

# Timing and expression of the angiopoietin-1–Tie-2 pathway in murine lung development and congenital diaphragmatic hernia

Adrienne Grzenda<sup>1</sup>, John Shannon<sup>2</sup>, Jason Fisher<sup>1</sup> and Marc S. Arkovitz<sup>1,3,\*</sup>

## SUMMARY

Congenital diaphragmatic hernia (CDH) is one of the most common congenital abnormalities. Children born with CDH suffer a number of comorbidities, the most serious of which is respiratory insufficiency from a combination of alveolar hypoplasia and pulmonary vascular hypertension. All children born with CDH display some degree of pulmonary hypertension, the severity of which has been correlated with mortality. The molecular mechanisms responsible for the development of pulmonary hypertension in CDH remain poorly understood. Angiopoietin-1 (Ang-1), a central mediator in angiogenesis, participates in the vascular development of many tissues, including the lung. Although previous studies have demonstrated that Ang-1 might play an important role in the development of familial pulmonary hypertension, the role of Ang-1 in the development of the pulmonary hypertension associated with CDH is poorly understood. The aim of this study was to examine the role of the Ang-1 pathway in a murine model of CDH. Here, we report that Ang-1 appears important in normal murine lung development, and have established its tissue-level expression and localization patterns at key time-points. Additionally, our data from a nitrofen and bisdiamine-induced murine model of CDH suggests that altered expression patterns of Ang-1, its receptor Tie-2 and one of its transcription factors (epithelium-specific Ets transcription factor 1) might be responsible for development of the pulmonary vasculopathy seen in the setting of CDH.

## INTRODUCTION

Congenital diaphragmatic hernia (CDH) affects approximately 1/4000 live births each year and is characterized by a diaphragmatic defect with resultant herniation of the abdominal viscera into the thoracic cavity (Doyle and Lally, 2004; Pober, 2007). The most serious morbidity in CDH is respiratory insufficiency, which affects both lungs and results from a combination of alveolar hypoplasia and pulmonary vascular hypertension (Stolar, 1996; Dillon et al., 2004). Most children born with CDH exhibit some degree of pulmonary hypertension, the severity of which has been correlated with mortality (Dillon et al., 2004).

Lung development requires the coordination of molecular, morphogenetic and mechanical events within the differentiating respiratory epithelium. A close, reciprocal relationship exists between blood vessels and airways throughout branching morphogenesis (Hislop, 2002). In early development, airways serve as a template for primary blood vessel formation and, later, capillary

beds guide alveolar formation. However, the regulatory mechanisms linking vascular development with alveolarization remain unclear. Vasculogenesis, the formation of new blood vessels from endothelial progenitor cells in previously avascular tissue, and angiogenesis, the remodeling and differentiation of primitive blood vessels, are both crucial to lung development (Parera et al., 2005). Vascular growth factors from the distal lung buds are thought to promote capillary expansion. Three receptor tyrosine kinase (RTK) pathways have been implicated in these activities: vascular endothelial growth factor (VEGF), ephrins and the angiopoietins (Gao and Raj, 2010).

Angiopoietin-1 (Ang-1) is an essential mediator of vascular remodeling and endothelial cell stabilization. Ang-1 is a secreted glycoprotein member of the angiopoietin growth factor family, which has both agonist and antagonistic members. Secreted Ang-1 ligand binds and phosphorylates Tie-2, a receptor tyrosine kinase expressed by the vascular endothelium, promoting endothelial cell migration and survival while inhibiting vascular permeability (Fukuhara et al., 2010). Knockout of Ang-1 in mice is lethal by embryonic day E12.5. *Ang1*<sup>-/-</sup> animals display a marked reduction in the complexity of vessel branching, fewer endothelial cells and reduced endothelium-matrix contacts (Suri et al., 1996). Ang-1 is known to be important in the pathophysiology of pulmonary hypertension (Du et al., 2003). However, the contribution of Ang-1 to the arteriopathy observed in CDH is still largely unknown. Using a teratogen-induced model of CDH, we demonstrate the temporal and spatial regulation of the Ang-1 pathway in normal fetal lung development. These patterns are disrupted in our model of CDH, implicating the pathway in the pathogenesis of pulmonary hypertension and other comorbidities.

<sup>1</sup>Charles Edison Laboratory for Pediatric Surgery Research, Department of Surgery, Division of Pediatric Surgery, Columbia University College of Physicians and Surgeons, New York, NY 10032, USA

<sup>2</sup>Division of Pulmonary Biology, Cincinnati Children's Hospital Medical Center, Cincinnati, OH 45229, USA

<sup>3</sup>Division of Pediatric Surgery, Tel Hashomer Hospital, Tel Aviv 52621, Israel

\*Author for correspondence (drarko@gmail.com)

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## TRANSLATIONAL IMPACT

### Clinical issue

Congenital diaphragmatic hernia (CDH) affects ~1/4000 live births and constitutes ~8% of all birth defects, making it one of the most common congenital abnormalities. CDH is characterized by abnormal diaphragm development that results in herniation of the abdominal contents into the thoracic cavity, compressing the developing lungs. CDH represents a major clinical problem because children affected with CDH have multiple significant morbidities affecting the gastrointestinal, musculoskeletal, cardiac and respiratory systems, as well as developmental delay. Historically, prognosis for newborns with CDH has been poor.

The etiology of CDH remains unknown. All CDH patients develop some degree of alveolar hypoplasia and pulmonary hypertension. Angiopoietin-1 (Ang-1) is an essential mediator of vascular remodeling and endothelial cell stabilization. Other work has demonstrated a significant role for the Ang-1 pathway in the development of non-familial pulmonary hypertension in adults. A role for Ang-1 in the pulmonary hypertension observed in CDH, however, has not been defined.

