



Shh and *Ptch* Expression in Mouse Embryonic Craniofacial Development

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Objective: To evaluate the particular expression of *Shh* and its receptor patched (*Ptch*) involved in mouse embryonic craniofacial development.

Methods: The expression patterns of *Shh* pathway genes during murine embryonic craniofacial development were investigated by applying in-situ hybridization studies of whole-mount and sections, immunohistochemistry, and reverse transcription polymerase chain reaction analysis.

Results: *Shh* was expressed in the mouse embryo at 11 and 12.5 days postcoitum (dpc); *Ptch* was expressed at 11, 12.5, and 14.5 dpc, but expression patterns were different. SHH protein could also be detected at 11, 12.5, and 14.5 dpc, confirming the gene expression studies.

Conclusion: *Shh* and *Ptch* expression patterns are characterized. The data suggest that *Shh* and *Ptch* are involved during craniofacial development and their roles may vary.

Key words: immunohistochemistry, in-situ hybridization (ISH), maxillofacial region development, *Shh*, *Ptch*

Hedgehog (HH) proteins, a family of secreted molecules first identified by a genetic screen in *Drosophila*, are involved in many patterning processes during development. Sonic hedgehog (SHH) is the most broadly expressed member of three mammalian HH homologues and is probably responsible for the major effects of this signalling pathway. Genes in the *Shh* sig-

nalling pathway include *Shh*, patched (*Ptch*), patched2 (*Ptch2*), smoothed (*Smo*), *Gli1*, *Gli2*, and *Gli3*. PTCH, the putative 12-pass transmembrane receptor for SHH, normally acts to inhibit SHH signalling by repressing the signalling activity of a seven-pass transmembrane protein, Smoothed (*SMO*). This inhibition is relieved when SHH binds to PTCH, possibly through changes in distribution or concentration of a small molecule¹ and allows a zinc-finger-type transcription factor GLI to enter the nucleus, where it regulates transcription. Compared with many reports about the expression of *Shh* and *Ptch* in the brain and limb²⁻⁵, there are few studies focusing on expression patterns in the oral maxillofacial region^{6,7}. Many studies indicate that *Shh* and *Ptch* play key roles in embryonic craniofacial development, but few studies address the particular expression of *Shh* and *Ptch* in the craniofacial region, and their functions in development remain unclear. In this study we use whole-mount and section in-situ hybridization (ISH), immunohistochemistry and reverse transcription polymerase chain reaction (RT-PCR) to study the gene expression patterns of the *Shh* signalling pathway during mouse embryonic craniofacial development.

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Materials and Methods

Plasmid construct of *Shh*, *Ptch*

cDNA fragments were amplified from mouse lung and brain cDNA. They were cloned into pGEM-T Easy plasmid (Invitrogen Inc., Carlsbad, CA, USA) and verified by DNA sequencing. The subcloned fragments were used as templates to generate sense or antisense digoxigenin (Dig)-labelled riboprobes for ISH using T7 or SP6 RNA polymerase (Roche Inc., Basel, Switzerland).

Whole-mount and section ISH analyses

To determine whether *Shh* and *Ptch* were involved in oral and maxillofacial development, we examined their expression patterns during facial development. Because at 11 dpc an embryo's maxillofacial and mandibular arches are separated, and at 14.5 dpc facial formation is almost complete and similar to the new-born, we selected 11, 12.5, and 14.5 dpc to observe the gene expression of *Shh* and *Ptch*. Murine embryos (11, 12.5, and 14.5 dpc) were dissected free from extra-embryonic membranes, fixed in 4% paraformaldehyde in DEPC-PBS overnight at 4°C, dehydrated in methanol, and processed for whole-mount ISH as described previously^{8,9}. Embryos of 11, 12.5, and 14.5 dpc were embedded with paraffin and sectioned (4–6 µm sagittal sections). ISH procedures were adopted from those of Ang and Rossant¹⁰. Hybridization was performed overnight at 63°C in hybridization solution containing equal amounts of digoxigenin-labelled sense (control group) or anti-sense riboprobes (experimental group) (0.5 µg/ml). The positive regions are blue.

