Experimental Lung Research

ISSN: 0190-2148 (Print) 1521-0499 (Online) Journal homepage: www.tandfonline.com/journals/ielu20

Angiogenesis-related gene expression profiling in ventilated preterm human lungs

Monique E. De Paepe, David Greco & Quanfu Mao

To cite this article: Monique E. De Paepe, David Greco & Quanfu Mao (2010) Angiogenesis-related gene expression profiling in ventilated preterm human lungs, Experimental Lung Research, 36:7, 399-410, DOI: 10.3109/01902141003714031

To link to this article: https://doi.org/10.3109/01902141003714031

Experimental Lung Research, 36, 399–410, 2010

ISSN: 0190-2148 print / 1521-0499 online
DOI: 10.3109/01902141003714031

informa
healthcare

Angiogenesis-related gene expression profiling in ventilated preterm human lungs

Monique E. De Paepe,[1] David Greco,[2] and Quanfu Mao[1]

[1]*Department of Pathology, Women and Infants Hospital, Providence, Rhode Island, USA; and the Department of Pathology and Laboratory Medicine, Warren Alpert Medical School of Brown University, Providence, Rhode Island, USA*

[2]*Department of Pathology, Women and Infants Hospital, Providence, Rhode Island, USA*

ABSTRACT

Preterm infants exposed to oxygen and mechanical ventilation are at risk for bronchopulmonary dysplasia (BPD), a multifactorial chronic lung disorder characterized by arrested alveolar development and nonsprouting, dysmorphic microvascular angiogenesis. The molecular regulation of this BPD-associated pathological angiogenesis remains incompletely understood. In this study, the authors used focused microarray technology to characterize the angiogenic gene expression profile in postmortem lung samples from short-term ventilated preterm infants (born at 24 to 27 weeks' gestation) and age-matched control infants. Microarray analysis identified differential expression of 13 of 112 angiogenesis-related genes. Genes significantly up-regulated in ventilated lungs included the antiangiogenic genes thrombospondin-1, collagen XVIII alpha-1, and tissue inhibitor of metalloproteinase-1 (TIMP1), as well as endoglin, transforming growth factor-α, and monocyte chemoattractant protein-1 (CCL2). Increased expression of thrombospondin-1 in ventilated lungs was verified by real-time polymerase chain reaction (PCR) and immunolocalized primarily to intravascular platelets and fibrin aggregates. Down-regulated genes included proangiogenic angiogenin and midkine, as well as vascular endothelial growth factor (VEGF)-B, VEGF receptor-2, and the angiopoietin receptor TEK/Tie-2. In conclusion, short-term ventilated lungs show a shift from traditional angiogenic growth factors to alternative, often antisprouting regulators. This angiogenic shift may be implicated in the regulation of dysmorphic angiogenesis and, consequently, deficient alveolarization characteristic of infants with BPD.

KEYWORDS bronchopulmonary dysplasia, chronic lung disease, endoglin, newborn infants, thrombospondin

Preterm newborns who require mechanical ventilation and supplemental oxygen are at risk for bronchopulmonary dysplasia (BPD), a chronic lung disease (CLD) of newborn infants associated with significant mortality and morbidity [1]. The dominant pathological finding in postsurfactant BPD is a disruption of alveolar development, resulting in large and simplified airspaces with varying degrees of interstitial fibrosis and inflammation [1–3]. Recent studies have shown that, in addition to arrested alveolar development, there is also impaired pulmonary microvascular development in infants with BPD [3–5] or in BPD-like animal models such as chronically ventilated premature baboons [6, 7]. In view of the intimate relationship between alveolar and microvascular development during pulmonary morphogenesis [8, 9], disruption of microvascular development in premature lungs has been implicated as a critical factor in the arrest of alveolar development that is characteristic of BPD [10, 11].

We have recently studied the growth kinetics of the pulmonary microvasculature in ventilated preterm infants [5] and determined that the first postnatal weeks of preterm infants are characterized by brisk endothelial cell proliferation, resulting in significant expansion of the pulmonary microvasculature [5]. In concordance with other studies [3, 4], we determined that the microvasculature in long-term ventilated lungs shows striking architectural abnormalities compared with age-matched nonventilated control lungs [5]. At term (36 to 40 weeks' gestation), normal human lungs are in the early alveolar stage of lung development and display thin alveolar septa with abundant secondary crest formation. Within the complex alveolar septa of normal-term lungs,

Received 13 December 2009; accepted 15 February 2010

Address correspondence to Monique E. De Paepe, MD, Women and Infants Hospital, Department of Pathology, 101 Dudley Street, Providence, RI 02905, USA. E-mail: mdepaepe@wihri.org

the microvasculature forms a delicate network, characterized by extensive capillary sprouting into secondary crests and alveolar septa. In contrast, the pulmonary microvasculature of long-term ventilated preterm infants at the same corrected postmenstrual age (36 to 40 weeks) appears to retain the primitive vascular pattern of canalicular/saccular lungs, characterized by a persistent dual capillary pattern and tortuous, nonbranching vessels [5].

