genes was downregulated (Table 1; Fig. 6A,B,E,F). The injection of *MOosr2* did not affect *wnt2ba* expression (Fig. 6C) but caused the downregulation of *wnt2bb* (Fig. 6G). The co-injection of both Osr MOs at half doses caused similar defects to those observed in *osr1* single morphants (Table 1; Fig. 6D,H). Our results therefore suggest that Wnt signaling may contribute to the effect of Osr depletion on *tbx5a* expression and fins development. To test this, we overexpressed this pathway in embryos co-injected with both Osr MOs. For that purpose, we injected an mRNA encoding an inducible form of β-catenin fused to the dexamethasone-regulated glucocorticoid receptor (GR) domain (Afouda et al., 2008). This allowed us, upon adding dexamethasone to the injected embryos at tailbud stage, to activate the pathway only after gastrulation, which prevented early defects associated with increased Wnt signaling during this crucial period of development. In embryos injected with 50 pg of this β-catenin-GR mRNA, the expression of the Wnt-target gene *lef1* was effectively induced, as determined by qRT-PCR, but only in the presence of the hormone (supplementary material Fig. S2). At the 13-somite stage, the expression of *tbx5a* was not affected in embryos injected with β-catenin-GR mRNA in the absence of dexamethasone but was apparently slightly expanded upon dexamethasone addition (69%, *n*=52; Fig. 6I,J). In embryos co-injected with both splicing-blocking Osr MOs and β-catenin mRNA, *tbx5a* was downregulated in the absence of dexamethasone (96%, *n*=77; Fig. 6K). However, *tbx5a* downregulation was observed in significantly fewer embryos upon adding this synthetic hormone (75%, *n*=67; Fig. 6L).

Finally, we examined the expression of *osr1* and *tbx5a* in embryos with depleted *wnt2ba* function. Gene expression was analyzed at the 13-somite stage, when *tbx5a* downregulation in Osr morphant embryos was first detected and *wnt2bb* was still not present. In this experiment, we used a MO that efficiently blocks the splicing of *wnt2ba* mRNA (supplementary material Fig. S1C). In 13-somite embryos injected with MO*wnt2ba*, *tbx5a* expression

