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Meckel's Cartilage Differentiation Is Dependent on Hedgehog Signaling

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Abstract
The hedgehog (Hh) signaling pathway has been shown to be essential for craniofacial development. Although mandibular arch derivatives are largely absent in *Shh* null mice, little is known about the role of Hh signaling during Meckel's cartilage development per se. Mandible development is dependent on the morphogenesis of Meckel's cartilage, which then serves as a template for subsequent skeletal differentiation. In this study, we examine the biological function of Hh signaling during Meckel's cartilage development in vivo and in vitro. E13.5 *Shh* null mice present a small mesenchymal condensation in the region of a presumptive Meckel's cartilage in the hypoplastic mandibular arch. By E15.5, the *Shh* mutant exhibits a mere remnant of the mandibular arch, without evidence of Meckel's cartilage differentiation. Further, wild-type embryonic (E11 or E12) mandibular explants cultured for up to 5 days in the presence of cyclopamine, a steroidal alkaloid that specifically disrupts the Hh signaling pathway, exhibit a stage-dependent inhibition of Meckel's cartilage chondroblast differentiation to mature chondrocytes. This phenotype can be rescued by exogenous FGF8, a downstream effector of Hh signaling. Taken together, our results indicate that the Hh signaling pathway is critical to Meckel's cartilage ontogenesis and the rate of chondrogenesis, but not to initial primordium formation. The reliance on Hh signaling is stage dependent.

Abbreviations used in this paper

BSA	bovine serum albumin
Cdk	cyclin-dependent kinase
E	embryonic day
FGF	fibroblast growth factor
Hh	hedgehog
Hh/PTHrP	hedgehog/parathyroid hormone-related protein
MB	mandibular bone
Mc	Meckel's cartilage
PBS	phosphate-buffered saline
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
Ptc	patch
Shh	sonic hedgehog
SMG	submandibular salivary gland
Smo	smoothened

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Introduction

Hedgehog (Hh) signaling plays critical morphoregulatory roles during embryonic development [see reviews by Ingham and McMahon, 2001; McMahon et al., 2003]. Hh participates in cell proliferation, survival, differentiation and pattern formation in numerous embryonic tissues, including the nervous system, limb, lung, and craniofacial structures. The Hh signal is transduced by two transmembranous proteins, Patch (Ptc), the Hh receptor, and smoothened (Smo). By E10 in the mouse, Hh, Ptc and Smo are expressed in the mandibular arch [Jeong et al., 2004]. In the absence of Hh binding, Ptc inhibits Smo activity. Hh binding to Ptc relieves Ptc's repression of Smo, thereby allowing Smo to induce downstream targets, including the cubitus interruptus/Gli family of transcription factors [see reviews by Ingham and McMahon, 2001; McMahon et al., 2003]. In vertebrates, there are three Gli proteins (Gli 1–3) which exhibit distinct yet partially redundant functions [Ruiz and Altaba, 1999; Ruiz et al., 2002]. Gli 1 acts as a transcription activator whereas Gli 2 and Gli 3 can act as activators or repressors of transcription, depending on the cellular conditions. Gli 2 and Gli 3 are the primary mediators of the Hh signal, with Gli 1 being secondarily activated by Hh-activated Gli 3 [Sasaki et al., 1997; Dai et al., 1999; Ruiz and Altaba, 1999; Sasaki et al., 1999; Ruiz et al., 2002].

Numerous gene targeting and in vitro studies have clearly demonstrated the importance of Hh signaling for the development of a wide variety of embryonic tissues [see reviews by Ingham and McMahon, 2001; McMahon et al., 2003]. Of particular note is the observation that *Shh* null mice exhibit complete absence of craniofacial skeletal structures [Chiang et al., 1996]. Significant reductions in mandibular development were also seen in chick embryos injected in vivo with anti-Shh antibody [Ahlgren and Bronner-Fraser, 1999] and in mouse embryos treated with jervine, a steroid alkaloid known to inhibit Shh signaling [ten Berge et al., 2001]. Although these results indicate that Hh signaling plays an important role during mandibular development, there remain questions regarding the precise relationship of Hh signaling and the initiation, growth, and chondrogenesis of Meckel's cartilage.

Mandibular development is dependent on the presence of Meckel's cartilage which serves as a template for subsequent differentiation of the bony mandible. Meckel's cartilage development starts as an aggregation of cranial neural-crest-derived mesenchymal cells at the molar tooth bud region [Ito et al., 2002]. Meckel's cartilage grows anteriorly and posteriorly to develop the 'wishbone-like' structure, with cranial neural crest-derived prechondrocytes being found at the chondrogenic front. Although cranial neural-crest-derived and non-neural-crest-derived cells contribute to Meckel's cartilage [Chai et al., 2000; Ito et al., 2002], it has become apparent that neural-crest-derived cells play the primary role in Meckel's cartilage initiation, growth and chondrogenesis.

The function of Hh signaling during Meckel's cartilage development is presently unclear. Jeong et al. [2004] removed Hh responsiveness specifically in the mouse neural crest cells. Their results indicate that Hh signaling is not necessary for the generation and migration of neural crest cells, but is critical for postmigratory craniofacial development. Thus, given that Shh signaling is essential for neural-crest-derived mesenchymal cell proliferation and survival [Seleck et al., 1998; Ahlgren and Bronner-Fraser, 1999; Testaz et al., 2001; Jeong et al., 2004] and that Meckel's cartilage development is dependent on cranial neural-crest-derived cells [Chai et al., 2000; Ito et al., 2002], it is reasonable to postulate that the absence of Meckel's cartilage in *Shh* null mice is due to an insufficient number of neural-crest-derived cells within the mandibular arch. The size of the ectomesenchymal condensation is critical to the initiation of chondrogenic or osteogenic differentiation [Hall and Miyake, 2000]. Jeong et al. [2004] have recently provided a likely mechanistic explanation for the *Shh* null mandibular phenotype: Shh supports cell survival during early stages (<E11) and promotes proliferation at later stages (>E11). Thus, increased apoptosis results in apparent aplasia of Meckel's cartilage, an early pathogenetic event. However, the observation of hypoplastic Meckel's cartilage development, not absence, with reduced Hh signaling in the *Smo* conditional mutant [Jeong et al., 2004], suggests that the Hh signaling pathway may also more directly regulate Meckel's chondrogenesis.

