

Suppression of Bmp4 signaling by the zinc-finger repressors Osr1 and Osr2 is required for Wnt/ β -catenin-mediated lung specification in *Xenopus*

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SUMMARY

Embryonic development of the respiratory system is regulated by a series of mesenchymal-epithelial interactions that are only partially understood. Mesenchymal FGF and Wnt2/Wnt2b signaling are implicated in specification of mammalian pulmonary progenitors from the ventral foregut endoderm, but their epistatic relationship and downstream targets are largely unknown. In addition, how *wnt2* and *wnt2b* are regulated in the developing foregut mesenchyme is unknown. We show that the Odd-skipped-related (Osr) zinc-finger transcriptional repressors Osr1 and Osr2 are redundantly required for *Xenopus* lung specification in a molecular pathway linking foregut patterning by FGFs to Wnt-mediated lung specification and RA-regulated lung bud growth. FGF and RA signals are required for robust *osr1* and *osr2* expression in the foregut endoderm and surrounding lateral plate mesoderm (lpm) prior to respiratory specification. Depletion of both Osr1 and Osr2 (Osr1/Osr2) results in agenesis of the lungs, trachea and esophagus. The foregut lpm of Osr1/Osr2-depleted embryos fails to express *wnt2*, *wnt2b* and *raldh2*, and consequently Nkx2.1⁺ progenitors are not specified. Our data suggest that Osr1/Osr2 normally repress *bmp4* expression in the lpm, and that BMP signaling negatively regulates the *wnt2b* domain. These results significantly advance our understanding of early lung development and may impact strategies to differentiate respiratory tissue from stem cells.

KEY WORDS: Lung, Osr, Wnt, BMP, *Xenopus*, Foregut, Endoderm

INTRODUCTION

The epithelial lining of the vertebrate trachea and lungs is derived from the ventral foregut endoderm. During embryogenesis, the earliest known marker of the respiratory epithelium is the homeodomain transcription factor Nkx2.1 (Ttf1) (Lazzaro et al., 1991). *Nkx2.1*^{-/-} mutant mice have severely hypoplastic lungs and tracheoesophageal fistula (Minoo et al., 1999). Two signaling pathways, FGF and Wnt/ β -catenin, have been implicated in specification of the respiratory lineage. In vitro studies using mouse foregut explants suggest that between embryonic days (E) 8 and 8.75 of development, FGF ligands from the cardiac mesoderm pattern the ventral foregut progenitors into lung, liver and pancreas fates in a concentration-dependent manner, with the highest levels of FGF promoting lung and *nkx2.1* expression (Sells et al., 2005). It is thought that multiple, redundant FGF ligands are required, although this remains to be validated. Genetic studies in mice have recently demonstrated that between E8.5 and E9.5 Wnt2/Wnt2b signals from the lateral plate mesoderm (lpm) are necessary and sufficient for lung specification. Compound *Wnt2*^{-/-};*Wnt2b*^{-/-} mutants or endoderm-specific conditional β -catenin^{-/-} mutants exhibit lung agenesis and fail to express *nkx2.1* (Goss et al., 2009; Harris-Johnson et al., 2009).

After pulmonary specification, further crosstalk between the Wnt, FGF, Shh, TGF β , BMP and retinoic acid (RA) pathways regulate trachea-esophagus separation, lung bud outgrowth,

branching morphogenesis and proximal-distal patterning (reviewed by Cardoso and Lu, 2006; Maeda et al., 2007; Morrissey and Hogan, 2010). For example, analyses of mouse embryos lacking the RA-synthesizing enzyme Raldh2 indicate that RA is required for outgrowth of the specified lung epithelium, in part by suppressing TGF β and promoting Wnt and FGF10 signaling (Desai et al., 2004; Wang et al., 2006; Chen et al., 2007). Recent evidence suggests that RA may also have an earlier role facilitating Wnt-mediated pulmonary specification by RA-mediated repression of the Wnt antagonist Dkk1 (Chen et al., 2010). BMP signaling also has multiple roles in respiratory development. Analysis of conditional *Bmp4*^{-/-}, *Smad1*^{-/-} and compound *Bmpr1a*^{-/-};*Bmpr1b*^{-/-} mutants indicates that BMP signaling is dispensable for pulmonary specification, restricts the location of lung bud formation, promotes tracheal fate, and is required for proper distal differentiation and branching morphogenesis (Eblaghie et al., 2006; Li et al., 2008; Sun et al., 2008; Domyan et al., 2011; Xu et al., 2011). Despite recent progress, there are still a number of important unanswered questions about early lung development. First, the molecular pathways upstream of Wnt2/Wnt2b are undefined. It is unclear if early FGF and Wnt2/Wnt2b act in parallel or in an epistatic fashion, and their specific target genes are largely unknown. Finally, the relationship between early FGF/Wnt-mediated lung specification and the pathways regulating lung bud growth are poorly understood.

Osr zinc-finger transcriptional repressors are homologous to the Odd-skipped family of proteins in *Drosophila*, which regulate early segmentation and gut development (Green et al., 2002; Johansen et al., 2003; Goldstein et al., 2005). Vertebrates have two odd-skipped-related genes, *Osr1* and *Osr2*, which in mice have partially overlapping embryonic expression in the developing heart, kidney, limb and lung, and in craniofacial structures (So and Danielian, 1999; Lan et al., 2001). Analyses of *Osr1*^{-/-} mutant mice have

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shown that *Osr1* is essential for heart and kidney development (Wang et al., 2005; James et al., 2006; Mudumana et al., 2008), whereas *Osr2*^{-/-} mutants show defective craniofacial and tooth development (Lan et al., 2004; Zhang et al., 2009). Interestingly, a knock-in of the *Osr1* cDNA into the *Osr2* locus rescues the *Osr2*^{-/-} phenotype, suggesting that *Osr1* and *Osr2* are functionally equivalent (Gao et al., 2009). Compound *Osr1*^{-/-};*Osr2*^{-/-} mutant mice have not been characterized, but in *Xenopus* and zebrafish both *Osr1* and *Osr2* are required for kidney development (Tena et al., 2007).

In this study, we show that *Osr1* and *Osr2* are redundantly required for *Xenopus* respiratory specification and foregut development. Our data demonstrate that early FGF and RA signaling control *osr1/osr2* expression in the foregut epithelium and lpm. Depletion of *Osr1/Osr2* results in lung, trachea and esophagus agenesis. We show that the *Osr1/Osr2*-depleted lpm fails to express *wnt2/wnt2b* and *raldh2* and ectopically expresses *bmp4*; consequently, *Nkx2.1*+ respiratory progenitors are not specified in the foregut. *Osr1/Osr2* are required to spatially restrict *bmp4* expression in the lpm and BMP signaling negatively regulates *wnt2b* and *raldh2*. Temporal activation of Wnt/ β -catenin signaling can rescue lung specification but not lung bud growth in *Osr1/Osr2*-depleted embryos, whereas temporal inhibition of BMP signaling can completely rescue lung specification and bud formation. Together, these data indicate that *Osr1* and *Osr2* are key components of a molecular pathway that connects early foregut patterning by FGFs to Wnt-mediated pulmonary specification and RA-regulated lung bud development.

MATERIALS AND METHODS

Embryo manipulations and microinjections

Culture and injection of *X. laevis* and *X. tropicalis* embryos were performed as previously described (Sive et al., 2000; Khokha et al., 2002). The morpholinos *Osr1*-MO (10 ng), *Osr2*-MO (10 ng) and *Wnt2b*-MO (20 ng) have previously been shown to generate specific loss of functions (Tena et al., 2007; Damianitsch et al., 2009). The *X. tropicalis* *Wnt2*-MO (20 ng) has the sequence 5'-ATGAGGAGGTAACAACAGCTCCCAT-3'. GR-m*Osr1* was constructed by fusing the hormone-binding domain or the human glucocorticoid receptor in-frame with the mouse *Osr1* cDNA (Open Biosystems #MMM1013-7512959). GR-m*Osr1*, GR-Smad1 (Kim and Han, 2011) and GR-DNXtcf3 (McLin et al., 2007) mRNAs were prepared using the Ambion SP6 mMessage mMachine kit. Embryos with clear dorsal-ventral pigmentation were selected for four- to eight-cell stage injections targeting anterior mesendoderm. Small molecules were dissolved in DMSO and working concentrations were as follows: 100 μ M PD173074 (Tocris Bioscience), 80 μ M SU5402 (Tocris Bioscience), 50 μ M XAV939 (Cayman Chemical), 10 μ M BMS493 (Tocris Bioscience), 10 μ M LDN193189 (Axon MedChem), 10 μ M BIO (Cayman Chemical) and 1–5 μ M all-trans retinoic acid (Sigma). Dexamethasone (1 μ M; Sigma) was used to activate hormone-inducible GR constructs. Culture buffers were refreshed with the inhibitors every 12–16 hours, and embryos were cultured in the dark as some inhibitors are known to be light sensitive. Luciferase assays were performed as described previously (Rankin et al., 2011).

In situ hybridization and Immunohistochemistry

Whole-mount in situ hybridization was performed as previously described (Sive et al., 2000) and details on specific clones and probe production are available upon request. For immunofluorescence, embryos were fixed in MEMFA for 1 hour at 4°C, washed in PBS (pH 7.4) and then transferred into Dent's post-fixative (80% methanol, 20% DMSO) and stored at -20°C for at least 48 hours. Embryos were rehydrated through a methanol series into PBS+0.1% Triton X-100 (PBSTr), bisected with a razor blade through the foregut region, blocked for 2 hours in PBSTr +10% goat serum + 1% BSA, followed by incubation with primary antibodies [rabbit-anti-phospho-Smad1/5/8, Cell Signaling #9511, 1:350; mouse anti-fibronectin 4H2, kindly

provided by D. DeSimone (University of Virginia, Charlottesville, VA, USA), 1:400; mouse anti-Nkx2.1, Thermo Scientific #8G7G3/1, 1:300; rabbit anti-Sox2, Abcam #ab97959, 1:500] in blocking solution overnight at 4°C. After washing in PBSTr, samples were incubated in goat-anti-rabbit DyLight488 and/or goat-anti-mouse DyLight649 (Jackson ImmunoResearch, used at 1:500 in PBSTr + 1%BSA) overnight at 4°C. After extensive washing in PBSTr, samples were dehydrated in 100% methanol, cleared in two parts benzyl benzoate/one part benzyl alcohol and imaged using a Zeiss LSM 510 confocal microscope. Standard immunohistochemical protocols were used to analyze cell proliferation and cell death.

