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ARTICLES

Sonic Hedgehog Is Essential for First Pharyngeal Arch Development

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ABSTRACT: The secreted protein sonic hedgehog (Shh) is essential for normal development of many organs. Targeted disruption of *Shh* in mouse leads to near complete absence of craniofacial skeletal elements at birth, and mutation of *SHH* in human causes holoprosencephaly (HPE), frequently associated with defects of derivatives of pharyngeal arches. To investigate the role of Shh signaling in early pharyngeal arch development, we analyzed *Shh* mutant embryos using molecular markers and found that the first pharyngeal arch (PA1) was specifically hypoplastic and fused in the midline, and remaining arches were well formed at embryonic day (E) 9.5. Molecular analyses using specific markers suggested that the growth of the maxillary arch and proximal mandibular arch was severely defective in *Shh*-null PA1, whereas the distal mandibular arch was less affected. TUNEL assay revealed an increase in the number of apoptotic signals in PA1 of *Shh* mutant embryos. Ectodermal expression of fibroblast growth factor (*Fgf*)-8, a cell survival factor for pharyngeal arch mesenchyme, was down-regulated in the PA1 of *Shh* mutants. Consistent with this observation, downstream transcriptional targets of Fgf8 signaling in neural crest–derived mesenchyme, including *Barx1*, *goosecoid*, and *Dlx2*, were also down-regulated in *Shh*-null PA1. These results demonstrate that epithelial-mesenchymal signaling and transcriptional events coordinated by Shh, partly *via* Fgf8, is essential for cell survival and tissue outgrowth of the developing PA1. (***Pediatr Res* 59: 349–354, 2006**)

Pharyngeal arches are bilaterally symmetric ventral structures that develop in a segmental fashion along the anteroposterior axis during embryogenesis (1). The first pharyngeal arch (PA1), which in mammals develops into jaws, lateral skull wall, teeth, middle ear, and part of the tongue and other soft tissue derivatives, is formed as the most rostral and earliest pharyngeal arch beginning at E8.25 in the mouse embryo. This arch rapidly increases in size as it is populated by mesenchyme derived from cranial NCC and develops into the mandibular and maxillary arches at E9.5.

Appropriate epithelial-mesenchymal signaling is essential for proper development of the pharyngeal arches. Genetic analyses in mice provide evidence that numerous homeobox genes, including *Msx, Dlx, goosecoid (Gsc)*, and *Prx*, and other transcription factors, such as *Hand2*, are expressed in pharyngeal arch mesenchyme and play essential roles in development of PA1 (1,2).

Members of the Fgf family, particularly Fgf8, are epithelial signals that regulate gene expression during PA1 development (3,4). Inactivation of mouse *Fgf8* specifically in PA1 epithelium revealed that Fgf8 promotes mesenchymal cell survival and induces a developmental program required for PA1 morphogenesis (5). Members of the Bmp family also have important roles in outgrowth of PA1 (4). PA1 development appears very sensitive to the level of Bmp signaling during the initial outgrowth phase, and the level of Bmp signaling is tightly regulated by various factors (6).

The secreted protein Shh, a vertebrate ortholog of the *Drosophila* segment polarity gene, *Hedgehog*, is essential for normal development of many organs and is implicated as a cause of HPE. *Shh* is expressed in the pharyngeal arch epithelium and targeted disruption of *Shh* in mouse leads to near complete absence of craniofacial skeletal elements along with multiple organ defects (7). Recent studies using chick and mouse embryos have suggested that Shh may play a role in NCC development and pharyngeal pouch patterning (8–10).

Here, we analyzed the developing pharyngeal arches in *Shh* mutant embryos and found that Shh may be required for outgrowth of PA1 by regulating epithelial-mesenchymal interactions partly *via* Fgf signaling pathways that ultimately promote cell survival of mesenchymal cells in PA1.

METHODS

Mouse genetic studies. Mice heterozygous for the *Shh* mutation or the *Pax3-Cre* transgene were previously described (7,11). *Pax3-Cre* transgenic mice were crossed with *lacZ* reporter mice (Rosa26 reporter: R26R) (12).

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Abbreviations: Bmp, bone morphogenic protein; **E,** embryonic day;**Fgf,** fibroblast growth factor; **HPE,** holoprosencephaly; **Gsc,** goosecoid; **NCC,** neural crest cells; **PA1,** first pharyngeal arch; **R26R,** Rosa26 reporter; **Shh,** sonic hedgehog; **SMMCI,** solitary median maxillary central incisor

These mice were crossed and pregnant mothers were killed at E8.5–9.5 to obtain *Shh*$^{-/-}$/*Pax3-Cre*:R26R and *Shh*$^{-/-}$ embryos. This study has been approved by University of Texas Animal Care and Use Committee.