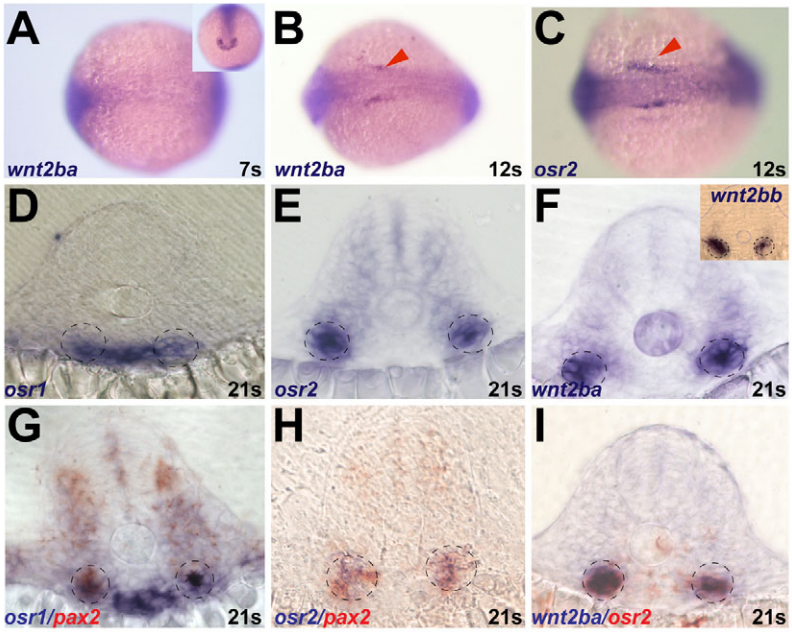

Fig. 5. Osr, *wnt2ba* and *pax2a* genes are co-expressed in the kidney anlage. (A-C) Dorsal views of embryos at the 7- (A) or 12- (B,C) somite stages, showing the expression of *wnt2ba* (A,B) and *ors2* (C), as revealed by mRNA in situ hybridization. (**A**) No expression of *wnt2ba* is visible at the 7-somite stage at the level of the intermediate mesoderm, whereas it is expressed at more anterior regions (inset). (**B**,**C**) The expression of *wnt2ba* is detected from the 12-somite stage (B, arrowhead) onwards in the kidney anlage, colocalizing with *osr2* expression (C, arrowhead). (D-I) Transverse sections of 21-somite stage embryos with dorsal at the top. (**D-F**) Embryos showing the expression of *osr1* (D), *ors2* (E) or *wnt2ba* (F) genes in the kidney anlage. Inset in F shows a similar expression pattern for *wnt2bb*. (**G-I**) Embryos double stained for *osr1* (G) or *ors1* (H) and *pax2a* (Osr genes in purple and *pax2a* in red) and *wnt2ba* (purple) and *osr2* (red) (I). The expression of all three genes overlaps in the pronephros (encircled). s, somite.

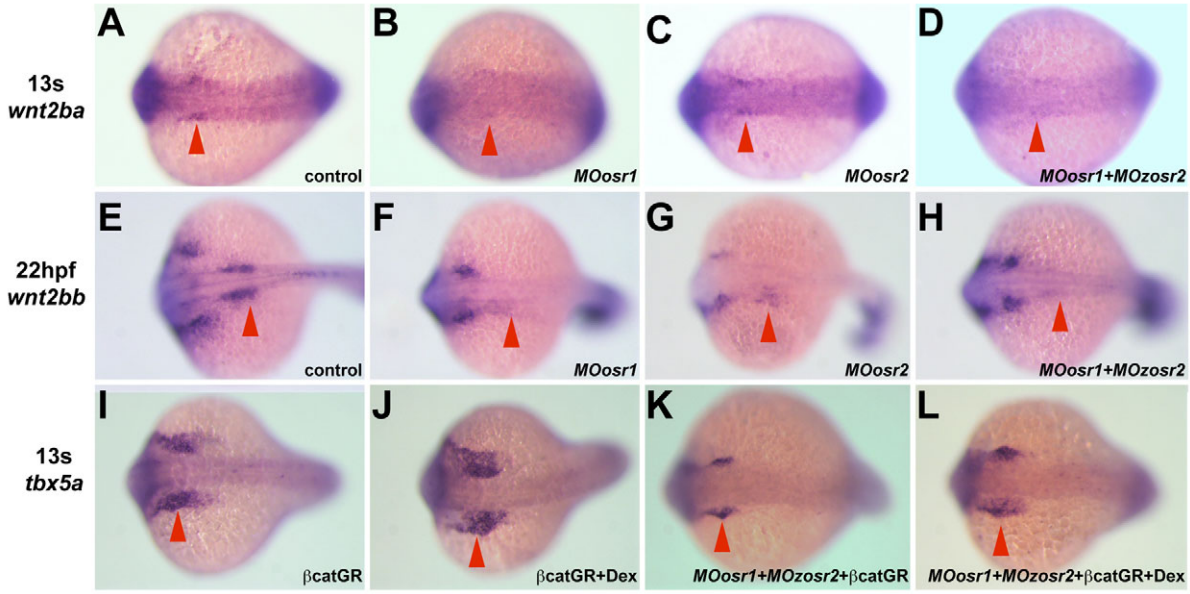

Fig. 6. *wnt2b* expression in the intermediate mesoderm requires Osr function. mRNA in situ hybridizations on 13-somite stage (*wnt2ba* and *tbx5a*) and 22 hpf (*wnt2bb*) embryos using riboprobes as indicated on the left. All panels show dorsal views with anterior towards the left. (**A-H**) *wnt2ba* (A-D) and *wnt2bb* (E-H) expression in the IM (arrowheads) in wild-type (A,E), single (B,C,F,G) and double (D,H) Osr-morphant embryos. (I-L) Increasing Wnt signaling in double Osr-morphant embryos rescues *tbx5a* expression in the limb buds at 13 somites. (**I**,**J**) Embryos injected with β-catenin-GR mRNA show normal (I) or slightly larger (J) *tbx5a* expression domains (arrowheads) in the absence or presence of dexamethasone, respectively. (**K**,**L**) Embryos co-injected with β-catenin-GR mRNA and the two Osr MOs show reduced (K, arrowhead) or rescued (L, arrowhead) *tbx5a* expression domains in the absence or presence of dexamethasone, respectively. hpf, hours postfertilization; s, somite.

was reduced (75%, *n*=90) whereas *osr1* expression was not affected (Fig. 7). Moreover, *tbx5a* reduction was similar to that found in Osr morphant embryos (compare Fig. 7D with Fig. 3F-H). These results strongly suggest that Wnt2b signaling is downstream of Osr factors and mediates, at least in part, the effect of Osr genes on *tbx5a* expression and limb development. Moreover, as the onset of *tbx5a* activation precedes that of the Wnt2b genes, this signaling pathway is probably required for maintaining, but not initiating, *tbx5a* expression, something we also found for the Osr genes.

