

# Hepatocyte growth factor (HGF) acts as a mesenchyme-derived morphogenic factor during fetal lung development

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## SUMMARY

Mesenchymal-epithelial tissue interactions are important for development of various organs, and in many cases, soluble signaling molecules may be involved in this interaction. Hepatocyte growth factor (HGF) is a mesenchyme-derived factor which has mitogenic, motogenic and morphogenic activities on various types of epithelial cells and is considered to be a possible mediator of epithelial-mesenchymal interaction during organogenesis and organ regeneration. In this study, we examined the role of HGF during lung development. In situ hybridization analysis showed HGF and the c-met/HGF receptor gene to be respectively expressed in mesenchyme and epithelium in the developing lung. In organ cultures, exogenously added HGF apparently stimulated branching morphogenesis of the fetal lung. In contrast, HGF translation arrest or neutralization assays resulted in clear inhibition of epithelial branching. These results suggest that HGF is a putative candidate for a mesenchyme-derived morphogen regulating lung organogenesis. We also found

that HGF is involved in epithelial branching, in collaboration with fibroblast growth factor (FGF) family molecule(s). In mesenchyme-free culture, HGF alone did not induce epithelial morphogenesis, however, addition of both HGF and acidic FGF (aFGF) or keratinocyte growth factor (KGF), ligands for the KGF receptor, induced epithelial branching more extensively than that was observed in explants treated with aFGF or KGF alone. In addition, the simultaneous inhibition of HGF- and FGF-mediated signaling using neutralizing antibody and antisense oligo-DNA resulted in drastic impairment of epithelial growth and branching. Possible interactions between HGF and FGFs or other growth factors in lung development is given consideration.

Key words: Hepatocyte growth factor (HGF), c-met, Epithelial-mesenchymal interaction, Branching morphogenesis, Fibroblast growth factor (FGF), Lung, Rat

## INTRODUCTION

During embryonic development, proper proliferation and morphogenesis of epithelial cells proceed to form most epithelial (parenchymal) organs, including lung, liver, intestines and kidney, through inductive interactions with mesenchyme. In general, organogenesis is initiated when the primitive epithelium interacts with specific mesenchymal tissues at defined stages of development, resulting in organ-specific structures and functions. Such tissue interactions are essential not only for ordered organ formation in fetal stages, but also for proper maintenance and repair in adult tissues. Soluble and/or membrane-bound mesenchymal factors have been suggested to be involved as signaling molecules mediating such tissue interactions (reviewed by Birchmeier and Birchmeier, 1993; Birchmeier et al., 1995; Gilbert, 1997). In most cases, precise molecular mechanisms remain to be determined.

Hepatocyte growth factor (HGF), which was originally purified and cloned as a potent mitogen for mature hepatocytes in primary culture (Nakamura et al., 1984; Nakamura et al., 1989), is a well-known cytokine produced by cells of

mesenchymal origin. The receptor for HGF is a membrane-spanning tyrosine kinase encoded by the c-met proto-oncogene (Bottaro et al., 1991; Naldini et al., 1991). During fetal development, HGF and its receptor (c-met/HGF-R) are expressed in a wide variety of developing organs, such as liver, lung, pancreas and kidney, and their transcripts were found to be localized in mesenchymal and epithelial tissues, respectively (Sonnenberg et al., 1993), thereby indicating an important role for HGF in epithelial-mesenchymal interaction during development. HGF exerts multiple biological activities on various types of epithelial cells (reviewed by Matsumoto and Nakamura, 1993; Zarnegar and Michalopoulos, 1995; Matsumoto and Nakamura, 1996). HGF has mitogenic effects on hepatocytes and also on a wide variety of epithelial cells and some mesenchyme-derived cells such as endothelial cells, hematopoietic cells and chondrocytes. Scatter factor, independently identified as a fibroblast-derived motility factor for epithelial cells (Stoker et al., 1987), is known to be a molecule identical to HGF (Weidner et al., 1991; Furlong et al., 1991; Konishi et al., 1991), a finding which indicates that HGF acts on cell dissociation and migration, as a potent motogen. In

addition, HGF elicits a unique morphogenic activity, e.g., induces kidney, or mammary gland-derived epithelial cells, to form branching ducts in three-dimensional collagen gels (Montesano et al., 1991; Soriano et al., 1995; Barros et al., 1995; Niranjani et al., 1995). It was also reported that HGF induces morphogenesis in diverse epithelial cells, such as colon, pancreas, prostate and lung (Brinkmann et al., 1995). Such mitogenic, motogenic and morphogenic activities are essential for organization, maintenance and remodeling of multicellular structures (tissues and organs). We have demonstrated that HGF acts as an 'organotrophic' factor in several types of tissue repair, such as liver and renal regeneration (reviewed by Matsumoto and Nakamura, 1993, 1996)

Lung organogenesis is well characterized as an extensive progression of branching tubulogenesis of epithelial tissues (Wessells, 1970). In rat, the lung primordium develops from endodermal buds from the foregut, at around 9-10 days postcoitum (d.p.c.). The epithelial rudiments, the lung buds, form columnar epithelial tubules and invade the surrounding mesenchyme and then progress through a series of repetitive branchings to give rise to the prospective bronchial and pulmonary tree. Fetal lung buds undergo similar branching morphogenesis in culture, and this is frequently used as a typical example for studying the indispensable role of mesenchymal tissues in epithelial morphogenesis. For example, when mesenchyme is removed from lung rudiments, branching morphogenesis does not occur (Wessells, 1970). Furthermore, transplantation of bronchial mesenchyme onto trachea epithelium caused ectopic branching, whereas transplantation of trachea mesenchyme onto bronchial epithelium resulted in disrupted morphogenesis (Shannon, 1994). The molecular nature of the mesenchymal signals controlling morphogenesis of lung epithelium is beginning to be clarified.

