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Tbx4-Fgf10 system controls lung bud formation during chicken embryonic development

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SUMMARY

The respiratory primordium is positioned and its territory is defined in the foregut. The visceral mesoderm of the respiratory primordium acquires the inducing potential that is necessary for endodermal budding morphogenesis and respiratory endoderm formation. *Tbx4*, a member of the T-box transcription factor gene family, was specifically expressed in the visceral mesoderm of the lung primordium. To analyze the function of Tbx4, we ectopically expressed Tbx4 in the visceral mesoderm of the foregut using in ovo electroporation. Ectopic Tbx4 induced ectopic bud formation in the esophagus by activating the expression of *Fgf10*. Conversely, interference of Tbx4 function resulted in repression of *Fgf10* expression and in failure of lung bud formation. In addition, ectopic Tbx4 or Fgf10 also induced ectopic expression of *Nkx2.1*, a marker gene specific for the respiratory endoderm, in the underlying esophagus endoderm. When the border of the *Tbx4* expression domain, which demarcates the respiratory tract and the esophagus, was disturbed by misexpression of *Tbx4*, formation of the tracheo-esophageal septum failed. These results suggested that *Tbx4* governs multiple processes during respiratory tract development; i.e. the initial endodermal bud formation, respiratory endoderm formation, and septation of the respiratory tract and the esophagus.

Key words: Lung bud, Tracheo-esophageal fistula, *Tbx4*, *Fgf10*, *Nkx2.1*, In ovo electroporation, Chick

INTRODUCTION

Many visceral organs develop from a distinct position in the respiratory-digestive tube along the anteroposterior (AP) and dorsoventral (DV) axis. Each visceral organ has a unique morphology and the appropriate cell types to effectively perform the assigned physiological function: food intake, digestion, water absorption, homeostasis maintenance and respiration. At an early stage in development, the primitive gut tube, which consists of the endoderm and visceral mesoderm, is subdivided into distinct domains along the AP axis and each domain gives rise to an organ primordium (Grapin-Botton and Melton, 2000; Roberts, 2000). During this process, AP positional information of the gut tube is translated into the organ-specific signaling systems within each subdomain and these signaling systems govern organ-specific morphogenesis and differentiation. In addition, organ-specific morphogenesis and cellular differentiation are controlled by the crosstalking signaling system between the visceral mesoderm and endoderm, i.e. the epithelial-mesenchymal interaction, and between the adjacent primodia (Smith et al., 2000; Yasugi, 1993). These crosstalking signaling pathways control the transfer of positional information (organ-specific information) from one tissue to another and the establishment of the borders between different organs.

It is proposed that positional information in the gut tube is reflected by the region-specific expression pattern of transcription factors in the endoderm and visceral mesoderm as in the CNS and paraxial mesoderm. Several transcription factors are expressed in the visceral mesoderm and/or the endoderm of a specific region of the gut tube along the AP axis: Hox genes, *Nkx2.5* and *Bapx1*, etc. in the visceral mesoderm; *Sox2*, *CdxA*, *CdxC* and *Pdx1*, etc. in the endoderm (Grapin-Botton and Melton, 2000; Roberts, 2000). The boundaries of the expression domains of these transcription factors correspond to the boundaries of the presumptive territories of different organ primordia. Actually, some of these genes control the region-specific or, in some cases, the organ-specific signaling systems for the specification of the presumptive organ territory and organ-specific morphogenesis (Grapin-Botton and Melton, 2000; Roberts, 2000). In addition, signaling crosstalk between the different tissues or organ primordia is mediated by secreted proteins such as Fgfs, Bmps and Wnts, etc. In this way, signaling systems that are spatiotemporally coordinated by transcription factors and secreted proteins are thought to control the region-specific organogenesis in the overall gut tube along the AP axis.

The lung originates from the ventral wall of the foregut of the esophagus-respiratory region, which lies between the pharynx and stomach. Lung development begins with the

establishment and swelling of the primordium in the ventral wall of the foregut. Subsequently, the lung primordium forms a pair of primary lung buds and then the lung bud endoderm (bronchus) repetitively undergoes budding and branching. Because of the characteristic budding and branching involved in lung morphogenesis, this process has been studied extensively by experimental embryology in which dissected and recombined lung primordia were cultured in vitro (Alescio and Cassini, 1962; Spooner and Wessels, 1970). These classical tissue recombination experiments revealed that: (1) the presumptive field of the lung primordium is already established in the early gut tube; (2) the tissue interaction between the mesoderm and the endoderm plays an essential role in budding and branching morphogenesis; and (3) lung morphogenesis consists of two independent sequential processes – the establishment of the lung primordium and primary lung bud formation in the early stage, and the stereotypic budding and branching morphogenesis at the following stage.

Recent molecular biological studies revealed that spatial- and temporal-coordinated signaling systems, which are mediated by transcription factors and secreted proteins, control the proper stereotypic morphological pattering and differentiation in lung development (Hogan, 1999; Warburton et al., 2000). The elongation of the bronchial bud and bronchial branching, the later processes of lung morphogenesis, are coordinated along the proximodistal (PD) axis. This later phase of lung morphogenesis is controlled by the region-specific signaling system along the PD axis and signaling crosstalk between the endoderm and mesoderm, which are mediated by several transcription factors, e.g. Foxf1 and Gli proteins, and secreted proteins, e.g. Fgfs, Bmp4 and Shh (Bellusci et al., 1997a; Bellusci et al., 1997b; Colvin et al., 2001; Lebeche et al., 1999; Litingtung et al., 1998; Mahlapuu et al., 2001; Motoyama et al., 1998; Pepicelli et al., 1998; Weaver et al., 2000; Weaver et al., 1999). To date, the mechanisms of bronchial branching and cytodifferentiation, the later processes of lung development, have been well studied. However, the mechanism of the early processes of lung development, the positioning/establishment of the lung primordium and primary lung bud formation, are less well understood.

The positioning and establishment of the lung primordium are expected to reflect the positional information of the gut tube along the AP and DV axes. In mice, *Nkx2.1*, which encodes a homeobox transcription factor, is specifically expressed in the ventral wall of the foregut endoderm: the presumptive respiratory endoderm (Lazzaro et al., 1991; Minoo et al., 1999). *Nkx2.1* knockout mice exhibit immature bronchial cytodifferentiation, abnormal branching, and the DV patterning defect of the foregut (tracheo-esophageal fistula) (Minoo et al., 1999). Therefore, *Nkx2.1* plays a role in the regulation of the lung morphogenesis specific pathway and the DV patterning of the foregut, which reflects the AP and DV positional information in the gut endoderm. However, the transcription factors that show lung-specific expression in the mesoderm have not been identified. Furthermore, as *Fgf10* and *Bmp4* are specifically expressed in the lung primordium mesoderm (Bellusci et al., 1997b; Weaver et al., 1999), transcriptional regulation specific for the lung primordium mesoderm is assumed. Moreover, homozygous *Fgf10*-null mice do not generate primary lung buds from the ventral foregut (Min et al., 1998; Sekine et al., 1999), indicating that Fgf10 acts as an essential component of the inductive signaling systems necessary for primary lung bud formation. From this evidence it is clear that the signaling systems specific for the lung primordium mesoderm, especially the mechanisms of transcriptional regulation of *Fgf10* expression in the lung primordium mesoderm, is very important for understanding how the primary lung bud develops from a distinct area of the gut tube.

