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Involvement of Hepatocyte Growth Factor in Formation of Bronchoalveolar Structures in Embryonic Rat Lung in Primary Culture

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To clarify the role of hepatocyte growth factor (HGF) in embryonic lung development, organoids from fetal rat lung were cultured in collagen gels with or without HGF antisense oligonucleotides. Cyst-like structures formed within 24 h in organoids isolated from fetuses after 14 days' gestation, but this was abolished by the oligonucleotide addition, apparently by interference with the endogenous expression of HGF. Electron microscopy revealed two types of structure: an alveolar type characterized by osmiophilic lamellar bodies in the cytoplasm and lumen, and a bronchial type consisting of epithelial cells bearing microvilli on their apical surfaces. HGF mRNA was detectable from day 14 in fetal lung by RT-PCR. Our results suggest that HGF plays, coordinately with its expression, a crucial role in the morphogenesis of both alveolar and bronchial epithelia in the rat fetal lung.

During embryonal development, histogenesis of epithelial organs is dependent on various soluble factors that induce epithelial growth, motility, and/or differentiation. For the lungs, an outgrowth of epithelial cells from the endoderm into the splanchnic mesoderm generates an anlage within which the primitive bronchial epithelium undergoes branching tubulogenesis. Subsequently, epithelial cells at the bronchial tips differentiate to form alveolar structures. Two decades ago, Douglas and Teel (1) had already described reorganization of fetal rat lung cells to form alveolar-like structures when cultured on a gelatin sponge matrix, but the molecular nature of the involved signals is still not clear.

Hepatocyte growth factor (HGF), one of the soluble factors acting via tyrosine kinase receptors, has been shown to be a potent stimulator of epithelial organ development. Originally identified as hepatocyte-specific, it is now considered to be multifunctional with mitogenic, motogenic, and morphogenic influences on a variety of epithelial cells (2, 3, 4). Recent studies have strongly suggested mitogenic and motogenic effects on pulmonary epithelial cells (5, 6, 7). However, it has remained unclear whether HGF also acts as a morphogen during lung development.

We have therefore evaluated the morphogenic activity of HGF with embryonic rat lung organoids grown in three-dimensional collagen matrices, interfering with endogenous expression of HGF with specific antisense HGF oligonucleotides.

MATERIALS AND METHODS

Culture of fetal lung organoids in collagen gels. Rat fetuses were taken from pregnant Wister rats of known gestational times (13 th to 19 th day of gestation) and their lungs were removed aseptically under a dissection microscope, washed in chilled RPMI 1640 medium (Nissui, Japan), and minced into 1 mm^3 cubes with sterile scissors. After digestion with 0.1 % trypsin and 0.1 % type II collagenase (Wako Pure Chemical, Japan) in RPMI 1640 medium at 37 °C for 20 min. The dispersed cell preparations were diluted with an equal volume of RPMI 1640 medium and repeatedly passed through a 18 gauge needle fitted to a sterile 5 ml syringe. The resulting suspensions were filtered through surgical gauze to eliminate undissociated tissue. In order to accumulate lung organoids, suspensions were subsequently filtered through 60 μm pore size sieve, and the cells that remained on the sieve were backwashed for collection into plastic syringes. Incubation of the lung organoid suspensions for 2 h at 37 °C in tissue culture flasks allowed attachment and subsequent removal of contaminating fibroblasts. Collagen gels (Rat tail type I, Collaborative Research, USA) were prepared by mixing 250 μl collagen solution with 40 μl 7.5 % sodium hydrogen carbonate and 210 μl of serum-free Eagle's MEM (Nissui, Japan) medium containing 10^5/ml of nonadherent organoids in 24-multiwell tissue culture plates and incubated at 37 °C for 30 min to allow gel formation. Then 500 μl of MEM medium supplemented with, 100 U/ml penicillin G, 100 μg/ml streptomycin, and 10 % fetal calf serum were added, and organoids were incubated in 5 % CO_2 and 95 % air. Covering media were exchanged daily.