RESULTS

osr1 and *osr2* are dynamically expressed in the developing *Xenopus* foregut

The expression of *Osr* transcription factors in *Xenopus* digestive and respiratory system development has not been characterized. In situ hybridization of *Xenopus* embryos revealed that *osr1* and *osr2* are dynamically expressed in overlapping domains of the endoderm and surrounding mesoderm during early organogenesis. After gastrulation (stage 15), *osr1* and *osr2* are expressed in the lpm (Tena et al., 2007) and posterior endoderm but are excluded from the ventral foregut (Fig. 1A,E). They first become expressed in the foregut prior to specification of organ lineages during stages 25–32, at which time they are also expressed in the hindgut, lpm, brain, branchial arches and developing kidney, as previously described (Fig. 1B,F) (Tena et al., 2007). At stage 33, prior to organ bud formation, *osr1* and *osr2* have overlapping expression patterns in the anterior foregut that gives rise to the esophagus, trachea and lungs (Fig. 1C,G). At this time, the anterior foregut cavity is still a single tube that has not yet separated into a distinct dorsal esophagus and a ventral trachea. *osr1* is expressed in both the presumptive pulmonary epithelium and surrounding lpm with lower dorsal expression in the presumptive esophagus (Fig. 1C; supplementary material Fig. S1). By contrast, *osr2* is robustly expressed in both the dorsal and ventral aspects of the anterior foregut endoderm and in lpm (Fig. 1G; supplementary material Fig. S1). In isolated stage 42 gut tubes *osr1* and *osr2* are both expressed in the esophagus, trachea, lungs and proximal stomach (Fig. 1D,H).

Osr1 and *Osr2* are redundantly required for respiratory specification and tracheal-esophageal separation

To determine the role of *Osr1* and *Osr2* in *Xenopus* foregut development, we injected established translation blocking morpholino oligos (MOs) (Tena et al., 2007) with a fluorescent tracer into vegetal cells of the eight-cell embryo, targeting the anterior mesendoderm. Injection of individual *Osr1*-MO or *Osr2*-MO had no detectable effect on early endoderm organogenesis (data not shown). However, co-injection of both the *Osr1*-MO and *Osr2*-MO (*Osr1/Osr2*-MO) into one side of the foregut mesendoderm resulted in lung bud agenesis on the injected side, as indicated by the lack of *surfactant protein c* (*sftpc*) and *nkx2.1* expression at stage 37, and the absence a lung bud of at stage 42 (Fig. 1I–N). Sectioning of the unilateral injected *Osr1/Osr2*-MO embryos revealed that the lung bud was missing on the injected side but the *nkx2.1*-expressing trachea was still present (Fig. 1N–N'). Liver, pancreas, thyroid, heart and intestine development was unaffected by *Osr1/Osr2* knockdown (supplementary material Fig. S2). Analysis of early mesendoderm markers also indicated that germ layer formation and anterior-posterior patterning were normal (supplementary material Fig. S2), but kidney development was impaired as described previously (Tena et al., 2007). Comparable results were obtained with *Osr1/Osr2*-MOs

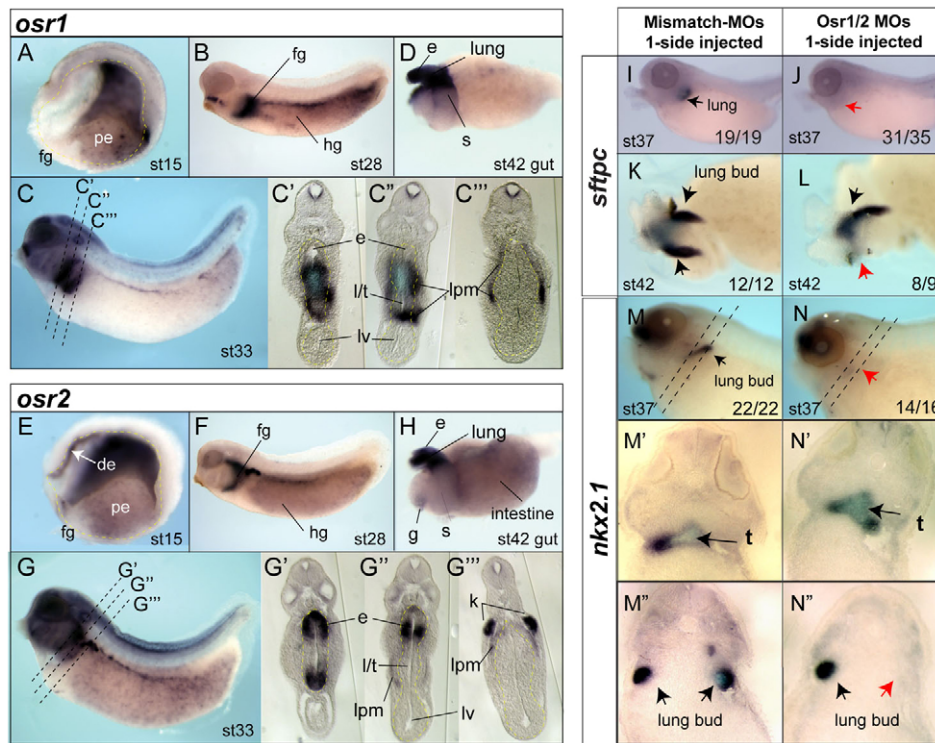


Fig. 1. *osr1* and *osr2* are dynamically expressed in the developing *Xenopus* foregut and are required for lung development. (A-G''') In situ hybridization of *osr1* (A-D) and *osr2* (E-H) expression in *Xenopus tropicalis* embryos and isolated gut tubes (D,H) at the indicated stages (st), anterior leftwards, dorsal upwards. Stage 15 embryos (A,E) are bisected and stage 33 embryos (C'-C''',G'-G''') are sectioned to show the endoderm (outlined in yellow in A,E). fg, foregut; hg, hindgut; de, dorsal endoderm; pe, posterior endoderm; e, esophagus; l/t, presumptive lung and trachea; lv, liver; lpm, lateral plate mesoderm; k, kidney; s, stomach. (I-N'') *surfactant protein c* (*sftpc*) (I-L) and *nkx2.1* (M,N'') expression in embryos injected on one side with *Osr1/Osr2*-MOs (10 ng each) or negative control mutated oligos MM-MO (20 ng) shows lung agenesis on the *Osr1/Osr2*-depleted side. (M'-N'') Sections. Numbers of embryos with the shown phenotype are indicated. Dorsal view of an isolated gut tube (K,L) shows the lung bud absent on the injected side (red arrow).

in both *Xenopus tropicalis* and *Xenopus laevis*, whereas control mismatch-MOs (MM-MO) with 5/25 nucleotides altered had no effect. We conclude that *Osr1* and *Osr2* are redundantly required for lung formation.

The lung bud agenesis could be due to a failure of respiratory specification or impaired lung bud outgrowth after specification. To determine which was the case, we injected *Osr1/Osr2*-MOs into both sides of the embryo to target the entire foregut (Fig. 2) and examined the expression of *nkx2.1*, the earliest marker of respiratory progenitors, which is first detectable by in situ hybridization in *Xenopus* embryos at stage 35 (Small et al., 2000). In over 90% of the *Osr1/Osr2*-depleted embryos, *nkx2.1* transcripts were undetectable (Fig. 2A,B). Confocal immunostaining showed that in stage 35/36 controls, the foregut epithelium is patterned into a dorsal region that upregulates Sox2 and a ventral *Nkx2.1*⁺ region with nascent lung buds (Fig. 2D,D'). By contrast, the *Osr1/Osr2*-MO-depleted foreguts had no detectable *Nkx2.1*⁺ cells and only a scattering of cells expressing a low level of Sox2 (Fig. 2E,E'). Analysis of cell proliferation and cell death at this stage revealed no differences between control and *Osr1/Osr2*-MO embryos (data not shown). We conclude that respiratory fate is not specified in the absence of *Osr1/Osr2*.

By stage 42, the MM-MO-injected control lung buds express *sftpc* and the Sox2⁺ esophagus and stomach are clearly distinct from the *Nkx2.1*⁺ trachea and lung buds (Fig. 2G,J,J'). By contrast, *Osr1/Osr2*-depleted embryos exhibited complete respiratory agenesis, with a single foregut tube and no detectable *sftpc* and no *Nkx2.1*⁺ trachea or lung buds (Fig. 2H,K,K'). The stomach of *Osr1/Osr2*-MO embryos was also severely hypoplastic with reduced Sox2 levels. Extensive apoptosis and reduced proliferation was detected in both the epithelium and lpm of the *Osr1/Osr2*-depleted stage 42 foregut (supplementary material Fig. S3; data not shown). To demonstrate the MO phenotype was specifically due to loss of *Osr* function, we co-injected the *Osr1/Osr2*-MOs with

mRNA encoding an inducible form of mouse *Osr1* (GR-m*Osr1*), which lacks the morpholino target sequence. Addition of dexamethasone (dex) to activate the GR-m*Osr1* at stage 20 completely rescued all aspects of the *Osr1/Osr2* phenotype (Fig. 2; supplementary material Fig. S3).

We conclude that *Osr1/Osr2* are required to specify respiratory fate in the developing foregut and for tracheal-esophageal separation. The remnant foregut appears to be blocked in an immature progenitor state, it does not adopt another foregut lineage (supplementary material Fig. S2) and eventually undergoes programmed cell death.

***Osr1/Osr2* are required for the expression of *wnt2b* and *raldh2* in the lateral plate mesoderm**

The pathways regulating respiratory development in *Xenopus* are largely unknown; therefore, we examined the expression of candidate genes required for pulmonary development in mice. We focused on stages 34-35 when *nkx2.1*⁺ progenitors can first be detected and used unilateral *Osr1/Osr2*-MO injections because the uninjected side serves as an internal control that allows the identification of where respiratory tissue should normally form. Consistent with the results of Fig. 2, *Osr1/Osr2*-depletion resulted in a loss of *nkx2.1* transcripts and a reduction of *sox2* (Fig. 3D-F), and their expression was rescued by GR-m*Osr1* (supplementary material Fig. S3). By contrast, other epithelial markers including *shh* and *foxa1* (Fig. 3G-I; data not shown) were expressed normally, suggesting that endoderm identity was maintained in *Osr1/Osr2*-MO embryos.