T-box transcription factor family genes (Tbx) function as key regulatory genes in many cases of vertebrate development (Bruneau et al., 2001; Chapman and Papaioannou, 1998; Rodriguez-Esteban et al., 1999; Takeuchi et al., 1999; Zhang et al., 1998). Among the T-box gene family, *Tbx4* is specifically expressed in the hindlimb, and misexpression of *Tbx4* in the forelimb induces a leg-like limb, indicating that this gene plays a crucial role in the specification of hindlimb patterning (Rodriguez-Esteban et al., 1999; Takeuchi et al., 1999). In addition, *Tbx4*, was briefly reported to be expressed in the lung mesoderm (Gibson-Brown et al., 1998), raising the possibility that *Tbx4* is involved in lung development.

In this study, we analyzed the expression profile of *Tbx4* in chick embryos in detail and found that *Tbx4* was specifically expressed in the visceral mesoderm of the lung primordium, and its expression overlapped with mesodermal *Fgf10* and endodermal *Nkx2.1* expression. There is a discrepancy between the *Tbx4* and *Fgf10/Nkx2.1* expression domains along the DV axis. Misexpression of Tbx4 induced ectopic *Fgf10* and *Nkx2.1* in the Tbx4-introduced mesoderm and only in the ventral region of the underlying endoderm, respectively, followed by ectopic endodermal bud formation. Our results suggest that: (1) *Tbx4* defines the anterior and posterior boundaries of *Fgf10* expression; and (2) *Tbx4* is involved in the demarcation of the posterior border of the *Nkx2.1* expression domain, but is independent from the system that determines the DV border of *Nkx2.1* expression.

MATERIALS AND METHODS

Probes

RNA probes for *Tbx4* (T. Kimura and A. K., unpublished), *Fgf10* (see below), *Bmp4* (Yokouchi et al., 1996) and *Nkx2.1* (Qiu et al., 1998) were prepared from the chicken cDNA clone. *Foxa2* (*HNF3β*) (Nishizaki et al., 2001) was prepared from the chicken genomic clone. A fragment of *Fgf10* cDNA (2.3 kb including coding and 3′ non-coding sequence) was amplified by PCR using *Fgf10*-specific (corresponding to nucleotides 228-252 of GenBank D8633) and vector primers. *Fgf10* probes were prepared from the entire coding sequence (0.6 kb) (Ohuchi et al., 1997) and the coding/3′ non-coding sequences (2.3 kb). The RNA probes were labeled with digoxigenin or fluorescein according to the manufacturer's protocol (Roche).

Whole-mount in situ hybridization

Whole-mount in situ hybridization was performed essentially according to Dietrich et al. (Dietrich et al., 1997). Prior to pre-hybridization treatment, the foreguts were dissected and isolated to allow penetration of the RNA probe. For two-color whole-mount in situ hybridization, one probe was labeled with digoxigenin (DIG) and the other was labeled with fluorescein (FITC). Alkaline phosphatase (AP)-conjugated anti-DIG and anti-FITC antibodies (Roche) were colored with Fast Red/Naphthol (Sigma) in NTMT (pH 8.0) (red), NBT (Roche, 3.5 μl/ml)/BCIP (Roche, 3.5 μl/ml) in NTMT (pH 9.5) (purple) and NBT (3.5 nl/ml)/BCIP (3.5 μl/ml) in NTMT (pH 9.5) (blue).

DNA constructs

Chicken *Tbx4* cDNA was cloned into the internal ribosome entry site (IRES)-carrying vector and then the IRES-*Tbx4* fragment was isolated and cloned into pCMV-Script (Stratagene). RCAS-*Tbx4* and RCAS-*Tbx4-EGFP* were kindly provided by Dr T. Ogura (Takeuchi et al., 1999). RCAS-*Fgf10* was kindly provided by Dr S. Noji. *Cla*I fragments of *Tbx4*, *Tbx4-EGFP* and *Fgf10* were isolated from RCAS-*Tbx4*, RCAS-*Tbx4-EGFP* and RCAS-*Fgf10*, respectively, and inserted into the *Cla*I site of a modified pCAGGS expression vector. The dominant-activator and dominant-repressor forms of Tbx4 (Tbx4-VP16, Tbx4-EnR) were generated by fusion of a truncated chick *Tbx4* fragment including the T-box (amino acid 1 to 273) with the activator domain of VP16 and with the repressor domain of the En2 protein (eh1 domain) (Matsunaga et al., 2000). The *Tbx4-VP16* and *Tbx4-EnR* fragment were cloned into the *Cla*I site of a modified pCAGGS expression vector.

In ovo electroporation

In ovo electroporation was performed according to Momose et al. (Momose et al., 1999). Eggs of HH stage 7-12 embryos were opened, and the vitelline membranes were removed to expose the embryo. A platinum electrode (0.5 mm diameter) was used as a positive electrode. A sharpened tungsten needle or a platinum electrode (0.5 mm diameter) was used as a negative electrode. A positive electrode was placed beneath the embryo at the prospective esophago-tracheobronchial region. A positive electrode was placed into the coelomic cavity (when using a sharpened tungsten needle) or on the embryo (when using a platinum electrode 0.5 mm diameter). Plasmid solutions (8-15 μg/μl in T1/4E/0.1-1% Fast Green) were injected into the coelomic cavity with a sharp glass pipette and an electric pulse was immediately applied [5-9 V (HH7-10), 9-14 V (HH10-12); pulse length 50-90 ms, once or twice times) with a CUY21 electroporator (Tokiwa). Eggs were sealed and incubated in a humidified incubator until analyzed further. Embryos were fixed with 4% paraformaldehyde at 4°C overnight. The foreguts were isolated and GFP fluorescence was photographed, and then the foreguts were processed for whole-mount in situ hybridization. Electroporation performed on total 1297 embryos, and 663 survival embryos (51.1%) were recovered 1-3 days after electroporation. Among the recovered embryos, 348 embryos (26.8%) exhibited efficient transfection. The efficiency indicated in Results is always based on the number of the embryos exhibited efficient transfection.

RESULTS

The first sign of respiratory tract morphogenesis is the swelling of the ventral wall of the foregut in the esophagus-respiratory region, which lies between the pharynx and stomach primordia (pre-budding stage, Fig. 1A). Then, the endoderm at the posterior edge of the respiratory primordium evaginates and buds out bilaterally forming a pair of primary buds, the so-called lung buds (budding stage, Fig. 1A). At a subsequent stage, each lung bud elongates to form the primary bronchi and subsequently the bronchi branch repetitively, forming the bronchial tree (branching stage, Fig. 1A). At the same time, at the bifurcation point of the bronchi, the primary gut tube separates anteriorly into the dorsal esophagus and the ventral trachea (budding stage and branching stage, Fig. 1A, blue arrow).