It was reported that HGF and c-met/HGF-R are expressed in the developing lung (Defrances et al., 1992; Sonnenberg et al., 1993). In addition, HGF is known to be a potent mitogen for lung epithelial cells in vitro (Mason et al., 1994a; Shiratori et al., 1995; Ohmichi et al., 1996). Furthermore, we recently demonstrated that HGF functions as a 'pulmotrophic' (lung regenerating) factor after lung injury (Yanagita et al., 1993; Ohmichi et al., 1996). Based on these findings, the possibility that HGF also participates in fetal lung development would have to be given attention. We hypothesized that HGF might be one of the mesenchyme-derived epithelial morphogens functioning in fetal lung development. In the present study, we show the gene expression of HGF and its receptor in the fetal lung and that HGF stimulates branching morphogenesis of developing lung in culture. Impairment of the HGF-mediated signaling system in culture results in retardation of branching morphogenesis of lung explants. Possible functional relationships between HGF and other polypeptide signaling molecules, such as fibroblast growth factor (FGF) family, on lung development are also examined.

## MATERIALS AND METHODS

### Materials

Recombinant human HGF was purified from culture medium of CHO cells transfected with plasmid containing the full size cDNA encoding human HGF (Nakamura et al., 1989; Seki et al., 1990). Recombinant

human acidic fibroblast growth factor (aFGF; Boehringer Mannheim, Hamburg, Germany) and recombinant human keratinocyte growth factor (KGF; Upstate biotechnology INC, New York, USA) were obtained commercially. Anti-rat HGF IgG was prepared according to methods described elsewhere (Honda et al., 1995; Yamada et al., 1995; Tabata et al., 1996). Briefly, the IgG fraction was purified from rabbit antisera raised against recombinant rat HGF, using a protein A column, followed an affinity column immobilized with recombinant rat HGF. Mono-specific anti-rat HGF IgG was digested with papain and the Fab fragment was separated using the carboxy methyl cellulose column system, and further dialyzed against sterilized PBS prior to addition to culture media.

### Organ culture

Time-mated Wistar rats (Nihon SLC, Shizuoka, Japan) were killed on day 13 of pregnancy and embryos were removed. Under a stereomicroscope, fetal lung rudiments were dissected, washed in Hanks' solution, and placed on a transwell polycarbonate membrane filter (12 mm in diameter, 8 µm pore size, Costar, Massachusetts, USA) in 12 separate wells of a culture plate containing 800 µl of culture medium. Dulbecco's modified Eagle's medium (DMEM) containing 0.38% (w/v) NaHCO<sub>3</sub>, 100 units/ml penicillin, 100 µg/ml streptomycin and 10% fetal bovine serum (GIBCO BRL, Tokyo, Japan) was used. For experiments using antisense oligo-DNA, serum-free medium (1:1 mixture of DMEM and Ham's F12) was used. All the explants were incubated at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> in air.

Mesenchyme-free epithelium culture was done essentially as described by Nogawa and Ito (1995) with minor modifications. The lung rudiments were isolated from 13 d.p.c. embryos, digested with Hanks' solution containing 1000 U/ml of dispase (Godo Shusei, Tokyo, Japan) at 37°C for 30 minutes, washed twice with Hanks' solution, and epithelial tissues were completely isolated. The absence of mesenchymal cells surrounding epithelial tissues was histologically confirmed using paraffin sections. Two or three pieces of epithelial tissues were embedded in 20 µl of Matrigel (Becton Dickinson, Massachusetts, USA) and cultured on polycarbonate membrane.

### Antisense oligo-DNA

Effects of HGF or KGF-R antisense oligo-DNA on lung development in culture were examined according to methods described in previous reports (Yang et al., 1995; Souza et al., 1995; Tabata et al., 1996; Post et al., 1996). A region of the rat HGF gene consisting of bp 143-157 (Tashiro et al., 1990) including the initiation codon for translation was chosen for the design of oligo-DNA. The sequence of 'antisense' oligo-DNA was 5'-GGT CCC CCA CAT -3'. In our culture condition, this 'antisense' oligo-DNA was more effective in impairing lung development than those described in previous reports (Yang et al., 1995; Tabata et al., 1996). However, several matches were found between this 15 mer sequence and the gene sequence listed in the GenBank database. Therefore, we used a 'rearranged', not 'sense', oligo-DNA as a control. The sequence of this oligo-DNA was 5'-GGA CCC CCT CTA CTA-3', with an altered A and T to each T and A in antisense oligo-DNA and closely resembles antisense oligo-DNA. 'Antisense' and 'sense' oligo-DNA specific for KGF-R has been described (Post et al., 1996). The phosphorotioate oligo deoxynucleotide, purified by HPLC, was prepared by Sawaday Technology Ltd (Tokyo, Japan).

### RT-PCR

Reverse transcription-polymerase chain reaction (RT-PCR) was done according to the standard protocol. Short, single strand cDNA was prepared from total RNA, using MuLV reverse transcriptase (GIBCO BRL). The following sets of primers were used. HGF: forward primer, 5'-TTG GCC ATG AAT TTG ACC TC-3'; reverse primer, 5'-ACA TCA GTC TCA TTC ACA GC-3'. c-met/HGF-R: forward primer, 5'-TGT GCA TTC ACT AAA TAT GT-3'; reverse primer, 5'-GTC CCA GCC ACA TAT GGT CA-3'. The identity of PCR products was

confirmed by sequencing. PCR conditions were as follows: denaturation at 94°C for 30 seconds, followed by annealing at 55°C for 60 seconds and extension at 72°C for 60 seconds.