The molecular regulation of this BPD-associated dysangiogenesis remains largely undetermined. Angiogenesis, here defined as the abluminal sprouting of new vessels from preexisting ones, is a complex and highly coordinated process. Successful angiogenesis requires precise temporal and spatial orchestration of endothelial cell activation, proliferation and directional migration, extracellular matrix generation and remodeling, recruitment of mural cells (pericytes and smooth muscle cells), and specialization of the vessel wall for regulation of vessel function [12, 13]. Coordination of these complex functions is regulated by various pro- and antiangiogenic growth factors, angiogenic chemokines/cytokines, matrix-degrading proteases, and cell-extracellular matrix interactions [12, 13].

We speculate that the framework for dysmorphic pulmonary microvascular development in infants with BPD is established during the early postnatal weeks. Consequently, elucidation of the molecular regulation of angiogenesis in short-term ventilated lungs may provide insight into the mechanisms underlying the microvascular, and ultimately alveolar, abnormalities in BPD. We recently used ribonuclease protection (RPA) and Western blot assays to study angiogenic gene expression in postmortem lung samples from short-term ventilated infants [14]. The aim of the present study was to investigate the pulmonary expression of a wider range of angiogenesis-related genes using a focused microarray approach, which allows more global interrogation of over 100 angiogenic genes. Our results suggest that short-term ventilated preterm infants display an 'angiogenic shift' from traditional soluble angiogenic regulators to alternative, often antiangiogenic or antisprouting, regulators. Altered angiogenic gene expression may contribute to the dysregulated angiogenesis and, consequently, to the disrupted alveolarization that is characteristic of infants with BPD.

METHODS

Patients

Lung samples from ventilated and control infants were obtained from the Women and Infants Hospital (Providence, RI) perinatal autopsy files. The protocols were approved by the institutional review board and informed consent was obtained in compliance with institutional guidelines. Medical and autopsy records of patients were reviewed. Infants with congenital, chromosomal, or cardiac anomalies or with other conditions potentially predisposing to pulmonary anomalies [15] were excluded. In addition, cases with documented lung hypoplasia, defined as a lung/body weight ratio below the 10th percentile for age [16], were excluded. Records were reviewed for postmenstrual age (PMA) at birth, postnatal age, and corrected postmenstrual age at death (gestational age at birth plus postnatal age). In all patients, the postmortem interval (i.e., time between death and autopsy) was recorded.

Angiogenic gene expression was studied in postmortem lung samples from short-term ventilated preterm infants (between 23 and 29 weeks' gestation corrected postmenstrual age at the time of death) who had lived for at least 3 days and had been ventilator-dependent throughout life. Control patients consisted of age-matched nonmacerated stillborns whose intrauterine demise immediately preceded delivery (intrapartum death or documented death less than 1 hour prior to delivery) or live born infants who had lived for less than 1 hour.

Lung processing

Autopsies were performed at Women and Infants Hospital according to standard methods. After thorough in situ examination, the lungs were dissected and weighed. Biopsies taken from the right upper lobe were treated with RNA*later* (Ambion, Austin, TX) for molecular analyses, as described previously [17]. The remainder of the right lung was immersed in formalin. The left lung was inflation-fixed with formalin at a standardized pressure of 20 cm H_2O. After overnight fixation, the tissues were embedded in paraffin, sectioned at a thickness of 4 μm, and stained with hematoxylin and eosin.

Analysis of angiogenesis-related gene expression by cDNA microarray

Angiogenic gene expression was studied by pathway-focused microarray, allowing simultaneous interrogation of >110 angiogenesis-related genes (OligoGEArray Human Angiogenesis Microarray; SuperArray Bioscience, Frederick, MD) (Table S1, Supplementary Data). Total RNA was isolated from lung homogenates using TRIzol reagent (Invitrogen, Carlsbad, CA). Reverse transcription, polymerase chain reaction (PCR) array analysis, and data analysis

were performed by the research staff at SuperArray Bioscience Corporation. For data analysis the $\Delta\Delta C_t$ method was used [18]. The analyzed annotated data after background subtraction and interquartile normalization of ventilated and control samples, provided by SuperArray, were compared by unpaired Student *t* test.