Expression of *Tbx4* in the developing respiratory system

First, we carefully analyzed the expression profiles of *Tbx4* during chick lung development. In chick, the lung primordium appears between 50 and 60 hours after egg laying (stage 15/16) as a swelling of the ventral wall and is recognized by the expression of *Nkx2.1*, the earliest marker gene specific for the respiratory endoderm (Lazzaro et al., 1991; Minoo et al., 1999). At stage 15 (25 somites), *Tbx4* was expressed in the visceral mesoderm of the distinct area of the esophagus-respiratory region covering the ventral swelling (Fig. 1B). *Tbx4* was expressed in both the ventral and dorsal regions (Fig. 1B) and the mesodermal *Tbx4* expression domain overlapped the *Nkx2.1*-expressing endoderm except for the anterior end region of the *Nkx2.1* domain (Fig. 1D, compare B with C). We could not detect *Tbx4* expression at stage 14 (21 somites), whereas *Nkx2.1* was already expressed in the ventral endoderm of the foregut in the esophagus-respiratory region (data not shown). On day 3 of incubation (stage 18-20), when the lung buds were evident, *Tbx4* was expressed in the mesoderm of the lung buds, but not in the esophagus (Fig. 1E,F). The anterior boundary of the endodermal *Nkx2.1* expression domain (purple staining in Fig. 1E) was clearly more anterior to the anterior boundary of the mesodermal *Tbx4* expression domain (mesodermal red staining in Fig. 1E). This expression profile of *Tbx4* implies that the *Tbx4* expression domain at stage 15 contribute to the posterior structure of the respiratory tract: the lung bud. To verify this possibility, we carried out cell lineage analysis of the foregut visceral mesoderm at stage 15 and confirmed that the ventral *Tbx4* expression domain contributes to the lung bud (data not shown). On day 4 of incubation (stage 24/25), when the separation of the trachea and the esophagus was progressing, *Tbx4* was expressed in the mesoderm of the respiratory tract, posterior to the bifurcation point of the main bronchus (Fig. 1G,H), but not in the esophagus (Fig. 1H).

We compared the *Tbx4* expression domain with the expression domain of *Fgf10* that is expressed in the lung primordium mesoderm (Bellusci et al., 1997b) and required for primary lung bud formation in mice (Min et al., 1998; Sekine et al., 1999). At stage 15 (25 somites), *Fgf10* was expressed in the visceral mesoderm of the chick lung primordium, as in the mouse embryo (Fig. 1I,J, purple staining). Both the anterior and posterior boundaries of the *Fgf10* domain overlapped with those of *Tbx4*, as judged from the relationship to the *Nkx2.1* domain (blue staining in Fig. 1I) and by the morphology (compare Fig. 1I,J with 1B,D). However, the *Fgf10* domain was restricted to the ventral region of the *Tbx4* domain where a pair of endodermal buds would arise (Fig. 1K, compare Fig. 1I with 1B).

Misexpression of Tbx4 caused ectopic endodermal bud formation

To analyze the role of *Tbx4* in lung development, we transiently misexpressed Tbx4 in the visceral mesoderm of the presumptive esophagus-respiratory region using in ovo electroporation (Momose et al., 1999). When the expression construct for the Tbx4-EGFP fusion protein (Takeuchi et al., 1999) or the Tbx4 expression plasmids together with GFP plasmids were electroporated into the prospective esophagus-respiratory region (Matsushita, 1995) at stage 7 to 12, evagination of the ectopic endodermal bud was observed [31/141 (22.0%)] (Fig. 2A, yellow arrowhead). By monitoring the expression of the foreign genes by GFP

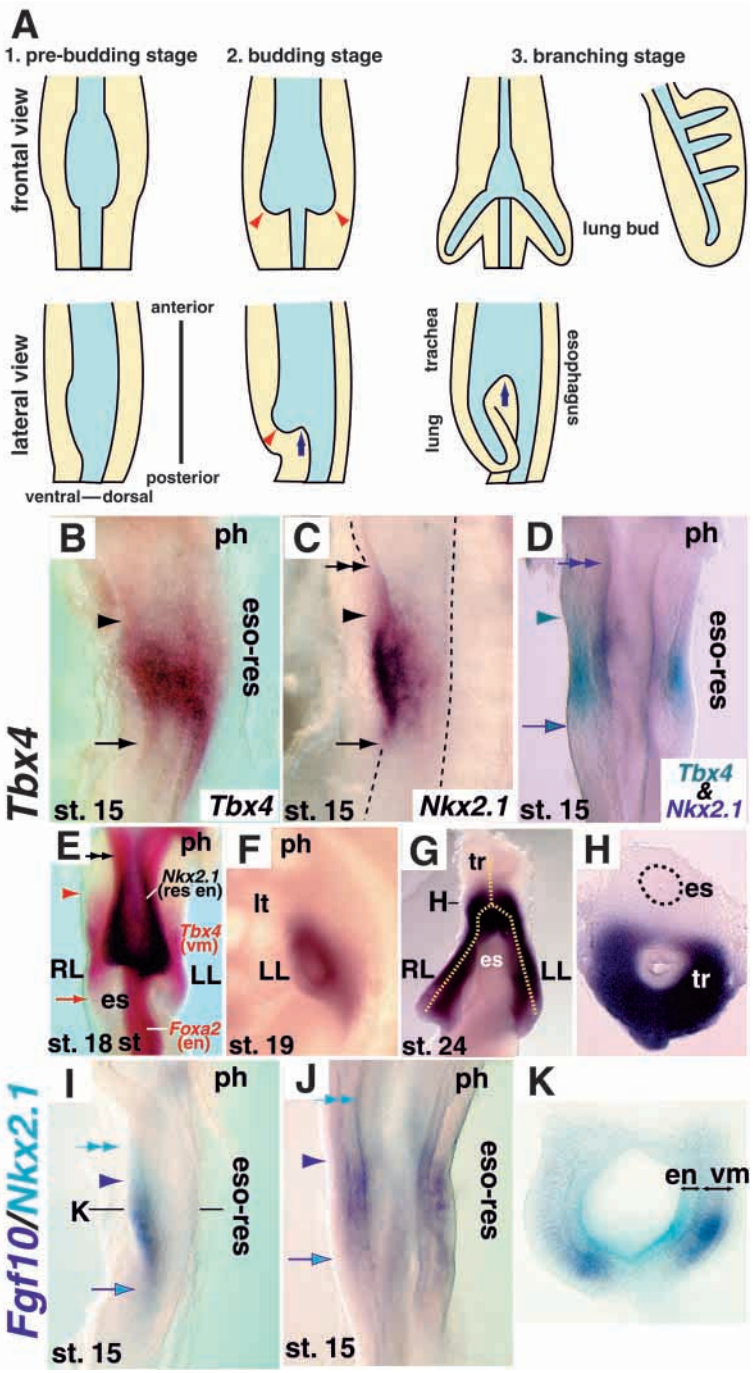

Fig. 1. Schematic representation of the chick respiratory tract development and expression of *Tbx4*, *Fgf10* and *Nkx2.1* in the respiratory tract of the chick embryo. (A) The chick respiratory tract development. (B-H) Expression of *Tbx4* (B,D-H) and *Nkx2.1* (C-E) in the chick embryonic foregut at stage 15 (B-D), 18 (E), 19 (F) and 24 (G,H) visualized by whole-mount in situ hybridization. (B,C,F), Views from the left side. (D,E,G), Frontal views. (H) Cross-section as indicated in G. (D) Two-color whole-mount in situ hybridization with probes for *Tbx4* (blue) and *Nkx2.1* (purple). (E) Two-color whole-mount in situ hybridization with probes for *Tbx4* (red), *Foxa2* (red) and *Nkx2.1* (purple). Endodermal red staining in E indicates the expression of *Foxa2*, a pan-endoderm marker gene. Note that the respiratory endoderm (*Nkx2.1*-expressing endoderm, purple stained endoderm) also expresses *Foxa2*. Arrowheads and arrows in B,C indicate the position of the anterior and posterior boundaries of *Tbx4* expression, respectively. Double arrow in C indicates the position of the anterior boundaries of *Nkx2.1* expression. Blue arrowhead in D and red arrowhead in E indicate the position of the anterior boundary of *Tbx4* expression. Blue arrow fringed with purple in D indicates the position of the posterior boundaries of *Tbx4* and *Nkx2.1* expression. Red arrow in E indicates the position of the posterior boundary of *Tbx4* expression. Purple double arrow in D and black double arrow in E indicate the position of the anterior boundaries of *Nkx2.1* expression. Broken lines in C indicate the outline of the endodermal tube. The broken yellow line in G represents the bifurcation pattern of the endodermal layer of the respiratory tract. (I-K) Expression of *Fgf10* (purple) and *Nkx2.1* (blue) in the chick embryonic foregut at stage 15. (I) View from the left side. (J) Frontal view. (K) Cross-section as indicated in I. Top of the K is the dorsal side. Purple arrowheads in I,J indicate the position of the anterior boundary of *Fgf10* expression. Blue arrows fringed with purple in I,J indicate the position of the posterior boundaries of *Fgf10* and *Nkx2.1* expression. Blue double arrows in I,J indicate the position of the anterior boundaries of *Nkx2.1* expression. en, endoderm; es, esophagus; eso-res, esophagus-respiratory region; res en, respiratory endoderm; st, stomach; lt, laryngotracheal groove; ph, pharynx; tr, trachea; vm, visceral mesoderm; LL, left lung bud; RL, right lung bud.

