Gene Expression Patterns 6 (2006) 826–834

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Comparative expression pattern of *Odd-skipped related* genes *Osr1* and *Osr2* in chick embryonic development

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Received 21 December 2005; received in revised form 3 February 2006; accepted 6 February 2006
Available online 22 March 2006

Abstract

Odd-skipped genes encode zinc-finger transcription factors with widespread roles in embryonic development. In *Drosophila*, *odd-skipped* acts as a pair-rule gene, while its orthologous gene in *Caenorhabditis elegans* is involved in gut development. In mammals two paralogs exist, *Osr1* and *Osr2*, with functions described in heart and urogenital, and in secondary palate development, respectively. As the chicken embryo is a widely used system for analysing gene function in vivo, we determined the expression pattern of the two chicken orthologues, *cOsr1* and *cOsr2*, during embryonic development. We demonstrate expression of both genes in a variety of organs and structures, such as kidney, eye, branchial arches and dermis. Both genes show a highly dynamic expression pattern with partially overlapping, but mostly distinct domains of expression. Special emphasis in this study was laid on the investigation of *cOsr1* and *cOsr2* in limb development, where we compared their expression pattern with the expression of *Osr1* and *Osr2* in the mouse.

Keywords: Odd-skipped; Osr1; Osr2; Chicken; Mouse; Kidney; Craniofacial development; Branchial arches; Somites; Endoderm; Limb

1. Results and discussion

In *Drosophila*, *odd-skipped* (*odd*) is part of a small gene family encoding C2H2 zinc-finger transcription factors. *Odd* was initially characterised as a pair-rule gene. Mutations in *odd* cause the loss of odd-numbered segments (Nusslein-Volhard and Wieschaus, 1980; Coulter and Wieschaus, 1988). In this context, Odd can function either as a repressor or activator of target gene transcription (Saulier-Le Drean et al., 1998). Beyond its function during segmentation, *odd* is expressed in several tissues during *Drosophila* embryogenesis (Ward and Coulter, 2000), including the gut, nephrocytes, the nervous system and pericardial cells (Ward and Skeath, 2000).

Odd homologous genes have been cloned and characterised from several other species, including *Caenorhabditis elegans* (Buckley et al., 2004), mouse (So and Danielian, 1999; Lan et al., 2001) and man (Debeer et al., 2002; Katoh, 2002).

In mammals, two *odd-skipped related* genes exist, *Osr1* and *Osr2*. Both genes exhibit a dynamic expression pattern during mouse embryogenesis, showing predominant expression in intermediate mesoderm, branchial arches, kidneys and limbs (So and Danielian, 1999; Lan et al., 2001). Functionally, *Osr1* and *Osr2* have been characterised in the mouse by gene targeting. Mice with inactivated alleles of *Osr1* show severe defects in embryonic heart and kidney development and die in utero (Wang et al., 2005). *Osr2* null mice on the other hand show specific defects in secondary palate development (Lan et al., 2004).

In the chick, it is so far only known that *cOsr1* is an early marker of the intermediate mesoderm showing expression laterally of the somites as early as Hamburger–Hamilton (HH) stage 10 (James and Schultheiss, 2005). No chicken orthologous gene for *Osr2* has been described to date. As the chicken is a powerful and widely used developmental model organism, we determined the expression pattern of

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doi:10.1016/j.modgep.2006.02.003