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Histology of colonies in collagen gels. Colonies in collagen gels were fixed for 24 h in 10 % neutral-buffered formalin, dehydrated, and embedded in paraffin. Sections were cut, deparaffinized with xylene, and stained with hematoxilin and eosin. The cells in gels were also prepared for electron microscopy as described previously (8). In brief, the organoids in gels were fixed for 120 min in chilled 2.0 % glutaraldehyde in Sorenson's phosphate buffer (0.1 M, pH 7.4) at 4 °C and, after rinsing, were postfixed in Caulfield's 2 % osmium tetroxide for 30 min. The cells were then rinsed, dehydrated, and embedded in Epon 812. Thin sections were cut with an LKB ultramicrotome, mounted on copper grids stained with lead citrate and uranyl acetate and examined under a Hitachi H-300 electron microscope.

Electroporation of cells with oligonucleotides and their effects on morphogenesis. Electroporation was performed with a Electro Cell Manipulator 600 system (BTX, USA). Organoids were electroporated in MEM containing 10% calf serum at a capacitance of 1200 μF and 100 V. Oligonucleotides were added, just before electroporation to a final concentration of 20 μM (9, 10). The 17 mer phosphorothioate oligonucleotides used were synthesized and purified by high-performance liquid chromatography (Boehringer Mannheim Biochemica, Japan): HGF antisense, 5′-TTGGTCCCCCACATCAT-3′: sense, 5′-ATGATGTGGGGGACCAA-3′ (11, 12).

The numbers of spherical cysts were counted in collagen gels at different time points at a 16-fold magnification (3 mm^2) using Nikon