### Results

This work applies a well-characterized nitrofen-based model of CDH and pulmonary hypertension to examine the role of the Ang-1 pathway in pathology, based on histological and morphologic analyses. The authors demonstrate that Ang-1 levels steadily increase during normal lung development and are restricted to the developing lung bud. By contrast, expression of the Ang-1 receptor, Tie-2, is localized to the vasculature in the surrounding mesenchyme, suggesting epithelial-to-endothelial crosstalk between ligand and receptor. Compared with age-matched controls, nitrofen-treated embryos with CDH and pulmonary hypertension displayed alveolar hypoplasia with associated reductions in Tie-2 and Ang-1 protein levels, as well as an abnormal Ang-1 expression pattern that resembles an earlier stage of development. In summary, this work suggests that alterations in the Ang-1–Tie-2 pathway play an important role in the development of pulmonary hypertension in the setting of CDH.

### Implications and future directions

This work contributes substantially to understanding of the Ang-1–Tie-2 pathway by providing a comprehensive examination of the pathway during normal and pathological development. Importantly, the experiments establish a paradigm for epithelial-to-endothelial crosstalk between ligand and receptor during lung development that appears disturbed in the setting of nitrofen-induced CDH, suggesting that the pathway is involved in the etiology of pulmonary hypertension associated with CDH. These data indicate that future investigation into the role of other components of the Ang-1 pathway under both normal and pathological conditions is warranted.

## RESULTS

### Ang-1 expression increases throughout development and is localized to the distal lung bud

To determine the temporal expression of Ang-1 during normal lung development, whole lung homogenates were prepared from embryos at three distinct stages of lung organogenesis: early canalicular stage (E15.5), saccular (E18.5) and alveolar (post-natal day 1, PN-1). Ang-1 protein levels significantly increased throughout all stages of development (Fig. 1A), whereas levels of Ang-1 transcript showed a plateau during the alveolar stage (Fig. 1B). Gene expression of the Ang-1 transcription factors, epithelium-specific Ets transcription factor 1 (ESE-1), peaked during the saccular stage and was significantly reduced during the alveolar stage (Fig. 1C). Protein levels of Tie-2, the Ang-1 receptor, significantly increased from the canalicular to saccular stages of physiological development but appeared to plateau thereafter (Fig.

1D); Tie-2 transcript significantly increased throughout development (Fig. 1E).

Immunohistochemistry of Ang-1 revealed a distinctive spatial pattern of expression (Fig. 2A). In the early, pseudoglandular and canalicular stages of development, Ang-1 was expressed exclusively in the epithelium of the distal lung bud and was completely absent from smooth muscle-lined airways and vasculature. During the later saccular and alveolar stages of development, Ang-1 expression shifted from the growing lung buds to the primary, central airways. Fluorescent co-labeling for Ang-1 and  $\alpha$ -smooth muscle actin ( $\alpha$ SMA; Fig. 2B,C) confirmed that the region of Ang-1 expression is separate from the central, smooth muscle-lined airways where  $\alpha$ SMA expression is highest in early stages of development. The complete shift of Ang-1 to the central airways by the alveolar (PN-1) stage of development was also evident. To determine which cell type might be responsible for expression of Ang-1, fluorescent co-labeling of Ang-1 and pro-surfactant C (Pro-C) was performed across all stages. Pro-C is a marker of distal respiratory epithelial cells, most probably early type II alveolar cells (Samadikuchaksaraei et al., 2006; Mondrinos et al., 2008). Ang-1 and Pro-C appeared to be colocalized throughout development, suggesting that these distal respiratory epithelial cells are responsible for a portion of Ang-1 production (Fig. 3A).

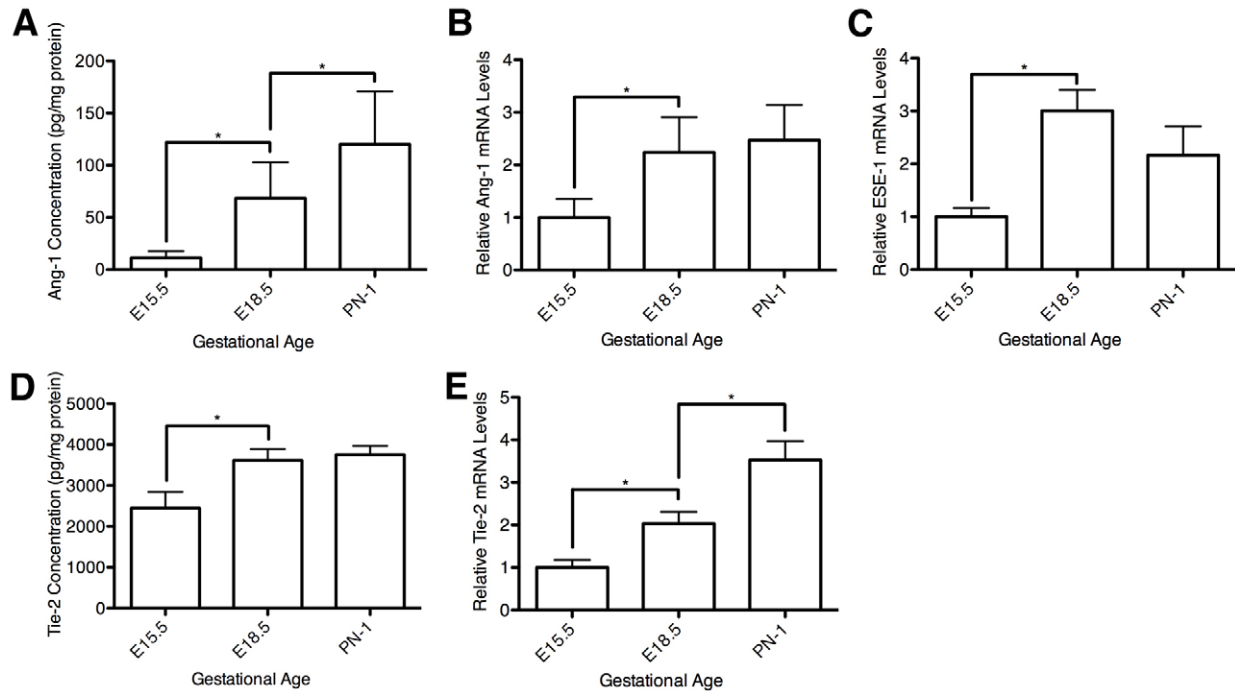
### Tie-2 expression increases throughout development and is localized to the developing vasculature