Immunohistochemistry and histology

Sections were prepared as for ISH and stained with haematoxylin and eosin. A polyclonal goat antibody against mouse *SHH* (Santa Cruz Inc., Santa Cruz, CA, USA) was used. Sections were incubated with the antibody overnight at 4°C in a 1:150–250 dilution and processed using a standard DAB detection system (Amersham Inc., Piscataway, NJ, USA). The control group of same time was incubated with PBS. The positive regions are yellow.

RT-PCR analysis

Total RNA was isolated from the mouse face-region tissues, including the maxillofacial and mandibular processes, at 11, 12.5, 13.5, 14.5, 15.5, and 17.5 dpc, new-born days 1 and 5, and adult. We prepared cDNA from 1 µg total RNA (Invitrogen Inc.), and performed PCR under

the following conditions: initial denaturation at 95°C for 15 min; denaturation at 94°C for 30 s, annealing at 59–60°C for 30 s, and extension at 72°C for 1 min for 35 cycles (Qiagen Inc., Valencia, CA, USA).

We designed primers using the Primer 3 software. Forward and reverse primers of *Shh* and *Ptch* were shown separately as *Shh*: 5'-ACTCGCAGCTGCTCTACCA-CAT-3' and 5'-ATCTTCCCTCACTGTGAGTACAG-3', *Ptch*: 5'-TGTCAGCAGGGTGATTCAAG-3' and 5'-GGTGCTGGATATTCGGGTAG-3'.

We used *Gapdh* as a control. Forward and reverse primers were: 5'-GGTGAAGTTCGGTGTGAACG-3' and 5'-CTCGCTCCTGGAAGATGGTG-3'.

Results

Expression of *Shh* signalling pathway genes in mouse craniofacial development

We detected a faint *Shh* expression signal in the maxillofacial process at 11 dpc (Fig 1A), and a more notable signal was detected in the maxillofacial process at 12.5 dpc (Fig 1B). However, at 14.5 dpc, *Shh* was not detectable using whole-mount ISH (Fig 2A). *Shh* expression detected by section ISH was similar to that detected in the whole-mount, except that *Shh* was also expressed in the mandible (Figs 1C, 1D and 2B), and there was no difference in expression intensity between the epithelium and the mesenchyme (Figs 1H and 1I). There were no signals in the control group (Figs 2C–H). However, for the SHH receptor, PTCH, we found that at 11 dpc (Figs 3A and 3D) there was faint *Ptch* expression in the face, and at 12.5 dpc (Figs 3B and 3E) there was a strong signal detected in the maxillofacial and mandibular regions, especially in the whisker follicle, which continued to 14.5 dpc (Figs 3C, 3F and 3I). The signal spread into both the epithelium and the mesenchyme, but at 11 dpc there was no difference in the signal between them. At 12.5 dpc, the signal in the mesenchyme was more dominant than in the epithelium; at 14.5 dpc there was again no difference in the signal between them (Figs 3G–3I). Also, we found no expressions in the area in the control group (Figs 4A–4F).

Immunohistochemistry analysis showed that SHH was expressed in the maxillofacial and mandibular processes from 11 to 14.5 dpc (Figs 1E–1G and 1J–1L), but expression in whisker follicle was faint (Fig 1L) compared with *Ptch*. No signals were found in the control group (Figs 2I–2K).