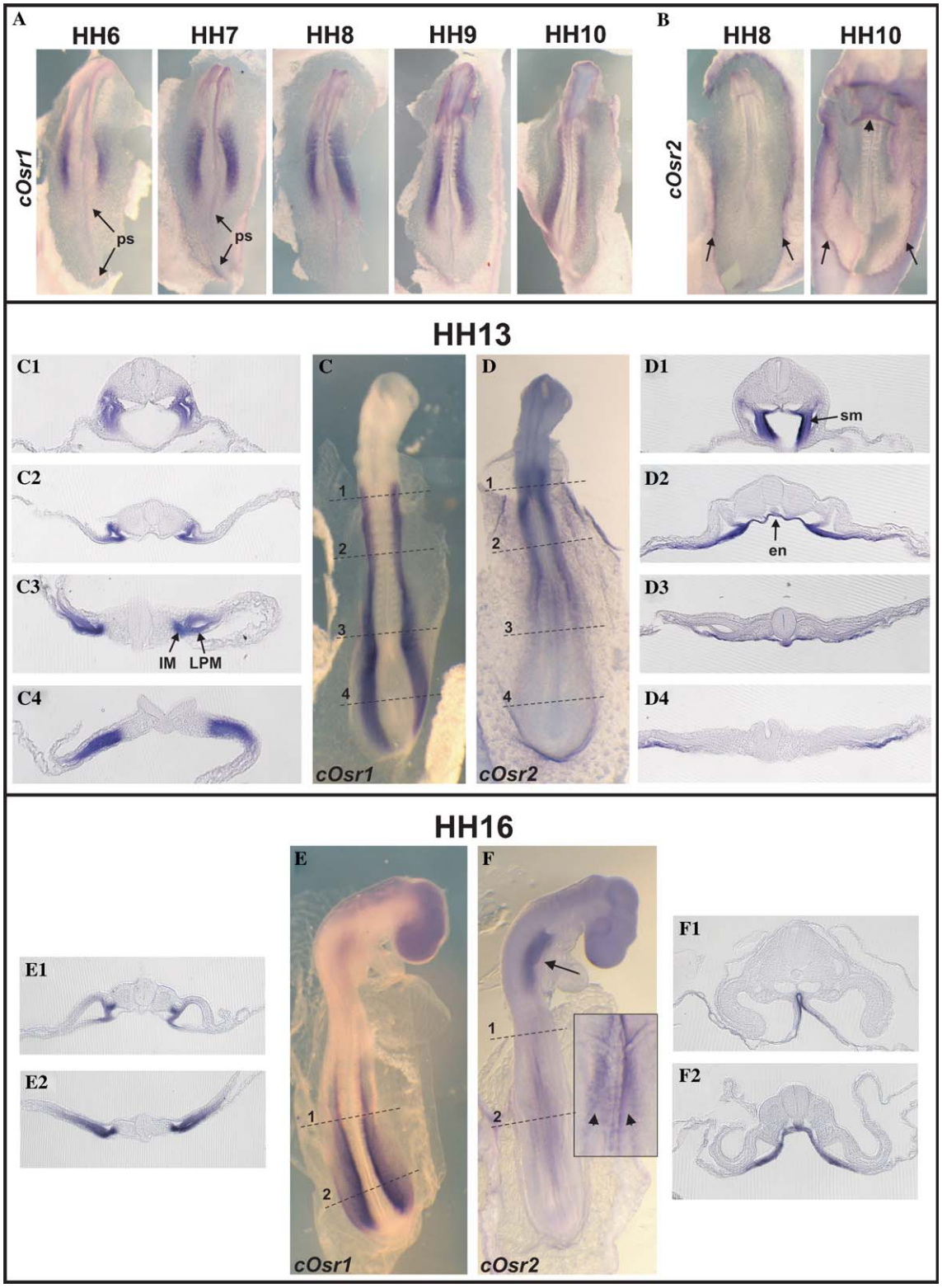


Fig. 1. Expression patterns of *cOsr1* and *cOsr2* at HH6-10 (A,B), HH13 (C,D) and HH16 (E,F). Whole-mount embryos were hybridised with antisense probes for *cOsr1* (A, C, and E) and *cOsr2* (B, D, and F). (A) Dorsal view of embryos at the indicated stages. *cOsr1* is expressed in the lateral mesoderm flanking neural tube and somites, but not in regions undergoing gastrulation (arrows). (B) Ventral view of embryos at stages indicated show expression of *cOsr2* at the margin of the area pellucida (arrows) and in the anterior intestinal portal (arrowhead). (C,D) Dorsal view of HH13 embryos. Note expression of *cOsr1* in the intermediate mesoderm (IM) and lateral plate mesoderm (LPM). (C1–4, D1–4) Transversal vibratome sections from planes indicated in (C) and (D). Note expression of cOsr2 in endoderm and splanchnic mesoderm. (E,F) Dorsal views of HH16 embryos. *cOsr1* is expressed in the IM and LPM in the posterior half of the embryo. *cOsr2* is expressed in the foregut (arrow). Ventral view (inset) shows weak expression of *cOsr2* in the endoderm (arrowheads). (E1,2 and F1,2) Vibratome sections from (E) and (F). en, Endoderm; IM, intermediate mesoderm; LPM, lateral plate mesoderm; ps, primitive streak; sm, splanchnic mesoderm.

the chicken orthologous genes *cOsr1* and *cOsr2* during embryonic development. Special emphasis was laid on embryonic limb development, where we compared the expression of *cOsr1* and *-2* to their mouse orthologues, for which no detailed limb expression pattern has been described so far.

We have used the BBSRC chicken EST database (Boardman et al., 2002) to identify ESTs containing partial sequences of chicken *Osr* genes. Two ESTs for cOsr1 matching the predicted coding sequence (XM_419967) were found and used for hybridisation. Both ESTs yielded identical results. For *cOsr2*, no ESTs were identified. We used the predicted sequence for chicken *Osr2* (XM_418353) as a template to generate specific probes. Two independent probes were used harbouring 5′-coding sequence without the zinc-finger coding region, or complete coding sequence. Both probes showed identical hybridisation signals.

In the early chick embryo, the expression of *cOsr1* is associated with the lateral mesoderm. We found first expression of *cOsr1* at HH6 in a medial domain at both sides of the neural fold (Fig. 1A). *cOsr1* shows no expression in the posterior part of the embryo, which is still undergoing gastrulation. During further development (HH8–10) the medial domain of *cOsr1* expression expands caudally, as the primitive streak regresses. *cOsr2* could first be detected at HH8 in a faint expression domain surrounding the outer margin of the area pellucida (Fig. 1B). This expression can still be seen at HH10, where *cOsr2* is also expressed in the anterior intestinal portal. At HH13, *cOsr1* shows strong expression lateral to neural tube and somites, thereby its expression domain is widening caudally (Fig. 1C). Vibratome sections demonstrate expression of *cOsr1* in intermediate mesoderm (IM) and lateral plate mesoderm (LPM). *cOsr2* on the other hand appears strongly expressed in the anterior part of the embryo (Fig. 1D). Sections reveal expression of *cOsr2* in the endoderm and, more anteriorly, also in the splanchnic mesoderm. At HH16, *cOsr1* expression is found in the IM and LPM in the posterior half of the embryo (Fig. 1E). The expression of *cOsr2* is weak at HH16, but still can be seen in a ventral view of the embryo (Fig. 1F, ventral view in inset). Sections show expression of *cOsr2* in the endoderm. Anteriorly, *cOsr2* becomes restricted to gut endoderm. Strong expression of *cOsr2* can be seen in the developing foregut (Fig. 1F, arrow), an expression that can still be seen at HH19 (Fig. 2B, arrow, and magnification in Fig. 2I). Expression of *cOsr2* later can also be found in other regions of the intestinal tract. Fig. 2F demonstrates expression in the developing caeca (yellow arrowhead) and the intestine (black arrowhead) at HH24. Vibratome sections reveal that *cOsr2* is expressed in superficial mesenchymal layers in both horns of the caecal branches (Fig. 2J) and in the epithelium of the intestine (Fig. 2K). In Fig. 2F expression of *cOsr2* cannot be seen in the hindgut. Interestingly, the endoderm of the presumptive hindgut region is also negative for *cOsr2* in earlier stages (not shown). Expression of *odd-skipped* genes in the intestinal tract appears to be an evolutionary conserved property, as expression in the gut was also demonstrated in *C. elegans* (Buckley et al., 2004) and in *Drosophila* (Ward and Coulter, 2000).