As observed, protein levels of Tie-2, the Ang-1 receptor, significantly increased from the canalicular to saccular stages of physiological development but appeared to plateau thereafter. CD34 is a known marker of early vascular development and is expressed by vascular progenitor cells (Asahara et al., 1997). Fluorescent co-labeling of CD34 with Ang-1 demonstrated that Ang-1 expression is confined to the developing respiratory epithelium and that CD34 is expressed by the developing vasculature in the interstitial mesenchyme (Fig. 3B). Finally, co-labeling of Tie-2 with CD34 demonstrated that expression of the Tie-2 receptor is colocalized to the endothelium of the developing vasculature during early development but shifts to the large vessels associated with central airways in late development, although some expression remains in the mesenchyme (Fig. 4A–C).

### Teratogen induction of CDH with associated pulmonary hypertension results in Ang-1 pathway disruption

Teratogen induction of CDH (Fig. 5A) in our study resulted in embryos with diaphragmatic defects consistent with previous characterizations of the model (Fig. 5B) and that mimic human CDH. The abnormalities observed included significantly reduced late-stage fetal weight (Fig. 5C), pulmonary hypoplasia and pulmonary hypertension, as evidenced by thickened arterioles (Fig. 5D), cleft of the skull and palate, a smaller number of embryos per pregnancy and dorsal body wall edema (Clugston et al., 2010; Clugston et al., 2006). Only embryos with diaphragmatic defect and associated pulmonary deficiencies were included for analysis. Compared with age-matched, untreated controls, Ang-1 and Tie-2 receptor protein levels were significantly decreased in CDH lungs throughout development (Fig. 6A,B).

Tie-2 transcript levels were also significantly reduced in CDH lungs compared with controls (Fig. 6D). However, Ang-1 transcripts



**Fig. 1. Ang-1, ESE-1 and Tie-2 expression levels during normal lung development.** (A-E) Whole lung homogenates were prepared from untreated embryos from the E15.5, E18.5 and PN-1 stages of lung development ( $n=8$  per time-point). Protein concentrations of Ang-1 and Tie-2 were determined by ELISA and expressed as pg of marker/mg of protein (A,D). RNA was extracted from the lungs of untreated embryos from the E15.5, E18.5 and PN-1 stages of development ( $n=8$  per time-point). qRT-PCR was used to assay the transcript levels of Ang-1 (B), ESE-1 (C) and Tie-2 (E). Expression is represented as fold change over the E15.5 baseline (1.0). Significance is assumed at  $*P<0.05$  for both assays.

were only significantly reduced in early development and remained relatively unchanged compared with controls in the saccular and alveolar stages (Fig. 6C). Surprisingly, levels of the Ang-1 transcription factor, ESE-1, were significantly increased in CDH lungs in late development compared with controls (Fig. 6E).

Although the hypoplastic nature of the CDH lung makes assessing the reduction of Ang-1 protein difficult to visualize, immunohistochemistry demonstrated that Ang-1 expression in CDH lungs remains significantly localized to the distal airways in the periphery, reminiscent of the canalicular stage and indicative of a possible developmental delay (Fig. 7A). Alterations in Ang-1 and Tie-2 localization and expression in CDH lungs appeared to be affected to an equal degree both ipsilateral and contralateral to the diaphragmatic defect (data not shown). The significant decrease in Tie-2 present at both the transcript and protein level in CDH lungs was evident in fluorescent co-labeling of CD34 and Tie-2 (Fig. 7B).

## DISCUSSION

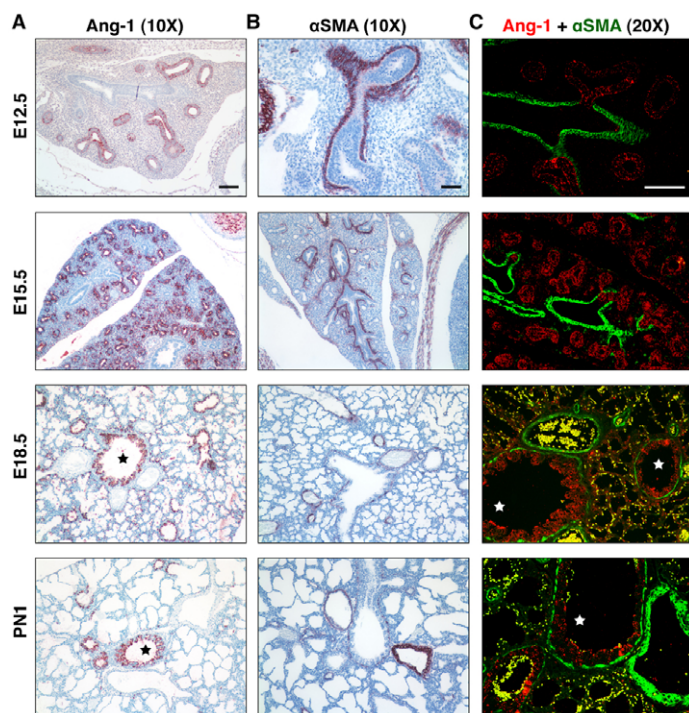
Ang-1 is a key mediator of angiogenesis, with demonstrated gene expression in a number of developing embryonic tissues, including the lung, pancreas and heart (Suri et al., 1996; Colen et al., 1999). Our data indicates that Ang-1 serves as a mediator of communication between the growing lung bud and the developing vasculature in the mesenchyme throughout normal lung development and that alterations in Ang-1 might be responsible for the vascular abnormalities seen in CDH. Fluorescent co-labeling of Ang-1 and  $\alpha$ SMA demonstrated that Ang-1 is localized

exclusively to the epithelium of the growing lung bud, possibly early type II pneumocytes, and is absent from the smooth muscle-lined airways and endothelium during the early stages of development. The receptor for Ang-1, Tie-2, is observed in the surrounding mesenchyme, an area that also expresses CD34, a marker of progenitor vascular endothelial cells. Therefore, we speculate that Ang-1 expressed by the developing lung bud is trophic for the development of the peri-alveolar vasculature in the surrounding embryonic mesenchyme through Tie-2-mediated signaling. Ang-1 appears to contribute first to the expansion and stabilization of the capillary network during early development and to the stabilization of primary blood vessels during late development (Fig. 8).