In the present study, we investigate the phenotypic outcomes of reduced Hh function on Meckel's cartilage development in vivo and in vitro. Analysis of craniofacial development in E13.5 *Shh* null mice demonstrates a small mesenchymal condensation within the hypoplastic mandibular arch. By E15.5, only a small remnant of the mandibular arch is seen without evidence of either Meckel's cartilage or mandibular bone being found – a true aplasia. To understand the role of Hh signaling in Meckel's cartilage chondrogenesis per se, we cultured E11 and E12 mandibular explants with and without cyclopamine, a steroid alkaloid that specifically inhibits the Hh pathway by interacting with Smo [Cooper et al., 1998; Incar-

dona et al., 1998; Taipale et al., 2000; Chen et al., 2002; Jaskoll et al., 2004a]. Cyclopamine treatment results in a substantial stage-dependent inhibition of Meckel's cartilage chondrocyte differentiation. This abnormal phenotype can be rescued by exogenous FGF8, a downstream effector of Hh signaling. It appears, then, that Hh signaling is critical to Meckel's cartilage ontogenesis and differentiation, but not initial primordium formation (mesenchymal condensation).

Materials and Methods

Characterization of Shh *Null Mice*

Shh+/– mice were purchased from The Jackson Laboratory, Bar Harbor, Me., USA (Stock Shh<tm1>, #JR3318) and mated. The resulting genotypes were verified by PCR as previously described [Mahlapuu et al., 2001]. The microscopic anatomy of E13.5, E15.5 and E18.5 *Shh* wild-type and null mice was determined by routine hematoxylin and eosin staining.

Culture System

Female B10A/SnSg mice, obtained from Jackson Laboratories (Bar Harbor, Me., USA), were maintained and mated as previously described [Jaskoll et al., 2002]; plug day = day 0 of gestation. Pregnant females were euthanized by cervical dislocation on days 11–12 of gestation (E11–E12). Embryos were dissected in cold phosphate-buffered saline (PBS) and staged according to Theiler [1989]. Mouse mandibles were cultured in serumless, chemically defined medium according to methods from our laboratory [Melnick et al., 2001]. Briefly, E11 and E12 mouse mandibular arches were dissected and explanted using a modified Trowell method. The medium consisted of BGJb (Life Technologies, Rockville, Md., USA) supplemented with 1% BSA, 0.5 mg ascorbic acid/ml and 50 units penicillin/streptomycin (Life Technologies), pH 7.2, and replicate cultures were changed every other day. Cyclopamine, a steroid alkaloid previously shown to block Hh signaling [Cooper et al., 1998; Incardona et al., 1998; Kim and Melton, 1998; Taipale et al., 2000; Chen et al., 2002], was used to abrogate Hh signaling. For these interruption studies, 250 μ*M* cyclopamine was dissolved in 100 μl 95% ethanol + 900 μl BGJb and then diluted in the enriched BGJb described above to yield a 10 μ*M* concentration. This concentration has been shown in our laboratory to be the optimal dose to interrupt embryonic salivary gland development [Jaskoll et al., 2004b]. Mandibles were cultured in 10 μ*M* cyclopamine or enriched BGJb for up to 5 days, with media changed every 2 days. For each experiment, we evaluated 3–6 explants per treatment. The explants were collected and processed for routine hematoxylin and eosin histology, whole-mount staining or cell proliferation analysis.

For the rescue experiment, E11 mandibular arches were cultured in 10 μ*M* cyclopamine for an initial period of 3 h and then cultured in 10 μ*M* cyclopamine, with or without FGF8 peptide (8 μg/ml or 0.8 μg/ml; R & D Systems, Minneapolis, Minn., USA) for 5 days. Control E11 + 5 explants were also concurrently cultured in enriched BGJb. Explants were collected and evaluated by whole-mount staining or histological analysis. Selected explants were sectioned and stained with hematoxylin and eosin or with alizarin red [Presnell et al., 1997]. For the rescue experiment, we evaluated 6–8 explants for each treatment.

Cell Proliferation Assay

Total cell number and cell proliferation index was determined as previously described [Melnick et al., 2001]. Briefly, E11 + 2 control and cyclopamine-treated explants were sectioned, incubated with anti-proliferating cell nuclear antigen (anti-PCNA) using the Zymed mouse PCNA kit, and counterstained with hematoxylin. Four sections per explant and 3 explants per group were photographed at 200× and the number of cells/mm^2 and proliferation index (PCNA-positive epithelial cells/total cells/mm^2) were determined. The data were arcsin transformed to insure normality and homoscedasticity, and the mean ratios compared by t test [Sokal and Rohlf, 1981].

Whole Mount

E11 + 5 control (n = 6), cyclopamine-treated (n = 6), and cyclopamine + FGF8 (n = 3) mandibles were examined employing whole-mount staining with Alcian blue which stains for cartilage and alizarin red which stains for bone basically as previously described [Jaskoll and Melnick, 1982]. For the first set of experiments, explants were rinsed in PBS, fixed and stained overnight in Alcian blue/95% ethyl alcohol/glacial acetic acid. The explants were then destained in graded ethanols, rinsed in distilled water, and stained/cleared overnight in 0.1% KOH containing 12 drops of alizarin red solution (2 g alizarin red in 100 ml distilled water). The explants were then further cleared and preserved in several changes of graded 1% KOH/glycerin solutions (50:50 KOH/glycerin, 30:70 KOH/glycerin, 5:95 KOH/glycerin) and stored in 100% glycerin. For the rescue experiments, we modified the protocol and prolonged the time of alizarin red staining so that these explants were stained for 48 h in 0.01% KOH containing 12 drops of the alizarin red.