We next examined the expression of signaling molecules in the lpm. We found that *wnt2b*, one of the earliest known inducers of respiratory fate in mouse, was absent on the *Osr1/Osr2*-MO injected side (Fig. 3J-L; supplementary material Fig. S3), as was *raldh2* (Fig. 3M-O), which has been shown to regulate RA-mediated lung bud growth. The specific FGF ligands that induce

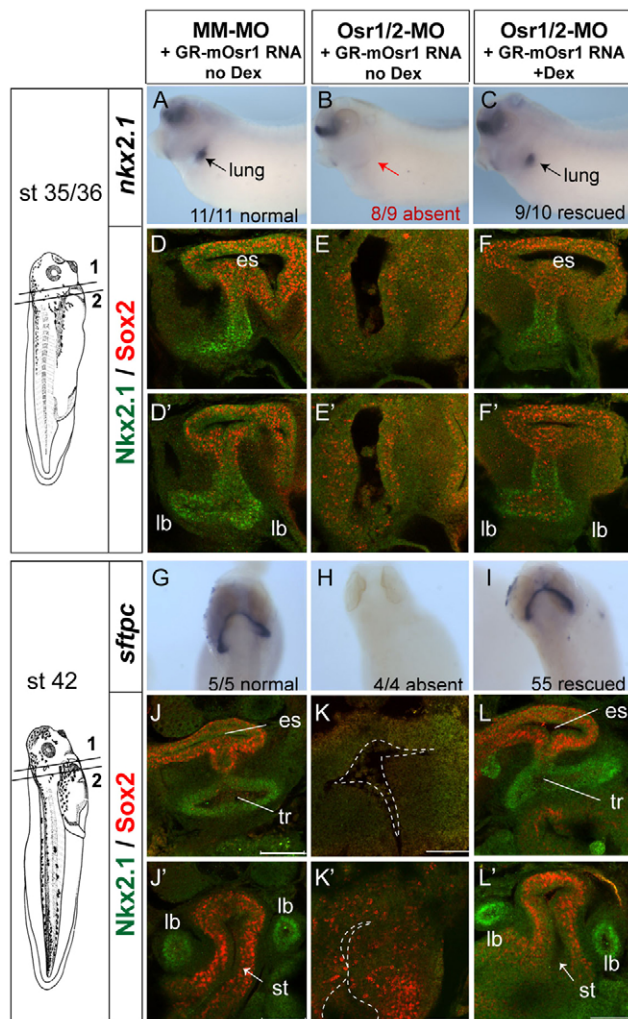


Fig. 2. Osr1/Osr2 are required for respiratory specification and tracheal-esophageal separation. (A-L') Embryos were co-injected with control MM-MOs or Osr1/Osr2-MOs and GR-mOsr1 RNA (250 pg) in both dorsal-vegetal blastomeres at the eight-cell stage (bilateral injection) and then cultured \pm dexamethasone from stage 20 onwards. (A-C, G-I) Analysis by in situ hybridization of *nkx2.1* at stage 34/35 (A-C) and *sftpc* at stage 42 (G-I). (D-F', J-L') Confocal immunofluorescence of Nkx2.1 (green) and Sox2 (red) at stage 35/36 (D-F) and stage 42 (J-L). es, esophagus; tr, trachea; lb, lung buds; st, stomach. Scale bars: 100 μ m.

lung fate during foregut patterning in mice are unknown and it is thought that multiple redundant ligands may be involved. Candidates include *fgf7* and *fgf10*, but these were unaffected in Osr1/Osr2-depleted embryos at stage 35, as were the mesenchymal transcription factors *foxf1*, *gli2* and *gli3* (Fig. 3P-R; supplementary material Fig. S4; data not shown). These data suggest that loss of respiratory cell fate in Osr1/Osr2-depleted embryos might be due to a loss of Wnt and RA signaling from the lpm.

Osr1/Osr2 regulate foregut development in a molecular pathway with FGF, Wnt and RA

Our analysis suggested that Osr1 and Osr2 might regulate foregut development downstream of FGF signaling but upstream of Wnt and RA signaling. To test this hypothesis, we inhibited

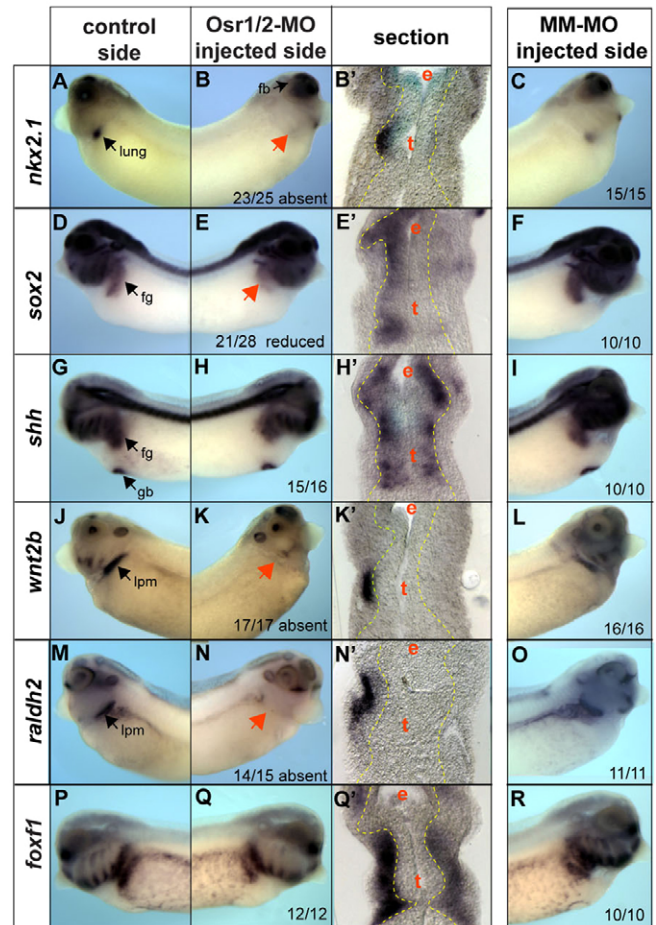


Fig. 3. Osr1/Osr2 are required for *wnt2b* and *raldh2* expression in the lpm. (A-X) Analysis of stage 34/35 unilateral-injected embryos shows that Osr1/Osr2 are required for expression of *nkx2.1* (A-C) and *sox2* (D-F) in the epithelium, whereas *shh* (G-I) is unaffected. In the lpm, *wnt2b* (J-L) and *raldh2* (M-O) are absent, while *foxf1* is unaffected (P-R). Black arrows indicate normal expression on the control side and red arrows indicate lack of expression on the Osr1/Osr2-MO-injected side (right). Endoderm outlined by a yellow line. Numbers of embryos with the shown phenotype are indicated. fg, foregut; hg, hindgut; de, dorsal endoderm; pe, posterior endoderm; e, esophagus; lt, presumptive lung and trachea; lv, liver; lpm, lateral plate mesoderm; k, kidney; s, stomach.

the FGF, Wnt and RA pathways, and asked to what extent this could either regulate *osr* expression or phenocopy Osr1/Osr2 loss of function. Evidence from mouse suggests that multiple redundant FGF/Wnt ligands and RA receptors act over a period of 1-2 days to regulate early lung development. To overcome this complication, we used cell-soluble small molecules to inhibit the FGF, Wnt and RA pathways. We cultured embryos from stages 25-35 because this is after early mesendoderm patterning (McLin et al., 2007; Deimling and Drysdale, 2009; Deimling and Drysdale, 2011), but prior to lung specification. This is also when *osr1* and *osr2* are first expressed in the foregut and previous studies have shown that during this time mesodermal signals are required for regional specification of the endoderm (Horb and Slack, 2001).

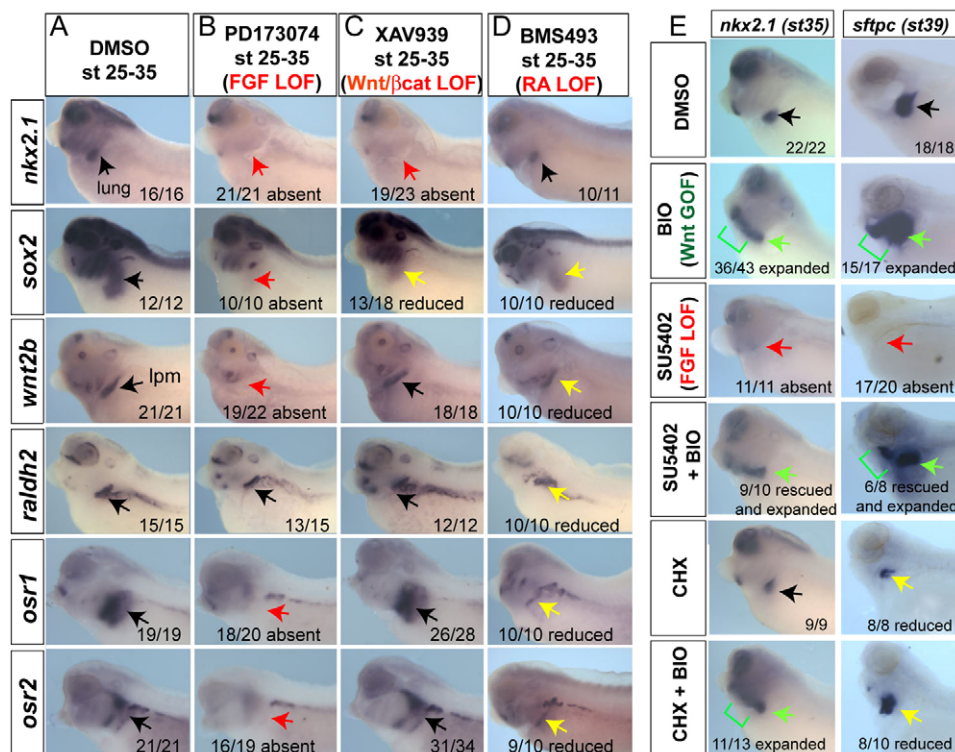


Fig. 4. FGF, Wnt/β-catenin and RA signaling regulate *Xenopus* lung development in a pathway with *Osr1/Osr2*. (A–D) Embryos were treated from stages 25–35 with DMSO vehicle (A), 100 μM PD173074 (B), 50 μM XAV939 (C) or 10 μM BMS493 (D), and assayed at stage 35 with the indicated probes. (E). Embryos were treated between stages 28 and 35 with vehicle (DMSO), 10 μM BIO, 80 μM SU5402, a combination of 80 μM SU5402 + 10 μM BIO, 10 μg/ml cycloheximide (CHX) or CHX + 10 μM BIO, and analyzed for *nkx2.1* at stage 35 and *sftpc* at stage 40. Black arrows indicate normal expression, red arrows indicate absent expression, yellow arrows indicate reduced expression and green arrows indicate expanded or rescued expression.

