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## The Zinc Finger Transcription Factors *Osr1* and *Osr2* Control Synovial Joint Formation

Yang Gao, Yu Lan, Han Liu, and Rulang Jiang\*

Center for Oral Biology and Department of Biomedical Genetics, University of Rochester School of Medicine and Dentistry, Rochester, New York 14642, USA

### Abstract

Synovial joints enable smooth articulations between different skeletal elements and are essential for the motility of vertebrates. Despite decades of extensive studies of the molecular and cellular mechanisms of limb and skeletal development, the molecular mechanisms governing synovial joint formation are still poorly understood. In particular, whereas several signaling pathways have been shown to play critical roles in joint maintenance, the mechanism controlling joint initiation is unknown. Here we report that *Osr1* and *Osr2*, the mammalian homologs of the odd-skipped family of zinc finger transcription factors that are required for leg joint formation in *Drosophila*, are both strongly expressed in the developing synovial joint cells in mice. Whereas *Osr1*<sup>-/-</sup> mutant mice died at midgestation and *Osr2*<sup>-/-</sup> mutant mice had only subtle defects in synovial joint development, tissue-specific inactivation of *Osr1* in the developing limb mesenchyme in *Osr2*<sup>-/-</sup> mutant mice caused fusion of multiple joints. We found that *Osr1* and *Osr2* function is required for maintenance of expression of signaling molecules critical for joint formation, including *Gdf5*, *Wnt4* and *Wnt9b*. In addition, joint cells in the double mutants failed to upregulate expression of the articular cartilage marker gene *Prg4*. These data indicate that *Osr1* and *Osr2* function redundantly to control synovial joint formation.

### Keywords

joint formation; joint fusion; limb development; Odd-skipped; *Osr1*; *Osr2*; synovial

### Introduction

The successful adaptation of vertebrate animals to a wide variety of ecological niches depended largely on the formation and function of synovial joints. Synovial joints facilitate smooth articulation between two or more skeletal elements and thus play essential roles in vertebrate movement. The tissues that make up the synovial joint include articular cartilages of the opposing skeletal elements, ligaments, synovium, and the fibrous capsule. These tissues are prone to injury and malfunction during natural aging, resulting in impaired mobility. Moreover, synovial joints are often targets of diseases, such as osteoarthritis and inflammatory rheumatoid arthritis, which are the most common causes of disability in the adult human population (Elders, 2000). Despite the obvious importance of synovial joints,

\*Author for correspondence: Rulang Jiang, Ph.D., Center for Oral Biology, University of Rochester, 601 Elmwood Avenue, Box 611, Rochester, New York 14642, USA, Phone: (585)273-1426, Fax: (585)276-0190, Rulang\_Jiang@urmc.rochester.edu.

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however, there is currently very limited understanding of the molecular mechanisms of joint development.

During embryogenesis, the limb skeleton initiates by condensation and differentiation of mesenchymal cells to form cartilage, which further undergoes endochondrial ossification to form bone. The various long bones of the limb do not initiate as discrete individual elements, but rather initially form as continuous condensed mesenchyme that is subsequently divided into distinct elements by segmentation. For example, the humerus, radius and ulna rudiments initially form as an uninterrupted Y-shaped prechondrogenic mesenchymal condensation marked by high levels of expression of Type II collagen (*Col2*) (Hinchliffe and Johnson, 1980; Craig et al., 1987; Lizarraga et al., 2002). The first overt sign of joint development is the appearance of the so-called interzone, consisting of densely packed flattened mesenchymal cells that provide a clear demarcation between the adjacent cartilaginous elements (Holder, 1977; Mitrovic, 1978; Pacifici et al., 2005). It has been proposed that initiation of joint formation involves inhibition of the interzone progenitor cells from differentiating into chondrocytes by downregulating or blocking *Col2* expression (Lizarraga et al., 2002; Später et al., 2006; Pitsillides and Ashhurst, 2008).

One of the earliest markers of joint interzone cells identified is *Gdf5*, a member of the TGF- $\beta$  family of signaling molecules. Mice lacking *Gdf5* exhibited multiple joint fusions, particularly involving interphalangeal joints and the carpal or tarsal bones (Storm et al., 1994, 1996). Joint defects are also associated with mutations in *GDF5* in humans (Francis-West et al., 1999b; Buxton et al., 2001). Genetic lineage tracing studies showed that the *Gdf5*-expressing interzone cells gave rise to most joint tissues including articular cartilage but contributed little to the growth plate cartilage (Koyama et al. 2008). However, application of exogenous *Gdf5* protein to the developing chick or mouse limb mesenchyme stimulated cartilage development (Storm and Kingsley, 1999), indicating that *Gdf5* signaling does not specify joint cell fate and that cellular responses to *Gdf5* signaling may be cell-type specific. Joint development failed to initiate in mice lacking *Noggin*, a secreted BMP antagonist (Brunet et al., 1998). *Noggin* mRNA is expressed throughout the prechondrogenic mesenchymal condensation, in both the cartilage anlagen and the presumptive joint cells. In addition to lacking joints, the *Noggin*<sup>-/-</sup> mutant mice exhibited gross skeletal hyperplasia, suggesting that failure of joint formation in these mutant mice was due to increased recruitment of the prechondrogenic limb mesenchyme into cartilage formation (Brunet et al., 1998). Indeed, implantation of agarose beads soaked with *Bmp2* or *Bmp4* adjacent to developing chick digital joints caused partial joint fusion (Archer et al., 2003).

Another signaling pathway that plays critical roles in joint development is the Wnt/ $\beta$ -catenin pathway. Hartmann and Tabin (2001) showed that *Wnt9a* (formerly *Wnt14*) mRNA was expressed early during interzone formation in the developing chick limb and that misexpression of *Wnt9a* induced ectopic expression of several interzone cell molecular markers including *Gdf5* and *Chordin*. Moreover, *Wnt9a* misexpression in primary chick sternal chondrocytes caused downregulation of *Col2* and upregulation of *Col3* (Später et al., 2006), mimicking the molecular events during interzone formation. Expression of either *Wnt9a* or a constitutively active form of  $\beta$ -catenin driven by the *Col2a1* gene promoter in transgenic mice also caused ectopic expression of joint cell markers in the developing limbs (Guo et al., 2004). However, targeted disruption of *Wnt9a* did not affect joint induction but resulted in synovial chondroid metaplasia in some joints (Später et al., 2006). Although the *Wnt4*<sup>-/-</sup>*Wnt9a*<sup>-/-</sup> double mutant newborn mice showed fusion of some carpal and tarsal elements, the fusion occurred after initiation of the joint interzones. Moreover, tissue-specific inactivation of  $\beta$ -catenin in the early developing limb mesenchyme did not block

joint interzone formation (Später et al., 2006), indicating that other mechanisms are involved in joint specification.

Previous studies have shown, in addition to the selected signaling molecules of the Gdf and Wnt families, that several transcription factor genes, including *Cux1*, *Erg*, *Osr1* and *Osr2*, are also expressed in highly restricted patterns during synovial joint formation (Dhordain et al., 1995; Lizarraga et al., 2002; Stricker et al., 2006; Iwamoto et al., 2007; Koyama et al., 2008). *Cux1*, encoding a large homeodomain-containing DNA-binding protein, is highly expressed at sites of incipient joint formation during limb development in chick (Lizarraga et al., 2002). Misexpression of *Cux1* mRNA suppressed chondrocyte differentiation in limb mesenchyme cultures. However, overexpression of *Cux1* did not induce expression of known joint markers and mice deficient in *Cux1* did not have limb developmental defects (Lizarraga et al., 2002; Ellis et al., 2001). Thus, the role of *Cux1* in joint development is unclear. *Erg*, encoding a member of the Ets family of transcription factors (Sharrocks, 2001), is co-expressed with *Gdf5* in the incipient joint cells during mouse limb development and exogenous *Gdf5* protein induced *Erg* mRNA expression in the developing limb mesenchyme (Iwamoto et al., 2007). Transgenic mice overexpressing *Erg* mRNAs throughout the cartilaginous skeleton did not induce ectopic joint formation but rather blocked chondrocyte maturation toward hypertrophy, suggesting that *Erg* may be an important factor downstream of *Gdf5* in the differentiation and maintenance of articular cartilage (Iwamoto et al., 2007). *Osr1* and *Osr2* are the vertebrate homologs of the *Drosophila* odd-skipped family of zinc finger proteins (So and Danielian, 1999; Lan et al., 2001; Gao et al., 2009). In *Drosophila*, all four odd-skipped family genes, *odd*, *sob*, *drm*, and *bowl*, are expressed in a segmental pattern in the developing leg imaginal discs. Ectopic expression of *odd*, *sob*, or *drm*, induced invaginations in the leg disc epithelium and morphological changes in the adult leg that are characteristic of joint cells (Hao et al., 2003). *Osr1* and *Osr2* mRNAs exhibit partially overlapping expression in the early limb bud and both are expressed at high levels in the incipient joints during limb development in chick and in mice (Stricker et al., 2006), suggesting that this family of transcription factors may be involved in joint cell fate specification in vertebrates as well. Since most mouse embryos lacking *Osr1* died in midgestation (Wang et al., 2005), we have generated mice with limb-specific inactivation of *Osr1* and demonstrate in this report that *Osr1* and *Osr2* function partially redundantly to control synovial joint formation in mice.