Our results corroborate those of Colen and co-workers, who demonstrated that Ang-1 is expressed in the developing lung from E9.5 through PN-1 (Colen et al., 1999). However, the investigators neither quantified nor localized Ang-1 expression. To better define the spatial (qualitative) and temporal (quantitative) expression of the crucial participants in the Ang-1 pathway during embryonic lung development, we collected lung tissue from mice at four representative stages: pseudoglandular stage (E12.5), early canalicular stage (E15.5), saccular stage (E18.5) and alveolar stage (PN-1). Additionally, we examined Ang-1 and Tie-2 expression at both the transcriptional and proteomic level to better assess pathway regulation.

Moreover, in our model of CDH we observed significant downregulation of Ang-1 and Tie-2, with associated defects in Ang-1 localization and lung morphology. The pattern of Ang-1



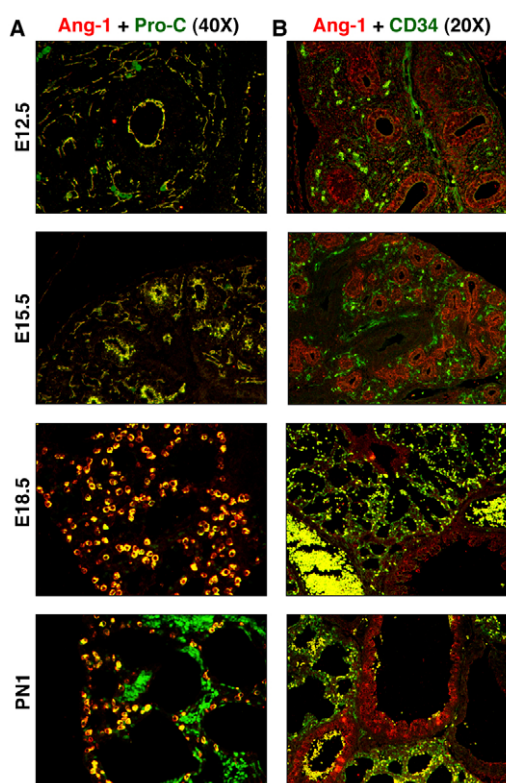


**Fig. 2. Ang-1 localization in normal lung development.** (A-C) Sections of 5  $\mu\text{m}$  were prepared from untreated paraffin-embedded embryos from the E12.5, E15.5, E18.5 and PN-1 stages of lung development. Immunohistochemistry was performed to assess expression and localization of Ang-1 (A) and  $\alpha\text{SMA}$  (B). Images are presented at 10 $\times$  magnification. (C) Fluorescent co-labeling of Ang-1 (green) and  $\alpha\text{SMA}$  (red) was used to determine colocalization of each antigen across development. Fluorescent images are presented at 20 $\times$  magnification. White and black stars denote large central airways. Scale bars: 100  $\mu\text{m}$ .

localization within the lung is reminiscent of an earlier stage, suggestive of developmental delay. From this, we speculate that disruption of the Ang-1 pathway contributes to development of the persistent pulmonary hypertension seen in CDH.

Only one study has examined Ang-1 in the setting of teratogen-induced CDH in mice. The investigators observed mildly increased levels of Ang-1 protein in CDH lungs compared with controls during late development, using a nitrofen-based mouse model (Chinoy et al., 2002). The differences between our results and those described by Chinoy and co-workers are interesting. In their study, protein levels of Ang-1 were measured using western blot. Levels of Ang-1 protein were mildly elevated early in gestation compared with controls. The authors speculated that increased Ang-1 contributed to the vascular pathology seen. Immunohistochemistry demonstrated very minor increases in Ang-1 expression at these time-points. No mRNA was analyzed and none of the transcription factors were studied. Perhaps more importantly, Tie-2 was not analyzed at all. Moreover, the study used nitrofen exclusively, but this model is difficult to reproduce successfully in mice. It is therefore difficult to draw any specific conclusions about the role of Ang-1 from their work.

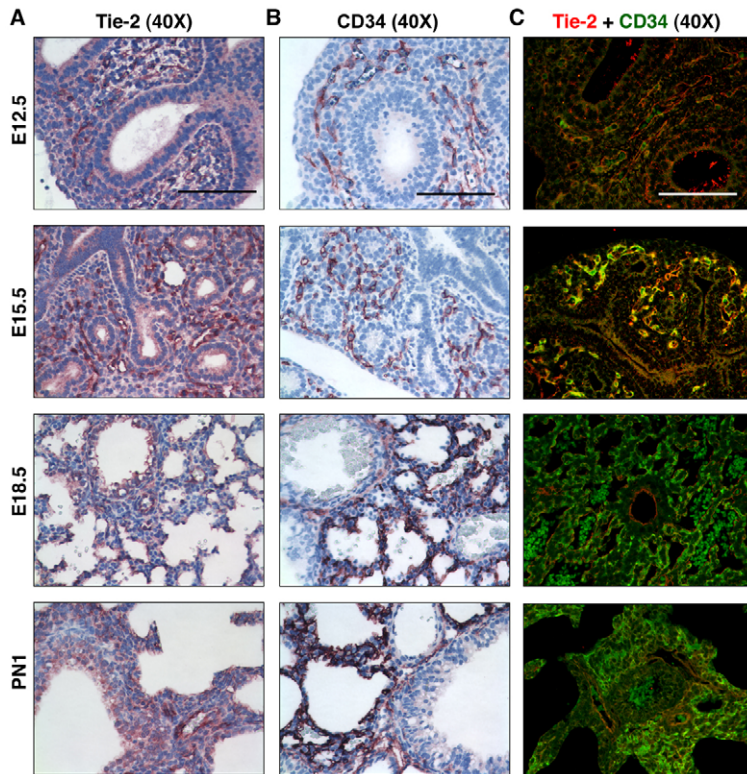
Our study utilized a multiple-teratogen model of CDH, which was first described, and published many times, by Greer (Allan and



