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Lrp6-mediated canonical Wnt signaling is required for lip formation and fusion

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Neither the mechanisms that govern lip morphogenesis nor the cause of cleft lip are well understood. We report that genetic inactivation of Lrp6, a co-receptor of the Wnt/β-catenin signaling pathway, leads to cleft lip with cleft palate. The activity of a Wnt signaling reporter is blocked in the orofacial primordia by Lrp6 deletion in mice. The morphological dynamic that is required for normal lip formation and fusion is disrupted in these mutants. The expression of the homeobox genes *Msx1* and *Msx2* is dramatically reduced in the mutants, which prevents the outgrowth of orofacial primordia, especially in the fusion site. We further demonstrate that *Msx1* and *Msx2* (but not their potential regulator *Bmp4*) are the downstream targets of the Wnt/β-catenin signaling pathway during lip formation and fusion. By contrast, a 'fusion-resistant' gene, *Raldh3* (also known as *Aldh1a3*), that encodes a retinoic acid-synthesizing enzyme is ectopically expressed in the upper lip primordia of Lrp6-deficient embryos, indicating a region-specific role of the Wnt/β-catenin signaling pathway in repressing retinoic acid signaling. Thus, the Lrp6-mediated Wnt signaling pathway is required for lip development by orchestrating two distinctively different morphogenetic movements.

KEY WORDS: Msx, Wnt, Cleft lip, Lip morphogenesis, Lrp6, Retinoic acid, Mouse

INTRODUCTION

Failure of the lip and/or roof of the mouth to fuse during embryogenesis will cause cleft lip with or without cleft palate (CLP), a type of common birth defect in humans with poorly understood mechanisms (Wilkie and Morriss-Kay, 2001). The prevalence of CLP varies with geographic and ethnic background, with an average rate of 1 in 700 newborns (Schutte and Murray, 1999). Several genes have been implicated in human CLP (Carinci et al., 2007), and studies in animal models have revealed that the Tgfβ/Bmp, Fgf and Shh morphogenetic pathways are involved in facial development and lip formation (Jiang et al., 2006). These signaling pathways control the proliferation, differentiation and migration of cranial neural crest cells and epithelial ectodermal cells that contribute to the formation of orofacial and neck complexes. Four paired prominences give rise to the vertebrate face: the medial nasal (mnp), lateral nasal (lnp), maxillary (maxp) and mandibular (manp) prominences, which are derived from the frontonasal prominence and the first pharyngeal (or branchial) arch (Helms et al., 2005). Mutations of the Tgfβ/Bmp and Fgf pathways can result in CLP by diminishing proliferation or increasing apoptosis in orofacial primordia (Ito et al., 2003; Liu et al., 2005; Pauws and Stanier, 2007; Riley et al., 2007).

Wnts are a large family of secreted proteins, with 19 members present in mammals. Wnt signaling in broad developmental processes involves 10 frizzled receptors and two low density lipoprotein receptor-related protein (Lrp 5 and Lrp6) co-receptors, as well as numerous upstream and downstream factors that act through the β-catenin-dependent canonical pathway and the β-catenin-independent Wnt/calcium and planar cell polarity pathways (Logan and Nusse, 2004; Veeman et al., 2003). In particular, Lrp6 is a key co-receptor for the canonical Wnt/β-catenin pathway (He et al., 2004; Pinson et al., 2000). Wnt signaling has recently been suggested to play a role in mid-facial development (Brugmann et al., 2007). However, the role of the Wnt/β-catenin pathway in lip morphogenesis and CLP remains unknown. Here, we present evidence that the Lrp6-mediated Wnt/β-catenin signaling pathway is required for lip formation and fusion by regulating the gene expression of Msx1/Msx2 positively and of Raldh3 negatively during early orofacial development.

MATERIALS AND METHODS

Animals

Conventional $Lrp6^{\beta geo}$ mice were generated by a gene-trap approach with the reporter gene of β-galactosidase fused with neomycin (Pinson et al., 2000) and maintained on a C57/B6 background for most of the experiments in this study. Conditional $Lrp6^{floxdel}$ mice were generated by crossing loxP-floxed Lrp6 mice with CMV-Cre mice (Zhou et al., 2009), which generated mutants with identical external phenotypes to those reported previously (Pinson et al., 2000; Zhou et al., 2008) and the cleft lip reported in this study. The TOPgal mice were generated by DasGupta and Fuchs (DasGupta and Fuchs, 1999), and are distributed by the Jackson Laboratory (Maine, USA). Mice were housed in the vivarium of the UC Davis School of Medicine (Sacramento, CA, USA). Pregnant, timed-mated mice were euthanized with CO_2 gas prior to cesarean section. The day of conception was designated embryonic day 0 (E0). All research procedures using mice were approved by the UC Davis Animal Care and Use Committee and conformed to NIH guidelines.

In situ hybridization, TUNEL, immunofluorescence and X-gal staining

Embryos were fixed in 4% paraformaldehyde (PFA). Whole-mount and section in situ hybridization were performed according to standard protocols using digoxigenin-labeled antisense RNA probes (Zhou et al., 2008; Zhou et al., 2004a). TUNEL assays were performed using the Dead End Fluorometric TUNEL System (Promega), following the manufacturer's instructions, on 10-μm frozen or paraffin-wax embedded tissue sections. Immunohistochemistry was carried out on sections using appropriate primary antibodies and Alexa fluorescence-conjugated secondary antibodies (Molecular Probes), according to standard protocols. The anti-BrdU antibody (1:50) developed by S. Kaufman (University of Illinois, Urbana,

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IL, USA) and anti-PY489-β-catenin antibody (1:50-1:200) developed by J. Balsamo and J. Lilien (University of Iowa, IA, USA) were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa (USA). Anti-phospho-histone H3 antibody (1:50; Cell Signaling) was also used. X-gal staining was carried out as follows. Embryos were fixed in 2% PFA for 10 minutes on ice, washed in phosphate-buffered saline (PBS) three times for 5 minutes each, and then subjected to standard X-gal staining for 3 hours for the lithium- or sodium-treated embryos, or overnight for the regular TOPgal embryos.

BrdU incorporation and maternal administration of lithium chloride

Acute BrdU labeling was performed by intraperitoneal injection of BrdU at 100 mg/kg body weight to the pregnant dams 1 hour prior to sampling (Zhou et al., 2004b). In vivo stimulation of the Wnt/β-catenin signaling pathway by lithium was carried out as described in a previous publication (Cohen et al., 2007) with slight modifications. Pregnant dams were injected intraperitoneally with 30 μl of a 600 mM LiCl or NaCl control solution on both E8.5 and E9.5. Embryos were collected at E10.5 for 3-hour X-gal staining, whole-mount in situ hybridization, or real-time RT-PCR.

Scanning electron microscopy

Embryos were fixed in 2.5% glutaraldehyde and 2% PFA and dehydrated in a 70% to 100% ethanol (200 proof) series. Hexamethyldisilazane (HMDS, Ted Pella, Redding, CA, USA) was used for the final drying of samples; embryos were infiltrated in a graded mixed ethanol and HMDS series with the final solution in the series being 100% HMDS. The treated embryos were mounted on aluminum stubs and then sputter coated with gold (Pelco Auto Sputter Coater, Ted Pella) for scanning electron microscopy (Phillips XL30 TMP, F.E.I. Company, Hillsboro, OR, USA).

RNA isolation and real-time quantitative PCR

Total RNA was isolated from orofacial tissue of E10.5 embryos. Semi-quantitative PCR was carried out according to the manual of the Mx3005P Real-Time PCR system using SYBR GREEN PCR master mix. The mRNA levels of *Msx1*, *Msx2*, *Bmp4*, *Raldh3*, *Wnt3* and *Wnt9b* were normalized to the mRNA level of glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) to allow comparisons among different experimental groups using the ΔC_t method (Goydos and Gorski, 2003). The forward 5′-TCGAGAGTGG-GAAGAAGGAA-3′, and the reverse 5′-AGAAGACGGTGGGTTTG-ATG-3′ primers were used for *Raldh3* mRNA detection. Primers for other genes were designed by the SuperArray Bioscience Corporation (Frederick, MD, USA).