## Materials and Methods

### Mouse strains and breeding strategies

The *Osr1*<sup>+/-</sup> and *Osr2*<sup>+/-</sup> mice, which carry in-frame fusion of the *lacZ* gene, have been described previously (Lan et al., 2004; Wang et al., 2005). Mice carrying a conditional *Osr1* allele (*Osr1*<sup>ff</sup>) were generated by inserting an *FRT*-flanked *neo* expression cassette together with a *loxP* sequence in the first intron and another *loxP* sequence in the second intron of the *Osr1* gene. The *Osr1*<sup>ff/ff</sup> homozygous mice appear normal and Cre-mediated germ line deletion of the *loxP*-flanked exon 2 recapitulated the *Osr1* null mutant mouse phenotypes (Lan et al., submitted). Other mouse strains used include the  $\beta$ -catenin conditional mice (*Catnb*<sup>ff</sup>) (Brault et al., 2001), the *Osr2*<sup>IresCre</sup> mice (Lan et al., 2007), and the *Prrx1-cre* transgenic mice (Logan et al., 2002).

To generate mice lacking both *Osr1* and *Osr2* in the developing limb mesenchyme, *Osr1*<sup>ff/ff</sup> homozygous mice were crossed with *Osr2*<sup>+/-</sup> mice to generate the *Osr1*<sup>ff/+</sup>*Osr2*<sup>+/-</sup> mice, which were then crossed with *Osr1*<sup>ff/ff</sup> mice to generate *Osr1*<sup>ff/ff</sup>*Osr2*<sup>+/-</sup> mice. *Prrx1-cre* transgenic mice were purchased from the Jackson Laboratory (Bar Harbor, ME). *Prrx1-cre* mice were bred to the *Osr1*<sup>ff/ff</sup>*Osr2*<sup>+/-</sup> mice to generate *Osr1*<sup>ff/+</sup>*Osr2*<sup>+/-</sup>*Prrx1-cre* mice. No gross morphological defects were found in these mice. Timed mating was set up between

*Osr1<sup>ff/+</sup>Osr2<sup>+/-</sup>Prrx1-cre* mice and *Osr1<sup>ff</sup>Osr2<sup>+/-</sup>* mice to generate the *Osr1<sup>ff</sup>Osr2<sup>-/-</sup>Prrx1-cre* mutant embryo for analysis.

To inactivate  $\beta$ -catenin in the developing limb joint cells, *Catnb<sup>ff</sup>* mice were crossed to *Osr2<sup>IresCre/+</sup>* mice to generate *Catnb<sup>ff/+</sup>Osr2<sup>IresCre/+</sup>* mice, which were then crossed to *Catnb<sup>ff</sup>* mice to generate *Catnb<sup>ff</sup>Osr2<sup>IresCre/+</sup>* mutant embryos for analysis.

### Skeletal preparations and histology

Timed-mating was set up for embryos with desired genotypes. Embryos were collected at E17.5. Tail DNA was extracted and genotypes of the embryos were determined by allele-specific PCR. Skeletal preparations were performed following the protocol described previously (Martin et al., 1995). For histology analysis, embryos were fixed in Bouin's fixative, dehydrated through graded ethyl alcohols, embedded in paraffin, sectioned at 7- $\mu$ m thickness and stained with alcian blue, hematoxylin and eosin.

### Detection of LacZ expression and section in situ hybridization

Timed mating was set up for embryos at desired stages. Tail DNA was extracted for genotyping with allele-specific PCR. X-gal staining of whole-mount embryos and cryostat sections were performed as described (Hogan et al, 1994). Sections were counterstained with eosin. For in situ hybridization, embryos were fixed in 4% paraformaldehyde in PBS overnight at 4 °C, washed with PBS, dehydrated through graded ethyl alcohols, embedded in paraffin and sectioned at 7- $\mu$ m thickness. Section in situ hybridization was carried out as previously described (Zhang et al., 1999) with digoxigenin-labeled antisense RNA probes.

### Dual color whole mount LacZ staining and in situ hybridization

E11.5 and E12.5 *Osr1<sup>+/-</sup>* and *Osr2<sup>+/-</sup>* embryonic limbs were dissected and fixed on ice for 2 hours with 4% paraformaldehyde in PBS and stained at 37°C for 2 hours in Salmon-gal (Biosynth International, Naperville, IL) staining solution. After Salmon-gal staining, samples were washed with PBS, post-fixed with 1% paraformaldehyde in PBS and processed for whole-mount in situ hybridization as described previously (Jiang et al, 1998).

## Results

### Expression patterns of *Osr1* and *Osr2* overlap in most developing synovial joints and *Osr2<sup>-/-</sup>* mice have mild joint defects that correlate with its distinct expression

It has been reported that *Osr1* and *Osr2* mRNAs are both expressed in the developing joint tissues (Stricker et al., 2006). Since *Osr1<sup>-/-</sup>* mutant embryos died in midgestation (Wang et al., 2005), we first examined the *Osr2<sup>-/-</sup>* mutant mice, which die shortly after birth due to craniofacial defects (Lan et al., 2004), for possible joint defects. We found that all *Osr2<sup>-/-</sup>* mutant mice had abnormally fused tarsal elements in the ankle region (Fig. 1A, B) while other joints in the limb appeared normal.

We investigated the expression patterns of *Osr1* and *Osr2* during limb development in detail using the *Osr1<sup>+/-</sup>* and *Osr2<sup>+/-</sup>* mice, which carry targeted in-frame fusion of the *lacZ* reporter gene to the *Osr1* and *Osr2* genes, respectively (Lan et al., 2004; Wang et al., 2005). From E13.5 to E18.5, both *Osr1* and *Osr2* are strongly expressed in the developing synovial joints, including interphalangeal, metacarpal-phalangeal, elbow and knee joints (Fig. 2A–F). While *Osr1* is more broadly expressed than *Osr2* in the developing limb mesenchyme surrounding the skeletal elements at E13.5, including inter-digital mesenchyme (Fig. 2A, D, G, J, M, R), expression of *Osr1* was much weaker than that of *Osr2* in the carpal and tarsal regions at this stage (Fig. 2G, J, M, P). At E15.5, while both *Osr1* and *Osr2* mRNA expression were clearly detected in the developing joint cells in between the carpal elements

in the wrist (Fig. 2I, L), strong *Osr2* mRNA expression was detected in the developing joints between the tarsal elements but little *Osr1* mRNA was expressed in that region (Fig. 2Q, R). The differential expression of *Osr1* and *Osr2* in the developing tarsal joints correlated very well with the joint defects in the *Osr2*<sup>-/-</sup> mutant mice (Fig. 1B). Together with the strong overlapping expression patterns in most developing joint tissues, these data suggest that *Osr1* and *Osr2* may play critical but partially redundant roles in synovial joint development.

### Defects in synovial joint development in *Osr1*<sup>ff</sup>*Osr2*<sup>-/-</sup>*Prrx1-cre* double mutant mice

Since *Osr1*<sup>-/-</sup> mutant mice died at midgestation due to heart defects, we crossed the *Osr1*<sup>ff</sup> mice with *Prrx1-cre* transgenic mice, which express the Cre recombinase throughout the developing limb mesenchyme as early as E9.5 (Logan et al., 2002), and generated *Osr1*<sup>ff</sup>*Prrx1-cre* mice to examine the role of *Osr1* in limb joint development. We found that the *Osr1*<sup>ff</sup>*Prrx1-cre* mice, lacking *Osr1* gene product in the developing limb mesenchyme, did not have obvious defects in synovial joint development (data not shown). To investigate further the roles of *Osr1* and *Osr2* in synovial joint development, we generated and analyzed limb development in *Osr1*<sup>ff</sup>*Osr2*<sup>-/-</sup>*Prrx1-cre* mice. The *Osr1*<sup>ff</sup>*Osr2*<sup>-/-</sup>*Prrx1-cre* mutant mice had the open-eyelid phenotype as found in *Osr2*<sup>-/-</sup> mutants (Lan et al., 2004). The wrist and ankle regions of the limbs in the double mutant were abnormally shaped, causing the limbs to wrap in front of the trunk (data not shown). Skeletal preparations of the E17.5 *Osr1*<sup>ff</sup>*Osr2*<sup>-/-</sup>*Prrx1-cre* and control embryos showed multiple joint defects in the double mutants: the humerus was fused with both the ulna and radius at the elbow region while the tibia was fused with the fibula at both the proximal and distal ends (Fig. 3, A–H). In addition, the double mutant mice exhibited delay in mineralization of digital bones (Fig. 3B, D). Histological sections further confirmed the fusion of the elbow joints and revealed additional joint defects in the wrist and ankle regions (Fig. 3, I–P). In the wrist region, the carpal elements were fused together in the double mutants (Fig. 3N). In the ankle region, in addition to the fusion between intermediate cuneiform (i.c.) and navicular (nc) bones and between lateral cuneiform (l.c.) and nc seen in the *Osr2*<sup>-/-</sup> mutants, the double mutants exhibited fusion between cuboidal (cub) and calcaneous (cal) bones (Fig. 3P). The interphalangeal and metacarpophalangeal joints were also disorganized in the double mutants in comparison with the control littermates (Fig. 3, Q–R). These data confirm that *Osr1* and *Osr2* play critical and partially redundant roles in synovial joint development.