Results

Analysis of Shh *Null Mice*

We evaluated craniofacial development in E13.5, E15.5 and E18.5 *Shh* wild-type *(Shh+/+)* and null *(Shh–/–)* mice (fig. 1, 2). On all days analyzed, craniofacial abnormalities are seen in *Shh* null mice, including holoprosencephaly, a severely hypoplastic mandibular arch, microstomia, and low-set ears (fig. 1, 2). Although the E13.5 wild-type mouse mandible is characterized by the presence of Meckel's cartilage, the tongue primordium, and tooth buds, only a small mesenchymal condensation is seen in the region of the mandibular arch in the *Shh* null mouse (compare fig. 1c–1e). By E15.5, mandibular bone has formed around Meckel's cartilage in the wild-type mouse; however, mandibular bone, Meckel's cartilage, the tongue primordium and tooth buds are absent from the null mice (compare fig. 1d–1f). By E18.5, only a remnant of the mandibular arch with no differen-

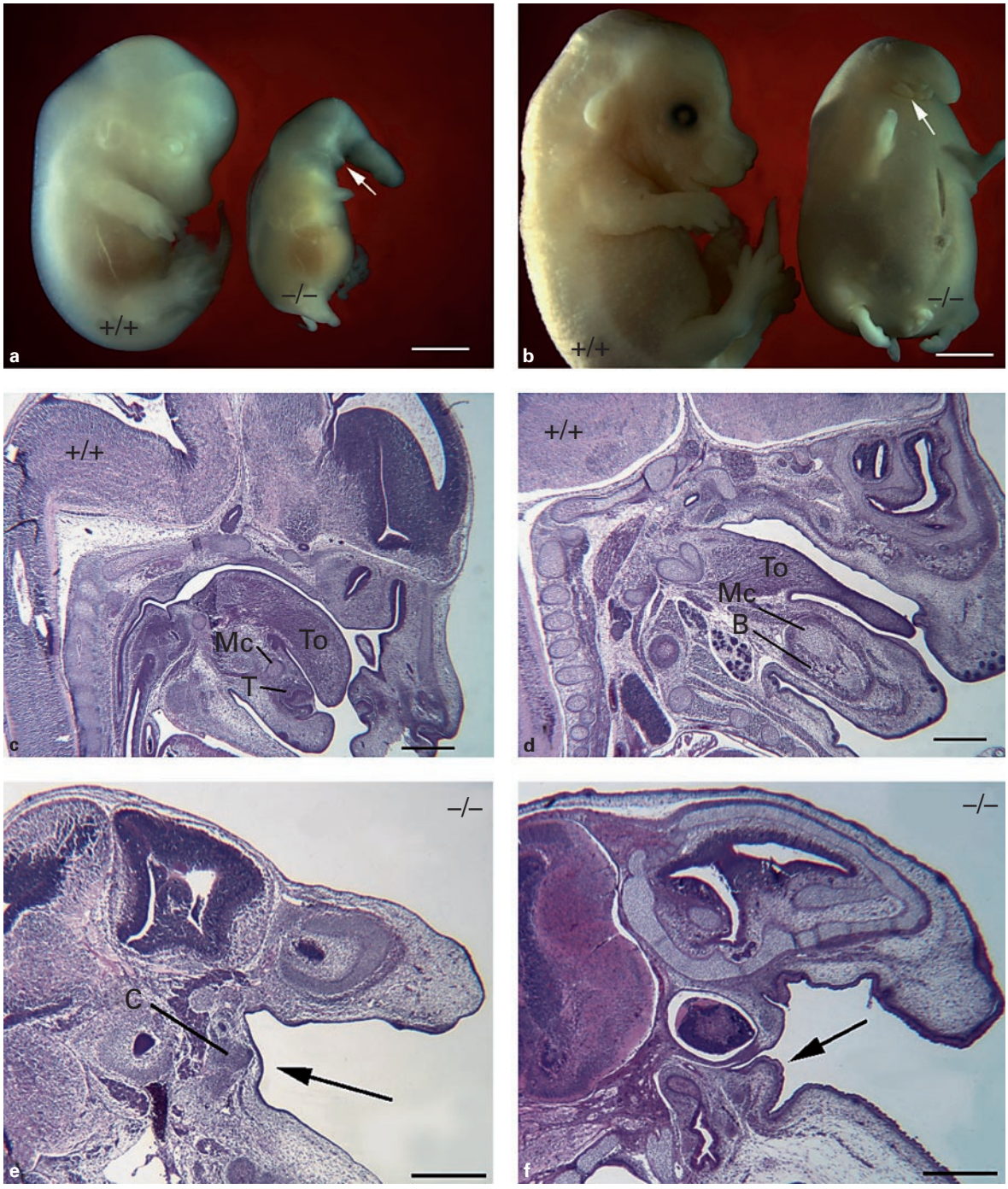


Fig. 1. *Shh* null mice exhibit severely abnormal craniofacial structures. **a, c** E13.5 wild-type *Shh*+/+ mouse. **a**, **e** E13.5 *Shh*–/– mouse. **b**, **d** E15.5 wild-type *Shh*+/+ mouse. **b**, **f** E15.5 *Shh*–/– mouse. In the *Shh*–/– mice (**a**, **b**, **e**, **f**), a small primitive mandibular arch is detected (arrow). In E13.5 *Shh* null mice (**e**), a small mesenchymal condensation (C) is seen in the primitive mandibular arch (arrow). By E15.5 (**f**), no mesenchymal condensation is seen within the *Shh* null primitive arch (arrow). This contrasts with the well-differentiated Meckel's cartilage (Mc) and bone (B) seen in the wild-type *Shh* mice (**d**). T = Tooth; To = tongue. **a**, **b** Bar: 200 mm. **c**–**f** Bar: 500 μm.