Quantitative real-time polymerase chain reaction analysis

For thrombospondin-1 (THBS1), the results obtained by microarray analysis were verified by quantitative reverse transcriptase (RT)-PCR (qRT-PCR) analysis. Total cellular RNA was extracted from whole lung using TRIzol reagent. Total RNA (2 μg) was DNase-treated (TURBO DNA-free kit; Ambion, Austin, TX) and reverse-transcribed using the RT^2 First Strand Kit (SuperArray BioScience) according to the manufacturer's protocols. The cDNA templates were amplified with mouse THBS1 and β-actin (SuperArray) primer pairs in independent sets of PCR using RT^2 Real-Time SYBR Green PCR master mix (SuperArray) on an Eppendorf Mastercycler ep realplex (Westbury, NY) according to the manufacturer's protocols. Each sample was run in triplicate, and mRNA levels were analyzed relative to the β-actin housekeeping gene. Relative gene expression ratios were calculated according to the SuperArray-recommended $\Delta\Delta C_t$ protocol [18].

Immunohistochemical analysis

Sections of left lung were processed for avidin-biotin-immunoperoxidase staining using anti-thrombospondin-1 antibody (Abcam, Cambridge, MA). Binding was detected with 3,3'-diaminobenzidine tetrachloride (DAB). Sections were lightly counterstained with Mayer's hematoxylin, cleared, and mounted. Controls for specificity consisted of omission of the primary antibody, which abolished all immunoreactivity.

For double immunofluorescence studies, tissue sections were stained sequentially with anti-thrombospondin-1 antibody (fluorescein-labeled, green), followed by either macrophage/monocyte-specific anti-CD68 antibody (Dako, Carpinteria, CA); dendritic cell–specific antibody (anti-DC-SIGN [C-type lectin dendritic cell–specific intercellular adhesion molecule-3 grabbing nonintegrin; CD209]) (Abcam Inc) [19] or endothelial cell–specific anti-CD31 antibody (Dako) (all Cy3-labeled, red). Controls consisted of omission of one or both primary antibodies, which abolished the respective immunoreactivities.

Data Analysis

Values are expressed as mean $\pm$ standard deviation (SD) or, where appropriate, as mean $\pm$ standard error of the mean (SEM). Microarray and qRT-PCR results of ventilated and control groups were compared by 2-tailed Student *t* test. The significance level was set at $P < .05$. Statview software (Abacus, Berkeley, CA) was used for all statistical work.

RESULTS

Patients

The age distribution, relevant clinical data, and general autopsy findings in control and ventilated infants studied by angiogenesis-focused microarray analysis are summarized in Table 1.

Lung histology

Lungs of control preterm infants showed histological features characteristic of the early saccular stage of lung development (Figure 1A–C). The peripheral lung parenchyma was composed of large-sized primitive acini, separated by relatively wide septa that showed focal septation of the acinar units by vascularized ridges ('secondary crests'). A well-developed capillary network was present within the peripheral mesenchyme, preferentially located in immediate subepithelial position. Compared with control lungs, the lungs of short-term ventilated infants showed wider and more cellular septa (Figure 1D–F). Focal pulmonary hemorrhages and interstitial emphysema were noted. The thickened septa contained abundant capillary structures, randomly arranged in subepithelial as well as central locations.

Although no formal morphometry or cell proliferation assays were performed in the present study, we previously demonstrated that the early postnatal period of ventilated preterm infants is characterized by marked endothelial cell proliferation and expansion of the microvascular network [5]. We speculate that these early vascular changes form the molecular and architectural blueprint for the dysmorphic microvasculature seen in infants with fully established BPD, justifying our selection of early ventilated preterm newborns to study the regulation of BPD-associated dysangiogenesis.

Angiogenesis-focused microarray analysis

Gene expression profiling identified 13/112 (12%) regulated genes included in the array (Table 2). In accordance with our previous studies [5, 14], microarray analysis confirmed significant up-regulation of

TABLE 1 Clinical Data of Patients Studied by Microarray Analysis

	Age at birth (weeks)	Postnatal age	Corrected age at death (weeks)	Gender	PMI) (hours)	BW at autopsy (g)	Clinical/autopsy diagnosis	Other
Control								
Patient 1	26	SB	26	F	24	900	Abruption	
Patient 2	24	<1 hour	24	M	14	518	PPROM, amniotic fluid infection syndrome	
Patient 3	25	<1 hour	25	F	35	698	PPROM, acute chorioamnionitis	Antenatal steroids
Mean ± SD	25.0 ± 1.0		25.0 ± 1.0		24.3 ± 10.5	705 ± 191		
Ventilated								
Patient 1	24	6 days	25	F	18	712	Early BPD, abruption and large placental chorangioma	Surfactant
Patient 2	25	3 days	25	M	42	1013	PPROM, acute chorioamnionitis, early BPD, GBS sepsis	Surfactant
Patient 3	27	4 days	27	F	47	717	Acute chorioamnionitis, early BPD, massive pulmonary hemorrhage	Surfactant
Mean ± SD	25.3 ± 1.5		25.7 ± 1.2		35.7 ± 15.5	814 ± 172		

Note. M = male; F = female; SB = stillborn; PPROM = preterm premature rupture of membranes; BPD = bronchopulmonary dysplasia; GBS = group B Streptococcus.
*Age and corrected age reflect postmenstrual age.