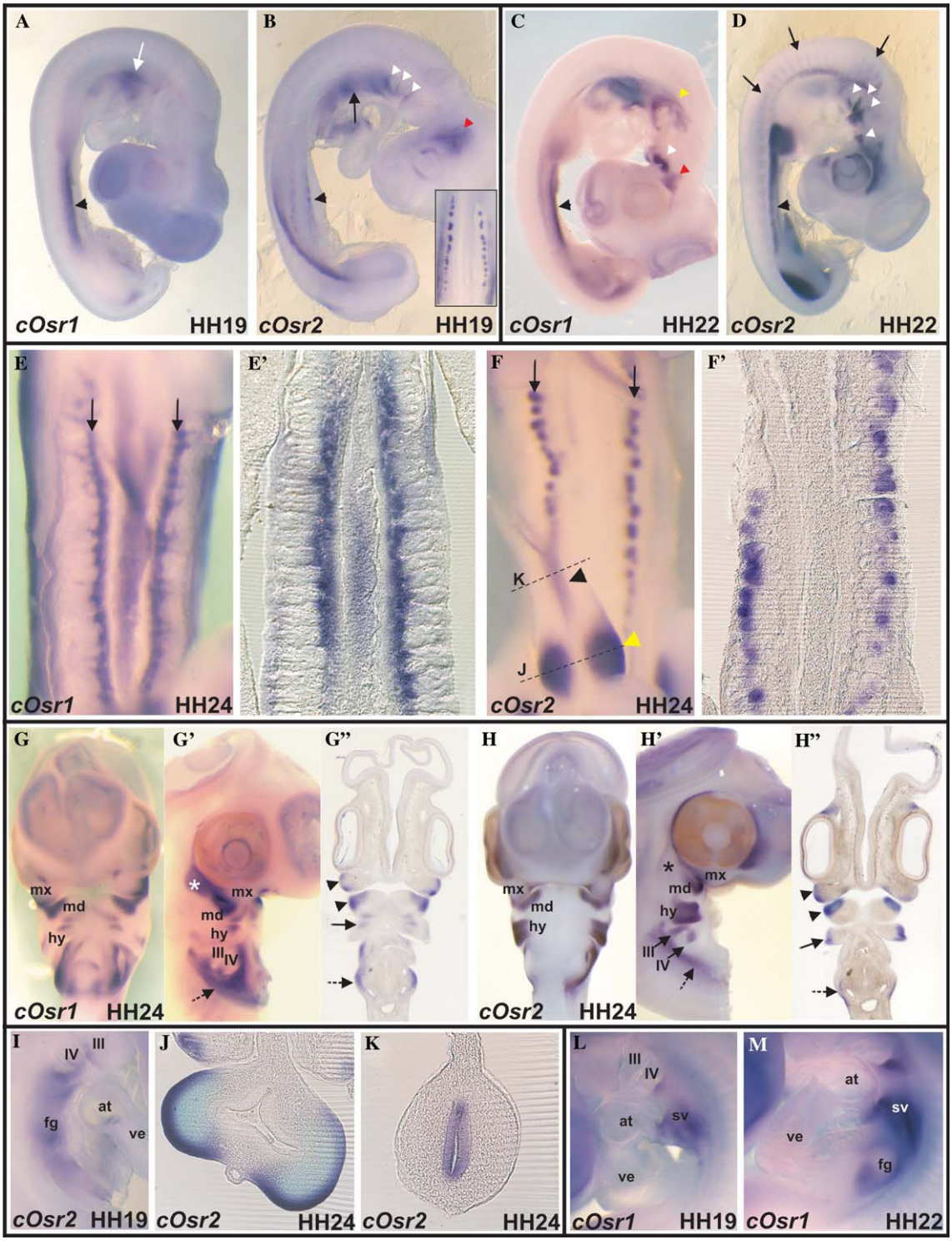
At HH19, both genes show expression associated with the developing mesonephros. While *cOsr1* is strongly expressed in a continuous domain, *cOsr2* shows a dotted pattern of expression, which also can be seen at HH22 (Figs. 2A–D, arrowheads). At HH24, we found expression of *cOsr1* in mesenchymal cells surrounding the mesonephric tubules (Fig. 2E and E′), while *cOsr2* is expressed within the tubular cells (Fig. 2F and F′). Interestingly, first detection of *cOsr2* in mesonephric tubules at HH19 apparently coincides with the time at which the mesonephric system starts to remove nitrogenous waste from the blood flow (Schoenwolf, 1995). It is known that the intermediate mesoderm cells give rise to all kidney tissues (Sainio and Raatikainen-Ahokas, 1999). *Osr1* is known to be one of the earliest markers for intermediate mesoderm in the mouse and also in the chick (Wilm et al., 2004; James and Schultheiss, 2005). Additionally, in Osr1-deficient mice kidney development is blocked in early stages (Wang et al.,

Fig. 2. Expression of *cOsr1* and *cOsr2* in kidney, craniofacial, gut and heart development. At HH19 (A,B), *cOsr1* and *-2* are expressed in the mesonephros (black arrowheads). Note expression of *cOsr2* in nephric tubules (see ventral view inset). *cOsr1* is expressed in the sinus venosus (white arrow, also compare (L)). *cOsr2* is expressed in branchial arches II–IV (white arrowheads). Additionally, *cOsr2* is expressed in a domain posterior of the eye (red arrowhead). At HH22 (C,D), *cOsr1* and *-2* are expressed in the mesonephros (black arrowheads) and the limbs. *cOsr1* now also shows expression posterior and ventral of the eye (red arrowhead) and is expressed in mesenchyme surrounding the branchial arches caudally (yellow arrowhead). *cOsr2* is expressed in all branchial arches, while *cOsr1* shows expression in the mandibular process only (white arrowheads). Additionally, *cOsr2* can be detected overlapping with anterior somites (arrows). (E,F) Expression of *cOsr1* and *-2* in the mesonephros at HH24. While *cOsr1* is expressed in a continuous domain, *cOsr2* is restricted to nephric tubules. Frontal vibratome sections (E′,F′) demonstrate expression of *cOsr1* in mesenchyme surrounding the nephric tubules, while *cOsr2* is expressed in the tubules. Additional expression of *cOsr2* (F) is seen in the intestine (black arrowhead) and in the caeca (yellow arrowhead). (G,H) and (G′,H′) Craniofacial expression of *cOsr1* and *-2* in frontal and lateral views of HH24 embryos. *cOsr1* is expressed in maxillary and mandibular processes, at the tip of the hyoid and the basis of arches III and IV. Strong expression is also seen ventrolateral of the eye (asterisk). *cOsr2* is expressed in maxillar and mandibular processes and in medial domains of arches II–IV. Vibratome sections (G″,H″) show coexpression of *cOsr1* and *-2* in maxillary and mandibular processes (black arrowheads), but separate domains in the hyoid (arrows). Expression in mesenchyme dorsal and caudal of the branchial arches can be seen for both genes (G′,H′, dotted arrows), but expression of *cOsr2* is restricted to superficial layers as compared to *cOsr1* (G″,H″, dotted arrows). Expression of *cOsr2* is associated with the intestinal tract; (I) expression in the foregut at HH19. (J,K) Vibratome sections as indicated in (F) reveal expression of *cOsr2* in superficial cells of caecal buds and in intestinal epithelium, respectively. Expression of *cOsr1* is associated with the developing heart. (L) Expression of *cOsr1* in the sinus venosus at HH19, (M) expression of *cOsr1* in the sinus venosus and in patches on the foregut. At, atrium; fg, foregut; hy, hyoid arch; md, mandibular process; mx, maxillar process; sv, sinus venosus; ve, ventricle.

