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Inhibition of *Wnt*/β-catenin pathway by Dikkopf-1 affects midfacial morphogenesis in chick embryo

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The development of the vertebrate face is regulated by complex interactions among several signaling pathways. Dickkopf-1 (Dkk-1), an inhibitor of the *Wnt*/β-catenin signaling pathway, can affect midfacial morphogenesis. The downstream target genes of the *Wnt*/β-catenin signaling pathway in morphogenesis of the developing upper jaw and lip remain unknown. To investigate the functional roles of *Wnt*/β-catenin signaling in facial development, we performed a loss-of-function experiment using local implantation of beads soaked with Dkk-1 during lip fusion at the maxillary prominence of chick embryos at stage 22(HH22). Antagonism of *Wnt*/β-catenin signaling by Dkk-1 induced deformities of the premaxilla and jugal bone, which are derived from the maxillary mesenchyme. Real-time and semi-quantitative RT-PCR analysis showed the significant reduction of *Lhx8, Msx1* and *Msx2* expression levels around the beads in the maxillary mesenchyme at 6 and 24 h after bead implantation. Time course experiments in the HH 22 embryos showed the effect of Dkk-1 on *Lhx8*, *Msx1* and *Msx2* expression was not significant after 48 h of the treatment. At HH 26 when the fusion of facial primordial started, Dkk-1 application did not exhibit any significant reduction of those genes. Our findings suggested that Dkk-1 regulates maxillary morphogenesis in chick embryos through *Lhx8*, *Msx1* and *Msx2* signals. *Wnt*/β-catenin signaling is responsible for intrinsic upper jaw development before the lip fusion.

[**Key words:** Dickkopf-1 (Dkk-1); *Lhx8*; *Wnt*/β-catenin signaling; Chick embryo; Craniofacial development]

Morphogenesis of the vertebrate face is a result of the outgrowth of several facial primordia (1). Cleft lip usually results directly from failure of fusion of medial nasal prominences with the lateral and maxillary prominences. Production of specific gene products or other cellular or regional characteristics can impact selective vulnerability. Several major morphogenetic signaling pathways are involved in dynamic facial morphogenesis. The β-catenin-dependent or canonical Wnt signaling (*Wnt*/β-catenin signaling) pathway is a prominent intercellular signaling pathway in animals and plays fundamental roles in cellular proliferation, regeneration, differentiation, and function. Coordinated activation and inhibition of *Wnt*/β-catenin signaling are essential for proper antero-posterior formation in the vertebrate embryo, and recent advances have revealed roles for the *Wnt*/β-catenin signaling in the development of oral tissues (2,3).

The *Wnt*/β-catenin signaling pathway functions by regulating the amount of the transcriptional co-activator β-catenin. Wnt ligands bind to a Frizzled receptor and its co-receptors, low-density lipoprotein receptor-related proteins (LRP5, LRP6). These events lead to the stabilization of β-catenin, which activates Wnt target gene expression (4–6). These activities are inhibited by Dickkopf-1 (Dkk-1), a secreted protein that specifically inhibits the *Wnt*/β-catenin signaling pathway by interacting with LRP5/6 co-receptors (7,8). Dkk-1 was originally identified as a strong head inducer in Xenopus, due to its potent anti-Wnt effect (9). Dkk-1 knockout mice display severe developmental phenotypes including head defects (10), suggesting that proper head formation in mouse requires an optimal level of *Wnt*/β-catenin signaling activity (11). Conditional knockout of β-catenin in mouse showed that the ablation of *Wnt*/β-catenin signaling in the oral epithelium blocks the formation of palatal rugae, which are associated with antero-posterior extension of the hard palate and precocious mid-line fusion (2). Studies with Lrp6-deficient mice suggest that lip formation and fusion requires *Wnt* signaling to regulate the expression of *Msx1* and *Msx2*, members of the *Msx* homeobox gene family that have been implicated in human cleft lip and palate (12,13). Although these lines of evidence suggest that the *Wnt*/β-catenin signaling pathway and its downstream target genes contribute to morphogenesis of the upper jaw, it is unlikely that decreased gene function can explain all of the mechanisms underlying cleft lip and palate.