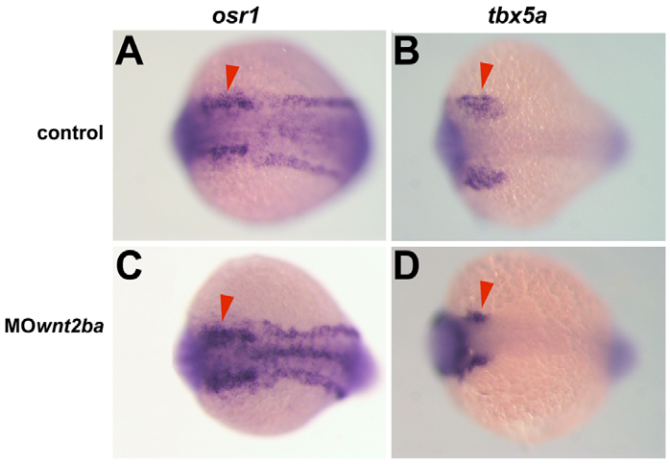

Fig. 7. Early *tbx5a* expression is reduced but not suppressed in *wnt2ba* morphant embryos. (**A-D**) Whole-mount in situ hybridization on 13-somite stage control embryos (A,B) or embryos injected with MO*wnt2ba,* using *osr1* (A,C) or *tbx5a* (B,D) riboprobes. Note that whereas *tbx5a* expression is reduced upon *wnt2ba* downregulation, *osr1* expression is not affected. Arrowheads highlight expression in the pronephros (A,B) or pectoral fin mesenchyme (B,D).

Retinoic acid is required for limb formation partially through Osr genes

Retinoic acid (RA) is essential for proper kidney development and also for limb formation through the regulation of *tbx5a* (Begemann et al., 2001; Cartry et al., 2006; Gibert et al., 2006; Grandel and Brand, 2010; Mic et al., 2004). As Osr genes are key regulators of both processes, we examined the mutual relationship between these genes and the RA pathway. We first determined whether the expression of *aldh1a2*, an enzyme required for RA production was affected in Osr morphant embryos. As expected for downstream factors of the RA pathway, the expression of *aldh1a2* was not affected in embryos injected with any of the Osr MOs or the combination of both of them (*MOosr1*, 3%, *n*=53; *MOosr2*, 2%, *n*=41; *MOosr1*+ *MOosr2*, 1%, *n*=40; Fig. 8A-D). We then manipulated RA signaling and examined Osr expression. To increase or reduce the RA signaling pathway, we incubated the embryos from tailbud to the 21- to 22-somite stages either with all-trans RA or with DEAB (4-diethylaminobenzaldehyde), a competitive reversible inhibitor of retinaldehyde dehydrogenases. Increasing RA signaling rescued the downregulation of the *tbx5a* expression promoted by DEAB treatment (48%, *n*=128; Fig. 8E-G), but was unable to rescue *tbx5a* expression in Osr morphant embryos (95% of the embryos with reduced *tbx5a* expression, *n*=47; Fig. 8H). This indicates that Osr genes are downstream of the RA pathway.

We then examined the effect of increasing or reducing the RA signaling pathway on the expression of the Osr genes. In embryos incubated with DEAB or RA from tailbud onwards, the *osr1* expression domain at the 21- to 22-somite stage was reduced (96%, *n*=25) or expanded (45%, *n*=31), respectively (Fig. 8I-K). By contrast, these treatments had, if any, minor effects on *osr2* expression (Fig. 8M-O). Therefore, during somitogenesis, only *osr1* depends on RA. Next, we determined whether the overexpression of