**Fig. 3. Localization of early type II alveolar cells and early vascular progenitor cells during normal lung development.** (A) Pro-C is a useful marker of distal respiratory epithelial cells, primarily early type II alveolar cells. Fluorescent co-labeling of Ang-1 (red) and Pro-C (green) in sections prepared from untreated embryos was used to determine colocalization. Images are displayed at 40 $\times$  magnification. Areas of colocalization generate a yellow signal. (B) CD34 is a marker of vascular progenitor endothelial cells. Fluorescent co-labeling for Ang-1 (red) and CD34 (green) was used to determine localization of cells expressing each antigen. Images are displayed at 20 $\times$  magnification.

Greer, 1997). This difference in technique could account for the differences seen in Ang-1 expression. This is an interesting point that needs to be emphasized. Though nitrofen provides a well-reproduced, well-described model of CDH in rats, it is not well accepted in mice. Our difficulty in getting this model to work in mice led us to discuss this issue with several other investigators, who reported similar issues. The Greer model is a very noxious and potent model that has overall global teratogenic effects and this must be taken into consideration when analyzing any data published using this model. In addition, our study was not a physiology study and although the pulmonary blood vessels did show evidence of medial hypertrophy and hyperplasia (data not shown) in our mice, we did not directly measure pulmonary hypertension. This is a shortcoming of the teratogen (nitrofen)-induced model of CDH: it is not a survival study and the embryos were all sacrificed during, or shortly after, the embryonic period.

The association between Ang-1 and the development of pulmonary hypertension has been described in both humans and rodents, although the data is often contradictory regarding the precise relationship between pathway dysfunction and disease.



**Fig. 4. Tie-2 localization in normal lung development.** (A-C) Immunohistochemistry for Tie-2 (A) and CD34 (B) combined with fluorescent co-labeling of both Tie-2 (red) and CD34 (green) (C) was used to determine the localization of Tie-2 during normal lung development. Images are displayed at 40 $\times$  magnification. Scale bars: 100  $\mu$ m.

Similar to our results, Chu and co-workers demonstrated that constitutive overexpression of Ang-1 in the lungs of rats resulted in hyperplasia of the vascular media and resultant pulmonary hypertension (Chu et al., 2004). These findings are supported by a study in human non-familial pulmonary hypertension (Du et al., 2003). The authors determined that expression of Ang-1 and Tie-2 increased in surgical lung samples from patients with pulmonary hypertension compared with normal controls. Taken together, these findings are suggestive of a causative role for Ang-1 in the development of non-familial pulmonary hypertension by promoting smooth muscle cell recruitment and proliferation leading to arteriolar constriction (Du et al., 2003).

By contrast, Zhao and co-workers have shown that constitutive overexpression of Ang-1 in the lungs has no effect on normal pulmonary vasculature (Zhao et al., 2003). Moreover, the overexpression of Ang-1 appears to be protective against the development of pulmonary hypertension in a monocrotaline-based model by inhibiting endothelial cell apoptosis and preventing arteriolar dropout, which also promotes pulmonary hypertension (Rudge et al., 2003). Similarly, Boucherat and co-workers attempted to look at the role of Ang-1 and the angiotensin pathway in human fetal pulmonary hypertension (Boucherat et al., 2010). In that study, there was no significant difference in Ang-1 activity in fetuses with pulmonary hypertension, whether from CDH or not, compared with controls. Interestingly, Ang-2 did appear to increase significantly with gestational age in fetuses with pulmonary hypertension compared with controls. Tie-2 activity did not appear to change. Taken together, these data highlight the importance of the angiotensin pathway in the development of fetal and newborn pulmonary hypertension, but do not definitively describe the relationship.