fluorescence, we found that the ectopic buds always underlie the Tbx4-transfected mesoderm (Fig. 2B,C). The ectopic buds were induced in both the ventral (Fig. 2A, yellow arrowhead) and dorsal (Fig. 2D, yellow arrowhead) endoderm. In some cases, the ectopic endodermal bud was induced between the primary bronchi, which resulted in trifurcation of the bronchi (Fig. 2E,F). Misexpression of Tbx4 caused endodermal budding, but this morphogenesis did not proceed to bud elongation and branching. In addition, misexpression of Tbx4 caused a failure of tracheo-esophageal septum formation, so-called tracheo-esophageal fistula [13/141 (9.2%)] (Fig. 2G). By contrast, misexpression of irrelevant genes, i.e. those encoding GFP or alkaline phosphatase, did not cause ectopic endodermal bud formation and tracheo-esophageal fistula [0/49 (0%)] (data not shown). Therefore, ectopic endodermal bud induction by electroporation was a specific effect of Tbx4 misexpression.

Ectopic Tbx4 induced ectopic *Fgf10* expression in the mesoderm

As Tbx4 is a transcription factor, downstream signaling molecule(s) of Tbx4 in the visceral mesoderm are likely to mediate budding morphogenesis in the underlying endoderm. The best candidate signaling molecule is Fgf10 because, in

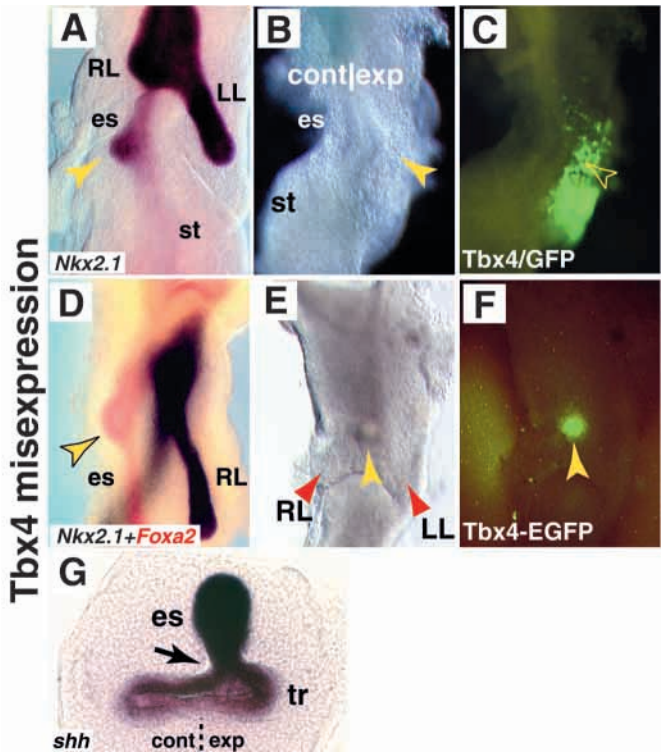

Fig. 2. Misexpression of Tbx4 causes ectopic endodermal budding and tracheo-esophageal fistula. Tbx4 misexpression induced ectopic endodermal budding in the underlying endoderm. (A-F) The foreguts from a Tbx4 misexpressed embryo 2 days after electroporation. (A,E,F) Frontal views. (B,C) Dorsal views. (D) View from the right. (A) Ectopic endodermal bud (yellow arrowhead) formed in the ventral distal esophagus. The endoderm was visualized by whole-mount in situ hybridization with a *Nkx2.1* probe. (B,C) Ectopic endodermal bud (yellow arrowhead) formed in the distal esophagus as in A. (C) Tbx4-transfected mesoderm monitored by GFP fluorescence. (D) Ectopic endodermal bud (yellow arrowhead) formed in the dorsal distal esophagus. The endoderm was visualized by whole mount in situ hybridization with probes for *Nkx2.1* (purple) and *Foxa2* (red), a pan-endoderm marker gene. (E,F) Ectopic endodermal bud formed between the primary bud, which results in trifurcation of the bronchi. (F) Tbx4-transfected mesoderm monitored by GFP fluorescence. (G) Cross-section of the foregut of the Tbx4 misexpressed embryo showing tracheo-esophageal fistula. The endoderm was visualized by whole-mount in situ hybridization with a *Shh* probe, followed by sectioning. The arrow indicates fusion of the esophagus and tracheal endoderm. cont, control; exp, experimental.

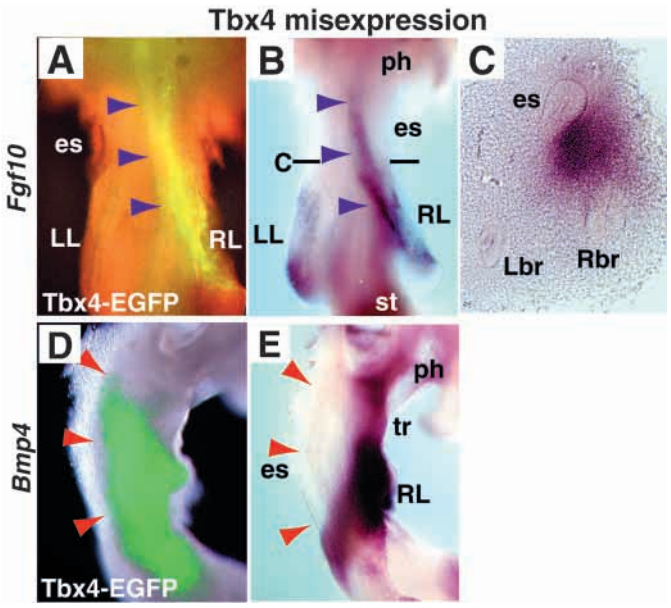

Fig. 3. *Fgf10* and *Bmp4* expression in the Tbx4 misexpressing foregut. (A-C) Induction of ectopic *Fgf10* expression in the Tbx4-misexpressing mesoderm. (A) The Tbx4-transfected mesoderm was monitored by GFP fluorescence from the dorsal view 2 days after electroporation. (B) *Fgf10* expression in the same specimen from the same viewpoint as in A. Blue arrowheads indicate the esophagus mesoderm misexpressing exogenous Tbx4. (C) Cross-section as indicated in B. (D,E) Tbx4 misexpression does not affect *Bmp4* expression. (D) The Tbx4-transfected region was monitored by GFP fluorescence 2 days after electroporation. (E) *Bmp4* expression in the same specimen from the same view point as in D. Red arrowheads in D,E indicate the esophagus mesoderm misexpressing exogenous Tbx4. es, esophagus; ph, pharynx; st, stomach; tr, trachea; LL, left lung; RL, right lung; Lbr, left main bronchus; Rbr, right main bronchus.

