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Augmented Indian hedgehog signaling in cranial neural crest cells leads to craniofacial abnormalities and dysplastic temporomandibular joint in mice

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Abstract

Extensive studies have pinpointed the crucial role of Indian hedgehog (Ihh) signaling in the development of the appendicular skeleton and the essential function of Ihh in the formation of the temporomandibular joint (TMJ). In this study, we have investigated the effect of augmented Ihh signaling in TMJ development. We took a transgenic gain-of-function approach by overexpressing *Ihh* in the cranial neural crest (CNC) cells using a conditional *Ihh* transgenic allele and the *Wnt1-Cre* allele. We found that *Wnt1-Cre*-mediated tissue-specific overexpression of *Ihh* in the CNC lineage caused severe craniofacial abnormalities, including cleft lip/palate, encephalocele, anophthalmos, micrognathia, and defective TMJ development. In the mutant TMJ, the glenoid fossa was completely absent, whereas the condyle and the articular disc appeared relatively normal with slightly delayed chondrocyte differentiation. Our findings thus demonstrate that augmented Ihh signaling is detrimental to craniofacial development, and that finely tuned Ihh signaling is critical for TMJ formation. Our results also provide additional evidence that the development of the condyle and articular disc is independent of the glenoid fossa.

Keywords

Indian hedgehog; Cranial neural crest; Temporomandibular joint; Condyle; Glenoid fossa

Introduction

The temporomandibular joint (TMJ), as a unique bilateral diarthrosis located between the mandible and the temporal bone, is essential for jaw movements, food intake, mastication, and speech. Its sophisticated structure surrounded by a fibrous capsule includes the temporal glenoid fossa, a mandibular condyle, and a specific articulating disc that divides the joint

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Compliance with ethical standards

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cavity into the upper and the lower parts (Sperber 2001; Gu and Chen 2013). The concave-shaped glenoid fossa is adapted to the head of the condyle, allowing its free horizontal and vertical movements, with the intervening fibrocartilaginous disc buffering the stress during function.

Both the condyle and the glenoid fossa originate from cranial neural crest (CNC) cells that initially develop into two distinct mesenchymal condensations (Gu et al. 2008). Subsequently, the condylar blastema grows rapidly towards the glenoid fossa and forms bone through endochondral ossification, whereas the glenoid fossa primordium undergoes intramembranous ossification (Sperber 2001; Gu and Chen 2013). In mice, TMJ development starts at embryonic day 13.5 (E13.5) as evidenced by the appearance of the mesenchymal condensation of the condyle with *Sox9* expression (Shibata and Yokohama-Tamaki 2008; Gu et al. 2008). The blastema of the glenoid fossa forms at E14.5 as a triangle structure. At E15.5, the shapes of glenoid fossa and condyle have been primarily established, and the gap between these two anlagen continuously narrows. At E16.5, a distinct articular disc begins to form, with a layer of flat-shaped cell condensation appearing at the apex of the condyle, and the upper synovial cavity becoming discernible (Frommer 1964). Subsequently at E17.5, the lower joint cavity appears after the disc separates from the condyle. Although the tissue structures of the TMJ have been well documented, the underlying molecular mechanism regulating this complicated multi-step developmental process remains elusive.

Most studies of molecular mechanisms underlying TMJ development depend largely on gene expression assays and gene expression manipulations including loss-of- or gain-of-function approaches in the mouse model. A number of transcription factors and growth factors have been reported with regard to their essential roles in TMJ morphogenesis and growth, such as Runx2, Sox9, Shox2, Spry1 and Spry2, Bmpr1A, Tgfb β 2, and Ndst1 (Shibata et al. 2004; Fukuoka et al. 2007; Mori-Akiyama et al. 2003; Wang et al. 2011; Gu et al. 2008, 2014; Li et al. 2014; Purcell et al. 2012; Oka et al. 2007; Yasuda et al. 2010). Among them, Indian hedgehog (Ihh), a signaling molecule that plays a pivotal role in the regulation of chondrocyte proliferation, maturation, and ossification both in long-bone development and digit joint formation (St-Jacques et al. 1999), has also been found to be essential for TMJ development (Shibukawa et al. 2007; Purcell et al. 2009; Gu et al. 2014). As is well documented, Ihh signaling promotes chondrocyte proliferation but prevents its hypertrophy via the Ihh-parathyroid hormone-related protein (PTHrP) negative-feedback mechanism in long-bone growth. Ihh, produced by newly postmitotic chondrocytes, upregulates PTHrP expression in the periarticular perichondrium, and the latter activates the expression of PTHrP receptor (PTHrPR) on proliferating cells, thus keeping these cells in the proliferative state and delaying their hypertrophy process (Lanske et al. 1996; Vortkamp et al. 1996). In the developing condyle, loss-of-function approaches have revealed that Ihh not only controls the proliferation and maturation of condylar chondrocytes, but also acts through its downstream effectors of hedgehog (Hh) signaling, such as Gli2, Gli3, and Smo, to regulate the formation of the TMJ articular disc and its splitting from the condyle (Purcell et al. 2009; Shibukawa et al. 2007). In addition, Ihh is also required for the maintenance of TMJ growth and proper organization, even after it forms (Ochiai et al. 2010).