β-Galactosidase staining. Embryos were dissected, fixed in 2% paraformaldehyde/PBS with phenol red, and stained in Xgal solution as described previously (13).

Whole-mount and section RNA in situ hybridization. Whole-mount RNA *in situ* hybridizations were performed using digoxigenin-labeled antisense riboprobes as described previously (13). Section RNA *in situ* hybridizations were performed on paraffin-embedded sections of mouse embryos as described previously (14).

TUNEL analysis and cell proliferation assay. TUNEL analysis was performed on paraffin sections using apoptosis detection kit (ApopTag, Intergen, Purchase, NY) following manufacturer's protocol with the blue fluorescent DAPI nucleic acid stain (Molecular Probes, Eugene, OR). Cell proliferation assay was performed on paraffin section by immunohistochemistry with purified mouse anti-human Ki-67 antibody (2.5 μg/mL) (BD Pharmingen, San Diego, CA) using VECTASTAIN ABC kit (Vector Laboratories, Burlingame, CA) following manufacturer's protocol.

Bead experiments on cultured first pharyngeal arch explants. The first pharyngeal arches were dissected in DMEM (Invitrogen, Carlsbad, CA) from E9.5 embryos and cultured on membrane filter (Transwell, Corning, Palo Alto, CA). Bead experiments were performed as described previously (15). Explants were washed in PBS and fixed in 4% paraformaldehyde overnight at 4°C, and then subjected to whole-mount *in situ* hybridization for *Fgf8* expression.

RESULTS

Hypoplasia and midline fusion of the first pharyngeal arches in Shh mutant mice. To examine how Shh signaling might regulate early pharyngeal arch development, we analyzed the developing pharyngeal arches in *Shh* mutant embryos. At E9.5, *Shh* mutant embryos were grossly thinner and smaller in the head with hypoplasia of PA1 compared with wild-type littermates, but were similar in length (cranial to caudal) (Fig. 1). Morphometrics of PA1 in wild-type *versus Shh* mutant embryos demonstrated sizes of 0.405 ± 0.033 mm *versus* 0.281 ± 0.039 mm along the dorsal-ventral axis, respectively, and 0.313 ± 0.019 mm *versus* 0.142 ± 0.015 mm along the anteroposterior axis, respectively ($p < 0.05$ along both axes). In addition to morphologic and histologic indications, hypoplasia of PA1 was demonstrated using a unique property of the *Pax3-Cre*/R26R transgenic mouse line. In this mouse line, a population of NCC and their progeny can be marked with *lacZ* by Cre-mediated recombination under control of the *Pax3* promoter, which drives transcription specifically in the early migratory NCC that populate the second to fourth pharyngeal arches, but not PA1 (Fig. 1, *B* and *C*). We crossed these transgenic mice into the *Shh*-null background, and stained the obtained embryos with X-gal. The hypoplastic arch in *Shh* mutants was PA1 as marked by exclusion of *lacZ* expression (Fig. 1*G*). In frontal view, PA1 of *Shh* mutants appeared as a single hypoplastic arch structure with the bilateral PA1s being fused in the midline anterior to the second pharyngeal arches (Fig. 1*H*). This analysis also demonstrated relatively normal size and patterning of second to fourth pharyngeal arches at E9.5, indicating that NCC migration into second to fourth arches was grossly normal in *Shh* mutants (Fig. 1, *G* and *H*). Histologic analyses suggested a small and fused single arch structure in the midline, absence of the maxillary component and hypoplasia of the mandibular component of PA1 in *Shh* mutants (Fig. 1*I*) compared with wild-type embryos (Fig. 1*D*), although bilaterally symmetric PA1 were initially developed at E8.5 in *Shh* mutant embryos (Fig. 1*J*).

To elucidate a mechanism for the PA1 defect in *Shh* mutant embryos, we performed *in situ* hybridization using markers specific for PA1 development. *Bmp4* is normally expressed in numerous embryonic domains, including the distal epithelium of the mandibular, maxillary, and frontonasal region, as well as the cardiac outflow tract at E9.5 (Fig. 2, *A* and *M*) (16,17). In *Shh* mutant embryos, the expression of *Bmp4* was specifically absent in the maxillary epithelium, whereas its expression in the mandibular and frontonasal epithelium, and the cardiac outflow tract was intact (Fig. 2, *D* and *P*). *Mhox* and

