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Id2 controls chondrogenesis acting downstream of BMP signaling during maxillary morphogenesis

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ABSTRACT

Maxillofacial dysmorphogenesis is found in 5% of the population. To begin to understand the mechanisms required for maxillofacial morphogenesis, we employed the inhibitors of the differentiation 2 (Id2) knock-out mouse model, in which Id proteins, members of the regulator of basic helix–loop–helix (bHLH) transcription factors, modulate cell proliferation, apoptosis, and differentiation.

We now report that spatially-restricted growth defects are localized at the skull base of Id2 KO mice. Curiously, at birth, neither the mutant Id2 KO nor wild-type (WT) mice differed, based upon cephalometric and histological analyses of cranial base synchondroses. In postnatal week 2, a narrower hypertrophic zone and an inhibited proliferative zone in presphenoid synchondrosis (PSS) and spheno-occipital synchondrosis (SOS) with maxillary hypoplasia were identified in the Id2 mutant mice. Complementary studies revealed that exogenous bone morphogenetic proteins (BMPs) enhanced cartilage growth, matrix deposition, and chondrocyte proliferation in the WT but not in the mutant model. Id2-deficient chondrocytes expressed more Smad7 transcripts.

Based on our results, we assert that Id2 plays an essential role, acting downstream of BMP signaling, to regulate cartilage formation at the postnatal stage by enhancing BMP signals through inhibiting Smad7 expression. As a consequence, abnormal endochondral ossification was observed in cranial base synchondroses during the postnatal growth period, resulting in the clinical phenotype of maxillofacial dysmorphogenesis.

Introduction

Temporal and spatial information is critical for craniofacial morphogenesis, especially between forming the mandible and complementary maxilla. In human craniofacial development, morphospatial disharmony between the maxillocranial and mandibular complex results in well-recognized jaw deformities, including maxillary hypoplasia, mandibular prognathism, mandibular micrognathism, and facial asymmetry [1]. Patients with severe jaw deformities present significant masticatory dysfunctions and severe psychosocial issues. Such patients require surgical correction and postsurgical rehabilitation. The prevalence of such jaw deformities ranges from 1 to 23% according to the ethnic background of study populations [2–5]. Jaw deformities become apparent after birth, as well as being associated with first and second branchial arch syndromes, including Treacher Collins syndrome, Pierre Robin syndrome, Crouzon syndrome, cleidocranial dysplasia (CCD), achondroplasia, and Pfeiffer syndrome. These relatively rare branchial arch syndromes are readily identified at birth, and these represent less than 5% of jaw deformity cases [1]. The vast majority of such cases become clinically evident during early postnatal growth and development. Overt manifestation of the postnatal jaw deformities may not appear until after adolescence, being generally associated with increased craniofacial growth. Both environmental and genetic factors have been identified as causes of postnatal jaw deformities [6,7], and available evidence suggests that genetic factors are the major determinants to the clinical phenotype [8–10]. However, the primary cause for maxillofacial dysmorphogenesis is not known.

In order to investigate growth impairment in postnatal jaw deformities, we identified the role of cartilages in the growth and development of the craniofacial complex. Available evidence suggests that SOS and nasal septal cartilage (NSC) are derived from the chondrocranium

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[11]. Multiple synchondroses separating bones of the skull base are assumed to function as growth sites during skull base expansion. Morphologically, a synchondrosis appears at two opposing cartilage growth plates. Therefore, analogous to endochondral growth plates in long bones, synchondroses of the skull base develop through aberrations of the temporal and spatial combination of chondrocyte proliferation and hypertrophy [7]. In a mouse model, both the PSS and SOS remain patent through adulthood. Similar to the epiphyseal growth plate in long bones, the cell proliferation, differentiation, and maturation rates of chondrocytes within synchondroses are critical for the longitudinal growth of the cranial base [12].

Id proteins are members of the regulator of helix–loop–helix (HLH) transcription factors [13–15]. Transcriptional regulators with a bHLH domain regulate a broad range of cellular differentiation processes including myogenesis, neurogenesis, and hematopoiesis [16]. In embryonic and adult tissues, Id proteins act as regulators of cell proliferation, differentiation, tumorigenesis, and neoplastic transformation [17–19]. Id expression is partially regulated by BMP-Smad signaling [20,21]. BMP regulates cell fate determination, differentiation, proliferation, maturation, hypertrophy, and apoptosis of chondrocyte cells [22]. The mechanisms by which BMPs control specific cell lineages and patterns have been found to be critical for subsequent stages of development [23]. The exogenous application of BMP2 or BMP4 to embryonic maxillary mesenchymal cells resulted in a significant upregulation of Id1, Id2, and Id3 mRNA [24] and the modulation of Id1, Id2, Id3, as well as Id4 protein levels [25]. BMP2 and BMP4 induced the transcription of Id1, Id2, and Id3 genes in ES cells as well as embryos by promoting the direct binding of the BMP-responsive Smads; Smad1 and Smad5 binding to promoters of these genes [26–28]. This body of evidence suggests important functions of Id proteins during postnatal jaw growth and development.

First, we identified maxillary hypoplasia in Id2 KO mice. We advanced the hypothesis that Id2 abrogation will interfere with the transduction of BMP signaling and, thereby, contributes to maxillary hypoplasia due to abnormal endochondrial ossification in the cranial base synchondrosis during the postnatal growth period. To test our hypothesis, we utilized the Id2 KO mouse model.