### In situ hybridization

Rat HGF and c-met cDNA, subcloned into pBluescript KS, were used as templates for in vitro transcription (Honda et al., 1995). Sense and antisense riboprobes were generated after linearization of plasmid DNA by restriction enzyme digestion and in vitro transcription by T3 or T7 RNA polymerase in the presence of <sup>35</sup>S-, or digoxigenin-labeled UTP. Alkaline hydrolysis was used to reduce the average size to 500 bp. In our preliminary studies using these probes, positive signals for HGF and c-met mRNA were confirmed in fetal rats (data not shown), in good accordance with the results in fetal mice described by Sonnenberg et al. (1993). Detection of HGF and c-met mRNA by in situ hybridization to whole mounts was essentially carried out according to the protocol described by Wilkinson and Nieto (1993). Hybridization of radio-labeled riboprobes in cross sections was done essentially as described by Honda et al. (1995).

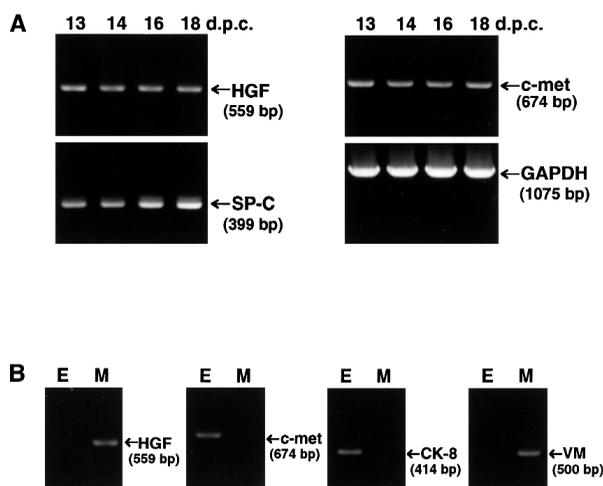
### Histology

After culture, lung explants of epithelial tissues were fixed overnight in 70% ethanol. After dehydration through a graded series of ethanol, samples were treated with xylene and embedded in paraffin. The tissues were then sectioned at a thickness of 4 µm, deparaffinized, and stained with hematoxylin and eosin.

## RESULTS

### Expression of HGF and its receptor mRNA in developing lung

It was reported that, HGF and c-met/HGF-R mRNA are expressed in fetal murine lung (Sonnenberg et al., 1993). However, temporal and spatial expression of those genes in the developing lung have not been described in detail. We first



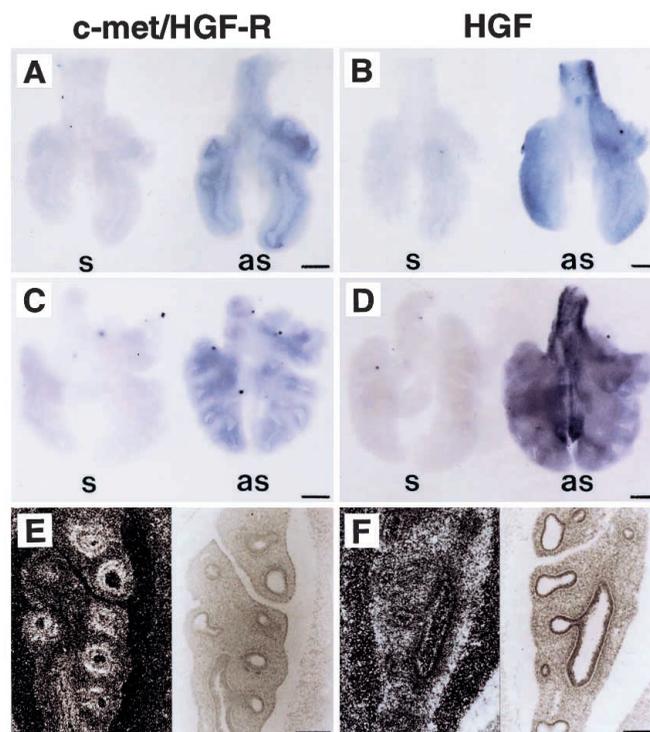
**Fig. 1.** Expression of HGF and c-met/HGF-R mRNA during lung development, determined by RT-PCR analysis. (A) Oligo-dT primed cDNA from indicated stages of fetal lung were used as templates. As a control, the amplifications of GAPDH fragment are shown. Surfactant protein-C (SP-C) is a marker, the expression of which increases in later stages of development. (B) Oligo-dT primed cDNA, prepared from 13 d.p.c. fetal rat lung epithelium (E) or mesenchyme (M), were used as templates. Purity of epithelial and mesenchymal tissues was confirmed by amplifications of cytokeratin 8 (CK-8) and vimentin (VM) (Post et al., 1996), respectively.

examined the temporal expression pattern of HGF and c-met/HGF-R mRNA during lung development, using RT-PCR. Both HGF and c-met/HGF-R genes were expressed in lung buds from 13 d.p.c. embryos, when the branching morphogenesis of epithelial tissues exclusively began (Fig. 1A). Expression of these genes was fairly constant throughout the development. Furthermore, HGF and c-met/HGF-R mRNA were separately detected in mesenchymal and epithelial tissues, respectively (Fig. 1B).

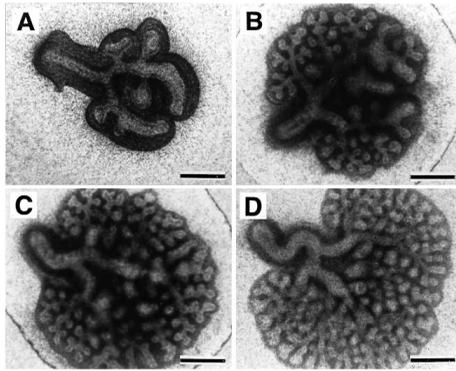
To elucidate the precise localization, in situ hybridization analysis was performed. In 13 d.p.c. fetal lung, c-met/HGF-R mRNA were localized in the main bronchus and branching end-buds, but not in the trachea (Fig. 2A,C). HGF transcripts were distributed in mesenchymal tissues (Fig. 2B,D). In cross sections, differences in spatial distribution of HGF and c-met/HGF-R could be seen more clearly. Specific signals for c-met/HGF-R were localized in tubular epithelial cell layers and HGF transcripts were detected throughout the mesenchymal region (Fig. 2E,F). In later stages of developing lungs (15 d.p.c. and thereafter), essentially the same expression pattern was observed (data not shown). These results suggest that HGF was derived from the mesenchyme and could act on the epithelium in the developing lung.