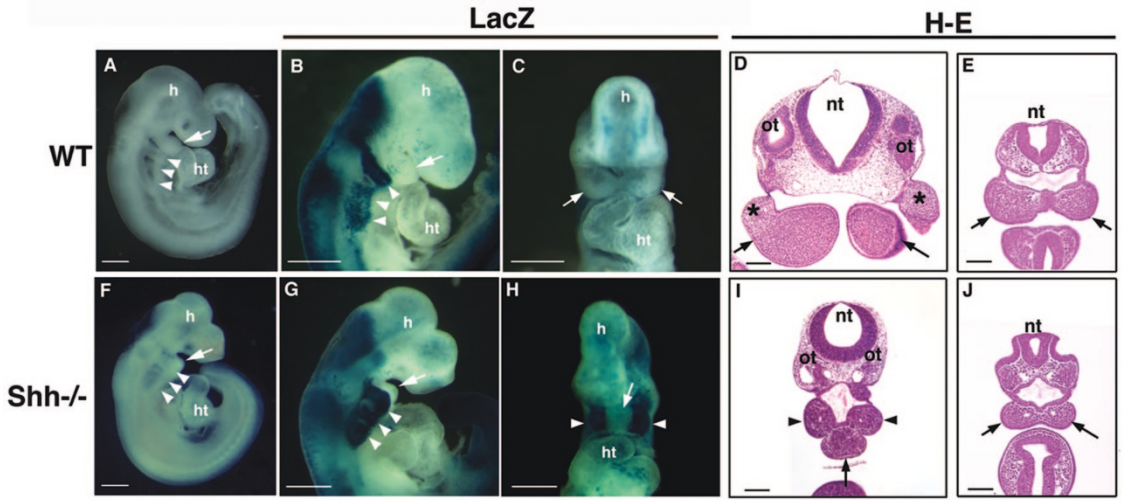

Figure 1. Hypoplastic PA1 in *Shh* mutant embryos. Wild-type (*A–E*) and *Shh*$^{-/-}$ embryos (*F–J*) at E9.5 (*A–D, F–I*) and E8.5 (*E, J*) are shown. PA1 was hypoplastic in *Shh*$^{-/-}$ embryos at E9.5 (*arrows* in *F–I*) compared with wild-type (*arrows in A–D*). In Pax3-Cre/R26R transgenic background, the second, third and fourth pharyngeal arches marked by LacZ (*arrowheads in A, B, F, G*) were comparable between wild-type and *Shh* mutants. Exclusion of *lacZ* expression demonstrates that the hypoplastic arch was PA1 in *Shh*$^{-/-}$ embryos (*arrow in G, H*). The PA1 of *Shh* mutants appeared to be a single small structure fused in the midline (*arrow in H, I*), anterior to the second arches (*arrowheads in H, I*) compared with wild-type PA1 (*arrows in C, D*). Hypoplasia of maxillary (*) and mandibular (*arrows*) of the PA1 was observed in *Shh*$^{-/-}$ embryos (*I*) compared with wild-type (*D*). The bilaterally symmetric PA1 were initially developed at E8.5 in *Shh*$^{-/-}$ embryos (*J*), similar to wild-type embryos (*E*), although they were smaller in size. *h,* Head; *ht,* heart; *nt,* neural tube; *ot,* otic vesicle. Scale bar, 0.5 mm (*A–C, F–H*); 0.1 mm (*D, E, I, J*).

