

# Differential gene expression in the distal tip endoderm of the embryonic mouse lung

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## Abstract

During the early development of the mouse lung a number of genes encoding signaling molecules are differentially expressed in the epithelium and mesenchyme of the distal buds. Evidence suggests they play a role in regulating the stereotypic processes of bud outgrowth and branching as well as proximal–distal patterning of both cell layers. To better understand the mechanisms underlying branching morphogenesis, a subtractive hybridization and differential screen was carried out for genes preferentially expressed in the epithelium at the tips of embryonic day 11.5 lung buds, versus more proximal regions. Twenty genes were identified, assigned to different categories based on sequence analysis, and their distal expression confirmed by whole-mount in situ hybridization. © 2002 Elsevier Science B.V. All rights reserved.

**Keywords:** Lung; Endoderm; Mouse; Embryo; Gene expression

## 1. Results and discussion

Lung branching morphogenesis is regulated by reciprocal interactions between the epithelial cells, the surrounding mesenchyme, and the outermost mesothelium, mediated by extracellular signaling molecules, cell membrane bound receptors and intracellular signaling pathways. Among the signaling molecules, it is known that members of several conserved families such as the FGF, Hh, Bmp/TGF $\beta$ , Wnt, EGF and PDGF families, are expressed specifically in the distal tips of the lung at the pseudoglandular stage (embryonic day E9.5–16.6) (reviewed by Hogan and Yingling, 1998; Warburton et al., 2000). The epithelial cells in the tips respond to signals from the mesenchyme in several ways, including increased proliferation, coordinated cell shape changes, and the generation of the precursors of the different cell types along the proximal–distal axis. In order to know more about the mechanisms underlying these changes, we compared gene expression in proximal and distal lung endoderm at E11.5 by subtraction hybridization and differential screening (Fig. 1 and Section 2). Twenty genes were identified as being preferentially transcribed in distal endoderm, and this allocation was confirmed by whole-mount in situ hybridization of E11.5 lungs. Some genes are only expressed in the distal endoderm

while others are expressed in distal endoderm as well as some portion of the distal mesoderm (Table 1 and Fig. 2).

The genes fell into several groups characteristic of different molecular functions (Table 1). Five encoded transcription factors, including Erm, Gabpb, Idb2, Sox 9 and Crebbp (Creb binding protein) (Fig. 2A–E). Erm (Etv5 or Ets variant gene 5) is an ETS domain transcription factor of the Pea3 subfamily (de Launoit et al., 1997). Gabpb (GA repeat binding protein b subunit) contains a C-terminal leucine zipper-like motif mediating the homodimerization necessary for activation of transcription, and four N-terminal tandem ankyrin repeats involved in heterodimerization with GABPa, an ETS domain protein (Suzuki et al., 1998; Chinenov et al., 2000). *Idb2* (inhibitor of DNA binding 2) encodes a helix–loop–helix (HLH) protein of the ID family which acts primarily as a negative regulator of basic HLH (bHLH) transcriptional factors to promote cell proliferation while inhibiting differentiation (Norton, 2000). At present, no bHLH protein is known to be expressed in the distal endoderm, but *Idb2* can also interact with the ETS domain transcription factor TCF (ternary complex factor) and Pea3 subfamily members (Yates et al., 1999), so raising the possibility that *Idb2* modulates Erm function in lung endoderm. In addition, in mouse embryonic stem cells, *Idb2* appears to be a downstream target of Bmp2/4 signaling, (Hollnagel et al., 1999), and Bmp4 signaling is also active in distal lung epithelium (Weaver et al., 1999). Sox9 belongs to the HMG (high mobility group) family of transcription factors (de Crombrughe et al., 2000), and

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plays an important role in vertebrate sex determination and in chondrogenesis, where its expression in chondrocytes may be induced by FGF and/or Bmp signaling (Capel, 1998; Healy et al., 1999; de Crombrughe et al., 2000). It is therefore not surprising that *Sox9* is expressed in proximal mesenchyme where tracheal cartilage rings are developing. However, its function and regulation in the distal endoderm remains to be determined. Creb binding protein (*Crebbp*) acts as a general coactivator of a number of transcription factors mediating cell proliferation and differentiation (Kwok et al., 1994).