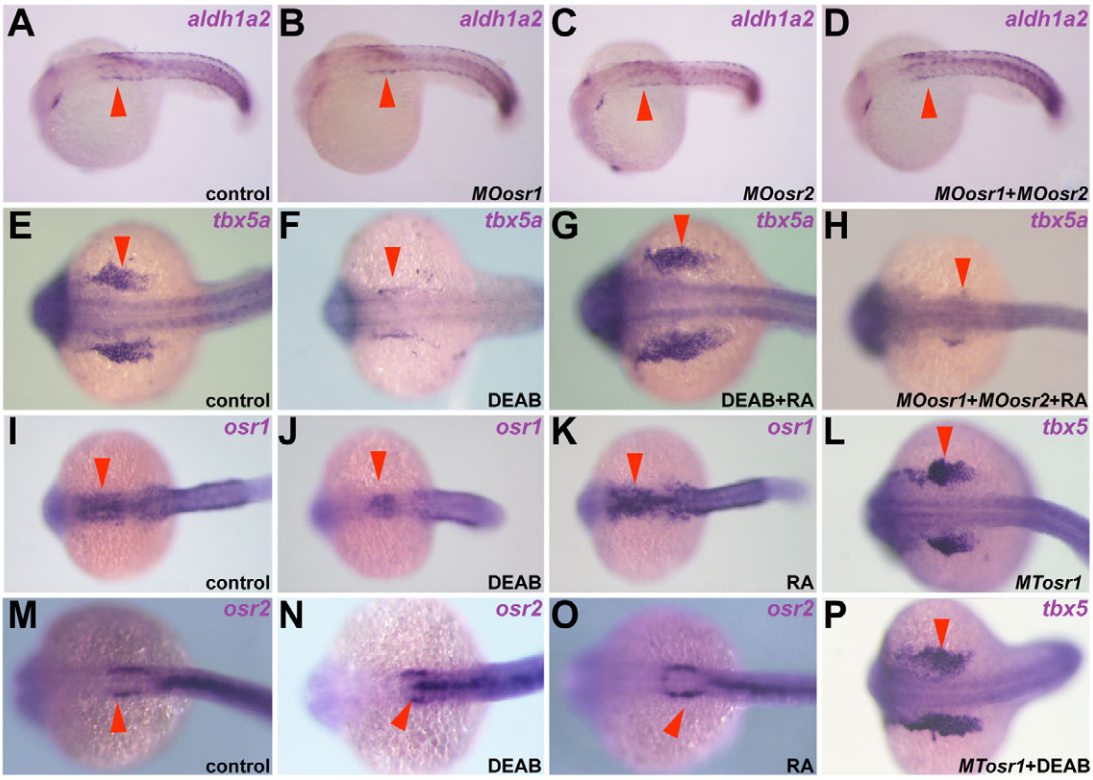

Fig. 8. Osr gene expression is regulated by retinoic acid signaling. Dorsal views of embryos at the 21- to 24-somite stage after whole-mount in situ hybridization against the genes indicated. Anterior is towards the left. Arrowheads indicate the expression of *aldh1a2* (A-D), *tbx5a* (E-H,L,P) and Osr (I-K,M-O) genes in the anterior somite, developing fin buds and pronephros, respectively. (**A-D**) *aldh1a2* expression is not affected in single or double Osr-morphant embryos. (**E-H**) Increasing RA signaling is able to rescue *tbx5a* downregulation caused by DEAB (E-G, arrowheads) but not Osr impairment (H). (**I-K**,**M-O**) Reducing (J,N) or increasing (K,O) RA signaling, reduced or expanded, respectively, the expression domain of *osr1* but not *osr2*. (**L**,**P**) Osr genes can rescue the loss of *tbx5a* expression when RA signaling is reduced.

osr1 could overcome the loss of *tbx5a* expression under conditions of reduced RA signaling. Indeed, this was the case as *tbx5a* expression was recovered in 30% of the embryos exposed to DEAB when injected with *osr1* mRNA (Fig. 8L,P).

Finally, we compared the temporal requirement of RA for the expression of Osr, *wnt2b* and *tbx5* genes. Embryos were incubated with DEAB from 50% epiboly, tailbud or 6-somite stages to the 21-somite stage, and subsequently fixed and processed for in situ hybridization. When RA was inhibited at 50% epiboly, the expression of all genes, including *osr2*, was strongly impaired, effects that became less pronounced when DEAB was added at tailbud or 6-somite stages (Fig. 9). These results indicate that, as has been shown for fin development (Gibert et al., 2006; Grandel and Brand, 2010), RA is required during gastrulation and early somitogenesis for Osr gene expression.