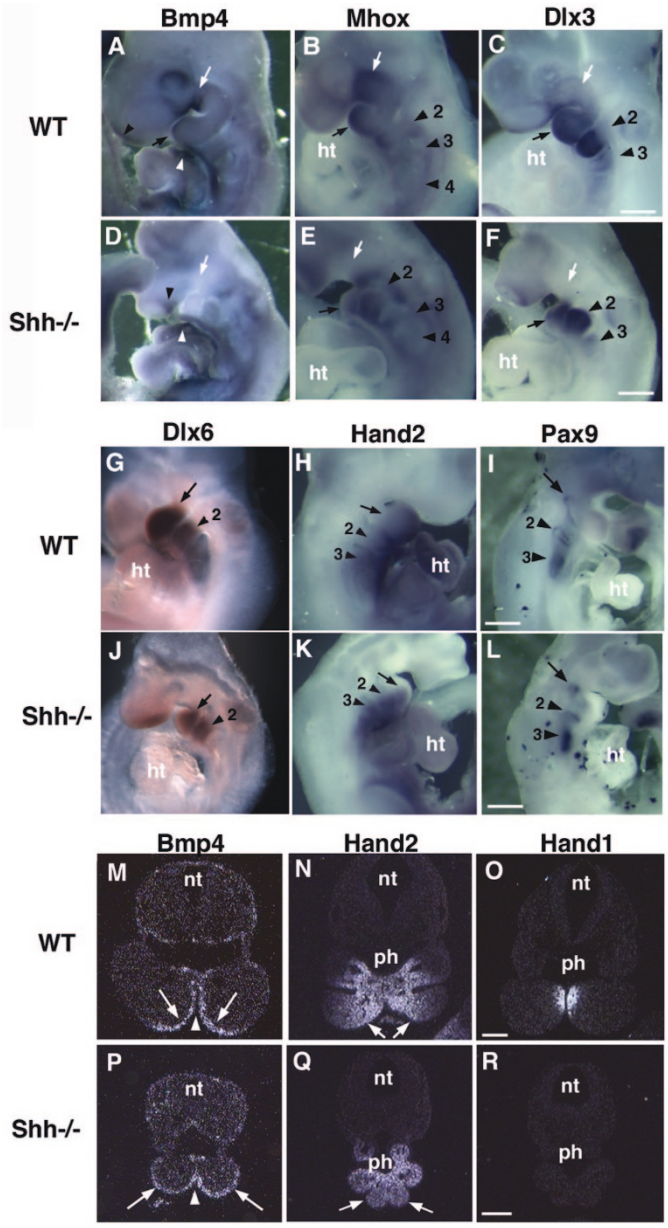

Figure 2. Severe hypoplasia of the maxillary and the proximal mandibular arches, and defective midline development of PA1 in *Shh* mutants. Whole-mount *in situ* hybridization (*A–L*) and radioactive section *in situ* hybridization (*M–R*) of molecular markers at E9.5 are shown. *Bmp4* was expressed in the distal epithelium of the mandibular (*black arrow*), maxillary (*white arrow*), frontonasal (*black arrowhead*) region and cardiac outflow tract (*white arrowhead*) in wild-type embryos (*A*), whereas the expression of maxillary epithelium was not detectable in *Shh*$^{-/-}$ embryos (*D*). *Mhox* and *Dlx3* were expressed in maxillary (*white arrow*) and mandibular (*black arrow*) mesenchyme derived from NCC of wild-type embryos (*B, C*). Their expressions were absent in the maxillary component, but intact in the mandibular component of *Shh*$^{-/-}$ embryos (*E, F*). Arrowheads with numbers denote the second to fourth pharyngeal arch mesenchyme derived from NCC. *Dlx6* and *Hand2* were similarly expressed in the mandibular mesenchyme (*arrow*) in *Shh*$^{-/-}$ embryos (*J, K*) compared with wild-type embryos (*G, H*). *Pax9* was normally detected in the pharyngeal endoderm (pharyngeal pouches) of *Shh* mutants at E9.5 (*L*) compared with wild-type embryos (*I*). In transverse section, *Bmp4* was expressed in the distal epithelium of the mandibular component of wild-type embryos (*arrows in M*). In *Shh*$^{-/-}$ embryos, *Bmp4* was expressed throughout the epithelium of the mandibular arch (*arrows in P*). Ectodermal epithelium of right and left mandibular arches appeared to be fused in the midline of *Shh*$^{-/-}$ embryos (*arrowhead in P*), in contrast to wild-type embryos where they are separate (*arrowhead in M*). *Hand2* is normally expressed in the mesenchyme of the distal (*arrows in H, N*), but not proximal, region of the mandibular arches (*N*). In *Shh*$^{-/-}$ embryos, the expression of *Hand2* was homogenously detectable in the fused mandibular arch (*arrows in Q*). *Hand1* is normally expressed in the medial mesenchyme of mandibular arches (*O*), but it was not detectable in *Shh*$^{-/-}$ embryos (*R*). *ht,* Heart; *nt,* neural tube; *ph,* pharynx. Scale bar, 0.3 mm (*A–L*); 0.1 mm (*M–R*).

Dlx3 are expressed in maxillary and mandibular mesenchyme derived from NCC in wild-type embryos at E9.5 (Fig. 2, *B* and *C*). Their expressions were undetectable in the maxillary component, but detectable in the mandibular component of *Shh* mutant embryos (Fig. 2, *E* and *F*). Consistent with these results, the expression of *Dlx6* and *Hand2*, detectable only in the distal mandibular mesenchyme of wild-type embryos at E9.5 (Fig. 2, *G* and *H*), were unaltered in *Shh* mutant embryos (Fig. 2, *J* and *K*). In transverse sections of the mandibular arch at E9.5, expression of *Bmp4* was detectable only in the distal epithelium of wild-type embryos (Fig. 2*M*), however, it was detected throughout the PA1 epithelium of *Shh* mutants (Fig. 2*P*), suggesting that the proximal mandibular component might be hypoplastic in the *Shh* mutant PA1. The *Bmp4* expression domain in the medial epithelium of PA1 was absent in mutants and the ectodermal epithelium of right and left PA1 appeared to be continuous in *Shh* mutant embryos (Fig. 2*P*), probably reflecting a midline defect of the *Shh* mutant PA1. A defective proximal mandibular component and midline structure of PA1 in *Shh* mutants was further demonstrated by the expression pattern of *Hand* genes in transverse sections at E9.5. *Hand2* is normally expressed in mesenchyme of the distal, but not proximal, region of the mandibular arch (Fig. 2, *H* and *N*). In *Shh* mutant embryos, the expression of *Hand2* was homogenously detectable in PA1 (Fig. 2*Q*), further suggesting that the proximal region of mandibular arch was severely hypoplastic in these mutants. *Hand 1* is normally expressed in the medial mesenchyme of PA1 (Fig. 2*O*). However, no expression of *Hand1* was detectable in *Shh* mutants (Fig. 2*R*), consistent with a loss of midline structure in PA1.