The second category of genes encoded signaling receptors and ligands. Among them, *Tgf $\beta$ 2* was previously shown to be specifically expressed in distal lung epithelium at E11.5, in contrast to *Tgf $\beta$ 1* and  $\beta$ 3, which are expressed in the mesenchyme at the same stage (Bragg et al., 2001). EphA4 is an Eph family receptor tyrosine that recognize membrane bound ephrin ligands (Holder and Klein, 1999)

(Fig. 2F,G). Eph/ephrin interactions do not have pronounced mitogenic activities but in a number of systems regulate cell migration, formation of boundaries between cell populations, and change in cell shape and adhesion (reviewed in Holder and Klein, 1999), all activities associated with distal endoderm. It was recently reported that *ephrinB2* is expressed in the epithelial cells of the developing ureteric bud as it extends into the nephrogenic mesenchyme (Takahashi et al., 2001) suggesting that Ephs and ephrins may be important common mediators of budding morphogenesis in several vertebrate organs. In addition, our finding that *Epha4* is strongly expressed specifically in the mesenchyme tip of the accessory lobe at E11.5 (Fig. 2G) suggests that these cells differ in some way from mesenchyme cells in other lobes. Only the accessory lobe extends across the midline at this time and one possibility is that *Epha4* plays a role in this process. Transcription of the gene