### *Osr1* and *Osr2* are expressed in the presumptive joint cells prior to and are critical for interzone formation

To understand the roles of *Osr1* and *Osr2* in synovial joint development, we focused on development of the elbow joint as that is the earliest joint to form in the developing limb and it has been well described at the morphological and cellular levels. It has been reported that the humerus, ulna and radius elements arise at E11.5 in mice as a continuous Y-shaped mesenchymal condensation with a seemingly uniform population of cells (Hinchliffe and Johnson, 1980). By combining whole mount detection of LacZ and mRNA expression in the *Osr1*<sup>+/-</sup> and *Osr2*<sup>+/-</sup> embryos, respectively, we found that expression of both *Osr1* and *Osr2* in the developing E11.5 forelimb overlapped with that of *Gdf5* (Fig. 4B, D), one of the earliest markers for joint formation. By performing in situ hybridization using sections through the presumptive elbow joint region, we found that both *Osr1* and *Osr2* mRNAs are expressed in the presumptive joint cells at E11.5 when the expression of *Col2* mRNA is continuous throughout the humerus, ulna and radius elements (Fig. 4E–H). By E12.5, formation of the interzones in the future elbow joint had been initiated, with specific and dramatic downregulation of *Col2* mRNA expression (Fig. 4I). At this stage, expression of both *Osr1* and *Osr2* mRNAs continued to overlap that of *Gdf5* mRNA in the interzone cells and are absent in the cartilage anlagen of humerus, ulna, and radius elements (data not



shown). These data indicate that *Osr1* and *Osr2* are expressed in the presumptive joint cells from the onset of joint formation.

We next investigated whether the elbow joint failed to initiate in *Osr1<sup>fl/fl</sup>Osr2<sup>-/-</sup>Prrx1-cre* mutant embryos. At E11.5, we found that *Gdf5* mRNA was expressed in the joint progenitor cells in the elbow region of the double mutant embryos at levels comparable to that of the control embryos (data not shown). At E12.5, the interzones between humerus and radius and between humerus and ulna elements are clearly demarcated by the loss of *Col2a1* expression in the control embryos (Fig. 4I). In contrast, *Col2a1* expression was only slightly downregulated in these prospective joint regions in the double mutants (Fig. 4J). Moreover, whereas *Gdf5* expression was strongly expressed in the interzone cells in the control embryos at this stage, it was dramatically downregulated in the double mutant littermates (Fig. 4K, L). At E13.5, as the interzone cells started to differentiate into a three-layered structure with the middle layer remaining negative for *Col2a1* expression and positive for *Gdf5* expression in the control embryos (Fig. 4M, O), the *Osr1<sup>fl/fl</sup>Osr2<sup>-/-</sup>Prrx1-cre* mutant limbs still lacked a clear *Col2a1*-negative interzone in the elbow region (Fig. 4N). In addition, *Gdf5* expression was lost in the joint progenitor cells in these double mutants by this stage (Fig. 4P).

We further investigated whether expression of *Wnt9a* and *Wnt4* were altered in developing limb in *Osr1<sup>fl/fl</sup>Osr2<sup>-/-</sup>Prrx1-cre* mutant embryos. At E12.5, *Wnt9a* is only weakly expressed in the elbow joint interzone cells in the control embryos and no significant difference was detected between the control and the *Osr1<sup>fl/fl</sup>Osr2<sup>-/-</sup>Prrx1-cre* mutant littermates (Fig. 5A, B). In contrast, strong *Wnt4* mRNA expression was detected in the cells surrounding the elbow joint interzone in the control embryos at E12.5 but *Wnt4* mRNA expression in the corresponding elbow joint cells in the *Osr1<sup>fl/fl</sup>Osr2<sup>-/-</sup>Prrx1-cre* mutant littermates was significantly reduced (Fig. 5E, F). By E13.5, *Wnt9a* mRNA expression was clearly detected in the interzone cells between the humerus and radius and between the humerus and ulna in the control embryos (Fig. 5C). However, *Wnt9a* expression was absent in between the humerus and radius elements but was clearly detected in a subset of cells in between the humerus and ulna elements in the E13.5 double mutant embryos (Fig. 5D). Interestingly, the domain of *Wnt9a* expression at this stage corresponded with the incomplete fusion of the humerus and ulna at E17.5 (Fig. 3H). Remarkably, while *Wnt4* mRNA continued to be expressed in the cells surrounding the elbow interzone in the control embryos at E13.5, its expression in the corresponding cells in the double mutants was almost completely downregulated (Fig. 5G, H). In contrast, *Wnt4* expression in the differentiating chondrocytes in the radius and ulna elements, which do not express either *Osr1* or *Osr2*, was similar in the control and *Osr1<sup>fl/fl</sup>Osr2<sup>-/-</sup>Prrx1-cre* mutant limbs (Fig. 5H). Taken together, these data indicate that joint formation was initiated, as marked by *Gdf5* expression at E11.5, but the cellular program of interzone formation was aborted in the absence of both *Osr1* and *Osr2* function, resulting in lack of separation of the humerus from the radius and ulna elements in the double mutants.

### Expression of *Osr1* and *Osr2* in the developing joint cells do not depend on Wnt/ $\beta$ -catenin signaling

It was previously proposed that Wnt/ $\beta$ -catenin signaling was necessary and sufficient for synovial joint formation (Guo et al., 2004). We investigated whether expression of *Osr1* and *Osr2* in the joint cells depended on Wnt/ $\beta$ -catenin signaling. We generated mice homozygous for the *loxP*-flanked  $\beta$ -catenin allele (*Catnb<sup>fl/fl</sup>*) (Brault et al., 2001) and heterozygous for the *Osr2<sup>IresCre</sup>* allele (Lan et al., 2007). Since Cre is active in the *Osr2*-expressing cells as early as E10.5 in the embryos, we expect the *loxP*-flanked  $\beta$ -catenin alleles to be inactivated in the joint progenitor cells. Histological analysis of E13.5 embryos showed that the interzone in the elbow joint region of the *Catnb<sup>fl/fl</sup>;Osr2<sup>IresCre/+</sup>* mutant

embryo was not as clearly organized as in the *Osr2<sup>IresCre/+</sup>* control embryos (Fig. 6A, B), consistent with a requirement for  $\beta$ -catenin in synovial joint formation. However, expression of *Osr1* and *Osr2* in the developing elbow joint regions was not significantly altered in the *Catnb<sup>ff</sup>;Osr2<sup>IresCre/+</sup>* mutant embryos in comparison with the *Osr2<sup>IresCre/+</sup>* control littermates at E13.5 (Fig. 6, C–H), indicating that expression of *Osr1* and *Osr2* in the developing joint cells do not depend on Wnt/beta-catenin signaling.

### ***Osr1<sup>ff</sup>Osr2<sup>-/-</sup>Prrx1-cre* double mutant mice exhibit defects in joint cell differentiation**

To maintain the normal function of the synovial joints, articular chondrocytes and synovial fibroblasts synthesize lubricin, which is a water-soluble glycoprotein encoded by *Prg4* gene and plays an important role in joint lubrication and synovial homeostasis (Swann and et al., 1981; Schumacher et al., 1994; Flannery et al., 1999; Jay et al., 2000). To investigate whether *Osr1* and *Osr2* regulate articular cartilage differentiation, we investigated whether expression of *Prg4* was altered in developing joints in *Osr1<sup>ff</sup>Osr2<sup>-/-</sup>Prrx1-cre* mutant embryos. At E15.5, while *Prg4* is strongly expressed in the presumptive articular cartilage in both the humerus-radius and the humerus-ulna joints in the elbow region of the control embryos (Fig. 7A, C). In comparison, expression of *Prg4* mRNA is dramatically reduced in the *Osr1<sup>ff</sup>Osr2<sup>-/-</sup>Prrx1-cre* mutant embryos, including in the region where humerus and ulna were not fused (Fig. 7B, D). These results suggest, in addition to an early function in interzone formation, that the function of *Osr1* and *Osr2* is also required for joint cell differentiation.