We also analysed RNA expression from 11 dpc to adult via RT-PCR and found the *Shh* expressed from 11 to 13.5 dpc, then faintly found in adult, which coincided with the ISH results (Fig 1M). Compared with *Shh*, *Ptch*

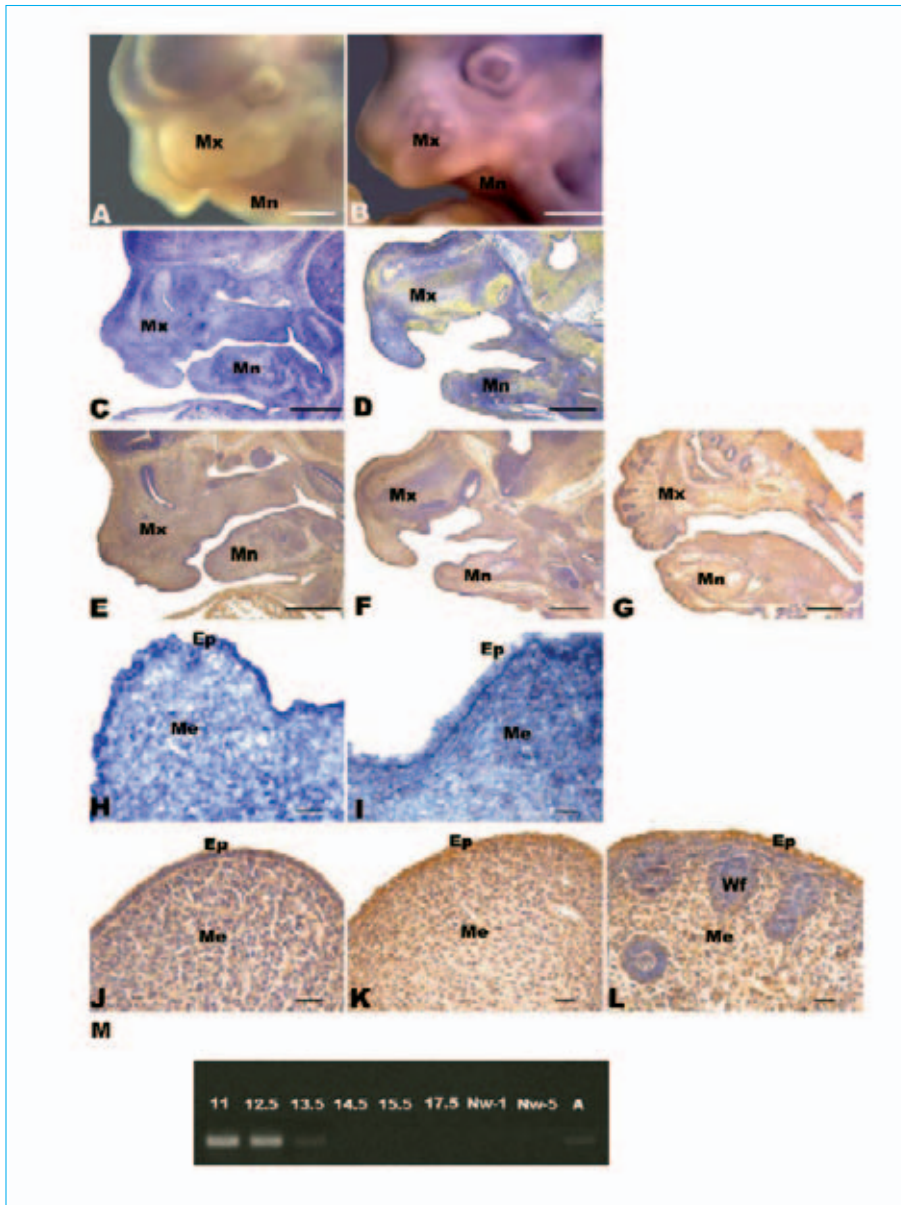


Fig 1 Expression of *Shh* in the maxillofacial region. *Shh* was expressed faintly in the maxillofacial process at 11 dpc (A) and was more obviously expressed in maxillofacial process at 12.5 dpc (B). The expression in section ISH was almost the same as expression detected in the whole-mount, except that expression in mandibular region was the same as in the maxilla (C, D). *SHH* protein was expressed from 11 to 14.5 dpc in the maxillofacial and mandibular processes (E–G). There were no differences in the intensity of expression between the epithelium and mesenchyme; expression in whisker follicle was faint (H–L). (A, B) 11 and 12.5 dpc embryo by whole-mount ISH; (C, D) 11 and 12.5 dpc embryo by section ISH; (E–G) 11, 12.5 and 14.5 dpc embryo by immunohistochemistry. Mx: maxillary prominence; Mn: mandibular prominence; Wf: whisker follicle. Bar: 500 μm. (H, I) 11 and 12.5 dpc embryo by section ISH; (J–L) 11, 12.5 and 14.5 dpc embryo by immunohistochemistry. Bar: 50 μm. M: RT-PCR of *Shh* in the face region from 11 dpc to adult. RT-PCR detected *Shh* mRNA in 11–13.5 dpc, and is faintly expressed in adult. Nw-1: newborn 1 day; Nw-5: newborn 5 days; A: adult.

were found from 11 dpc to adult, which not only accords with the ISH results, but also supplies other information about the function of *Ptch* in the maxillofacial region.

Discussion

Sporadic and inherited mutations in the human *SHH* and *PTCH* genes have been shown to cause craniofacial malformation, such as holoprosencephaly^{11,12}, nevoid basal cell carcinomas (NBCCs)^{13–15}, solitary median maxillary central incisor¹⁶ and many syndromes such as

Smith–Lemli–Opitz (OMIM 270400) and Pallister–Hall syndrome (OMIM 146510), which are associated with facial abnormalities. The mechanisms of these diseases remain unclear. The face forms from five facial prominences (swellings) that ultimately fuse together. Developmental dysfunction of any of them may result in facial malformation.

In previous studies, the expressions of *Shh* and *Ptch* were evaluated one gene at a time, and the expression patterns in maxillofacial and mandibular regions were not resolved^{2,3}. There have been no systematic studies