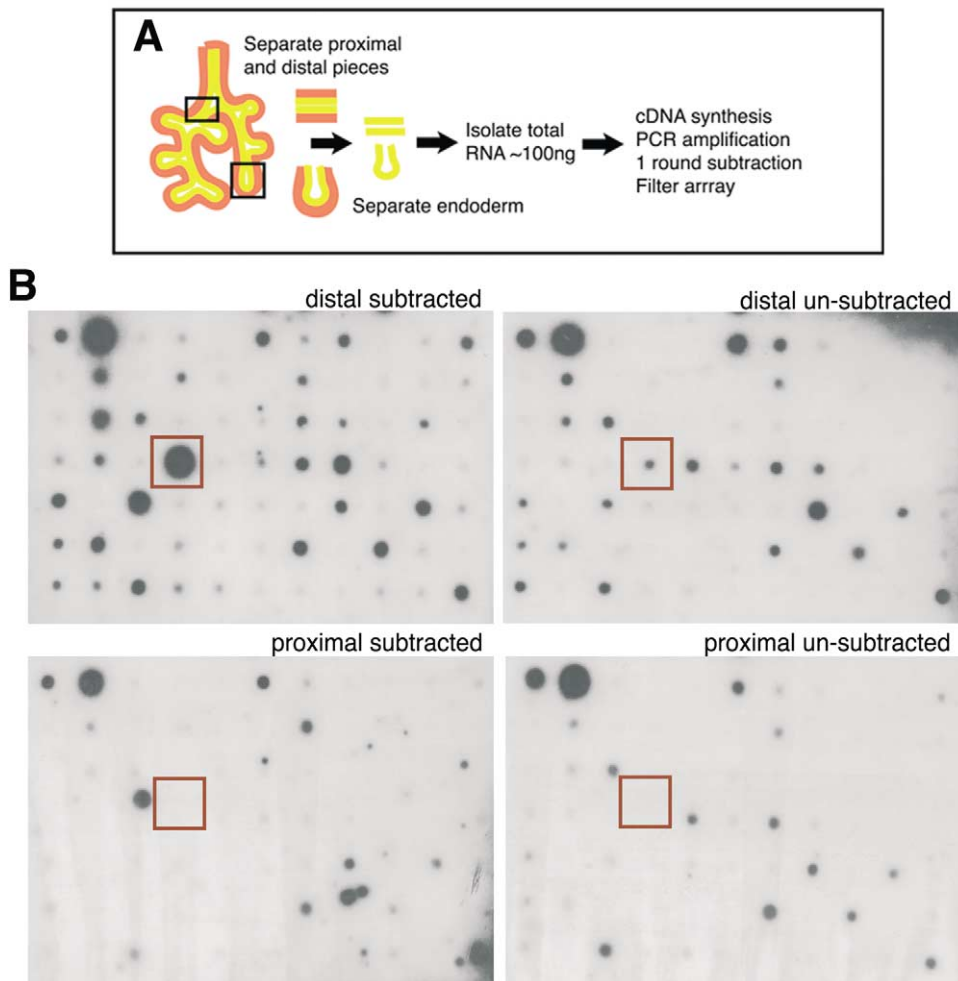


Fig. 1. Procedure for subtractive hybridization and differential screening. (A) Lungs were dissected from E11.5 ICR mice. Proximal and distal samples about 100  $\mu$ m in length, corresponding to future primary bronchi and terminal buds, respectively, were separated into endoderm and mesoderm. About 100 ng total RNA was isolated from each pool. Total RNA was subjected to SMART amplification and subtractive hybridization as described in Section 2. Equal amounts of the PCR amplified inserts from about 350 clones, roughly corresponding to 2.5% of the total subtracted distal cDNA, were printed onto four identical copies of nylon filter. (B) An example of the results of the differential screen. Four copies of filters were hybridized with  $^{32}$ P-labeled probes as indicated. Red box indicates an example of a positive clone selected. This example corresponded to *Erm*.

Table 1  
Genes preferentially expressed in distal endoderm

Candidate genes	Accession no.	Protein encoded	Times appeared	Previously known to be enriched in distal endoderm?
<b>Transcription factors</b>				
<i>Etv5(Erm)</i>	AY004174	Ets-related transcription factor	4	Yes (Chotteau-Lelievre et al., 1997)
<i>Gabpb</i>	NM_010249	GA repeat binding protein beta	2	No
<i>Idb2</i>	BC006921	Inhibitor of DNA binding 2	3	Yes (Jen et al., 1996)
<i>Sox9</i>	AF421878	SRY sex determining region Y-box 9 protein	1	No
<i>Crebbp</i>	S66385	CREB-binding protein	1	No
<b>Signaling receptors and ligands</b>				
<i>Calcr1</i>	AF209905	Calcitonin receptor-like receptor	2	No
<i>Epha4</i>	NM_007936	Eph receptor A4	1	No
<i>Tgfb2</i>	NM_009367	Transforming growth factor beta 2	1	Yes (Bragg et al., 2001)
<b>Chaperone; Heatshock protein; Channel</b>				
<i>Sec61g</i>	NM_011343	Sec61 gamma subunit	1	No
<i>Hspa5</i>	NM_022310	Heat shock 70 kDa protein 5	1	No
<i>Hsc70</i>	BC006722	Heat shock cognate protein 70	2	No
<i>ERp99</i>	J03297	ER transmembrane protein ERp99	1	No
<b>Isomerase</b>				
<i>ERp60</i>	BC003285	Disulfide isomerase ER-60	2	No
<i>Protein disulfide isomerase</i>	BC006865	Similar to protein disulfide isomerase-related protein(p5)	2	No
<b>Motor protein</b>				
<i>Dncic2</i>	NM_010064.1	Dynein, cytoplasmic intermediate chain 2	1	No
<b>Structural protein</b>				
<i>Gjal</i>	BC006894	Gap junction membrane channel protein alpha 1(connexin43)		No
<b>Other</b>				
<i>Thbs1</i>	M87276	Thrombospondin 1	4	No
<i>Arl6 interacting protein</i>	AK008381	Hypothetical protein similar to Arl6 interacting protein	2	No
<b>ESTS</b>	BC004677; NM_009186		2	No

for calcitonin-receptor like protein (Calcr1), which is a seven-transmembrane G protein coupled receptor (Pondel, 2000), is tightly restricted to the most distal lung endoderm (Fig. 2H).