## **Discussion**

Initiation of joint formation in the developing vertebrate limb involves both specification of the joint cell fate and suppression of the cartilage molecular program in the joint progenitor cells. Whereas recent studies have identified several signaling molecules that are specifically expressed in the presumptive joint cells (reviewed by Archer et al., 2003; Pacifici et al., 2005), little is known about the transcription factors controlling joint formation. In this report, we show that expression of the *Osr1* and *Osr2* genes are both activated in the presumptive joint cells at the onset of joint initiation and that they play critical roles in regulating synovial joint formation.

*Osr1* and *Osr2* were initially identified as mammalian homologs of the *Drosophila* odd-skipped family zinc finger transcription factors (Lan et al, 2001; So and Danielian, 1999). The odd-skipped family in *Drosophila* consists of four distinct genes, *odd*, *bowl*, *sob*, and *drm* (Coulter et al., 1990; Green et al., 2002; Hart et al., 1996; Wang and Coulter, 1996). *Odd* encodes a protein containing four C2H2-type zinc finger motifs, whereas *bowl* and *sob* each encode a protein containing five zinc finger motifs highly homologous to ODD. In contrast, *drm* encodes a small protein containing only two zinc finger motifs, of which only the first finger conforms to the canonical C2H2 sequence and shows high sequence identity to the first zinc finger in the other Odd-skipped family proteins (Green et al., 2002). Mutations in *odd*, *bowl*, and *drm* each resulted in distinct developmental defects (Coulter et al., 1990; Green et al., 2002; Wang and Coulter, 1996). Interestingly, although there is significantly more extensive amino acid sequence identity between ODD and BOWL than that between ODD and DRM proteins, ectopic expression of *bowl* almost always resulted in different phenotypes from that of expression of *odd* under the same conditions, whereas ectopic expression of *odd* and *drm* in the same tissues often resulted in similar developmental defects (Green et al., 2002; Hao et al., 2003; de Celis Ibeas and Bray, 2003; Hatini et al., 2005; Bras-Pereira et al., 2006). These data suggested that the odd-skipped family transcription factors in *Drosophila* have largely distinct biochemical functions in vivo that are most likely due to functional domains outside of the zinc finger motifs. In contrast to the *Drosophila* odd-skipped family, *Osr1* and *Osr2* are the only members of this

family in mammals and they share extensive amino acid sequence identity with each other throughout the length of the protein products (Lan et al., 2001). Although targeted null mutations in *Osr1* and *Osr2* in mice resulted in distinct phenotypes, with heart and urogenital defects in *Osr1*<sup>-/-</sup> mice and with cleft palate and open-eyelids in *Osr2*<sup>-/-</sup> mice, respectively, (Lan et al., 2004; Wang et al., 2005), we recently demonstrated that expression of *Osr1* from the *Osr2* locus through targeted gene replacement rescued the craniofacial developmental defects in the *Osr2*<sup>-/-</sup> mice (Gao et al., 2009). Thus, we hypothesized that the distinct phenotypes of the *Osr1*<sup>-/-</sup> and *Osr2*<sup>-/-</sup> mutant mice are most likely due to the distinct developmental expression patterns of these two genes. In this study, we found that expression of both *Osr1* and *Osr2* are activated in most presumptive joint cells and persist throughout joint development in the developing limbs except that *Osr2*, but not *Osr1*, is highly expressed in the joint cells in between several tarsal elements in the ankle region. The corresponding tarsal elements were fused in the *Osr2*<sup>-/-</sup> mice whereas all other limb joints developed apparently normally in either the *Osr2*<sup>-/-</sup> mice or the *Osr1*<sup>fl/fl</sup> *Prrx1-cre* mice. However, multiple joints failed to form properly in the *Osr1*<sup>fl/fl</sup> *Osr2*<sup>-/-</sup> *Prrx1-cre* mice. These data confirm that *Osr1* and *Osr2* function redundantly in the tissues where they are co-expressed during mouse development. The overlapping expression of *Osr1* and *Osr2* in most developing joints ensures proper development and function of the major synovial joints.

Joint fusion phenotypes partially overlapping with that observed in the *Osr1*<sup>fl/fl</sup> *Osr2*<sup>-/-</sup> *Prrx1-cre* mutant mice have been reported in mice lacking *Gdf5*, *Gdf6*, or both *Wnt9a* and *Wnt4* (Settle Jr. et al., 2003; Storm and Kingsley, 1996; 1999; Storm et al., 1994). *Gdf5*<sup>-/-</sup>, *Gdf6*<sup>-/-</sup>, *Gdf5*<sup>-/-</sup> *Gdf6*<sup>-/-</sup>, and *Wnt9a*<sup>-/-</sup> *Wnt4*<sup>-/-</sup> mutant mice all displayed fusion of the carpal and tarsal elements in the wrist and ankle regions, respectively, but they did not have fusion of the long bone elements at the elbow or knee joints as observed in the *Osr1*<sup>fl/fl</sup> *Osr2*<sup>-/-</sup> *Prrx1-cre* mutant mice. Expression of *Gdf5*, *Wnt4*, and *Wnt9a* were all detected in the presumptive joint or peri-joint cells at the onset of joint initiation in the *Osr1*<sup>fl/fl</sup> *Osr2*<sup>-/-</sup> *Prrx1-cre* mutant embryos, indicating that *Osr1* and *Osr2* are not required for the specification of the joint progenitor cells or for activating expression of these signaling molecules. However, in the absence of *Osr1* and *Osr2*, most of the interzone cells continued to express high levels of *Col2* mRNA, suggesting that *Osr1/Osr2* function is required for the interzone cells to fully commit to the joint fate. Interestingly, while *Gdf5* expression in the joint progenitor cells and *Wnt4* expression in the peri-joint cells were downregulated in the *Osr1*<sup>fl/fl</sup> *Osr2*<sup>-/-</sup> *Prrx1-cre* mutant elbow region, a subset of cells in the humerus-ulna joint region maintained *Wnt9a* mRNA expression (Fig. 5D). Nevertheless, even in the region of the humerus-ulna joint where expression of *Wnt9a* persisted in the *Osr1*<sup>fl/fl</sup> *Osr2*<sup>-/-</sup> *Prrx1-cre* mutant embryos, those cells failed to upregulate the articular cartilage marker *Prg4* (Fig. 7B), suggesting that *Osr1/Osr2* plays a critical role in joint cell differentiation. The persistence of *Wnt9a* expression in some joint progenitor cells in the *Osr1*<sup>fl/fl</sup> *Osr2*<sup>-/-</sup> *Prrx1-cre* mutant embryos, together with our data showing normal expression of *Osr1* and *Osr2* in the *Catmb*<sup>fl/fl</sup>; *Osr2*<sup>IresCre/+</sup> mutant embryos, suggests that *Osr1/2* function in parallel with the *Wnt9a* signaling pathway during joint interzone formation. On the other hand, the downregulation of *Wnt4* in the peri-joint cells in the *Osr1*<sup>fl/fl</sup> *Osr2*<sup>-/-</sup> *Prrx1-cre* mutant embryos suggests that *Osr1/2* may be involved in the maintenance of the peri-joint cells and indirectly modulate the *Wnt* signaling pathway during joint formation. The differences in severity of the phenotype in the different joints may be due to differences in spatiotemporal activation of other molecular pathways, such as the *Wnt* pathway, during joint development.

Multiple joint fusions, including fusion of the humerus with the ulna and the radius at the elbow region, have also been reported in mice lacking either *Noggin* or *Zeb1* (Brunet et al., 1998; Takagi et al., 1998). *Noggin*<sup>-/-</sup> mutant embryos had excessive cartilage formation at the expense of the residual limb mesenchyme, suggesting that the failure of joint formation



in this mutant mouse strain resulted from increased chondrogenesis of the limb mesenchyme due to elevated Bmp signaling (Brunet et al., 1998). The Zeb1 protein, containing homeodomain and zinc finger domains, has been shown to interact with Smad proteins and to modulate TGF $\beta$  signaling (Postigo, 2003; Nishimura et al., 2006). Interestingly, we recently found that *Bmp4* expression was enhanced and expanded in the developing tooth mesenchyme in the *Osr2*<sup>-/-</sup> mutant mice (Zhang et al., 2009). One possibility is that *Osr1* and *Osr2* may function to modulate the activity of the Bmp or TGF $\beta$  signaling pathway in the joint progenitor cells. Identification of direct downstream target genes of *Osr1* and *Osr2* and elucidation of the molecular interactions involving these and other factors in the joint progenitor cells will provide new insights into the molecule mechanisms of joint development.