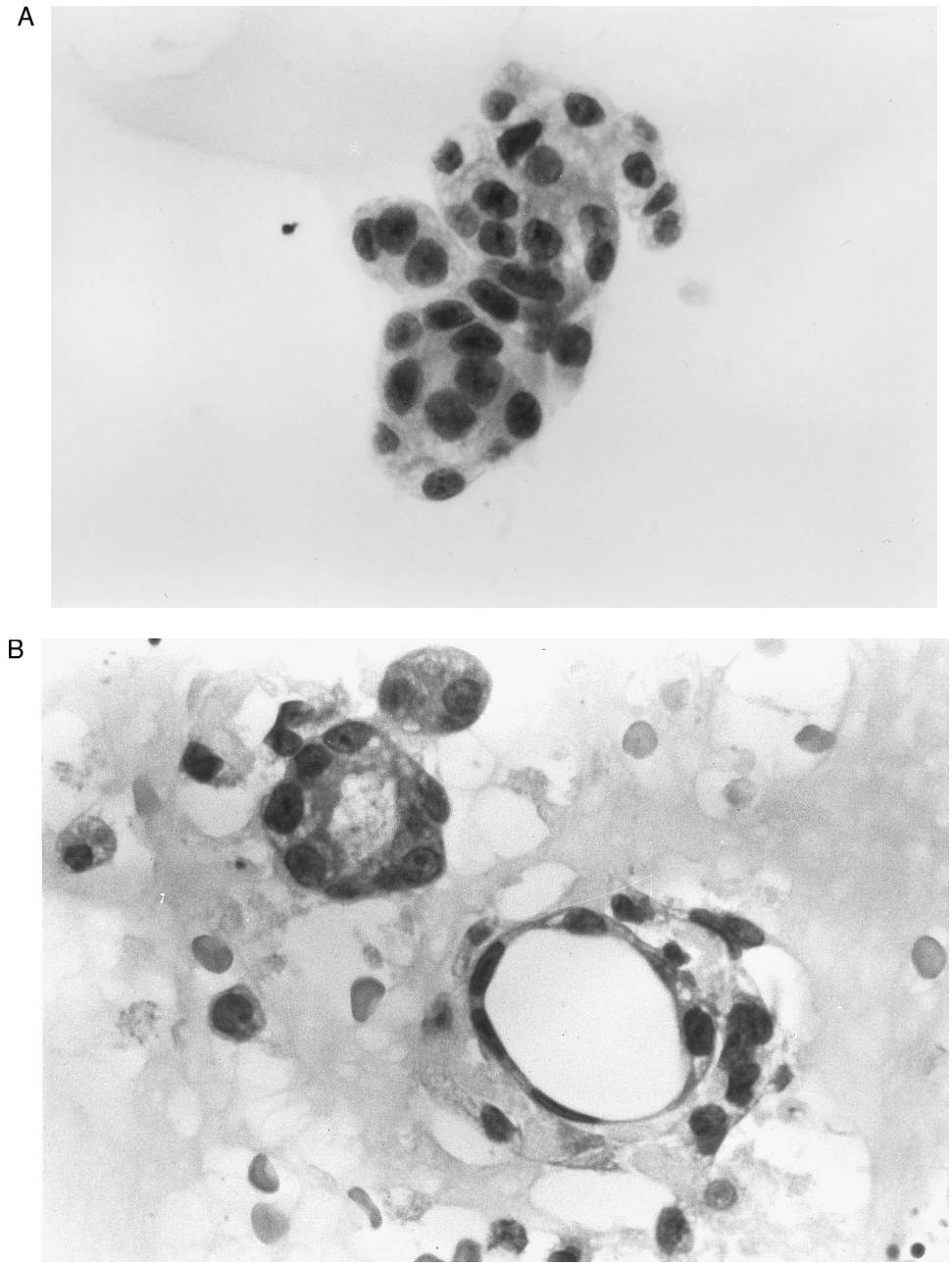

FIG. 1. Morphological appearance of embryonic rat lung organoids cultured in collagen gels. The light micrograph displays simple cell cluster in lung organoids isolated from fetal rat at 18 days' gestation (A). The lung at 18 days' gestation represent cyst-like formation after 48 h culture (B). (original magnification: × 800)