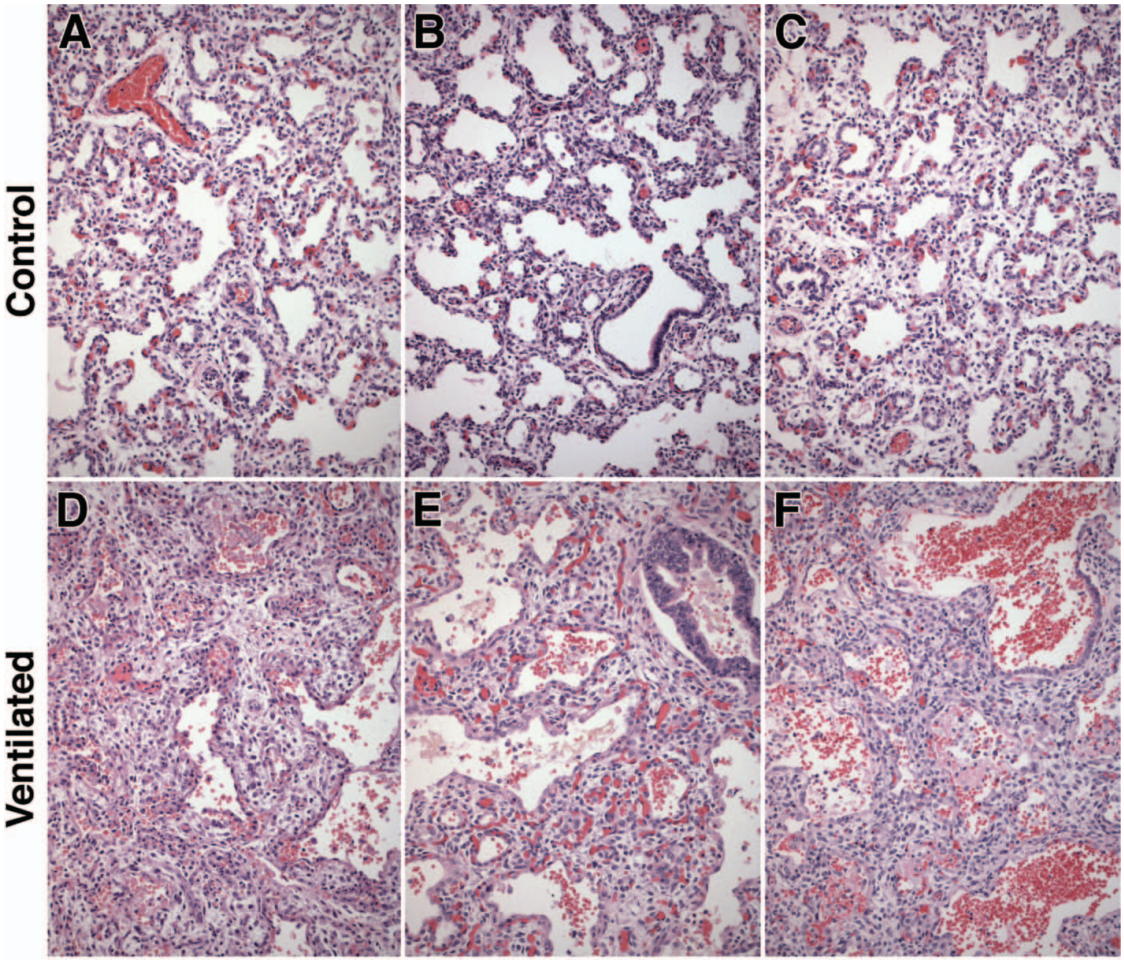

FIGURE 1 Morphology of lungs studied by microarray analysis. (**A**–**C**) Control infants. (**D**–**F**) Short-term ventilated infants. (Hematoxylin-eosin stain, original magnification ×200.)

TABLE 2 Angiogenesis-Related Genes Differentially Expressed in Postmortem Ventilated Preterm Lungs*