### HGF stimulates branching morphogenesis in developing lung

To assess the biological function of HGF in lung development,



**Fig. 2.** In situ hybridization analysis of HGF and c-met/HGF-R mRNA expression in fetal lungs. Whole-mount (A-D) and radioactive (E,F) in situ hybridization analysis was carried out in 13 d.p.c. (A, B) and 14 d.p.c. (C-F) fetal lung. (A, C, E) c-met/HGF-R expression. (B, D, F) HGF expression. In A-D, positive signals were not observed in rudiments hybridized with sense (s) probe. In E and F, dark- (left) and bright-field photographs are shown. Scale bar, 200 µm (A, B, E, F), and 25 µm (C, D).

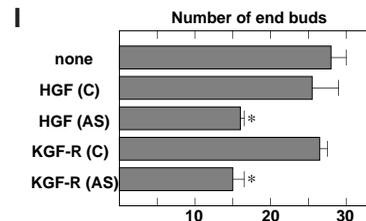
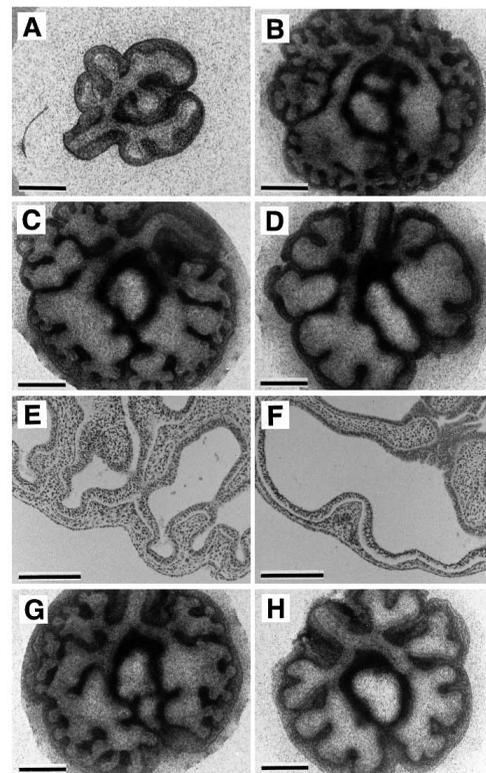


**Fig. 3.** Effects of recombinant HGF on fetal lung development in culture. (A) Lung rudiments isolated from 13 d.p.c. rat fetus were cultured for 3 days in the absence (B) or presence of (C) 200 ng/ml or (D) 500 ng/ml HGF. Scale bar, 500  $\mu$ m.

embryonic lungs were cultured in the presence of HGF. Lung buds isolated from 13 d.p.c. have a right and left main bronchus with a few rudimentary branches (Fig. 3A). After 3 days in culture, the lung rudiments had increased in size and the main bronchus branched to form the putative alveolar region (Fig. 3B). In this culture condition, the addition of 200 ng/ml of HGF stimulated the growth and branching of the lung explants (Fig. 3C). At a dose of 500 ng/ml HGF, the stimulatory effects on branching morphogenesis were more prominent, the peripheral epithelial tubules showed a more complex branching pattern (Fig. 3D) and the number of terminal airway buds markedly increased to over 1.6-fold higher than that in the untreated control (mean  $\pm$  s.d.;  $61 \pm 4.1$  vs.  $101 \pm 8.5$  in untreated- and HGF-treated explants, respectively.  $P < 0.05$ ). No apparent morphological change was seen in the cultured explants treated with other growth factors, such as EGF, TGF- $\alpha$ , PDGF, bFGF and IGF-I (data not shown).

#### Abrogation of endogenous HGF action results in impaired branching morphogenesis

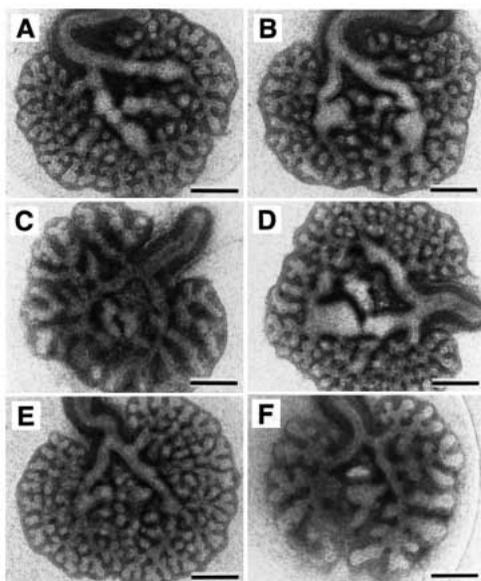
Next, we examined the effects of antisense oligo-DNA specific for HGF, which may block translation of endogenous HGF. In lung rudiments cultured in serum-free condition, several buds were newly formed around the lateral side of the main bronchus (Fig. 4B). Although addition of control DNA led to no morphological changes, explants treated with antisense HGF oligo-DNA showed significantly less branching (Fig. 4C,D). Compared with the control, little difference was observed in the size of the main bronchus, but the number of tubules protruding from the main bronchus was significantly reduced (about 60% of control, Fig. 4I). Histologically, many small epithelial tubules with newly formed end-buds, were frequently evident at the periphery of control explants (Fig. 4E), whereas fewer tubules were observed in explants treated with antisense HGF oligo-DNA (Fig. 4F). To determine the degree of inhibition of endogenous HGF production by antisense HGF oligo-DNA, HGF protein contents in cultured explants were measured by enzyme-linked immunosorbent assay (ELISA). The treatment with HGF antisense DNA significantly reduced the total amount of HGF in cultured explants (mean  $\pm$  s.d.;  $0.22 \pm 0.03$  ng in control vs.  $0.10 \pm 0.01$  ng in antisense DNA treated,  $P < 0.05$ ). Such inhibitory effects