DISCUSSION

Novel function of Osr genes during pectoral fin induction

Our work demonstrates an essential function of zebrafish Osr1 and Osr2 transcription factors during pectoral fin formation. Osr genes are required at initial stages of fin outgrowth, for maintaining *tbx5* expression, which is the earliest marker involved in limb bud formation (Agarwal et al., 2003; Ahn et al., 2002; Garrity et al., 2002; Rallis et al., 2003). Interestingly, this requirement occurs at a developmental stage in which the Osr genes are not expressed in the fin bud primordia, but in the adjacent IM. A role for the IM during limb development has been previously been reported by others (Geduspan and Solursh, 1992; Stephens and McNulty, 1981; Abu-Daya et al., 2011). Our data further support an important function of the kidney anlage for limb bud formation.

As it has been previously found during early kidney formation in zebrafish and *Xenopus* (Tena et al., 2007), both Osr genes seem to be partially redundantly required for pectoral fin formation. A similar redundancy has also been shown during mouse joint formation (Gao et al., 2011) and in other knock-in studies (Gao et al., 2009). Nevertheless, during pectoral fin formation, we systematically observed that loss of *osr1* function seems to produce stronger defects than the reduction of *osr2* activity. This may be due to broader and earlier expression of *osr1* in the IM territory in most vertebrates, and correlates with a stronger inhibition of Wnt2b signaling by *osr1* gene silencing (Lan et al., 2001; So and Danielian, 1999; Stricker et al., 2006; Tena et al., 2007). Therefore, as proposed recently (Gao et al., 2009), the distinct developmental requirement exhibited by both genes in this and other processes is probably the result of divergence of the cis-regulatory regions that control the spatiotemporal expression of these genes, although the functional potential of both gene products is likely to be very similar.

Our results demonstrate that Osr genes are required in the IM territory to promote pectoral fin development indirectly. This indirect action could be explained, at least in part, by the diffusible molecule Wnt2b, a factor essential for *tbx5a* expression (Ng et al.,