2005). However, Osr2-deficient mice show no kidney abnormalities (Lan et al., 2004).

Osr1-null mice additionally show severe heart defects (Wang et al., 2005). As in the mouse, we found *Osr1* expressed during chick heart development. At HH19, *cOsr1* is expressed in the sinus venosus of the developing heart (Fig. 2A, white arrow; magnification in Fig. 2L). At HH22, there is still apparent strong expression in the sinus venosus, additionally patches of positive staining can be seen in the foregut (Fig. 2M).

From HH19 on, *cOsr2* is expressed in the branchial arches II–IV (Fig. 2B, white arrowheads). *cOsr1* is not expressed in the branchial arches at this stage. At HH22, *cOsr2* is strongly expressed in all four branchial arches (Fig. 2D, white arrowheads), while *cOsr1* shows high expression in the maxillary and mandibulary processes



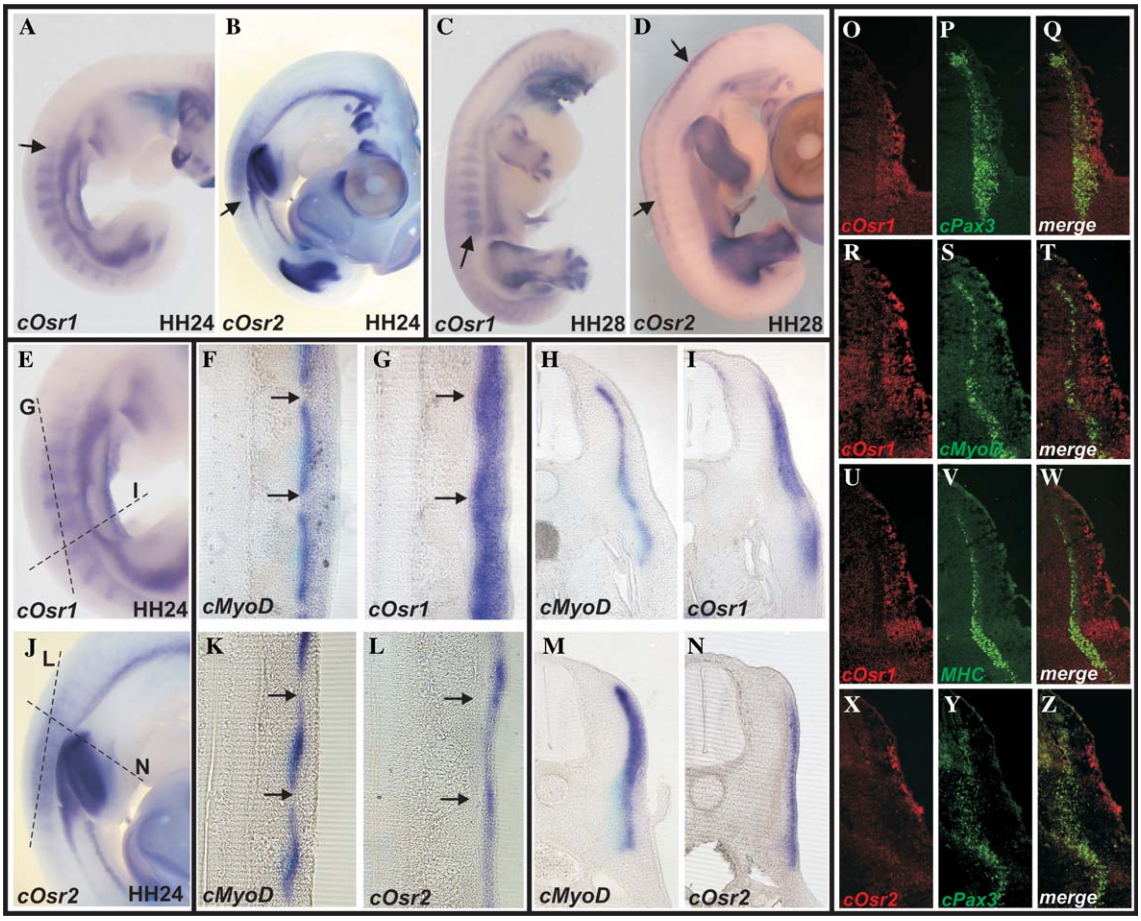
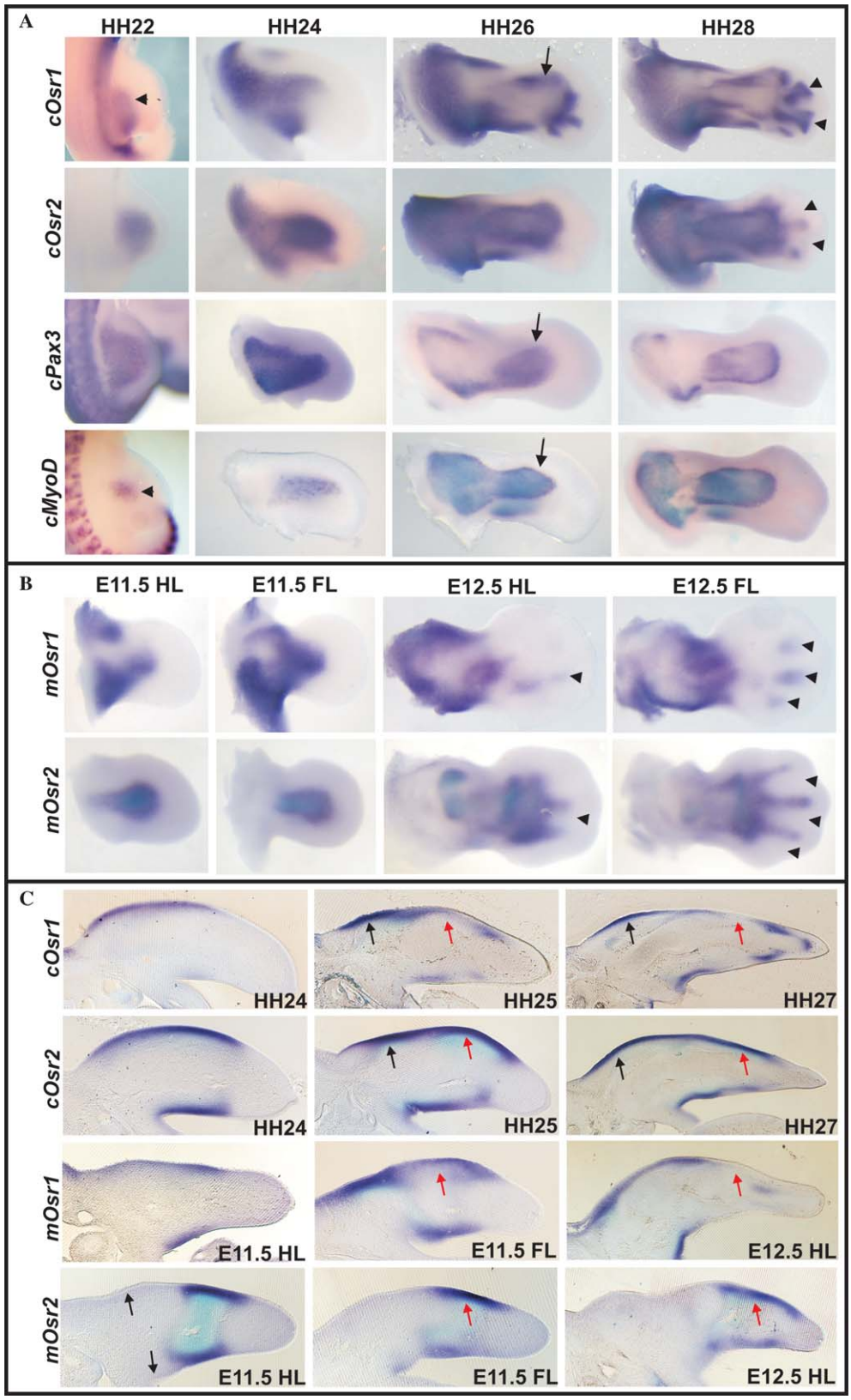