normal development, *Fgf10* is expressed in the visceral mesoderm of the lung primordium, overlapping with *Tbx4* (Fig. 1I,J compare with 1B,D). In addition, *Fgf10* is essential for budding morphogenesis of the primary bud and bronchial branching (Bellusci et al., 1997b; Mailleux et al., 2001; Min et al., 1998; Sekine et al., 1999). To examine this possibility, we analyzed *Fgf10* expression in the Tbx4 misexpressed embryos. In the Tbx4 misexpressed embryo, ectopic *Fgf10* expression was observed in the visceral mesoderm at precisely the same locations as exogenous Tbx4 expression (Fig. 3A,B, blue arrowheads). Tbx4 misexpression induced ectopic *Fgf10* expression in the mesoderm irrespective of the DV territory (Fig. 3C). We also examined another signaling molecule, *Bmp4*, which is specifically expressed in the respiratory mesoderm of chicks (Sakiyama et al., 2000) and plays role in the proximodistal patterning of mouse lung development (Weaver et al., 1999). Unlike *Fgf10*, *Bmp4* expression was not affected by Tbx4 misexpression (Fig. 3D,E, red arrowheads). These results indicate that Tbx4 promotes *Fgf10* expression, but not *Bmp4* expression.

Ectopic Fgf10 induced ectopic endodermal buds

To examine whether Fgf10 has endodermal bud inducing activity, we introduced Fgf10 expression plasmids together with GFP plasmids into the visceral mesoderm of the prospective esophagus-respiratory region. As a result, ectopic Fgf10 induced ectopic budding morphogenesis in the endoderm underlying the Fgf10-transfected mesoderm [14/26 (53.8%)] (Fig. 4A,B, yellow arrowheads). Fgf10 also induced ectopic endodermal buds in both the dorsal (Fig. 4A, yellow arrowheads) and ventral (Fig. 4C, yellow arrowhead) region. These results indicate that Fgf10 has endodermal bud inducing activity. Fgf10 misexpression also caused ectopic *Tbx4* expression in the esophagus mesoderm (Fig. 4E,F, red arrowheads). Ectopic *Tbx4* expression in the esophagus, however, was weaker than its endogenous expression in the lung bud mesoderm (Fig. 4F). These observations indicate the

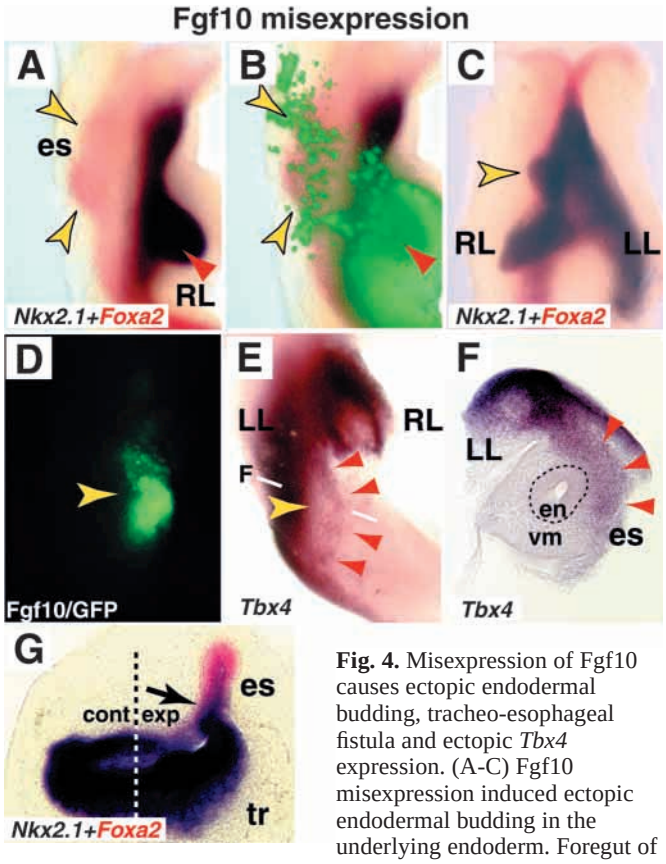

Fig. 4. Misexpression of Fgf10 causes ectopic endodermal budding, tracheo-esophageal fistula and ectopic *Tbx4* expression. (A-C) Fgf10 misexpression induced ectopic endodermal budding in the underlying endoderm. Foregut of the Fgf10 misexpressing embryo 2 days after electroporation. The endoderm was visualized by whole-mount in situ hybridization with *Nkx2.1* (purple) and *Foxa2* (red) probes. (A,B) Views from the right. (C) Frontal view. (A,B), Ectopic endodermal buds (yellow arrowheads) formed in the dorsal distal esophagus viewed from the right. The Fgf10-transfected mesoderm is visualized by merging with the picture of GFP fluorescence (green) in B. (C) Ectopic endodermal bud (yellow arrowhead) formed in the ventral distal esophagus. (D-F) Fgf10 misexpression induced ectopic *Tbx4* expression. Foregut of the Fgf10 misexpressing embryo 2 days after electroporation. (D) The Fgf10-transfected mesoderm monitored by GFP fluorescence. (E) *Tbx4* expression in the same specimen as D. Red arrowheads indicate ectopic *Tbx4* expression. Yellow arrowhead indicates the Fgf10-transfected mesoderm. (F) Cross-section as indicated in E. (G) Cross-section of the foregut of the Fgf10 misexpressed embryo showing tracheo-esophageal fistula. The endoderm was visualized by whole-mount in situ hybridization with *Nkx2.1* (purple) and *Foxa2* (red) probes, followed by sectioning. Arrow indicates fusion of the esophagus and tracheal endoderm. en, endoderm; es, esophagus; tr, trachea; vm, visceral mesoderm; LL, left lung bud; RR, right lung bud; cont, control; exp, experimental.

presence of a feedback loop between *Tbx4* and *Fgf10* in the regulation of lung development. Furthermore, misexpression of Fgf10 also caused tracheo-esophageal fistula [15/26 (57.7%)] (Fig. 4G).

