

# Different thresholds of fibroblast growth factors pattern the ventral foregut into liver and lung

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## Summary

Cell fate and morphogenesis within the embryo is dependent upon secreted molecules that transduce signals between neighboring tissues. Reciprocal mesenchymal-epithelial interactions have proven essential during branching morphogenesis and cell differentiation within the lung; however, the interactions that result in lung specification from the foregut endoderm, prior to lung bud formation, are poorly understood. In this study, we investigate the tissue requirements and signals necessary for specification of a pulmonary cell fate using embryo tissue explants. We show that NKX2.1, an early transcription factor crucial for lung development, is expressed in the ventral foregut endoderm shortly after albumin and Pdx1, early markers of the liver and pancreas lineages, respectively. Similar to hepatic specification, direct contact of cardiac mesoderm with ventral endoderm is required to induce *in vitro* expression of NKX2.1 and downstream lung target genes including surfactant protein C and Clara cell secretory protein. In the absence of

cardiac mesoderm, ventral foregut endoderm explants respond to exogenous fibroblast growth factor (FGF) 1 and FGF2 in a dose-dependent manner, with lower concentrations activating liver specific genes and higher concentrations activating lung specific genes. This signaling appears to be instructive, as the prospective dorsal midgut endoderm, which predominantly gives rise to the intestinal tract, is competent to respond to FGFs by inducing NKX2.1. Furthermore, the temporal expression and selective inhibition of FGF receptors 1 and 4 present within the endoderm implies that signaling through FGFR4 is involved in specifying lung versus liver. Together, the findings suggest that a concentration threshold of FGFs emanating from the cardiac mesoderm are involved in patterning the foregut endoderm.

Key words: Lung, Liver, Endoderm, Heart, NKX2.1, FGF signaling, Mouse

## Introduction

The lung emerges as a ventral diverticulum of the foregut endoderm, appearing in mouse on embryonic day 9-9.5, slightly later than the liver (Kaufman, 1992). The resulting lung bud undergoes branching morphogenesis and cell differentiation into the surrounding mesenchyme to form both the conducting airways and terminal alveoli necessary for respiration at birth. Gene targeting experiments have elucidated several signaling pathways that are important in lung morphogenesis, which are functionally conserved through evolution. Specifically, pathways mediated by fibroblast growth factors (FGFs), epidermal growth factors, transforming growth factor  $\beta$ , bone morphogenetic proteins and the sonic hedgehog family have all been implicated in lung branching and pattern formation within the developing airways (for a review, see Warburton et al., 2000). However, with the exception of a compound null *Gli2*<sup>-/-</sup>/*Gli3*<sup>-/-</sup> mutation, in which the early lung anlage fails to arise from the embryonic foregut (Motoyama et al., 1998), the specific tissue interactions

and signaling events that commit the multipotent endoderm to a pulmonary cell fate prior to morphogenesis are not well understood.

Lung specification, along with its neighboring foregut derivatives (including pancreas, liver, thyroid, esophagus, stomach), occurs within the domain of FOXA2 (formally designated HNF3 $\beta$ ) gene expression; members in a family of developmentally regulated transcription factors that play a significant role in regional differentiation of the gut endoderm (Sasaki and Hogan, 1993; Monaghan et al., 1993; Ang et al., 1993). *In vitro*, FOXA2 (HNF3 $\beta$ ) activates transcription of the homeodomain protein NKX2.1 (Ikeda et al., 1996) and is thought to cooperate with NKX2.1 to define cell lineage within the pulmonary epithelium (Guazzi et al., 1990; Kimura et al., 1996; Minoo et al., 1999). NKX2.1 [also named thyroid transcription factor 1 (TTF1) and thyroid enhancer binding protein (T/ebp)] is the earliest known marker of the respiratory epithelium, with reported onset of expression in the developing lung, thyroid and ventral forebrain at 9-9.5 days mouse gestation (Lazzaro et al., 1991; Minoo et al., 1999). Early in

morphogenesis, NKX2.1 is expressed in all pulmonary epithelial cells, but becomes progressively restricted to the distal alveolar type II cells and proximal Clara cells with development (Lazzaro et al., 1991; Ikeda et al., 1995). Targeted disruption of the gene has demonstrated that although NKX2.1 is essential for lung branching and terminal cell differentiation (including expression of surfactant proteins SP-A and SP-C, and Clara cell secretory protein CC10), it is not required for the initial specification of the lung or thyroid (Kimura et al., 1996; Minoo et al., 1999; Kimura et al., 1999). The early onset and essential requirement for NKX2.1 in lung development implies that this transcription factor is activated by the primary signals that induce pulmonary specification from the endoderm.

Experimental systems using embryo tissue isolation and culture have proven valuable in defining inductive interactions crucial for endoderm patterning as well as liver and pancreas specification (Gualdi et al., 1996; Jung et al., 1999; Wells and Melton, 2000; Deutsch et al., 2001; Rossi et al., 2001; Kumar et al., 2003; Bort et al., 2004). Chick embryo transplant studies and mouse embryo explants have established that in liver development, cardiac mesoderm is an absolute requirement for activation of liver-specific genes from the ventral foregut endoderm and for subsequent morphogenesis of the hepatic bud (LeDouarin, 1975; Houssaint, 1980; Fukuda-Taira, 1981; Gualdi et al., 1996). FGF1 and FGF2, diffusible signals secreted from cardiac mesoderm at the time of liver specification in the mouse embryo, can efficiently induce hepatic differentiation within isolated ventral endoderm explants (Jung et al., 1999). Within the same assay, the absence of cardiac mesoderm and FGF signaling leads to expression of genes specific to the pancreas lineage (Deutsch et al., 2001), confirming the multipotent capacity of the ventral foregut endoderm and the ability to study cell specification *in vitro*.

In the developing lung bud, FGF receptor type 2 isoform IIIb and its high affinity mesenchymal ligands (notably FGF10, FGF7 and FGF1) are essential for patterning and morphogenesis of the respiratory tract, after specification has occurred (Peters et al., 1994; Bellusci et al., 1997; Celli et al., 1998; De Moerloose et al., 2000). Disruption of FGFR3b signaling in transgenic mice completely blocks airway branching and distal lung differentiation; the persistence of NKX2.1 expression indicates that specification of the lung remains unperturbed (Peters et al., 1994; Celli et al., 1998). Mice deficient in FGF10, which is expressed in the early pulmonary mesenchyme at E9.5 (Bellusci et al., 1997), have a similar phenotype with normal trachea but no mainstem bronchi (Min et al., 1998; Sekine et al., 1999). Although these models underscore the integral requirement of FGF signaling in early branching morphogenesis, they fail to define which signals are important in initiating a lung pathway from multipotential endoderm.

In the present study we assess the tissue interactions necessary for the initial specification of a pulmonary cell fate, using embryonic tissue cultures. The results indicate that FGF signaling from the cardiac mesoderm is a crucial early determinant of lung specification, which functions in an instructive rather than permissive manner. In an *in vitro* system, induction of a liver versus lung cell fate is controlled by FGFs acting at different concentration thresholds and upon selective activation of FGFR1 and FGFR4 expressed in the ventral

foregut endoderm. Together, the findings imply that temporally specific FGF signaling from the developing cardiac domain plays a role in patterning pluripotent endoderm during embryogenesis.

## Materials and methods

### Embryo tissue isolation, culture and RNA analysis

After mating C3H mice, noon of the day of the appearance of a vaginal plug was considered to be 0.5 post coitum. Embryos were harvested at E8-9.5 and staged according to number of somites present. Embryo tissues were dissected in 1×PBS under a 60× dissecting microscope, using fine polished tungsten needles (Point Technologies, Boulder, CO). Presumptive ventral endoderm, dorsal endoderm (foregut and midgut), mesoderm and ectoderm control tissues were isolated as previously described (Gualdi et al., 1996). In an attempt to produce endoderm cultures free of adjacent mesoderm, the tissue was divided into smaller segments and then gently pipetted, which produced several colonies for analysis. To generate single cell suspensions ventral endoderm was incubated in PBS containing 20 µg/ml dispase for 5 minutes followed by a 5 minute incubation in PBS containing 0.05 mg/ml trypsin (Chiang and Melton, 2003). Vortexed samples were used to generate cytospin slides with Cytospin 3 (Shandon, Pittsburgh, PA) according to the manufacturer's instructions. Tissues for culture were placed in glass microwell slides (Nalgene) coated with rat-tail type I collagen (BD Biosciences, Bedford, MA). They were cultured in 0.4 ml of DMEM (BRL) containing 10% defined calf serum (Hyclone) at 37°C in a humidified 5% CO<sub>2</sub> incubator. Colonies were monitored daily by microscopy for growth rate and presence of beating cardiac mesoderm cells. On each day, images of the explants was documented by a SPOT RT digital camera (Diagnostic Instruments) mounted on a Leitz DMIRB inverted phase-contrast research microscope; the beating domain when present was designated on the captured image using the SPOT annotation software. After 2 days of culture, RNA was isolated and subjected to RT-PCR as previously described (Wells and Melton, 2000; Gualdi et al., 1996). The number of cycles was determined for each primer pair as required to reach mid-logarithmic phase of amplification. The sequences of oligonucleotides and expected sizes of RT PCR products were: 5' NKX2.1, 5'-CCAGGACACCATGCGGAACA-3'; 3' NKX2.1, 5'-GGCCATGTTCTTGCTCACGT-3', 163 bp (Katoh et al., 2000); 5' SP-C, 5'-CATACTGAGATGGTCCTTGAG-3'; 3' SP-C, 5'-GTGCTAGATATAGTACAGTGG-3', 399 bp (Minoo et al., 1999); 5' CCSP, 5'-GCCTCCAACCTCTACCATGA-3'; 3' CCSP, 5'-CTCTTGTGGGAGGGTATCCA-3', 241 bp; 5' albumin, 5'-CCC-CACTAGCCTCTGGCAAAT-3'; 3' albumin, 5'-CTTAAACC-GATGGGCGATCTCACT-3', 127 bp (Gualdi et al., 1996); 5' β-actin, 5'-AAAGACCTGTACGCCAACACACAGTC-3'; 3' β-actin, 5'-GT-CATACTCCTGCTTGCTGATCCA-3', 219 bp (Jung et al., 1999); 5' PDX1, 5'-AATCCACCAAAGCTCACGCGTGG-3'; 3' PDX1, 5'-CCCGTACTACGTTTCTTATCTTCC-3', 279 bp (Deutsch et al., 2001); 5' FOXA2, 5'-CCATCCAGCAGAGCCCAACA-3'; 3' FOXA2, 5'-GTCTGGGTGCAGGGTCCAGAA-3', 169 bp (Clotman et al., 2002); 5' cardiac troponin I, 5'-ACGTGGAAGCAAAGT-CACC-3'; 3' cardiac troponin I, 5'-CCTCCTTCTTCACCTGCTTG-3', 201 bp). For each experiment, controls were routinely performed in which reverse transcriptase was omitted from the cDNA reaction mixture and in which template DNA was omitted from the PCR reaction.

