BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **225,** 161–166 (1996)
ARTICLE NO. 1147

# Spatial Expression of *Sonic Hedgehog* in the Lung Epithelium during Branching Morphogenesis

Koko Urase,* Takeshi Mukasa,* Hiroshi Igarashi,† Yasuo Ishii,‡ Sadao Yasugi,‡ Mariko Y. Momoi,† and Takashi Momoi*,[1]

**Division of Development and Differentiation, National Institute of Neuroscience, NCNP, Kodaira, Tokyo 187, Japan; †Department of Pediatrics, Jichi Medical School, Minamikawach, Kawachi-gun, Yakushiji, Tochigi 329-04, Japan; and ‡Department of Biology, Faculty of Science, Tokyo Metropolitan University, 1-1 Minamiosawa, Hachioji, Tokyo 192-03, Japan*

Received June 7, 1996

*Sonic hedgehog (Shh),* a homologue of *Drosophila hedgehog,* was specifically expressed in lung epithelium during branching morphogenesis, but was not uniformly expressed in lung epithelium. *Shh* was intensely expressed in the distal tips of the bronchial tubes during branching morphogenesis, and *Shh* was localized on the apical side of the epithelium. On the other hand, *Bmp-4,* one of the target genes of *Shh,* was also specifically expressed in the epithelium at the branching point. These results suggest that *Shh* and *Bmp-4* are involved in the branching morphogenesis of lung epithelium. 

Interactions between the mesenchyme and the epithelium are required for the development of many organs, including those of the gastrointestinal, integumental, urogenital, and respiratory systems (1-9). Development of the lung primodium occurs by the growth and branching of the primitive respiratory epithelium (the endodermal tubule) into the surrounding mesenchyme to form the bronchial tree. This process is known as branching morphogenesis and is considered the most active period of epithelial-mesenchymal interactions. Branching morphogenesis does not occur in the absence of bronchial mesenchyme. Throughout branching morphogenesis, development and regional specification of the respiratory system is dependent on a complex network of interactions among cells and their products, such as cytokines, ECM components, and cell surface receptors (7).

*Sonic hedgehog* (*Shh*) is a vertebrate homologue of *hedgehog* (*hh*), one of the segment polarity genes of *Drosophila* (10), which is involved in the organizing pattern within the parasegment of *Drosophila* in early developmental stage, and in the patterning the imaginal disk limb and eye in later developmental stages (11,12). In vertebrate development, *Shh* is specifically expressed in the notochord, floor plate in the early developmental stages, and ZPA in the limb buds (10, 13-17). *Shh* is involved in the digits pattern formation in the limbs (14,15) and in the induction of mortar neurons (17). *Shh* also expresses in the endodermal epithelial organs, including lung and digestive tract (18,19).

In the present study, we analyzed the distribution of *Shh* mRNA and Shh proteins in the rat lung at different developmental stages by the method of whole mount *in situ* and *in situ* hybridization of frozen sections, and by immunostaining using anti-Shh antisera, respectively. We demonstrate that the expression of *Shh* is restricted to the epithelium, but *Shh,* as well as its target gene, is not uniformly expressed in the lung epithelium during branching morphogenesis. *Shh* is specifically located in the apical side of the epithelium at the branching points.

[1] To whom all correspondence should be addressed. Fax: 0423-46-1754.



0006-291X/96 $18.00

## MATERIALS AND METHODS

*RT-PCR.* The expression of *Shh* in tissues of 15.5-day rat embryos was examined by RT-PCR and southern blot analysis. The following primers were used: 5′-CCAATTACAACCCCGACATC-3′ and 5′-TCATAGTAGACCCAG-TCGAA-3′ for *Shh.* Total RNA was prepared from lung, liver, heart and brain of 15.5-day rat embryos according to the guanidium thiocyanate method (20). The total RNA (1 $\mu$g) was subjected to RT-PCR as described in the manual from Perkin-Elmer, 1 cycle at 95°C for 2 min, 30 cycles at 95°C for 1 min and at 60°C for 2 min, and 1 cycle 60°C for 7 min. The PCR products were subjected to 3% Nusieve gel (Takara, Kyoto) and blotted to nylon filter. The filters were hybridized with [$^{32}$P]-labeled *Shh* cDNA fragment prepared as described below.