Luciferase reporter assay

The 333-bp promoter region of the mouse *Msx2* gene, which contains a conserved Tcf/Lef-binding site, and the same promoter region with the binding site mutated by site-directed mutagenesis were amplified by PCR and cloned into the pGL2-basic vector to acquire the pMsx2-Luc and pMsx2-mut-Luc constructs, respectively (Fig. 5). The DE region with or without a Tcf/Lef-binding site of the mouse *Msx1* gene was amplified and cloned into the luciferase reporter O-Fluc upstream of the minimal c-fos promoter, and the resulting plasmids were designated pMsx1DE-Luc and pMsx1-DE-del-Luc, respectively (Fig. 6). Transient transfection was performed in L cells and primary orofacial cells with Lipofectamine 2000 reagent following the manufacturer's instructions (Invitrogen). L cells were treated with 0-75% Wnt3a-containing conditioned media (Wnt3a CM) solely or 5% Wnt3a CM with 0 to 400 ng/ml Dkk1 proteins (EMD Biosciences, CA, USA). Wnt3a CM from a Wnt3a-expressing L cell line was collected from cultures grown to confluence and centrifuged at 1,000 rpm for 10 minutes. Primary orofacial cells were prepared from wild-type embryos by dissecting the tissue in cold PBS, which was then digested at 37°C in Hank's solution containing 0.025% trypsin (Gibco BRL), centrifuged at 1455 ***g*** for 10 minutes, cultured in 10% fetal bovine serum (FBS) for 12 hours, and treated with 12 nm LiCl. L cells were also transiently transfected with pMsx2-Luc/pMsx2-Mut-Luc or pMsx1-DE-Luc/pMsx1-DE-del-Luc in combination with either a control expression vector (pcDNA3) or Lef1 and constitutively active β-catenin expression constructs (Lin et al., 2007). Twenty-four hours after transfection, luciferase activities were assayed using the Dual-Luciferase assay kit (Promega, Madison, WI, USA) as described previously (Song et al., 2007).

Chromatin immunoprecipitation (ChIP)

For in vivo and in vitro ChIP experiments, extracts were prepared from wild-type orofacial tissue of E10.5 mouse embryos and a Wnt3a-expressing L cell line. Embryos were dissected in ice-cold PBS. After gentle pipetting, tissue or cells were cross-linked with 2% formaldehyde for 10 minutes at room temperature. Chromatin extraction and immunoprecipitation were performed according to the manufacturer's protocols using a ChIP assay kit (17–295; Upstate Biotechnology, Lake Placid, NY, USA). An antibody against β-catenin was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit IgG was used as a negative control. The 333-bp *Msx2* promoter region was amplified with the forward primer 5′-CTATTAGTAAAGATGCTGCTATT-3′ and the reverse primer 5′-AGTCTCATTTCTTGTCTTTTAAC-3′. The distal element (DE) of the endogenous mouse *Msx1* promoter was recovered by PCR using the forward primer 5′-AAAGAGAGGGGAACTCGGG-3′ and reverse primer 5′-AATTCGTGGGGGTTGAGGG-3′. The *Bmp4* promoter region with a Tcf-binding site was amplified by primers as previously described (Shu et al., 2005).

Statistical evaluation

Two to five mutant embryos or littermate controls were used for each non-quantitative experiment; each produced identical results. At least three mutant embryos or littermate controls were examined in each quantitative analysis for statistical significance. Student's *t*-test was used for statistical comparisons when appropriate, and differences were considered significant at $P<0.05$.

RESULTS

Genetic deletion of Lrp6 caused full penetrant cleft lip with cleft palate in mice

The conventional Lrp6$^{\beta geo/+}$ mice contained a *lacZ* gene (Pinson et al., 2000) with a ubiquitous expression pattern in early embryos (see Fig. S1 in the supplementary material). We examined whether lip formation and fusion are disrupted in homozygous Lrp6$^{\beta geo/\beta geo}$ mice, which have previously been shown to express defects in the neural tube, eye, brain, limb and bone, and which thereby represent the combined phenotypes seen in multiple individual Wnt mutants (Kelly et al., 2004; Pinson et al., 2000; Zhou et al., 2006; Zhou et al., 2008; Zhou et al., 2004a; Zhou et al., 2004b). At E13.5, a day after lip fusion is completed in normal mice (Fig. 1A), we found dramatic defects including bilateral clefts and hypoplasia in the upper lip of Lrp6 knock out (Lrp6-KO) mice (Fig. 1B). We also detected hypoplasia and midline-cleft of the mutant mandible (Fig. 1D). In addition, the mutant mice had a cleft palate (see Fig. S2 in the supplementary material), which is frequently associated with cleft lip in humans. At E16.5, when palate fusion is completed in control mice (see Fig. S2A in the supplementary material), the primary palate was absent in the anterior mouth of the mutant embryos (Fig. S2B in the supplementary material). The rostral-most maxillary and mandibular structures were both hypoplastic (Fig. 1B; see also Fig. S2B in the supplementary material). We observed a full penetrance of CLP and mandibular defects in Lrp6-deficient mice (20/20 mutant embryos examined). These orofacial defects in the Lrp6 mutants closely coincide with the Wnt/β-catenin signaling activation sites demonstrated in TOPgal mice (DasGupta and Fuchs, 1999) during early orofacial formation and fusion processes (Brugmann et al., 2007; Jiang et al., 2006).

Lrp6 was required for Wnt/β-catenin signaling in orofacial primordia

We also observed intense TOPgal activity (visualized by X-gal staining for the enzymatic activity of β-galactosidase encoded by the *lacZ* gene) in the frontonasal, maxillary and mandibular primordia. It