**Fig. 4.** Effects of antisense HGF or KGF receptor oligo-DNA on fetal lung development in culture. (A) Lung rudiments isolated from 13 d.p.c. rat fetus were cultured in serum-free medium for 2 days (B). (C,D,G,H) Appearance of whole culture or (E,F) histological view of lung explants treated with control HGF oligo-DNA (C,E), antisense HGF oligo-DNA (D,F), sense KGF-R oligo-DNA (G), and antisense KGF-R oligo-DNA (H). Scale bar, 500  $\mu$ m (A-D,G,H), and 200  $\mu$ m (E,F). The number of end-buds in cultured explants was counted and shown in I. Bars represent mean  $\pm$  s.d. More than four lung explants were used in each experiment. Concentration of all oligo-DNAs was 30  $\mu$ M. \*Statistically different from non-treated control (none) at  $P < 0.05$ .

of antisense HGF oligo-DNA on branching tubulogenesis were comparable to those of KGF-R. The addition of antisense KGF-R oligo-DNA to the culture caused significant impairment of epithelial branching in lung rudiments (Fig. 4G-I), in good agreement with the data of Post et al. (1996).

More apparent inhibitory effects were confirmed using neutralizing antibody specific for HGF. The lung explants treated with anti-HGF IgG showed a drastic inhibition of epithelial branching. The structure of distal parts of the bronchus in anti-HGF IgG treated-rudiments was obviously different from that of the control; many bud-like structures were seen at the periphery of control explants, whereas the same region was populated by long tubules with poor

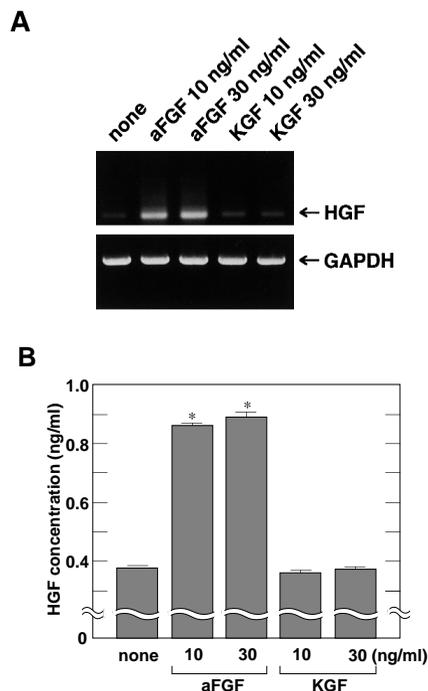


**Fig. 5.** Effects of anti-HGF neutralizing antibody on fetal lung development in culture. The lung rudiments isolated from 13 d.p.c. rat fetus were cultured for 3 days (A). Appearance of whole lung explants treated with normal rabbit IgG (B), anti-HGF IgG (C), anti-HGF IgG and 200 ng/ml recombinant HGF (D), 200 ng/ml of aFGF and normal rabbit IgG (E), and 200 ng/ml of aFGF and anti-HGF IgG (F). Concentration of antibodies used was 30  $\mu$ g/ml. Scale bar, 500  $\mu$ m.

branchings in anti-HGF IgG treated rudiments (Fig. 5B,C). These inhibitory effects of anti-HGF IgG on branching morphogenesis were abrogated by an excess amount of exogenous HGF (Fig. 5D). In this culture system, the addition of aFGF, but not KGF and bFGF (data not shown), resulted in markedly stimulated branching morphogenesis in lung explants (Fig. 5E), a finding comparable to that observed in HGF-treated explants (Fig. 3C,D). In the presence of anti-HGF antibody, however, stimulatory effects on epithelial branching caused by aFGF almost completely disappeared, and a significant reduction was observed in end-bud formation (Fig. 5F). These results suggest the possibility that aFGF, at least in part, elicits lung morphogenesis via induction of HGF production in mesenchyme. In fact, aFGF, not KGF, significantly stimulated HGF expression and production in fetal lung mesenchymal cells in vitro (Fig. 6).

### Synergistic action of HGF with FGFs in lung morphogenesis

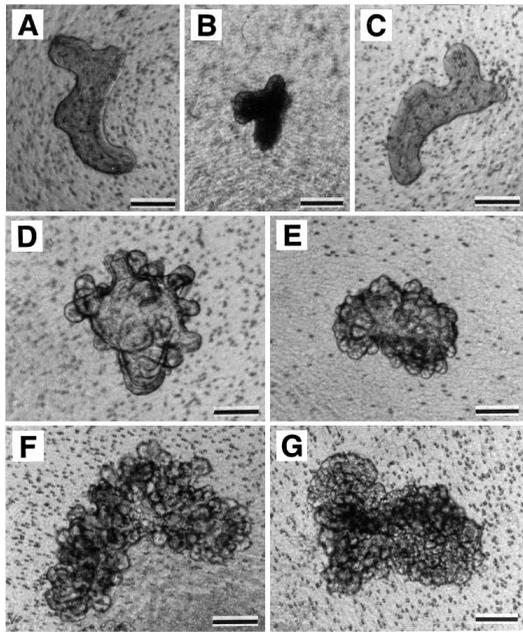
Using a mesenchyme-free epithelium culture system, the direct effects of HGF on epithelial morphogenesis were examined. In this culture condition, it was reported that aFGF stimulated branching morphogenesis of isolated lung epithelium in mice (Nogawa and Ito, 1995). The ductal epithelium were enzymatically separated from lung buds (Fig. 7A) and cultured in Matrigel. When no growth factors were added, the epithelial structures decreased in size and did not keep their intact morphology (Fig. 7B). Furthermore, many necrotic cells were observed inside the lumen. In the presence of HGF, however, no morphological changes were observed (Fig. 7C). The tubular structure of epithelium was well preserved, grew