*In situ hybridization.* The cDNA of Shh and Bmp-4 for *in situ* hybridization was prepared by RT-PCR. The following primers were used: 5′-TGATGTGTGGGCCCGGCAGGGGGTTT-3′ and 5′-TCAGCCGCCGGATTTGGC-CGCCACG-3′ for *Shh,* 5′-AAGAATTCATGATTCCTGGTAACCGAA-3′ and 5′-GAAGGAATTCGGGCTCAC-ATCGAAAG-3′ for *Bmp-4.* The PCR products were cloned into pCRII vector by a TA cloning kit (Invitrogen, San Diego, CA ). The DNA sequence of these PCR products was analyzed by a fully automated DNA sequencer (Pharmacia, Milwaukee, WI). Sense and anti-sense digoxigenin-labeled RNA probes were transcribed from linearized pCRII plasmid containing rat *Shh* and rat *Bmp-4* by Sp6 or T7 RNA polymerase *in vitro* according to the manufacturer's manual (DIG RNA Labeling Kit Sp6/T7, Boehringer Mannheim, DFG). Rats (Sprague-Dawlye) at 13.5-, 14.5-, and 15.5-day of gestation points were obtained from Clea Japan, Inc. *In situ* hybridization was performed as described by Wilkinson (21). In brief, explants and tissues were fixed with 4% paraformaldehyde in phosphate buffered saline. Frozen sections (10 $\mu$m thick ) were cut by a cryostat and attached to slides coated with VECTABOND reagent (Vector Laboratories, CA). Samples were treated with proteinase K (1 $\mu$g/ml) at 37°C for 10 min, refixed in 4% paraformaldehyde, and hybridized overnight with a digoxigenin-labeled RNA probe. The hybridized RNA was detected by alkaline phosphatase-conjugated anti-digoxigenin according to the procedure described by Wilkinson (21).

*Immunostaining.* The distribution of Shh in lung of 15.5-day rat embryos was examined by immunostaining using anti-Shh antisera. Anti-Shh antisera which react with C-terminal region of Shh (ShhC) and N-terminal region of Shh (ShhN) were prepared by using QSATEARGAEPTAGIC, synthetic peptide of C-terminal region of Shh and RLAVEAGFDWVYYESC, synthetic peptide of N-terminal region of Shh as antigens, respectively. These synthetic peptides of Shh were conjugated with KLH and were injected into rabbits with Freud's complete adjuvants. Anti-ShhC and anti-ShhN antibodies were purified by peptides-affinity column chromatography. Detailed characterization of the anti-ShhC and anti-ShhN will be published elsewhere. The 15.5-day rat embryos were fixed with 4 % paraformal-dehyde and embedded into paraffin. The sections (10 $\mu$m) were subjected to immunostaining using anti-ShhC and anti-ShhN. The immunoreactivities on the section of lung tissues were detected by peroxidase-conjugated avidin-biotin kit (Vector Laboratories, CA).

## RESULTS

*Shh* was expressed in lung and brain of 15.5-day rat embryos. A 290 bp fragment of *Shh* cDNA was detected in lung as well as brain by RT-PCR and southern blot analysis (Figure 1a). The overall distribution of *Shh* mRNA in lung was investigated by whole mount *in situ* hybridization (Figure 1b). *Shh* was already expressed in the endodermal epithelial tube in the primitive esophagus and in the primitive lung bud of 13.5-day rat embryos (Figure 1b-A), which had initiated its own branching morphogenesis to form the bronchial tree. *Shh* was intensely expressed in the distal tip of the bronchial tube. Other part of the bronchial tube was weakly positive and the mesenchyme was negative. The epithelium branched into the sur-rounding mesenchyme and formed secondary bronchi in the lung of 14.5-day rat embryos (Figure 1b-B) and the bronchial tree in the lung of 15.5-day rat embryos (Figure 1b-C). *Shh* was also intensely expressed in the epithelium of the distal tip of secondary bronchial tubes or bronchial tree. The expression of *Shh* in the trachial epithelium was weaker than that in the bronchial epithelium. No positive signal was detected by whole mount *in situ* hybridization using a sense probe of *Shh* (Figure 1b-D).