Gene symbol	Gene ID no.	Control (3) (mean ± SD)	Early ventilated (3) (mean ± SD)	*P*	Fold change	Description
Genes up-regulated in ventilated lungs						
Growth factors and receptors						
TGFA	NM_003236	0.41 ± 0.11	1.01 ± 0.24	.017	2.46	Transforming growth factor, alpha
ENG	NM_000118	28.96 ± 8.79	55.57 ± 4.35	.009	1.92	Endoglin (CD105)
Adhesion molecules						
THBS1	NM_003246	0.65 ± 0.14	3.56 ± 1.25	.016	5.48	Thrombospondin 1
COL18A1	NM_030582	4.03 ± 0.88	9.72 ± 0.51	<.001	2.41	Collagen, type XVIII, alpha-1
Cytokines and chemokines						
CCL2	NM_002982	12.23 ± 0.89	35.16 ± 8.26	.005	2.87	Chemokine (C-C motif) ligand 2
Proteases, inhibitors and other matrix proteins						
TIMP1	NM_003254	42.54 ± 15.75	114.54 ± 5.83	.002	2.69	Tissue inhibitor of metalloproteinase 1 (erythroid potentiating activity, collagenase inhibitor)
Genes down-regulated in ventilated lungs						
Growth factors and receptors						
FGFR3	NM_000142	8.38 ± 4.19	1.52 ± 0.29	.047	5.51	Fibroblast growth factor receptor 3
EFNA-1	NM_182685	40.25 ± 16.59	8.90 ± 0.81	.031	4.52	Ephrin-A1
KDR	NM_002253	3.38 ± 0.76	1.18 ± 0.46	.013	2.86	Kinase insert domain receptor (a type III receptor tyrosine kinase)
VEGFB	NM_003377	21.94 ± 4.69	10.68 ± 5.23	.049	2.05	Vascular endothelial growth factor B
TEK	NM_000459	4.16 ± 0.34	3.05 ± 0.40	.021	1.36	TEK tyrosine kinase, endothelial (venous malformations, multiple cutaneous and mucosal)
Cytokines and chemokines						
MDK	NM_002391	0.73 ± 0.22	0.27 ± 0.11	.030	2.70	Midkine (neurite growth-promoting factor 2)
Transcription factors and others						
ANG	NM_001145	4.07 ± 0.99	0.58 ± 0.12	.004	7.02	Angiogenin, ribonuclease, RNase A family, 5

*$P < .05$ versus control, Student *t* test.
Values are analyzed annotated densitometry data after background subtraction and interquartile normalization, as provided by SuperArray Inc. Minimal intensity was used for background subtraction.

endoglin expression ($P < .01$) and significant down-regulation of expression of kinase insert domain receptor (KDR; also known as fetal liver kinase-1 [Flk-1] or vascular endothelial growth factor receptor-2 [VEGFR-2]; $P < .02$) and TEK tyrosine kinase, endothelial (also known as Tie-2, angiopoietin receptor; $P < .05$) in ventilated lungs. Transcript levels of transforming growth factor (TGF)-α were more than 2-fold higher in ventilated lungs compared with controls ($P = .017$), consistent with previous reports documenting increased TGF-α immunoreactivity in lungs of infants with BPD [20, 21]. Novel genes found to be up-regulated in ventilated lungs included the adhesion molecules thrombospondin-1 and collagen XVIII alpha, the chemokine (C-C motif) ligand 2 (CCL2; also known as human monocyte chemoattractant protein-1 [MCP-1]), and tissue inhibitor of metalloproteinase-1 (TIMP1). Conversely, expression of fibroblast growth factor receptor 3, ephrin-A1, VEGF-B, midkine, and angiogenin was significantly down-regulated in ventilated preterm lungs.

Pulmonary transcripts were scored as 'present but not significantly changed' ($P \geq .05$ between ventilated and control lungs) for 49/112 (44%) angiogenesis-related genes. Among these, transcript levels of the traditional angiogenic growth factors, VEGF and angiopoietin, showed a 1.4- fold and 1.3-fold decrease in ventilated lungs, respectively. Although this downward trend was in agreement with our previous RNase-based study [5], the differences in VEGF and angiopoietin transcript levels between ventilated and control lungs did not reach statistical significance in this smaller study.

Pulmonary mRNA was scored as 'absent' in ventilated and/or control lungs for 50 angiogenesis-related genes (50/112; 45% of total). We found no obvious correlation between the length of the postmortem interval and gene expression levels or absence/presence of gene transcripts. Some gene transcripts, such as those of transforming growth factor-β (TGF-β), fibroblast growth factors (FGF), and hepatocyte growth factor (HGF) were absent in all samples, regardless of the duration of the postmortem interval. Conversely, transcripts of other genes, such as endoglin and thrombospondin-1, were present in all samples, suggesting rates of postmortem mRNA degradation are gene-specific.

Quantitative real-time polymerase chain reaction analysis of thrombospondin-1 gene expression

The glycoprotein thrombospondin-1 was among the more highly overexpressed genes in ventilated lungs (5.5-fold up-regulated; $P < .02$). The role of this potent angiostatic regulator in the pathogenesis of BPD remains undetermined. Based on its high level of expression in early-ventilated lungs, its known robust antiangiogenic functions, and the promise of discovering a novel molecular target for therapeutic approaches, further studies were focused on thrombospondin-1. First, we verified the thrombospondin microarray results by real-time PCR analysis. As shown in Figure 2, thrombospondin-1 mRNA levels were more than 6-fold higher in ventilated lungs than in controls ($P < .05$), confirming the significant increase detected by microarray analysis.