Given the essential role of *Ihh* signaling in TMJ development, as manifested primarily by loss-of-function studies, we considered the determination of the consequence of elevated *Ihh* signaling in the developing TMJ to be important. For such a purpose, we generated a conditional *Ihh* transgenic allele and targeted it to overexpress *Ihh* in CNC cells. We showed that augmented *Ihh* signaling in CNC cells caused severe craniofacial abnormalities including defective TMJ formation, indicating a requirement for finely tuned *Ihh* signaling in TMJ development.

Materials and methods

Animal and sample collection

Conditional *Ihh* transgenic mice (*pMes-Ihh*) were generated by pronuclear injection of the *pMes-Ires-Egfp* vector containing the full-length cDNA of mouse *Ihh*, as described previously (Xiong et al. 2009; He et al. 2010). The *pMes-Ihh* transgenic vector was constructed by inserting the *Ihh* sequence into the site downstream of a *LoxP*-flanked *STOP* cassette under the control of the β -*actin* promoter and upstream of the *Ires-Egfp* sequences. *Wnt1-Cre* transgenic mice (Danielian et al. 1998) and the *R26R* conditional reporter line (Soriano 1999) were obtained from Jackson Laboratories (USA). *Wnt1-Cre;pMes-Ihh* embryos were obtained by mating *Wnt1-Cre* mice with *pMes-Ihh* mice; embryos of *Wnt1-Cre;R26R* mice and *Wnt1-Cre;pMes-Ihh;R26R* mice were also obtained. Embryos were collected from the time-mated pregnant females, and embryonic day 0.5 (E0.5) was defined as being that on the morning of which the vaginal plug was discovered. Embryonic heads were harvested in ice-cold phosphate-buffered saline, fixed individually in 4 % paraformaldehyde (PFA) with diethyl pyrocarbonate (DEPC) at 4 °C overnight, and subsequently embedded in paraffin after dehydration. Tail samples were used for genotyping by methods based on the polymerase chain reaction (PCR). The animal treatments and procedures involved in this study were approved by the Institutional Animal Care and Use Committee (IACUC) of Tulane University.

Skeletal preparation, histological analyses, and LacZ staining

Skeletal preparation of the heads was performed by staining with Alizarin red and Alcian blue, as described previously (Zhang et al. 2000). For histological analysis, coronal sections of mutant and control heads were cut at 6 μ m and subjected to standard hematoxylin and eosin (H&E) staining or Azoncarmine G/Aniline blue staining, as described in Presnell and Schreibman 1997. For whole-mount β -galactosidase staining, samples were fixed with 0.2 % glutaraldehyde solution and immediately subjected to LacZ staining according to the standard protocol (Chai et al. 2000). For the β -galactosidase staining of sections, samples were fixed with 0.2 % glutaraldehyde solution and embedded in O.C.T. after sucrose dehydration. Frozen sections were obtained from the samples and then subjected to LacZ staining.

In situ hybridization, immunofluorescence, and TUNEL assay

For in situ hybridization assay, paraffin sections (10 μ m thickness) were pretreated with proteinase K and hybridized with appropriate digoxigenin-labeled probes, as described previously (St Amand et al. 2000). Transcripts were detected by color reaction with BM

purple (Roche) and counterstaining with eosin. Immunofluorescence was performed on sections of 6 μ m in thickness by using anti-Ki67 antibody (Cell Signaling Technologies, #9129) and MF-20 antibody (Developmental Studies Hybridoma Bank), respectively, following the manufacturers' instructions. After incubation with secondary antibodies (Alexa Fluor 594 goat anti-rabbit IgG or goat anti-chicken IgY, Invitrogen), the samples were counterstained with 4,6-diamidino-2-phenylindole (DAPI), and visualized under a fluorescence microscope. Ki-67-positive cells at the condyle and glenoid fossa region were counted and presented as the percentage of total cells within the defined areas. Student's *t*-test was applied to determine the significance of difference between wild-type controls and mutants ($n = 3$ for each genotype). Apoptosis analysis (Tunel) of TMJ was conducted by using the In Situ Cell Death Detection Kit (Roche) and alkaline phosphatase staining ($n = 3$ for each genotype).