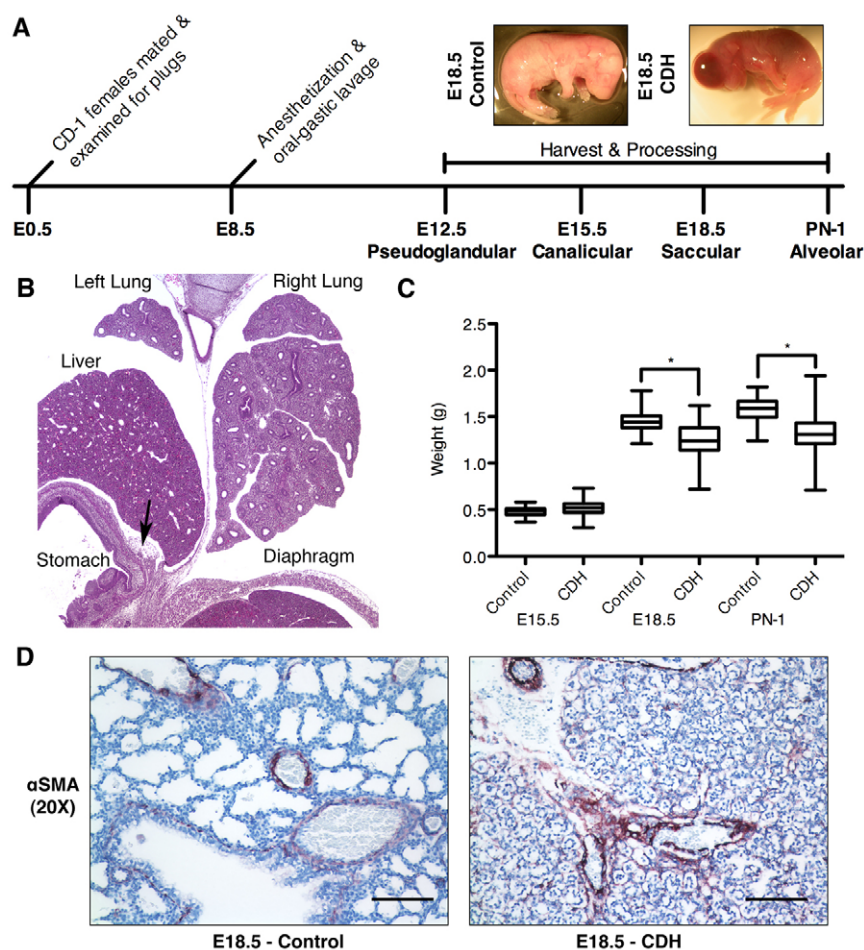
Our data suggest that the Ang-1–Tie2 pathway is important in the development of normal pulmonary vasculature. Although we show an association between Ang-1 expression from the developing lung buds and Tie-2 expression from the vascular mesenchyme, we could not demonstrate a direct causative relationship or mechanism. Future studies are needed to assess the direct relationship between Ang-1 and vascular development at the cellular level as well as the role of the three transcription factors, ESE-1, acute myeloid leukemia 1 (AML-1) and core-binding factor  $\beta$  (CBF- $\beta$ ). Further studies into the role of the Ang-1 pathway in the development of the vascular abnormalities seen in CDH could help provide direction for future treatment.

## METHODS

### CDH model and specimen harvest

All experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Columbia University College of Physicians and Surgeons under protocol #AC-AAAA571. Female CD-1 mice (Charles River Laboratories, Wilmington, MA) were mated overnight and examined for the presence of a vaginal plug the following morning; the presence of the plug indicated embryonic day 0.5 (E0.5) of gestation. On day E8.5 of gestation, pregnant dams were briefly anesthetized with 2-4% isoflurane. Adapting the protocol from Greer (Allan and Greer, 1997), 15 mg of nitrofen (Wako Chemicals, Richard, VA) and 10 mg of bisdiamine (Acros Organics, Morris Plains, NJ) were administered in 400  $\mu$ l of olive oil via oral-gastric lavage to induce hernia. Control animals were gavaged with olive oil alone. Tissues were harvested on days E12.5 (pseudoglandular stage), E15.5 (early canalicular stage), E18.5 (saccular stage) and PN-1 (alveolar stage). Pregnant dams and neonates were euthanized with carbon dioxide. Embryos were





**Fig. 5. Model of teratogen-induced CDH.**

(A) Administration of nitrofen and bisdiazine yields a high rate of diaphragmatic defects in embryonic mice. Pregnant dams were anesthetized on day E8.5 of gestation and delivered a solution of 15 mg of nitrofen and 10 mg of bisdiazine in 400  $\mu$ l of olive oil. Controls were administered olive oil alone. Administration of this solution resulted in the induction of hernia in 73% of embryos (data not shown). Representative images of E18.5 control and CDH embryos are shown. (B) Posterior view of a left-sided fetal CDH at gestational day E15.5 (H&E staining, 4 $\times$  magnification). The diaphragmatic defect is highlighted with a black arrow. (C) The weight of embryos exposed to the nitrofen-bisdiazine solution. Significance is assumed at  $*P < 0.05$ . (D) Immunohistochemistry of control and CDH (E18.5) sections with anti- $\alpha$ SMA confirm thickening of the pulmonary arteries characteristic of pulmonary hypertension. Images are displayed at 20 $\times$  magnification. Scale bars: 100  $\mu$ m.

rapidly harvested via caesarean section and placed in ice-cold Hanks' balanced salt solution. Sternotomy was performed to check for the presence of a diaphragmatic defect. The defect was detected in approximately 73% of teratogen-treated embryos. Embryos without diaphragmatic defect were discarded.