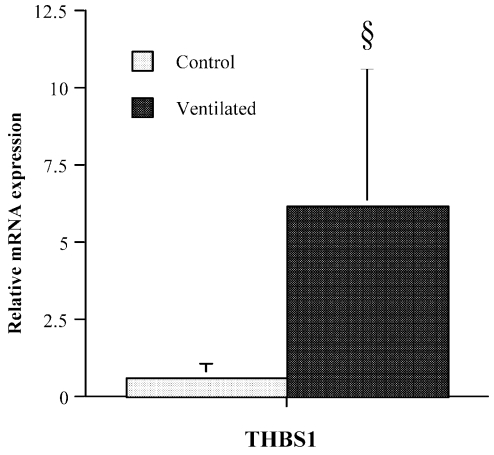

FIGURE 2 Quantitative real-time polymerase chain reaction analysis of thrombospondin-1 gene expression. Analysis of thrombospondin-1 (THBS1) mRNA expression in lung homogenates of control or short-term ventilated infants. $P < .05$ versus control lungs. Data represent mean $\pm$ SD.

Immunohistochemical analysis of thrombospondin-1 protein expression

We then determined the cellular origin of thrombospondin-1 expression in preterm lungs by immunohistochemical analysis of short-term ventilated and control lung samples. Control lungs showed only infrequent scattered foci of thrombospondin-positivity in the interstitium (Figure 3A). In contrast, ventilated lungs displayed more abundant areas of thrombospondin immunoreactivity, which varied in size and staining intensity (Figure 3B). Thrombospondin staining was limited to the interstitium; alveolar and bronchial epithelial cells were devoid of staining. Thrombospondin-positive platelets were only rarely seen in control lungs but appeared more abundant in ventilated lungs (Figure 3B). Omission of the thrombospondin antibody abolished all

immunoreactivity (Figure 3C), confirming the staining specificity.

To determine the cellular phenotype of the thrombospondin-positive structures, we performed immunohistochemical double labeling using anti-thrombospondin antibodies in conjunction with various cell-specific antibodies. First, we combined thrombospondin-1 labeling with immunohistochemical identification of endothelial cells using anti-CD31 (platelet endothelial cell adhesion molecule [PECAM]) antibody. We found no evidence of thrombospondin-1 staining in endothelial cells. However, CD31 staining provided clear delineation of microvascular structures and allowed unequivocal localization of virtually all thrombospondin-1 immunoreactivity to the lumen of small- or medium-sized vascular structures (Figure 3D–F). Whereas small-sized, coarsely granular immunoreactive structures could readily be identified as platelets, larger aggregates of thrombospondin-positive material appeared more consistent with intravascular fibrin/platelet conglomerates (Figure 3D–F). Although the vast majority of thrombospondin-1 protein was associated with platelets and fibrin aggregates, a small minority (<1%) of alveolar macrophages and interstitial dendritic cells displayed thrombospondin-1 immunoreactivity as well (Figure 3G–I and Figure 3J–L, respectively). In agreement with our immunoperoxidase stains, immunofluorescence showed no thrombospondin staining in alveolar or bronchial epithelium.

DISCUSSION

Focused gene expression profiling of postmortem lung tissues of short-term ventilated and nonventilated age-matched control preterm infants revealed differential expression of 13 angiogenesis-related genes, comprising growth factors and their receptors, adhesion molecules, cytokines/chemokines, protease inhibitors, and transcription factors. In concordance with our previous Western blot and ribonuclease protection assay studies [14] and studies by others in infants with BPD [4], microarray analysis identified down-regulation of several (soluble) angiogenic growth factors traditionally implicated in the regulation of postcanalicular microvascular development, such as VEGFB, VEGFR2 (KDR/Flk-1), and the angiopoietin-1 receptor, TEK/Tie-2. Similarly, transcript levels of VEGF (VEGFA) and angiopoietin tended to be lower in ventilated lungs, although the difference between ventilated and control lungs did not reach statistical significance in this relatively small study.

Microarray analysis demonstrated significant up-regulation ($P < .009$) of endoglin (CD105) in short-term ventilated lungs, confirming our recent study performed by ribonuclease protection assay and Western blot analysis [14]. Endoglin is a hypoxia-inducible accessory receptor for transforming growth factor-β (TGF-β) that is predominantly expressed in proliferating endothelial cells in culture and in angiogenic blood vessels in vivo [22, 23]. Although endoglin has been implicated as an important regulator of cardiovascular development, angiogenesis and vascular remodeling [24, 25], its exact functional contributions to angiogenesis in ventilated preterm lungs remain to be determined.