Inhibition of the FGF receptor kinase with PD173074 (or SU5402) (Beenken and Mohammadi, 2009) abolished *nkx2.1* expression at stage 35 and embryos completely lacked lung buds at stage 40 (Fig. 4A,B; data not shown). In addition, these embryos failed to express *osr1*, *osr2*, *sox2* and *wnt2b* in the foregut region (Fig. 4B). This indicates that FGF signaling between stages 25 and 35 is required for *Xenopus* respiratory specification, similar to recent findings (Wang et al., 2011) and supports a model in which FGF acts upstream of *Osr1/Osr2* and Wnt. Moreover, these data indicate that FGF signaling impacts both the presumptive pulmonary epithelium as well as the pulmonary-inducing lpm.

Inhibition of canonical Wnt signaling by XAV939, which promotes β-catenin degradation (Huang et al., 2009; Karner et al., 2010), resulted in loss of *nkx2.1* and lung agenesis but had no impact on *wnt2b*, *raldh2*, *osr1* or *osr2* expression (Fig. 4C). To confirm the temporal requirement of Wnt/β-catenin signaling in *Xenopus* lung specification, we injected RNA encoding a hormone-inducible dominant-negative Tcf3 (GR-dnTcf3) construct, which represses β-catenin/Tcf target genes when activated by dex (Molenaar et al., 1996; McLin et al., 2007). Activation of GR-dnTcf3 from stages 28–35 resulted in a loss of *nkx2.1* but had no impact on *osr1/osr2* expression (supplementary material Fig. S5A). Finally, we tested the possibility that endogenous Wnt2 and Wnt2b were redundantly required for lung specification in *Xenopus* as they are in mice. In situ hybridization showed that *wnt2* was weakly expressed in a strip of lpm (Garriock et al., 2007), similar to *wnt2b*; furthermore, neither *wnt2* nor *wnt2b* was expressed in *Osr1/Osr2*-depleted embryos (supplementary material Fig. S5B). As predicted, co-injection of Wnt2-MO and Wnt2b-MOs resulted in a loss of *nkx2.1* at stage 35 and lung bud agenesis at stage 42, whereas liver, thyroid, pancreas and duodenum appeared unaffected (supplementary material Fig. 5C). Knockdown of either Wnt2 or Wnt2b alone did not inhibit *nkx2.1* induction (data not shown). Consistent with XAV939-treated or dNXTCF3-injected embryos,

the expression of *osr1/osr2* was unchanged in Wnt2/Wnt2b-MO embryos. Together, these results suggest that *Osr1/Osr2* act upstream of Wnt2/Wnt2b-mediated lung specification in *Xenopus*.

RA signaling is well known to regulate lung bud development in mice (Desai et al., 2004) and recent data suggest that RA might facilitate Wnt-mediated specification by repressing expression of the Wnt-antagonist Dkk1 (Chen et al., 2010); these data raise the possibility that the lack of *raldh2* might also contribute to the *Osr1/Osr2*-MO phenotype. To test the role of RA during *Xenopus* lung development, we treated embryos with the pan-retinoic acid receptor antagonist BMS493 (Desai et al., 2004) during stages 25–35. Specification of *nkx2.1*+ lung progenitors was unperturbed in these embryos (Fig. 4D), even though the Wnt-antagonist *dkk1* was upregulated throughout the embryo (supplementary material Fig. S6A), similar to *raldh2*-deficient mouse embryos (Chen et al., 2010). Inhibition of RA signaling did, however, reduce *osr1*, *osr2*, *sox2*, *wnt2b* and *raldh2* levels (Fig. 4D), and retarded lung bud growth at later stages (supplementary material Fig. S6B). Therefore, although RA signaling between stages 25 and 35 contributes to maintaining *osr1*, *osr2* and *wnt2b* levels, it is not absolutely required for their expression and is dispensable for lung specification. However, RA signaling is clearly required for later lung bud growth, and *Osr1/Osr2* might contribute to this through regulation of *raldh2* expression.

These data demonstrate that *Xenopus* respiratory development is regulated by FGF, Wnt and RA signaling in a molecular pathway remarkably conserved with mammals, and that *Osr* factors are novel players in this process.

Wnt/β-catenin signaling is sufficient to specify respiratory fate downstream of FGF

Our results suggest, for the first time in any species, an epistatic relationship where early FGF signaling acts upstream of canonical Wnt/β-catenin to control respiratory specification. To test this, we

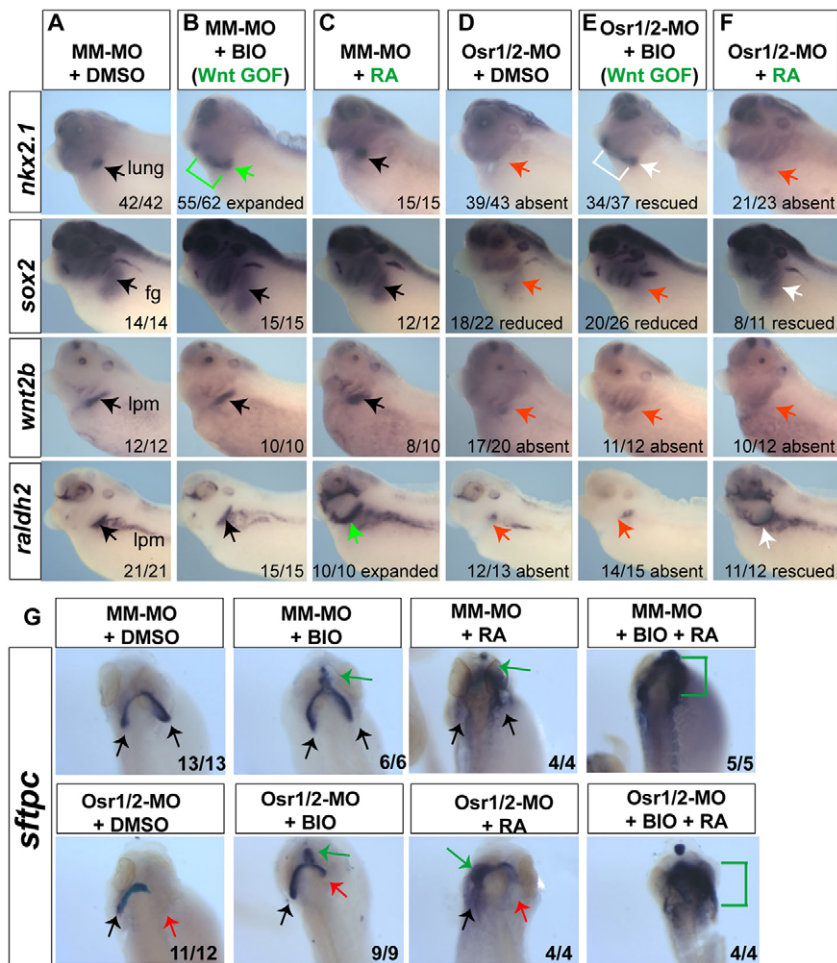


Fig. 5. Osr1/Osr2 act upstream of Wnt-mediated pulmonary specification and RA-regulated lung bud growth. (A-G) Osr1/Osr2-MO- and MM-MO-injected embryos were treated at stages 25-35 with DMSO vehicle, 10 μ M BIO or 1 μ M all-trans-retinoic acid (RA), and analyzed for *nkx2.1*, *sox2*, *wnt2b* and *raldh2* at stage 35 (A-F) and *sftpc* at stage 42 (dorsal view) (G). Black arrows indicate normal expression, red arrows show absent expression, green arrows and lines indicate expanded expression and white arrows indicate rescued expression.

determined whether activation of Wnt/ β -catenin signaling could rescue lung specification in embryos where FGF signaling was inhibited. Treatment of control embryos at stage 28-35 with the small molecule BIO, which inhibits Gsk3 and causes stabilization of β -catenin (Sato et al., 2004), was sufficient to expand *nkx2.1* and *sftpc* expression throughout the pharyngeal region (Fig. 4E), without affecting *osr1*, *osr2*, *wnt2b*, *raldh2* or *sox2* (data not shown). This confirms that that activation of the canonical Wnt/ β -catenin pathway is sufficient to induce pulmonary fate in *Xenopus* similar to the conditional activation of β -catenin in mouse embryos (Goss et al., 2009; Harris-Johnson et al., 2009). Importantly BIO treatment also rescued (and expanded) *nkx2.1* and *sftpc* expression in embryos where FGF receptor signaling was blocked by PD173074 or SU5402 (Fig. 4E; data not shown), indicating that Wnt/ β -catenin is sufficient to control *Xenopus* lung specification downstream of FGF. We note that although *sftpc* was expressed in SU5402+BIO embryos, there were no distinct lung buds, consistent with mouse studies demonstrating that FGF10 is required for bud formation after specification (Sekine et al., 1999). BIO was also able to expand *nkx2.1* expression in the presence of the translation inhibitor cycloheximide (Fig. 4E), suggesting that *nkx2.1* is a direct transcriptional target of β -catenin signaling in *Xenopus*. Although we cannot exclude the possibility that FGF can act in parallel with Wnt to induce *nkx2.1*, these data, along with the observation that FGF is required for *osr1/osr2* and *wnt2b* expression, support an FGF→Osr1/Osr2→Wnt2/Wnt2b→Nkx2.1 pathway that specifies respiratory fate.

Temporal activation of Wnt/ β -catenin rescues lung specification but not lung bud growth in Osr1/Osr2-depleted embryos

The observation that Osr1/Osr2-depleted embryos fail to express *wnt2b* or *raldh2* suggested that the lung agenesis might be due to loss of Wnt and/or RA signaling. We tested whether activation of Wnt/ β -catenin by BIO or addition of exogenous RA could rescue lung development in Osr1/Osr2-MO unilateral-injected embryos. BIO treatment from stages 25-35 was sufficient to rescue *nkx2.1* in Osr1/Osr2-MO embryos (Fig. 5), but did not rescue *sox2* nor restore *wnt2b* or *raldh2* expression in the mesoderm. Interestingly, although BIO treatment resulted in ectopic *sftpc* expression in the rostral foregut at stage 42 (Fig. 5G; green arrow), BIO only partially rescued the absent lung bud on the Osr1/Osr2-depleted side, which remained hypoplastic and failed to grow (Fig. 5G). This suggests that Osr1/Osr2 might also have a later role in lung bud growth that is independent of Wnt activation, which based on the inhibitor experiments (supplementary material Fig. S6B) is likely to be mediated by RA.