Results

Forced expression of *Ihh* in CNC lineage leads to severe craniofacial abnormalities

Ihh is expressed in the developing condyle, and its mediated signaling has been shown to be required for the formation of the articular disc and for the maintenance of TMJ growth and proper organization (Ochiai et al. 2010; Purcell et al. 2009; Shibukawa et al. 2007). To further investigate the role of *Ihh* signaling in craniofacial development, especially of the TMJ, we generated a *pMes-Ihh* conditional transgenic mouse line. To achieve overexpression of *Ihh* in the CNC lineage and its derived tissues, we compounded the *Wnt1-Cre* allele with the *pMes-Ihh* allele to produce *Wnt1-Cre;pMes-Ihh* mice. All mutant mice died perinatally. Gross morphological examination revealed severe craniofacial abnormalities in the mutants as early as E9.5, including neural tube dysraphism, encephalocele, anophthalmos, cleft lip, and micrognathia (Fig. 1a–h). Alizarin red and Alcian blue staining of the mutant heads at E17.5 further revealed the loss of cranial bones, including the front bone, parietal bone, interparietal bone, and temporal bone, and hypoplasia of the maxillary and mandibular bones (Fig. 1i–l). The lack of parietal bone, which is of mesodermal origin (Jiang et al. 2002), indicates a non-cell autonomous effect of *Ihh* overexpression in CNC cells.

Histological examination showed that the size and structure of the lower incisors and molars were not changed in *Wnt1-Cre;pMes-Ihh*, as compared with the controls, but that the upper incisors appeared poorly developed, probably because of the cleft lip defect (Fig. 2a–f). Interestingly, although eyes were not observed by external view, histological sections identified internally located but poorly developed eyes, with 42.8% (18/42) of mutants exhibiting undetached eyes located in the medial axis region, 31.0 % (13/42) showing eyes detached to various degrees, and 26.2 % (11/42) with bilaterally located eyes (Fig. 2g–i). In addition, mutant mice also exhibited 100 % penetrance of cleft lip (Fig. 2b) and anterior clefting of the secondary palate (Fig. 2k, m; 51/51). However, among the mutant mice examined, 31.4 % (16/51) exhibited complete clefting of the secondary palate (data not shown).

Agenesis of glenoid fossa of TMJ in *Wnt1-Cre;pMes-Ihh* mice

The severe craniofacial defects in *Wnt1-Cre;pMes-Ihh* mice prompted us to determine whether they could be attributed to defective CNC cell migration. We compounded *Wnt1-Cre;pMes-Ihh* alleles with the *R26R* reporter allele. LacZ staining revealed comparable staining patterns and strength in CNC-derived tissues in the craniofacial region in both control and *Wnt1-Cre;pMes-Ihh;R26R* embryos at E10.5 (Fig. 3a, b), suggesting normal migration of CNC cells in the mutants. Because the major components of the TMJ, namely the condyle and glenoid fossa, are CNC derivatives (Gu et al. 2008) and the original focus of this study, we also examined CNC cells in the developing TMJ at E15.5 at which time both condyle and glenoid fossa become discernible. As shown in Fig. 3c, in control, both condyle and glenoid fossa were populated with LacZ-labeled cells. In the mutants, the condyle was present and was similarly populated by CNC cells, but the glenoid fossa was absent (Fig. 3d). The observation that the CNC-derived cells but not mesoderm-derived cells were populating the region in which the glenoid fossa was supposed to form further supported normal CNC cell migration in *Ihh*-overexpressing mice (Fig. 3d), as evidenced by the in situ hybridization assay showing an enhanced level of *Ihh* transcripts in the TMJ, especially in the condyle, as compared with controls (Fig. 3e, f). Immunofluorescence with MF-20, a muscle-specific antibody, revealed the normal differentiation of myogenic cells surrounding the dysplastic TMJ with disrupted patterning in the mutants, as compared with controls (Fig. 3g, h).

We next conducted a time-course histological examination to determine developmental defects that contributed to the dysplastic TMJ. The primordia of both condyle and glenoid fossa appeared at E14.5, as shown in controls (Fig. 4a). At this time in the mutants, although the condylar condensation appeared at the right position, and although the size was comparable with that of the controls, a definite glenoid fossa primordium failed to form, with a small condensed cell mass existing in the putative region of the glenoid fossa (Fig. 4b). Subsequently, at E15.5 and E16.5 in mutants, the glenoid fossa remained absent, but the development of the condyle seemed relatively normal as compared with controls (Fig. 4c–f). At E17.5, a well defined TMJ appeared in controls, with the ossifying glenoid fossa encompassing the apex of the condyle, and the formation of the upper and lower synovial cavities occurring separated by a compact articular disc (Fig. 4g). However, in mutants, the glenoid fossa was completely absent, with a few sporadic bony-like tissues in the presumptive glenoid fossa position (Fig. 4h). Interestingly, despite the lack of the glenoid fossa, the condyle continued to develop, and a distinct articular disc formed and became separated from the apex of the condyle, producing a discernible lower synovial cavity (Fig. 4h).