To investigate the distribution of *Shh* in lung epithelium more precisely, we attempted to detect *Shh* mRNA by *in situ* hybridization of lung frozen sections (Figure 2). The expression of *Shh* was more intense in the epithelium of the branching regions than in the bronchi (Figure 2A, 2B and 2D). Expression of *Shh* was localized in the apical side of epithelial cells (Figure 2C), and throughout the developmental stages, the expression of *Shh* was restricted to the

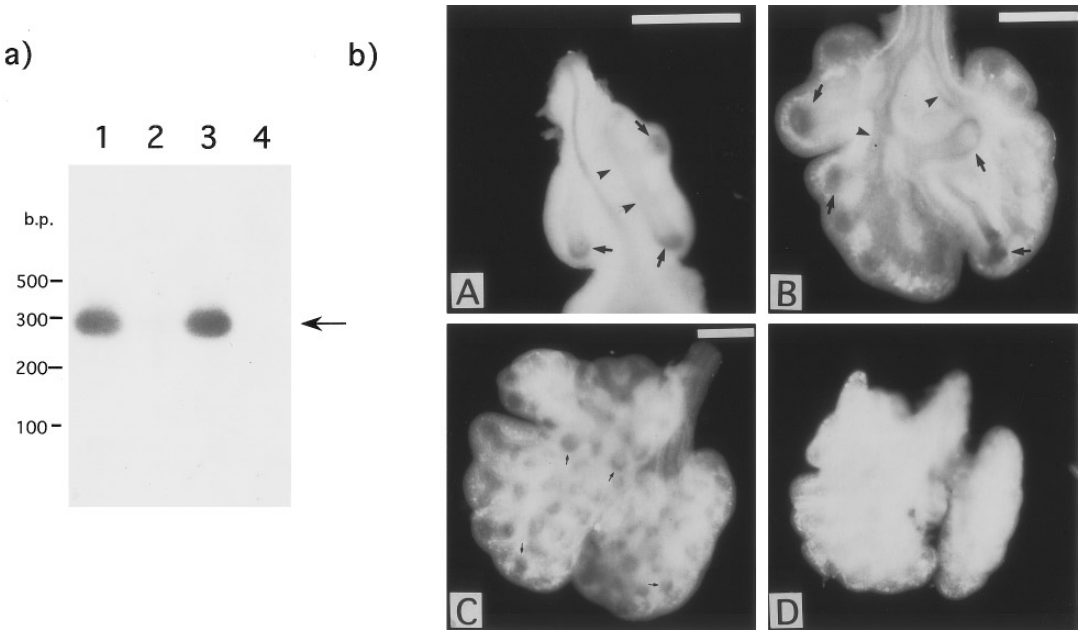



**FIG. 1.** The expression of *Shh* in lung of 15.5-day rat embryos. (a) Detection of *Shh* by RT-PCR and southern blot analysis. Lane 1, lung; lane 2, liver; lane 3, brain; and lane 4, heart. (b) Whole mount *in situ* hybridization of *Shh* in lung of rat embryos. (A) 13.5-day, (B) 14.5-day, (C and D) 15.5-day rat embryo. (A, B, and C) Anti-sense probe; (D) sense probe. The expression of *Shh* in the distal tip of the epithelium of lung bud (arrows) was more intense than in bronchi (arrowheads). The scale bars indicate 500 $\mu$m.

epithelia. No positive signal was detected in the lung by *in situ* hybridization using a sense probe of *Shh* (Figure 2E).

To confirm the expression of *Shh* in lung, we examined the distribution of Shh protein in lung of rat embryos by immunostaining using anti-ShhC and anti-ShhN antisera. Anti-ShhC and anti-ShhN reacted with a 45 kDa band in the extracts of 12.5-day rat embryos (unpublished observation). The distribution of Shh in lung of 15.5-day rat embryos shown by immunostaining using anti-ShhC and anti-ShhN was essentially similar. The distribution of Shh protein in lung had the same tendency as that of *Shh* mRNA. Shh protein was positive in the epithelia, but not uniformly. The epithelium of bronchi at the branching points was more intensely positive than other parts, and Shh protein was located in the apical side of the epithelium (Figure 3).