Several genes were identified that encode proteins associated with protein trafficking, transport, and secretion, including molecular chaperons, heatshock proteins, and factors involved in protein translocation. This class also includes two members of the protein disulfide isomerase family localized at the endoplasmic reticulum that may be responsible for the correct folding and modification of secreted and extracellular matrix proteins (Fig. 2I,J). In

addition, one gene encoded a putative protein that potentially binds ADP-ribosylation-like factor 6 (Arl6), a small G protein of the ras super family thought to be involved in protein transport between organelles (Ingley et al., 1999) (Fig. 2K). The differential expression of these genes probably reflects the fact that distal endoderm cells are rapidly proliferating and actively synthesizing secreted proteins such as Bmp4, Wnt7b and Shh.

Distally expressed genes involved in cell structure include *Dncic2*, encoding a cytoplasmic intermediate chain 2 of dynein, a microtubule-based motor protein mediating the trafficking of numerous intracellular orga-

nelles. Transcripts for the gap junction protein, *Gja1* (connexin 43 or gap junction membrane channel protein alpha 1), also appear to be enriched in distal lung buds (Fig. 2L).

The localized distal expression of *Thbs1*, which encodes the multimeric, multidomain glycoprotein, Thrombospondin1, is shown in Fig 2M. The protein is associated with the extracellular matrix and potentially influences many different cell functions, such as migration and shape change, proliferation and apoptosis (for review see Adams, 2001). One possible function for distal *Thbs1* at E11.5 is to activate latent TGF $\beta$ 1, which is expressed in the mesoderm (see above). This idea is supported by the fact that *Thbs1* null mutant mice die postnatally and have an abnormal lung

phenotype very similar to that of *Tgf $\beta$ 1* null mutants (Crawford et al., 1998)

During early lung development, distal epithelial cells are engaged in numerous biological activities, including a high rate of proliferation, maintenance of an undifferentiated phenotype, and frequent cytoskeletal and cell shape changes related to budding, outgrowth and branching. In addition, the distal endoderm is both responding to morphogenetic signals from the mesoderm, and secreting factors into it that regulate mesodermal proliferation, survival and differentiation. The screen described in this paper has identified differentially expressed genes in several functional classes that throw new light on the regulatory networks acting in distal lung buds.