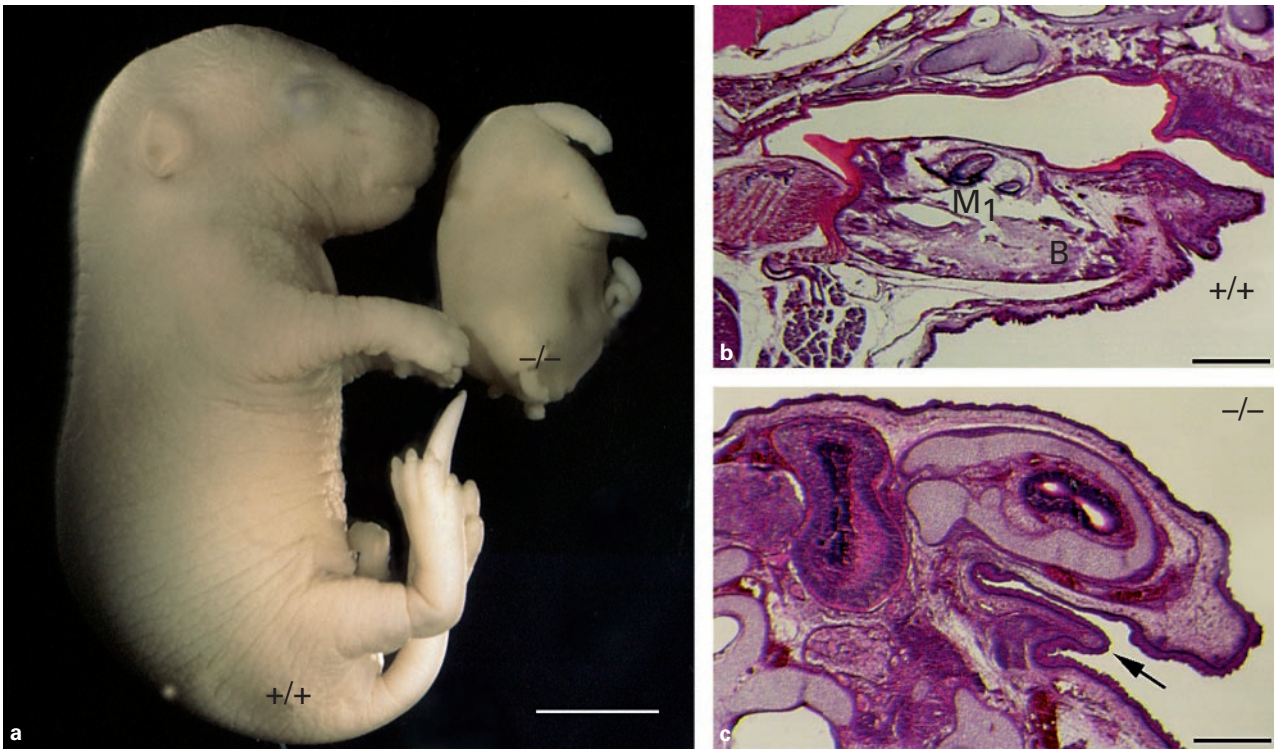


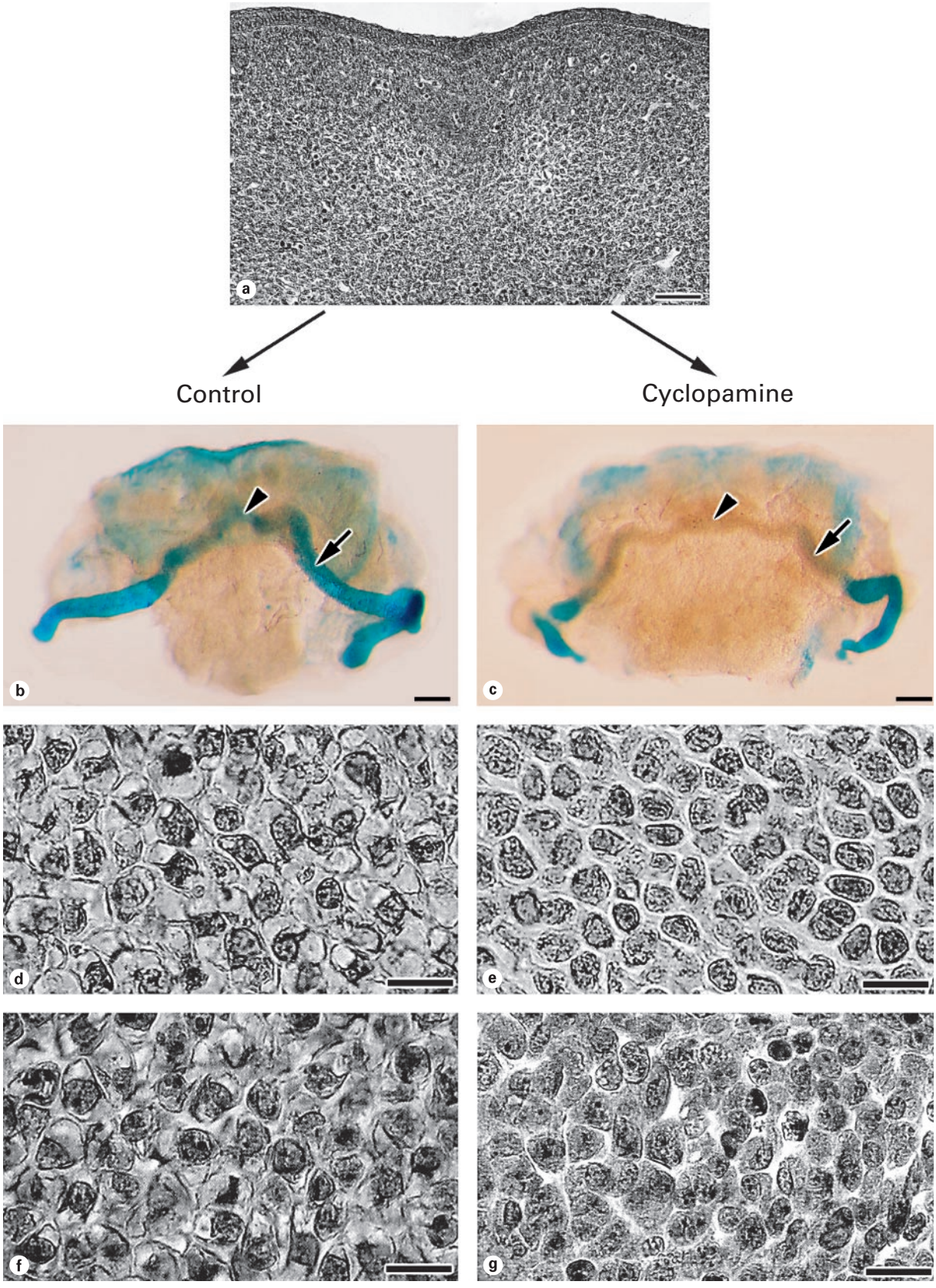
Fig. 2. Mandibular derivatives are absent from E18.5 *Shh* null mice. **a**, **b** E18.5 wild-type *Shh*+/+ mouse. **a**, **c** E18.5 *Shh*–/– mouse. In the E18.5 wild-type mouse (**b**), the lower jaw is characterized by the presence of mandibular bone (B), tongue, and tooth bud (M1). By contrast, these structures are all absent from the *Shh* null mouse (**c**); instead, there remains a largely undifferentiated remnant of the mandibular arch (arrow). **a** Bar: 2 mm. **b**, **c** Bar: 500 μm.

tiated structures is seen in the *Shh* null mouse; the *Shh* wild-type mouse has a well-differentiated bony mandible and teeth (compare fig. 2b–2c). These results indicate that Shh signaling plays an essential role in the development of mandibular derivatives, including the development of Meckel's cartilage. Importantly, the mesenchymal condensation associated with subsequent Meckel's cartilage formation (the primordium) is present even in the absence of Shh signaling, even though it is ultimately resorbed – a true aplasia.