The expression of *BMP-4*, one of the target genes of Shh, was also investigated during lung branching morphogenesis. As shown in Figure 4, *BMP-4* was mainly expressed in the epithelium. The expression of *BMP-4*, as well as that of *Shh*, in the epithelium was not uniform; *BMP-4* was especially localized at the branching point in the epithelium (Figures 4A and 4C). No positive signal was detected by *in situ* hybridization using a sense probe of *BMP-4* (Figure 4B).

## DISCUSSION

In our study, *Shh* mRNA was intensely expressed in the distal tip of the epithelium of lung buds during branching morphogenesis and located in the apical side of the epithelial cells (Figures 1b and 2), suggesting a close relationship between the biological function of Shh and branching morphogenesis. Recently, it has been shown that Shh protein is a secreted protein and functions by an autocrine or paracrine system (22). As shown in Figure 3, Shh protein as well as *Shh* mRNA was preferentially expressed in the epithelium at branching points and

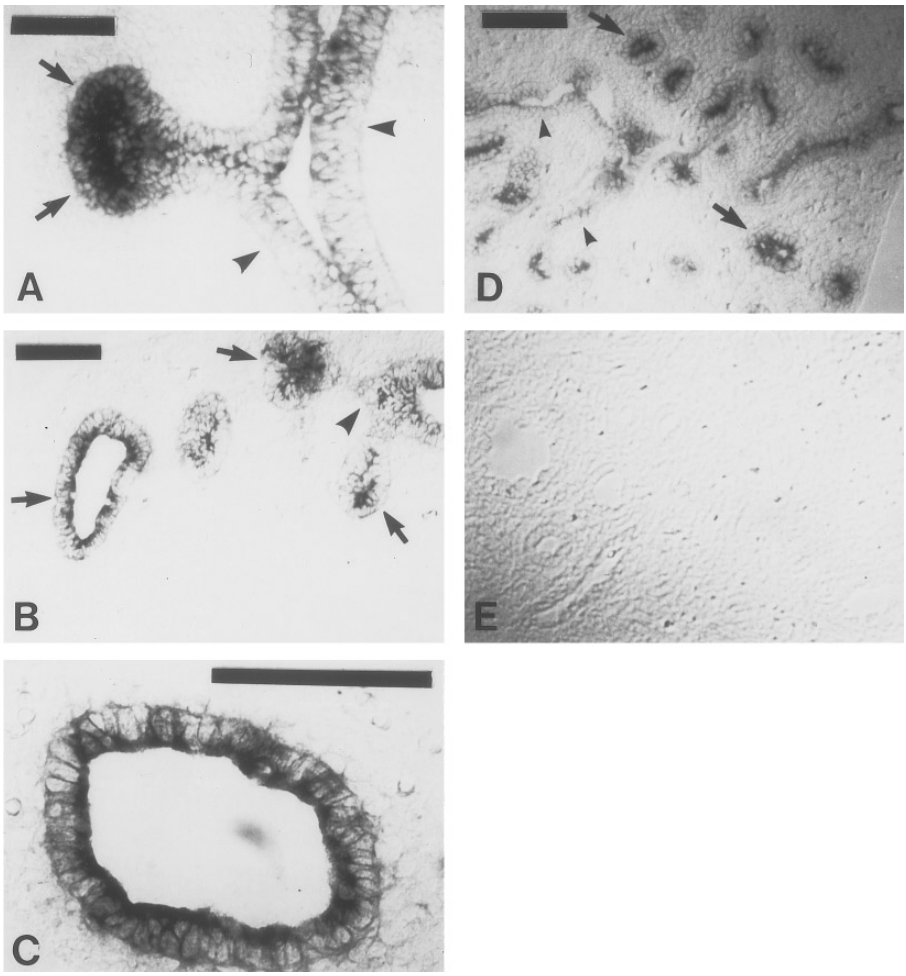
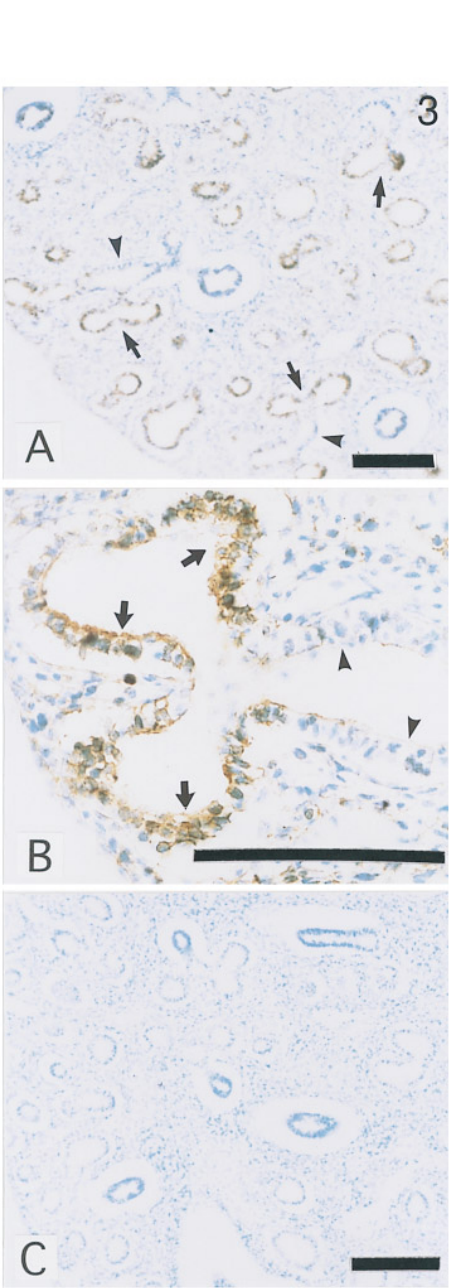