In spite of the PA1 defect, expression of the endodermal marker, *Pax9*, was normally detected in the pharyngeal endoderm (pharyngeal pouches) of *Shh* mutants at E9.5 (Fig. 2*L*) compared with wild type embryos (Fig. 2*I*), suggesting that initial endodermal development and pharyngeal patterning were unaffected in mouse embryos lacking *Shh* by E9.5. Significant expressions of the NCC-derived mesenchymal cell markers, *Mhox* (Fig. 2*E*), *Dlx3* (Fig. 2*F*), *Dlx6* (Fig. 2*J*) and *Hand2* (Fig. 2*K*) were observed in PA1 as well as other arches of *Shh* mutant embryos at E9.5, indicating that NCC could, at least in part, migrate and differentiate in PA1. Taken together, Shh signaling may be critical for proper outgrowth of PA1, especially the maxillary arch and the proximal region of mandibular arch, in addition to establishment of the midline structure during PA1 development.

Shh is required for survival of mesenchymal cells in the first pharyngeal arch. To test whether the failure of PA1 outgrowth in *Shh* mutant embryos might be the result of a cell survival defect, we performed TUNEL assays on tissue sections to mark apoptotic cells. Little apoptosis was detected in PA1 of wild type at E9.0–9.5 (Fig. 3, *A–D*). In contrast, a

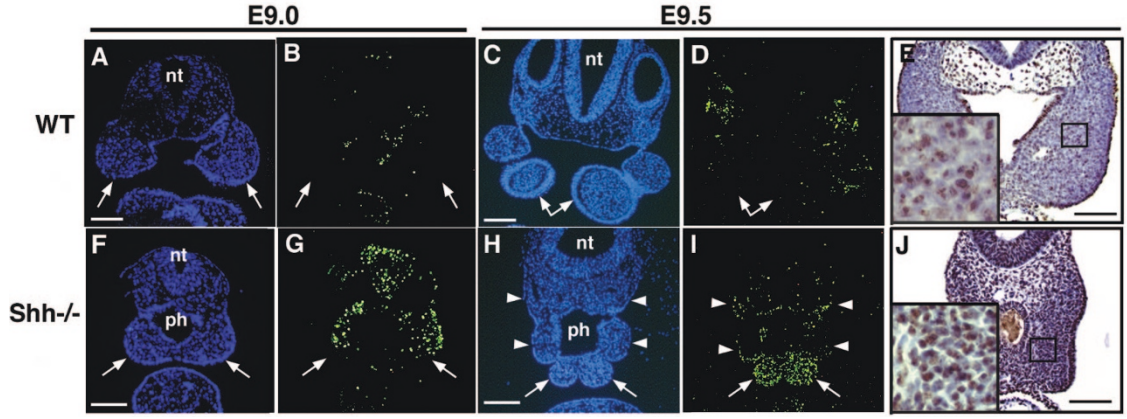

Figure 3. Enhanced apoptosis in the PA1 of *Shh* mutant embryos. Transverse sections of wild-type (*A–E*) and *Shh*$^{-/-}$ embryos (*F–J*) at E9.0 (*A, B, F, G*) and E9.5 (*C–E, H–J*) were analyzed by TUNEL assay (*B, D, G, I*) and counter-stained with DAPI (*A, C, F, H*), and assayed for cell proliferation by immunohistochemistry using anti-Ki-67 antibody (*E, J*). Enhanced apoptotic signals were observed in mesenchyme of *Shh*$^{-/-}$ PA1 (*arrows*), but not in the second or third pharyngeal arches (*arrowheads*), beginning from the maxillary and the proximal mandibular region at E9.0 (*G*) and extending throughout the PA1 at E9.5 (*I*), compared with wild type (*B* and *D*, respectively). Cell proliferation appeared relatively normal in PA1 of *Shh* mutants (*J*) compared with wild-type embryos (*E*). Inset is a higher magnification of the boxed area. *nt,* Neural tube; *ph,* pharynx. Scale bar: 0.1 mm