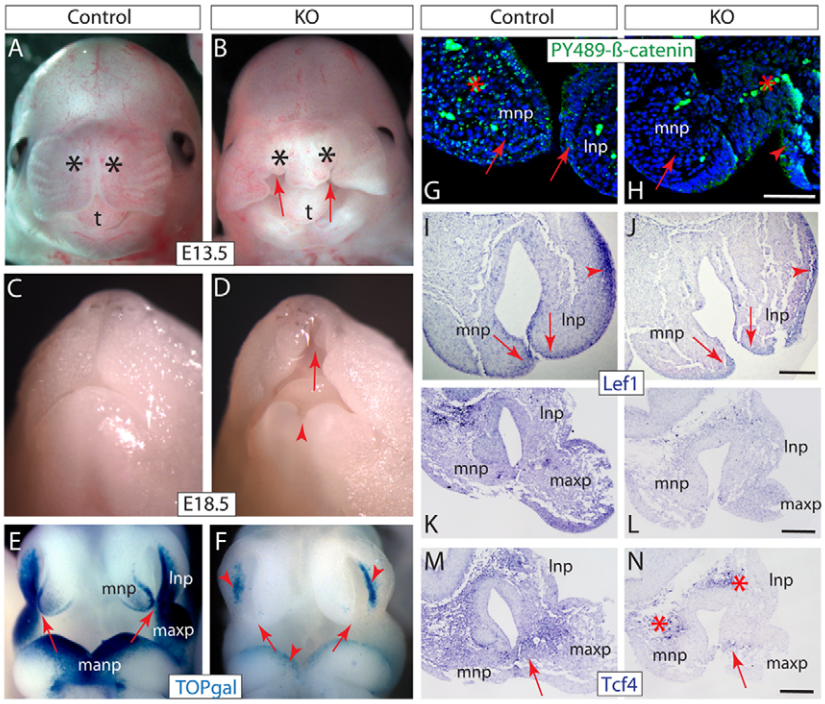

Fig. 1. Cleft lip and diminished Wnt/β-catenin signaling activity in Lrp6-deficient mice. (**A**,**B**) Front facial views of a littermate wild-type (WT) embryo and an Lrp6-KO embryo at E13.5. Arrows indicate the bilateral cleft lip, and asterisks indicate the hypoplastic maxillary prominence in the mutant embryo. (**C**,**D**) Views from the lower front looking up at the lips of a normal control and an Lrp6-KO embryo at E18.5. Arrow indicates the cleft lip with a severe defect on one side of the upper lip and arrowhead indicates a midline cleft of the mandible in the mutant embryo. (**E**,**F**) Front facial views of X-gal-stained whole embryos at E10.5 showing the Wnt reporter TOPgal in normal control and Lrp6$^{floxdel/floxdel}$ mice. Arrows indicate the fusion site of the upper lip; arrowheads indicate the residual activity of TOPgal in Lrp6-KO embryos. (**G**,**H**) Immunolabeling for nuclear β-catenin (green) in the mnp and lnp at E10.5. Arrows in G indicate positive labeling in the fusion epithelium and mesenchyme; arrow in H indicates weak or negative labeling in the mutant mesenchyme; arrowhead in H indicates some strong labeling in the lnp epithelium; asterisk indicates non-specific labeling for blood cells. (**I-L**) In situ hybridization for Lef1 on rostral (I,J) and caudal (K,L) front facial sections. Arrows indicate the lip fusion site. Arrowheads indicate the mesenchyme and adjacent surface ectoderm in the lnp. (**M**,**N**) Section in situ hybridization for Tcf4. Arrows indicate the fusion site. Asterisks in N indicate some restricted intense signals in the mutant mesenchyme adjacent to the olfactory epithelium. lnp, lateral nasal prominence; mnp, medial nasal prominence; manp, mandibular primordium; maxp, maxillary prominence; t, tongue. Scale bars: 50 μm.

was predominantly restricted to the ventral rostral region of the lnp and the ventral tip of the mnp at E10.5 (Fig. 1E), when upper lip fusion occurs in normal control mice. We then crossed the TOPgal reporter mice with Lrp6-deficient mice. Because the conventional Lrp6^{βgeo} mice contained a *lacZ* gene (Pinson et al., 2000), we generated a novel mutant mouse line Lrp6^{floxdel} (which has no *lacZ* reporter gene) by ubiquitous deletion of the exon 2-floxed Lrp6 using CMV-Cre (Zhou et al., 2009). The homozygous Lrp6$^{floxdel/floxdel}$ mice reproduced the representative phenotypes seen in Lrp6$^{βgeo/βgeo}$ mice. X-gal staining was significantly attenuated in the Lrp6$^{floxdel/floxdel}$ orofacial primordia (Fig. 1F); the signal was dramatically reduced in the lnp and the manp, and no signal was observed in the fusing lip sites, including in the mnp and the maxp. The anti-PY489-β-catenin antibody can detect active β-catenin in the nucleus (Rhee et al., 2007), which binds to the transcription factor Tcf/Lef and regulates the gene expression of Wnt/β-catenin target genes. We found dominant nuclear β-catenin immunolabeling in both epithelial and mesenchymal cells, particularly in the mnp of E10.5 control embryos (Fig. 1G); however, labeling was diminished dramatically in mutant primordia at the same age (Fig. 1H). In addition, expression of the Wnt signaling effectors Lef1 and Tcf4 was also obviously decreased in the mutant primordia (Fig. 1I-N). These results demonstrate that Lrp6 is a key receptor for transducing the canonical Wnt/β-catenin signaling pathway during orofacial formation and fusion.

Scanning electron microscopy revealed two types of lip clefts in Lrp6-KO embryos

The morphology of the developing upper lip in normal mouse embryos is still understudied. We employed scanning electron microscopy (SEM) to examine lip formation and fusion in both control and mutant embryos (Fig. 2). In the E9.5 control embryo, we found that the mnp and the lnp had become distinguishable from the nasal placode (Fig. 2A), by the formation of rich microvilli on the surface of the nasal placode (Fig. 2F; data not shown), but that they were not yet obviously rising up. Several hours later, the nasal pit became evident owing to the uprising of the horseshoe-like mnp and lnp from both sides. However, a shallow gap still existed between the slower-rising ventral tips of the mnp and the lnp (Fig. 2B,C,G,H). At E10.5, the ventral tip of the rapid-growing mnp extended further rostrally and ventrolaterally, and it was clearly joined with both the lnp and the maxp at the medial end of their boundary junction (Fig. 2D,I). At E11.5, the ventral mnp was widely connected with both the lnp and the maxp, whereas the boundary lines on the surface ectoderm between these prominences were still recognizable in the wild-type embryos (Fig. 2J).

In Lrp6-KO embryos, the orofacial primordia and the nasal pit were evident at E10 (Fig. 2K,L). At E10.5, although the mutant prominences were smaller than those seen in the controls, the ventral tip of the mutant mnp was apparently joined with the lnp and the