**Fig. 6.** The induction of HGF mRNA expression (A) and HGF production (B) in fetal lung mesenchymal cells treated with aFGF or KGF. Mesenchymal cells ( $5 \times 10^4$  cells) were isolated from 15 d.p.c. fetal lung, seeded, and cultured in 250  $\mu$ l of culture medium for 24 hours. The expression of HGF in cultured cells were determined by RT-PCR. Concentrations of HGF in culture supernatants were measured by ELISA (Yamada et al., 1996). Bars represent mean  $\pm$  s.d. \*; Statistically different from non-treated control (none) at  $P < 0.05$ .

moderately in size, and dead cells were rare inside the lumen, but there was no branching. In comparison, remarkable branching occurred in rat lung epithelium in the presence of aFGF (Fig. 7D), consistent with reported data (Nogawa and Ito, 1995). Furthermore, KGF, but not bFGF (data not shown), also showed prominent morphogenic activity on lung epithelium. KGF-treated rudiments had a much larger number of branches compared to aFGF-treated rudiments (Fig. 7E). Whereas HGF alone did not induce budding, HGF could synergistically stimulate epithelial morphogenesis induced by aFGF or KGF (Fig. 7F,G). The explants treated with HGF and KGF, or aFGF increased in size considerably and showed a more complex, uneven budding structure, compared to explants treated with aFGF or KGF alone (Fig. 8). These results suggested that, during lung development, HGF may be significantly involved in epithelial branching, in concert with FGF family molecules.

Effects of simultaneous inhibition of HGF- and FGF-mediated signaling on lung development were then examined. As described above, treatment with either anti-HGF IgG or antisense KGF-R oligo-DNA apparently, but not completely, impaired branching morphogenesis in cultured lung explants (Fig. 9B,C). In combination with antisense KGF-R oligo-DNA, the addition of anti-HGF IgG completely inhibited lung development (Fig. 9D,E). Compared to rudiments prior to culture (Figs 3A, 4A), the main bronchus of treated lung did not grow in length and branching was scarcely observed, although the surrounding mesenchymal tissues were thickened



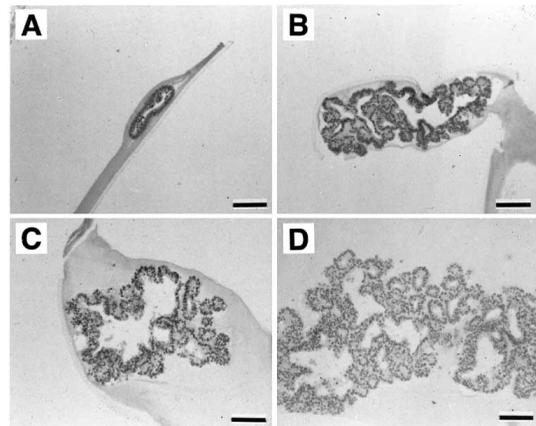
**Fig. 7.** Effects of HGF and FGFs on morphogenesis of fetal lung epithelium in mesenchymal-free culture. Lung epithelium isolated from 13 d.p.c. rat fetus (A) was cultured in Matrigel for 3 days in the absence (B) or presence of HGF (C). In the explant treated with HGF, the tubular structure is completely preserved. The epithelial branching (budding) is observed in explants treated with aFGF (D) or KGF (E). Note that addition of HGF led to more prominent epithelial morphogenesis in explants treated with aFGF (F) or KGF (G). Factors were added at concentrations as follows: HGF, 500 ng/ml; aFGF, 200 ng/ml; and KGF, 500 ng/ml, which were optimal doses in our culture condition. Scale bar, 200  $\mu$ m.

and enlarged. Such inhibition was specific as incubation of explants with an equivalent dose of non-immune IgG and control oligo-DNA had no effects (Fig. 9A).

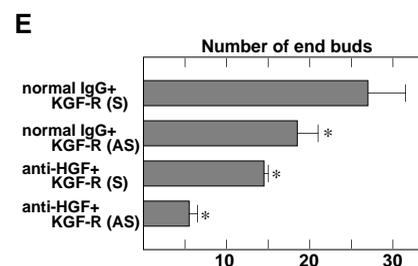
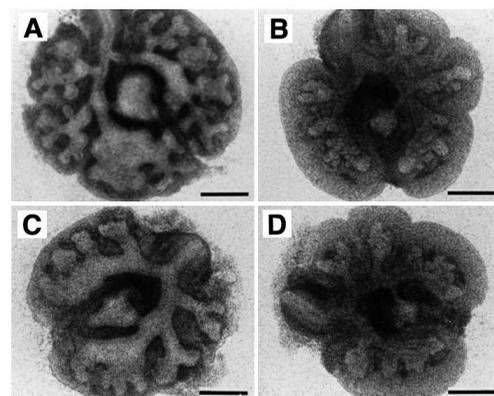
## DISCUSSION

### HGF as a mesenchymal regulator of lung branching morphogenesis

There is now growing evidence for the functional involvement of HGF in mesenchymal-epithelial interaction during various types of organ formation and tissue development. Mice deficient in HGF or *c-met*/HGF-R gene functions, which are embryonic lethal, display severe defects of liver and placenta (Schmidt et al., 1995; Uehara et al., 1995). Similar results were also observed in *Xenopus*; injection of a dominant-negative form of *c-met*/HGF-R mRNA into *Xenopus* embryos resulted in depletion of liver and gross underdevelopment of intestine and kidney (Aoki et al., 1997). In addition, the functional contribution of HGF in development of kidney (Santos et al., 1994), mammary gland (Niranjan et al., 1995; Yang et al., 1995), and tooth germ (Tabata et al., 1996) has been demonstrated. Besides epithelial organs, it was also reported that HGF is indispensable for muscle development (Bladt et al., 1995; Maina et al., 1996), and plays an important role in neural development (Ebens et al., 1996) and chondrocyte formation (Takebayashi et al., 1995).