Fig. 3. Expression of *cOsr1* and *cOsr2* in trunk dermis compared to muscle markers *cMyoD* and *cPax3*. At HH24 (A,B), expression of *cOsr2* is intensified overlapping with the somites at forelimb level, while *cOsr1* is strongly expressed in the flank caudal of the *cOsr2*-domain (arrows). Spatially separated expression of *cOsr1* and *cOsr2* in the body flank is maintained at HH28 (C,D), where *cOsr1* is expressed in a lateral domain (arrow), while *cOsr2* is expressed dorsally (arrows). (E,J) Overview of *cOsr1* and *cOsr2* expression in the body flank with vibratome section orientation indicated by broken lines. Vibratome sections show staining for *cOsr1* (G,I), *cOsr2* (L,N) and *cMyoD* (F, H, K, and M). Anterio-posterior extension of myotome is indicated by arrows in (F) and (K). Note stronger staining for *cOsr1* and *cOsr2* at intersomite level (arrows in (G) and (L)). (I,N) Transverse sections showing *cOsr1* and *cOsr2* expression in the dermis lateral to the myotome. Note that ventral expansions of *cOsr1* and *cOsr2* domains coincide with the ventral expansion of *cMyoD* staining. (O–T) Two-colour fluorescent in situ hybridisations with *cOsr1* and *cPax3* or *cMyoD*, respectively. Note that *cOsr1* is expressed in a broad domain, where cells expressing *cOsr1* intermingle with premyogenic cells expressing *cPax3*. Coexpression of *cOsr1* and *cPax3* or *cMyoD* is not observed. (U–W) Costaining of *cOsr1* and myosin heavy chain (MHC) demonstrates no expression of *cOsr1* in myocytes. (X–Z) *cOsr2* is expressed in a more superficial domain exhibiting no contact with myogenic cells.