### Explant cultures with growth factors, FGF receptor inhibition assays, analyses for cell proliferation and apoptosis

Immediately upon placing explants in culture, different concentrations of recombinant FGFs were added to culture medium containing heparin sulfate proteoglycan (50 ng/ml) and 0.1% bovine

serum albumin. The following concentrations of FGFs were used: human FGF1 and human FGF2 (Boehringer Mannheim) at 5, 15, 30, 50, 125, 250, 500 and 1000 ng/ml; and mouse FGF8B (R&D Systems) at 5, 50 and 500 ng/ml. Control cultures without FGFs were always grown in parallel. Growth and changes in morphology were monitored daily.

For FGF receptor inhibition assays antiserum to FGFR1 or FGFR4 (Santa Cruz Biotechnology; final concentration 1  $\mu$ g/ml) or recombinant human soluble FGFR1 (IIC)/Fc and FGFR4/Fc complexes (obtained from Axxora, LLC, San Diego, CA; final concentration 500 ng/ml) were added to the culture medium containing heparin sulfate (100 ng/ml) prior to tissue isolation. The same concentration of rabbit IgG was used as a control.

SU5402 (Calbiochem, La Jolla, CA; final concentration 25 or 50  $\mu$ M) was added to the culture medium immediately after tissue isolation. Control explants were cultured with an equal volume of the vehicle DMSO.

For statistical analysis of FGF-treated and inhibition assays the Fisher's exact test or chi-square test were used to compare control with experimental groups; the Student's *t*-test (two-tailed) was also used in the inhibition assays. *P* values of less than 0.05 were considered to indicate significant differences.

BrdU incorporation was detected using the BrdU labeling and Detection Kit II (Roche). At the end of a 24-hour culture period, BrdU was added at a concentration of 10  $\mu$ M for 1 hour. BrdU immunostaining was carried out using the manufacturer's protocol. Apoptotic cells were detected using the In Situ Cell Death Detection Kit (Roche).

### Whole-mount and section immunofluorescence

Embryos and endoderm cultures were fixed overnight at 4°C in 4% paraformaldehyde buffered to pH 7.4 with PBS, washed three times with PBS then sequentially dehydrated and stored in 100% methanol at -20°C until used for whole-mount or sectioned immunostaining. Embryo and explant sections were generated by embedding the rehydrated tissue in tissue freezing medium, and serially sectioned in a Leitz cryostat at 8  $\mu$ m at -20°C. The following antibodies and dilutions were used for both whole-mount double immunostaining and embryo/tissue section double immunofluorescence: mouse anti-TTF1 (1:150, Neomarkers, Fremont, CA); sheep anti-albumin (1:300, The Binding Site, Birmingham, UK); sheep anti-HNF3 $\beta$  (FOXA2) 1:100, Upstate Biotechnology, Lake Placid, NY); muscle-specific mouse actin (1:500, Neomarkers); goat anti-SP-C and anti-CC10; rabbit anti-FGFR1 and FGFR4 (all 1:100, Santa Cruz Biotechnology). The rabbit PDX1 antibody was generously provided by C. Wright (dilution 1:1500). In all experiments, normal serum was used as a control (the latter giving no signals; data not shown). All antibody incubations took place overnight at room temperature. Immunofluorescence of embryo and explant sections was detected with fluorescein or Texas Red anti-sheep, anti-mouse, anti-rabbit or anti-goat IgG and mounted with DAPI media (all from Vector). All results were photographed with a SPOT RT digital camera (Diagnostic Instruments) mounted on a Nikon E400 microscope.

## Results

### Endodermal NKX2.1 activation in vivo occurs in close temporal and spatial proximity to liver and pancreas

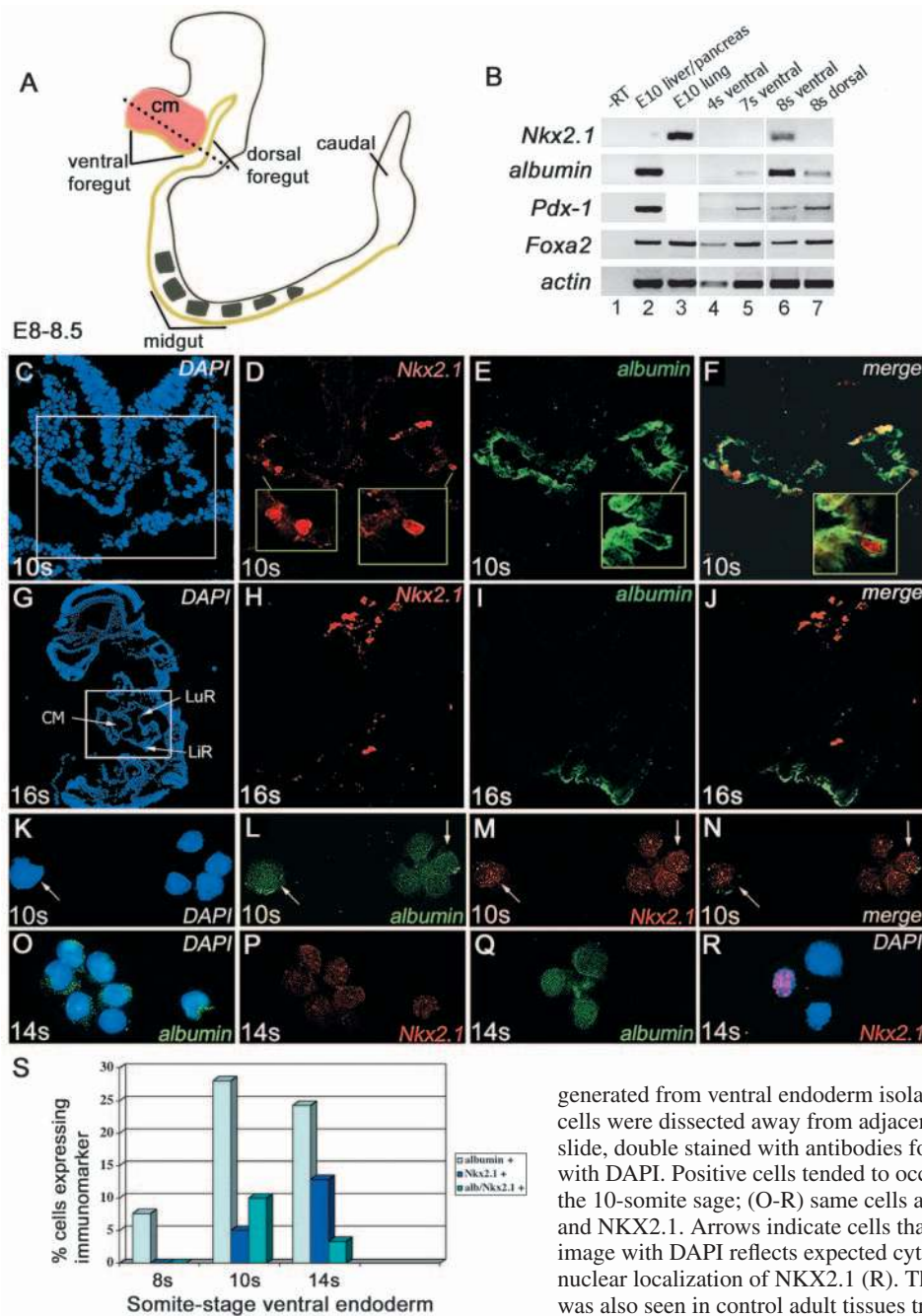
The onset of lung specification within the endoderm in vivo was assessed by using NKX2.1 as a molecular marker. Prior studies that detail NKX2.1 expression in the lung and thyroid primordium have used in situ hybridization and immunohistochemistry (Lazzaro et al., 1991; Minoo et al., 1999), less sensitive methods than RT-PCR analysis at