RA treatment from stages 25-35 was unable to restore *nkx2.1* or *wnt2b* in Osr1/Osr2-depleted embryos (Fig. 5F). However, exogenous RA did rescue *raldh2* (a known RA-target) as well as *sox2*. At stage 42, RA treatment caused ectopic *sftpc* expression throughout the anterior foregut and head of both MM-MO and Osr1/Osr2-MO embryos (Fig. 5G). Despite this, ectopic *sftpc* expression RA did not rescue outgrowth of a discrete lung bud on the Osr1/Osr2-MO injected side. We next tested whether a

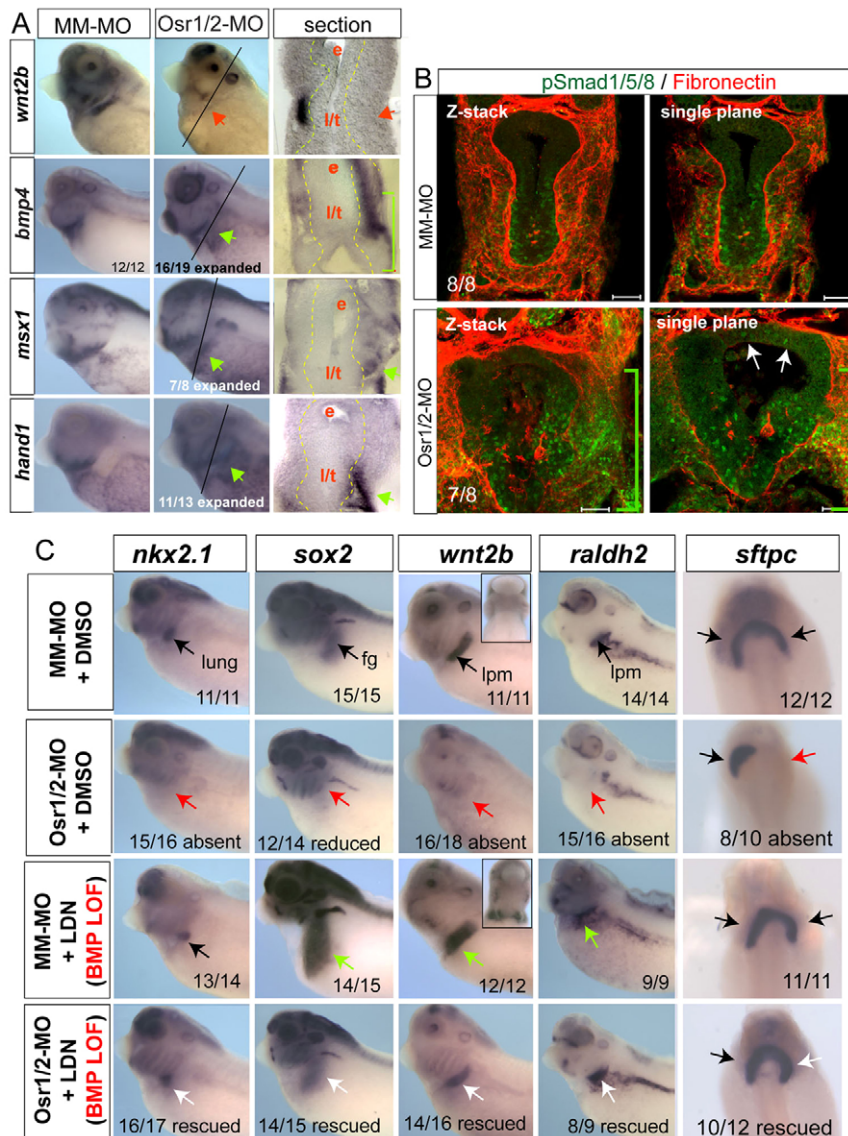


Fig. 6. Osr1/Osr2 promotes lung specification and growth by restricting BMP signaling. (A) Analysis of Osr1/Osr2-MO and control MM-MO stage 35 embryos indicated that expression of *bmp4* and BMP-target genes *msx1* and *hand1* are expanded into the 'wnt2b-domain' of the lpm on the Osr1/Osr2-depleted side. Black arrows indicate normal expression, red arrows indicate absent expression and green arrows indicate enhanced expression. (B) Confocal immunostaining of stage 35 embryos for phospho-Smad1/5/8 (green) and fibronectin (red). Scale bars: 50 μ m. White arrows in B indicate ectopic pSMAD1 expression in dorsal foregut endoderm. (C) Analysis of *nkx2.1*, *sox2*, *wnt2b* and *raldh2* at stage 35 or *sftpc* at stage 41 in embryos injected with Osr1/Osr2-MOs or MM-MOs and treated at stages 25-35 with either DMSO (vehicle control) or 10 μ M LDN193189. Insets show ventral views of *wnt2b* expression. For analysis of *sftpc* expression at stage 41, embryos were removed from the LDN193189 at stage 35 and further cultured in DMSO vehicle until stage 41. Black arrows indicate normal expression, red arrows indicate absent expression, green arrows indicate expanded expression and white arrows indicate rescued expression.

combination of BIO and RA could rescue both specification and growth. We found that BIO and RA together resulted in a massive upregulation of ectopic *sftpc* expression throughout the foregut and head tissue; however, we never detected a discrete lung bud on the Osr1/Osr2-MO injected side.

Overall, these results suggest that Osr1/Osr2 regulate both Wnt-mediated lung specification and RA-mediated lung growth, via regulating expression of *wnt2/wnt2b* and *raldh2*.

BMP/Smad1 signaling is elevated Osr1/Osr2-depleted embryos

Osr factors are generally thought to act as transcriptional repressors (Goldstein et al., 2005; Tena et al., 2007) so Osr1/Osr2 depletion (loss of a repressor) probably caused the loss of *wnt2b* and *raldh2* transcripts indirectly. A candidate Osr target came from recent mouse studies where *Osr2*^{-/-} embryos exhibited ectopic *bmp4* expression in the developing tooth mesenchyme (Zhang et al., 2009). We found that, in Osr1/Osr2-MO-injected embryos, *bmp4* transcripts were ectopically upregulated in the foregut lpm, which should normally express *wnt2/wnt2b* (Fig. 6A), and the ectopic *bmp4* could be abolished by co-injection of GR-mOsr1 RNA

(supplementary material Fig. S3). Phospho-Smad1/5/8 (pSmad1) immunostaining and analysis for the BMP-target genes *msx1* and *hand1* confirmed that BMP signaling was upregulated in both the endoderm and lpm in Osr1/Osr2-MO unilateral- (Fig. 6A,B) and bilateral-injected embryos (supplementary material Fig. S7A). Sox2 is severely downregulated in Osr1/Osr2-MO-injected embryos (Figs 2, 3), and numerous studies have shown BMP signaling to be inhibitory to Sox2 expression (Matsushita, 2008; Domyan et al., 2011; Green et al., 2011). These data led us to hypothesize that, during normal development, Osr1/Osr2 negatively regulate *bmp4* expression levels and that BMP signaling in turn restricts *wnt2b* and *raldh2* expression to a thin strip of lpm adjacent to the presumptive pulmonary epithelium. However, in Osr1/Osr2 morphants, elevated BMP signaling would inhibit *wnt2b* expression, resulting in a failure of lung induction. Consistent with this hypothesis, during stages 25-37, *osr1*, *osr2* and *bmp4* have largely exclusive expression patterns, although there is some overlap in the lpm (Fig. 6; supplementary material Fig. S1). Most importantly, *bmp4* is clearly not robustly expressed in the *wnt2/wnt2b*-positive lpm prior to and during lung specification stages.

Osr1/Osr2 promotes lung specification by restricting BMP signaling

To test the hypothesis that BMP signaling negatively regulates *wnt2b* expression and lung specification, we injected an inducible GR-Smad1 construct (Kim and Han, 2011) into one side of four-cell stage embryos. GR-Smad1 activation by addition of dex between stages 25–35 resulted in a severe reduction of *wnt2b* and a loss of *nkx2.1* and *sox2* on the injected side, with no detectable increase in cell death (supplementary material Fig. S7B,C). Therefore, high levels of BMP/Smad1 in the foregut during stages 25–35 are inhibitory to lung specification.

To test whether the Osr1/Osr2-MO phenotype was due to elevated BMP signaling, we treated control and Osr1/Osr2-MO-injected embryos with the BMP type I receptor antagonist LDN193189 (Yu et al., 2008) from stages 25 to 35. Consistent with BMP signaling normally restricting *wnt2b* and *raldh2* expression, LDN193189 treatment of control embryos caused an expansion of the *wnt2b*, *sox2* and *raldh2* expression domains (Fig. 6C, green arrows). However, LDN193189 treatment did not increase *nkx2.1* expression, indicating that suppression of BMP signaling is not sufficient to induce lung fate. Importantly, LDN193189 treatment completely rescued expression of *wnt2b*, *raldh2*, *sox2* and specification of *nkx2.1*-expressing lung progenitors in Osr1/Osr2 morphants (Fig. 6D, white arrows) and abolished the ectopic pSMAD1 in the foregut (supplementary material Fig. S7D). When rescued embryos were taken out of the BMPR inhibitor at stage 35 and allowed to develop until stage 42, *sfpc* expression and lung bud growth was also rescued. If embryos were left in the BMPR inhibitor after lung specification between stages 35 and 42, we observed tracheal degeneration and hypoplastic lung buds (supplementary material Fig. S8), consistent with mouse studies demonstrating a role for BMP in maintenance of trachea fate and lung bud morphogenesis (Domany et al., 2011; Lie et al., 2008). These data indicate that the failure of respiratory specification in Osr1/Osr2-depleted embryos is primarily due to elevated BMP signaling. We conclude that Osr1 and Osr2 are required to restrain BMP/pSmad1 activity in order to allow expression of *wnt2b*, *raldh2* and *nkx2.1*, and thus promote lung specification and development.