Fig. 4. Limb expression of *Osr1* and *Osr2* in chick and mouse. (A) Whole-mount in situ hybridisation on chicken embryo hindlimbs at stages indicated. *CPax3* and *cMyoD* are shown for comparison. At HH22, *cOsr1* and *cOsr2* are expressed in partially overlapping domains. Thereby, *cOsr1* expression partially overlaps with the region of muscle differentiation marked by *cMyoD* (arrowheads), while the expression of *cOsr2* overlaps with part of the *cPax3* domain. Note coincidence of the distal expansion of *cOsr1* and *cOsr2* expression at HH24 with *cMyoD* and *cPax3* expression, respectively. At HH26, expression domains of *cOsr1* and *cOsr2* split; *cOsr1* is expressed in mesenchyme surrounding the premuscle masses distally (arrows), *cOsr2* is still expressed overlapping with *cPax3* and *cMyoD*. At HH28, separation of *cOsr1* and *cOsr2* domains can still be seen. In the autopod, *cOsr1* shows expression in interdigital mesenchyme (arrowheads) while *cOsr2* is expressed overlapping with condensations. (B) Analysis of *Osr1* and *Osr2* limb expression in the mouse. Forelimbs (FL) and hindlimbs (HL) of 11.5- and 12.5-day embryos are shown. Note partially overlapping expression of *mOsr1* and *mOsr2* in a medial domain in E11.5 fore- and hindlimbs. In E12.5 limbs, expression domains of *mOsr1* and *mOsr2* have split comparable to the situation in the chick at HH26. Note expression of *mOsr1* in interdigital mesenchyme (arrowheads) and expression of *mOsr2* overlapping digit condensations. (C) Longitudinal vibratome sections of chick and mouse limbs stained for *Osr1* or *Osr2* at stages indicated. Sections reveal expression of both, *Osr1* and *Osr2*, in chick and mouse in superficial domains dorsal and ventral of the premuscle masses. Comparison of chick and mouse expression patterns shows a high degree of similarity between both species. Note that *mOsr2* in the E11.5 hindlimb is lacking the proximal domain present in the chick hindlimb at HH24 (arrows; also compare with (B)). At HH25, there is remarkable coexpression of *cOsr1* and *-2* in the proximal domain. The distal expression domains of *Osr1* and *-2* start to separate in the chick hindlimb at this developmental stage. This is also observed in the mouse E11.5 FL (red arrows). At HH27, *Osr1* and Osr2 appear to be mutually exclusive in distal regions in chick and mouse (red arrows). Coexpression is still seen in the proximal domain in the chick (black arrows).

(Fig. 2C, white arrowhead) but is not expressed in arches II–IV. Additionally, *cOsr1* is expressed in mesenchyme surrounding the branchial arches caudally (Fig. 1C, yellow arrowhead). From HH24 on, both genes are robustly expressed in all branchial arches (Figs. 2G and H). Strong expression of *cOsr1* is seen in the maxillary and mandibular processes. Weaker expression is seen at the tip of the hyoid (II) and at the base of arches III and IV (Fig. 2G and G′).



In the maxillary and mandibulary processes, *cOsr2* shows expression in a domain reminiscent of the *cOsr1* expression (Fig. 2H and H′). In arches II–IV *cOsr2* is expressed in medial areas, thereby appearing mutually exclusive with *cOsr1* (Fig. 2G and G′, and H and H′). This is also demonstrated on frontal vibratome sections, where overlapping staining can be seen in the maxillary and mandibular processes (Fig. 2G″ and H″, arrowheads), but exclusive expression is seen in the hyoid arch (Figs. 2G″ and H″, arrows). *cOsr1* and *cOsr2* are also expressed in mesenchymal cells flanking the basis of the branchial arches (Figs. 2G′ and H′, dotted arrows), where cOsr1 shows a more widespread expression than *cOsr2*. Vibratome sections show that *cOsr2* is expressed mainly in most superficial mesenchymal cell layers, while the expression of *cOsr1* comprises deeper cell layers. As in the caudal branchial arches, both genes appear mutually exclusive in this expression domain (compare Figs. 2G″ and H″, dotted arrows).

Both genes also show expression associated with the developing eye. At HH24, *cOsr1* is expressed in a domain at the caudal side of the eye; this domain is negative for *cOsr2*, which is expressed in a layer of mesenchymal cells surrounding almost the whole eye (Figs. 2G′ and H′, asterisks). Expression associated with the eye can be seen from HH22 on for *cOsr1* (Fig. 2C, red arrowhead), and from HH19 on for *cOsr2* (Fig. 2B, red arrowhead).

At HH24, both genes are expressed overlapping with the developing somites at the flank of the embryo. Here, *cOsr1* and *-2* are expressed in mutually exclusive domains along the anterior–posterior axis of the embryo. While the expression of *cOsr1* starts caudally of the forelimbs, *cOsr2* is expressed in the flank area at the level of the forelimb with its most caudal domain at the point where the expression of *cOsr1* starts (Figs. 3A and B, arrows). While expression of *cOsr1* in the flank starts at HH24, *cOsr2* can be detected overlapping with somites cranial of the forelimb already at HH22 (Fig. 2D, arrows). A mutual exclusive pattern of expression is maintained during further development, with expression of *cOsr1* situated at the lateral flank of the somites and expression of *cOsr2* at the dorsalmost edges of the somites (Figs. 3C and D, arrows). Section analysis revealed expression of *cOsr1* in the dermis lateral to the myotome marked by *cMyoD*, stronger expression is seen between somites than adjacent to somites (Figs. 3F and G). Transverse sections at interlimb level show the ventral expansion of the *cOsr1* expression domain overlapping with the extension of *cMyoD* positive myoblasts (Figs. 3H and I). Thus, *cOsr1* appears to be expressed in intimate contact with myotomal cells. To see, whether *cOsr1* is expressed in different types of myotomal cells, we performed two-colour fluorescence-labelled in situ hybridisation on transverse sections. Hybridisations demonstrate that *cOsr1* expressing cells are in close spatial contact and even intermingling with the *cPax3* positive myogenic precursors (Figs. 3O–Q). Double labelling for *cOsr1* and *cMyoD* shows no expression of *cOsr1* in committed myogenic precursors (Figs. 3R–T). Costaining for *cOsr1* and myosin heavy chain (MHC) demonstrates that *cOsr1* is not expressed in differentiated myocytes (Figs. 3U–W).