detecting low levels of gene expression. We isolated RNA from dissected ventral and dorsal foregut endoderm (Fig. 1A) in 8- to 9.5-day gestation embryos, and subjected it to RT-PCR. Our results have demonstrated that the *Nkx2.1* gene is reliably activated within the ventral endoderm, but not dorsal endoderm, in the 8.25 day embryo (E8.25), starting at the eight-somite stage (Fig. 1B, lane 6) – approximately a full day before the formation of the laryngo-tracheal groove from which the lungs arise and half day before a thyroid rudiment is apparent (Kaufman, 1992). This finding validates NKX2.1 as the earliest marker of lung and thyroid specification, well before morphogenesis occurs. We have previously demonstrated (Deutsch et al., 2001) that serum albumin and PDX1, which are specific to the hepatic and pancreatic lineages respectively, can be detected at seven to eight somites in the same domain of cells. In repeating the above using embryos staged by precise somite number, we reproducibly observed an RT-PCR product for albumin and *Pdx1* mRNA at the seven-somite stage, shortly before NKX2.1 was detectable at the eight-somite stage (Fig. 1B, lane 5). Significantly activation of these tissue specific genes occurs soon after the ventral foregut endoderm becomes closely apposed to the cardiogenic mesoderm in the five- to six-somite stage embryo (Fig. 1A; Fig. 2A,B) (Kaufman, 1992). This close approximation diminishes with further morphogenetic movement of the heart and foregut at later somite stages.

To examine the spatial relationship between albumin and NKX2.1 in situ, detailed immunohistochemical analysis was performed on embryo tissue sections from E8.0-E9.5. Single cells positive for albumin protein were observed as early as the eight-somite stage in the foregut (data not shown); by contrast, NKX2.1-positive cells were first seen at 9-10 somites, located in ventral endoderm adjacent to the developing cardiac region (Fig. 1D). Double-labeled sections at this stage revealed many more cells positive for albumin protein than for NKX2.1 protein in the foregut endoderm. We also observed sporadic albumin-positive cells in the dorsal endoderm; definitive staining for NKX2.1 in the same cells was not seen (Fig. 1C-F; see figure legend). Surprisingly, in numerous sections examined, ventral endoderm cells with NKX2.1-positive nuclei frequently appeared to co-localize with cells positive for albumin in their cytoplasm (Fig. 1F, boxed inset). Between the 11- and 14-somite stages, there was an expansion of NKX2.1-positive cells throughout the ventral foregut endoderm and albumin-positive cells in the dorsal endoderm were rarely visualized (data not shown). By E9.5, albumin and NKX2.1 expression ceased to co-localize, and was largely confined to the respective liver and lung bud regions (Fig. 1G-J).

To better resolve the expression pattern seen within sectioned embryos, single cell cytospin slides were generated from dissected ventral foregut endoderm at 8, 10 and 14 somites and analyzed by double immunofluorescence for NKX2.1 and albumin (Fig. 1K-S). Quantification of the material confirmed the early onset of albumin versus NKX2.1 protein expression in the ventral foregut, as well as the existence of co-expressing cells that diminished with advancing somite age (Fig. 1S). The co-expression of NKX2.1 and albumin cells within the foregut endoderm in vivo led us to hypothesize that the cardiac signals that induce liver specification may impact lung specification as well.





### Lung specification is induced by adjacent cardiac mesoderm

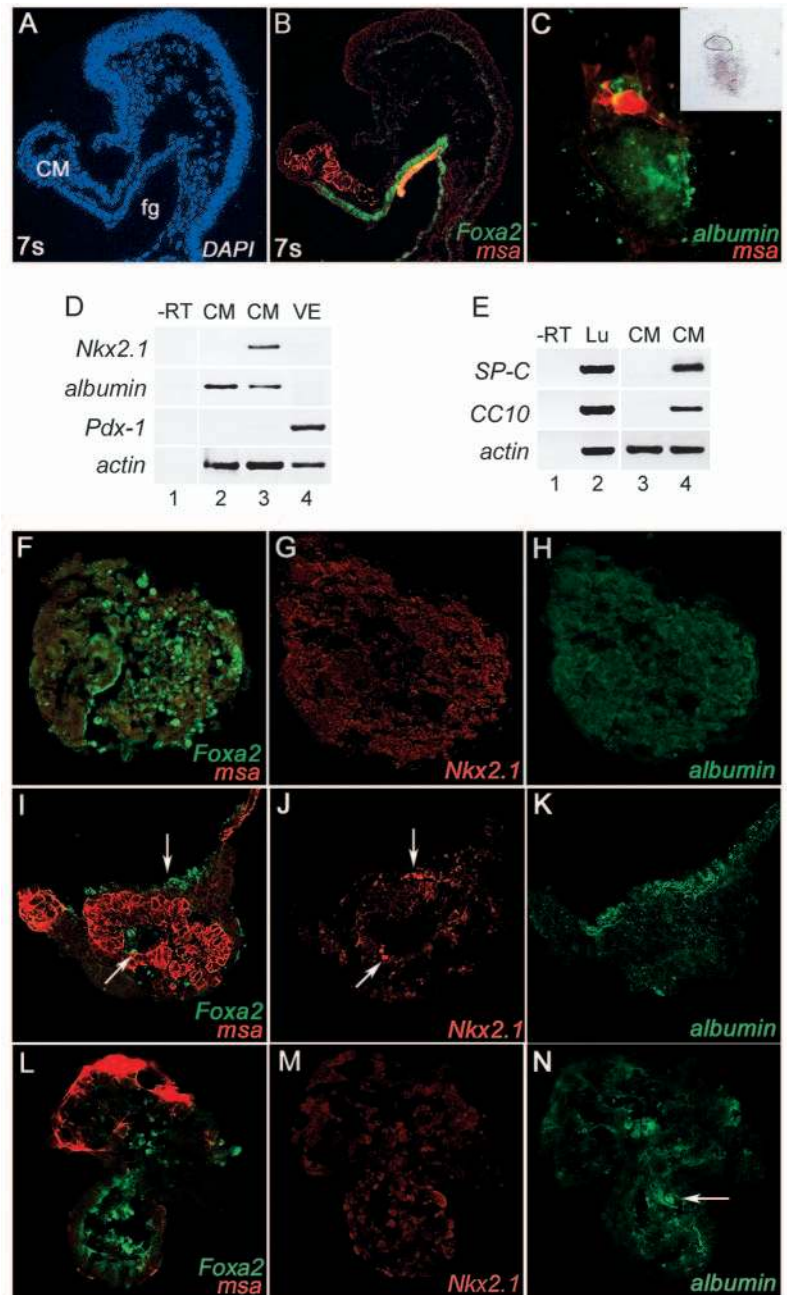
To examine the potential relationship between lung and liver specification, we used an embryo explant assay. Hepatic specification can be reconstituted *in vitro* by culturing ventral endoderm from the two- to five-somite stages in close proximity to cardiac mesoderm (Gualdi et al., 1996). Cardiac mesoderm cultivated in DMEM + 10% calf serum progresses to the beating stage within 1 day of culture and is thus readily distinguishable from co-cultured endoderm fragments; we also stained explants for expression of muscle specific actin (MSA) which delineates the cardiac domain (Fig. 2B). Ventral

**Fig. 1.** NKX2.1 activation in foregut endoderm occurs in close proximity to liver specification. (A) Sagittal view of a mouse embryo at the 6-somite stage, demonstrating tissue regions isolated for RT-PCR analysis and used for explant assays. Broken line indicates plane of embryo sections in C-F. (B) RT-PCR analysis of total RNA from designated tissue regions at different somite stages. Tissue microdissected from day 10 embryos (E10), with and without reverse transcriptase (–RT), used as positive and negative controls, respectively. Actin probe used as control for total RNA integrity; FOXA2 probe is used to ensure actin levels reflected isolated endoderm. (C–J) Embryo sections at designated somite stages demonstrating expression of albumin (green) and NKX2.1 (red) in mouse foregut endoderm by double immunofluorescence. (C–F) Transverse embryo section at the 10-somite stage encompassing foregut and region of cardiac mesoderm. (C) Boxed area in DAPI image (to detect nuclei) is magnified in D–F. Yellow signal in merged image (F) reflects nonspecific (present in all filters) fluorescent staining of acellular material frequently seen in the foregut pocket (also seen in Fig. 2B) (Kaufman, 1992; Jung et al., 1999). (D,E) More cells in the foregut endoderm express albumin than NKX2.1 protein. Markers (boxed magnification) are colocalized in merged image (F). (G–J) Albumin and NKX2.1 expression in E9.5 sagittal embryo section. Boxed area in G (DAPI image) is magnified in H–J and encompasses the cardiac and presumptive lung (LuR) and liver (LiR) regions.