Our microarray study further revealed differential expression of several novel genes not previously described in the context of BPD. Interestingly, several genes that were significantly up-regulated in ventilated lungs are involved in extracellular matrix remodeling and have known antisprouting functions. Such up-regulated matrix-modulating genes included thrombospondin-1 [26], collagen XVIII alpha-1 [27, 28], and tissue inhibitor of metalloproteinase-1 (TIMP1) [29]. Conversely, significantly down-regulated genes included potent inducers of neovascularization, such as angiogenin, as well as genes involved in extracellular matrix resolution, such as midkine. Angiogenin is one of the most potent inducers of neovascularization in experimental models in vivo described [30, 31].

Midkine is a retinoic acid–responsive, heparin-binding growth factor expressed in various cell types during embryogenesis [32]. Midkine has been implicated in a range of biological functions in vitro, including angiogenesis [33], cell growth [34], and cell migration [35]. The exact biological role of midkine in lung morphogenesis is not fully understood. Midkine is expressed in developing mouse lungs in a bimodal pattern, with high levels of expression between embryonal days 13 to 15.5 and postnatal days 5 to 12 [36]. Postnatal midkine expression coincides with the timing of intense alveolar and pulmonary microvascular remodeling. Based on its potential role in angiogenesis and its spatiotemporal pattern of pulmonary expression, midkine is believed to play a role in the regulation of the development of the pulmonary vascular bed [37]. Midkine expression is regulated by hypoxia and influences pulmonary vascular remodeling, at least in part by inducing increased muscularization of small pulmonary arteries [37].

Short-term ventilated lungs exhibited increased mRNA levels of the CC chemokine monocyte chemotactic protein (MCP)-1 (CCL2), consistent with monocyte infiltration of the lungs. Tracheal aspirate concentrations of CCL2 and related cytokines