Cell proliferation and apoptosis in TMJ of *Wnt1-Cre;pMes-Ihh* mice

In order to investigate the cellular mechanisms underlying the agenesis of the glenoid fossa of the TMJ, the indexes of cell proliferation and apoptosis were assayed in the TMJ region at E14.5, because at this stage, the definite primordia of both condyle and glenoid fossa became visible in wild-type, and the histological phenotype of the glenoid fossa was first detected in mutants (Fig. 4). TUNEL assay revealed comparable patterns and numbers of apoptotic cells in the developing TMJ of control and mutant mice (Fig. 5a, b). However, cell

proliferation assay by using anti-Ki67 antibody staining showed that, although the cell proliferation indexes in the condyles did not exhibit a statistically significant difference between controls and mutants, the cell proliferation index in the presumptive glenoid fossa region of mutants was significantly lower than that in the control glenoid fossa (Fig. 5c–e). These results suggest that a reduced level of cell proliferation could contribute to glenoid fossa agenesis.

Delayed cell differentiation in the condyle of *Wnt1-Cre;pMes-Ihh* mice

The elongation of the mandibular ramus relies on the growth and endochondral ossification of the condyle. The condyle, although classified as the secondary cartilage, organizes its structure similar to that of the growth plate of the long bone that is subdivided along its main axis into four distinct zones: a fibrous articular cell layer, a polymorphic progenitor cell zone, a flattened chondrocyte zone, and a hypertrophic chondrocyte zone (Luder et al. 1988). Although we did not observe any obvious histological defect in the condyle of *Wnt1-Cre;pMes-Ihh* mice, the critical role of *Ihh* in the regulation of chondrocyte maturation prompted us to examine chondrocyte differentiation closely. We conducted in situ hybridization to examine the expression of the following markers: *Sox9*, a molecular marker for chondroprogenitors and immature chondrocytes; *Col II*, a marker for immature chondrocytes; *Col X*, a marker for hypertrophic chondrocytes; and *Runx2*, which is expressed in both the perichondrial region and hypertrophic chondrocytes. We chose to examine gene expression at E15.5 when chondrocyte maturation begins and at E16.5 when a well-differentiated condyle is present (Gu et al. 2008). At E15.5 in controls, *Sox9* expression was seen in the polymorphic and flattened cell layers at the apex side of the condyle, and *Col II* and *Col X* were detected in the immature and hypertrophic chondrocytes simultaneously (Fig. 6a, c, e), a unique feature of the rapid differentiation of progenitor cells to hypertrophic chondrocytes of the secondary cartilage (Beresford 1975). At the same stage, *Runx2* expression was observed in the perichondral region and the hypertrophic chondrocytes, and *Col I* expression was largely restricted to the perichondral region (Fig. 6g, i). In mutants at this stage, *Sox9* was much more strongly expressed in a larger domain at the apex side of the condyle (Fig. 6b). Although the expression levels of *Col II* and *Col X* in mutants appeared comparable with those of controls, the *Col II* expression domain was expanded in the mutant as compared with controls (Fig. 6c–f). In addition, although the *Runx2* expression domain and level in mutants appeared similar to those of controls, *Col I* expression exhibited an altered pattern in the mutant condyle in which *Col I* was not expressed in the perichondral region at the apex side where stronger *Sox9* expression was observed (Fig. 6b, g–j). At E16.5, similar altered gene expression patterns were retained in the mutant condyle, including persistent stronger *Sox9* expression in the apex side, an enlarged *Col II* expression domain, and a lack of *Col I* expression in the perichondral region at the apex side, as compared with controls (Fig. 6k–n, s, t). At this time point, while *Col X* retained its expression in the mutant condyle, the longer distance between the condylar apex and the beginning of the hypertrophic zone, as compared with controls, indicated a delayed terminal hypertrophy of chondrocytes (Fig. 6o, p). The reduced *Runx2* expression in the differentiating chondrocytes and increased thickness of immature progenitor cell layer (polymorphic cell layer) at E18.5 further supported a delayed hypertrophic process in the mutant condyle (Fig. 6q, r, u, v).

Discussion

Overexpression of *Ihh* in CNC cells leads to severe craniofacial abnormalities

CNC cells are multi-potential progenitor cells that give rise to a diverse array of cell types for craniofacial morphogenesis. The migration of CNC cells, following the innate pathways towards their ventral-lateral destination (Chai et al. 2000; Jiang et al. 2002), is a crucial event in the formation of craniofacial skeletons and organs. As is well documented, the cells at the lateral edge of the prosencephalon and a portion of mesencephalic region migrate to the frontonasal mass, and those emigrating from both the mesencephalon and rhombomere A migrate to the first branchial arch to construct the maxillary and mandible (Osumi-Yamashita et al. 1994; Trainor and Tam 1995). Also of CNC origin are the skull skeletal elements including the frontal bone, alisphenoid bone, part of the interparietal bone, the squamous part of the temporal bone, and the interfrontal and coronal suture mesenchyme (Rice 2008; Jiang et al. 2002). In the current study, although CNC cell migration seemed not to be affected in *Wnt1-Cre;pMes-Ihh* embryos, overexpressed *Ihh* in the CNC lineage caused a number of craniofacial abnormalities, including neural tube dysraphism manifested as encephalocele and loss of the frontal bone, parietal bone, interparietal bone, and temporal bone and hypoplasia of the maxillary and mandibular bones. Since CNC cells do not contribute to the formation of the parietal bone, overdosed *Ihh* in the CNC lineage appeared to exert both cell autonomous and nonautonomous effects on craniofacial skeletogenesis. Although the molecular and cellular basis underlying the absence of these craniofacial bones is currently unknown, the normal population of CNC cells and the lack of obviously increased cell death in the TMJ-forming site of *Wnt1-Cre;pMes-Ihh* mice suggest a failed conversion of mesenchymal cells to osteoblasts. The malformation of upper incisors (but not the lower incisors and molars), cleft lip, and cleft palate phenotypes in mutant mice indicate the sensitivity of these organs to the altered activity of Hh signaling during development.