## Acknowledgments

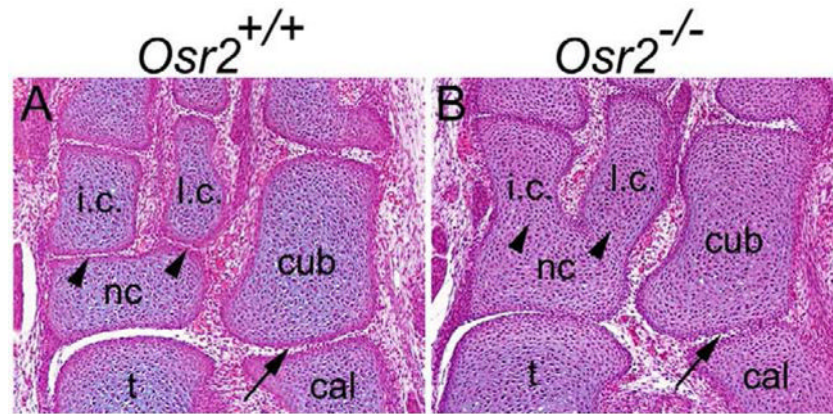
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## References

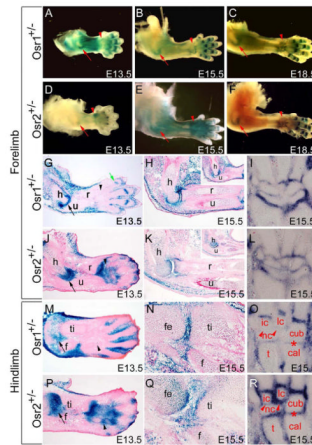
- Archer C, Dowthwaite G, Francis-West P. Development of synovial joints. *Birth Defects Res C Embryo Today* 2003;69:144–155. [PubMed: 12955858]
- Bras-Pereira C, Bessa J, Casares F. Odd-skipped genes specify the signaling center that triggers retinogenesis in *Drosophila*. *Development* 2006;133:4145–4149. [PubMed: 17021046]
- Brault V, Moore R, Kutsch S, Ishibashi M, Rowitch D, McMahon A, Sommer L, Boussadia O, Kemler R. Inactivation of the  $\beta$ -catenin gene by Wnt1-Cre-mediated deletion results in dramatic brain malformation and failure of craniofacial development. *Development* 2001;128:1253–1264. [PubMed: 11262227]
- Brunet L, McMahon J, McMahon A, Harland R. Noggin, cartilage morphogenesis, and joint formation in the mammalian skeleton. *Science* 1998;280:1455–1457. [PubMed: 9603738]
- Buxton P, Edwards C, Archer C, Francis-West P. Growth/differentiation factor-5 (GDF-5) and skeletal development. *J Bone Joint Surg Am* 2001;83A:S23–S30. [PubMed: 11263662]
- Coulter DE, Swaykus EA, Beran-Koehn MA, Goldberg D, Wieschaus E, Schedl P. Molecular analysis of odd-skipped, a zinc finger encoding segmentation gene with a novel pair-rule expression pattern. *EMBO J* 1990;9:3795–3804. [PubMed: 2120051]
- Craig F, Bentley G, Archer C. The spatial and temporal pattern of collagens I and II and keratan sulphate in the developing chick metatarsophalangeal joint. *Development* 1987;99:383–391. [PubMed: 2958266]
- de Celis Ibeas JM, Bray SJ. Bowl is required downstream of Notch for elaboration of distal limb patterning. *Development* 2003;130:5943–5952. [PubMed: 14573519]
- Dhordain P, Dewitte F, Desbiens X, Stehelin D, Duterque-Coquillaud M. Mesodermal expression of the chicken *erg* gene associated with precartilaginous condensation and cartilage differentiation. *Mech Dev* 1995;50:17–28. [PubMed: 7605748]
- Elders MJ. The increasing impact of arthritis on public health. *J Rheumatol Suppl* 2000;60:6–8. [PubMed: 11032095]
- Ellis T, Gambardella L, Horcher M, Tschanz S, Capol J, Bertram P, Jochum W, Barrandon Y, Busslinger M. The transcriptional repressor CDP (*Cutl1*) is essential for epithelial cell differentiation of the lung and the hair follicle. *Genes Dev* 2001;15:2307–2319. [PubMed: 11544187]
- Flannery C, Hughes C, Schumacher B, Tudor D, Aydelotte M, Kuettner K, Caterson B. Articular cartilage superficial zone protein (SZP) is homologous to megakaryocyte stimulating factor precursor and is a multi-functional proteoglycan with potential growth-promoting, cytoprotective, and lubricating properties in cartilage metabolism. *Biochem Biophys Res Commun* 1999;254:535–541. [PubMed: 9920774]

- Francis-West P, Abdelfattah A, Chen P, Allen C, Parish J, Ladher R, Allen S, Macpherson S, Luyten F, Archer C. Mechanisms of GDF-5 action during skeletal development. *Development* 1999;126:1305–1315. [PubMed: 10021348]
- Gao Y, Lan Y, Ovitt CE, Jiang R. Functional equivalence of the zinc finger transcription factors *Osr1* and *Osr2* in mouse development. *Dev Biol* 2009;328:200–209. [PubMed: 19389375]
- Green RB, Hatini V, Johansen KA, Liu XJ, Lengyel JA. Drumstick is a zinc finger protein that antagonizes *Lines* to control patterning and morphogenesis of the *Drosophila* hindgut. *Development* 2002;129:3645–3656. [PubMed: 12117814]
- Guo X, Day T, Jiang X, Garrett-Beal L, Topol L, Yang Y. Wnt/ $\beta$ -catenin signaling is sufficient and necessary for synovial joint formation. *Genes Dev* 2004;18:2404–2417. [PubMed: 15371327]
- Hao I, Green R, Dunaevsky O, Lengyel J, Rauskolb C. The odd-skipped family of zinc finger genes promotes *Drosophila* leg segmentation. *Dev Biol* 2003;263:282–295. [PubMed: 14597202]
- Hart MC, Wang L, Coulter DE. Comparison of the structure and expression of odd-skipped and two related genes that encode a new family of zinc finger proteins in *Drosophila*. *Genetics* 1996;144:171–182. [PubMed: 8878683]
- Hartmann C, Tabin C. Wnt-14 plays a pivotal role in inducing synovial joint formation in the developing appendicular skeleton. *Cell* 2001;104:341–351. [PubMed: 11239392]
- Hatini V, Green RB, Lengyel JA, Bray SJ, DiNardo S. The Drumstick/*Lines*/*Bowl* regulatory pathway links antagonistic Hedgehog and Wingless signaling inputs to epidermal cell differentiation. *Genes Dev* 2005;19:709–718. [PubMed: 15769943]
- Hinchliffe, J.; Johnson, D. The development of the vertebrate limb. New York: Oxford University Press; 1980. Limb development in tetrapods; p. 72-83.
- Hogan, B.; Beddington, R.; Costantini, F.; Lacy, E. Manipulating the Mouse Embryo: A Laboratory Manual. 2. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press; 1994.
- Holder N. An experimental investigation into the early development of the chick elbow joint. *J Embryol Exp Morphol* 1977;39:115–127. [PubMed: 886251]
- Iwamoto M, Tamamura Y, Koyama E, Komori T, Takeshita N, Williams JA, Nakamura T, Enomoto-Iwamoto M, Pacifici M. Transcription factor *ERG* and joint and articular cartilage formation during mouse limb and spine skeletogenesis. *Dev Biol* 2007;305:40–51. [PubMed: 17336282]
- James R, Kamei C, Wang Q, Jiang R, Schultheiss T. Odd-skipped related 1 is required for development of the metanephric kidney and regulates formation and differentiation of kidney precursor cells. *Development* 2006;133:2995–3004. [PubMed: 16790474]
- Jiang R, Lan Y, Norton CR, Sundberg JP, Gridley T. The *Slug* gene is not essential for mesoderm or neural crest development in mice. *Dev Biol* 1998;198:277–285. [PubMed: 9659933]
- Jay G, Britt D, Cha C. Lubricin is a product of megakaryocyte stimulating factor gene expression by human synovial fibroblasts. *J Rheumatol* 2000;27:594–600. [PubMed: 10743795]
- Koyama E, Shibukawa Y, Nagayama M, Sugito H, Young B, Yuasa T, Okabe T, Ochiai T, Kamiya N, Rountree R, Kingsley D, Iwamoto M, Enomoto-Iwamoto M, Pacifici M. A distinct cohort of progenitor cells participates in synovial joint and articular cartilage formation during mouse limb skeletogenesis. *Dev Biol* 2008;316:62–73. [PubMed: 18295755]
- Lan Y, Kingsley P, Cho E, Jiang R. *Osr2*, a new mouse gene related to *Drosophila* odd-skipped, exhibits dynamic expression patterns during craniofacial, limb, and kidney development. *Mech Dev* 2001;107:175–179. [PubMed: 11520675]
- Lan Y, Ovitt C, Cho E, Maltby K, Wang Q, Jiang R. Odd-skipped related 2 (*Osr2*) encodes a key intrinsic regulator of secondary palate growth and morphogenesis. *Development* 2004;131:3207–3216. [PubMed: 15175245]
- Lan Y, Wang Q, Ovitt C, Jiang R. A unique mouse strain expressing Cre recombinase for tissue-specific analysis of gene function in palate and kidney development. *Genesis* 2007;45:618–624. [PubMed: 17941042]
- Lizarraga G, Lichtler A, Upholt W, Kosher R. Studies on the role of *Cux1* in regulation of the onset of joint formation in the developing limb. *Dev Biol* 2002;243:44–54. [PubMed: 11846476]
- Logan M, Martin J, Nagy A, Lobe C, Olson E, Tabin C. Expression of Cre Recombinase in the developing mouse limb bud driven by a *Prxl* enhancer. *Genesis* 2002;33:77–80. [PubMed: 12112875]