Effect of Endogenous Hh Signaling on Meckel's Chondrogenesis

To investigate the functional role of Hh during Meckel's cartilage chondrogenesis, we cultured embryonic mandibles and inhibited endogenous Hh signaling with cyclopamine, an antagonist which specifically binds to Smo to abrogate Hh signal transduction [Cooper et al., 1998; Incardona et al., 1998; Taipale et al., 2000]. Cyclopamine has previously been used to successfully interrupt the development of other mandibular derivatives (e.g., tongue and submandibular gland) in vitro [Hall et al., 2003; Mistretta et al., 2003; Jaskoll et al., 2004a; Liu et al., 2004]. In the first set of experiments, E11 mandibles

Fig. 3. Abrogated Hh signaling in vitro inhibits Meckel's cartilage differentiation. **a** Histological section demonstrating the absence of mesenchymal condensations in the wild-type E11 mandibular arch. **b**, **d**, **f** Control E 11 + 5 explants. **c**, **e**, **g** Cyclopamine-treated E11 + 5 explants. **b**, **c**. Whole-mount control (**b**) and cyclopamine-treated (**c**) explants stained with Alcian blue. Meckel's cartilage in the control explant (**b**) appears as a 'wishbone-like' structure completely stained with Alcian blue. In contrast, cyclopamine treatment (**c**) explants are characterized by the absence of Alcian blue staining in the anterior two thirds and the presence of Alcian blue staining in the posterior third of Meckel's cartilage. **d**–**g** Histological sections of the region indicated by the arrow head (**d**, **e**) and arrow (**f**, **g**). Meckel's cartilage of E11 + 5 control explant is composed of chondrocytes (**d**, **f**); the cyclopamine-treated E11 + 5 explant exhibits chondroblasts (**e**, **g**). **a** Bar: 25 μm. **b**, **c** Bar: 60 μm. **d**–**g** Bar: 25 μm.



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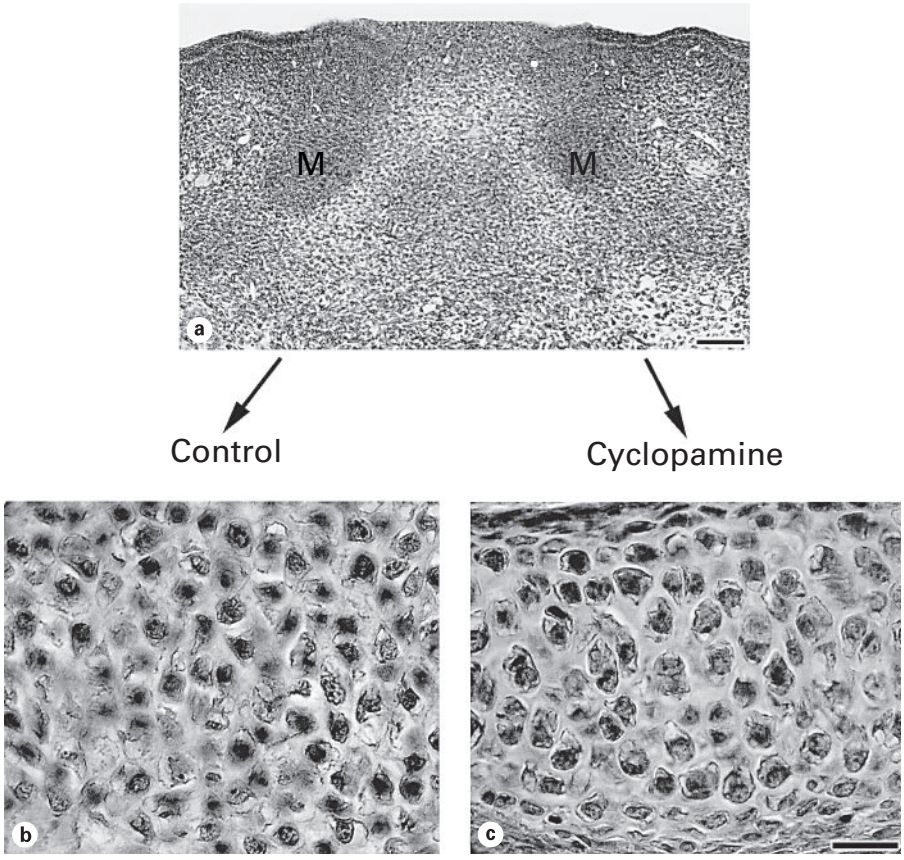

Fig. 4. Analysis of E12 + 4 control and cyclopamine-treated explants. **a** Mesenchymal condensations (M) are found in the E12 mandibular arch. **b**, **c** Chondrogenesis is seen in both E12 + 4 control (**b**) and cyclopamine-treated (**c**) explants. Bar: 25 μm.

were cultured in control or cyclopamine-supplemented medium for 5 days and the development of Meckel's cartilage was assayed. At E11, no mesenchymal condensation is seen (fig. 3a). In the control E11 + 5, Meckel's cartilage displays the characteristic 'wishbone-like' structure which is completely stained with Alcian blue (fig. 3b). In contrast, cyclopamine treatment results in a marked inhibition of Meckel's cartilage differentiation (fig. 3c). Although a seemingly appropriate mesenchymal condensation in a 'wishbone-like' formation is seen, Alcian blue stain is only found in the posterior one third of the presumptive Meckel's cartilage. Histological analysis of control explants demonstrates that Meckel's cartilage is composed throughout of chondrocytes (fig. 3d, f). In cyclopamine-treated explants, only chondroblasts are found in the anterior two thirds (fig. 3e, g) of the presumptive Meckel's cartilage. These results suggest that Hh signaling is essential for the transition of chondroblasts to chondrocytes in the anterior, but not more posterior, region of Meckel's cartilage.

To determine if the influence of Hh signaling on Meckel's cartilage differentiation is stage dependent, we then cultured E12 mandibular arches in control or cyclopamine-supplemented medium for 4 days and compared their phenotypes to that seen in E11 + 5 explants. We chose E12 because mesenchymal condensation of Meckel's is seen on that gestational day (fig. 4a) [Miyake et al., 1996]. Chondrogenesis is seen in both E12 + 4 control and cyclopamine-treated explants, with no differences being seen between control and cyclopamine-treated explants (fig. 4). Thus, we see a notable stage-dependent effect of cyclopamine treatment: after 4 days, E12 explants exhibit a normal cartilage phenotype compared with the abnormal Meckel's cartilages seen in E11 + 5 explants (compare fig. 3e, 4c).