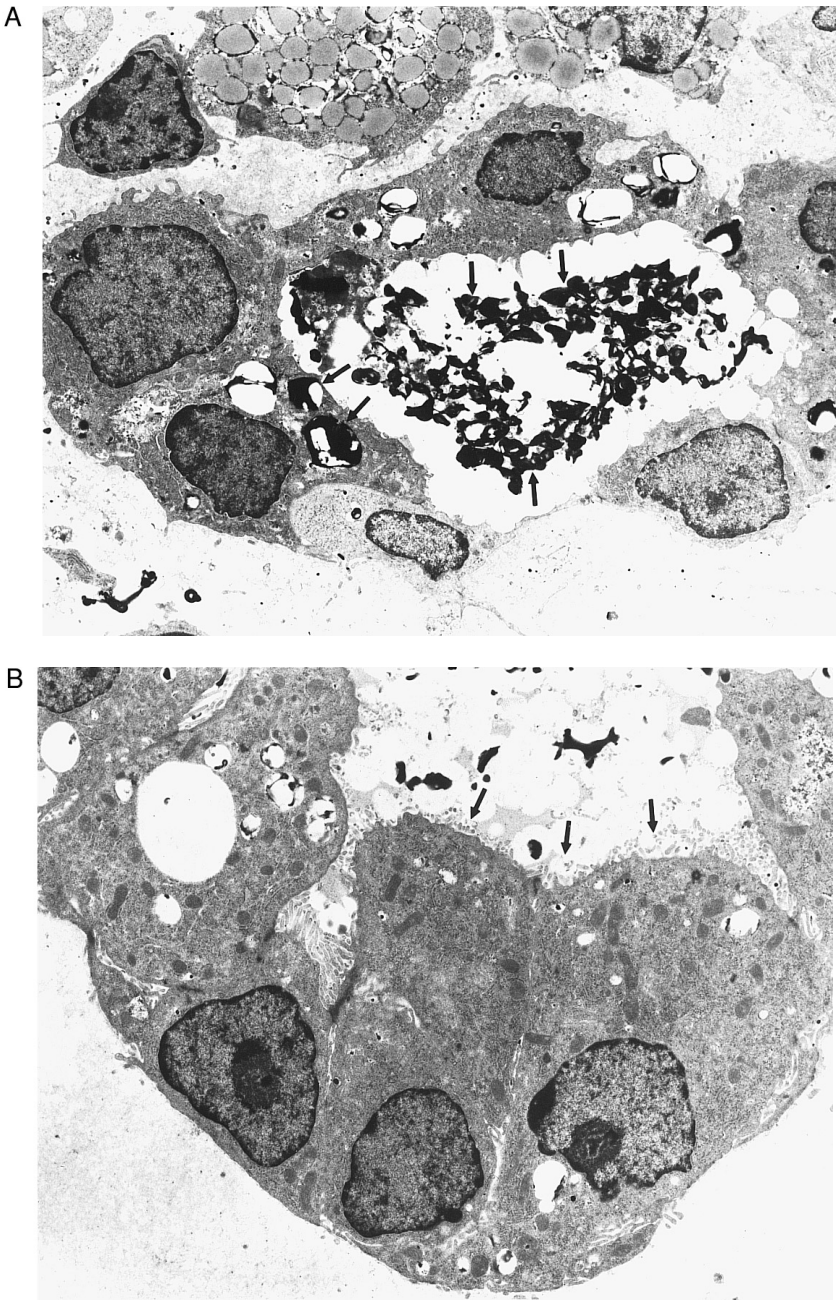

FIG. 2. Transmission electron micrograph of the ultrastructure of cyst like formed organoids at 18 days' gestation after 48 h culture. Osmiophilic lamellar bodies (arrow) are shown in the cytoplasm and in the lumen of the cyst-like structure (original magnification: × 2500) (A). Microvilli (arrow) are present on the apical surface of the cells (original magnification: × 3000) (B).

phase-contrast microscope. Experiments were performed in sextuplicate and the results were expressed as the mean number of organoids per 3 mm^2 ± SD. Comparisons were performed using the Student's *t* test. With statistical significance concluded at $P < 0.05$.

Immunoblot analysis. To detect endogenous HGF, 50 μg cultured cell pellets, were lysed in lysate buffer. Western blot analysis was performed by electrophoretic transfer to nitrocellulose sheets (Hybond-C super, Amersham, England) after SDS-PAGE using 10 % polyacrylamide gels. The sheets were incubated with a polyclonal antibody against rat-HGF α-chain (Institute of Immunology, Japan) and immunoreactive proteins were stained using an ABC kit (Vectastain; Vector Laboratories, USA) as previously described (13).

Reverse-transcriptase polymerase chain reaction. Embryonic rat lungs at different gestational stages (13 to 19 days of gestation) were isolated and messenger RNA was extracted by the guanidinium thiocyanate method using an mRNA purification kit (Pharmacia LKB Sweden). For each reaction, 1 μg of poly (A^+) RNA was used to prepare cDNAs using M-MuLV reverse transcriptase (Pharmacia LKB Sweden), and the resulting cDNA were subjected to 25 to 35 cycles of polymerase chain reaction (Perkins-Elmer Cetus, USA) using gold AmpliTaq DNA polymerase. The PCR products were electro-