Activity of Tbx4 was necessary for *Fgf10* expression in ovo

For further analysis on the regulatory role of Tbx4 on *Fgf10* expression, we designed misexpression assays of a variant form of Tbx4. Tbx4-VP16 and Tbx4-EnR were constructed as a constitutive active form and a dominant-negative form of Tbx4, respectively. Electroporation was carried out at stage 7-10 in order to express transgenes prior to endogeneous *Tbx4* and *Fgf10* expression. Then, *Fgf10* expression was examined 24-30 hours after electroporation when development of the manipulated embryos reached stage 17-19. Tbx4 misexpression in the presumptive esophagus-respiratory region (Fig. 5A, bracket) resulted in a marked expansion of the *Fgf10* expression domain throughout the Tbx4-transfected mesoderm (100%, *n*=6) (Fig. 5B). In the case of Tbx4-VP16 misexpression, the *Fgf10* expression domain expanded throughout the Tbx4-VP16-transfected mesoderm as in the case of Tbx4 misexpression (26%, *n*=19) (Fig. 5D, bracket), but the level of *Fgf10* expression was lower than normal in the lung bud on the contralateral side (Fig. 5D, compare RL side with LL side). Tbx4-EnR misexpression resulted in significant reduction or elimination of *Fgf10* expression in the Tbx4-EnR-transfected mesoderm (62%, *n*=21) (Fig. 5F, bracket). In severely affected cases, budding morphogenesis of the primary bud was blocked (11/30 [36.7%]) (Fig. 5F,H). Surprisingly, Tbx4-EnR misexpression caused ectopic *Fgf10* expression in the distal esophagus mesoderm posterior adjacent to the Tbx4-EnR-transfected mesoderm (Fig. 5F,G, red arrow). Corresponding to this ectopic *Fgf10* induction, 2 days after electroporation of Tbx4-EnR, an ectopic endodermal bud was observed in the distal esophagus (Fig. 5H, arrowheads) even though Tbx4-EnR was transfected into the mesoderm of the lung field (Fig. 5H, green). In control experiments in which GFP was electroporated, ectopic expression of *Fgf10* was never observed (0%, *n*=27), and downregulation of *Fgf10* expression was rarely observed (22%, *n*=27) (Fig. 5I,J).

Tbx4 and Fgf10 induced ectopic *Nkx2.1* expression in the underlying endoderm

During gut organogenesis, the visceral mesoderm influences both endodermal morphogenesis and endodermal differentiation (Yasugi, 1993). It is likely that the lung primordium mesoderm influences differentiation of the respiratory endoderm. As described above, the *Tbx4*-expressing mesoderm overlies the *Nkx2.1*-expressing endoderm (Fig. 1D). To examine whether *Tbx4* influences differentiation of the underlying endoderm, we analyzed the expression of *Nkx2.1* in the Tbx4 misexpressed embryo. When Tbx4 was misexpressed throughout the esophagus-respiratory region, the expression domain of *Nkx2.1* expanded posteriorly (Fig. 6A,B, compare with 6G). The ectopic *Nkx2.1* domain was observed in the distal esophagus endoderm that underlies the Tbx4-transfected mesoderm (Fig. 6A), but ectopic *Nkx2.1* expression was restricted to the ventral wall of the distal esophageal endoderm of the Tbx4-transfected side (Fig. 6C). The ectopic bud in the ventral esophagus expressed *Nkx2.1* at levels as high as in the normal lung bud (Fig. 2A, Fig. 6B, yellow arrowhead). By contrast, the ectopic bud in the dorsal esophagus did not express *Nkx2.1* (Fig. 2D, yellow arrowhead). Fgf10 misexpression also caused ectopic *Nkx2.1* expression in the ventral endoderm, but not in the dorsal endoderm, as seen in Tbx4 misexpression (Fig. 6E,F compare with 6H).

DISCUSSION

The role of *Tbx4* during the respiratory tract development

The territories of the presumptive organ primordia in the early gut tube are considered to be marked by the region-specific expression of transcription factors (Grapin-Botton and Melton, 2000; Roberts, 2000). The lung primordium is placed and established in the ventral region of the esophagus-respiratory region located between the pharynx and the stomach. We found that *Tbx4*, a member of the T-box transcription factor family of genes, was specifically expressed in the mesoderm of the presumptive area of the lung primordium (Fig. 1B,D).

The primordia of the visceral organs are established through a regionalization process of the gut tube along the AP axis. During region specific gut morphogenesis, a restriction of cell intermingling within a single organ primordium occurs by approximately stage 15-16 in chicks (Smith and Tabin, 2000). Thus, it is likely that the compartment boundaries of the organ primordia and the compartment-specific, i.e. organ-specific, identities are established by approximately stage 15-16 in the developing chick gut tube. *Tbx4* expression initiates in the mesoderm of the presumptive lung primordium area around stage 14-15 (21-25 somites) (Fig. 1B,D). Thus, the expression of *Tbx4* is coincident with the establishment of the compartment of the lung primordium field, indicating that *Tbx4* is involved in the initial specification process of the territory for the lung primordium mesoderm.

Once the lung primordium is established, the lung primordium mesoderm acquires an inductive capability for the initial budding morphogenesis of primary lung buds (Spooner and Wessels, 1970). *Fgf10* is specifically expressed in the lung primordium mesoderm (Bellusci et al., 1997b) and acts as an essential component of the inductive signal for the initial budding morphogenesis of primary lung buds (Min et al., 1998; Sekine et al., 1999). In our study, misexpression of Tbx4 in the esophagus mesoderm induced ectopic *Fgf10* expression in the mesoderm (Fig. 3A-C) and subsequent ectopic budding morphogenesis in the underlying esophagus endoderm (Fig. 2A-F). The results of the Tbx4 misexpression studies suggest that Tbx4 is able to trigger the specific signaling pathway for endodermal budding morphogenesis in the ectopic area of the esophagus. Furthermore, misexpression of Tbx4 in the esophagus mesoderm induced the expression of *Nkx2.1*, a specific marker for the respiratory endoderm, in the underlying esophagus endoderm (Fig. 6A; discussed below). Taken together, *Tbx4* plays a role in the acquisition of the inductive capability that is specific for the lung primordium mesoderm.

At later stage, *Tbx4* was still expressed in the lung bud mesoderm and the expression boundary of *Tbx4* clearly demarcated the respiratory tract (*Tbx4*-expressing) and the esophagus (*Tbx4*-non-expressing) (Fig. 1H). When the border of the *Tbx4* expression domain was disturbed by misexpression of *Tbx4*, separation of the trachea and esophagus failed (Fig.