**FIG. 2.** *In situ* hybridization of *Shh* of the frozen section of lung of rat embryos during branching morphogenesis. (A) 13.5-day, (B and C) 14.5-day, and (D and E) 15.5-day rat embryos. (A–D) Anti-sense probe; (E) sense probe. Expression of *Shh* was seen only in the epithelium. *Shh* was more intensely expressed in the branching sites (arrows) than in other bronchial epithelia (arrowheads). The expression of *Shh* is localized in the apical side of the epithelial cell (C). The scale bars indicate 100 $\mu$m.

located in the apical side of the epithelium, strongly suggesting that Shh is secreted from the apical side of epithelial cells at branching points during lung branching morphogenesis.

If secreted Shh functions by the autocrine or paracrine system, the target genes must appear in the region near the cells secreting Shh protein. *Drosophila decapentaplegic* (*dpp*), which is a homologue of vertebrate *BMP-4*, a member of TGF-$\beta$ family, is activated by *hh* in many organ patterning fields including eye (23) and wing (24). Recently, the distribution of *Shh* family and *BMP* genes has been investigated in many fields of epithelial-mesenchymal interaction in the late developmental stage of mouse embryos (18). *Bmp-2* and/or *Bmp-4* are either in the mesenchyme underlying the site of *Shh* mRNA expression or in the epithelial cells near or even coincident with the cells that express *Shh*.

In lung, *Bmp-4* as well as *Shh* was expressed in the epithelium, but not uniformly. The expression of *Bmp-4* was restricted to the distal tip of the epithelium of lung buds (Figure 4),