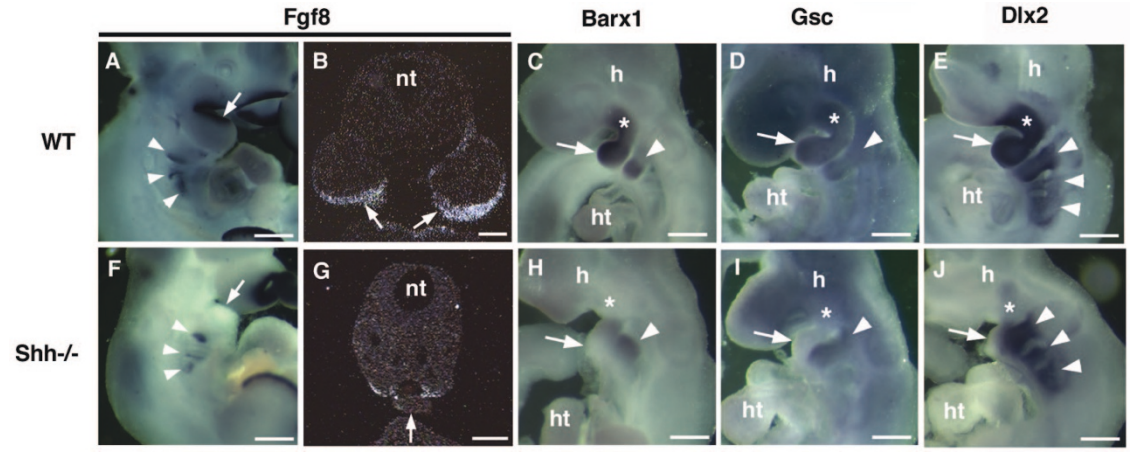

Figure 4. Requirement of Shh for Fgf signaling during epithelial-mesenchymal interactions in the PA1. Whole-mount and section *in situ* hybridization of wild-type (*A–E*) and *Shh*$^{-/-}$ embryos (*F–J*) at E9.5 are shown. *Fgf8* was expressed in the PA1 ectoderm of wild-type embryos (*arrows in A, B*) but was down-regulated in *Shh* mutants (*arrows in F, G*), with normal expression in the second to fourth pharyngeal arches (*arrowheads in A, F*). *Barx1*, *Gsc*, and *Dlx2* were normally expressed in mesenchymal cells in the PA1 (*arrows in C–E*) and other pharyngeal arches at E9.5 (*arrowheads in C–E*). In *Shh*$^{-/-}$ embryos, they were specifically down-regulated in PA1 (*arrows in H–J*), with normal expression in other arches (*arrowheads in H–J*). Down-regulation of *Barx1, Gsc*, and *Dlx2* were noted in the maxillary component (*) of mutant PA1 (*H–J*) compared with wild type (*C–E*). *h,* Head; *ht,* heart; *nt,* neural tube. Scale bar: 0.3 mm (*A, C–F, H–J*), 0.1 mm (*B, G*).

progressive increase in apoptosis was detected in *Shh* mutants, mainly in the proximal region at E9.0 (Fig. 3, *F* and *G*), and throughout PA1 mesenchyme at E9.5 (Fig. 3, *H* and *I*). Enhanced apoptotic signals were detectable only in PA1, but not in the second and third arches at E9.5 (Fig. 3, *H* and *I*). In contrast, cell proliferation assayed by immunohistochemistry using anti-Ki-67 antibody appeared relatively normal in PA1 of *Shh* mutants at E9.5 (Fig. 3*J*) compared with wild-type embryos (Fig. 3*E*). These results suggest that hypoplasia of PA1 in *Shh* mutants at E9.5 is mainly due to apoptosis of a substantial proportion of the cells that normally give rise to PA1.

Shh is required for epithelial-mesenchymal signaling in the first pharyngeal arch. In addition to *Bmp4, Fgf8* is a critical signaling molecule that transmits survival signals from the epithelial cells to the adjacent mesenchyme (4–6). In contrast to *Bmp4*, which is expressed in the epithelia, albeit in an abnormal pattern (Fig. 2, *D* and *P*), *Fgf8* was specifically down-regulated in the ectodermal epithelium of PA1 in *Shh* mutants at E9.5 (Fig. 4, *F* and *G*), coinciding with the extensive apoptosis observed. The expression of *Fgf8* in the second and third pharyngeal regions was normal at this stage.

Because *Fgf8* was down-regulated in *Shh* mutants, we examined expression of several transcription factors that are downstream of Fgf8 signaling in the pharyngeal arch mesenchyme (2,5). Genes encoding the homeobox transcription factors *Barx1*, *Gsc*, and *Dlx2* are expressed in NCC-derived mesenchymal cells in the PA1 and other pharyngeal arches (Fig. 4, *C–E*), and their expression is dependent upon Fgf8 signaling. Consistent with down-regulation of *Fgf8*, expression of *Barx1*, *Gsc*, and *Dlx2* were decreased in the PA1 of *Shh* mutants at E9.5, whereas expressions in other arches were normal (Fig. 4, *H–J*). These results indicate that the epithelial-

mesenchymal interactions mediated by Fgf8 signaling were affected in PA1 of *Shh* mutant embryos.