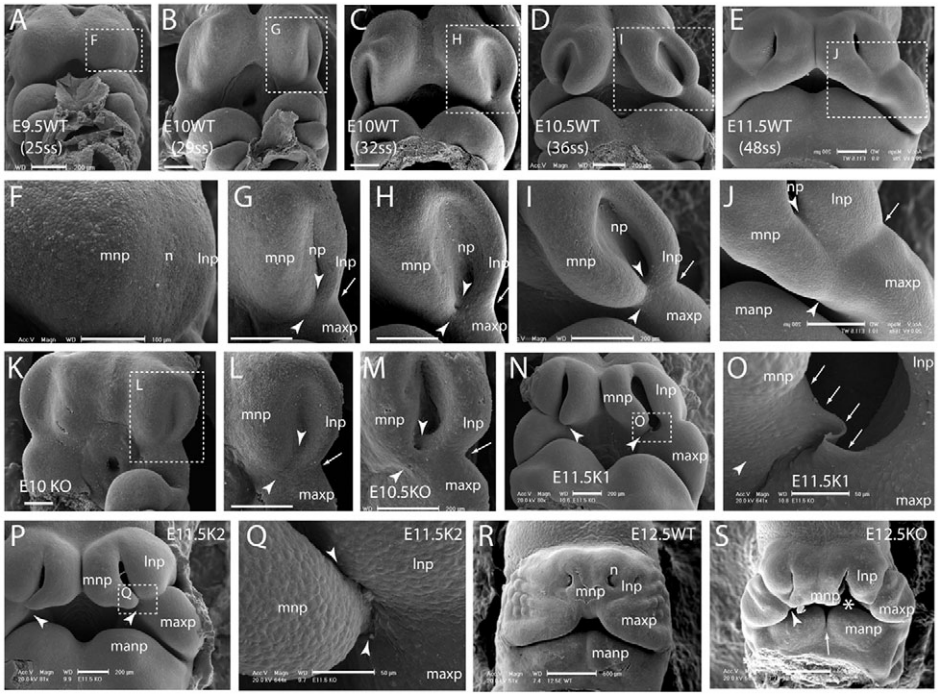

Fig. 2. Scanning electron microscopy of the upper lip morphogenesis. (**A-J**) Front facial views of normal control embryos from E9.5 to E11.5 show the key steps of the upper lip formation. Arrowheads indicate the fusion sites of the mnp with the lnp and the maxp. Arrows indicate the boundary line between the lnp and the maxp. ss, somite stage. Scale bars: 200 μm in A-E,G-J; 100 μm in F. (**K-Q**) Upper lip morphogenesis in Lrp6-KO embryos from E10 to E11.5. Note that at E10.5 the ventral tip of the mnp meets with the boundary site of the lnp and the maxp in the mutant embryo (arrow in M). Arrowheads and arrow in L indicate the boundary sites of the upper lip primordia. At E11.5, varied lip clefts are seen. Bilateral lip clefts (arrowheads in N) in a mutant embryo with a large gap (about 50 μm; arrows in O) existed between the ventral tip of the mnp and the boundary site of the lnp and the maxp. Within the cleft there is a folded and thin epithelium (arrows in O). Another mutant embryo shows a different type of lip cleft (arrowheads in P), in which the mnp is attached, barely, to the lnp only (arrowheads in Q). Scale bars: 200 μm in K-N,P; 50 μm in O,Q. (**R**) An E12.5 littermate control embryo shows completed formation of the upper lip. n, nostril. Scale bar: 500 μm. (**S**) An E12.5 Lrp6-KO embryo shows defective orofacial prominences, a large cleft (asterisk) in one side of the upper lip, and a milder cleft (arrowhead) on the other side, with no attachment of the mnp to the maxp. A midline cleft in the mandible is also seen (arrow). Scale bar: 500 μm.

maxp at this age (Fig. 2M). At E11.5, two types of clefts were seen in the mutant upper lips (Fig. 2N-Q). Bilateral lip clefts with a large gap (about 50 μm) existed between the ventral tip of the mnp and the medial end of the lnp/maxp boundary (Fig. 2N,O); within the cleft there was a folded and thin epithelium (Fig. 2O). Some mutant embryos showed a different type of lip cleft, in which the ventral tip of the mnp attached barely and only to the lnp at E11.5 (Fig. 2P,Q). At E12.5, the littermate control embryos exhibited complete formation and fusion of the upper lip, in which, morphologically, no boundary lines between these prominences were distinguishable (Fig. 2R). By contrast, the E12.5 mutant embryos exhibited both types of clefts in the upper lip and defective growth of the prominences (Fig. 2S). In addition, a midline cleft in the mandible was evident at this age.

Proliferation and apoptosis were altered in the lip epithelium of the mutants

To address whether cell proliferation and apoptosis were altered in the lip fusion site and related orofacial primordia of Lrp6-KO embryos, we performed immunolabeling of phospho-histone H3 (pHH3) for mitotic cells and TUNEL assay for apoptotic cells during upper lip formation and fusion (Fig. 3). We found that the number of mitotic cells was significantly reduced in the mutant mesenchyme but not in the epithelium of the mnp/lnp at E10.5 (Fig. 3A-C). This fits well with our observation of retarded growth of the mesenchyme in the mutant orofacial primordia. We also performed BrdU incorporation and detection assays and found high levels of $BrdU^+$ cells in the orofacial primordia of control embryos without obvious changes in the mutants at E10.5 and E11.5 (see Fig. S3 in the supplementary material). However, $BrdU^+$ cells were not detectable in the fusion epithelium of control embryo at E11.5 (Fig. S3C in the supplementary material), but were observed in the corresponding epithelium of the mutant embryo (see Fig. S3D in the supplementary material).

Some apoptotic cells detected by TUNEL assay were clustered in the epithelium of the putative fusion site and scattered in the mesenchyme of both normal and mutant orofacial primordia at E10.5 (Fig. 3D,E). The apoptotic epithelial seam was evident during normal lip fusion at E11.5 (Fig. 3F), but it was not seen in the corresponding region of the mutant epithelium (Fig. 3G). This could be a consequence of the failure of the initial fusion in the mutant lip primordia.

Msx1 and Msx2 but not Bmp4 were positively targeted by the Lrp6-mediated Wnt/β-catenin pathway in orofacial primordia

To address the underlying molecular mechanisms of orofacial defects in Lrp6-KO mice, we examined several factors that are known to be important in early orofacial primordia around E10.5. The homeobox genes *Msx1* and *Msx2* have been reported to play

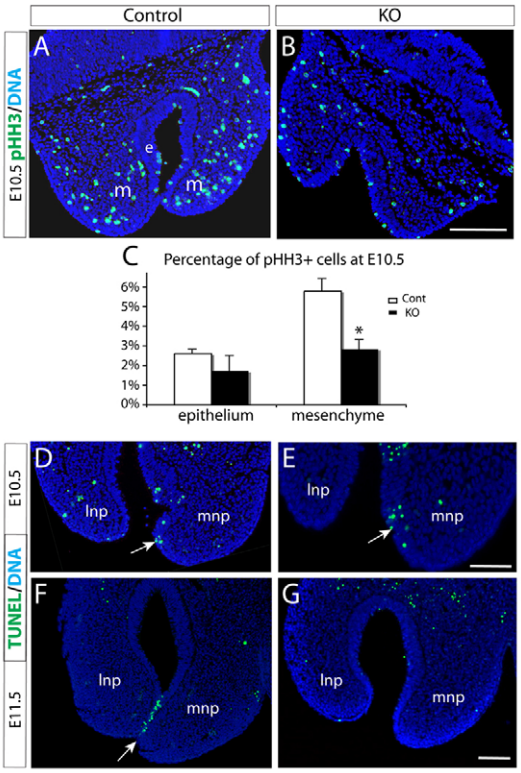

Fig. 3. Proliferation and apoptosis in the lip fusion site. (**A-C**) Mitotic cells in the lip/nasal primordia at E10.5. The number (A,B) and ratio (C) of dividing cells (green) immunolabeled for phospho-histone H3 (pHH3) are significantly reduced in the mesenchyme (m) but not in the epithelium (e) of the mutant lip/nasal primordia compared with the littermate control. The pHH3+ cells (in total 200 to 400 orofacial primordial cells) were counted in each of three sections of each embryo (in total three embryos for each genotype) for calculating the proliferation rate. **P*<0.05. (**D-G**) TUNEL detection for cell death during lip formation and fusion at E10.5 and E11.5. Arrows indicate apoptotic epithelial cells. Note the apoptotic epithelial seam in the normal control (arrow in F) but not in the mutant embryos at E11.5 during the lip fusion process. Cell nuclei were counterstained by DAPI (blue). Scale bars: 50 μm.

important roles in murine craniofacial development (Ishii et al., 2005; Satokata et al., 2000; Satokata and Maas, 1994), and have been implicated in human CLP (Vieira et al., 2005). By using whole-mount in situ hybridization, we observed dramatically diminished expression of both *Msx1* (Fig. 4A,B; see also Fig. S4A-C in the supplementary material) and *Msx2* (Fig. 4C,D; see also Fig. S4D-F in the supplementary material) in the mnp, lnp and maxp of Lrp6-deficient embryos. Their expression was also significantly reduced in the manp, but this was less severe than that which we observed in the upper lip/nasal prominences mentioned above. By contrast, *Bmp4* expression was relatively conserved in the mutant orofacial primordia (Fig. 4E,F; see also Fig. S4G-I in the supplementary material). The statistical significances of *Msx1*, *Msx2* and *Bmp4* mRNAs isolated from the control and mutant orofacial tissues were demonstrated by real-time quantitative PCR (see Fig. S4C,F,I in the supplementary material). *Sostdc1* (also known as Ectodin or Wise) is a known Bmp antagonist (Kassai et al., 2005) that also binds Lrp6 directly to regulate Wnt signaling activities (Itasaki et al., 2003). We found that *Sostdc1* expression was dramatically attenuated in the surface ectoderm of Lrp6-KO embryos at E10.5 (Fig. 4G-J). Both *Wnt3* and *Wnt9b* are expressed in the orofacial region and may be linked to CLP (Carroll et al., 2005; Lan et al., 2006; Niemann et al., 2004). We observed a restricted expression pattern of *Wnt3* in the interface between the lnp, maxp and manp of the control embryos; we found a dramatic reduction of *Wnt3* in the same regions of Lrp6-KO mice when detected by in situ hybridization but not when detected by the real-time quantitative PCR (see Fig. S5A-C in the supplementary material). We also could not detect any obvious differences in the expression of *Wnt9b*, *AP2* (which might be slightly upregulated in the mutants) or *Fgf8* (see Fig. S4D-J in the supplementary material).