As has been extensively reported, Sonic Hedgehog (Shh), secreted from the prechordal mesoderm, plays a critical role in dividing the single eye anlage into the two symmetric eye fields (Chiang et al. 1996; Chow and Lang 2001). In addition to the above-mentioned gross craniofacial defects, eyes were lacking in *Wnt1-Cre;pMes-Ihh* mice. Histological examination revealed internally located eyes, with close to half of the mutants bearing undetached eyes in the medial axis region. Hence, although the initial single eye field was bisected, under the influence of augmented *Ihh* signaling, it failed to separate and move to the lateral side. *Ihh* and *Shh* are homologous proteins in mammals (Ingham and McMahon 2001). Thus, despite Hh signaling being essential for the splitting of the single eye anlage, elevated Hh signaling prevents the separation of the split eye fields.

Enhanced *Ihh* signaling inhibits CNC cell condensation of glenoid fossa primordium

The development of the condyle and glenoid fossa begins as two distinct CNC-derived mesenchymal cell condensations, which then grow rapidly towards each other (Gu et al. 2008). Whereas the blastemal formation of these two major components of the TMJ occurs independently, the presence of the condyle is required for the sustained development of the glenoid fossa (Wang et al. 2011; Gu et al. 2014). However, the TMJ condyle and disc can develop in the absence of the glenoid fossa (Purcell et al. 2012). Although previous studies

of TMJ development have been largely focused on the condyle and the articular disc, developmental defects in the glenoid fossa have been reported only in a few genetically modified mouse models (Wang et al. 2011; Purcell et al. 2012; Gu et al. 2014). In these mouse models, with an absent or dislocated condyle, inactivation of *Spry1* and *Spry2*, or elevated BMPR-1A mediated signaling, the primordia of the glenoid fossa form nevertheless but became degenerated. However, in our current study, despite the normal population of CNC cells within the TMJ-forming site, the glenoid fossa primordium failed to form in *Wnt1-Cre;pMes-Ihh* mice, suggesting that augmented *Ihh* signaling inhibits the initial cell condensation of the glenoid fossa primordium. Interestingly, the condylar primordium and its subsequent development appeared normally in *Wnt1-Cre;pMes-Ihh* mice, at least at the morphological level. The distinct effects of enhanced *Ihh* signaling on the mesenchymal cell condensation of the condyle and glenoid fossa are probably attributable to the different osteogenetic processes of these two tissues, with the condyle undergoing endochondral ossification and the fossa undertaking intramembranous ossification. The lack of skull bones in *Wnt1-Cre;pMes-Ihh* mice further strengthens the idea that elevated *Ihh* signaling has specific detrimental effects on the formation of intramembranous bones.

Delayed chondrocyte differentiation in *Wnt1-Cre;pMes-Ihh* condyle

In the developing condyle of the mouse, mesenchymal condensation appears at E13.5, chondrogenic differentiation occurs at E14.5, and hypertrophy is initiated at E15.5 (Gu et al. 2008). The chondroprogenitor cells in the apical polymorphic zone are reported to be responsible for appositional growth of the condyle with rapid proliferation and chondrogenesis (Kantomaa et al. 1994), whereas the chondrocytes in lower zones undergo maturation and hypertrophy and are replaced by endochondral bone (Silbermann and Frommer 1972). Cells in the polymorphic progenitor zone and flattened zone of the condyle undergo active proliferation, are characterized by the expression of *Sox9* and *Col II* (Shibukawa et al. 2007), and might differentiate into *Col X*-expressing hypertrophic chondrocytes through a *Col II*-expressing cell intermediate (Chen et al. 2012).

In our *Wnt1-Cre;pMes-Ihh* mice, enhanced *Ihh* signaling led to distinctly up-regulated *Sox9* and *Col II* expression and the down-regulation of *Col I* in the apical polymorphic and flattened cell zones. Simultaneously, *Runx2* expression in the hypertrophic domain of the developing condyle was downregulated, and so was the increased distance between the apex of the condyle and the beginning of the *Col X* expression domain, the sign for delayed chondrocyte maturation. Indeed, consistent with the enhanced expression of *Sox9* and *Col II*, the thickness of the polymorphic layer of the mutant condyle was obviously increased, further indicating that enhanced *Ihh* signaling delays chondrogenic differentiation in the developing TMJ. Since the polymorphic zone is a major target of *Ihh* signaling activity and action (Ochiai et al. 2010), these observations are consistent with the established function of *Ihh* signaling in promoting chondrocyte proliferation but preventing chondrocyte hypertrophy via the *Ihh*-PTHrP negative-feedback mechanism (Lanske et al. 1996; Vortkamp et al. 1996). Furthermore, our work provides an additional piece of strong evidence for the development of the condyle and articular disc independently of the glenoid fossa