To determine whether Shh can activate *Fgf8* expression, we attempted to induce Fgf8 mRNA with Shh-soaked beads in cultured PA1. Compared with the control PA1 cultured with BSA-soaked beads, the expression level of *Fgf8* was elevated in PA1 cultured with Shh-soaked beads (Fig. 5*A*). Despite the limitations of this experiment using cultured tissues, it supports the observation in *Shh* mutants that Fgf8 may function downstream of Shh signaling during PA1 development.

DISCUSSION

Shh signaling plays a primary role for early PA1 development. In this report, we analyzed pharyngeal arch development in *Shh* mutant embryos, focusing on PA1, and demonstrated that bilateral PA1 initially form, but they become hypoplastic, resulting in a single fused structure in the midline by E9.5. Exposure to the Shh inhibitor, jervine, at E7.5 frequently leads to only forebrain defects, but no mandibular defects, whereas susceptibility for mandibular defects is highest when pregnant mice are treated around E9.5 (18). A recent study demonstrated that mice lacking Hh-responsiveness specifically in cranial NCC showed a hypoplastic PA1 without mid- or forebrain defects at E11.5 (9). Taken together, the PA1 defect in *Shh* mutants at E9.5 presented in this study is likely to be a primary defect resulting from lack of Shh signaling.

Our analyses did not address whether NCC migration into PA1 was altered in *Shh* mutant embryos. Significant expression of the NCC-derived mesenchymal cell markers, *Mhox*, *Dlx3*, *Dlx6*, and *Hand2*, was observed in PA1 as well as other arches of *Shh* mutant embryos at E9.5, indicating that NCC could, at least in part, migrate and differentiate in PA1. However, it remains to be elucidated whether appropriate amounts of NCC migrate into PA1 in absence of Shh signaling, inasmuch as Smoak *et al.* (19) suggested that NCC were migrating along abnormal pathways in *Shh* mutant embryos based on the expression of NCC markers, *CrabP1* and *AP2α*.

Our analyses revealed the severe hypoplasia of the maxillary component and the proximal mandibular component, and defective midline development of PA1 in *Shh* mutants (Fig. 5*C*). The distal mandibular component where *Hand2*, *Mhox*, *Dlx3*, and *Dlx6* are highly expressed was less affected. In the current concept of PA1 or jaw development, it has been proposed that "maxillary" and "mandibular" precursors are from distinct origins (20,21), and that identities of "maxillary," "proximal mandibular," and "distal mandibular" components are controlled by specific sets of transcription factors and signaling molecules (4). Shh signaling appears to be, directly or indirectly (see discussion below), more critical for development of maxillary and proximal mandibular region, and establishment of midline structure than identities of the distal mandibular region.

Enhanced apoptosis and altered epithelial-mesenchymal interactions lead to severe hypoplasia of PA1 in Shh mutants. Our data suggest that the hypoplastic PA1 in *Shh* mutant embryos is mainly as a result of enhanced apoptosis in PA1 mesenchyme, indicating that Shh signaling functions in survival of PA1 mesenchymal cells. This is consistent with the observation that blocking Shh signaling in chick embryos by anti-Shh antibody results in significant increase in apoptosis in NCC-derived mesenchyme in pharyngeal arches (8), and recent experiments using lysotracker red (molecular probes) (10,19). Enhanced apoptosis of mesenchymal cells was also documented in PA1 of *Wnt1-Cre; Smo*$^{n/c}$ mouse embryos that were created by crossing mice harboring the *Wnt1-Cre* transgene with those that contain loxP sites around the Shh receptor *Smoothened* (*Smo*) to remove Hh-signaling specifically in the cranial NCC lineage (9).