Section analysis performed in the region of *cOsr2* expression (i.e., the somites at forelimb level) revealed a pattern of expression partially similar to that of *cOsr1* flanking the caudal somites. Frontal sections show that *cOsr2* is expressed in the dermis lateral to the myotome, but apparently in a more superficial domain than it was seen for *cOsr1* (Figs. 3K and L). Transverse sections show that the ventral expansion of the *cOsr2* domain also overlaps with the ventral expansion of the myotome labelled by *cMyoD*, as it was the case for *cOsr1* (Figs. 3M and N). Concordant with the superficial expression seen in the vibratome sections, *cOsr2* expressing cells show less close spatial contact to Pax3 expressing cells than it was the case for *cOsr1*. Two-colour fluorescence-labelled in situ hybridisation (Figs. 3X–Z) demonstrates expression of *cOsr2* in a superficial layer of cells clearly separated from the Pax3 expressing myogenic precursors.

From HH22 on, strong expression of both *cOsr1* and *-2* can be seen in the developing limbs. Fig. 4A shows expression of *cOsr1* and *-2* in developing chick hindlimbs. As we found a close spatial conjunction between the expression of *cOsr* genes and muscle development related genes in the trunk, we compared the limb expression patterns of *cOsr1* and *-2* to the expression of *cPax3* and *cMyoD*, labelling migrating and differentiating muscle cells of the limb premuscle masses. Hindlimbs have been chosen for the expression analysis, as they are anatomically more related to mammalian limbs than the forelimbs, which give rise to the highly specialised structures of the wing. Generally, vertebrate limb development starts with the outgrowth of tissue from the lateral plate mesoderm forming the limb bud. The nascent limb bud mainly consists of two different types of cells, undifferentiated mesenchymal cells and ectodermal cells covering the bud. Early thereafter, the tissues that will later form all components of the limb start to differentiate. While cartilage, tendons and connective tissue arise within the limb from mesenchymal cells derived from lateral plate mesoderm, the cells forming muscles and blood vessels migrate into the limb bud from the somites (Chevallier et al., 1977; Christ and Brand-Saberi, 2002; Akiyama et al., 2005).

Expression of *cOsr1* can be traced at HH22 in the posterior mesenchyme and in the medial portion of the chick hindlimb bud, while *cOsr2* is expressed in a broad, medially located domain in the limb mesenchyme (Fig. 4A). Interestingly, the medial part of the *cOsr1* expression domain shows spatial overlap with the differentiating premuscle cells labelled by *cMyoD* (Fig. 4A, arrowheads). The *cOsr2* expression resembles the domain of *cPax3* positive migrating and proliferating cells, however, it encompasses a smaller domain than *cPax3*. At HH24, there is strong expression of *cOsr1* surrounding the limb basis. The domain in the middle of the limb has expanded distally. *cOsr2* shows weaker expression at the limb basis, but intense staining can be seen in its distal portion. This distal expression domain, which is shared by both genes, shows considerable

spatial overlap with migrating (*cPax3*) and for *cOsr1* especially differentiating muscle precursors (*cMyoD*). As in HH22 hindlimbs, the domain of *cOsr2* expression is broader than that of *cOsr1*. At HH26, the initial coexpression of *cOsr1* and *-2* undergoes dramatic change. The expression domains of both genes split and become mutually exclusive, with the exception of the limb basis. The *cOsr1* domain has expanded and is now distally surrounding the expression domains of *cPax3* and *cMyoD* (arrows). Contrasting to this, *cOsr2* remains expressed in the domain overlapping with the migrating/differentiating muscle. At HH28, the *cOsr1*-domain surrounding the distal margin of the premuscle mass can still be seen, while *cOsr2* expression remains associated with the developing muscles. In the autopod, *cOsr1* is now expressed in interdigital regions (arrowheads), while the expression of *cOsr2* is confined to mesenchymal areas overlapping with the digit condensations.

The embryonic limb is a well-characterised developmental model system for the investigation of patterning, tissue specification and differentiation. To evaluate the evolutionary conservation of *odd-skipped* gene expression in vertebrate limb development, we re-analysed expression of *mOsr1* and *-2* in the mouse limb at comparable differentiation stages to those used in the chick. Overall, in the mouse limb, the expression patterns of both genes are highly similar to the chick (Fig. 4B). For days 11.5 and 12.5 fore- and hindlimbs are shown, as the development of the hindlimb is lagging behind the development of the forelimb, thus both represent different phases of limb development. At E11.5, expression of *mOsr1* in fore- and hindlimb is comparable to the chick hindlimb at HH24 and 25 (latter not shown), with expression at the limb basis and also in the medial part. *Osr2* in the mouse is also strongly expressed in a medial domain in the limb mesenchyme, which is overlapping the medial domain of *mOsr1* expression. However, the domain of *Osr2* expression surrounding the limb basis present in the chick is lacking in the mouse. At E12.5, expression of *mOsr1* in the hindlimb still resembles the pattern observed in the chick at HH26, showing expression at the limb base and distally coinciding with muscle migration/differentiation. However, the characteristic domain of *cOsr1* expression surrounding the premuscle mass distally cannot be seen in the mouse. As in the chick, at this stage the expression domains of *mOsr1* and *-2* split and apparently exclude each other. As observed in the chick between HH26 and 28, autopod expression of *mOsr1* is also confined to the interdigital spaces in the E12.5 hind- and forelimbs (arrowheads), while *mOsr2* is expressed overlapping with digit condensations.

To refine the relationship between *Osr1* and *Osr2* in chick and mouse, we compared vibratome sections of corresponding developmental stages. Chick HH24 hindlimb was compared to mouse E11.5 hindlimb, chick HH25 hindlimb to mouse E11.5 forelimb and chick HH27 hindlimb to mouse E12.5 hindlimb (Fig. 4C). At HH24, *cOsr1* is expressed in superficial cell layers in the dorsal and ventral mesenchyme of the chick leg bud. A highly similar pattern can be seen in the mouse leg bud. As suggested by the whole-mount ISH, at this stage *cOsr2* shows an expression pattern largely overlapping with that of *cOsr1*, while in the mouse expression can be seen only in a domain overlapping with the distalmost expansion of the *Osr1* domain. In both species, the expression domains of *Osr2* appear to extend more distally than those of *Osr1*. At HH25/E11.5 (forelimb), the beginning subdivision of expression domains of *Osr1* and *Osr2* in chick and mouse already becomes apparent by section analysis. While in the proximal mesenchyme, *cOsr1* and *-2* are still coexpressed in superficial cell layers (black arrows), in the distal portions of their expression *Osr1* and *Osr2* in chick and mouse appear to occupy at least in part different domains of mesenchymal cells (red arrows). At HH27/E12.5 (hindlimb), separation of *Osr1* and *-2* domains has advanced in both species, with exception of the proximal area in the chick (black arrows). In distal areas, almost complete exclusive expression of *Osr1* and *Osr2* can be observed in limb mesenchyme in chick and mouse (red arrows).