To test our hypothesis that *Msx1* and *Msx2* are targeted by the canonical Wnt/β-catenin signaling pathway in orofacial primordia, we searched the promoter region of these genes for Tcf/Lef-binding sites or Wnt/β-catenin-responsive elements and found multiple binding sites in both putative promoter regions (Fig. 5A, Fig. 6A). We first screened the two proximal Tcf/Lef-binding sites in both genes by luciferase reporter assays and found that these promoter sites in *Msx2*, but not in *Msx1*, are highly responsive to Wnt3a stimulation (data not shown). The 333-bp promoter region of mouse *Msx2*, which is highly conserved in the human *MSX2* gene (Fig. 5A), demonstrated luciferase activation stimulated by Lef1 and the constitutively active β-catenin; however, the site-directed mutant *Msx2* promoter showed no activation under the same conditions (Fig. 5B). In addition, this *Msx2* promoter region demonstrated a dose-dependent response to Wnt3a (Fig. 5C), which was significantly diminished by Dkk1 (Fig. 5D), an inhibitory ligand for Lrp6. The mutant promoter showed response to neither Wnt3a in L cells (Fig. 5E) nor lithium in primary orofacial cells (Fig. 5F). Furthermore, we performed ChIP on in vivo orofacial tissues and in vitro L cells, and recovered by PCR the 333-bp *Msx2* promoter region from chromatin immunoprecipitated with the β-catenin antibody (Fig. 5G).

The *Msx1* promoter has proximal and distal elements (PE and DE) that regulate its activity in a tissue-specific manner (Fig. 6A), and the DE drives gene expression specifically in orofacial primordia (MacKenzie et al., 1997). We identified a Tcf/Lef-binding site in the DE region of the *Msx1* promoter (Fig. 6A), which demonstrated Lef1/β-catenin-responsive activity in the luciferase assay (Fig. 6B). We also recovered by PCR the DE, but not the PE, region from the ChIP assay in vivo (Fig. 6C). By contrast, a published Wnt-responsive element in the *Bmp4* promoter region was recovered by PCR in the Wnt3a-expressing L cell line, but not in the orofacial tissue, by our ChIP assay (Fig. 6D), which indicates that *Bmp4* is not a target of Wnt/β-catenin signaling in this region.

Moreover, we intraperitoneally administered lithium, an antagonist for Gsk3β, which is a key intracellular component of Wnt/β-catenin signaling (Berger et al., 2005), for 2 days to E8.5 female mice and detected a significant induction of both Wnt reporter TOPgal signaling activity (Fig. 8A,B) and levels of *Msx1* (Fig. 8C-E) and *Msx2* (Fig. 8F-H) in orofacial primordia at E10.5. Together, our results demonstrate that *Msx1* and *Msx2* are target genes of the Wnt/β-catenin pathway in orofacial primordia.

Raldh3 expression was significantly expanded in the orofacial surface ectoderm of Wnt signaling mutants

The expression of the retinoic acid-synthesizing enzyme *Raldh3* is normally restricted to the olfactory pit/ventral olfactory epithelium adjacent to the surface ectoderm of the mnp and the lnp at E10.5 (Fig. 7A,C). In contrast to the downregulation of *Msx1* and *Msx2* genes, we unexpectedly found that *Raldh3* expression had expanded

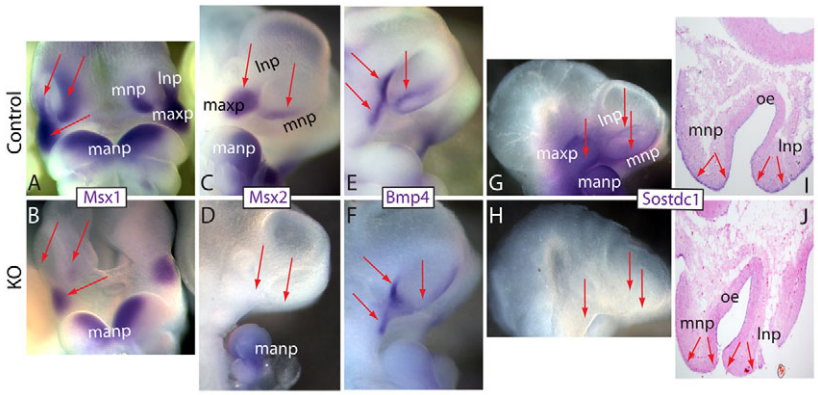

Fig. 4. Expression of *Msx1*, *Msx2*, *Bmp4* and *Sostdc1* in the orofacial primordia at E10.5. (**A**,**B**) *Msx1* expression is dramatically reduced in the mnp, lnp and maxp (arrows) of Lrp6-KO. By contrast, it is mildly reduced in the mutant manp. (**C**,**D**) *Msx2* expression is specifically lost in the mnp, lnp and maxp (arrows) of Lrp6-KO. By contrast, it is mildly reduced in the mutant manp. (**E**,**F**) *Bmp4* expression is not significantly altered in the upper lip/nasal primordia (arrows) of Lrp6-KO. (**G**,**H**) *Sostdc1* is dramatically lost in the mnp, lnp and maxp (arrows) of Lrp6-KO. (**I**,**J**) Expression signals of *Sostdc1* are present on the surface ectoderm (arrows) of the normal control but not the mutant embryo.

into the fusion region in all upper lip/nasal prominences of mutant embryos at the same age (Fig. 7B,D). At E11.5, *Raldh3* expression was still high in the cleft site (arrows) and the lnp/maxp boundary (asterisks) of the mutant embryo (Fig. 7E,F). The expansion of *Raldh3* expression was also demonstrated by real-time PCR at E10.5 (Fig. 7G). Conversely, significant repression of *Raldh3* expression by Wnt activation was demonstrated by the gain-of-function of Wnt signaling in L cells activated by Wnt3a (Fig. 7H) and in E10.5 orofacial tissues stimulated by the Wnt agonist lithium (Fig. 8I-K). Together, these results demonstrate that the Wnt/β-catenin pathway represses *Raldh3* expression in the upper lip primordia during normal development.

DISCUSSION

Orofacial defects in Wnt signaling mutants

The current study has shown that genetic deletion of Lrp6 in mice resulted in CLP with full penetrance. Our findings suggest a potential role of Lrp6 and other Wnt signaling genes in human CLP. In support of this, a recent study has reported that single-nucleotide polymorphisms in *WNT3A*, *WNT5A* and *WNT11* are significantly associated with non-syndromic CLP in humans, and that gene-gene interactions are observed between *WNT3A* and both *WNT3* and *WNT5A* (Chiquet et al., 2008). In addition, a nonsense mutation in human *WNT3* causes Tetra-amelia with CLP (Niemann et al., 2004). In mice, Wnt9b-knockout mice have been shown to have kidney defects together with incomplete penetrance of CLP (Carroll et al., 2005). To date, however, no other Wnt signaling gene mutations have been shown to cause CLP in either humans or animal models. This might be partially due to functional redundancy of many Wnt signaling genes in lip morphogenesis, or to the loss of head structures and early embryonic lethality in several important Wnt signaling mutants. Dkk1 is a ligand for Lrp6 to negatively regulate the Wnt/β-catenin pathway, and its knock out results in mice that lack entire craniofacial structures (Mukhopadhyay et al., 2001). Conditional deletion of β-catenin in Wnt1-expressing neural crest lineage cells leads to the failure of craniofacial development (Brault et al., 2001). Together, these data demonstrate the significance of Wnt signaling genes in the pathogenesis of CLP and related orofacial malformations.