(K–T) Immunostaining of single cells generated from ventral endoderm isolated at 10- and 14-somite stages. Endoderm cells were dissected away from adjacent mesoderm, dispersed, spun down onto a slide, double stained with antibodies for albumin and NKX2.1, and counterstained with DAPI. Positive cells tended to occur in aggregates. K–N reflect the same cells at the 10-somite stage; (O–R) same cells at 14-somite stage double-stained with albumin and NKX2.1. Arrows indicate cells that express both albumin and NKX2.1. Merged image with DAPI reflects expected cytoplasmic localization of albumin (O) and nuclear localization of NKX2.1 (R). The granular nature of the fluorescent signal was also seen in control adult tissues treated identically. (S) Quantification of albumin-positive, NKX2.1-positive and albumin/NKX2.1-positive ventral endoderm cells relative to total cells on cytospin slides at 8-, 10- and 14-somite stages.

endoderm from the two- to five-somite embryo, cultured in the absence of cardiac mesoderm for 48 hours and assayed by RT-PCR or immunostaining, survived and expressed PDX1 but failed to express NKX2.1 ( $n=24$ ) or albumin ( $n=17$ ) (summarized in Table 1; Fig. 2D, lane 4; Fig. 2F–H; see Fig. 5A). This finding was anticipated by prior studies (Deutsch et al., 2001; Rossi et al., 2001), which demonstrated that ventral endoderm, in the absence of signals required for hepatic specification, activates pancreas-specific genes. Ventral endoderm co-cultured with beating cardiac mesoderm predictably expressed albumin protein and mRNA in 53 of 56 explants (95%), but also expressed NKX2.1 in 45 of 82

**Fig. 2.** Close proximity and sufficient quantity of cardiac mesoderm is required to reconstitute lung specification in vitro. (A,B) Sagittal view of the seven-somite embryo with foregut endoderm and cardiogenic mesoderm designated by immunostaining for FOXA2 (HNF3 $\beta$ ) and muscle-specific actin (*msa*), respectively. (C-N) Explants containing ventral endoderm (VE) or ventral endoderm with cardiac mesoderm (CM) were isolated from 2- to 5-somite embryos and cultured for 2 days. (C) Circled area in boxed inset denotes beating cardiac cells in captured phase image prior to fixation of colony. Antibody staining for *msa* was used to delineate the cardiac domain in sectioned explants and to confirm the absence of contaminating cardiac mesoderm in nonbeating endoderm cultures. (D,E) RT-PCR analysis of RNA from cultured ventral endoderm explants either with (CM) or without (VE) beating cardiac cells, using designated lung (NKX2.1, SP-C, CC10), liver (albumin) and pancreas (PDX1) markers. Endoderm or CM mesoderm cultured alone never expressed albumin or NKX2.1. In contrast to albumin, which is expressed in 95% of ventral endoderm/cardiac mesoderm co-cultures, lung markers are induced in 56% of explants with cardiac mesoderm. PDX1 is typically expressed in isolated ventral endoderm. (F-N) Endoderm explants were serially sectioned and the same section (G,H,J,K) or adjacent sections (M,N) were double-labeled for NKX2.1 and albumin using indirect immunofluorescence. (F,I,L) Merged image for FOXA2 and MSA reflects location of ventral endoderm and cardiac cells, respectively. Bright signal denotes positive immunoreactivity. NKX2.1-positive cells, when present, are within endoderm contiguous with the cardiac domain (J), while albumin-expressing cells were frequently seen in more distal locations (C,N). No appreciable positive staining is seen in G,H,M. Arrows in I,J indicate overlapping NKX2.1 and FOXA2 signal; arrow in N indicates albumin-expressing cells distant from the cardiac domain.



explants (55%) (Fig. 2C,D, lanes 2-3; Fig. 2I-N; Table 1). Examination for the activation of NKX2.1 by immunostaining (19 positive of 32 explants, 59%) yielded similar findings to analysis by RT-PCR (28 positive of 52 explants, 54%). Double-labeling assays corroborated that the cells positive for NKX2.1 overlapped with FOXA2 signal (Fig. 2I,J), a marker of definitive endoderm (Sasaki and Hogan, 1993; Ang et al., 1993; Monaghan et al., 1993). The specificity of cardiac mesoderm as an inducer of NKX2.1 was tested by co-culturing ventral endoderm with other ectodermal/mesodermal tissues, including isolated neural tube, optic lobes and caudal segments, comprising allantois and extremity of neural plate (Fig. 1A). These explants survived and grew but failed to express NKX2.1 by immunostaining after 2 days in culture (data not shown). Furthermore, isolated mesodermal and lateral ectodermal (non-NKX2.1 expressing in vivo) tissues cultured under identical conditions never expressed NKX2.1.

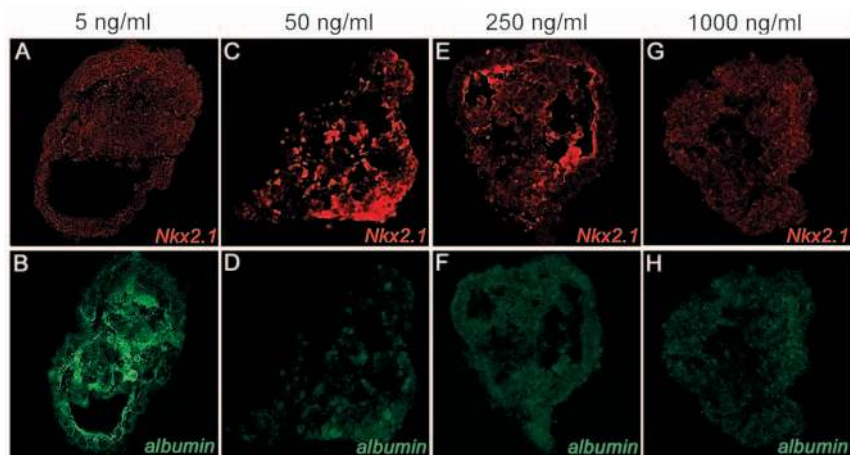
The expression of downstream targets of NKX2.1, including SP-C, a marker of distal pulmonary epithelium, and CC10, a marker of proximal pulmonary epithelium, were assayed for by RT-PCR and immunostaining. In vivo by RT-PCR, we have detected the transcript for SP-C, but not CC10, in the endoderm by the 20-somite stage, 9.5 days gestation (data not shown);

CC10 is first expressed in the developing rat lung at E14 by RT-PCR (Meneghetti et al., 1996). Similar to the proportion of explants positive for NKX2.1, we detected mRNA or protein for SP-C and CC10 in 18 out of 40 (45%) and in 10 of 22 (45%) ventral endoderm/cardiac mesoderm explants, respectively (Fig. 2E, lanes 3-4; Table 1). Neither lung-specific marker was detected within isolated ventral endoderm or control cultures (Fig. 5B,C; Table 1). From these findings we conclude that cardiac mesoderm has the capacity to activate a lung-specific pathway within the ventral endoderm.

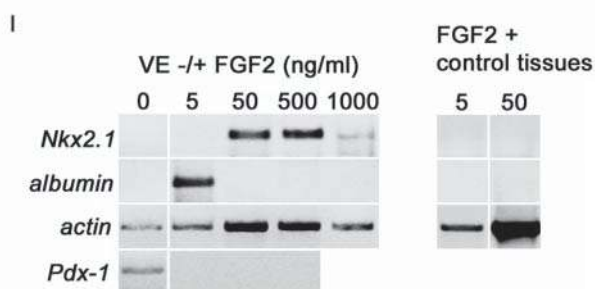
#### Activation of a lung pathway is related to quantity of and proximity to cardiac mesoderm

In contrast to liver specification, cardiac mesoderm did not consistently induce lung markers in cultured ventral endoderm in vitro, doing so in ~50% of explants tested (Table 1). The in





**Fig. 3.** Dose-dependent induction of NKX2.1 and albumin by FGF2. Ventral endoderm (VE) explants were initiated with a single dose of FGF2 and cultured for 24 or 48 hours; similar results were obtained from 1 or 2 days of exposure. (A-H) Immunostaining for NKX2.1 and albumin in serial sections (A,B) or the same explant (C-H). Bright signal indicates positive staining. (I) RT-PCR analysis of RNA from cultured ventral endoderm with heparin sulfate (50 ng/ml) and the designated concentration of FGF2. Control explants (embryonic neural tissue, midsection, tail) failed to express albumin or NKX2.1 in the presence of FGF2, at all doses. (J) Number of explants (positive signal/total tested), at indicated concentration of FGF2, which expressed NKX2.1 or albumin protein (immunostaining, I) or mRNA (RT-PCR, R).



**J**

		Positive signal/total tested at indicated concentration of FGF2 (ng/ml)				
		0	5	10-125	250-500	1000
Nkx2.1	I	0/4	0/3	13/14	6/6	1/5
	R	0/6	0/6	3/5	4/6	1/4
albumin	I	0/7	7/7	3/13	0/4	0/4
	R	0/5	3/4	1/4	0/5	0/2

I=immunostaining  
R=RT-PCR

vivo expression of NKX2.1, which was limited to endoderm neighboring the developing heart, implied that lung specification might require close proximity and/or high levels of signaling from the cardiac domain. Our data support this, showing that explants in which NKX2.1, as well as SP-C and CC10, were activated had more cardiac mesoderm than did explants in which only albumin was detected (Fig. 2I-N; data not shown). Furthermore, in explants in which NKX2.1 was expressed, the signal was invariably confined to endoderm that was directly adjacent to the beating cardiac domain, (Fig. 2J), whereas albumin expression often extended well beyond the beating areas (Fig. 2C,K,N) (Gualdi et al., 1996; Deutsch et al., 2001). These results suggested that induction of a hepatic versus pulmonary cell fate within the ventral endoderm rests on the extent of signal emanating from the cardiac mesoderm.