To analyse the expression of *Osr* genes in later limb development, we performed section ISH analysis on chicken HH35 hindlimbs and mouse E13.5 forelimbs. *Osr1* is continuously expressed in interdigital mesenchyme in chick and mouse (Figs. 5A and C, arrowheads). Furthermore, it is revealed that both genes in chick and mouse are

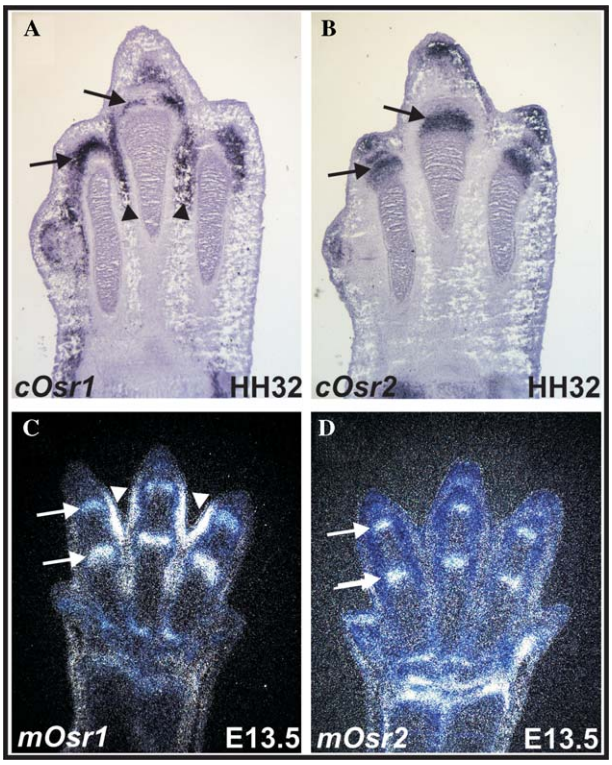


Fig. 5. Expression of *Osr1* and *Osr2* in synovial joints in chick and mouse. (A,B) Digoxygenin-labelled section in situ hybridisation on HH35 hindlimbs showing expression of *cOsr1* and *cOsr2* in the interzone of synovial joints (arrows). *cOsr1* is still expressed in interdigital mesenchyme (arrowheads). (C,D) P^{33}-labelled section in situ hybridisation on E13.5 mouse forelimbs revealing expression of *mOsr1* in interdigital mesenchyme (arrowheads) and both, *mOsr1* and *mOsr2*, in joints (arrows).

expressed in the interzone of developing joints in the autopod (Figs. 5A–D, arrows) and also in other synovial joints in the limb (not shown).

Summarising, we identified expression of chicken *Osr1* and *Osr2* in the developing kidney, heart, gut, eye, branchial arches, the trunk dermis and the limbs. Altogether, these domains of expression appear remarkably conserved between chick and mouse, making the chick embryo an attractive model for further functional studies of *Osr* genes. Expression of *odd-skipped* genes in domains shared with non-vertebrates, e.g., in the gut, suggests ancestral as well as newly acquired functions of *Osr* genes in vertebrates.

2. Experimental procedures

Whole-mount in situ hybridisation (ISH) was carried out as described previously (Schwabe et al., 2004). Section ISH using digoxygenin-labelled riboprobes was carried out as described in Seemann et al. (2005). Radioactive section ISH was carried out as in Stricker et al. (2002). Fluorescence-labelled two-colour ISH on fresh frozen sections was performed essentially as described by Tylzanowski et al. (2003). Instead of biotin-labelled probes we used FITC-labelled probes. For detection anti-FITC-POD (Roche 1426346) was used. For tissue permeabilisation, we used proteinase k digestion at 1 μg/ml for 5–8 min.

Immunohistochemistry subsequent to ISH was carried out as follows: slides were washed three times in PBS and then incubated in 3% BSA/0.1% saponin in PBS for 1 h. Primary antibody (anti-myosin heavy chain MY-32, Sigma) was applied at 1:200 in the same solution for 1 h at room temperature. Slides were washed three times in PBS and then secondary antibody (goat anti-mouse coupled to AlexaFluor-488, Molecular Probes) was applied (1:250 in 5% BSA/PBS) for 1 h at room temperature. After three washes in PBS slides were mounted in Fluoromount.

For *cOsr1*, we used EST clones 812m23 and 3k9 from the BBSRC ChickEST-Database (Boardman et al., 2002). Both ESTs showed an identical pattern. For *cOsr2*, two probes were designed based on the predicted sequence XM_418353. We amplified the 5′-coding region without the zinc-finger domain and the full-length ORF for use as probes, respectively, using the primers cOsr2-F: ATGGGCAGCAAGGCGCTGC; cOsr2-R1: GGTTGGCGAAGTCGAAGCG; and cOsr2-R2: TCAGAAGTCCTG CCGCGGGGT. Both probes were PCR amplified from 5-day whole chicken embryonic cDNA and cloned into pCRII-TOPO (Invitrogen). Both probes yielded identical results.

Probes for mouse *Osr1* and *Osr2* were made by PCR amplification from E12.5 total cDNA using the primers Osr1-F: TTTCCGGAGGCAA GACCAC; Osr1-R: GGAAGGCCGCACACTCAACTC; Osr2-F: GCA GACATCAAGCCCTACAGCTG; Osr2-R: GAGCCGTGAATATCT ACAAGGATC. All PCR products were cloned into pCRII-TOPO (Invitrogen).

Additional probes used were: *cMyoD* (Mennerich and Braun, 2001) and *cPax3* (Goulding et al., 1993).

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