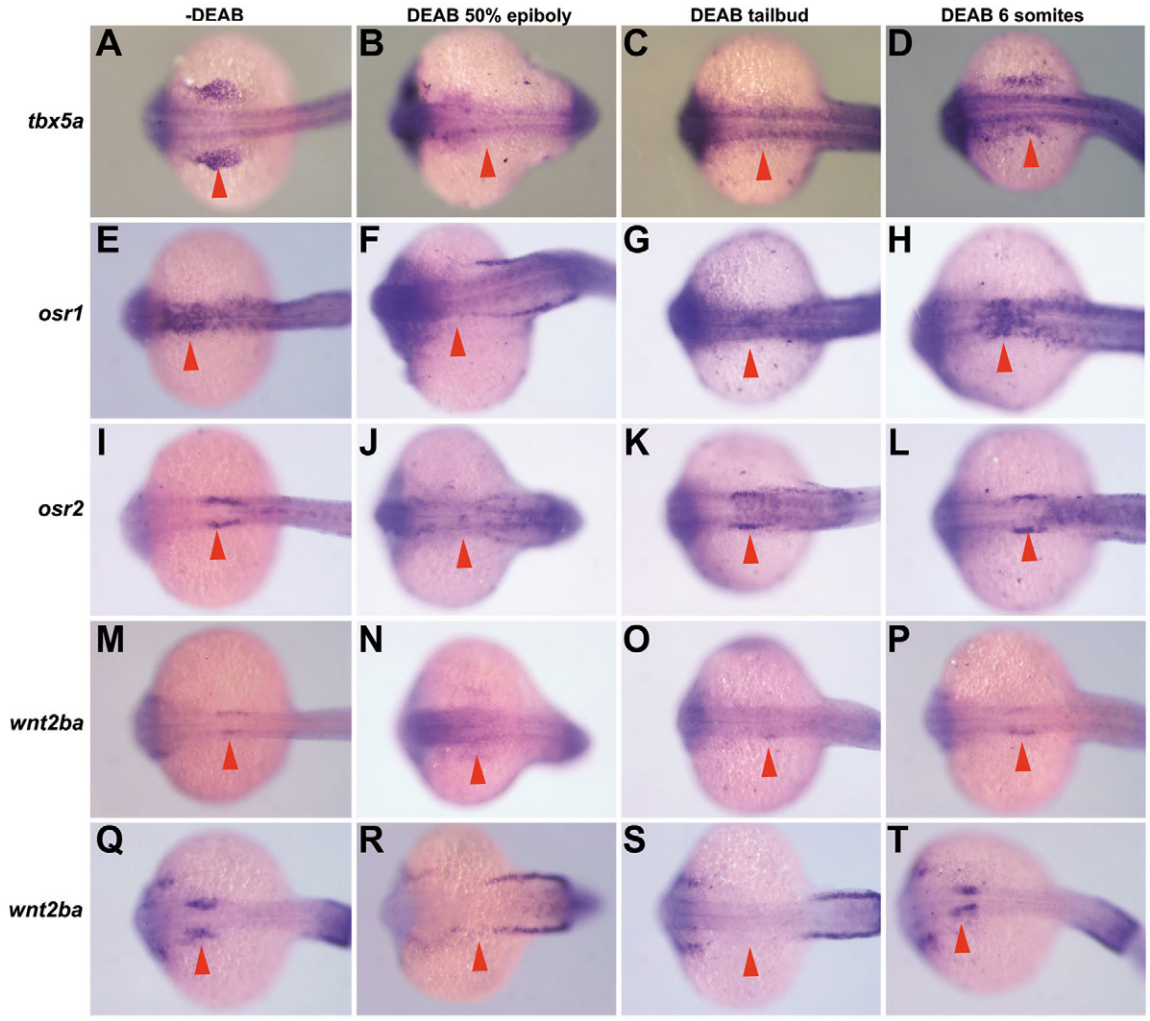

Fig. 9. Temporal requirement of RA for *tbx5a*, *wnt2b* and Osr expression. Dorsal views of embryos at the 21- to 24-somite stage treated with DEAB from the time shown above the panels showing the expression of genes indicated on the left. (**A**,**E**,**I**,**M**,**Q**) Control embryos incubated with DMSO. (**B**,**F**,**J**,**N**,**R**) Interference with RA signaling from 50% epiboly caused strong downregulation of all genes examined. (**C**,**G**,**K**,**O**,**S**) Incubation with DEAB from tailbud caused strong downregulation of *tbx5a*, *osr1*, *wnt2ba* and *wnt2bb* (C,G,O,S, respectively) while not affecting *osr2* expression (K). (**D**,**H**,**L**,**P**,**T**) Culturing embryos with DEAB from the 6-somite stage only caused some *tbx5a* downregulation (D). Arrowheads indicate the *tbx5a*-expressing domain (A-D) and the kidney anlage (E-T).

2002). Accordingly, we demonstrate that Wnt2b and Osr genes show overlapping expression domains in the IM, and that Wnt2b expression depends on the function of Osr transcription factors. Moreover, Wnt signaling partially rescues the *tbx5a* downregulation observed in Osr morphant embryos and the timing of *wnt2ba* activation at 12 somites coincides with the developmental period in which *tbx5a* expression became affected by Osr depletion. All these data suggest that the Wnt2 signaling from the kidney anlage mediates the Osr requirement for maintaining *tbx5a* expression and proper pectoral fin development. It should be nevertheless mentioned that the requirement of Osr factors for Wnt2b expression is probably indirect as these factors have been shown to behave as repressors (Tena et al., 2007).

Positioning Osr genes in the signaling cascade involved in fin induction

The earliest known player of the signaling cascade that leads to pectoral fin induction is the RA signaling pathway. RA signaling during gastrulation has been shown to be important for the establishment of a *tbx5a*-positive fin field (Grandel and Brand, 2010; Grandel et al., 2002). During somitogenesis, RA derived from the somites is involved in maintaining and expanding the *tbx5*-positive limb precursors (Begemann et al., 2001; Gibert et al., 2006; Linville et al., 2004; Mercader et al., 2006). RA is also required for kidney formation (Cartry et al., 2006) and has been shown to be able to activate the expression of Osr genes when *Xenopus* animal caps or mouse ES cells differentiated to IM or kidney identities (Drews et al., 2011; Mae et al., 2010). Here, we show that in zebrafish embryos RA signaling is also required for Osr expression. We therefore propose a model in which RA signaling is required to activate the expression of Osr genes in the IM. These transcription factors are then essential at the IM for renal organ formation but also for indirectly promoting pectoral fin development by controlling Wnt2b expression in the kidney anlage. Wnt2b, produced in the Osr-expressing domain, then might diffuse to the LPM to maintain *tbx5a* expression and promote pectoral fin formation (Fig. 10). In this genetic cascade, in which the RA signaling is relayed through three different tissues, the Osr genes in the IM play an essential linker function that responds to a signal

