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Retinoic acid regulates *Lhx8* expression via FGF-8b to the upper jaw development of chick embryo

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Expression of the LIM homeodomain transcription factor *Lhx8* is restricted to and up-regulated in the mesenchyme of the upper face prominence before lip fusion. Msx1/2 acts in early development to control cell proliferation and differentiation. Deficiency of these genes is associated with nonsyndromic cleft lip with/without cleft palate. Since retinoid is a potential patterning influence on the developing face, we have examined whether retinoic acid (RA) signaling regulated *Lhx8*, *Msx1* and *Msx2* transcription through fibroblast growth factor (FGF) signals in the maxillary prominence. Application of exogenous RA caused severe defects of the maxilla. Citral also induced a specific loss of derivatives from the maxillary prominences by blocking RA synthesis. Real-time RT-PCR and semi-quantitative RT-PCR analysis of the maxillary mesenchyme revealed that the expressions of *Lhx8*, *Msx1* and *Msx2* were significantly down-regulated by RA as well as by citral. The downregulated *Lhx8* was rescued by combined treatment with FGF-8b, which indicated a downstream of RA signaling. FGF-8b induced up-regulated *Lhx8* expression whereas SU5402, a pan-FGF family antagonist, down-regulated and caused defective maxillary morphogenesis and cleft lip. Our data suggest that *Lhx8* is regulated by RA signaling through FGF signals and the level window of RA and FGF-8b could control the upper jaw morphogenesis.

[**Key words:** Chick embryo; LIM homeodomain; *Lhx8*; Retinoic acid; Fibroblast growth factor; Craniofacial development]

Clefts lip and palate is one of the most common of all birth defects. The mechanisms that cause orofacial clefts are, however, still not completely understood. The development of the facial structure involves a complex network of transcriptional factors and signaling pathways (1). The critical signaling molecule, retinoic acid (RA), has a lot of roles in the facial development and has long been associated with craniofacial defects (2,3). Exogenous RA applied to vertebrate species including monkeys and rodents results in embryonic defects similar to non-syndromic cleft lip/palate. On the other hand, disruption of retinoid homeostasis by means of organic chemical manipulation in the maternal diet can result in similar anomalies. Experimental inhibition of retinoid receptor signaling also produces a phenocopy of the cleft lip/palate (4).

Members of the LIM homeodomain (LIM-HD) transcription factor family are essential for tissue patterning and cell type specification during embryonic development (5). *Lhx8* is a member of the LIM-HD family and is localized in the maxillary and rostral mandibular processes (6–8). *Lhx7*, which is the same as *Lhx8*, is one of the key components of signaling cascades that control proliferation and differentiation of mandibular mesenchyme (9,10). A targeted mutation of the gene *Lhx8* is associated with isolated secondary cleft palate in mice (11). We found that *Lhx8* was strongly expressed at the opposing edges of the frontonasal mass and maxillary process prior to their fusion, and that its expression in tissue explants was induced by FGF-8b and TGF-β3 (6). We are interested in signaling cascades upstream and downstream of *Lhx8*, which is involved in differentiation and morphogenesis of the facial skeleton. FGF-8b has already been shown to induce *Lhx8* expression in grafted mesenchyme of the maxillary process, but there remain several additional candidates that may regulate *Lhx8* (6). Endogenous retinoid is likely to promote cell survival by regulating the level of fibroblast growth factors (FGFs) (12). Msx1/2 acts during early development to control the formation of facial structure. Mutation of these genes has been associated with non syndromic cleft lip with or without cleft palate (13). A study at Xenopus suggests RA regulate *Lhx8* and *Msx2* directly or indirectly in the upper lip and primary palate

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development (14). We investigated whether RA signaling controls *Lhx8* expression in craniofacial development. We also examined the effects of FGF-8b on *Lhx8* expression because endogenous RA signaling might act upstream of FGF (12). Our results indicate that *Lhx8* is a component of the RA signaling pathway through FGF-8b in the developing upper jaw.

MATERIALS AND METHODS

Embryos and bead implantation All procedures followed protocols that were approved by Nara Medical University. Fertilized white leghorn eggs were purchased from Takeuchi Farm (Nara, Japan) and incubated at 38.0°C to the appropriate stage (HH) (15).

RA-soaked beads were prepared by immersing the beads in 10 mg/ml of all-*trans*-retinoic acid (Sigma, MO, USA) in DMSO. SM2 beads (Bio-Rad, CA, USA) were soaked in 0.1 g/ml of citral (Sigma) for 10 min (12). SU5402 (Calbiochem, NJ, USA and EMD Biosciences, UK) was dissolved in dimethyl sulfoxide (DMSO). AG1X-2 beads (Bio-Rad; format form, 200 μm diameter) were soaked in 50 mM of SU5402 for 1 h with a drop of 0.01% Fast Green added for bead visualization. Heparin Acrylic beads (Sigma) were soaked in 1 mg/ml recombinant mouse FGF-8b (R&D Systems, MN, USA) for a minimum of 1 h at room temperature. Control beads were soaked in DMSO. In all cases, the beads were inserted with small incisions in the maxillary process at HH stage 22 of chicken embryo and their final position was recorded (8).

Quantitative RT-PCR Total RNA was isolated from chicken maxilla 6 h after implantation of the beads using Sepazol (R)-RNA1 super (Nacalai Tesque, Japan). We synthesized cDNA using a QuantiTect Reverse Transcription kit (Qiagen, Germany) and chicken cDNA was prepared as previously described (6). The polymerase chain reaction (PCR) primers were used as previously described.