Cell Proliferation and Meckel's Cartilage Morphogenesis

Many studies have indicated that the size of the mesenchymal condensation (i.e. the number of cells within it) is critical to the initiation of chondrogenesis [see re-

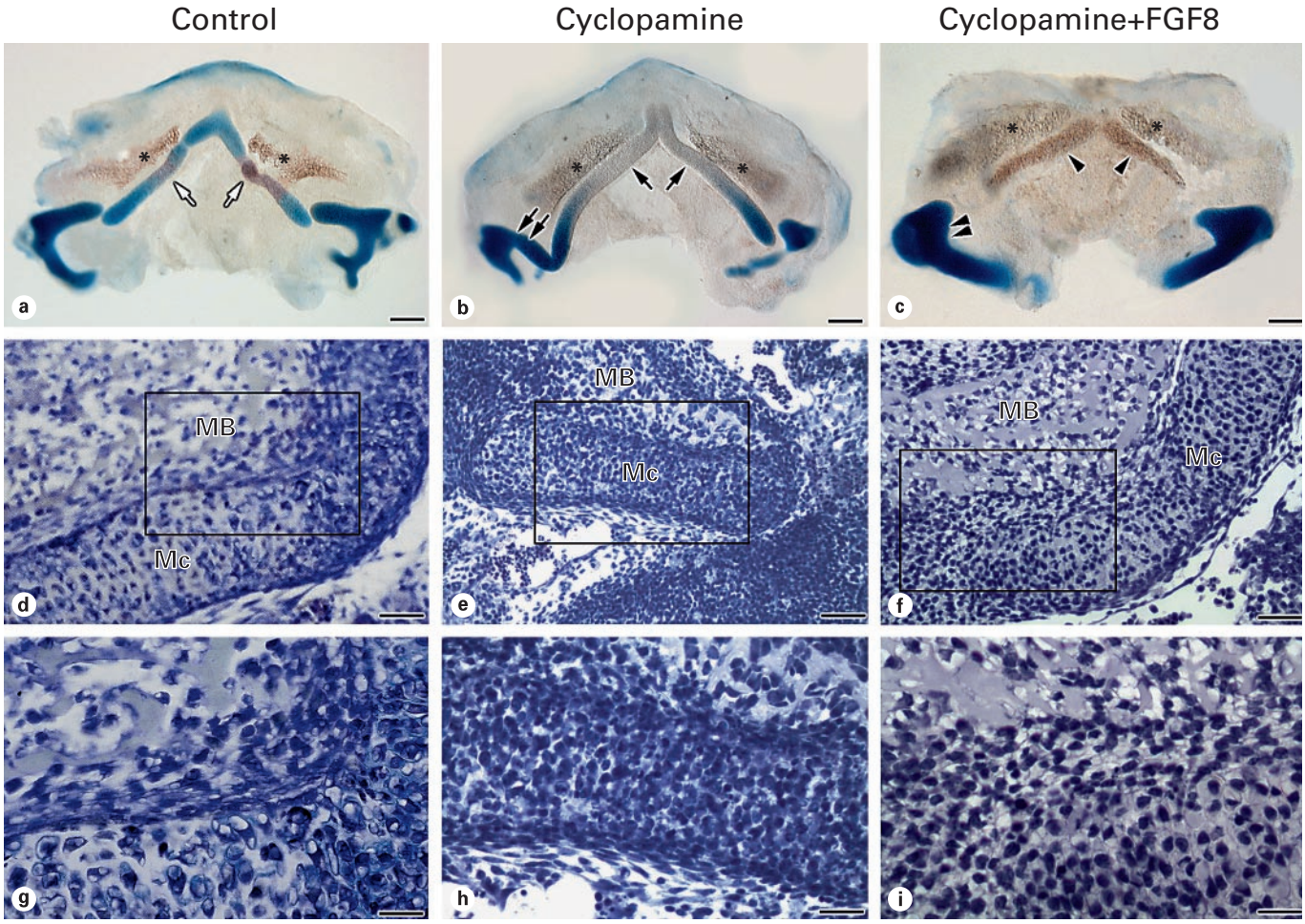

Fig. 5. FGF8 rescue of cyclopamine-treated E11 + 5 explants. **a–c** Whole-mount staining of control (**a**), cyclopamine-treated (**b**), and cyclopamine + FGF8-treated (**c**) explants. In control explants (**a**), Meckel's cartilage exhibits Alcian blue staining throughout, with weak Alizarin red staining seen in the mid region (white arrow). By contrast, Alcian blue staining is absent from the anterior two thirds (arrow) and present in the posterior third (double arrows) of Meckel's cartilage in explants treated with cyclopamine alone (**b**). The addition of FGF8 (**c**) results in alizarin red staining in the anterior two thirds of Meckel's cartilage (arrowhead), as well as a larger Alcian-blue-stained posterior cartilage (double arrowheads). * = Mandibular bone. **d–i** Histological analysis of E11 + 5 explants. **d** Meckel's cartilage (Mc) of control explants is composed of chondrocytes, with mandibular bone (MB) being seen adjacent to Meckel's cartilage perichondrium. **g** Higher magnification of the region shown in box in **d**. **e** Meckel's cyclopamine-treated explants are composed of chondroblasts. **h** Higher magnification of the region shown in box in **e**. **f** Meckel's cartilage of cyclopamine + FGF8-treated explants is composed of hypertrophic and calcified cartilage, with trabecular bone expanding from mandibular bone to intrude into it. **i** Higher magnification of region shown in box in **f**. **a–c** Bar: 60 μm. **d–f** Bar: 50 μm. **g–i** Bar: 25 μm.

view by Hall and Miyake, 2000]. Since Hh has been shown to act as a mitogenic factor [Cobourne et al., 2001; Long et al., 2001; Gritli-Linde et al., 2002; Jaskoll et al., 2004a], we cultured E11 mandibular explants for 2 days (E11 + 2) in the presence or absence of cyclopamine treatment and used PCNA-positive nuclei as an indicator of cell proliferation. The cell number (cells/ mm^2) and proliferation index (PCNA-positive cells/total cells/mm^2) of Meckel's cartilages were calculated for control and cyclopamine-treated explants. No differences in mean cell number ($p > 0.5$) or cell proliferation index ($p > 0.1$) were seen between control and cyclopamine-treated explants (data not shown).