- Martin J, Bradley A, Olson E. The paired-like homeobox gene *Mhox* is required for early events of skeletogenesis in multiple lineages. *Genes Dev* 1995;9:1237–1249. [PubMed: 7758948]
- Mitrovic D. Development of the diarthrodial joints in the rat embryo. *Am J Anat* 1978;151:475–485. [PubMed: 645613]
- Nishimura G, Manabe I, Tsushima K, Fujiu K, Oishi Y, Imai Y, Maemura K, Miyagishi M, Higashi Y, Kondoh H, Nagai R.  $\delta$ EF1 mediates TGF-beta signaling in vascular smooth muscle cell differentiation. *Dev Cell* 2006;11:93–104. [PubMed: 16824956]
- Pacifici M, Koyama E, Iwamoto M. Mechanisms of synovial joint and articular cartilage formation: recent advances, but many lingering mysteries. *Birth Defects Res C Embryo Today* 2005;75:237–248. [PubMed: 16187328]
- Pitsillides A, Ashhurst D. A critical evaluation of specific aspects of joint development. *Dev Dyn* 2008;273:2284–2294. [PubMed: 18729226]
- Postigo A. Opposing functions of ZEB proteins in the regulation of the TGF $\beta$ /BMP signaling pathway. *EMBO J* 2003;22:2443–2452. [PubMed: 12743038]
- Schumacher B, Block J, Schmid T, Aydelotte M, Kuettner K. A novel proteoglycan synthesized and secreted by chondrocytes of the superficial zone of articular cartilage. *Arch Biochem Biophys* 1994;311:144–152. [PubMed: 8185311]
- Settle S, Rountree R, Sinha A, Thacker A, Higgins K, Kingsley D. Multiple joint and skeletal patterning defects caused by single and double mutations in the mouse *Gdf6* and *Gdf5* genes. *Dev Biol* 2003;254:116–130. [PubMed: 12606286]
- So P, Danielian P. Cloning and expression analysis of a mouse gene related to *Drosophila* odd-skipped. *Mech Dev* 1999;84:157–160. [PubMed: 10473132]
- Später D, Hill T, O'sullivan R, Gruber M, Conner D, Hartmann C. Wnt9a signaling is required for joint integrity and regulation of *Ihh* during chondrogenesis. *Development* 2006;133:3039–3049. [PubMed: 16818445]
- Storm E, Huynh T, Copeland N, Jenkins N, Kingsley D, Lee S. Limb alterations in brachypodism mice due to mutations in a new member of the TGF- $\beta$  superfamily. *Nature* 1994;368:639–643. [PubMed: 8145850]
- Storm E, Kingsley D. Joint patterning defects caused by single and double mutations in members of the bone morphogenetic protein (BMP) family. *Development* 1996;122:3969–3979. [PubMed: 9012517]
- Storm E, Kingsley D. GDF5 coordinates bone and joint formation during digit development. *Dev Biol* 1999;209:11–27. [PubMed: 10208739]
- Stricker S, Brieske N, Haupt J, Mundlos S. Comparative expression pattern of Odd-skipped related genes *Osr1* and *Osr2* in chick embryonic development. *Gene Expr Patterns* 2006;6:826–834. [PubMed: 16554187]
- Swann DA, Slayter HS, Silver FH. The molecular structure of lubricating glycoprotein-I, the boundary lubricant for articular cartilage. *J Biol Chem* 1981;256:5921–5925. [PubMed: 7240180]
- Takagi T, Moribe H, Kondoh H, Higashi Y.  $\delta$ EF1, a zinc finger and homeodomain transcription factor, is required for skeleton patterning in multiple lineages. *Development* 1998;125:21–31. [PubMed: 9389660]
- Wang L, Coulter DE. *bowel*, an odd-skipped homolog, functions in the terminal pathway during *Drosophila* embryogenesis. *EMBO J* 1996;15:3182–3196. [PubMed: 8670819]
- Wang Q, Lan Y, Cho E, Maltby K, Jiang R. Odd-skipped related 1 (*Odd1*) is an essential regulator of heart and urogenital development. *Dev Biol* 2005;288:582–594. [PubMed: 16223478]
- Zhang Y, Zhang X, Hu Y, Ramamurthy RTA, Qiu M, Chen Y. *Msx1* is required for the induction of *Patched* by Sonic hedgehog in the mammalian tooth germ. *Dev Dyn* 1999;215:45–53. [PubMed: 10340755]
- Zhang Z, Lan Y, Chai Y, Jiang R. Antagonistic actions of *Msx1* and *Osr2* pattern mammalian teeth into a single row. *Science* 2009;323:1232–1234. [PubMed: 19251632]



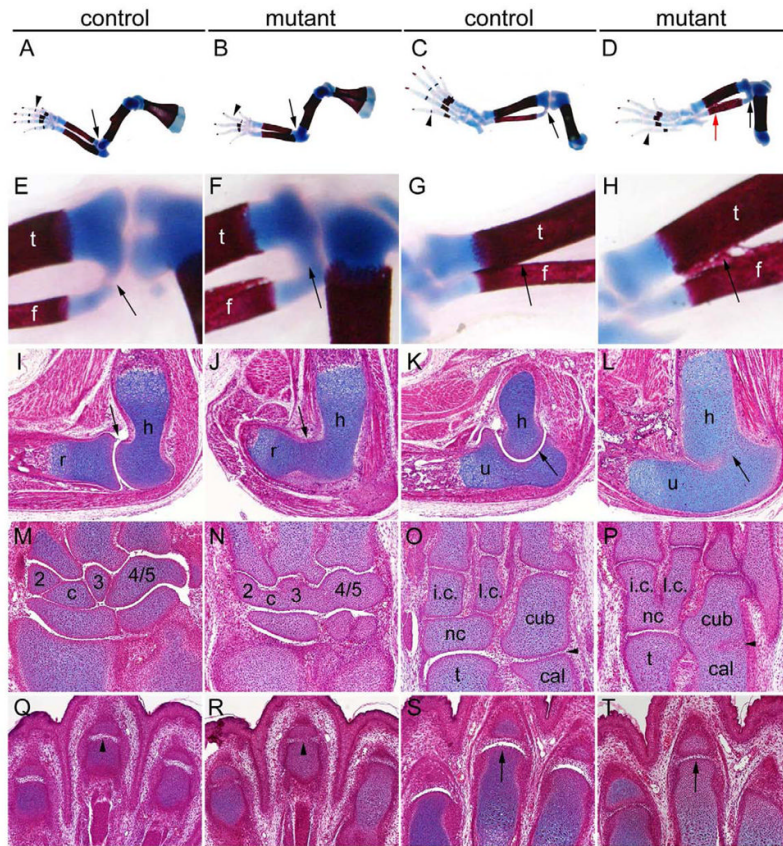
**Fig. 1.** *Osr2*<sup>-/-</sup> mutant mice have fused tarsal elements. (A) In the E17.5 wildtype embryo, tarsal elements are separated by synovial joints. (B) In the E17.5 *Osr2*<sup>-/-</sup> embryo, both intermediate cuneiform and lateral cuneiform are fused with the navicular bone (arrowheads in B). Cuboidal and calcaneus bones in *Osr2*<sup>-/-</sup> embryos remain separate (arrow in B). cal, calcaneus bone; cub, cuboid bone; i.c., intermediate cuneiform; l.c., lateral cuneiform; nc, navicular bone; t, talus bone.