### Dosage effect of FGF signaling on endoderm cell differentiation

As indicated in the Introduction, prior work has demonstrated that FGF1 or FGF2 can substitute for the cardiac mesoderm as an inducer of liver-specific genes within the ventral endoderm

(Jung et al., 1999). Both FGF1 and FGF2 bind with high affinity to FGF receptor 1 (FGFR1) and FGFR4 (Ornitz et al., 1996), which are expressed in the E8.5 ventral endoderm at the time of hepatic specification (see Fig. 7A-C) (Stark et al., 1991; Sugi et al., 1995) (data not shown). To investigate whether the concentration of FGF ligand determines a liver versus lung fate, we treated ventral endoderm explants at a range of FGF concentrations. Ventral foregut endoderm was obtained from two- to five-somite embryos and cultured with either recombinant FGF1 or FGF2 (5-1000 ng/ml tested). Over 48 hours of monitoring, we did not observe a significant difference in the morphological appearance or growth of explants treated with low versus high dose of FGF1 or FGF2; analyses for cell proliferation (BrdU incorporation) and apoptosis (TUNEL staining) confirmed this impression (data not shown). After one or two days of culture, the expression of NKX2.1 and albumin were assayed for by RT-PCR or double immunofluorescence. Using both methods we observed a dose-dependent expression of NKX2.1 and albumin in response to exogenous FGF2 and

**Table 1. Positive signal by immunostaining or RT-PCR**

		Ventral endoderm (VE)*	VE+ cardiac <sup>†</sup>	Control tissues <sup>‡</sup>
Albumin	I	0/8	19/21	0/10
	R	0/8	34/35	0/8
Nkx2.1	I	0/13	19/32	0/16
	R	0/10	28/52	0/14
SP-C	I	0/6	12/21	0/9
	R	0/5	§6/19	0/12
CC10	I	0/3	4/9	0/6
	R	0/5	6/13	0/2

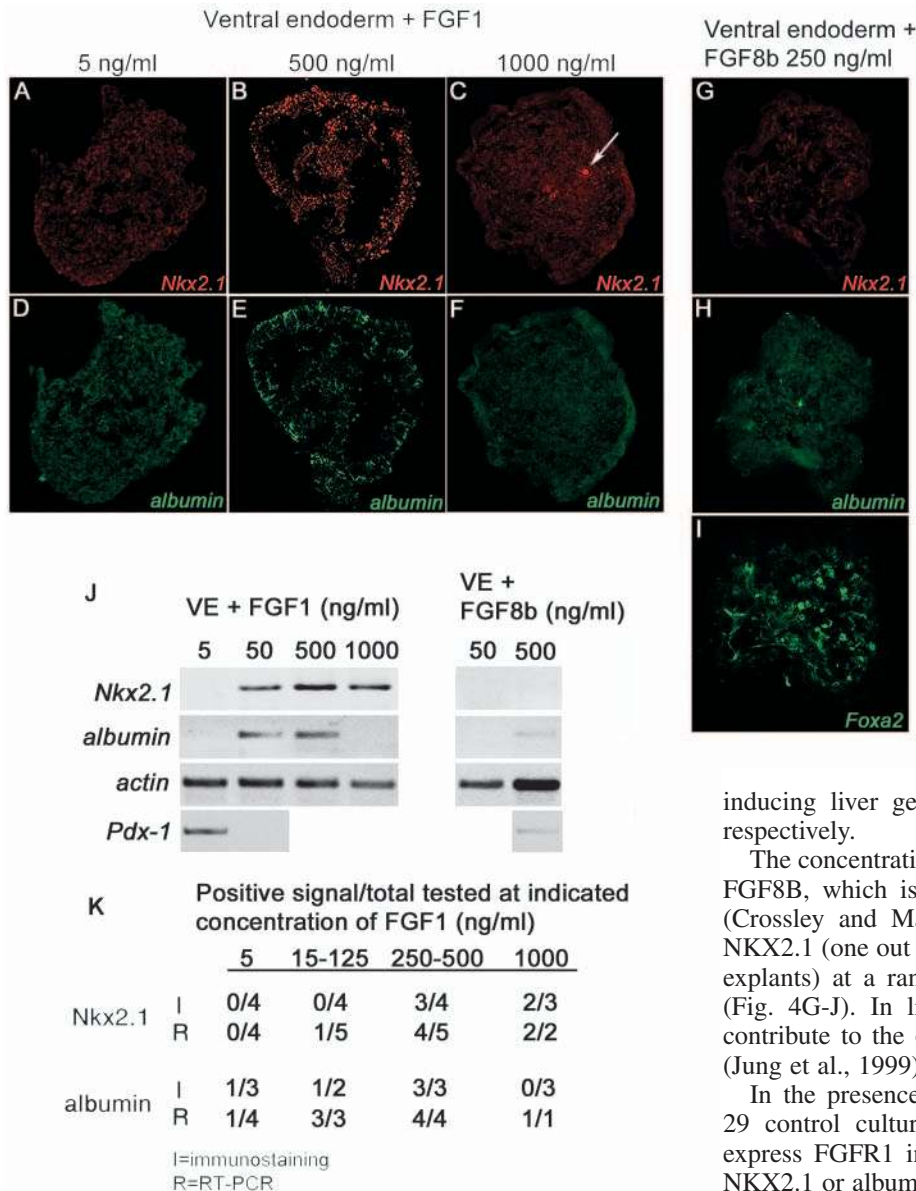
Shown are the number of colonies (positive signal/total tested), as designated, from 2- to 5-somite mouse embryos that either expressed Nkx2.1, albumin, SP-C or CC10 protein (I) or mRNA (R) after 2 days of culture.

\*Nineteen out of 31 cultured in the presence of heparin sulfate as controls for FGF treatments.

<sup>†</sup>Six out of 88 were cultured in the presence of DMSO as controls for SU5402 treatments.

<sup>‡</sup>Includes head and caudal regions, including those cultured in the presence of FGF1, FGF2 and FGF8b.

<sup>§</sup>Four positive signals were faint.



**Fig. 4.** Dose-dependent induction of NKX2.1 and albumin by FGF1. Induction of NKX2.1 or albumin protein (I) or gene (R) expression in ventral endoderm treated with recombinant FGF1, at designated concentrations, within 2 hours of isolation. Double-immunostaining (A-H) or RT-PCR analysis (J) of explants performed after 1-2 days of culture. Arrow indicates focal positive expression. (G-I) Ventral endoderm treated with FGF8B, at similar concentrations, did not consistently induce NKX2.1 or albumin; adjacent section stained with FOXA2 as marker of ventral endoderm. (K) Number of positive explants/total tested, at indicated concentration of FGF1.

to 1000 ng/ml were the most effective at inducing NKX2.1, doing so in 11 out of 14 explants (Fig. 4B,C,J,K). In the same assays, albumin was activated in eight out of 11 colonies; the three explants that failed to express albumin were treated with 1000 ng/ml of FGF1 (Fig. 4F,J,K). The above results are in agreement with the aforementioned studies of Jung et al. (Jung et al., 1999), who found that the optimal doses of FGF2 and FGF1 in

inducing liver genes were 5 ng/ml and 50 or 500 ng/ml, respectively.

The concentration effect of FGF1 and FGF2 was specific as FGF8B, which is also expressed by the cardiac mesoderm (Crossley and Martin, 1995), failed to consistently induce NKX2.1 (one out of nine explants) or albumin (two out of six explants) at a range of concentrations tested (5-500 ng/ml) (Fig. 4G-J). In liver development, FGF8B is theorized to contribute to the organ's morphogenesis versus specification (Jung et al., 1999).

In the presence of all doses of FGFs tested, none of the 29 control cultured ectodermal/mesodermal tissues, which express FGFR1 in vivo (Yamaguchi et al., 1992), activated NKX2.1 or albumin (Fig. 3I; Table 1).

### FGF signaling is sufficient to activate downstream targets of NKX2.1

Concentrations of FGF2 (500 ng/ml) that reliably activated NKX2.1 from the ventral endoderm in vitro also elicited expression of SP-C and CC10 (Fig. 5B-F), as well as of thyroglobulin, expression of which is restricted to the thyroid lineage (data not shown). From this we conclude that FGF signaling is sufficient to induce not only NKX2.1 but also the lung and thyroid genes that it regulates within the foregut.

### FGF signaling is instructive in lung specification

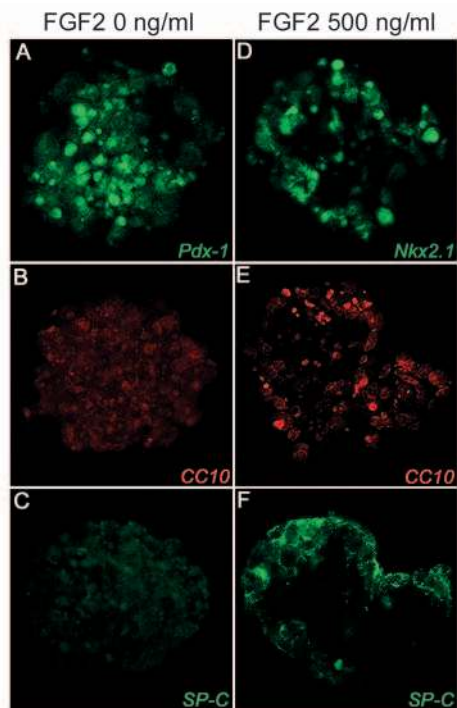
Although dorsal endoderm of the mid- and hindgut gives rise to the small and large intestine, studies have demonstrated that the 8.5-13.5 day dorsoposterior endoderm is competent to adopt a liver fate when isolated from adjacent mesoderm that inhibits albumin expression in co-culture assays (Gualdi et al., 1996; Bossard and Zaret, 2000).