FGF8 Rescue of Cyclopamine-Treated Explants

Analysis of mutant mice indicates that FGF8, a putative downstream target of Hh signaling, plays an important role in the development of most mandibular arch

derivatives [Trumpp et al., 1999; Abu-Issa et al., 2002; Frank et al., 2002; Macatee et al., 2003; Jaskoll et al., 2004b; Jaskoll, Melnick and Moon, unpubl. obs.]. Importantly, Meckel's cartilage is absent or severely hypoplastic in *Fgf8* hypomorphs [Abu-Issa et al., 2002] or mutant mice with ablation of *Fgf8* from the first branchial arch [Trumpp et al., 1999] or oral epithelium [Macatee et al., 2003; Jaskoll, Melnick and Moon, unpubl. obs.]. In addition, a recent study demonstrates that *Shh* and *Fgf8* act synergistically to promote cartilage outgrowth of the craniofacial skeletal components [Abzhanov and Tabin, 2004]. Further, exogenous FGF8 peptide was able to rescue the abnormal phenotype seen in cyclopamine-treated submandibular salivary glands (SMG), another mandibular arch derivative [Jaskoll et al., 2004a]. Given this, we postulated that exogenous FGF8 peptide could rescue the Meckel's cartilage phenotype seen in cyclopamine-treated explants. We cultured E11 mandibular primordia for 5 days in the presence of cyclopamine, with and without exogenous FGF8 peptide. In this set of experiments, the whole-mount staining protocol was modified to extend the time period of alizarin red staining.

In the presence of 8 μg/ml FGF8 peptide, with the exception of the symphysial portion, the anterior two thirds of Meckel's cartilage stained with alizarin red (fig. 5c), an indicator of mineralization. This result markedly differs from that seen with cyclopamine treatment alone in which the anterior region stained with neither alizarin red nor Alcian blue (fig. 5b). This also differs from that seen in control E11+ 5 explants in which Meckel's cartilage primarily stained with Alcian blue, and only in the mid region with alizarin red (compare fig. 5a–c). In addition, the posterior region of Meckel's cartilage, which consistently stains with Alcian blue, is substantially larger in FGF8-rescued explants than in control or cyclopamine-treated only explants (compare fig. 5c and 5a, b). Since the posterior region of Meckel's cartilage appeared normal in cyclopamine-treated explants, the increased size in the posterior region is likely due to the stimulatory effect of exogenous FGF8 [Jaskoll et al., 2004b]. No differences were seen between cyclopamine-treated explants and explants treated with cyclopamine + 0.8 μg/ml FGF8 (data not shown).

Histological analysis demonstrates that FGF8 treatment induced Meckel's cartilage differentiation (fig. 5f, i); with FGF8 treatment, trabecular bone has also expanded from mandibular bone to intrude into Meckel's cartilage. This phenotype markedly differs from that seen with cyclopamine treatment alone (compare fig. 5f, i and 5e, h); FGF8-treated Meckel's cartilages also appear more developmentally advanced than control Meckel's cartilage which exhibit intact perichondria (compare fig. 5f, i and 5d, g).

Discussion

Hh signaling is essential to the development of many embryonic tissues, including the limb, nervous system, tooth, tongue, and salivary gland [Hardcastle et al., 1998; Dassule et al., 2000; Sarkar et al., 2000; Cobourne et al., 2001; Ingham and McMahon, 2001; McMahon et al., 2003; Mistretta et al., 2003; Jaskoll et al., 2004a; Liu et al., 2004]. Significantly, the absence of craniofacial skeletal components in *Shh* null mice [Chiang et al., 1996] indicates that Shh signaling is essential for their development. Meckel's cartilage formation is dependent on the proper differentiation of cranial neural-crest-derived cells [Chai et al., 2000; Ito et al., 2002]. Inactivation of Hh signaling in neural crest cells in *Wnt1-Cre;Smo-/c* mice results in substantial apoptosis and decreased cell proliferation within the pharyngeal arches [Jeong et al., 2004], suggesting a pathogenetic mechanism for the hypoplastic Meckel's cartilage and bony mandible seen in these mice. It has also been observed that reduced Hh signaling in vivo in chick embryos injected with anti-Shh antibody [Ahlgren and Bronner-Fraser, 1999], jervine-treated E9.5 mouse embryos [ten Berge et al., 2001], transgenic mice overexpressing the *Ptc* gene [ten Berge et al., 2001], and *Prx1–/–/Prx2–/–* mutant mice [ten Berge et al., 2001] all result in abnormal mandibular development. These results further suggest that the Hh signal may directly regulate Meckel's chondrogenesis. In this paper, we have investigated the functional role of Hh signaling during Meckel's cartilage development.

We first analyzed mandibular arch development in the *Shh* null mice initially reported by Chiang et al. [1996]. Although they report that the 'craniofacial bones are severely affected and are almost entirely absent despite the presence and nearly normal appearance of the branchial arches at E9.5', little detail is given. In fact, the *Shh* mutant embryos shown in their figure 2E and our figure 2a resemble the well-described agnathia-holoprosencephaly syndrome in humans [Siebert et al., 1990], all cases of which are sporadic. The mandible is either absent or severely hypoplastic, and most often accompanied by cyclopia. A similar phenotype associated with a recessive lethal mutation in C57BL mouse strains was reported 20 years ago by Juriloff et al. [1985].

The embryopathogenesis of the agnathia is that of aplasia, a failure of differentiation from an extant initial primordium. Although a mesenchymal condensation in the presumptive mandibular arch in E13.5 *Shh* mutants is present (fig. 1e) by E15.5, this mesenchymal condensation is absent (fig. 1f). As Jeong et al. [2004] show, Shh supports cell survival in early embryonic stages and promotes cell proliferation at later stages, thus controlling the size of craniofacial primordia. Condensation is the pivotal event in the development of cartilage and other mesenchymal tissues [Hall and Miyake, 2000]. The size of the condensation (cell number) is key; reduce a condensation below a critical threshold, and chondrogenesis, for example, will not be initiated [Hall and Miyake, 2000]. The absence of Meckel's cartilage in E15.5 and older null mice indicates that chondrogenesis failed to progress beyond the initial stage of condensation. Indeed, the most parsimonious explanation is a significant decline of Shh-mediated cell survival. A similar result has also been seen for another mandibular arch derivative, the submandibular gland [Jaskoll et al., 2004a]. The presence of a severely 'pedomorphic' submandibular gland in E18.5 *Shh* null mice indicated that branching morphogenesis and differentiation, but not initial bud formation, is dependent on Shh signaling.