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References

- Beresford WA. Schemes of zonation in the mandibular condyle. *Am J Orthod.* 1975; 68:189–195. [PubMed: 1056706]
- Chai Y, Jiang X, Ito Y, Bringas PJ, Han J, Rowitch DH, Soriano P, McMahon AP, Sucov HM. Fate of the mammalian cranial neural crest during tooth and mandibular morphogenesis. *Development.* 2000; 127:1671–1679. [PubMed: 10725243]
- Chen J, Utreja A, Kalajzic Z, Sobue T, Rowe D, Wadhwa S. Isolation and characterization of murine mandibular condylar cartilage cell populations. *Cells Tissues Organs.* 2012; 195:232–243. [PubMed: 21646777]
- Chiang C, Litingtung Y, Lee E, Young KE, Corden JL, Westphal H, Beachy PA. Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function. *Nature.* 1996; 383:407–413. [PubMed: 8837770]
- Chow RL, Lang RA. Early eye development in vertebrates. *Annu Rev Cell Dev Biol.* 2001; 17:255–296. [PubMed: 11687490]
- Danielian PS, Muccino D, Rowitch DH, Michael SK, McMahon AP. Modification of gene activity in mouse embryos in utero by a tamoxifen-inducible form of Cre recombinase. *Curr Biol.* 1998; 8:1323–1326. [PubMed: 9843687]
- Frommer J. Prenatal development of the mandibular joint in mice. *Anat Rec.* 1964; 150:449–461. [PubMed: 14248316]
- Fukuoka H, Shibata S, Suda N, Yamashita Y, Komori T. Bone morphogenetic protein rescues the lack of secondary cartilage in Runx2-deficient mice. *J Anat.* 2007; 211:8–15. [PubMed: 17555546]
- Gu, S.; Chen, Y. Temporomandibular joint development. In: Huang, GT-J.; Thesleff, I., editors. *Stem cells in craniofacial development and regeneration.* New York: Wiley-Blackwell; 2013. p. 71-85.
- Gu S, Wei N, Yu L, Fei J, Chen Y. Shox2-deficiency leads to dysplasia and ankylosis of the temporomandibular joint in mice. *Mech Dev.* 2008; 125:729–742. [PubMed: 18514492]
- Gu S, Wu W, Liu C, Yang L, Sun C, Ye W, Li X, Chen J, Long F, Chen Y. BMPRIA mediated signaling is essential for temporomandibular joint development in mice. *PLoS One.* 2014; 9:e101000. [PubMed: 25093411]
- He F, Xiong W, Wang Y, Matsui M, Yu X, Chai Y, Klingensmith J, Chen Y. Modulation of BMP signaling by Noggin is required for the maintenance of palatal epithelial integrity during palatogenesis. *Dev Biol.* 2010; 347:109–121. [PubMed: 20727875]
- Ingham PW, McMahon AP. Hedgehog signaling in animal development: paradigms and principles. *Genes Dev.* 2001; 15:3059–3087. [PubMed: 11731473]
- Jiang X, Iseki S, Maxson RE, Sucov HM, Morriss-Kay GM. Tissue origins and interactions in the mammalian skull vault. *Dev Biol.* 2002; 241:106–116. [PubMed: 11784098]
- Kantomaa T, Tuominen M, Pirttiniemi P. Effect of mechanical forces on chondrocyte maturation and differentiation in the mandibular condyle of the rat. *J Dent Res.* 1994; 73:1150–1156. [PubMed: 8046103]
- Lanske B, Karaplis AC, Lee K, Luz A, Vortkamp A, Pirro A, Karperien M, Defize LH, Ho C, Mulligan RC, Abou-Samra AB, Jüppner H, Segre GV, Kronenberg HM. PTH/PTHrP receptor in early development and Indian hedgehog-regulated bone growth. *Science.* 1996; 273:663–666. [PubMed: 8662561]
- Li X, Liu H, Gu S, Liu C, Sun C, Zheng Y, Chen Y. Replacing Shox2 with human SHOX leads to congenital disc degeneration of the temporomandibular joint in mice. *Cell Tissue Res.* 2014; 355:345–354. [PubMed: 24248941]
- Luder HU, Leblond CP, Mark K von der. Cellular stages in cartilage formation as revealed by morphometry, radioautography and type II collagen immunostaining of the mandibular condyle from weanling rats. *Am J Anat.* 1988; 182:197–214. [PubMed: 3213819]