RT-PCR was performed in a total volume of 25 μl containing 1.0 μl of template cDNA, 0.2 mM of dNTPs, 0.25 U of AmpliTaq DNA polymerase, and 2.5 μl of 10×PCR buffer (AmpliTaq, Applied Biosystems), using 10 mM of the PCR primer sets as previously described (6). Nucleic acids were denatured at 94°C for 2 min, and then amplified by 25 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min followed by a final extension at 72°C for 7 min. The PCR products were resolved by electrophoresis through 8% polyacrylamide gels and then stained with ethidium bromide.

For quantitative RT-PCR, SYBR Green Realtime PCR Master Mix Plus (Toyobo, Japan) was used according to the manufacutre's instructions. The Delta−delta Ct method was used for analysis, and the ratio of gene expression for each sample was calculated by normalizing the values for comparative quantitation to those of GAPDH.

Skeletal staining To study the morphological changes of bone and cartilage, embryos were fixed at HH stage 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 (6).

Statistical analysis Statistical analysis was carried out by *t*-test using Microsoft Excel. A value of $p < 0.05$ was considered significant.

RESULTS

Exogenous RA down-regulates the expression of *Lhx8* *Lhx8* is mainly expressed on the medial aspects of the maxillary process mesenchyme, partially at lateral frontonasal mass at HH stage 22 (6). The lip fusion event involves reciprocal epithelial−mesenchymal interactions underlying pattern formation of the upper jaw. RA can interfere with the process of pattern formation of the upper beak (16). To examine whether RA is involved in *Lhx8* expression in the lip fusion event, we applied RA-soaked beads to the maxillary process of HH stage 22. We analyzed *Lhx8* mRNA expression semi-quantitatively by real-time RT-PCR. After total RNA extraction and generation of cDNA at 6 h after bead application, real-time RT-PCR for *Lhx8* and GAPDH was performed. The *Lhx8* mRNA level in maxillary tissues treated with exogenous RA was significantly lower than that in the control treated with DMSO (Fig. 1A, B). The results indicated that excess amount of exogenous RA could down-regulate *Lhx8* transcription. The embryos at HH stage 38 (7 days after the treatment) showed severe inhibition of maxillary growth and cleft lip (Fig. 2D−F), and the overall length of the upper beak and growth of the maxilla were reduced ($n = 4$). The premaxilla was truncated bilaterally. Skeletal staining showed absence of the forward growth of the maxilla and lack of an egg tooth. Most of the DMSO-treated embryos developed normally, indicating that surgical procedures per se (insertion of the beads and cutting into the maxillary process) had no effect on development ($n = 6$; Fig. 2A−C).

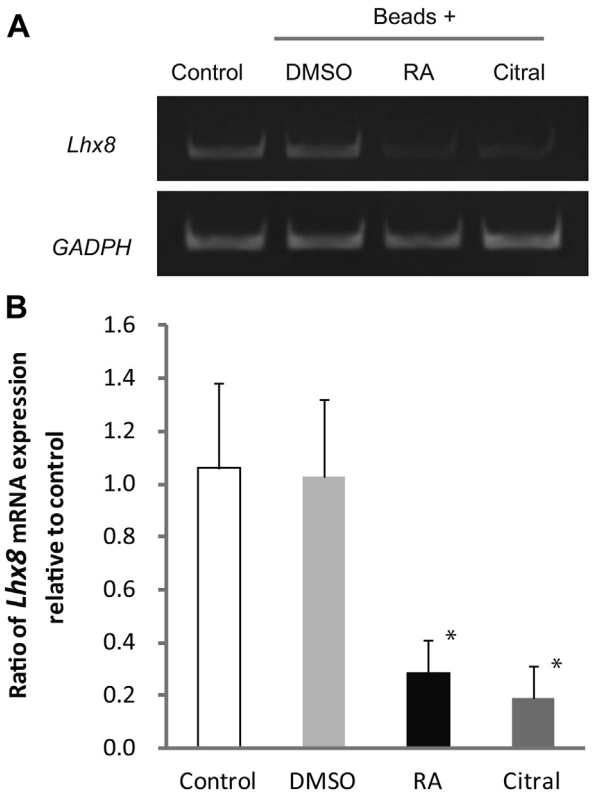

FIG. 1. RT-PCR and quantitative RT-PCR analysis of *Lhx8* mRNA in chick maxillary mesenchyme treated with RA- and citral-soaked beads. (A) Representative images of RT-PCR analysis of *Lhx8* mRNA. GAPDH is shown as a loading control. These experiments were repeated at least three times with similar results. (B) Real-time RT-PCR analysis of *Lhx8* mRNA. Values are the mean ± S.D. in separate experiments using five animals. * $p < 0.05$, significantly different from the corresponding the value of DMSO beads.

To investigate the effects of RA deficiency on maxillary morphogenesis, we implanted beads soaked with citral that inhibits a number of dehydrogenase enzymes necessary for RA synthesis. The *Lhx8* mRNA level in maxillary tissues treated with 0.1 g/ml citral was significantly lower than that in the control treated with DMSO (Fig. 1A, B). The level of mRNA was further suppressed than that in RA treated maxillary mesenchyme. The embryo at HH stage 38 showed the ablated upper beak, including lateral nasal prominence derivatives and maxillary prominence derivatives ($n = 9$; Fig. 2G−I). Significant abnormalities were found in the maxillary, palatine, and jugal bones. The eye dropped into the defect due to lack of an orbital ridge. These severe defects of the maxillary prominence derivates may be correlated with the decreases of *Lhx8* expression.

Although inhibition of *Lhx8* expression by excess RA or citral likely resulted in the retardation of normal development of the maxillary process, an alternative explanation was that mechanical injury with beads implantation caused an increase in cell death. The previous study supported transient cell death around the beads after implantation, but not induction of massive cell death (data not