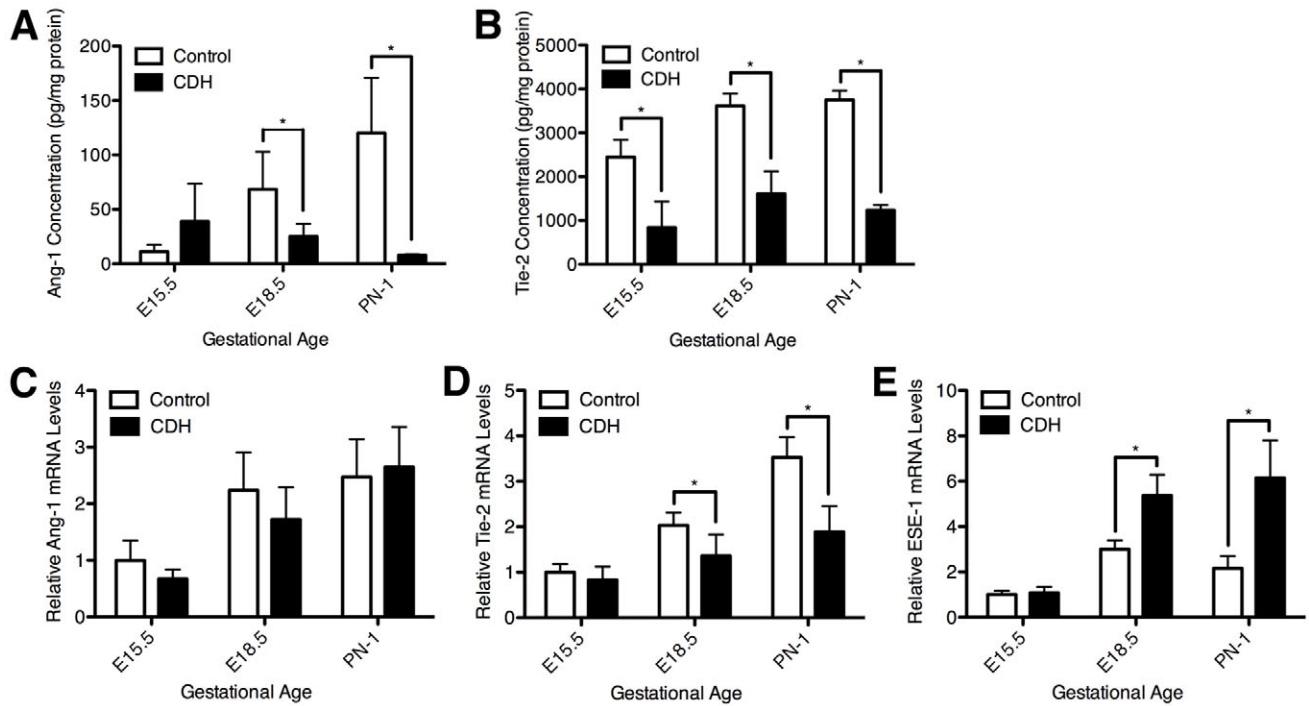
#### Immunohistochemistry

Paraformaldehyde-fixed sections (5  $\mu$ m) were deparaffinized with xylenes and rehydrated through a graded series of ethanols. Where necessary, antigen retrieval was performed. Endogenous peroxidase activity was quenched in 0.3% hydrogen peroxide in methanol for 20 minutes. Endogenous biotin was reduced via an avidin-biotin blocking kit (Vector, Burlingame, CA). Sections were incubated in universal CAS Block (Zymed, Carlsbad, CA) for 1 hour at room temperature prior to the application of primary antibodies. The primary antibodies used were Ang-1 (1:50; Santa Cruz Biotechnology, Santa Cruz, CA),  $\alpha$ SMA (1:10,000; Sigma-Aldrich, St Louis, MO), CD34 (1:100; Abcam, Cambridge, MA), Tie-2 (1:75; Santa Cruz Biotechnology) and pro-surfactant Protein C (1:500; Chemicon, Temecula, CA). Following overnight incubation (4 $^{\circ}$ C) with primary antibody, appropriate biotinylated secondary antibodies were applied for 30 minutes at room temperature. For chromogenic development, sections were then incubated in horseradish peroxidase (HRP)-streptavidin (Zymed) for 30 minutes, developed with either Nova Red (Vector) or AEC solution

(Invitrogen), and counterstained in hematoxylin. For fluorescent multilabeling, conjugates of streptavidin with Alexa Fluor 555 and Alexa Fluor 488 were used (1:200; Invitrogen) along with a Hoechst 33342 nuclear counterstain. All microscopy imaging was performed using a Nikon Eclipse E600 apparatus.

#### Enzyme-linked immunosorbent assay

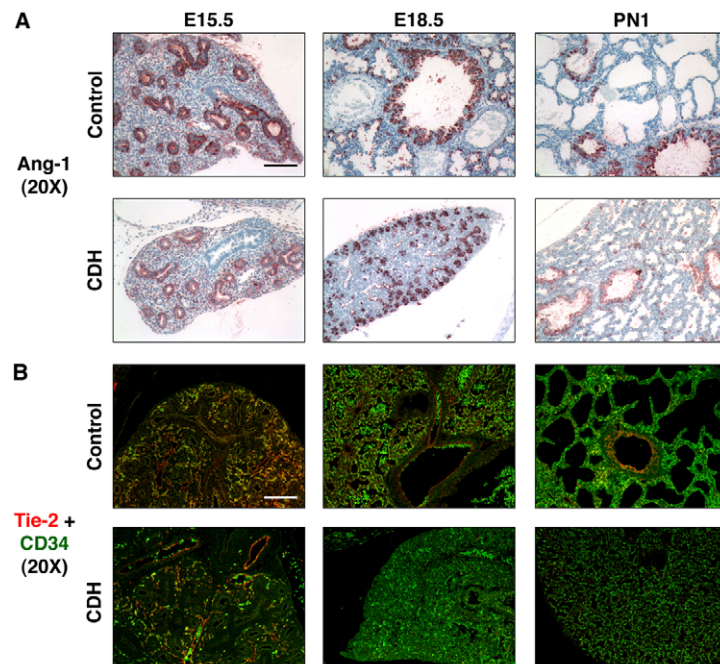
Tissue protein extracts were obtained from fresh homogenized fetal lung tissue at gestational days E15.5, E18.5 and PN-1. Lungs from littermates were pooled to form one sample; a total of eight litters were present in each control and CDH group per time-point examined. Protein was extracted using a Tris-based lysis buffer supplemented with the Complete Mini<sup>TM</sup> EDTA-free protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) and 10  $\mu$ l/ml of phenylmethylsulphonyl fluoride. Total protein concentrations were determined using the Bradford protein assay (Bio-Rad, Hercules, CA). Sandwich enzyme-linked immunosorbent assays were performed using Quantikine<sup>R</sup> ELISA systems (R&D Systems, Minneapolis, MN) specific for Ang-1 and Tie-2, according to the manufacturer's instructions. Briefly, standardized concentrations of mouse Ang-1 or mouse Tie-2, along with tissue protein extracts from all experimental groups, were added onto a 96-well microplate precoated with monoclonal antibodies raised against recombinant mouse Ang-1 or mouse Tie-2. A secondary mouse Ang-1 or mouse Tie-2 monoclonal