Lrp6 is a key mediator for Wnt/β-catenin signaling in orofacial primordia

We has also shown here that Lrp6 is required to activate the Wnt/β-catenin signaling pathway in the orofacial primordia; genetic deletion of Lrp6 results in the inactivation of a Wnt/β-catenin signaling reporter, TOPgal, in these regions. Only limited signals are seen in the lnp and the manp of Lrp6-deficient embryos, which might be transduced by other two Wnt co-receptors, Lrp5 or Ryk, upstream of β-catenin (He et al., 2004; Lu et al., 2004). However, TOPgal has been shown to be active in some retinal precursor cells in the absence of β-catenin (Fuhrmann et al., 2009), suggesting that some undetermined signals other than those of the Wnt/β-catenin pathway can also activate TOPgal in orofacial primordia. Nevertheless, our results indicate that TOPgal activity in the majority of orofacial primordia, particularly in the entire mnp and the lip fusion site of the lnp and the maxp, is solely mediated by Lrp6.

Previous studies have shown the presence of Lef1 and Tcf4 in orofacial primordia (Brugmann et al., 2007), suggesting that they are the downstream effectors of the Lrp6-mediated Wnt/β-catenin pathway during lip morphogenesis. We showed that both Lef1 and Tcf4 expression were diminished in the mutant fusion epithelium and adjacent mesenchyme, indicating that the inactivation of Wnt/β-catenin signaling might also diminish the expression level of the Wnt transcription factors. Both Wnt3 and Wnt9b are expressed in these regions (Lan et al., 2006), suggesting they are potentially the ligand molecules that bind to Lrp6 to control lip development. Interestingly, Sostdc1, a non-Wnt ligand that binds to Lrp6 to regulate the Wnt/β-catenin pathway, is also expressed in orofacial primordia. We showed that both Wnt3 and Sostdc1 are lost in the orofacial primordia of Lrp6-deficient embryos, suggesting a feedback regulation of the Lrp6-mediated Wnt/β-catenin signaling pathway during orofacial development.

Morphological dynamics during normal lip formation and a potential mechanism for lip cleft

The vertebrate upper lip is thought to form from the mnp, the lnp and the maxp that initially freely project (Jiang et al., 2006). From our SEM observations, we find no free projections of these prominences at any steps of normal lip morphogenesis. The ventral end of the lnp is the dorsal boundary of the maxp from the initial stage of lip development at E9.5. Compared with other parts within the same prominence at E10, the ventral tip of the mnp arises slower. The mesenchymal growth in the mnp is apparently faster than in both the lnp and the maxp from E10, which pushes the ventral tip of the mnp to extend rostrally and ventrolaterally, joining with both the lnp and the maxp at the medial end of the lnp/maxp boundary junction at E10.5. From E11.5, all three prominences grow rapidly towards each other, and the upper lip fuses widely at their boundary sites. The morphological association between the mnp and the lnp/maxp in mice is identical to the pattern of lip formation observed in primate embryos (Senders et al., 2003).

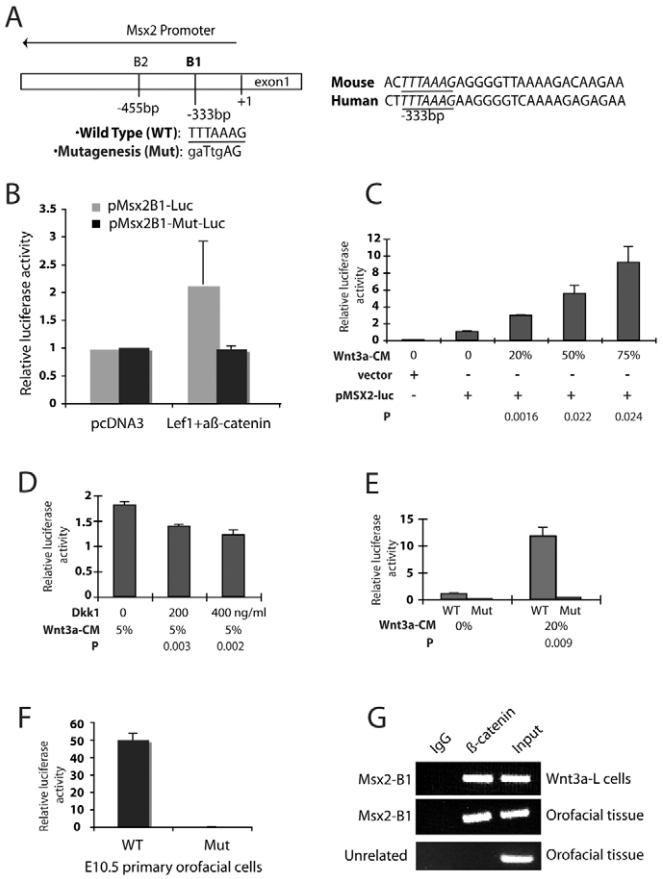

Fig. 5. *Msx2* is targeted by the Wnt/β-catenin pathway in orofacial primordia. (**A**) Two proximal Tcf/Lef-binding sites (B1 and B2) are shown in the putative promoter region of the *Msx2* gene. The site-directed mutagenesis (Mut) of B1 is shown. B1 is highly conserved in mice and humans. (**B**) Luciferase assay results for L cells transfected with pMsx2-B1-Luc or pMsx2-B1-Mut-Luc constructs in combination with either pcDNA3 or Lef1 and active β-catenin constructs; $P<0.05$. (**C**) Luciferase assay results for L cells transfected with the reporter vector pGL-basic or pMsx2-B1-Luc and stimulated by Wnt3a. The luciferase activity of pMsx2-B1-Luc without Wnt3a conditional media (Wnt3a CM) stimulation was defined as 1 unit. (**D**) pMsx2-B1-Luc transfected cells were treated with 5% Wnt3a CM and 0 to 400 ng/ml Dkk1 for 24 hours. The luciferase activity of the cultured cells without Dkk1 treatment was defined as 1.8 units. (**E**) Wnt3a failed to upregulate the expression of the reporter driven by the mutant Msx2-B1 promoter. (**F**) Wnt activation failed to upregulate the expression of the reporter driven by the mutant Msx2-B1 promoter that was transfected into primary orofacial cells isolated from E10.5 embryos and stimulated by lithium. Data were averaged from three independent cultures; error bars indicate s.d. $P<0.05$ was considered significant. (**G**) Chromatin immunoprecipitation (ChIP) assay on extracts from Wnt3a-expressing L cells and E10.5 orofacial tissue immunoprecipitated by β-catenin antibody indicated the in vitro and in vivo recruitment of β-catenin to the *Msx2* promoter. The negative control was carried out using an Msx2-unrelated primer pair.