Materials and methods

Mice

Id2 mutant mice [14], with a 129/Sv genetic background, were bred under a specific pathogen-free condition and used in this study. All experimental procedures were carried out according to the guidelines for animal experiments regulated by Kyoto University Graduate School of Medicine.

Image analysis of skulls

Neonatal and postnatal mice at the ages of 0, 2, and 12 weeks, respectively, were sacrificed with carbon dioxide gas. The skulls were then analyzed employing an X-ray microtomography method (SMX-100CT-SV3, Shimadzu Co., Kyoto, Japan). The means of the three-dimensional coordinates of these landmarks were used for image analyses of the skull. EDMA was used to measure localized differences between Id2 KO mice and the control group, as described previously [29]. A nonparametric statistical technique was used to evaluate the significance of differences [29].

Analysis of cell proliferation and apoptosis

BrdU (5-Bromo-2′-deoxyuridine, 05650) was injected intraperitoneally at a concentration of 50 μg/g body weight 2 h before sacrifice. Target skeletal tissues were harvested, fixed overnight at 4 °C in a 4% paraformaldehyde solution, and then decalcified in a 0.5 M EDTA solution for 2 weeks. Decalcified samples were embedded in paraffin and sectioned. BrdU-positive cells were detected with BrdU antibody. The rate calculated by expressing the number of BrdU-positive nuclei as a percentage of the total number of nuclei was defined as the proliferation index. Data are presented as the mean ± SD, and further examined with Student's *t*-test. Significance was set at 5%. Apoptotic cells were visualized and identified with the ApopTaq Plus Fluorescein In Situ Apoptosis Detection Kit S7111 (CHEMICON, USA and Canada).

In situ hybridization

In situ hybridization was performed as described previously [30]. Mouse cDNA clones were: Id2 (nt.650-939; NM010496); collagenX (nt.2893-3550; NM009925); and osteopontin (nt.486-844;NM009263).

Immunohistochemistry

Paraffin-embedded sections were subjected to immunostaining with goat polyclonal antibodies directed against Col2 (1:100) (code: 1320-01/SBA), rabbit serum against Col10 (1:200) (code: LB-0092/Lot: 812021/LSL), and primary rabbit antibodies against phosphorylated Smad 1/5/8 (1:100) (Cell Signaling Technology, MA, USA) [31].

Semi-quantitative RT-PCR analysis

Total RNA was cultured with TRIzol (Gibco-BRL, Gaitherburg, MD, USA), according to the manufacturer's instructions, and then quantitated with A260. Oligo(dT)-primed cDNA was prepared with a reverse transcriptase. For the purpose of semiquantitation, 50 ng of cDNA was serially diluted and subjected to PCR amplification with primer pairs. These primer pairs were: Id2, sense, 5′-AGCATCCCCCAGAACAAGAAGGTG-3′ and antisense, 5′-ATCGTCTTGCCCAGGTGTCGTTCT-3′; GAPDH, sense, 5′-CCATCACATCTTCCAGGAG-3′ and antisense, 5′-CCTGCTTCACCACCTTCTTG-3′; BMPR1, sense, 5′-CCTGTTGTTATAGGTCCGTTCTTTG-3′ and antisense, 5′-CGCCATTTACCCATCCATACTT-3′; BMPR2, sense, 5′-CTAACTGGAAATCGGCTGGTG-3′ and antisense, 5′-TGGGTCTCTGCTTCTCTCTGG-3′; Smad1, sense, 5′-AGCCTCTGGAATGCTGTGAGTT-3′ and antisense, 5′-TGGTTGGGGAGTGAGGGTAG-3′; Smad5, sense, 5′-TATGCCAGAACCACAGAAAGGA-3′ and antisense, 5′-ACAGCAAGAGAGGCAGGACTATG-3′; Smad6, sense, 5′-TGCTCAGCAAGGAGCCAGAC-3′ and antisense, 5′-CTGTGGTTGTTGAGTAGGATCTCCA-3′; Smad7, sense, 5′-TGCAGGCTGTCCAGATGCT-3′ and antisense, 5′-CTTGATGGAGAAACCAGGGAAC-3′. All PCR products were examined employing an electrophoretical technique that used 2% agarose gel and ethidium bromide staining. These bands were quantitated with a Bio-image analyzer (Fujix BAS2000, Fuji Photo Film, Tokyo, Japan). All the PCR data were representative from three independent experiments.

Microdissection and organ culture

P7 wild-type mice cranial base structures were dissected in Dulbecco's PBS (pH = 7.4) under a stereomicroscope. The dissected structures were cultured on Nucleopore filters at 37 °C, under a 5% carbon dioxide atmosphere, in a trowel-type organ culture containing BGJb supplemented with 200 ng/ml of BMP-2, BMP-4, and BMP-7 (R&D Systems, MN, USA). The culture medium was renewed every 2 days [31]. After the culture, explants were fixed in a 4% para-formaldehyde and formalin solution, and then processed for histological and immunohistochemical examinations.

Results

Id2 abrogation results in retarded postnatal growth of the maxillofacial complex

Adult Id2-deficient mice showed a shorter maxillofacial profile (Figs. 1A–I). Id2 KO mice present severe clinical phenotypes with