**Fig. 2.**

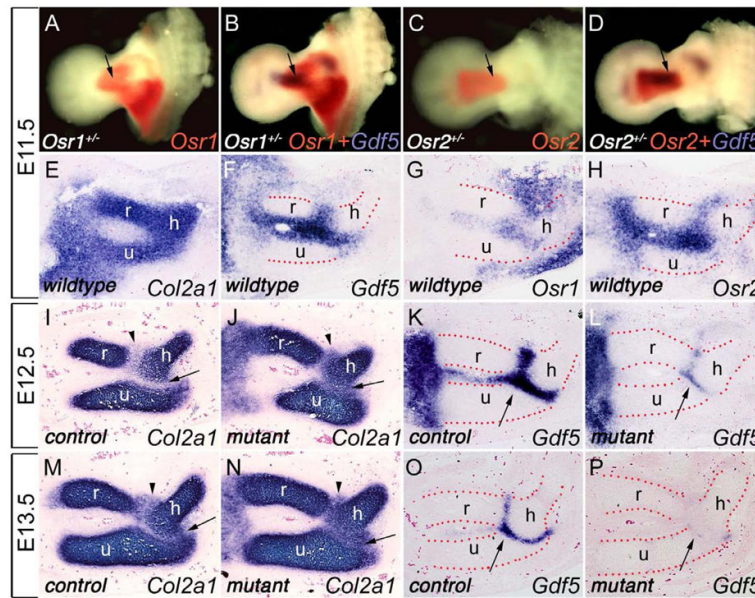
Expression patterns of *Osr1* and *Osr2* during synovial joint development. (A–F) Whole-mount X-gal staining of the forelimbs of E13.5 to E18.5 *Osr1*<sup>+/-</sup> and *Osr2*<sup>+/-</sup> embryos. Both *Osr1* and *Osr2* are strongly and highly specifically expressed in the developing interphalangeal, metacarpal-phalangeal, and elbow joints (arrows in A–F). In the carpal region, expression of *Osr1* is weak (arrowhead in A) as compared with that of *Osr2* (arrowhead in D) at E13.5. At E15.5 and E18.5, both *Osr1* and *Osr2* are strongly expressed in the carpal joints. (G, H) X-gal-stained sections of the forelimbs of E13.5 and E15.5 *Osr1*<sup>+/-</sup> embryos, respectively. Black arrow points to the elbow joint, green arrow points to the developing metacarpophalangeal joint in the digit, and black arrowhead points to the carpal region. (I) Section in situ hybridization of E15.5 wildtype embryos detected *Osr1* mRNA expression in the carpal joints. (J, K) X-gal stained sections of the forelimbs of E13.5 and E15.5 *Osr2*<sup>+/-</sup> embryos, respectively. Strong lacZ activity was detected in between the carpal elements (arrowhead in J) and in the elbow joint (arrowhead in K). (L) Section in situ hybridization detected *Osr2* mRNA expression in the carpal joint cells in the E15.5 wildtype embryos. (M, N) X-gal stained sections of the hindlimbs of E13.5 and E15.5 *Osr1*<sup>+/-</sup> embryos, respectively. (P, Q) X-gal stained sections of the hindlimbs of E13.5 and E15.5 *Osr2*<sup>+/-</sup> embryos, respectively. Expression of *Osr1* (arrowhead in M) is much weaker than that of *Osr2* (arrowhead in P) in the tarsal region at E13.5. However, both were expressed strongly in the knee joint at E13.5 and E15.5. (O, R) Section in situ hybridization detected difference in expression of *Osr1* (O) and *Osr2* (R) in the developing tarsal joints at E15.5. Strong *Osr2* mRNA expression was detected in the developing joints between the tarsal elements (R) while little *Osr1* mRNA was detected in between intermediate cuneiform and navicular bones and in between lateral cuneiform and navicular bones (arrowheads in O). Both *Osr1* and *Osr2* mRNAs were detected in between the cuboidal and calcaneus bones (asterisk in O and R). cal, calcaneus bone; cub, cuboid bone; f, femur; fe, femur; h, humerus; i.c., intermediate cuneiform; l.c., lateral cuneiform; nc, navicular bone; r, radius; t, talus bone; ti, tibia; u, ulna.





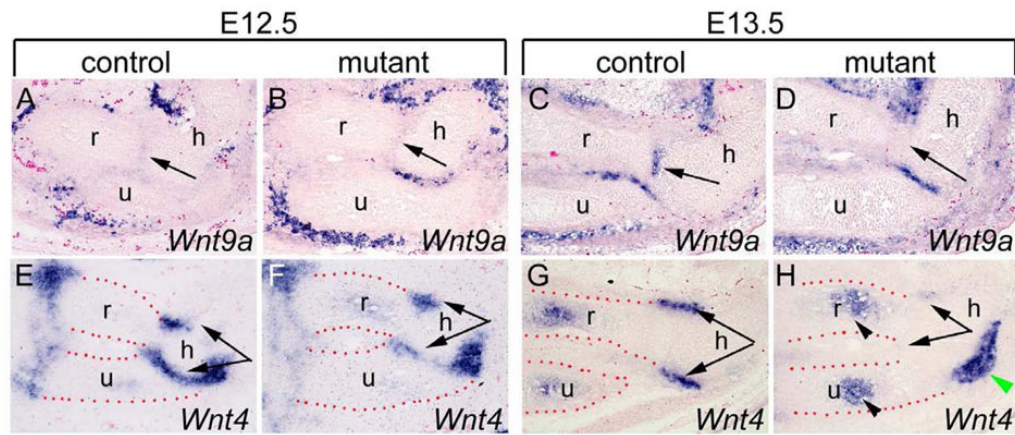
**Fig. 3.**

Joint defects in *Osr1<sup>ff</sup>Osr2<sup>-/-</sup>Prrx1-cre* double mutant mice. (A–D) Alizarin red and alcian blue stained skeleton preparations of E17.5 limbs of *Osr1<sup>ff</sup>Prrx1-cre* (A, C) and *Osr1<sup>ff</sup>Osr2<sup>-/-</sup>Prrx1-cre* mutant (B, D) embryos. The double mutants showed delay in mineralization of phalanges (arrowheads in B and D), fusion of the elbow joints (arrow in B), and fusion between tibia and fibula in both the distal (red arrow in D) and proximal (black arrow in D) regions. (E–H) High magnification views of proximal and distal regions of the tibia and fibula in control (E, G) and mutant (F, H) hindlimbs. Arrows point to the aberrant fusion between these two elements in the mutants and corresponding regions in the control littermates. (I–L) *Osr1<sup>ff</sup>Prrx1-cre* embryo had normal synovial joint development in the elbow (arrows in I and K) while in *Osr1<sup>ff</sup>Osr2<sup>-/-</sup>Prrx1-cre* mutant embryo humerus is fused to radius (arrow in J) and to ulna (arrow in H). (M, N) Carpal elements are demarcated clearly by the synovial joints in E17.5 *Osr1<sup>ff</sup>Prrx1-cre* littermate (M), while they are fused together in the E17.5 *Osr1<sup>ff</sup>Osr2<sup>-/-</sup>Prrx1-cre* mutant embryo (N). (O, P) Tarsal elements are separated by synovial joints in the E17.5 *Osr1<sup>ff</sup>Prrx1-cre* embryo (O), while the intermediate cuneiform and lateral cuneiform were fused respectively with the navicular bone in the *Osr1<sup>ff</sup>Osr2<sup>-/-</sup>Prrx1-cre* mutant (P). In addition, the cuboidal and calcaneus bones were also fused in *Osr1<sup>ff</sup>Osr2<sup>-/-</sup>Prrx1-cre* mutant embryo (arrowhead in P). (Q–T) Histological sections through the interphalangeal (Q, R) and metacarpophalangeal (S, T) joint regions in the E17.5 forelimbs of control (Q, S) and *Osr1<sup>ff</sup>Osr2<sup>-/-</sup>Prrx1-cre* mutant (R, T) embryos. Arrowheads in Q and R point to corresponding interphalangeal joints, whereas arrows in S and T point to the corresponding metacarpophalangeal joints in the control and mutant littermates. cal, calcaneus bone; cub, cuboid bone; h, humerus; i.c., intermediate cuneiform; l.c., lateral cuneiform; nc, navicular bone; t, talus bone; r, radius; u, ulna.