Lhx8 belongs to the LIM-homeodomain (LIM-HD) transcription factor family, and its expression overlaps with that of *Msx1* and *Msx2*. *Lhx7* (an alias of *Lhx8*) is expressed in the mesenchyme of the first pharyngeal arch and its derivatives, the maxilla and mandible, but not in the epithelium (14–16). Disruption of *Lhx8* gene function in mice causes impairments in palatal shelf contact and fusion that lead to the formation of a cleft secondary palate (17). *Msx1* and *Msx2* are expressed in the maxillary and median nasal prominence (18), and act in a variety of cell types to control cell proliferation and

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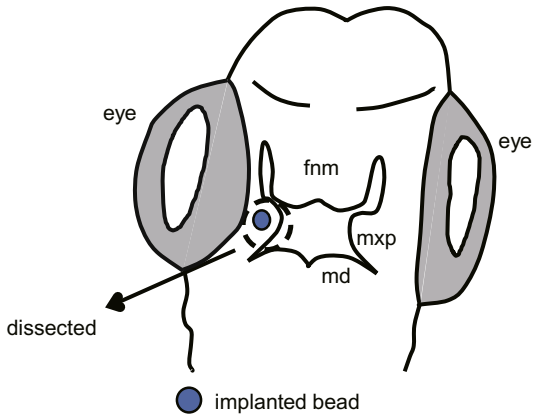

FIG. 1. Schematic representation of Dkk-1 bead implantation and dissection of the maxillary process.

differentiation (19,20). Mice with mutations in both *Msx1* and *Msx2* have severe craniofacial malformations including cleft palate and agenesis of teeth (21). Transcription factors such as *Lhx8*, *Msx1* and *Msx2* can mediate the organizing and patterning effects of the oral ectoderm.

Facial morphogenesis is a result of the patterning of neural crest-derived mesenchyme that is regulated by these orchestrated signals. However, the detailed roles and the downstream target genes of the *Wnt*/β-catenin signaling pathway in morphogenesis of the developing upper jaw and lip remain unknown. The chick embryo develops relatively fast and offers a very accessible system in molecular studies. In this study, we used chick embryos and present evidence that *Wnt*/β-catenin signaling is required for upper jaw morphogenesis by regulating the expression of *Lhx8*, *Msx1* and *Msx2* during lip fusion.

MATERIALS AND METHODS

Embryos and beads implantation Fertilized White leghorn eggs were purchased from Takeuchi Farm (Nara, Japan) and incubated at 38°C until embryos reached the appropriate stage. Embryos were treated with Dkk-1- or 2% BSA-soaked beads. Affigel Blue beads (BioRad) were soaked in 0.1 mg/ml Dkk-1 (DKK-1, Abcam) with 2% bovine serum albumin (BSA). Sham operations were performed using beads soaked in 2% BSA. In all cases, a small slit was carefully made in the right side of the maxillary prominence and the beads were inserted into the slit at HH 22 (22) (before the fusion of the frontonasal prominence and maxillary prominence) under a microscope (Leica, Germany). The left side of the maxillary prominence served as the control site (Fig. 1). Animal procedures were approved by the Nara Medical University Animal Care and Use committee.

RNA isolation, real-time RT-PCR and semi-quantitative RT-PCR Maxillary prominences were dissected 6 h after bead implantation. Total RNA was isolated with Sepasol-RNA I Super G (Nacalai Tesque, Japan) from the dissected processes. We then synthesized cDNA using the QuantiTect Reverse Transcription kit (QIAGEN, Germany) with PCR primers listed in Supplementary Table S1. The cDNA synthesized from the left sides of the maxillary prominence served as control. *Lhx8* chicken cDNA was prepared as previously described (16). *Msx1* and *Msx2* cDNAs were kindly provided by J.M. Richman (23).

Real-time RT-PCR was carried out according to the LightCycler supplier's manual (Roche) using SYBR Green Realtime PCR Master Mix Plus (TOYOBO, Japan). The ratio of the mRNA levels for each sample was calculated by normalizing the comparative quantitation values to those of *GAPDH* mRNA.