Hh signaling is known to mediate the differentiation of chondrogenic precursor cells into mature chondrocytes, as well as regulate chondrocyte proliferation [Enomoto-Iwamoto et al., 2000; Long et al., 2001]. Thus, we also conducted a series of in vitro experiments to determine the effect of Hh signaling at stages subsequent to mandibular arch ectomesenchymal condensation, that is, Meckel's chondrogenesis per se. We cultured embryonic (E11 and E12) mandibles under chemically defined conditions and used cyclopamine to interrupt endogenous Hh signaling. Cyclopamine treatment has successfully been used to interrupt the development of two other mandibular arch derivatives, embryonic tongue and submandibular gland [Hall et al., 2003; Mistretta et al., 2003; Jaskoll et al., 2004a; Liu et al., 2004]. Since the mesenchymal condensation of Meckel's cartilage is found in E12 mandibular arches and not in E11, our experiments were designed to determine if Hh regulation of chondrogenesis is stage dependent. Our results indicate that endogenous Hh signaling does regulate Meckel's cartilage chondrogenesis in a stage-dependent manner, cyclopamine treatment having a substantially lesser effect on chondrocyte differentiation the older the starting explant (fig. 3, 4). Although the Meckel's cartilage primordium (prechondroblastic mesenchyme) differentiated regardless of the age of the starting tissue, in E11 + 5 cyclopamine-treated explants the anterior Alcian-blue-poor region consists mostly of chondroblasts, not chondrocytes. The relative normality of the posterior region, as well as the decreased effect of cyclopamine treatment on E12 mandibular arches, suggests that Hh signaling is most essential to early stages of anterior Meckel's cartilage development. Our results also indicate that Hh signaling regulates the rate of chondroblast differentiation into chondrocytes, but is not critical to the initiation of chondrogenesis per se (fig. 3).

In mammals, the intramandibular portion of Meckel's cartilage, apart from a limited segment at the rostral end (symphysis), disappears [Bhaskar et al., 1953; Frommer and Margolies, 1971]. It is now clear that the entire intramandibular portion of Meckel's cartilage (excluding the rostral end) undergoes matrix calcification and is ultimately replaced by endochondral-type bone [Ishizeki et al., 1999]. Mature hypertrophic chondrocytes mineralize their surrounding matrix. Vascularization of the mineralized cartilage delivers osteoclasts and osteoblast precursors: the osteoclasts remove mineralized matrix and the osteoblasts differentiate between the dying hypertrophic chondrocytes to eventually deposit bone matrix [Blair et al., 2002].

FGF signaling appears to play many essential roles in both endochondral and intramembranous bone development [see review by Ornitz and Marie, 2002]. One such role is the promotion of terminal hypertrophic differentiation of chondrocytes, a key step in endochondral bone formation. In this context, FGF negatively regulates the cell cycle by Cdk inhibition and other pathways, and positively regulates hypertrophic cartilage differentiation via the Hh/PTHrP pathway [Dailey et al., 2003].

Moreover, analysis of *Fgf8* loss of function mutant mice have clearly demonstrated the importance of FGF8 signaling for the development of mandibular arch derivatives. *Fgf8*$^{neo/-}$ hypomorphic embryos have absent or severely hypoplastic Meckel's cartilage, absent malleus and incus, absent mandibular molars, and severely defective mandible and tympanic ring [Abbu-Issa et al., 2002]. Of particular note is the observation of agnathia and the complete absence of Meckel's cartilage in mutant mice with *Fgf8* ablation in the first branchial arch (*Fgf8/Nes-cre* mutants) [Trumpp et al., 1999] or oral epithelium (*Fgf8:AP2*α*-IRESCre* mutants) [Macatee et al., 2003].

Importantly, Shh and FGF8 act synergistically to promote cartilage outgrowth of the craniofacial skeleton [Abzhanov and Tabin, 2004]. FGF8 is a positive regulator of *Hh* expression [Moon and Capecchi, 2000] and Shh has been shown to regulate *Fgf8* expression, either by induc-

ing its expression [Aoto et al., 2002] or by maintaining its expression domain [Ohkubo et al., 2002]. The latter is supported by our studies of SMG ontogenesis: with FGF8 loss of function, SMG development is only partially rescued by enhanced Shh expression [Jaskoll et al., 2004b]; with Shh loss of function, SMG development is fully rescued by enhanced FGF8 expression [Jaskoll et al., 2004a]. In the present study, we successfully rescue the cyclopamine-mediated inhibition of Meckel's cartilage chondrocyte differentiation with exogenous FGF8 treatment. In these treated explants, enhanced FGF8 mediates the terminal hypertrophic differentiation of chondrocytes, the attending cartilage matrix mineralization, and the putative initiation of endochondral-type ossification (fig. 5), despite the Hh loss of function.

It is critical to remember that *Hh* expression and its cognate Gli-responsive genes are part of a much larger genetic network. Organogenesis is the programmed expression of regulatory genes coupled to downstream structural genes and epigenetic events. Specific signaling pathways are parallel and largely functionally redundant; that is, several pathways differentially and combinatorially compensate for the dysfunction of a given individual pathway. There are some pathways, however, that have unique and nonredundant functions. One has always to ask two key questions: Is our pathway of interest functionally redundant or nonredundant? Will a broadly related, not independent, pathway compensate for the dysfunction of our pathway of interest?

In the present study, the introduction of exogenous FGF8 in vitro was able to more than compensate for the loss of Hh function in differentiating Meckel's cartilages (fig. 5). This is not surprising since the FGF/FGFR signaling pathway has broad relations with many other pathways controlling proliferation, survival, and differentiation (e.g. ERK, JNK, Stat3)[see review by Ornitz and Itoh, 2002]. *Shh* loss of function *(Shh–/–)* is ultimately epistatic to the epigenome under normal physiologic conditions (i.e. no other gene mutations nor untoward environments). However, since the epistasis associated with declining *Shh* function is a nonlinear emergent property of the complete functional epigenotype, it can be manipulated in vitro in the manner reported here. Rescue experiments have always to be only a proof of this principle, not a mimic of the in vivo condition. What remains to be determined is the functional relationship between the Hh signal transduction pathway and other key downstream signaling pathways, and how these pathways are integrated during Meckel's cartilage development to compose the functional epigenome.

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