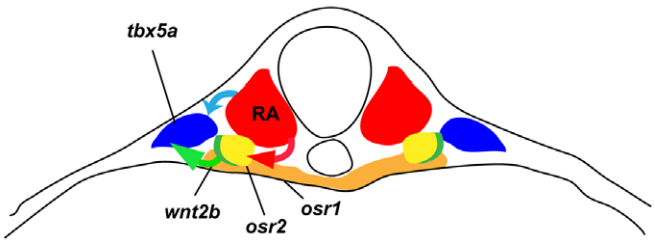

Fig. 10. Schematic representation of the relay mechanism that triggers pectoral fin development in zebrafish embryos. Retinoic acid, generated at the somites (red), activates the Osr genes (orange, *osr1*; yellow, *osr2*) in the pronephric territory. Osr transcription factors are then required for the expression of the signaling molecule *wnt2b* expression (green) to maintain *tbx5a* expression (blue) in the adjacent lateral plate mesoderm, giving rise to the fin bud primordium. Retinoic acid is also required in a Osr-independent way to initiate *tbx5a* expression (blue arrow).

from the somites and transmitting it to the LPM. Moreover, by activating the Osr genes, RA is capable of controlling in a coordinated way two different developmental processes, kidney formation and pectoral fin development. Nevertheless, the RA signaling pathway is probably also controlling limb development in an Osr-independent way as the initiation of fin bud development requires RA but seems not to depend on Osr genes (Begemann et al., 2001; Gibert et al., 2006; Grandel and Brand, 2010; Grandel et al., 2002).

How much of this genetic cascade is conserved during tetrapod limb development?

Neither *Osr1* nor *Osr2* knockout mouse embryos have been reported to present major limb defects (James et al., 2006; Lan et al., 2004; Wang et al., 2005). This could be due to a partial redundancy of both genes in the activation of *Wnt2b*, or another similar Wnt ligand, at the kidney anlage. A very recent report has examined the consequences of removing both genes at the early limb bud (Gao et al., 2011). In this study the impairment of both genes produced only late developmental defects in joint formation but it did not impair early limb bud development (Gao et al., 2011). However, in these experiments *Osr1* was still present in the IM. To examine Osr requirement precisely in the activation of limb bud formation, it would be necessary to eliminate both Osr genes from the early kidney anlage. Nevertheless, there are several reasons to believe that Osr genes are also likely to be necessary for limb bud formation in other vertebrates. First, in all vertebrates examined, proper kidney and limb formation require RA signaling (Begemann et al., 2001; Cartry et al., 2006; Gibert et al., 2006; Grandel and Brand, 2010; Grandel et al., 2002; Linville et al., 2004; Mercader et al., 2006; Mic et al., 2004; Negishi et al., 2010; Niederreither et al., 1999; Zhao et al., 2009). Second, as we report here in zebrafish, this signaling pathway also controls Osr expression in other vertebrates (Drews et al., 2011; Mae et al., 2010). Third, it has also been reported that the kidney anlage is required for limb formation in other vertebrates (Geduspan and Solursh, 1992; Stephens and McNulty, 1981), and kidney formation depends on Osr function in all vertebrates (James et al., 2006; Mudumana et al., 2008; Tena et al., 2007; Wang et al., 2005). Finally, Osr expression at the IM precedes and overlaps that of *Wnt2b* in all vertebrates examined (Kawakami et al., 2001; Mercader et al., 2006; Mudumana et al., 2008; Ng et al., 2002; Stricker et al., 2006; Tena et al., 2007). Therefore, in all vertebrates, Osr expression is in the right place (the kidney anlage) and at the right time to relay RA signaling from the somites to the LPM, at least partially, through *Wnt2b*. Functional experiments would be required to demonstrate whether Osr genes also participate in the genetic cascade required for limb bud formation in other vertebrates.

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Competing interests statement
The authors declare no competing financial interests.

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