Semi-quantitative RT-PCR was performed as described in the manufacture's protocol. The PCR products were resolved by electrophoresis through an 8% polyacrylamide gel and then stained with ethidium bromide. At least six processes were contained in one sample, and three sets of samples were used and examined in each quantitative analysis for statistical significance.

Skeletal staining To study the morphological changes of bone and cartilage, embryos were fixed at HH 38 (after 12 days' incubation) and kept in 100% ethanol for 4 days. The embryos were then infiltrated with acetone for 4 days, stained with Alcian blue and Alizarin red for up to 10 days, and cleared with KOH as previously described (23).

Immunohistochemistry and apoptosis assay Heads of chick embryos were fixed in 4% paraformaldehyde overnight. The heads were washed with 30% sucrose after the fixation and frozen in OCT for coronal sections. Analysis of apoptosis was done using cleaved caspase-3 antibody (1:500, rabbit polyclonal, Cell Signaling Technology) (24).

Statistical analysis The delta−delta CT method and ANOVA using Microsoft Excel were carried out for statistical comparisons and a value of $p < 0.05$ was considered significant.

RESULTS

External and skeletal changes of the chick face after inhibition of *Wnt*/β-catenin signaling Dkk-1 is one of the inhibitor of the canonical *Wnt*/β-catenin signaling whose receptors are expressed and activated around facial prominences (25). To determine whether direct antagonism of *Wnt*/β-catenin signaling can affect upper jaw development, we applied Dkk-1-soaked beads to the maxillary prominence of HH 22 chick embryos, which were then incubated until they reached HH 38. The embryo heads showed severe defect of the maxilla and collapsed eye at the treated side (Fig. 2D, $n = 14$). Skeletal staining with

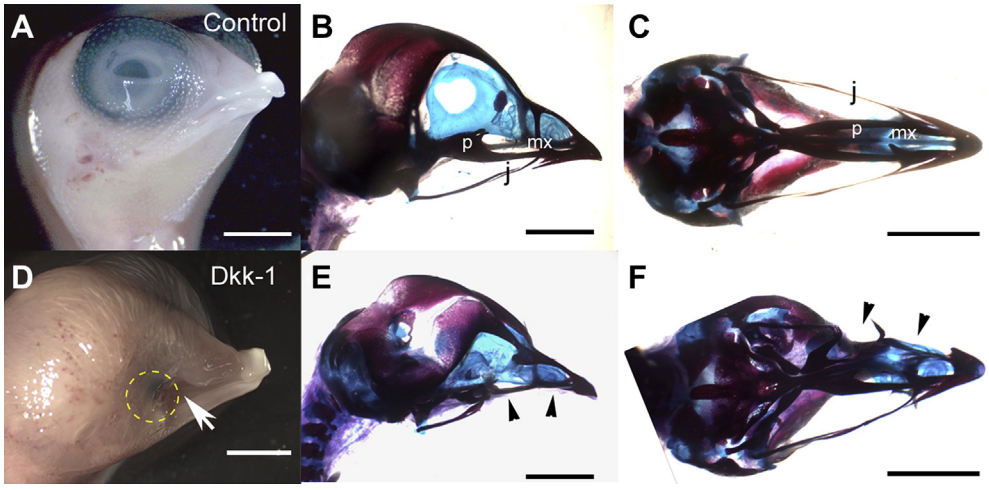

FIG. 2. Chick embryos at HH 38 after Dkk-1-soaked bead implantation. Lateral (B, E) or axial (C, F) views of head skeletons stained with Alizarin red and Alcian blue are shown. (A–C) Control embryos treated with 2% BSA-soaked beads. (D–F) Dkk-1-soaked beads caused defect of the maxilla (white arrow) and the eye became embedded into the maxilla (yellow dotted line). Hypoplasia of the premaxilla and palatine bone and a defect of the jugal bone occurred (E and F; black arrowheads). p: palatine bone; mx: maxillary bone; j: jugal bone. Bars: 5 mm.