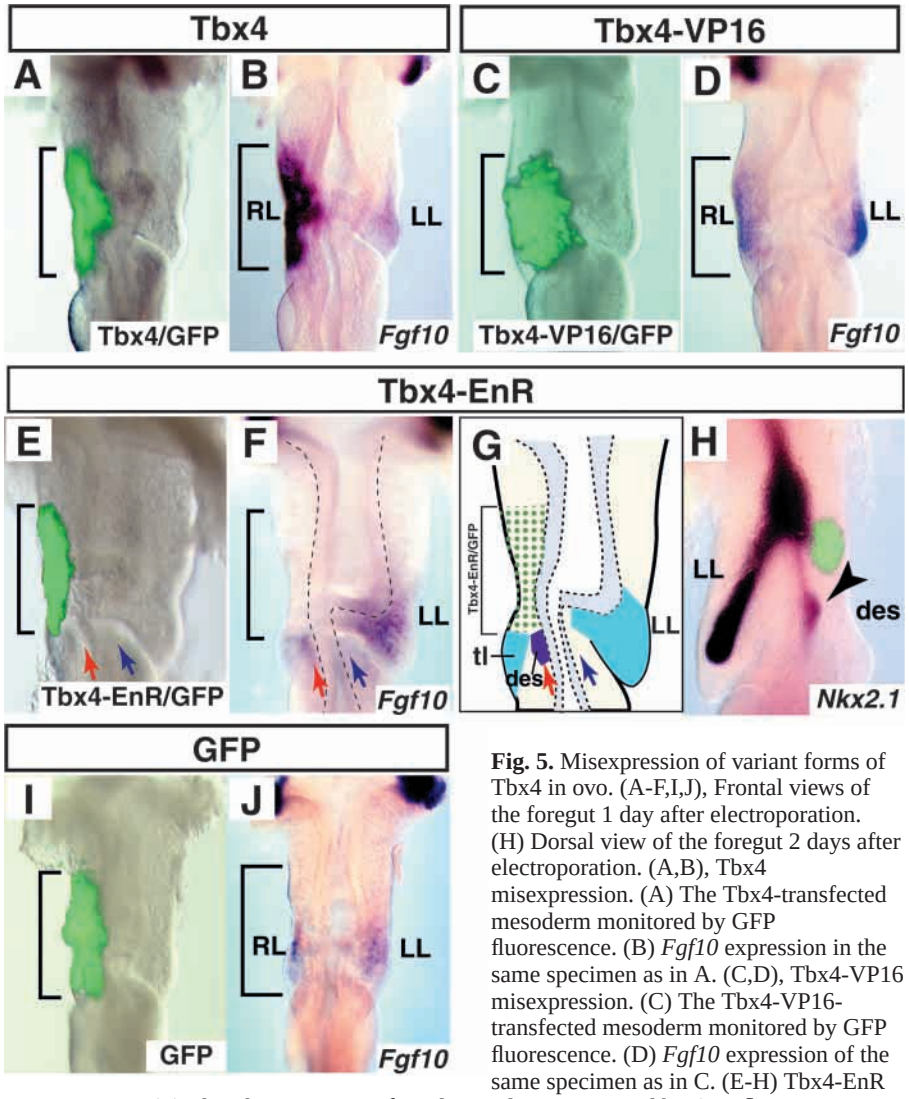

Fig. 5. Misexpression of variant forms of Tbx4 in ovo. (A-F,I,J), Frontal views of the foregut 1 day after electroporation. (H) Dorsal view of the foregut 2 days after electroporation. (A,B), Tbx4 misexpression. (A) The Tbx4-transfected mesoderm monitored by GFP fluorescence. (B) *Fgf10* expression in the same specimen as in A. (C,D), Tbx4-VP16 misexpression. (C) The Tbx4-VP16-transfected mesoderm monitored by GFP fluorescence. (D) *Fgf10* expression of the same specimen as in C. (E-H) Tbx4-EnR misexpression. (E) The Tbx4-EnR-transfected mesoderm monitored by GFP fluorescence. Red arrows in E-G indicate the distal esophagus mesoderm, which expresses ectopic *Fgf10*. Blue arrows in E-G indicate the distal esophagus mesoderm on the contralateral side. (F) *Fgf10* expression in the same specimen as in E. (G) Schematic representation of F. Dark-blue area indicates ectopic *Fgf10* expression in the distal esophagus mesoderm. Light blue areas indicate endogenous *Fgf10* expression. Green stippled area indicates Tbx4-EnR/GFP-expressing mesoderm. (H) *Nkx2.1* expression. The Tbx4-EnR-transfected mesoderm is visualized by merging with the picture of GFP fluorescence (green). Arrowhead indicates ectopic endodermal bud in the distal esophagus. (I,J) GFP misexpression. (I) The GFP-transfected mesoderm monitored by GFP fluorescence. (J) *Fgf10* expression in the same specimen as in I. des, distal esophagus; tl, tip of the lung; LL, left lung bud; RL, right lung bud.

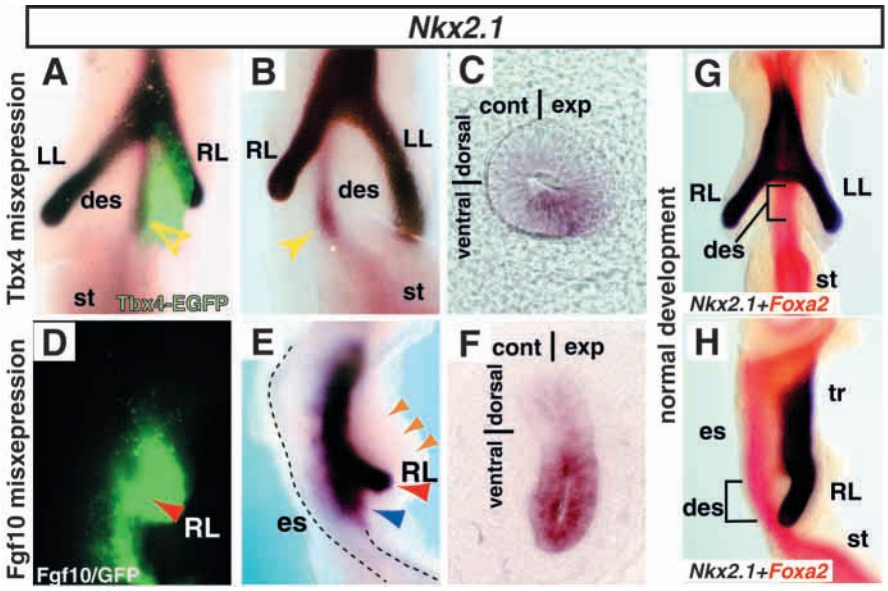

Fig. 6. *Nkx2.1* expression in the Tbx4 or Fgf10 misexpressing foregut. (A-F) *Nkx2.1* expression in the foregut 2 days after Tbx4 or Fgf10 electroporation. (A-C) Tbx4 misexpression induces ectopic *Nkx2.1* expression in the distal esophagus endoderm. (A,B) The same specimen viewed from the dorsal (A) and ventral (B) sides. The Tbx4-transfected mesoderm is visualized by merging with the picture of GFP fluorescence (green) in A. Yellow arrowheads in A,B indicate ectopic endodermal buds. (C) Cross-section of the distal esophagus showing that ectopic *Nkx2.1* expression is restricted to the ventral endoderm on the Tbx4-transfected side. (D-F) Fgf10 misexpression induces ectopic *Nkx2.1* expression in the distal esophagus endoderm. (D) The Fgf10-transfected mesoderm monitored by GFP fluorescence from the right side. (E) *Nkx2.1* expression in the same sample and from the same viewpoint as in D. Blue arrowhead indicates ectopic *Nkx2.1* expression in the distal esophagus endoderm. Orange arrowheads indicate the overproliferation of the mesoderm. Broken lines indicate the outline of the endodermal tube. (F) Cross-section of the distal esophagus showing that ectopic *Nkx2.1* expression is restricted to the ventral endoderm. (G,H) Normal *Nkx2.1* (purple) and *Foxa2* (red) expression at stage 20. (G) Frontal view. (H) View from the right side. des, distal esophagus; es, esophagus; st, stomach; tr, trachea; LL, left lung bud; RL, right lung bud; cont, control; exp, experimental.

2G). Fgf10 misexpression also caused an identical phenotype (Fig. 4G). Therefore, the respiratory-specific expression of *Tbx4* is crucial for tracheo-esophageal septum formation, the structural separation of the respiratory tract and the esophagus through *Fgf10* activation. However, Tbx4 or Fgf10 misexpression did not cause bud elongation following budding morphogenesis (Fig. 2A,D, Fig. 4A,C). One interpretation of this result is that another cascade(s) independent of the Tbx4-Fgf10 system (e.g. the Bmp4 cascade) may be necessary for bud elongation and bronchial branching. For bud elongation process, dynamic and localized *Fgf10* expression around the tip of the endodermal bud is crucial (Bellusci et al., 1997b; Weaver et al., 2000). In our Tbx4 misexpression study, ectopic *Fgf10* was expressed throughout the transfected mesoderm. If restriction of the Fgf10 source around the tip of the endodermal bud is crucial for normal development, such a situation was difficult to reproduce by our gene transfer method.