**Fig. 8.** Histological analysis of mesenchyme-free epithelium treated with HGF and FGFs. Cross sections of mesenchyme-free lung epithelium cultured in the presence of HGF (A), aFGF (B), KGF (C) and aFGF plus HGF (D). Concentrations of growth factors used were the same as in Fig. 7. Scale bar, 100  $\mu$ m.



**Fig. 9.** Combined effects of anti-HGF neutralizing antibody and antisense KGF-R oligo-DNA on end-bud formation of fetal lung explants. Appearance of whole lung explants treated with normal rabbit IgG plus sense KGF-R oligo-DNA (A), anti-HGF IgG plus sense KGF-R oligo-DNA (B), normal IgG plus antisense KGF-R oligo-DNA (C), and anti-HGF IgG plus antisense KGF-R oligo-DNA (D). Note that the epithelial structure of the explant in D is almost comparable to that prior to culture (cf. Figs 3A and 6A). Scale bar, 500  $\mu$ m. The number of end-buds in cultured explants were counted and are shown in E. Bars represent mean  $\pm$  s.d. More than four lung explants were used in each experiment. \*Statistically different from non-treated control (none) at  $P < 0.05$  or  $0.01$ , respectively. Concentrations of antibody and oligo-DNA used were 30  $\mu$ g/ml and 30  $\mu$ M, respectively.

In the present report, we show the involvement of HGF in fetal lung development. RT-PCR and in situ hybridization studies clearly showed that HGF mRNA is distributed in mesenchyme of developing lung, while their receptors, c-Met/HGF-R are expressed in tubular epithelium (Figs 1, 2). Such appositional localization of HGF and c-met/HGF-R mRNA was commonly observed in other epithelial organs, such as liver, pancreas, intestine and kidney (Sonnenberg et al., 1993). Immunohistochemical study also showed the distribution of HGF in fetal lung mesenchyme (Defrances et al., 1992). In organ culture, exogenously added recombinant HGF stimulated branching morphogenesis of the fetal lung (Fig. 3), whereas other growth factors, such as EGF, PDGF, bFGF and KGF, which are thought to be involved in fetal and/or adult lung epithelial cells, show no such stimulatory action. Our HGF translation arrest and neutralization experiments clearly indicated that endogenous HGF plays an important role in lung development (Figs 4, 5). In the presence of antisense HGF oligo-DNA or anti-HGF antibody, epithelial branching was significantly inhibited. Accordingly, we consider that HGF is a possible candidate for a mesenchyme-to-epithelium soluble factor regulating branching morphogenesis of the developing lung.

Since branching morphogenesis, especially end-bud formation, cannot proceed without epithelial proliferation (Goldin et al., 1984), HGF, at least in part, is involved in enhancement of epithelial growth. In fact, HGF is a potent mitogen for lung epithelial cells (Mason et al., 1994a; Shiratori et al., 1995; Ohmichi et al., 1996). In addition, HGF is known to elicit 'morphogenic' activity, which induces various types of organoid formation of epithelial cells (Montesano et al., 1991; Johnson et al., 1993; Niranjan et al., 1995; Brinkmann et al., 1995). We further demonstrated that HGF is responsible for regeneration of airway and alveolar epithelium after lung injury, as a 'pulmotrophic' factor (Yanagita et al., 1993; Ohmichi et al., 1996). From these previous and present findings, it is considered that HGF ensures proper growth and rearrangement of the epithelial mass of developing lung, as a mediator of mesenchymal-epithelial interactions.

### **Synergistic action of HGF with FGFs on lung epithelial branching**

From several lines of evidence, it is now clear that FGF family molecules are the major mediator of mesenchymal-epithelial interactions during lung development, causing normal epithelial branching. First, a targeted expression of a dominant-negative form of KGF-R in fetal lung epithelium resulted in drastically impaired bronchial branching (Peters et al., 1994). It was reported that antisense KGF or KGF-R oligo-DNA clearly inhibited branching morphogenesis of developing lung in culture (Post et al., 1996), and aFGF and KGF could induce epithelial branching under mesenchyme-free conditions (Nogawa and Ito, 1995 and this study). During early lung development, aFGF and KGF are distributed in mesenchyme, while their receptors, KGF-R, are localized exclusively in epithelium (Fu et al., 1991; Mason et al., 1994b; Finch et al., 1995; Post et al., 1996). It should be noted that such developmental expression is similar to that of HGF and c-met/HGF-R.