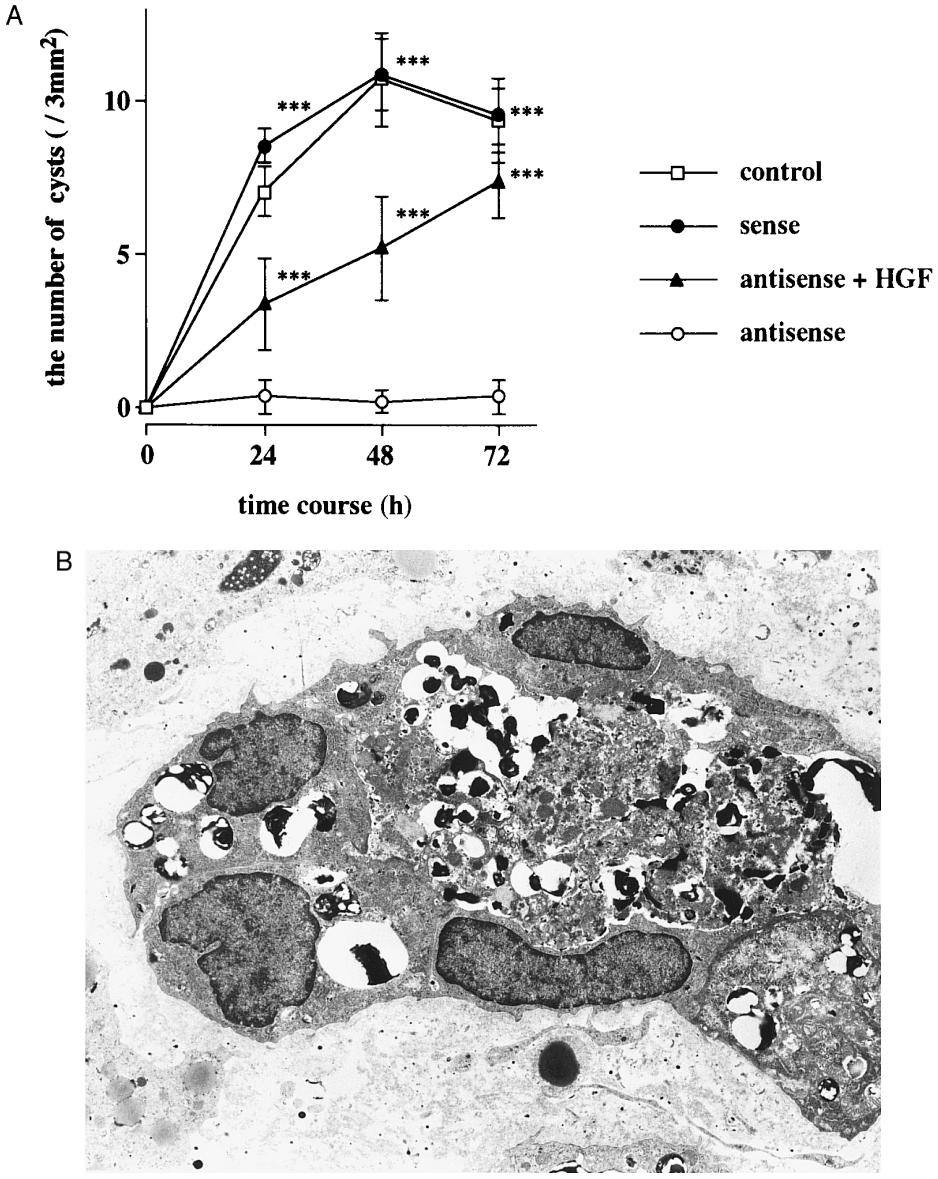

FIG. 3. Effects of HGF antisense oligonucleotides in organoids culture. Morphogenesis was assessed by determining the number of cysts along with time course: Organoids were electroporated in the presence of 20 μM sense oligonucleotides (sense); 20 μM antisense oligonucleotides (antisense); or 20 μM antisense oligonucleotides supplemented with 10 nM of rHGF (antisense + HGF). Electroporation was also performed without oligonucleotides (control) (A). (mean ± SD, ***$P<$ 0.005 vs. antisense) Ultrastructure of electroporated organoids with antisense oligonucleotides at 18 days' gestation after 48 h culture (original magnification: × 2500) (B).

phoresed through 2.0 % agarose gels in 1 × TBE buffer. Primers specific for reverse-transcriptase polymerase chain reaction of rat HGF gene were as follows: 5′-primer: 5′-CCAACACAAACAACA-GTGG-3′; 3′-primer: 5′-AACAATGACACCAAGAACCA-3′; size of the amplified fragment, 585 bp (14).

RESULTS

Changes in organoid structure within collagen gels. The isolated fetal lung organoids were composed of simple cell clusters before culture (Fig. 1A). Within collagen gels those from fetuses after 14 days' gestation formed spherical, cyst-like structures within 24 h. These then persisted for several days after seeding (Fig. 1B). Cyst formation was not noted in organoids isolated from 13 days fetuses. Two kinds of spherical cyst were recognized according to the electron microscope findings. In alveolar-like organoids type II cells, with osmiophilic lamellar bodies in the cytoplasm and lumen, predominated (Fig. 2A). The other, bronchial type consisted of epithelial cells bearing microvilli on their apical surfaces (Fig. 2B).

Effects of HGF antisense oligonucleotides. Formation of cyst-like structures from both alveolar cell dominant

and bronchial cell dominant organoids was inhibited to by the addition of HGF antisense oligonucleotides. As shown in Fig. 3A, the number of cyst-like structures was unchanged in cultures supplemented with antisense oligonucleotides whereas was obvious increase after 48 h in the cultures with sense oligonucleotides or antisense oligonucleotides complemented with recombinant HGF. The interference with cyst formation was accompanied by the appearance of osmiophilic lamellar bodies scattered in the cytoplasm (Fig. 3B).

Suppression of HGF protein by antisense oligonucleotides. Cultured organoids treated with antisense oligonucleotides were harvested and subjected to immunoblotting. As shown in Fig. 4, two major bands corresponding to the single-chain form of HGF (85 kDa) and the α-chain of HGF (56 kDa) were observed just after organoid preparation. However, these bands disappeared after 24 h of incubation and did not reappear thereafter.