Osr1/Osr2 can repress *bmp4* transcription

To test whether overexpression of Osr1/Osr2 could repress endogenous *bmp4* and induce ectopic *nkx2.1*+ respiratory tissue, we injected a high dose of GR-mOsr1 RNA (750 pg) and induced the construct at stage 20, after gastrulation and early AP patterning are complete. This resulted in a dramatic downregulation of endogenous *bmp4*, *msx1* and *hand1* transcripts in the lpm and pharyngeal mesenchyme at stage 35 (Fig. 7A). The induced GR-mOsr1 also resulted in upregulated/ectopic *wnt2b*, similar to LDN treatment and consistent with BMP signaling negatively regulating the *wnt2b* expression domain. However, similar to LDN-treated embryos, GR-mOsr1 was unable to expand *nkx2.1* expression (Fig. 7A).

An analysis of the *Xenopus laevis* *bmp4* promoter DNA sequence revealed two putative Osr-binding sites (Meng et al., 2005) clustered at approximately –2kb from the transcription start site (Fig. 7B). To test whether Osr1/Osr2 might repress the *bmp4* promoter, we injected a –2123bp *bmp4:luciferase* reporter construct that has previously been shown to drive *bmp4* expression (Schuler-Metz et al., 2000) and tested the ability of Osr factors from *Xenopus*, mouse and *Drosophila* to repress the reporter. Luciferase assays at late gastrula stage (st12) indicated that the

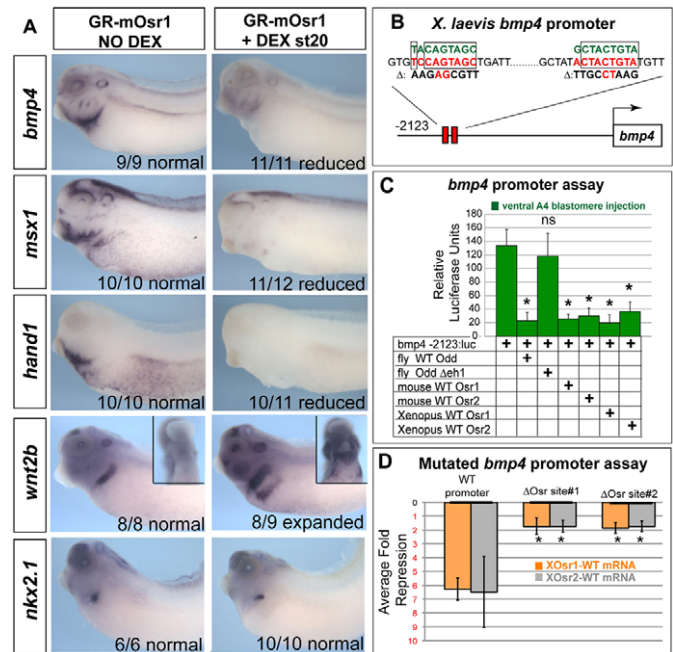


Fig. 7. Osr1/Osr2 can repress *bmp4* expression. (A) GR-mOsr1 RNA (750 pg) was injected into both dorsal-ventral cells at the eight-cell stage and embryos were dexamethasone treated at stage 20 to induce the construct and analyzed at stage 34/35 for expression of the indicated genes. Insets show ventral views of *wnt2b* expression. (B) The *X. laevis* *bmp4* promoter construct, showing two putative odd-skipped-binding sites. Green sequence indicates consensus odd skipped-binding site, red sequence is the exact *bmp4* promoter sequence, and mutations to these sites are shown below indicated by 'Δ' and in black. (C) RNA (750 pg) encoding *Xenopus*, mouse or *Drosophila* Osr factors was co-injected with the –2123 *bmp4:luciferase* reporter at the 32-cell stage and luciferase activity was assayed at stage 12. Average relative activity \pm s.d. (D) *Xenopus* Osr1 or Osr2 RNA (750 pg) was co-injected with the wild-type *bmp4* reporter or the mutated reporter constructs indicated in B, and the resulting average fold repression \pm s.d., of the luciferase reporter from three independent experiments was determined. * $P < 0.005$.

–2123 *bmp4:luc* construct was robustly expressed in animal caps and that this expression was dramatically downregulated by co-injection of RNA encoding *Xenopus* or mouse Osr1 and Osr2, as well as by the *Drosophila* ortholog Odd (Fig. 7C). By contrast, the *Drosophila* Odd construct lacking the Groucho interaction domain (OddΔeh1) failed to suppress the reporter (Fig. 7C), in agreement with studies showing Osr factors require Groucho co-factors in order to repress their targets (Goldstein et al., 2005). Finally, mutation of either the Osr-binding site sequences dramatically impaired the ability of *Xenopus* Osr1 or Osr2 to repress the *bmp4* reporter (Fig. 7D). These data are consistent with the conclusion that Osr1/Osr2 normally repress *bmp4* transcription in the *wnt2/wnt2b*-expressing lpm during respiratory development.

DISCUSSION

We have identified a novel foregut function for the zinc-finger transcriptional repressors Osr1 and Osr2, and show that they are key components of an epithelial-mesenchymal signaling cascade linking foregut patterning, respiratory specification and lung bud growth. Our data support a model (Fig. 8) in which foregut

patterning by early FGF signaling is required to promote *osr1* and *osr2* expression in the foregut endoderm and surrounding lpm prior to lung specification. *Osr1* and *Osr2* are redundantly required for the specification of *Nkx2.1*⁺ respiratory progenitors and they act primarily by limiting *bmp4* expression to the pharyngeal lpm just anterior to the presumptive lung. Our data indicate that in *Osr1/Osr2* morphants elevated BMP/pSmad1 activity inhibits *wnt2b*, *raldh2*, *sox2* and *nkx2.1*, and results in a failure of respiratory specification with trachea and lung agenesis. Downstream of *Osr1/Osr2*, canonical Wnt2/Wnt2b signaling is necessary and sufficient to induce pulmonary fate. Finally, our data suggest that *Osr1* and/or *Osr2* also act after specification to promote RA-regulated lung bud growth by maintaining *raldh2*.

A novel feature of our model is the importance of transcriptional repression in early lung development. *Osr1* and *Osr2* are known to interact with groucho co-repressors (Goldstein et al., 2005; Tena et al., 2007) and although future transgenic and ChIP studies will be needed to confirm that *Osr1/Osr2* directly repress *bmp4* transcription in the lpm, our data support this possibility. It is possible that BMP signaling indirectly suppresses *wnt2/wnt2b* expression through transcriptional repressors such as *Msx1*, which is a direct BMP target (Maeda et al., 1997) and is upregulated in *Osr1/Osr2* morphants.

It is important to point out that our model focuses on the specification of respiratory fate that we have tested and is not meant to show all possible interactions. For example, the exact FGF ligands involved are unknown and we cannot exclude the possibility that, in addition to acting via *Osr1/Osr2*, FGF signaling also directly contributes to promoting *nkx2.1* or *wnt2b* expression. Although our data suggest that *Osr1/Osr2* act in the mesoderm, they are also expressed in the foregut endoderm and might impact the epithelial response to mesenchymal signals. Preliminary studies preferentially targeting the *Osr1/Osr2*-MOs to either the endoderm or mesoderm support a function in both tissues, but the mesoderm function of *Osr1/Osr2* is essential (S.A.R., unpublished).

FGF, Wnt and BMP signals have multiple roles in later lung bud growth, differentiation and morphogenesis (Cardoso and Lu, 2006; Maeda et al., 2007; Morrissey and Hogan, 2010), and *Osr1/Osr2* might similarly have multiple temporally distinct roles. For example, our data suggest that RA may function in a positive regulatory loop acting both upstream and downstream of *Osr1/Osr2*. Early RA signaling between stages 25–35 is required for robust *osr1* and *osr2* expression, whereas *Osr1/Osr2* are essential for *raldh2* expression, and RA signaling is required for later lung bud outgrowth. While our study was in review, Drysdale and colleagues demonstrated that RA inhibition at stage 20 in *Xenopus* resulted in a more dramatic loss of lung fate than we observed with RA inhibition at stage 25 (Wang et al., 2011). Further studies could define temporally distinct RA-*Osr* interactions. Similarly FGFs have multiple roles in lung bud growth after specification, which we have not addressed. For example FGF and WNT act in a feedback loop that controls growth of both lung epithelium and mesenchyme with *Wnt2/Wnt2b* being required for FGF10 expression (Yin et al., 2008; Goss et al., 2009; Goss et al., 2011). Future temporal studies are needed to determine whether *Osr* factors are involved in this crosstalk after specification.

Finally, there are almost certainly other players not yet incorporated into the model. For example, the winged-helix DNA-binding factor *Rfx6* is required for *Xenopus* lung bud development, but how this fits into the known paradigms is unknown (Pearl et al., 2011). *Shh* signaling is also crucial in early mouse foregut development (Litingtung et al., 1998) and

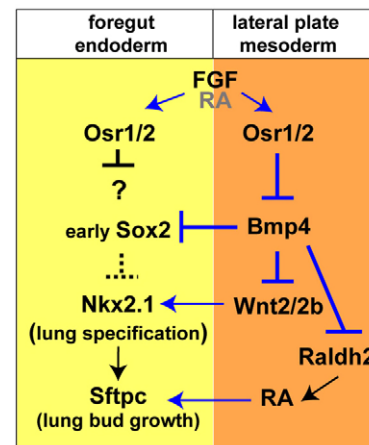


Fig. 8. Model of the molecular pathway regulating *Xenopus* lung development. *Osr1/Osr2* are key components of a molecular pathway regulating respiratory specification in *Xenopus*. Blue lines indicate relationships tested in this study and black lines indicate relationships predicted from published mouse studies.

Gli2^{−/−}; *Gli3*^{−/−} double mutants have agenesis of the lungs, trachea and esophagus (Motoyama et al., 1998) similar to depletion of *Osr1/Osr2*. We found that *shh*, *gli2* and *gli3* expression were unchanged in *Osr1/Osr2* morphants, suggesting that perhaps *Shh* also acts up stream of *Osr*. Consistent with this possibility, Hedgehog signaling is required for *Osr2* expression in the embryonic mouse palate (Lan and Jiang, 2009), and inhibition of hedgehog signaling in *Xenopus* downregulates *osr1/osr2* expression (S.A.R., unpublished).