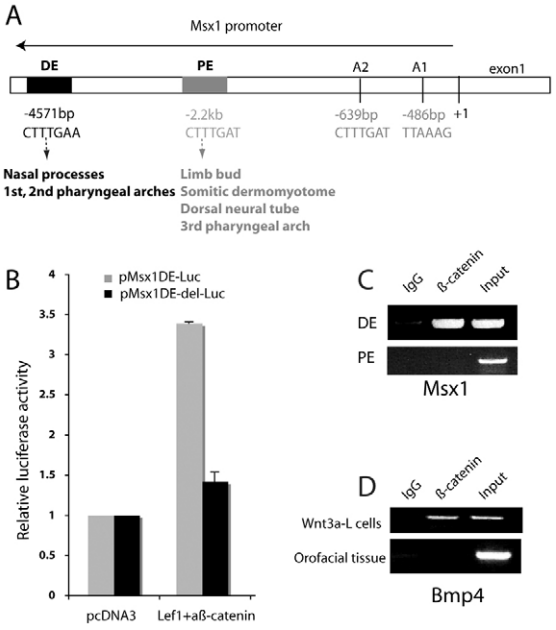

Fig. 6. A distal element (DE) of the *Msx1* promoter is specifically targeted by the Wnt/β-catenin pathway in orofacial primordia. (**A**) We identified a Tcf/Lef-binding site (CTTTGAA) in the highly conserved DE of the putative *Msx1* promoter (about 5 kb). Distal and proximal (PE) elements drive gene expression restricted to different organ/tissues, as reported previously (MacKenzie et al., 1997). (**B**) Luciferase assay results for L cells transfected with pMsx1-DE-Luc or pMsx1-DE-del-Luc constructs in combination with either pcDNA3 or Lef1 and active β-catenin constructs. (**C**) ChIP assays on extracts from E10.5 orofacial tissue immunoprecipitated by β-catenin antibody indicated the in vivo recruitment of β-catenin specifically to DE, but not to PE, of the *Msx1* promoter. (**D**) ChIP assays demonstrate the recruitment of β-catenin to the *Bmp4* promoter in vitro, but not in the orofacial tissue.

By contrast, the ventral tip of the mnp in Lrp6-KO embryos is connected with the lnp and the maxp at E10.5, but a lip cleft occurs around E11.5. A thin and folded epithelium is exhibited in the cleft at this age, indicating a lack of mesenchymal growth beneath the epithelium in the cleft region. Thus, the upper lip cleft seems to be caused by a continuing outgrowth of the ventral mnp rostrally but not ventrolaterally, which leads to either a free-ending projection of the ventral mnp or a weak connection with only the lnp. Together with the proliferation defects in the mesenchyme of the mutant prominences, our results suggest that the Lrp6-mediated Wnt/β-catenin pathway controls the morphological dynamics by regulating the growth speed and direction of mesenchymal cells in the upper lip primordia, particularly in the mnp.

Lrp6 might regulate Msx expression in orofacial primordia independently of Bmp signaling

Our results have clearly shown that the homeobox genes *Msx1* and *Msx2* are dramatically downregulated in the mesenchyme of the Lrp6-deficient orofacial primordia, and that both genes are positive targets of the Wnt/β-catenin pathway during lip formation, as demonstrated by in vivo and in vitro approaches. Both Msx genes have also been shown to be downstream of the Wnt/β-catenin signaling pathway in human embryonic carcinoma cells (Willert et al., 2002). *Msx1* mutations were found in 2% of cases of human CLP (Jezewski et al., 2003). Msx1-KO mice exhibit a cleft palate and abnormalities of the cranial skeleton (Satokata and Maas, 1994). Mutations in *Msx2* also cause craniofacial defects (Satokata et al., 2000). These Msx mutants have a major defect in facial mesenchymal proliferation. However, no cleft lip was reported in these gene-deficient mice, suggesting a redundant role of Msx1 and Msx2 in lip development. *Msx1; Msx2*

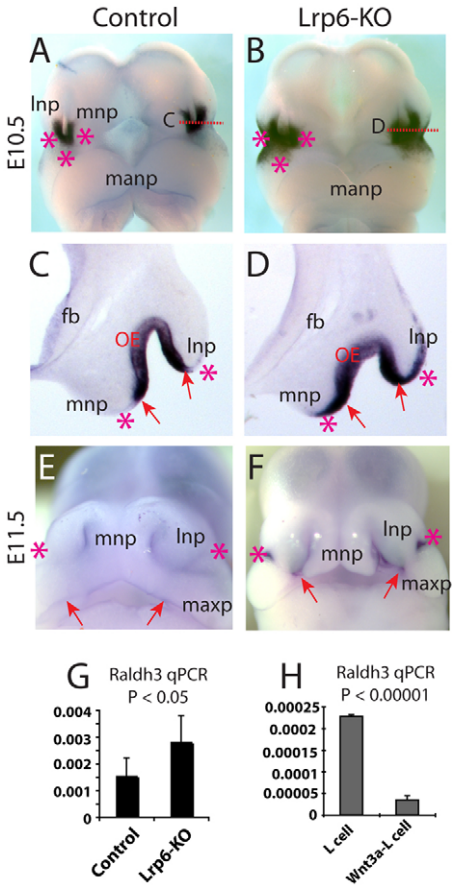

Fig. 7. *Raldh3* expression is negatively regulated by the Wnt/β-catenin pathway. (**A-D**) *Raldh3* expression, which is normally restricted to the olfactory epithelium (OE; indicated between arrows in C) at E10.5, was significantly expanded into the surface ectoderm, particularly in the fusion region of the orofacial primordia of Lrp6-KO embryos (asterisks in B,D). C and D are the vibratome sections cut through the mnp and the lnp, as indicated by the dashed lines in A and B. fb, forebrain. (**E**,**F**) *Raldh3* expression in control (E) and Lrp6-KO (F) embryos at E11.5 (front facial view). Arrows indicate the fusion site or lip clefts; asterisks indicate the lateral boundary between the lnp and the maxp. (**G**) Real-time RT-PCR shows the upregulation of *Raldh3* mRNA in the mutant orofacial tissue at E10.5. (**H**) Real-time RT-PCR shows the downregulation of *Raldh3* mRNA in L cells stimulated by Wnt3a.

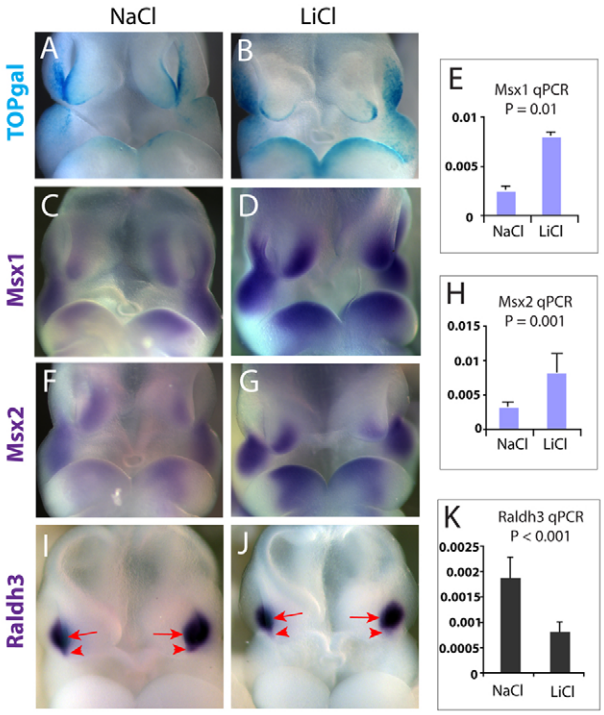

Fig. 8. In vivo stimulation of Wnt/β-catenin signaling promotes *Msx1* and *Msx2* and represses *Raldh3* gene expression. (**A**,**B**) X-gal-stained TOPgal signals were increased in orofacial prominences at E10.5 after 2-day maternal injections of lithium chloride but not sodium chloride. (**C-H**) *Msx1* and *Msx2* expression was significantly upregulated by lithium, as demonstrated by either in situ hybridization or real-time RT-PCR. (**I-K**) *Raldh3* expression was significantly repressed by lithium, as demonstrated by either in situ hybridization or real-time RT-PCR. Arrowheads in J indicate reduced expression in the ventral tip of the *Raldh3*-expressing domain (arrows).

double knock-out mice (Ishii et al., 2005) exhibit an extremely severe disruption in which the lip and other orofacial structures do not develop, indicating a key role of Msx genes in controlling rapid cell growth of the orofacial primordia. These data suggest that the local downregulation of both *Msx1* and *Msx2* genes in the upper lip primordia is a major cause of CLP in Lrp6-deficient mice (Fig. 9). The mandibular defects in Lrp6-KO embryos might share the same mechanism owing to the downregulation of Msx genes. In addition, we detected a significant reduction of mitotic cells (M phase immunolabeled for pHH3) in mutant orofacial primordia. This could be the consequence of the downregulation of Msx genes in the Lrp6 null orofacial primordia. By contrast, we did not see an obvious change in BrdU incorporation in cells in mutant tissue. This indicates that although the majority of the mutant orofacial cells are still able to incorporate BrdU during their S phase, these mutant cells might have to go through a longer G2 phase to enter into M phase owing to the downregulation of Msx genes or a direct effect of the inactivation of Wnt/β-catenin signaling. Notably, hedgehog inhibition has been shown to prolong G1 and G2 length and to slow down cell division (Locker et al., 2006). Wnt/β-catenin signaling might play a similar role in the regulation of cell cycle length. These issues will be addressed in future studies.