- Mori-Akiyama Y, Akiyama H, Rowitch DH, Crombrughe B de. Sox9 is required for determination of the chondrogenic cell lineage in the cranial neural crest. *Proc Natl Acad Sci U S A*. 2003; 100:9360–9365. [PubMed: 12878728]
- Ochiai T, Shibukawa Y, Nagayama M, Mundy C, Yasuda T, Okabe T, Shimono K, Kanyama M, Hasegawa H, Maeda Y, Lanske B, Pacifici M, Koyama E. Indian hedgehog roles in post-natal TMJ development and organization. *J Dent Res*. 2010; 89:349–354. [PubMed: 20200412]
- Oka K, Oka S, Sasaki T, Ito Y, Bringas PJ, Nonaka K, Chai Y. The role of TGF-beta signaling in regulating chondrogenesis and osteogenesis during mandibular development. *Dev Biol*. 2007; 303:391–404. [PubMed: 17204263]
- Osumi-Yamashita N, Ninomiya Y, Doi H, Eto K. The contribution of both forebrain and midbrain crest cells to the mesenchyme in the frontonasal mass of mouse embryos. *Dev Biol*. 1994; 164:409–419. [PubMed: 8045344]
- Presnell, J.; Schreibman, M. Humason's animal tissue techniques. 5th. Baltimore: The Johns Hopkins University Press; 1997.
- Purcell P, Joo BW, Hu JK, Tran PV, Calicchio ML, O'Connell DJ, Maas RL, Tabin CJ. Temporomandibular joint formation requires two distinct hedgehog-dependent steps. *Proc Natl Acad Sci U S A*. 2009; 106:18297–18302. [PubMed: 19815519]
- Purcell P, Jheon A, Vivero MP, Rahimi H, Joo A, Klein OD. Spry1 and spry2 are essential for development of the temporomandibular joint. *J Dent Res*. 2012; 91:387–393. [PubMed: 22328578]
- Rice DP. Developmental anatomy of craniofacial sutures. *Front Oral Biol*. 2008; 12:1–21. [PubMed: 18391492]
- Shibata S, Yokohama-Tamaki T. An in situ hybridization study of Runx2, Osterix, and Sox9 in the anlagen of mouse mandibular condylar cartilage in the early stages of embryogenesis. *J Anat*. 2008; 213:274–283. [PubMed: 18624832]
- Shibata S, Suda N, Yoda S, Fukuoka H, Ohyama K, Yamashita Y, Komori T. Runx2-deficient mice lack mandibular condylar cartilage and have deformed Meckel's cartilage. *Anat Embryol (Berl)*. 2004; 208:273–280. [PubMed: 15156401]
- Shibukawa Y, Young B, Wu C, Yamada S, Long F, Pacifici M, Koyama E. Temporomandibular joint formation and condyle growth require Indian hedgehog signaling. *Dev Dyn*. 2007; 236:426–434. [PubMed: 17191253]
- Silbermann M, Frommer J. The nature of endochondral ossification in the mandibular condyle of the mouse. *Anat Rec*. 1972; 172:659–667. [PubMed: 5027572]
- Soriano P. Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nat Genet*. 1999; 21:70–71. [PubMed: 9916792]
- Sperber, G. Craniofacial development. Hamilton: Decker; 2001.
- St Amand T, Zhang Y, Semina EV, Zhao X, Hu Y, Nguyen L, Murray JC, Chen Y. Antagonistic signals between BMP4 and FGF8 define the expression of Pitx1 and Pitx2 in mouse tooth-forming anlage. *Dev Biol*. 2000; 217:323–332. [PubMed: 10625557]
- St-Jacques B, Hammerschmidt M, McMahon AP. Indian hedgehog signaling regulates proliferation and differentiation of chondrocytes and is essential for bone formation. *Genes Dev*. 1999; 13:2072–2086. [PubMed: 10465785]
- Trainor PA, Tam PP. Cranial paraxial mesoderm and neural crest cells of the mouse embryo: co-distribution in the craniofacial mesenchyme but distinct segregation in branchial arches. *Development*. 1995; 121:2569–2582. [PubMed: 7671820]
- Vortkamp A, Lee K, Lanske B, Segre GV, Kronenberg HM, Tabin CJ. Regulation of rate of cartilage differentiation by Indian hedgehog and PTH-related protein. *Science*. 1996; 273:613–622. [PubMed: 8662546]
- Wang Y, Liu C, Rohr J, Liu H, He F, Yu J, Sun C, Li L, Gu S, Chen Y. Tissue interaction is required for glenoid fossa development during temporomandibular joint formation. *Dev Dyn*. 2011; 240:2466–2473. [PubMed: 21953591]
- Xiong W, He F, Morikawa Y, Yu X, Zhang Z, Lan Y, Jiang R, Cserjesi P, Chen Y. Hand2 is required in the epithelium for palatogenesis in mice. *Dev Biol*. 2009; 330:131–141. [PubMed: 19341725]

- Yasuda T, Mundy C, Kinumatsu T, Shibukawa Y, Shibutani T, Grobe K, Minugh-Purvis N, Pacifici M, Koyama E. Sulfotransferase Ndst1 is needed for mandibular and TMJ development. *J Dent Res*. 2010; 89:1111–1116. [PubMed: 20554886]
- Zhang Z, Yu X, Zhang Y, Geronimo B, Lovlie A, Fromm SH, Chen Y. Targeted misexpression of constitutively active BMP receptor-IB causes bifurcation, duplication, and posterior transformation of digit in mouse limb. *Dev Biol*. 2000; 220:154–167. [PubMed: 10753507]

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Fig. 1.