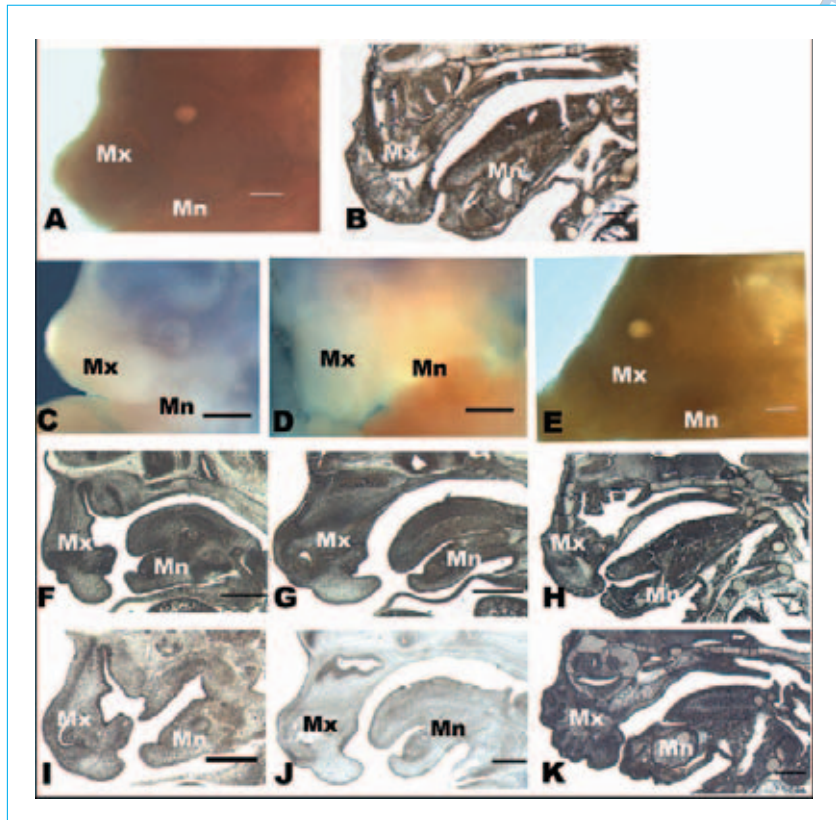


Fig 2 There were no *Shh* signals in maxillofacial region at 14.5 dpc and control group. (A) 14.5 dpc embryo by whole-mount ISH; (B) 14.5 dpc embryo by section ISH; (C–E) control group at 11, 12.5, and 14.5 dpc embryo by whole-mount ISH; (F–H) control group at 11, 12.5, and 14.5 dpc embryo by section ISH; (I–K) control group at 11, 12.5, and 14.5 dpc embryo by immunohistochemistry. Mx: maxillary prominence; Mn: mandibular prominence. Bar: 500 μ m.

of *Shh* and *Ptch* expressions in the maxillofacial region. In our study, we examined in detail the temporal and spatial expression patterns of *Shh* and *Ptch* during the mouse embryonic craniofacial development, paying special attention to the stage where the facial prominences merge to form a normal maxillofacial structure. Our findings on *Shh* and *Ptch* expressions in the mouse embryo were in partial concord with previous studies of the maxillofacial region^{2,3}. Our new findings will provide a guide for further investigation of *Shh* and *Ptch* function during craniofacial development. *Shh* was found in both epithelium and mesenchyme during 11 to 13.5 dpc. Compared with *Shh*, the expression of *Ptch* was more robust and more protracted, and its expression pattern in the epithelium and mesenchyme during face development did not coincide with that of *Shh*, which suggests

that *Ptch* may not only pass signals of *Shh*, but also act as receptor of *Ihh*, another component of Hh families. Mutations in *Ptch* have been associated with craniofacial malformation in nevoid basal cell carcinoma patients^{13–15}, which may indicate that *Ptch* plays an important role during embryonic face development. In a previous report, transgenic mice overexpressing SHH in the skin develop many features of basal cell nevus syndrome¹⁷. Mutated SHH may function as an oncogene in basal cell carcinomas which associate with the face. The marked expressions of *Ptch* and faint signal of *Shh* detected by RT-PCR suggest that these genes play key roles in the maxillofacial region even after birth, and what roles they play at adult stage still need more research.