Expression of HGF mRNA during lung development in vivo. To examine whether HGF could have the suggested function in vivo, we assessed HGF mRNA expression during lung development by RT-PCR (Fig. 5). The predicted 585-bp HGF cDNA fragments were detectable from 14 days of gestation.

DISCUSSION

The present study demonstrated that a distinct pattern of alveolar and bronchial morphogenesis occur pulmonary organoids isolated from rat fetuses after day 14 of gestation when they are cultured within collagen gels, and that this is abolished by the presence of specific antisense HGF oligonucleotides, apparently by interference with endogenous expression of HGF.

Rat embryonic lung organoids are well known to form alveolar-like structures when cultured within collagen gels (1). In the present study, two different types could be distinguished by electron microscopy. The finding of

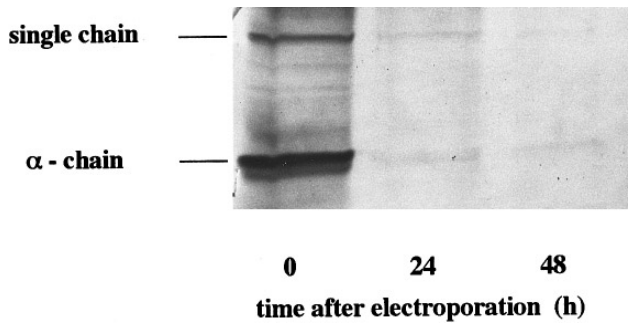

FIG. 4. Suppression of HGF protein by HGF antisense oligonucleotides in lung organoids. Immunoblot analysis was performed for lung organoids undergoing electroporation with oligonucleotides. Transferred protein were probed using a polyclonal antibody to rat HGF.

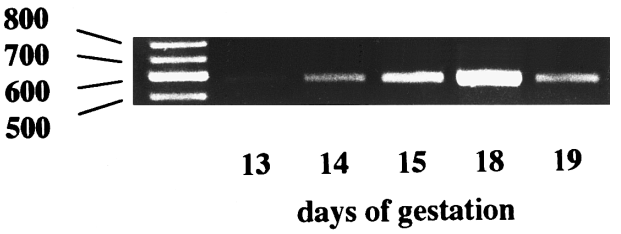

FIG. 5. Expression of HGF mRNA in embryonic rat lung. RT-PCR analysis was carried out on 1 μg of poly (A^+) RNA from embryonic rat lung. Ethidium bromide-stained agarose gel showing amplified HGF product (585bp).

osmiophilic lamellar bodies in the alveolar type suggested active production of pulmonary surfactant. The bronchial type was also highly differentiated as evidenced by the microvilli on the apical surfaces of the cells. These functional and morphologic features, which were abolished by interfering with expression of HGF, are typical of their respective in vivo counterparts. The present results thus strongly suggest that HGF makes an essential contribution to morphogenesis of both alveolar and bronchial epithelia in the rat fetal lung.

HGF mRNA was detectable from day 14 in the fetal lung by RT-PCR. This is consistent with previous immunohistochemical findings (15). The fact that formation of spherical, cyst-like structures was also only observed from 14 days of gestation provides further chronological evidence that the expression of HGF mRNA is closely related with morphological change.

Morphogenic activity of HGF was initially identified in Madin-Darby canine kidney cells in collagen gels (16). Subsequently, similar effects were recognized in a wide variety of epithelial cell lines stably transfected with HGF cDNA (17) and mammary glands in culture (11). HGF is currently considered to act as a mesenchymal derived mediator in epithelium-mesenchyme interactions (18, 19, 20). Consistent with this HGF receptor mRNA is specifically expressed in epithelial cells in the developing lungs, whereas HGF mRNA is localized to mesenchymal cells (21, 22). Concerning morphogenetic properties of HGF in lung development, Brinkmann et al (17) reported expression of transfected HGF cDNA in LX-1 lung carcinoma induced alveolar-like structures and Matsumoto et al. (22) described antisense HGF oligonucleotides to specifically inhibit epithelial branching morphogenesis in cultured lung rudiments isolated from rat embryos, although in the latter report, it is unclear how airway branching or alveolarization was affected. In conclusion our results along with the available literature, suggest that HGF plays, a crucial role in morphogenesis of both alveolar and bronchial epithelia in the rat fetal lung.

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