We used midgut endoderm to establish whether FGF signaling is instructive, rather than permissive, in activating a

FGF1, which was statistically significant ( $P < 0.0001$ , Fisher's exact test). A low concentration of FGF2 (2-5 ng/ml), which effectively suppresses the expression of PDX1 (Fig. 3I) (Deutsch et al., 2001), consistently induced albumin expression (10 out of 11 explants) but not expression of NKX2.1 (none of nine explants) (Fig. 3A,B,I,J). By contrast, 10 to 500 ng/ml of FGF2 induced NKX2.1 expression (26 out of 31 explants) but typically not expression of albumin (four out of 26 explants) (Fig. 3C-E,I,J). Neither NKX2.1 (two out of nine explants) nor albumin (none of six explants) was reliably activated in the presence of 1000 ng/ml of FGF2, suggesting an upper limit of dose responsiveness (Fig. 3G-J).

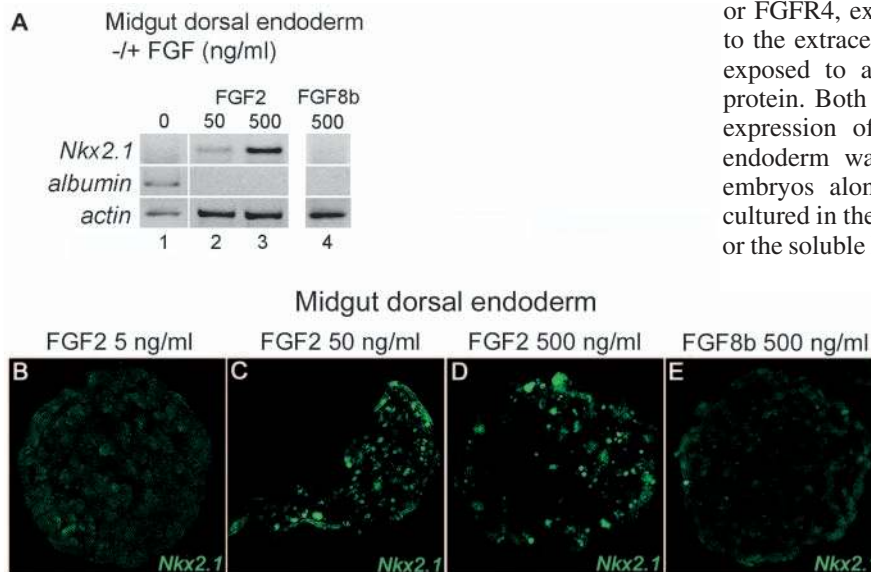
The outcome of endoderm treated with exogenous FGF1 was similar, although at a higher range of concentrations (Fig. 4). FGF1 at 5 ng/ml was inefficient at inducing NKX2.1 or albumin (none of eight and two out of seven explants, respectively); 15-125 ng/ml activated albumin expression in four out of five explants but NKX2.1 was observed in only one out of nine explants. Concentrations of FGF1 ranging from 250





**Fig. 5.** FGFs are sufficient to activate proximal and distal lung markers. Ventral endoderm explants cultured alone (A-C) or in the presence of 500 ng/ml FGF2 (D-F) for 1 day. Adjacent sections were immunostained with the designated markers.

lung pathway in endoderm fated to become the intestinal tract. Dorsal endoderm from the prospective midgut region (Fig. 1A) was dissected from two- to five-somite embryos, and treated with FGFs at a range of concentrations. In the absence of FGF, dorsal endoderm isolated from the prospective midgut region and cultured for 2 days did not activate NKX2.1 expression by RT-PCR analysis (none of four explants) or immunostaining (none of seven explants), although it did activate albumin (Fig. 6A, lane 1) (Gualdi et al., 1996; Bossard and Zaret, 2000). However, dorsal midgut endoderm treated with exogenous



**Fig. 6.** FGF signaling in dorsal midgut endoderm. (A) Midgut dorsal endoderm cultured alone (A, lane 1) or exposed to the designated concentration of FGF (A, lanes 2-4; B-E) for 2 days. Explants were analyzed for the activation of the designated marker by RT-PCR analysis (A) or immunofluorescence (B-E).

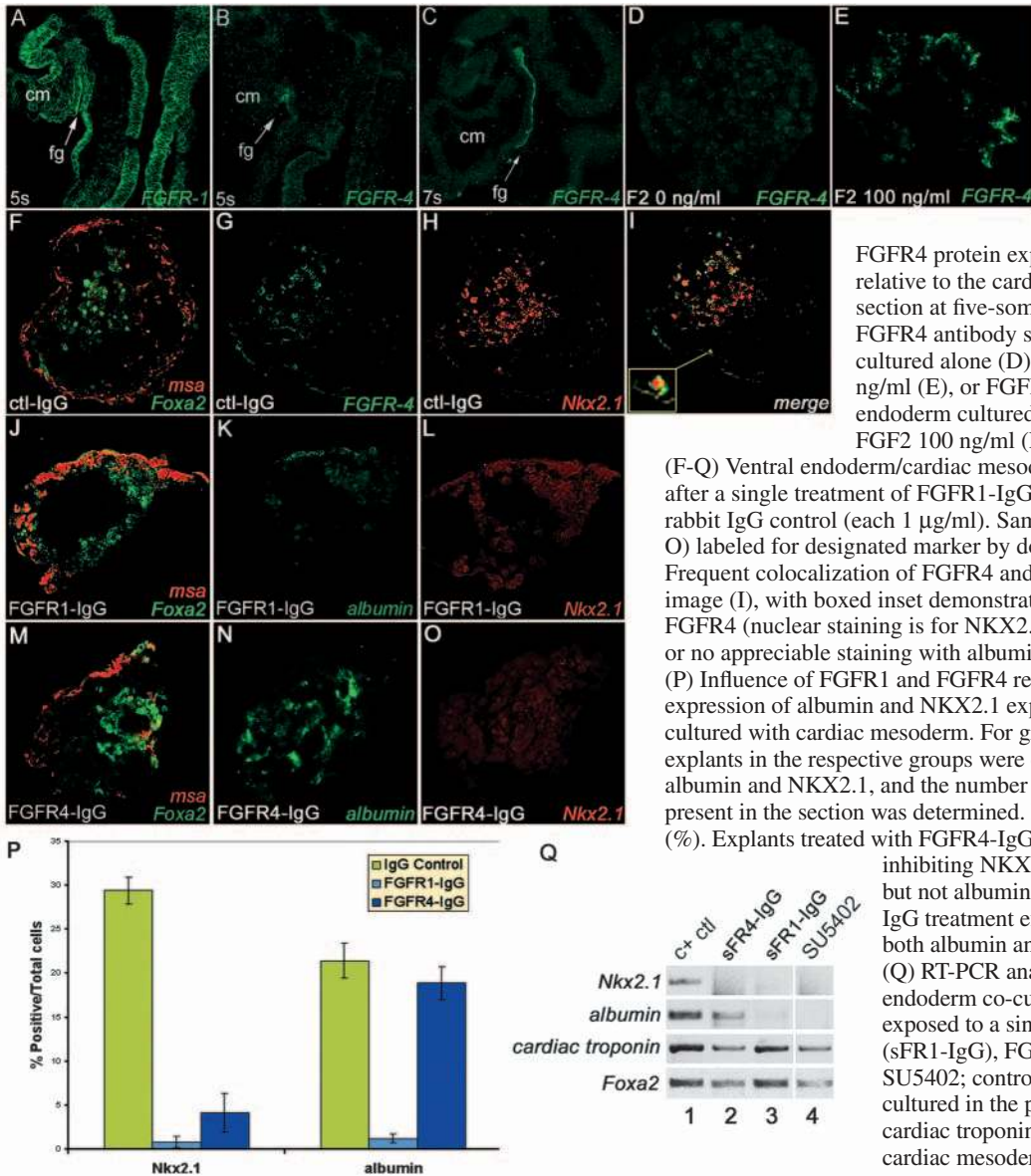
FGF2 exhibited a concentration-dependent induction of NKX2.1 in a manner analogous to the ventral endoderm. Specifically, NKX2.1 was efficiently induced at high (50 and 500 ng/ml) (six out of six explants) rather than low concentrations of FGF2 (1-5 ng/ml) (none of three explants) and inefficiently by FGF8B at identical concentrations (two out of six explants) (Fig. 6A, lanes 2-4; 6B-E). Furthermore, exposure of dorsal midgut endoderm to high concentrations of FGF2 suppressed activation of albumin in all five isolates tested, similar to what was observed with ventral foregut endoderm.