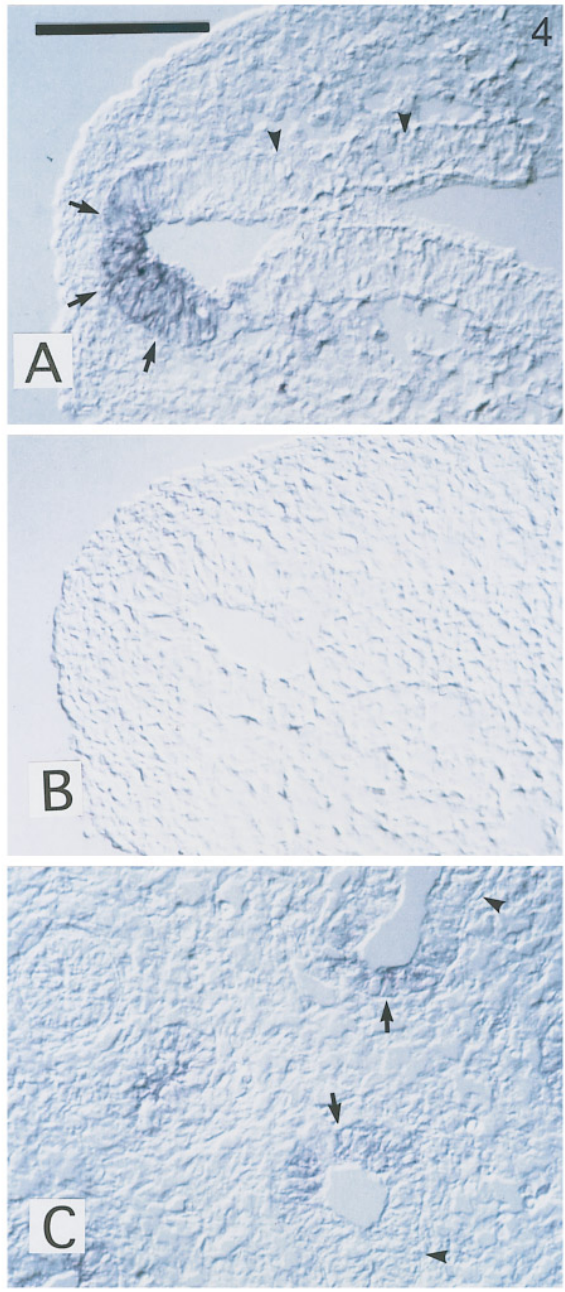




**FIG. 3.** Immunostaining of Shh protein in lung of rat 15.5-day embryos. (A) Distribution of Shh protein shown by immunostaining using anti-ShhC. The distribution of Shh shown by anti-ShhC and anti-ShhN was essentially similar. Arrows indicate the Shh-intensely positive epithelia in the branching regions and arrowheads indicate Shh-weakly positive or negative bronchial epithelia. (B) The distribution of Shh protein at branching points. Shh was intensely positive in the apical side of epithelium at the branching points. (C) Immunostaining using non-immune serum. The scale bars indicate 100 $\mu$m.

**FIG. 4.** *In situ* hybridization of *Bmp-4* of the frozen section of lung of 13.5- and 15.5-day rat embryos. *In situ* hybridization of *Bmp-4* in lung of 13.5-day rat embryos using sense probe (A) and anti-sense probe (B). *In situ* hybridization of *Bmp-4* in lung of 15.5-day rat embryos using sense probe (C). *Bmp-4* was intensely expressed at the branching points of the bronchial epithelium (arrows), but not in other parts of the bronchial epithelium (arrowheads). The scale bar indicates 100 $\mu$m.

where *Shh* was also intensely expressed. Thus, for the most part, the expression of *Shh* and *Bmp-4* overlapped in the epithelium of lung during branching morphogenesis, strongly suggesting that Shh, which secreted from the apical side of the epithelium, was involved in the epithelial branching morphogenesis by an autocrine or paracrine system.

However, Bitgood *et al* reported that *Shh* is uniformly expressed in lung epithelium of mouse embryos unlike *Bmp-4* (18). The discrepancy between our results and theirs may be due to a difference in developmental stages or species examined or a difference in the sections used. If lung sections not including the branching region were used for the *in situ* hybridization for *Shh* mRNA, the expression of *Shh* may be apparently uniform in the epithelium.

At present, it is not yet clear about the biological roles of *Shh* and *BMP-4* in the branching morphogenesis. Lung epithelia can give rise to branching morphogenesis in a serum free medium containing ECM and aFGF (25), suggesting that the spatial expression of *Shh* in lung epithelium is regulated by FGFs. Shh secreted from epithelial cells may cooperatively act on the branching morphogenesis with Bmp-4. Involvement of Shh or Bmp-4 in the branching morphogenesis and the relationship between a FGF and Shh must be clarified by studying the *in vitro* system for branching morphogenesis.

## ACKNOWLEDGMENTS

This work was supported in part by Research Grants 5A-1 for Nervous and Mental Disorders from the Ministry of Health and Welfare of Japan and by Grants-in-Aid for Scientific Research on Priority Areas (No. 05277102) from the Ministry of Education, Science, and Culture of Japan.

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