Wnt1-Cre;pMes-Ihh mice exhibit severe craniofacial abnormalities. **a–h** Whole-mount images show craniofacial abnormalities in *Wnt1-Cre;pMes-Ihh* embryos as compared with controls (+/+) at embryonic day 9.5 (E9.5; **a–d**) and E16.5 (**e–h**), including encephalocele, anophthalmos, cleft lip, and micrognathia (*white arrows* encephalocele, *black arrows* anophthalmos, *asterisks* cleft lip). **i–l** Skeletal preparations show malformed and absent craniofacial bones in the mutant line (*E* eye, *T* tongue, *fr* frontal bone, *ip* interparietal bone, *Ma* mandible, *Mx* maxillary, *na* nasal bone, *ppa* prominencia pars anterior, *pr* parietal bone, *te* temporal bone)

Fig. 2. Tooth, cleft lip/palate, and eye defects in *Wnt1-Cre;pMes-Ihh* mice. **a–f** Comparison of teeth in controls and mutants showing dysplasia of upper incisors but not lower incisors and molars in mutants. **g–i** Histology and statistical illustration showing abnormally formed and mis-located eyes in *Wnt1-Cre;pMes-Ihh* mice. **j–p** Whole-mount and histological images showing cleft lip and anterior clefting of the secondary palate (*black arrow*, *asterisk* clefting of the palate, *E* eye, *P* palate, *T* tongue, *li* lower incisor, *lm* lower molar, *ns* nasal septum, *pp* primary palate, *ui* upper incisor, *um* upper molar). Bars 100 μ m

Fig. 3. Migration of CNC cells and *Ihh* overexpression in the developing TMJ of *Wnt1-Cre;pMes-Ihh* mice. **a–d** Whole-mount and section images of LacZ staining of both *Wnt1-Cre;R26R* and *Wnt1-Cre;pMes-Ihh;R26R* embryos at E10.5 (**a, b**) and E15.5 (**c, d**) show comparable migration pattern of CNC cells to the craniofacial region and the TMJ-forming site. **e, f** In situ hybridization shows *Ihh* expression in the control condyle and overexpression of *Ihh* in the TMJ region of *Wnt1-Cre;pMes-Ihh* embryos. **g, h** Immunostaining with MF-20 antibody reveals that the cells in the presumptive region of the mutant glenoid fossa are not of myogenic origin (*C* condyle, *BA1* the first branchial arch, *Gf* glenoid fossa, *LP* lateral pterygoid muscle, *Mb* midbrain and *Tm* temporal muscle). Bars 100 μ m

Fig. 4.
a–h Agenesis of the glenoid fossa in the *Wnt1-Cre;pMes-Ihh* TMJ. Hematoxylin and eosin staining of histological sections of control and *Wnt1-Cre;pMes-Ihh* mice at various time points showing failed formation of glenoid fossa primordium and development of the condyle and articular disc in mutants (*black arrows* articular disc, *asterisks* residual mesenchymal cell condensation, *C* condyle, *M* Meckel's cartilage, *Gf* glenoid fossa, *lc* lower cavity, *LP* lateral pterygoid muscle, *uc* upper cavity). *Bars* 100 μ m

Fig. 5. Defective cell proliferation but unaltered apoptosis in the mutant TMJ. **a, b** Tunel assay shows unaltered cell apoptosis in the TMJ region of E14.5 *Wnt1-Cre;pMes-lhh* mice as compared with controls (*black arrows* apoptotic cells). **c–e** Immunostaining (*red*) for Ki-67 (*white boxes* higher magnification views in *bottom insets*, *blue* nuclear staining with DAPI, *C* condyle, *M* Meckel’s cartilage, *Gf* glenoid fossa) and statistical analysis showing reduced cell proliferation in the glenoid-fossa-forming region but not in the condyle of mutants as compared with controls (*ns* no statistical difference); **P*<0.001. *Bars*100 μm

Fig. 6. Altered gene expression in developing condyle of *Wnt1-Cre;pMes-Ihh* mice. **a–j** In situ hybridization assay showing gene expression in condyle of controls and mutants at E15.5. **k–t** In situ hybridization assay showing gene expression in condyle of controls and mutants at E16.5 (*double-head arrows* distance from apex of condyle to hypertrophic zone). **u, v** Histological sections showing increased thickness of polymorphic progenitor cell zone

(*black line*) in mutant condyle as compared with controls at E18.5. Bars 100 μm (**a–t**), 20 μm (**u, v**)

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