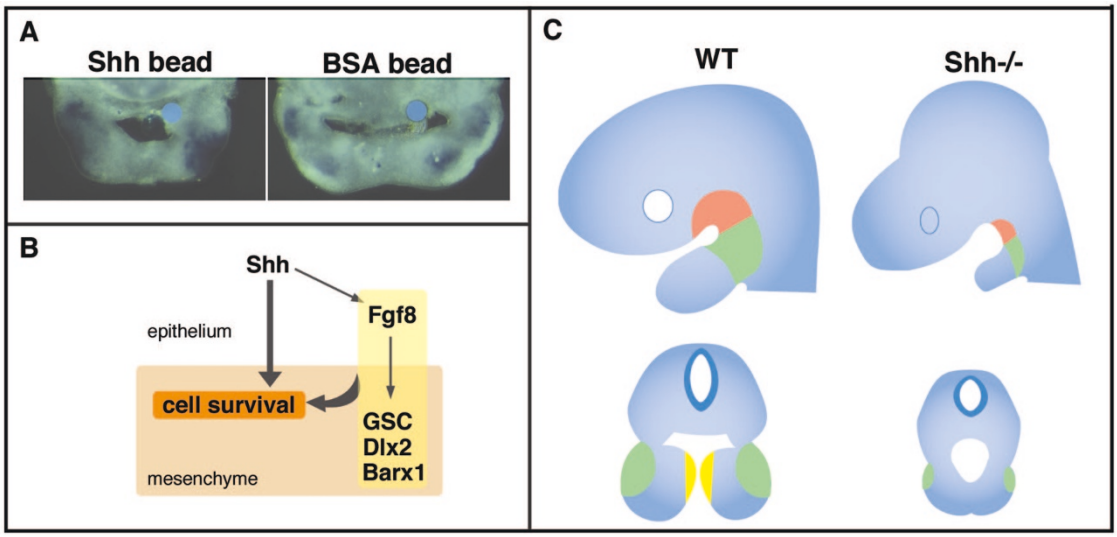

Figure 5. Shh activation of Fgf8 and proposed model for Shh signaling in PA1 development. (*A*) Representative *Fgf8* expression in cultured PA1 explants after application of a Shh-soaked bead or control (BSA-soaked) bead. Higher level of *Fgf8* expression was observed in the explants containing Shh-soaked bead. (*B*) Proposed model for Shh-Fgf signaling in PA1 development. *Shh* from epithelium may play a role in survival of mesenchymal cells in PA1 partly *via* Fgf8 signaling. (*C*) Schematic drawing of the maxillary (*pink*) and the proximal mandibular (*green*) components and the midline structure (*yellow*) are shown in right lateral views and transverse section of embryos at E9.5. Loss of Shh signaling results in severe hypoplasia of the maxillary and the proximal mandibular components, and defective midline development of PA1.

Whether Shh directly acts as a survival factor for mesenchymal cells in PA1 remains to be studied. Mesenchymal cells in PA1 can directly respond to Shh signaling, as they express the Shh receptors, *Smo* and *Patced* (Ptc), and the *Gli* family of transcription factors that transduce Shh signaling (4,22). Jeong *et al.* (17) have proposed a model in which Shh signaling directly regulates growth of pharyngeal arches *via* combinatorial expression of several forkhead (Fox) transcription factors, and we have previously reported that Foxc2 can mediate Shh signaling in craniofacial and cardiovascular development (23). These observations suggest that Shh signaling may directly play a role in survival of mesenchymal cells and growth of PA1 through epithelial-mesenchymal interaction.

Alternatively, it is also possible that Shh signaling may promote mesenchymal cell survival in PA1 by regulating the expression of other growth factors. Our molecular analysis revealed that Fgf8 expression was down-regulated in PA1 ectoderm of *Shh* mutants at E9.5. Tissue-specific inactivation of *Fgf8* by Cre-mediated recombination in the ectoderm of the PA1 leads to mesenchymal cell death around E9.5 and impairs development of PA1 (5), suggesting that Fgf8 is required, directly or indirectly, for survival of neural crest–derived mesenchyme in PA1. We also observed down-regulation of pharyngeal mesenchyme markers, *Barx1*, *Gsc*, and *Dlx2*, that are putative targets of Fgf8 signaling in PA1 of *Shh* mutant embryos. Fgf8 is normally able to induce expression of these genes, whereas Shh alone is not sufficient (3–5). In addition, other mesenchymal markers, including *Hand2*, *Mhox,* and *Dlx3* were not down-regulated in *Shh* mutants, suggesting that there was not a generalized defect of the pharyngeal arch mesenchyme. Consistent with the idea that Shh signaling functions upstream of Fgf8 in PA1 development, *Shh* mutants have more severe PA1 defects than those that result from tissue-specific loss of *Fgf8* function in PA1, where only the proximal region of PA1 is most severely affected (5). Our result that *Fgf8* is activated by Shh-soaked beads in PA culture supports this idea. We therefore favor the interpretation that the PA1 phenotype of *Shh* mutants, at least in part, reflects a lack of Fgf8 signaling during critical epithelial-mesenchymal interactions (Fig. 5*B*), although we still cannot rule out the possibility that Fgf8 expression is decreased secondary to the morphologic defect.

Implication in human diseases. Mild midline defects of PA1 may lead to SMMCI. Interestingly, SMMCI is suggested as a characteristic finding in HPE patients with *SHH* mutations (24). In addition, *SHH* mutations have been identified not only in SMMCI with HPE, but also SMMCI without HPE (24,25). These findings are consistent with a primary role of Shh in PA1 as suggested in our study.

Severe hypoplasia of PA1 in humans, on the other hand, leads to agnathia or micrognathia. Agnathia alone occurs very rarely, and is often associated with HPE and sometimes with situs inversus totalis (25,26), all of which occur in the setting of *SHH* disruption in mouse and human. Moreover, Shh lies upstream of *Tbx1*, a major genetic determinant of DiGeorge/22q11.2 deletion syndrome that is often associated with micrognathia (15,23). It is intriguing to speculate that Shh is not only a disease gene for HPE but also a genetic modifier of other human syndromes associated with agnathia/micrognathia.

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