A number of lines of evidence suggest that the model we propose is conserved in mammals: (1) key molecules in lung development (Hyatt et al., 2007; Yin et al., 2010) and the roles of FGF, Wnt and RA are remarkably conserved between *Xenopus* and mice; (2) mouse *Osr1* and *Osr2* are expressed at the right time and place to have a similar role. *Osr1* is expressed in the lpm at E7.5 and in the foregut epithelium and surrounding splanchnic mesoderm at E9.5 (So and Danielian, 1999; Lan et al., 2001; Wang et al., 2005; Grieshammer et al., 2008). *Osr2* is also weakly expressed in the foregut beginning E9.5 and later in the lung bud mesenchyme (Lan et al., 2001; Lan et al., 2004; Lan et al., 2007); (3) although *Osr1*^{−/−} and *Osr2*^{−/−} mutant embryos have lungs, the *Osr1* mutants do exhibit lung hypoplasia (Rulang Jiang, personal communication), which has not been characterized, suggesting that *Osr1* and *Osr2* might redundantly regulate lung specification in mice. Consistent with this possibility, murine *Osr1* and *Osr2* are partially redundant in joint formation where they regulate *Wnt4* and *Wnt9b* expression (Gao et al., 2011), and *Osr2* is required to negatively regulate *Bmp4* and *Msx1* during tooth development (Zhang et al., 2009). Furthermore, we have recently found that, in zebrafish, *Osr1/Osr2* also regulates *wnt2b* expression in lpm during fin development (Neto et al., 2012), suggesting that this regulatory cassette is employed in many tissues.

Our finding that FGF signaling is required for *Xenopus* lung specification in vivo is consistent with in vitro mouse embryonic foregut explants where exogenous FGF ligands induce pulmonary fate and FGF antagonists inhibit lung development (Serls et al., 2005). Importantly, we show that early FGF acts not only in the epithelium but also in the mesoderm and that FGF functions

upstream of *Osr1/Osr2* and Wnt-mediated lung specification. Our observation that β -catenin signaling is necessary and sufficient to induce *nkx2.1*⁺ lung progenitors in *Xenopus* is identical to reports in mice in which β -catenin is genetically deleted or activated (Goss et al., 2009; Harris-Johnson et al., 2009). Moreover, our data suggesting that *nkx2.1* is a direct transcriptional target of canonical Wnt signaling are consistent with a recent report that β -catenin/Tcf directly stimulated *Nkx2.1* transcription in human thyroid carcinoma cells (Gilbert-Sirieix et al., 2011).

An important novel finding in our study is the need for BMP repression to allow Wnt-mediated respiratory specification. Genetic studies in mice agree with this and show that BMP signaling is dispensable for lung specification, promotes later tracheal development and separation of the foregut tube (Eblaghie et al., 2006; Que et al., 2006; Li et al., 2008; Sun et al., 2008; Xu et al., 2011). During this process in mice, *Sox2* is initially expressed throughout the foregut epithelium and then around E9.5 BMP signaling suppresses *Sox2* and esophagus fate in the ventral foregut. Mutual repression between *Sox2* and *Nkx2.1* then resolves the two lineages (Que et al., 2006; Li et al., 2008; Domyan et al., 2011). Our data suggest that BMP has a similar role in *Xenopus*; BMP signaling was not required for *Xenopus* lung specification but is necessary to maintain later tracheal fate (supplementary material Fig. S8). Most importantly, we found that excessive BMP signaling in *Osr1/Osr2* morphants (or overexpression of *Smad1*) reduced *wnt2b*, *nkx2.1* and early pan-foregut *sox2* expression. Interestingly, we found that BMP inhibition or *Osr* overexpression could expand *wnt2b*; however, this was not sufficient to induce ectopic *nkx2.1*⁺ respiratory tissue. In both these conditions, we found that *Sox2* was also highly upregulated (S.A.R., unpublished), and this may explain why *nkx2.1* was unchanged, as *Sox2* is known to be inhibitory to *Nkx2.1* (Que et al., 2007; Domyan et al., 2011).

Finally, the results of this study may impact efforts to direct the differentiation of respiratory lineages from human stem cells. The best successes in making human tissue in vitro have come from recapitulating embryonic paradigms in culture (Spence and Wells, 2007; Green et al., 2011), which is an approach that requires a detailed understanding of the temporal-spatial dynamics of cell signaling during normal organogenesis. Our results suggest that co-culture of *Osr*-expressing mesenchymal cells and suppression of BMP in addition to Wnt at the right time may facilitate induction of pulmonary progenitors.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material available online at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.078220/-DC1>

References

- Beenken, A. and Mohammadi, M. (2009). The FGF family: biology, pathophysiology and therapy. *Nat. Rev. Drug Discov.* **8**, 235-253.
- Cardoso, W. V. and Lü, J. (2006). Regulation of early lung morphogenesis: questions, facts and controversies. *Development* **133**, 1611-1624.

- Chen, F., Desai, T. J., Qian, J., Niederreither, K., Lü, J. and Cardoso, W. V. (2007). Inhibition of Tgf beta signaling by endogenous retinoic acid is essential for primary lung bud induction. *Development* **134**, 2969-2979.
- Chen, F., Cao, Y., Qian, J., Shao, F., Niederreither, K. and Cardoso, W. V. (2010). A retinoic acid-dependent network in the foregut controls formation of the mouse lung primordium. *J. Clin. Invest.* **120**, 2040-2048.
- Deimling, S. J. and Drysdale, T. A. (2009). Retinoic acid regulates anterior-posterior patterning within the lateral plate mesoderm of *Xenopus*. *Mech. Dev.* **126**, 913-923.
- Deimling, S. J. and Drysdale, T. A. (2011). Fgf is required to regulate anterior-posterior patterning in the *Xenopus* lateral plate mesoderm. *Mech. Dev.* **128**, 327-341.
- Desai, T. J., Malpel, S., Flentke, G. R., Smith, S. M. and Cardoso, W. V. (2004). Retinoic acid selectively regulates Fgf10 expression and maintains cell identity in the prospective lung field of the developing foregut. *Dev. Biol.* **273**, 402-415.
- Domyan, E. T., Ferretti, E., Throckmorton, K., Mishina, Y., Nicolis, S. K. and Sun, X. (2011). Signaling through BMP receptors promotes respiratory identity in the foregut via repression of *Sox2*. *Development* **138**, 971-981.
- Eblaghie, M. C., Reedy, M., Oliver, T., Mishina, Y. and Hogan, B. L. (2006). Evidence that autocrine signaling through Bmpr1a regulates the proliferation, survival and morphogenetic behavior of distal lung epithelial cells. *Dev. Biol.* **291**, 67-82.
- Gao, Y., Lan, Y., Ovitt, C. E. and Jiang, R. (2009). Functional equivalence of the zinc finger transcription factors *Osr1* and *Osr2* in mouse development. *Dev. Biol.* **328**, 200-209.
- Gao, Y., Lan, Y., Liu, H. and Jiang, R. (2011). The zinc finger transcription factors *Osr1* and *Osr2* control synovial joint formation. *Dev. Biol.* **352**, 83-91.
- Garriock, R. J., Warkman, A. S., Meadows, S. M., D'Agostino, S. and Krieg, P. A. (2007). Census of vertebrate Wnt genes: isolation and developmental expression of *Xenopus* *Wnt2*, *Wnt3*, *Wnt9a*, *Wnt9b*, *Wnt10a*, and *Wnt16*. *Dev. Dyn.* **236**, 1249-1258.
- Gilbert-Sirieix, M., Makoukji, J., Kimura, S., Talbot, M., Caillou, B., Massaad, C. and Massaad-Massade, L. (2011). Wnt/ β -catenin signaling pathway is a direct enhancer of thyroid transcription factor-1 in human papillary thyroid carcinoma cells. *PLoS ONE* **6**, e22280.
- Goldstein, R. E., Cook, O., Dinur, T., Pisanté, A., Karandikar, U. C., Bidwai, A. and Paroush, Z. (2005). An eh1-like motif in odd-skipped mediates recruitment of Groucho and repression in vivo. *Mol. Cell. Biol.* **25**, 10711-10720.
- Goss, A. M., Tian, Y., Tsukiyama, T., Cohen, E. D., Zhou, D., Lu, M. M., Yamaguchi, T. P. and Morrissey, E. E. (2009). *Wnt2/2b* and β -catenin signaling are necessary and sufficient to specify lung progenitors in the foregut. *Dev. Cell* **17**, 290-298.
- Green, M. D., Chen, A., Nostro, M. C., d'Souza, S. L., Schaniel, C., Lemischka, I. R., Gouon-Evans, V., Keller, G. and Snoeck, H. W. (2011). Generation of anterior foregut endoderm from human embryonic and induced pluripotent stem cells. *Nat. Biotechnol.* **29**, 267-272.
- Green, R. B., Hatini, V., Johansen, K. A., Liu, X. J. and Lengyel, J. A. (2002). Drumstick is a zinc finger protein that antagonizes lines to control patterning and morphogenesis of the *Drosophila* hindgut. *Development* **129**, 3645-3656.
- Grieshammer, U., Agarwal, P. and Martin, G. R. (2008). A Cre transgene active in developing endodermal organs, heart, limb, and extra-ocular muscle. *Genesis* **46**, 69-73.
- Harris-Johnson, K. S., Domyan, E. T., Vezina, C. M. and Sun, X. (2009). β -Catenin promotes respiratory progenitor identity in mouse foregut. *Proc. Natl. Acad. Sci. USA* **106**, 16287-16292.
- Horb, M. E. and Slack, J. M. (2001). Endoderm specification and differentiation in *Xenopus* embryos. *Dev. Biol.* **236**, 330-343.
- Huang, S. M., Mishina, Y. M., Liu, S., Cheung, A., Stegmeier, F., Michaud, G. A., Charlat, O., Wiellette, E., Zhang, Y., Wiessner, S. et al. (2009). Tankyrase inhibition stabilizes axin and antagonizes Wnt signalling. *Nature* **461**, 614-620.
- Hyatt, B. A., Resnik, E. R., Johnson, N. S., Lohr, J. L. and Cornfield, D. N. (2007). Lung specific developmental expression of the *Xenopus laevis* surfactant protein C and B genes. *Gene Expr. Patterns* **7**, 8-14.
- James, R. G., Kamei, C. N., Wang, Q., Jiang, R. and Schultheiss, T. M. (2006). Odd-skipped related 1 is required for development of the metanephric kidney and regulates formation and differentiation of kidney precursor cells. *Development* **133**, 2995-3004.
- Johansen, K. A., Green, R. B., Iwaki, D. D., Hernandez, J. B. and Lengyel, J. A. (2003). The *Drm-Bowl-Lin* relief-of-repression hierarchy controls fore- and hindgut patterning and morphogenesis. *Mech. Dev.* **120**, 1139-1151.
- Karner, C. M., Merkel, C. E., Dodge, M., Ma, Z., Lu, J., Chen, C., Lum, L. and Carroll, T. J. (2010). Tankyrase is necessary for canonical Wnt signaling during kidney development. *Dev. Dyn.* **239**, 2014-2023.
- Khokha, M. K., Chung, C., Bustamante, E. L., Gaw, L. W., Trott, K. A., Yeh, J., Lim, N., Lin, J. C., Taverner, N., Amaya, E. et al. (2002). Techniques and probes for the study of *Xenopus tropicalis* development. *Dev. Dyn.* **225**, 499-510.