### Inhibition of FGFR1 and FGFR4 signaling abrogates induction of NKX2.1

Considering that inhibitors of FGF signaling block hepatic induction by cardiac mesoderm (Jung et al., 1999), we anticipated that FGF signaling is also necessary for cardiac-mediated activation of NKX2.1. Earlier studies have established that the E8.5 ventral endoderm co-expresses FGFR4 and FGFR1 mRNA (Stark et al., 1991; Sugi et al., 1995), the latter of which is present in both cardiac and endoderm regions. Our immunohistochemical analysis revealed that FGFR1 is expressed throughout the early somite stages (Fig. 7A). By contrast, FGFR4 was barely detectable in the foregut endoderm of the five-somite embryo (Fig. 7B); by the seven- to eight-somite stages, this expression was much more robust (Fig. 7C) and at the 10-somite stage was seen throughout the ventral and dorsal endoderm (data not shown) (Stark et al., 1991). The onset and expansion of FGFR4 expression in the five- to seven-somite foregut endoderm directly preceded that of albumin and NKX2.1, implying that functional activity of this FGF receptor might define the temporal and spatial domain of liver and lung specification. In support of this interpretation, we have observed an increase in FGFR4 levels in ventral endoderm co-cultured with cardiac mesoderm or FGF2, relative to ventral endoderm alone, as well as frequent overlap in FGFR4 and NKX2.1 expression (Fig. 7D-I; data not shown).

To test the necessity of FGF signaling and possible receptor specificity for NKX2.1 activation by the cardiac mesoderm, we used several approaches. To perturb signaling through FGFR1 or FGFR4, explants were treated with an antibody that binds to the extracellular, ligand-binding domain of the receptor or exposed to a soluble recombinant FGFR/IgG-Fc chimeric protein. Both approaches yielded a comparable effect on the expression of albumin and NKX2.1 (see below). Ventral endoderm was dissected from four- to five-somite mouse embryos along with its associated cardiac mesoderm and cultured in the presence of IgG to FGFR1 or FGFR4 (1  $\mu$ g/ml) or the soluble FR-IgG complex (500 ng/ml), and heparin sulfate





**Fig. 7.** Selective inhibition of FGFR1 and FGFR4 diminishes induction of NKX2.1 in ventral endoderm co-cultured with cardiac mesoderm. (A-C) Sagittal sections of mouse embryos at five- (B) and seven- (C) somite stages, demonstrating the onset and expansion of

FGFR4 protein expression (arrows) in the foregut (fg) relative to the cardiac domain (cm); (A) adjacent section at five-somite stage, stained with FGFR1. FGFR4 antibody staining of foregut endoderm cultured alone (D) or in the presence of FGF2 100 ng/ml (E), or FGFR4 antibody staining of foregut endoderm cultured alone (D) or in the presence of FGF2 100 ng/ml (E) or cardiac mesoderm (G).

(F-Q) Ventral endoderm/cardiac mesoderm explants cultured for 2 days after a single treatment of FGFR1-IgG or FGFR4-IgG, compared with rabbit IgG control (each 1  $\mu$ g/ml). Same or adjacent sections (F-I;J-L;M-O) labeled for designated marker by double immunofluorescence. Frequent colocalization of FGFR4 and NKX2.1 is shown in a merged image (I), with boxed inset demonstrating membranous staining for FGFR4 (nuclear staining is for NKX2.1). Explants in K,L,O have limited or no appreciable staining with albumin (green) or NKX2.1 (red). (P) Influence of FGFR1 and FGFR4 receptor inhibition on protein expression of albumin and NKX2.1 expression in ventral endoderm co-cultured with cardiac mesoderm. For graphic representation, four out of 10 explants in the respective groups were cryosectioned, immunostained for albumin and NKX2.1, and the number of positive cells out of total cells present in the section was determined. Results expressed as mean $\pm$ s.e.m. (%). Explants treated with FGFR4-IgG exhibit a significant difference in inhibiting NKX2.1 expression ( $t$ -test:  $P < 0.0001$ ) but not albumin expression ( $t$ -test:  $P < 0.4$ ). FGFR1-IgG treatment effectively diminishes expression of both albumin and NKX2.1 ( $t$ -test;  $P < 0.0001$ ).

(Q) RT-PCR analysis of RNA from ventral endoderm co-cultured with cardiac mesoderm and exposed to a single dose of soluble FGFR1/IgG-Fc (sFR1-IgG), FGFR4/IgG-Fc (sFR4-IgG) or SU5402; control explants for SU5402 assay cultured in the presence of DMSO. Expression for cardiac troponin and FOXA2 reflect integrity of the cardiac mesoderm and endoderm, respectively.

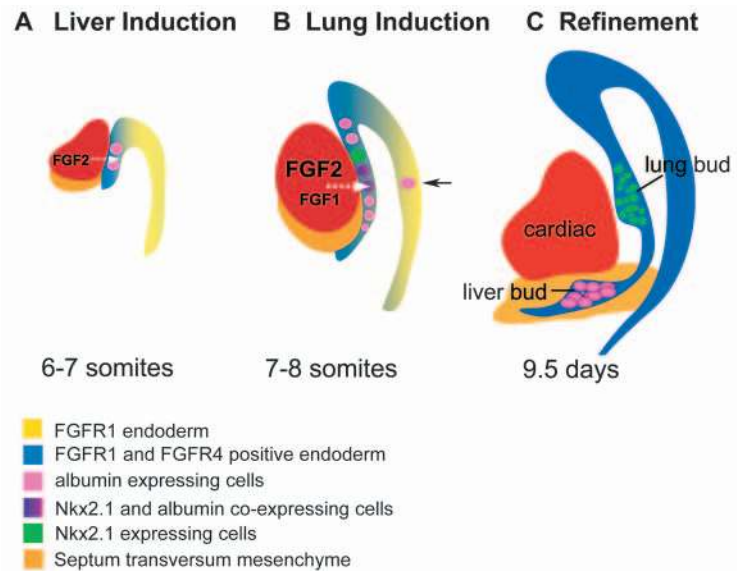
at 100 ng/ml. As a control, we used rabbit IgG with heparin sulfate. We modified our dissection technique to ensure that all explants contained an increased proportion of cardiac mesoderm to ventral endoderm. Over 48 hours, we did not see a significant difference between the treatment groups in the growth or viability of cultures. However, compared with controls treated with rabbit IgG, there was a 3- to 4-hour delay in the onset of beating within explants exposed to FGFR-IgG and soluble FR-IgG; by 18 hours this disparity in activity of the cardiac domain between groups was no longer evident. After 2 days, the presence and amount of NKX2.1 and albumin signal were assessed by sectioned double immunostaining and RT-PCR. Compared with the consistent induction of albumin and NKX2.1 within the control group (10/10 and 9/10 positive explants, respectively) (Fig. 7H,Q, lane 1), FGFR1-IgG and soluble FR1-IgG effectively diminished cardiac induction of albumin (two out of 10 and two out of seven positive explants, respectively) and NKX2.1 (one out of 11 and two out of seven positive explants, respectively), with only few labeled cells by immunostaining when present (Fig. 7K,L,P,Q, lane 3)

( $P < 0.0003$ , Fisher's exact test). Explants treated with a single dose of SU5402, a FGFR1 tyrosine kinase inhibitor, yielded comparable findings with only two out of nine explants expressing albumin and NKX2.1 (Fig. 7Q, lane 4). Notably, interfering with signaling through FGFR1 did not result in an appreciable defect of cardiac or endoderm viability, confirmed by morphology and gene expression (Fig. 7Q, lanes 1-4).

In agreement with prior work (Jung et al., 1999), FGFR4-IgG and soluble FR4-IgG were less effective than FR1-IgG in diminishing albumin expression (eight out of 10 and four out of seven explants positive, respectively), and we expected analogous results for NKX2.1. Surprisingly, compared with the controls, FGFR4-IgG and soluble FR4-IgG efficiently inhibited the expression of NKX2.1, with only two out of 10 and one out of seven explants demonstrating limited expression in cryosections and by RT-PCR (Fig. 7O-Q, lane 2); this was significantly different from that observed with albumin ( $P < 0.0019$ , chi-square test).

These data support the hypothesis that the cardiac mesoderm is the endogenous activator of NKX2.1 expression in the

**Fig. 8.** A model for patterning of the foregut endoderm through threshold of FGF activity. Schematic representation of embryo expression data shown in Figs 1 and 7, using sagittal cross-section through cardiac and foregut regions. (A) The onset of FGF2 expression in the five- to six-somite cardiac mesoderm (Jung et al., 1999) (data not shown) occurs just prior to albumin activation in the foregut endoderm, which predominantly expresses FGFR1. This threshold of FGF2 (broken arrow) is sufficient for liver but not lung induction. (B) Coincident with NKX2.1 activation in the seven- to eight-somite ventral endoderm, is the expansion of FGFR4 expression and the addition of FGF1 with FGF2 in the developing heart (Jung et al., 1999). The dose of FGF signaling (broken arrow) is now sufficient to induce lung specification, with frequent co-expression of NKX2.1 and albumin in the same cells. Broken arrows reflect that induction of liver and lung by cardiac signaling may be direct or indirect (see Discussion). Endogenous FGF2 in the adjacent notochord (arrow) may induce albumin expression in dorsal foregut endoderm. High doses of FGF, inhibitory to albumin *in vitro*, could contribute to refinement of cell commitment (C). Functional data (Fig. 7) indicates that induction of NKX2.1, but not albumin, relies on intact FGFR4 activity. (C) Once cell specification occurs, presumed positive and negative feedback between neighboring germ layers, including the mesenchyme of the septum transversum, maintain and refine cell commitment. Dark red indicates cardiac region.



endoderm and that this induction is mediated, at least in part, by FGFs. Furthermore, the ability to effectively suppress NKX2.1 expression by selective inhibition of both FGFR1 and FGFR4 implies that the FGF receptor composition on the surface of the endoderm cell is as important in lung versus liver specification as is the concentration of mesoderm-derived ligand.