During neural crest specification, *Msx1* and *Msx2* are regulated by Bmp signaling (Tribulo et al., 2003), particularly by *Bmp4*, which is known to be regulated by the canonical Wnt pathway in other tissues (Shu et al., 2005; Zhou et al., 2008). Conditional deletion of either *Bmp4* or *Bmpr1a* (a Bmp type I receptor) in orofacial primordia also caused cleft lip (Liu et al., 2005). However, we found no significant changes in *Bmp4* expression in the mutant orofacial primordia. One might suspect that the downregulation of *Msx* genes is also regulated by a Bmp antagonist, for instance, Sostdc1 (Kassai et al., 2005). Although Sostdc1 is also a ligand for Lrp6 to positively or negatively regulate Wnt signaling activities (Itasaki et al., 2003), its expression is repressed in the orofacial primordia of Lrp6-deficient embryos, suggesting that *Sostdc1* expression is directly or indirectly regulated by the Lrp6-mediated Wnt/β-catenin pathway. This is consistent with a recent report that *Sostdc1* expression is suppressed in cells with high Wnt signaling activity but induced in adjacent cells, perhaps by a secreted factor downstream of Wnt/β-catenin, during tooth development (Liu et al., 2008). Together, these results suggest that the downregulation of Msx genes in Lrp6 mutant

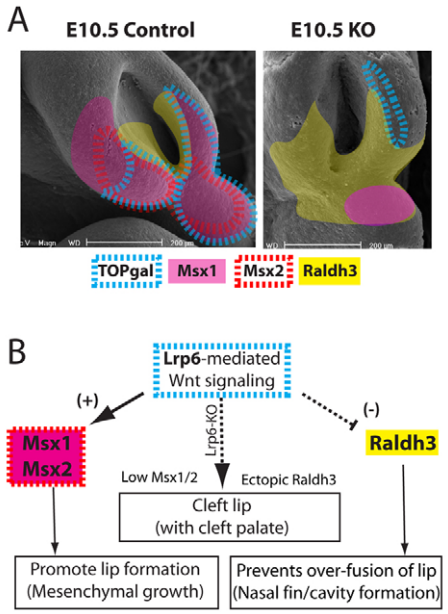

Fig. 9. Summary of the role of Lrp6 and its mediated Wnt/β-catenin signaling pathway in the activation of *Msx1*/*Msx2* and the repression of *Raldh3* during lip formation and fusion. (**A**) The spatial correlations of the Wnt reporter TOPgal, the homeobox genes *Msx1* and *Msx2*, and the retinoic acid-synthesizing gene *Raldh3* in the upper lip/nasal primordia at E10.5 during normal lip formation, and their dramatic alterations in the Lrp6-KO embryo. (**B**) The Lrp6-mediated Wnt/β-catenin pathway directly activates *Msx1* and *Msx2* expression, and directly or indirectly represses *Raldh3* expression during normal lip formation and fusion.

orofacial primordia is independent or downstream of the Bmp pathway; however, Lrp6 and Bmp signaling might also regulate Msx gene expression in a parallel fashion. In-depth analyses of the Msx genes in Bmp signaling in mutant orofacial primordia might address these possibilities.

In addition, we showed that the expression of another important morphogenetic molecule, *Fgf8*, and of a neural crest regulator, *AP2*, were not significantly diminished (and that *AP2* expression might even be upregulated) in orofacial primordia of Lrp6-deficient mice. The Wnt/β-catenin signaling pathway has been shown to play important roles in neural crest development (Garcia-Castro et al., 2002; Wu et al., 2003). Conditional deletion of β-catenin in *Wnt1*-expressing crest lineage cells resulted in the absence of entire craniofacial tissues (Brault et al., 2001). Conditional deletion of Lrp6 using either Wnt1-Cre or Pax3-Cre mice (Brault et al., 2001; Li et al., 2000) might clarify whether the lineage-specific inactivation of the Lrp6-mediated Wnt/β-catenin pathway in neural crest cells will cause CLP.

The role of Raldh3 in orofacial development and lip cleft

We have shown the significant upregulation of *Raldh3* in the orofacial epithelium of Lrp6-deficient mice. These results suggest a repressive role of canonical Wnt signaling on *Raldh3* expression in these regions and indicate that tissue patterning had been altered in the orofacial epithelium of Lrp6-KO mice. Raldh3 has been demonstrated to play a primary role in retinoic acid (RA) synthesis in the nasal and ocular regions (Dupe et al., 2003). Raldh3-deficient mice have RA-rescuable malformations restricted to these regions, in particular the lethal choanal atresia caused by the persistence of nasal fins, whose rupture is required for communication between nasal and oral cavities in the respiratory system. Notably, Raldh3-deficient mice were reported to have an over-fusion phenotype in the upper lip (Dupe et al., 2003), suggesting a 'fusion-resistant' effect of Raldh3 in the orofacial region during normal development. Our results suggest that Lrp6 is required to restrict the expression of Raldh3 to the nasal epithelium, and that expansion of Raldh3 expression to the surface ectoderm/fusion epithelium of the orofacial primordia is also a cause of CLP in Lrp6-KO embryos (Fig. 9).

Signaling crosstalk during vertebrate orofacial development

In the chicken model, it has been shown that the exogenous application of RA with a simultaneous block of Bmp signaling by Noggin may transform the maxp into a second frontonasal mass and result in a duplicated beak (Lee et al., 2001). A similar fate change might not occur in Lrp6-KO embryos owing to the sole expansion of Raldh3 and the absence of downregulation of Bmp4 in the mutant orofacial primordia. However, we might see a similar fate change in *Lrp6; Bmp4* double mutant embryos. It is noteworthy that Msx1 expression was expanded and not decreased in chicken orofacial primordia treated with Noggin either alone or combined with RA (Lee et al., 2001). This further suggests that Bmp inhibition is not required for the downregulation of Msx expression in Lrp6-KO orofacial primordia, and that Bmp signaling is also not upstream of Lrp6 during lip development. By contrast, blocking RA synthesis in chicken orofacial primordia decreased rather than increased Msx1, Msx2, Bmp4 and Fgf8 expression (Song et al., 2004). Our current study has demonstrated the upregulation of Raldh3, the downregulation of Msx1/Msx2, and no significant alteration of Bmp4 and Fgf8 in the Lrp6-KO primordia, suggesting a complex interaction between these signaling pathways. The compound deficiency of Lrp6 with Raldh3, Bmp4 or Fgf8 might provide new insights into these signaling interactions during orofacial development.

In conclusion, our findings suggest that Lrp6 regulates the balance of two opposing driving forces in the adjacent tissues for normal lip formation and fusion through the modulation of Msx1 and Msx2 (which promote orofacial mesenchymal growth) and Raldh3 (which prevents over-fusion of the upper lip epithelium and controls the size of nostril) expression (Fig. 9B).

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/136/18/3161/DC1

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