- Kim, H. and Han, J. K. (2011) Rab3d is required for *Xenopus* anterior neurulation by regulating Noggin secretion. *Dev. Dyn.* **240**, 1430-1439.
- Lan, Y. and Jiang, R. (2009). Sonic hedgehog signaling regulates reciprocal epithelial-mesenchymal interactions controlling palatal outgrowth. *Development* **136**, 1387-1396.
- Lan, Y., Kingsley, P. D., Cho, E. S. and Jiang, R. (2001). Osr2, a new mouse gene related to *Drosophila* odd-skipped, exhibits dynamic expression patterns during craniofacial, limb, and kidney development. *Mech. Dev.* **107**, 175-179.
- Lan, Y., Ovitt, C. E., Cho, E. S., Maltby, K. M., Wang, Q. and Jiang, R. (2004). Odd-skipped related 2 (Osr2) encodes a key intrinsic regulator of secondary palate growth and morphogenesis. *Development* **131**, 3207-3216.
- Lan, Y., Wang, Q., Ovitt, C. E. and Jiang, R. (2007). A unique mouse strain expressing Cre recombinase for tissue-specific analysis of gene function in palate and kidney development. *Genesis* **45**, 618-624.
- Lazzaro, D., Price, M., de Felice, M. and Di Lauro, R. (1991). The transcription factor TTF-1 is expressed at the onset of thyroid and lung morphogenesis and in restricted regions of the foetal brain. *Development* **113**, 1093-1104.
- Li, Y., Gordon, J., Manley, N. R., Litingtung, Y. and Chiang, C. (2008). Bmp4 is required for tracheal formation: a novel mouse model for tracheal agenesis. *Dev. Biol.* **322**, 145-155.
- Litingtung, Y., Lei, L., Westphal, H. and Chiang, C. (1998). Sonic hedgehog is essential to foregut development. *Nat. Genet.* **20**, 58-61.
- Maeda, R., Kobayashi, A., Sekine, R., Lin, J. J., Kung, H. and Maéno, M. (1997). Xmsx-1 modifies mesodermal tissue pattern along dorsoventral axis in *Xenopus laevis* embryo. *Development* **124**, 2553-2560.
- Maeda, Y., Davé, V. and Whitsett, J. A. (2007). Transcriptional control of lung morphogenesis. *Physiol. Rev.* **87**, 219-244.
- Matsushita, S., Urase, K., Komatsu, A., Scotting, P. J., Kuroiwa, A. and Yasugi, S. (2008). Foregut endoderm is specified early in avian development through signal(s) emanating from Hensen's node or its derivatives. *Mech. Dev.* **125**, 377-395.
- McLain, V. A., Rankin, S. A. and Zorn, A. M. (2007). Repression of Wnt/beta-catenin signaling in the anterior endoderm is essential for liver and pancreas development. *Development* **134**, 2207-2217.
- Meng, X., Brodsky, M. H. and Wolfe, S. A. (2005). A bacterial one-hybrid system for determining the DNA-binding specificity of transcription factors. *Nat. Biotechnol.* **23**, 988-994.
- Minoo, P., Su, G., Drum, H., Bringas, P. and Kimura, S. (1999). Defects in tracheoesophageal and lung morphogenesis in Nkx2.1(-/-) mouse embryos. *Dev. Biol.* **209**, 60-71.
- Molenaar, M., van de Wetering, M., Oosterwegel, M., Peterson-Maduro, J., Godsave, S., Korinek, V., Roose, J., Destree, O. and Clevers, H. (1996). XTcf-3 transcription factor mediates beta-catenin-induced axis formation in *Xenopus* embryos. *Cell* **86**, 391-399.
- Morrissey, E. E. and Hogan, B. L. (2010). Preparing for the first breath: genetic and cellular mechanisms in lung development. *Dev. Cell* **18**, 8-23.
- Mudumana, S. P., Hentschel, D., Liu, Y., Vasilyev, A. and Drummond, I. A. (2008). odd skipped related1 reveals a novel role for endoderm in regulating kidney versus vascular cell fate. *Development* **135**, 3355-3367.
- Neto, A., Mercader, N. and Gomez-Skarmeta, J. L. (2012). The Osr1 and Osr2 genes act in the pronephric anlage downstream of retinoic acid signaling and upstream of Wnt2b to maintain pectoral fin development. *Development* **139**, 301-311.
- Pearl, E. J., Jarikji, Z. and Horb, M. E. (2011). Functional analysis of Rfx6 and mutant variants associated with neonatal diabetes. *Dev. Biol.* **351**, 135-145.
- Que, J., Choi, M., Ziel, J. W., Klingensmith, J. and Hogan, B. L. (2006). Morphogenesis of the trachea and esophagus: current players and new roles for noggin and Bmps. *Differentiation* **74**, 422-437.
- Que, J., Okubo, T., Goldenring, J. R., Nam, K. T., Kurotani, R., Morrissey, E. E., Taranova, O., Pevny, L. H. and Hogan, B. L. (2007). Multiple dose-dependent roles for Sox2 in the patterning and differentiation of anterior foregut endoderm. *Development* **134**, 2521-2531.
- Rankin, S. A., Kormish, J., Kofron, M., Jegga, A. and Zorn, A. M. (2011). A gene regulatory network controlling hhex transcription in the anterior endoderm of the organizer. *Dev. Biol.* **351**, 297-310.
- Sato, N., Meijer, L., Skaltsounis, L., Greengard, P. and Brivanlou, A. H. (2004). Maintenance of pluripotency in human and mouse embryonic stem cells through activation of Wnt signaling by a pharmacological GSK-3-specific inhibitor. *Nat. Med.* **10**, 55-63.
- Schuler-Metz, A., Knöchel, S., Kaufmann, E. and Knöchel, W. (2000). The homeodomain transcription factor Xvent-2 mediates autocatalytic regulation of BMP-4 expression in *Xenopus* embryos. *J. Biol. Chem.* **275**, 34365-34374.
- Sekine, K., Ohuchi, H., Fujiwara, M., Yamasaki, M., Yoshizawa, T., Sato, T., Yagishita, N., Matsui, D., Koga, Y., Itoh, N. et al. (1999). Fgf10 is essential for limb and lung formation. *Nat. Genet.* **21**, 138-141.
- Serls, A. E., Doherty, S., Parvatiyar, P., Wells, J. M. and Deutsch, G. H. (2005). Different thresholds of fibroblast growth factors pattern the ventral foregut into liver and lung. *Development* **132**, 35-47.
- Sive, H. L., Grainger, R. M. and Harland, R. M. (2000) *Early Development of Xenopus laevis: A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
- Small, E. M., Vokes, S. A., Garriock, R. J., Li, D. and Krieg, P. A. (2000). Developmental expression of the *Xenopus* Nkx2-1 and Nkx2-4 genes. *Mech. Dev.* **96**, 259-262.
- So, P. L. and Danielian, P. S. (1999). Cloning and expression analysis of a mouse gene related to *Drosophila* odd-skipped. *Mech. Dev.* **84**, 157-160.
- Spence, J. R. and Wells, J. M. (2007). Translational embryology: using embryonic principles to generate pancreatic endocrine cells from embryonic stem cells. *Dev. Dyn.* **236**, 3218-3227.
- Sun, J., Chen, H., Chen, C., Whitsett, J. A., Mishina, Y., Bringas, P., Jr, Ma, J. C., Warburton, D. and Shi, W. (2008). Prenatal lung epithelial cell-specific abrogation of Alk3-bone morphogenetic protein signaling causes neonatal respiratory distress by disrupting distal airway formation. *Am. J. Pathol.* **172**, 571-582.
- Tena, J. J., Neto, A., de la Calle-Mustienes, E., Bras-Pereira, C., Casares, F. and Gómez-Skarmeta, J. L. (2007). Odd-skipped genes encode repressors that control kidney development. *Dev. Biol.* **301**, 518-531.
- Wang, J. H., Deimling, S. J., D'Alessandro, N. E., Zhao, L., Possmayer, F. and Drysdale, T. A. (2011). Retinoic acid is a key regulatory switch determining the difference between lung and thyroid fates in *Xenopus laevis*. *BMC Dev. Biol.* **11**, 75.
- Wang, Q., Lan, Y., Cho, E. S., Maltby, K. M. and Jiang, R. (2005). Odd-skipped related 1 (Odd 1) is an essential regulator of heart and urogenital development. *Dev. Biol.* **288**, 582-594.
- Wang, Z., Dollé, P., Cardoso, W. V. and Niederreither, K. (2006). Retinoic acid regulates morphogenesis and patterning of posterior foregut derivatives. *Dev. Biol.* **297**, 433-445.
- Xu, B., Chen, C., Chen, H., Zheng, S. G., Bringas, P., Jr, Xu, M., Zhou, X., Chen, D., Umans, L., Zwijsen, A. et al. (2011). Smad1 and its target gene Wif1 coordinate BMP and Wnt signaling activities to regulate fetal lung development. *Development* **138**, 925-935.
- Yin, A., Winata, C. L., Korzh, S., Korzh, V. and Gong, Z. (2010). Expression of components of Wnt and Hedgehog pathways in different tissue layers during lung development in *Xenopus laevis*. *Gene Expr. Patterns* **10**, 338-344.
- Yu, P. B., Deng, D. Y., Lai, C. S., Hong, C. C., Cuny, G. D., Bouxsein, M. L., Hong, D. W., McManus, P. M., Katagiri, T., Sachidanandan, C. et al. (2008). BMP type I receptor inhibition reduces heterotopic ossification. *Nat. Med.* **14**, 1363-1369.
- Zhang, Z., Lan, Y., Chai, Y. and Jiang, R. (2009). Antagonistic actions of Msx1 and Osr2 pattern mammalian teeth into a single row. *Science* **323**, 1232-1234.