Tbx4 defines the *Fgf10* expression domain during early lung development

During normal development, the anterior and posterior boundaries of *Tbx4* and *Fgf10* expression in the lung primordium mesoderm were overlapping (compare Fig. 1D with 1J). When the Tbx4 expression domain was expanded beyond the lung primordium field by Tbx4 misexpression, the *Fgf10* expression domain expanded throughout the Tbx4-misexpressing mesoderm, including the outside of the lung primordium field (Fig. 3A,B, Fig. 5A,B). These observations suggest that the lung primordium-specific transcription factor Tbx4 defines the *Fgf10* expression domain, especially at both the anterior and posterior boundaries, by activating *Fgf10* expression in a cell-autonomous manner. Because misexpression of Tbx4-VP16, a dominant-activator form of Tbx4, caused ectopic activation of *Fgf10* expression equal to that of full-length Tbx4 (Fig. 5D), Tbx4 acts as a transcription activator for *Fgf10* expression. Conversely, interference with the transcription activation function of endogenous Tbx4 by misexpression of Tbx4-EnR, a dominant-repressor form of Tbx4, resulted in repression of the endogenous *Fgf10* expression in the lung primordium mesoderm (Fig. 5F). Therefore, the transcription activation function of Tbx4 in the lung primordium mesoderm is necessary for *Fgf10* expression. We could not discriminate, however, whether Tbx4 is necessary for initiation or maintenance of *Fgf10* expression under the strict criteria. The results of our misexpression studies do not exclude the possibility that exogenous Tbx4 activates and Tbx4-EnR blocks the maintenance circuit for *Fgf10* expression. An answer to this issue will be provided by the analysis of molecular mechanism for *Fgf10* transcription regulation.

Although Tbx4-VP16 construct exhibited stronger transcriptional stimulation than native Tbx4 in reporter transcription assay (data not shown), Tbx4-VP16 construct induced ectopic *Fgf10* expression but to a lesser extent than native Tbx4 in the embryo (Fig. 5D). For constructing Tbx4-VP16, we have eliminated the region downstream of the T-box and replaced with VP-16 transcriptional activation domain. These results imply that Tbx4 may be not a simple transcriptional activator and behave as a repressor depend on the context of regulatory element of the target genes. It is possible that both activities are required for effective *Fgf10* transcription through complex transcriptional network.

Interestingly, we found that misexpression of Tbx4-EnR induced ectopic *Fgf10* expression in the distal esophagus mesoderm in the posterior region adjacent to the Tbx4-EnR-transfected mesoderm (Fig. 5F,G, red arrow). This result indicates that *Fgf10* is capable of being expressed in the distal esophagus mesoderm if the transcription activation function of

Tbx4 in the lung primordium mesoderm is disrupted. The simplest interpretation of this result is that Tbx4 normally activates the target genes that repress *Fgf10* expression in a non-cell-autonomous manner as a form of lateral inhibition. If this lateral inhibition system malfunctions, the distal esophagus, the posterior neighbor to the lung field, may develop an additional *Nkx2.1*-expressing endodermal bud as observed during Tbx4-EnR misexpression (Fig. 5H). Taken together, Tbx4 regulates *Fgf10* expression by binary pathways: a cell-autonomous activation pathway and a non-cell-autonomous repression pathway, and these dual regulation pathways of Tbx4 precisely define the *Fgf10* expression domain within the lung primordium mesoderm.

During normal development, the *Fgf10* expression domain was restricted to the ventral half of the *Tbx4* expression domain where the primary bud would arise (compare Fig. 1I with 1B). This discrepancy between *Tbx4* and *Fgf10* expression domains suggests two mechanisms for *Fgf10* activation. First possibility is that the dosage of Tbx proteins is crucial for *Fgf10* activation. *Tbx2*, *Tbx3* and *Tbx5* have been briefly reported to be expressed in the lung bud mesoderm of chick and mouse (Chapman et al., 1996; Gibson-Brown et al., 1998). In chicken foregut mesoderm, *Tbx2*, *Tbx4* and *Tbx5* showed overlapping and slightly different expression pattern. Among them, only *Tbx4* exhibited common anterior and posterior boundaries with the *Fgf10* expression domain (J. S. and A. K., unpublished). It is possible that the *Fgf10* expression domains may be defined by redundant/cooperative action of Tbx4 and other Tbx protein(s), which is exclusively expressed in the ventral mesoderm. The second possibility is the presence of the co-factor(s) that activate *Fgf10* transcription synergistically with Tbx4 on the ventral side and/or the presence of the antagonist of Tbx4 that represses *Fgf10* expression on the dorsal side. Our results showed that Tbx4 misexpression alone induced the expression of *Fgf10*. In this case, it is likely that the high dose of the foreign Tbx4 exceeds the threshold for *Fgf10* activation in the absence of a co-factor and/or in the presence of an antagonistic factor.

During limb development, *Tbx4* and *Tbx5* are also expressed in the hindlimb and forelimb mesenchyme, respectively, and this pattern is conserved in the tetrapod and fish (Gibson-Brown et al., 1996; Gibson-Brown et al., 1998; Tamura et al., 1999). Mesenchymal *Fgf10* expression is also crucial for growth of the limb bud (Min et al., 1998; Sekine et al., 1999). Recently, it has been reported that Tbx5 is necessary for *Fgf10* expression and fin bud formation in zebrafish (Ng et al., 2002). Thus, Tbx-Fgf system may be extensively used in the bud formation during vertebrate embryogenesis.

The Tbx4-Fgf10 system defines the *Nkx2.1* expression domain in the underlying endoderm

Previous studies did not mention whether the lung primordium mesoderm induced the underlying endoderm to differentiate into the respiratory endoderm (Spooner and Wessels, 1970). We found that Tbx4 or Fgf10 misexpression induced ectopic *Nkx2.1* expression in the underlying endoderm (Fig. 6A,E). Tbx4-Fgf10 system acts as a signaling component for the inductive interactions specific to the lung primordium mesoderm. Thus, results of Tbx4 or Fgf10 misexpression studies indicate that the lung primordium mesoderm also influences respiratory endoderm differentiation. As the expression of *Nkx2.1* in the ventral endoderm precedes *Tbx4* and *Fgf10* expression (data not shown), the Tbx4-Fgf10 system does not appear to be involved in the initiation of *Nkx2.1* expression or the initiation of respiratory endoderm differentiation. Once the expression of *Tbx4* and *Fgf10* is initiated, the posterior boundaries of the *Tbx4*, *Fgf10* and *Nkx2.1* expression domains are localized to the same position in the respiratory-esophagus region of the foregut (Fig. 1B-D,I,J). When the posterior boundaries of the *Tbx4* or *Fgf10* expression domains were shifted posteriorly by Tbx4 or Fgf10 misexpression, the *Nkx2.1* expression domain expanded posteriorly to the distal esophagus endoderm underlying the Tbx4- or Fgf10-miexpressing mesoderm (Fig. 6A,E). This coincidence of the posterior boundaries of *Tbx4/Fgf10* and *Nkx2.1* in the normal and Tbx4 or Fgf10 misexpressed embryos suggests that the Tbx4-Fgf10 system defines the posterior boundary of the *Nkx2.1* expression domain, i.e. the posterior boundary of the respiratory endoderm, in the maintenance pathway. However, misexpression of Tbx4 or Fgf10 induced ectopic *Nkx2.1* expression only in the ventral endoderm (Fig. 6C,F). These results suggest that another system(s) independent of Tbx4-Fgf10 regulates the ventral restricted *Nkx2.1* expression.

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