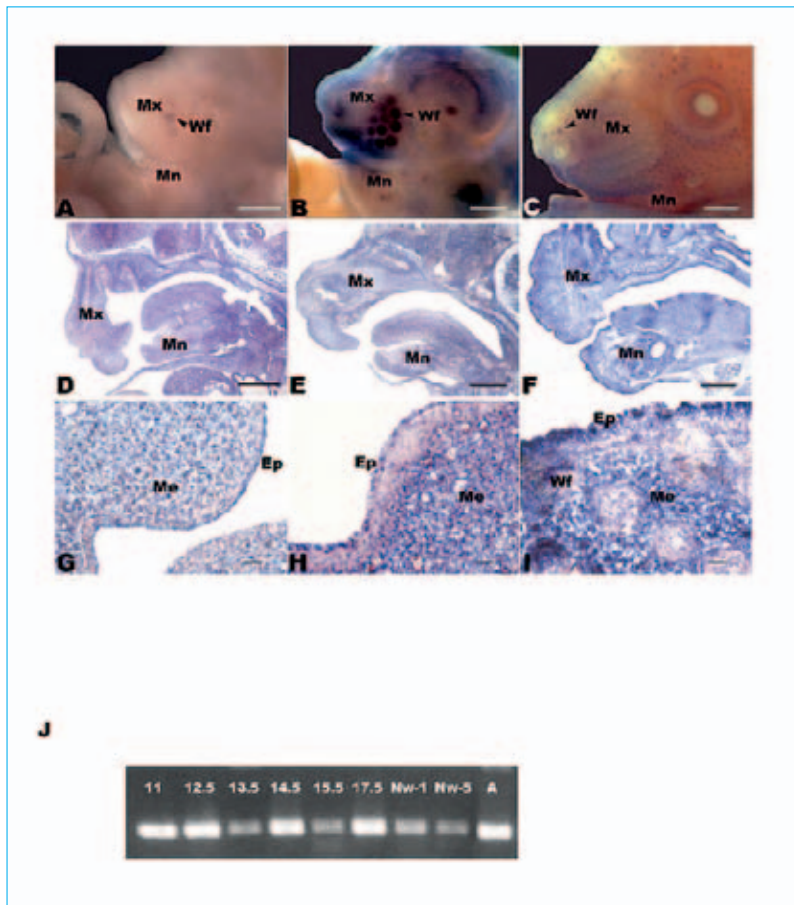


Fig 3 Expression of *Ptch* in the maxillo-facial region. At 11 dpc there was faint expression in the face (A, D), and at 12.5 dpc, a strong colour signal was detected in the maxillofacial and mandibular region, especially in the whisker follicle (black arrowhead; B, E), which continued through 14.5 dpc (C, F, I). The signal spread in both epithelium and mesenchyme, but at 11 dpc there was no difference in the epithelial and mesenchymal signals (G); at 12.5 dpc, the signal in mesenchyme was stronger than in the epithelium (H); at 14.5 dpc, there was again no difference of the signal between the epithelial and mesenchymal (I). (A–C) 11, 12.5, and 14.5 dpc embryo by whole-mount ISH; (D–F) 11, 12.5, and 14.5 dpc embryo by section ISH. Mx: maxillary prominence; Mn: mandibular prominence; Wf: whisker follicle. Bar: 500 μ m. (G–I) 11, 12.5, and 14.5 dpc embryo by section ISH. Bar: 50 μ m. (J) RT-PCR of *Ptch* in the face region from 11 dpc to adult. RT-PCR detected *Ptch* mRNA from 11 dpc to adult. Nw-1: newborn 1 day; Nw-5: newborn 5 days; A: adult.

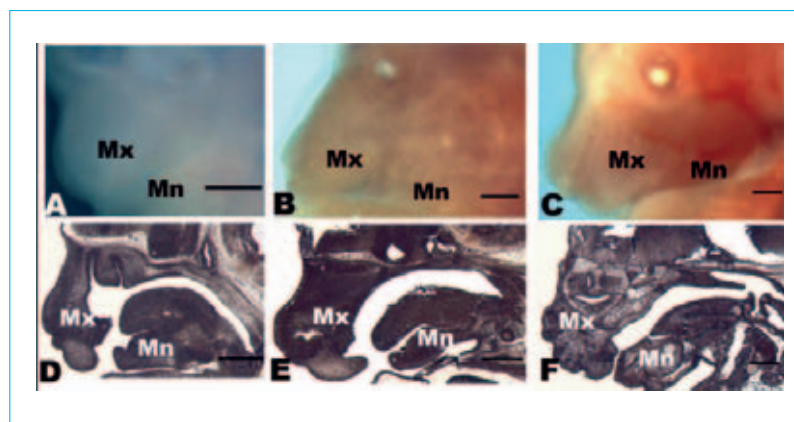


Fig 4 No *Ptch* signals in maxillofacial region in control group. (A–C) Control group at 11, 12.5, and 14.5 dpc embryo by whole-mount ISH; (D–F) control group at 11, 12.5, and 14.5 dpc embryo by section ISH. Mx: maxillary prominence; Mn: mandibular prominence. Bar: 500 μ m.



Fig 5 Expression of *Gapdh* by RT-PCR. *Gapdh* was detected from 11 dpc to adult. Nw-1: newborn 1 day; Nw-5: newborn 5 days; A: adult.

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