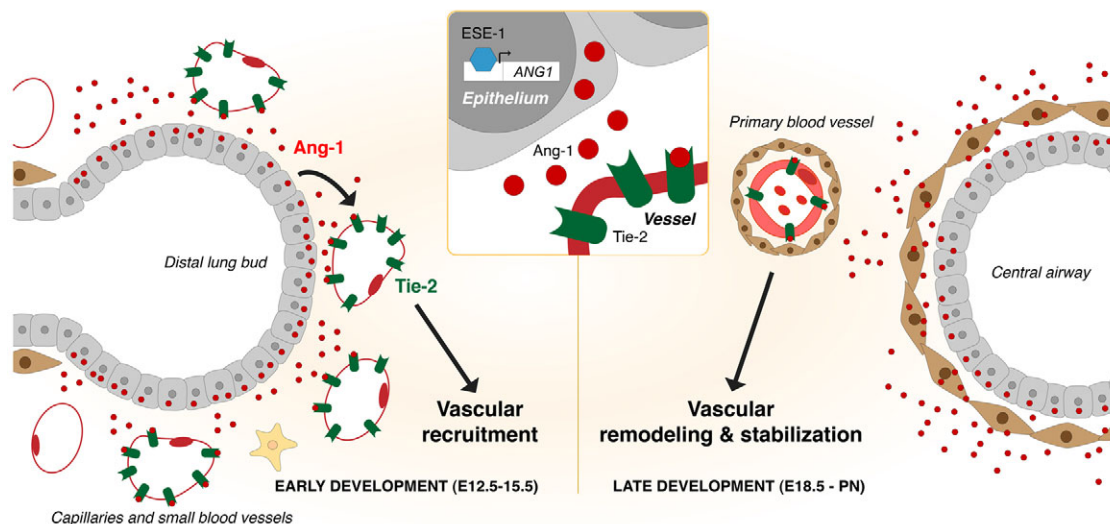
**Fig. 6. Ang-1, ESE-1 and Tie-2 expression levels during teratogen-induced CDH.** (A,B) Levels of Ang-1 and Tie-2 protein in teratogen-exposed embryos compared with untreated controls were determined by ELISA. (C-E) qRT-PCR was used to assess the transcript levels of Ang-1 (C), Tie-2 (D) and ESE-1 (E). Expression is represented as fold change over the E15.5 baseline (1.0). Significance is assumed at  $*P < 0.05$  for both assays.

antibody conjugated with HRP was subsequently added to each well, and developed with 1:1 mixture of hydrogen peroxide and tetramethylbenzidine. Colorimetric optical densities proportional to the concentration of Ang-1 or Tie-2 present in each sample were measured using a microplate reader set to 450 nm, with

wavelength correction at 570 nm. Final Ang-1 and Tie-2 concentrations were extrapolated from standards curves and normalized to total protein concentration. Normalized values for each experimental group are expressed as means  $\pm$  s.d. Significant differences within this non-normally distributed data set were



**Fig. 7. Localization of Ang-1 and Tie-2 during teratogen-induced CDH.** (A) Immunohistochemistry of control and CDH embryos was used to determine expression and localization of Ang-1 during teratogen-induced CDH (bottom) compared with untreated controls (top). (B) Fluorescent co-labeling of Tie-2 (green) and CD34 (red) was used to assess expression and localization of Tie-2 during teratogen-induced CDH (bottom) compared with untreated controls (top). All images are displayed at 20X magnification. Scale bars: 100  $\mu$ m.



**Fig. 8. Model of the Ang-1 pathway in early lung development.** Proposed model of the Ang-1 pathway in early lung development (E12.5 to E15.5) hypothesizes that Ang-1 (red) secreted by the distal lung bud acts in a trophic fashion on progenitor vascular endothelial cells expressing receptor Tie-2 (green) in the mesenchyme to induce downstream signaling and stabilization of the nascent vasculature. In later development (E18.5 to postnatal), the relationship between Ang-1 and Tie-2 contributes to the stabilization of the primary blood vessels associated with the central airways in the maturing lungs. PN, post-natal.

determined using Mann-Whitney *U* testing, with significance assumed at  $P < 0.05$ .

#### Quantitative real-time PCR

For quantitative real-time PCR (qRT-PCR), tissue RNA was obtained from fresh homogenized fetal lung tissue at gestational days E15.5, E18.5 and PN-1 using the ToTALLY RNA kit (Ambion, Austin, TX) followed by RNeasy (Qiagen, Valencia, CA) purification. Lungs from littermates were pooled to form one sample; a total of eight litters were present in each control and CDH group per time-point examined. cDNA was synthesized from 4  $\mu$ g total RNA using SuperScript II reverse transcriptase (Invitrogen). Gene expression was analyzed using mouse probe-primer sets for Ang-1 (mm00456503\_m1), Tie-2 (mm00443242\_m1) and ESE-1 (mm00468224\_m1) on an Applied Biosystems 7300 Real-time PCR System (Applied Biosystems, Foster City, CA). Two housekeeping genes, encoding mouse  $\beta$ -actin (4352341E) and GAPD (4352339E), were used to normalize the target gene data. Data were calculated using the  $2^{-\Delta\Delta Ct}$  method as described by the manufacturer and normalized to controls. Expression levels are expressed as the fold increase or decrease over E15.5 control expression level (set at 1.0). Significant differences were determined using ANOVA or Tukey, with significance assumed at  $P < 0.05$ .

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#### COMPETING INTERESTS

The authors declare that they do not have any competing or financial interests.

#### AUTHOR CONTRIBUTIONS

A.G. assisted in the study design, carried out all experimental procedures (tissue acquisition, immunohistochemistry and RT-PCR experiments) as well as drafted and edited the manuscript. J.F. carried out the ELISA assays. J.S. consulted on the design of the study. M.S.A. conceived of the study, assisted in its design and edited the manuscript. All authors read and approved the final manuscript.

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