Accordingly, we compared the functional significance of HGF in lung development with that of FGFs. In our organ culture, antisense HGF oligo-DNA significantly inhibited

branching morphogenesis of lung explants, comparable to those treated with antisense KGF-R oligo-DNA (Fig. 4). In addition, although aFGF could increase end-bud formation of lung explants, further addition of anti-HGF antibody almost abolished stimulatory actions caused by aFGF (Fig. 5). These findings suggest that FGFs are essential, but not sufficient for proper branching morphogenesis in the absence of endogenous HGF. Alternatively, aFGF, at least in part, stimulates epithelial branching via modulation of HGF production, since aFGF can enhance expression and production of HGF in fetal lung mesenchymal cells in vitro (Fig. 6), as described previously (Gohda et al., 1994; Nakamura et al., 1997). KGF did not show such a stimulatory action, which might be consistent with the finding that KGF did not induce branching morphogenesis in organ culture (Simonet et al., 1995; Siratori et al., 1996; Zhou et al., 1996b and our unpublished data). The simultaneous inhibition of both HGF- and FGF-mediated signaling systems drastically impaired early lung development (Fig. 9). In the presence of both anti-HGF neutralizing antibody and antisense KGF-R oligo-DNA, epithelial growth and branching of lung explants was almost arrested, whereas either factor alone showed fewer inhibitory effects. Of particular interest is that, in a mesenchyme-free epithelial culture system, HGF synergistically stimulated epithelial branching induced by aFGF or KGF (Figs 7, 8). In the present culture condition, HGF itself did not induce epithelial branching, although HGF is known to induce branching morphogenesis of various epithelial cells. However, HGF did preserve the intact tubular structure of epithelium with a small, but apparent stimulation of growth. Nogawa and Ito (1995) suggested that, in the morphogenesis of lung epithelium, the lumen (or tubular) formation and the branching (or budding) are different biological events. If this notion is correct, HGF seems to contribute to 'tubulogenesis' of lung epithelium rather than 'branching', which may be mainly induced by FGFs. Consistently, it was reported that, in the presence of HGF, LX-1 lung carcinoma cells developed a prominent lumen when cultured in collagen gels (Brinkmann et al., 1995).

'Branching' signal would be mainly mediated by KGF-R, since aFGF and KGF, which bind to KGF-R, induce epithelial branching in mesenchyme-free culture, but not bFGF, which binds to almost all known FGF receptors except for KGF-R (reviewed by Goldfarb, 1996). It is important to note that we cannot conclude at present that KGF and/or aFGF is a true in vivo ligand, since Post et al. (1996) found that aFGF was not so effective in epithelial branching as KGF, and KGF exposure induced a cystic, abnormal epithelial structure in transgenic experiments and organ culture assays (Simonet et al., 1995; Shiratori et al., 1996; Zhou et al., 1996b and our unpublished data). Another candidate ligand is FGF-10, a recently cloned FGF family member (Yamasaki et al., 1996). FGF-10 is closely related to FGF-7, and is specifically expressed in adult and fetal lungs. Its biological function and receptor specificity remain to be elucidated.

### **Interactions between HGF and other cytokines in lung morphogenesis**

In addition to FGFs, many soluble factors are reported to be involved in branching morphogenesis of developing lung. Among these cytokines, the functional relationships between HGF and PDGF, or transforming growth factor (TGF)- $\beta$  are of

interest, since PDGF is a typical inducer of HGF production, while TGF- $\beta$  is a most potent suppressor (Matsumoto et al., 1992). Post and colleagues showed that PDGF-AA is implicated in branching morphogenesis as an epithelium-to-mesenchyme regulator (Souza et al., 1995). On the contrary, TGF- $\beta$  inhibits epithelial cell proliferation of developing lung, resulting in less branching (Serra et al., 1994; Zhou et al., 1996a). Interestingly, HGF could release lung epithelial cells from growth arrest induced by TGF- $\beta$  in vitro (Shiratori et al., 1995; Taipale and Keski-Oja, 1996) and, on the contrary, TGF- $\beta$  inhibits HGF-induced branching tubulogenesis in vitro (Santos and Nigam, 1993). Branching morphogenesis is a combination of the growth of epithelial buds and the formation of epithelial clefts, due to differential rates of epithelial cell division. Based on these findings, at the distal region of epithelial buds, PDGF, FGFs, or other HGF-inducing factor might induce and/or maintain HGF production in mesenchyme, resulting in proper epithelial branching. In mesenchyme along the proximal airway and underlying cleft points of branching, however, accumulation of TGF- $\beta$  (Heine et al., 1990) and subsequent depletion of HGF might result in diminished branching of the adjacent epithelium. The functional significance of EGF receptor-mediated signals on lung development has also been reported (Miettinen et al., 1997), while the interactions between HGF and EGF family molecules remain to be elucidated.

Furthermore, Hogan and colleagues reported that bone morphogenetic protein (BMP)-4 and Sonic hedgehog (Shh), well known factors functioning in diverse developmental process (Fietz et al., 1994; Hogan, 1996), play an important role in lung organogenesis (Bellusci et al., 1996, 1997). Both Shh and BMP-4 were exclusively expressed at distal terminals of the growing epithelium and, in mice overexpressing Shh or BMP-4 throughout the distal epithelium, abnormal alveolar formation was observed in developing lung. The functional relationship between HGF and these morphogenic factors, albeit yet unknown, might be significant since they are co-expressed in various developing tissues and organs, such as somite, limb bud, spinal cord, cartilage and tooth (Sonnenberg et al., 1993; Fietz et al., 1994; Bitgood and McMahon, 1995; Takebayashi et al., 1995; Goldfarb, 1996; Hogan, 1996; Tabata et al., 1996).

In summary, we propose that HGF is an important regulator that transduces the morphogenic signal from mesenchyme to epithelium. HGF signaling via c-met/HGF-R synergistically induces epithelial morphogenesis in collaboration with FGFs, which may be indispensable for epithelial branching. An alternative important role of FGFs as an HGF-inducer is also suggested. In addition to FGFs, HGF production in mesenchyme might be controlled by other cytokines such as PDGF and TGF- $\beta$ , as described above. Our findings described in this report provide clues for concurrent analysis of molecular mechanisms of lung organogenesis, where complex interactions of many factors are involved, and will greatly facilitate our understanding of the role of HGF as a general epithelial morphogen with a comprehensive function in various types of organogenesis.

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