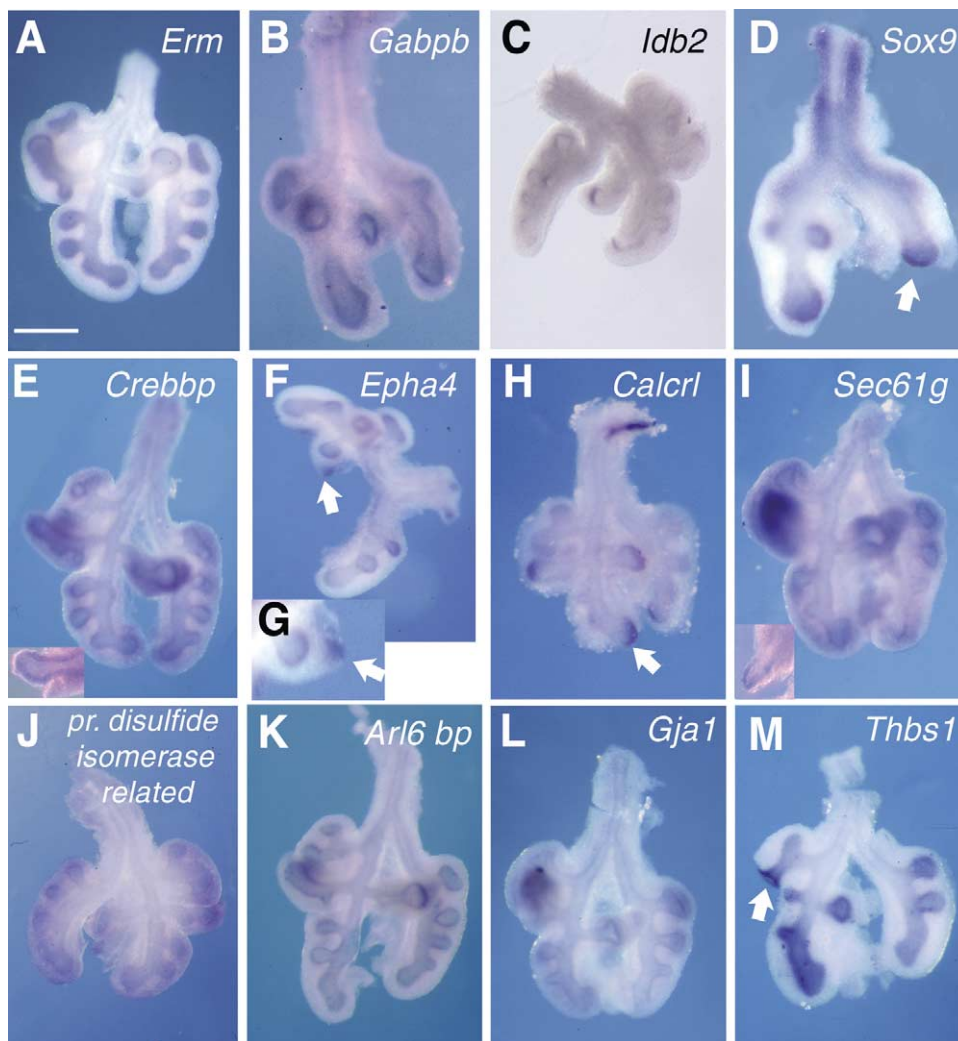


Fig. 2. Examples of whole-mount in situ hybridization of E11.5 lung using the genes identified from the screen as antisense riboprobes. Note that *Id2* and *Calcl* are only expressed at the very tip of the endoderm. *Erm* and *Thbs1* are expressed in distal endoderm but covering a larger domain than *Id2* and *Calcl*, while some others are expressed in distal endoderm as well as some portion of distal mesoderm. (A) *Erm*, (B) *Gabpb*, (C) *Id2*, (D) *Sox9*, arrow shows the distal endoderm expression while mesoderm was removed. (E) *Crebbp*, (F) *Epha4*, (G) the accessory lobe of (F). Arrow shows the *Epha4* expression in accessory lobe mesenchyme. (H) *Calcl*, (I) *sec61g*, (J) gene encoding for a disulfide bond isomerase related protein, (K) gene encoding an Arl6 binding protein, (L) *Gja1*, (M) *Thbs1*. The distal endoderm expression was particularly clear when mesoderm was removed, either accidentally during dissection (arrows in D, H and M) or after whole-mount in situ (insets in E and I). Scale bar: 250  $\mu$ m for A–M.

## 2. Experimental procedures

### 2.1. Isolation and amplification of total RNA

Lungs from one litter of E11.5 ICR embryos were dissected into proximal and distal endoderm fragments as described (Weaver et al., 1999) (Fig. 1A). Total RNA was amplified using the SMART kit designed to selectively amplify full-length cDNA (Clontech). The optimal number of polymerase chain reaction (PCR) cycles to ensure linear amplification was determined following manufacturer's instructions.

### 2.2. Subtractive hybridization and differential screen

Single round subtractions were carried out between the two cDNA populations using the PCR-Select cDNA subtraction kit (Clontech). A differential screen was carried out using the PCR-Select differential screen kit (Clontech). Four sets of <sup>32</sup>P-labeled probes were made from equal amounts of distal subtracted and un-subtracted, and proximal subtracted and un-subtracted, cDNAs. Eighty positive clones were sequenced. The number of times a specific gene was isolated within this 80 is shown in Table 1. Protein categorization was guided by the Bioknowledge database at <http://www.incyte.com/proteome>.

### 2.3. In situ hybridization

Whole-mount in situ hybridization was performed as described (Bellusci et al., 1997). *Epha4*, *Gabpb* and *Sox9* probes were generously provided by Drs. David Wilkinson, Mark Martin and Blanche Capel, respectively. For each probe, at least two lungs were examined.

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