## Discussion

The foregut endoderm gives rise to diverse epithelial cell lineages, which eventually organize into the liver, ventral pancreas, lung, thyroid and proximal segment of the gastrointestinal tract. Extensive investigation into liver and pancreas specification has provided a framework for understanding the inductive processes which promote cell fate decisions within the endoderm. In this paper, we provide evidence that FGF signaling from the cardiac mesoderm induces lung as well as liver specification in the foregut, and that this signaling functions along a threshold to define individual cell fate in the multipotential endoderm (Fig. 8).

The *in vivo* and *in vitro* studies presented here support a common mechanism for patterning of the endoderm. We have determined that NKX2.1, a homeodomain transcription factor necessary for lung morphogenesis, is activated within the ventral foregut endoderm in a subset of cells expressing albumin, implying that the inducing signals are derived from the same mesodermal source. An embryo explant culture system confirms this hypothesis, as close proximity to a sufficient quantity of cardiac mesoderm is required to reconstitute activation of a lung pathway *in vitro*, with expression of both proximal and distal pulmonary cell lineages, downstream of NKX2.1 transcription (Minoo et al., 1999). Explant experiments further indicate that cardiac induction and repression of cell fate within the multipotent endoderm is dose dependent; treatment of isolated foregut endoderm with low concentrations of cardiac-derived FGFs activates albumin but

not NKX2.1, whereas higher concentrations produce lung differentiation at the exclusion of liver. This response is mediated, at least in part, by the activity of FGFR1 and FGFR4, which are dynamically expressed in the endoderm, *in vivo* and *in vitro*, synchronous with cell specification (Fig. 7) (Jung et al., 1999).

Supportive evidence that cell fate within the foregut endoderm is driven by a threshold of FGF signaling comes from a detailed knowledge of FGF ligand expression within the developing heart. Jung et al. (Jung et al., 1999) determined that FGF2 mRNA is present, but barely detectable in the cardiac mesoderm of the five-somite stage embryo; by the seven- to eight-somite stages FGF1 first appears while FGF2 levels are now robust (Fig. 8). Additionally, a low level of FGF8 persists in an area of the cardiac mesoderm bordering ventral endoderm (Jung et al., 1999). Thus, the intensity of total FGF expressed in the heart *in vivo* positively correlates with the successive induction of albumin and NKX2.1 in the adjacent endoderm. The onset and expansion of FGF2 and FGF1 in the developing heart also parallels the temporal and spatial expression of FGFR4 in the endoderm, upon which NKX2.1 is functionally dependent in our *in vitro* assay (Fig. 7). Our data suggests that expression of FGFR4 within endoderm is induced by FGF from cardiac mesoderm. Ventral endoderm explants cocultured with cardiac mesoderm or with exogenous FGF1 or FGF2 show elevated levels of FGFR4 expression by immunostaining and RT-PCR (Fig. 7D-G; data not shown) implying that FGFR4 is regulated by FGF signaling. Induction of NKX2.1 in the dorsal endoderm by FGF2 may also be mediated through FGFR4. Further studies will be required to assess the precise relationship between cardiac signaling and the FGFR4 responsive endoderm in promoting a pulmonary cell fate. However, the data indicate that the total level of FGF signaling, both at the ligand and receptor level, is necessary for lung specification (Fig. 8).

Despite the described influence of FGF1 and FGF2, as well as FGFR4 *in vitro*, mice lacking these genes are viable and



have no major abnormalities in organ development (Ortega et al., 1998; Dono et al., 1998; Weinstein et al., 1998; Miller et al., 2000; Yu et al., 2000). However, the presence and requirement for FGFR1 during embryogenesis (Deng et al., 1994; Yamaguchi et al., 1994) as well as FGF4, FGF8 and FGF17 expressed in the cardiac mesoderm at the time of liver and lung specification (Crossley and Martin, 1995; Zhu et al., 1996; Maruoka et al., 1998) indicate that related molecules can probably function in a redundant capacity.

We acknowledge that the threshold effect of exogenous FGF on cell differentiation might be related to mesoderm cells (FOXA2 negative), which are frequently present within our endoderm isolates. Such cells could be a source of FGF, or another as yet unidentified inducer, and thus induction of albumin versus NKX2.1 may be an indirect phenomenon (see Fig. 8). Although the poor viability of individual endoderm cells in culture precludes systematic analysis at the single cell level, we have observed isolated and small clusters of endoderm cells expressing albumin in the presence of low FGF and NKX2.1 in the presence of high FGF (data not shown), supporting primary induction.

Interestingly, FGF1 and FGF2 lack a definitive signal sequence and are inefficiently secreted into the extracellular environment by nonclassical pathways (for a review, see Powers et al., 2000), implying that FGFs may pattern the ventral foregut through short-range signaling.

The dorsoposterior endoderm of the midgut is competent to activate NKX2.1 by FGF when isolated from adjacent mesoderm and ectoderm (Fig. 6). Prior studies (Gualdi et al., 1996; Bossard and Zaret, 2000) have demonstrated that dorsal endoderm is also able to activate liver-specific genes when cultured alone. This competency, which is lost at E13.5 prior to intestinal differentiation, is theorized to be endowed by the transient occupancy of FOXA2 and GATA4 factors bound to the albumin enhancer (Bossard and Zaret, 2000). The authors surmise that active repression of the gut mesoderm on a foregut fate involves a secreted factor, as fragments of dorsal mesoderm and ectoderm, in tissue recombination assays, inhibits liver genes in ventral endoderm co-cultured with cardiac mesoderm (Gualdi et al., 1996). Elucidating the nature of repressive mesodermal interactions in patterning the endoderm is of considerable interest, especially as the posterior mesoderm expresses high levels of FGFs (Crossley and Martin, 1995; Maruoka et al., 1998; Wells and Melton, 2000; Karabagli et al., 2002).

Low levels of FGF2 expressed by the notochord (Zuniga Mejia Borja et al., 1996; Hebrok et al., 1998) might explain the paradoxical finding that rare dorsal endoderm cells in the foregut, at least transiently, express albumin *in vivo* (Fig. 1B,E). This expression could be lost with the midline fusion of the paired dorsal aortas, which separate notochord from the foregut slightly later in development (Hebrok et al., 1998).

We have found that the ability of the endoderm to be programmed by FGF signaling is temporally restricted, for a 6 hour delay in exposure of explant cultures to FGF2 results in activation of PDX1 at low doses, and albumin at high doses, with minimal expression of NKX2.1 (data not shown). This latter data supports prior *in vitro* studies, which indicate that in the absence of cardiac mesoderm or FGF, the default fate of the early somite ventral foregut endoderm is to activate a pancreas gene program (Gamer and Wright, 1995; Deutsch et

al., 2001). This finding brings into question whether endoderm cells retain plasticity in fate after specification has occurred.

It is intriguing that early genes specific to the liver and lung transiently co-express within the ventral endoderm soon after hepatic and pulmonary initiation in the mouse embryo. It unclear at this time what refines the expression pattern of these genes by E9.5 (Fig. 1G-J,O-S), but multiple factors, including continued induction, lack of induction and/or suppression, particularly from adjacent germ layers, may play a role (Fig. 8). Prior work, using an identical *in vitro* system, has implicated BMP signaling from the septum transversum, cardiac/FGF-induced sonic hedgehog and Hex-dependent cell proliferation as contributing to hepatic versus pancreatic induction (Rossi et al., 2001; Deutsch et al., 2001; Bort et al., 2004). Within the developing respiratory tract of the chick embryo the TBX4-FGF10 system regulates early lung bud formation and defines the posterior boundary of NKX2.1 expression (Sakiyama et al., 2003). We are currently investigating the possibility that after NKX2.1 is activated combinatorial interactions with pathways implicated in lung development establish a pulmonary fate in the endoderm.

NKX2.1 is an early marker, as well as transcriptional regulator, of the thyroid as well as the lung (Lazzaro et al., 1991; Kimura et al., 1996; Kimura et al., 1999), and data drawn from the isolated expression of NKX2.1 cannot distinguish between the two. In preliminary studies, we have found that ventral endoderm explants require cardiac mesoderm or FGF to activate the thyroid specific marker thyroglobulin (G.H.D., unpublished). How these requirements modify specification of one organ versus the other remains to be determined.

The model described here for patterning of the ventral endoderm (Fig. 8) is reminiscent of the developmental processes involved in generating the limb bud, which is the source of soluble FGFs that confer proximodistal identity to the skeletal elements (reviewed by Martin, 1998). Re-examination of the vertebrate limb model (Sun et al., 2002; Dudley et al., 2002) supports early specification of the proximal and distal limb domains, of which the respective precursor populations expand and differentiate with subsequent development. Likewise in the ventral endoderm, cell fate choice is not related to the degree of cell proliferation or cell death (Deutsch et al., 2001) (data not shown); however, these processes might be involved in acquisition of a definitive fate after specification occurs. For example, the dose of FGF8 directly regulates cell survival in the developing forebrain (Storm et al., 2003). Defining lineage relationships and the factors that restrict cell fates in the embryonic foregut endoderm may facilitate the identification and manipulation of progenitor cells to treat congenital and adult diseases.

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