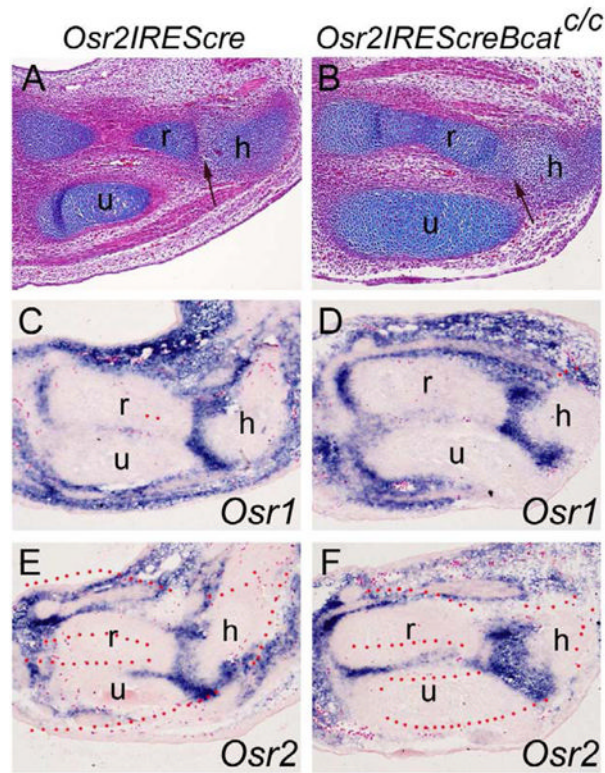
**Fig. 4.**

*Osr1* and *Osr2* are expressed in nascent joint cells and control joint formation. (A–D) Salmon-gal staining and combined Salmon-gal staining with *Gdf5* in situ hybridization in limbs of E11.5 *Osr1*<sup>+/-</sup> (A and B) and *Osr2*<sup>+/-</sup> (C and D) embryos. *Osr1* and *Osr2* share overlapping expression patterns in the presumptive elbow joint (arrows) with *Gdf5*. (E) Section in situ hybridization of E11.5 wildtype forelimb showing continuous *Col2a1* mRNA expression in the prospective humerus, ulna and radius anlagen. (F–H) Section in situ hybridization of E11.5 wildtype limb detected overlapping expression of *Gdf5* (F), *Osr1* (G), and *Osr2* (H) expression in the presumptive elbow joint cells. (I, J) At E12.5, joint interzone formation in the elbow region of the control embryo (I) was clearly demarcated by dramatically decreased *Col2a1* mRNA expression, while the presumptive joint cells in the *Osr1*<sup>fl/fl</sup>*Osr2*<sup>-/-</sup>*Prrx1-cre* mutant littermate showed persistent high levels of *Col2a1* mRNA expression (J). Arrows point to the prospective humerus-ulna joint and arrowheads point to the prospective humerus-radius joint. (K, L) At E12.5, *Gdf5* was strongly expressed in the interzone cells in the control embryo (arrow in K) but was significantly reduced in the elbow region in the *Osr1*<sup>fl/fl</sup>*Osr2*<sup>-/-</sup>*Prrx1-cre* mutant embryo (arrow in L). (M, N) At E13.5, as the interzone cells in the control embryos starts to differentiate into a three-layered structure with the middle layer remaining negative for *Col2a1* expression (M), the *Osr1*<sup>fl/fl</sup>*Osr2*<sup>-/-</sup>*Prrx1-cre* mutant limbs still lack a clear *Col2a1*-negative interzone in the elbow region (N). (O, P) At E13.5, the joint progenitor cells remain positive for *Gdf5* mRNA expression in the control embryo (arrow in O), while the expression of *Gdf5* was almost completely downregulated in the joint progenitor cells in *Osr1*<sup>fl/fl</sup>*Osr2*<sup>-/-</sup>*Prrx1-cre* double mutants (arrow in P). h, humerus; r, radius; u, ulna.

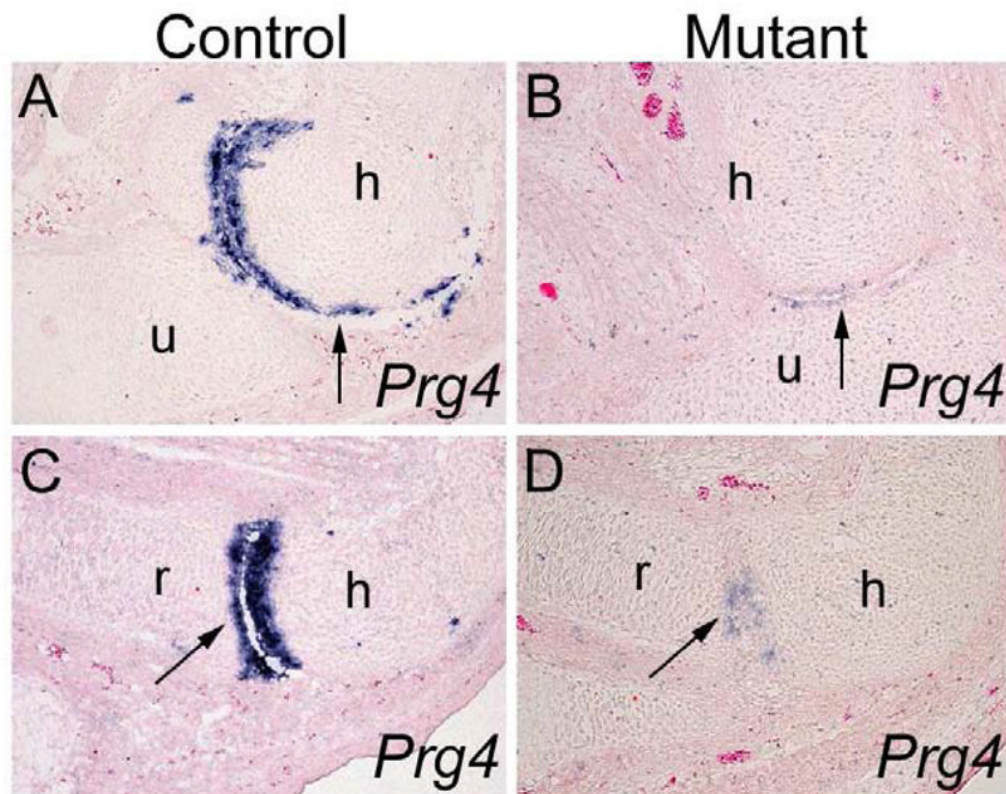


**Fig. 5.** *Osr1* and *Osr2* acts upstream of Wnt/ $\beta$ -catenin signaling during joint development. (A, B) At E12.5, *Wnt9a* was weakly expressed in the presumptive elbow joint cells in both the control and the *Osr1<sup>fl/fl</sup>Osr2<sup>-/-</sup>Prrx1-cre* mutant littermates (arrows). (C, D) At E13.5, *Wnt9a* mRNA expression was clearly detected in the interzone cells between the humerus and radius and between the humerus and ulna in the control embryos. *Wnt9a* mRNA expression was absent in between the humerus and radius elements (arrow in D) but was clearly detected in between the humerus and ulna elements in the E13.5 double mutant embryos. (E–H) Strong *Wnt4* mRNA expression was detected in mesoderm cells surrounding the elbow joint interzone in E12.5 control embryos (E) while lower levels of *Wnt4* expression was detected in the corresponding cells in the E12.5 *Osr1<sup>fl/fl</sup>Osr2<sup>-/-</sup>Prrx1-cre* mutant littermates (F). (G, H) At E13.5, *Wnt4* mRNA was still expressed in the mesoderm cells surrounding the elbow interzone in the control embryo (G), while its expression in the corresponding cells in the *Osr1<sup>fl/fl</sup>Osr2<sup>-/-</sup>Prrx1-cre* mutant appeared almost completely downregulated (H). *Wnt4* expression in the differentiating chondrocytes in the radius and ulna elements (arrowheads in H) was similar in the control and *Osr1<sup>fl/fl</sup>Osr2<sup>-/-</sup>Prrx1-cre* mutant embryos. Green arrowhead in H points to a *Wnt4*-expressing tendon tissue that was absent from the control section in G due to differences in the plane of sections. h, humerus; r, radius; u, ulna.





**Fig. 6.** Expression of *Osr1* and *Osr2* in the developing joint cells was not altered by loss of Wnt/ $\beta$ -catenin signaling. (A, B) HE-stained histological sections through the elbow region of E13.5 *Osr2<sup>IREScre/+</sup>* control (A) and *Catnb<sup>ff</sup>;Osr2<sup>IREScre/+</sup>* mutant (B) embryos. Arrow points to the developing joint between humerus and radius, which is disorganized in the mutant (B). (C–F) Section in situ hybridization with *Osr1* and *Osr2* probes in the elbow region of E13.5 *Osr2<sup>IREScre/+</sup>* (C, E) and *Catnb<sup>ff</sup>;Osr2<sup>IREScre/+</sup>* mutant (D, F) embryos. No significant differences in expression of *Osr1* or *Osr2* were detected in the developing limbs between control and *Catnb<sup>ff</sup>;Osr2<sup>IREScre/+</sup>* mutant embryos. h, humerus; r, radius; u, ulna.



**Fig. 7.** Comparison of *Prg4* expression in the developing elbow joints in the *Osr1<sup>ff</sup>Osr2<sup>-/-</sup>Prrx1-cre* mutant and control embryos. (A–D) While differentiating articular cartilage cells in the elbow joints in the control embryos (A, C) express high levels of *Prg4* mRNA at E15.5, only few low *Prg4*-expressing cells were detected in the elbow joint region in the *Osr1<sup>ff</sup>Osr2<sup>-/-</sup>Prrx1-cre* littermates (B, D). h, humerus; r, radius; u, ulna.