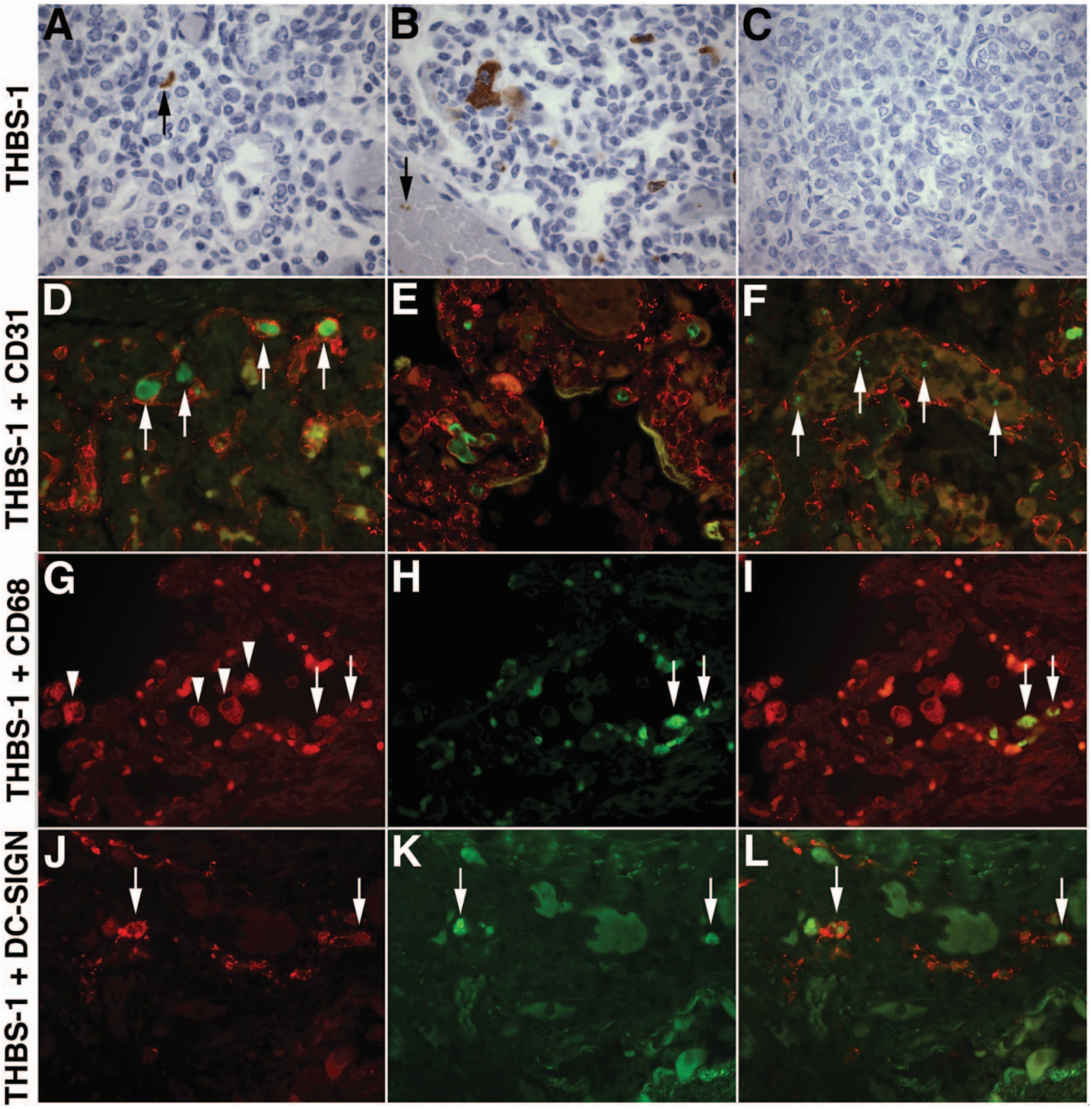

FIGURE 3 Immunohistochemical analysis of thrombospondin-1 protein expression. (**A**) Nonventilated control lung showing a single focus of thrombospondin-positive material in the interstitium. (**B**) Short-term ventilated lung showing abundant thrombospondin-positive structures of varying size, shape, and staining intensity in the alveolar septa. Immunoreactive platelets are present in the adjacent blood vessel (*arrows*). (**C**) Negative control (omission of primary antibody) showing absence of immunoreactivity. (**D**) Short-term ventilated lung showing large-sized aggregates of finely granular thrombospondin-positive material, consistent with fibrin, plugging tortuous capillary vessels (*arrows*). (**E**) Short-term ventilated lung showing thrombospondin-positive material of varying sizes in septal capillaries. Weak basement membrane staining of large-sized vessel is noted. (**F**) Short-term ventilated lung showing coarse granular thrombospondin-positive structures, consistent with platelets, in capillaries (*arrows*). (**G**–**I**). Short-term ventilated lung showing thrombospondin-containing alveolar macrophages (*arrows*) and alveolar macrophages devoid of thrombospondin immunoreactivity (*arrow heads*). (**J**–**L**) Short-term ventilated lung showing the presence of thrombospondin-immunoreactive granular material in the cytoplasm of several perivascular interstitial dendritic cells (*arrows*). **A**–**C**: Anti-thrombospondin-1 immunohistochemistry; 3,3′-diaminobenzidine tetrachloride (DAB) with hematoxylin counterstain; original magnification, ×600. **D**–**F**: Anti-thrombospondin-1 (fluorescein isothyocyanate, *green*) and anti-CD31 (PECAM) (Cy3, *red*) double immunofluorescence; original magnification, ×400. **G**–**I**: Anti- thrombospondin-1 (fluorescein isothyocyanate, *green*) and anti-CD68 (Cy3, *red*) double immunofluorescence; original magnification, ×400. **J**–**L**: Anti-thrombospondin-1 (fluorescein isothyocyanate, *green*) and anti-DC-SIGN (Cy3, *red*) double immunofluorescence; original magnification, ×400. THBS1 = thrombospondin-1.

MCP-2 and MCP-3 have been shown to be increased in infants with respiratory distress syndrome [38]. Increased tracheal aspirate concentrations of these cytokines correlate with pulmonary hemorrhage and the development of BPD [38].

The net biological outcome of the concurrent expression patterns of the various matrix-regulating genes revealed by the present microarray remains undetermined. However, it is tempting to speculate that increased expression of molecules depositing extracellular matrix around developing vessels (such as thrombospondin-1, collagen XVIII, and TIMP1), coregulated with decreased expression of genes that regulate perivascular matrix resolution (such as midkine), may contribute to the nonsprouting angiogenic phenotype that is seen in ventilated preterm lungs.

We focused our attention in this study on thrombospondin-1, which was one of the highest overexpressed genes in ventilated lungs. The thrombospondins are a family of five 'matricellular' calcium-binding extracellular glycoproteins that regulate cell-cell and cell-matrix interactions [39, 40]. Thrombospondin-1 (THBS1, TSP1, Thsp-1), the first identified and prototypic member of the family [41], has been studied most intensively and was the first protein to be recognized as a critical endogenous inhibitor of angiogenesis. Thrombospondin-1 is a major component of platelet α-granules from which it is secreted upon platelet activation [42]. In addition to blood-borne platelets, thrombospondin-1 is expressed at varying levels in many if not all tissues [39] and has broad but distinct tissue expression patterns during development and through adulthood [43–45]. A wealth of data both in vitro and in vivo supports that the main function of thrombospondin-1 as potent inhibitor of angiogenesis, modulating angiogenesis by acting on endothelial cells as well as vascular smooth muscle cells [46–48].

Despite vast evidence of widespread pulmonary expression of thrombospondin-1 during development and beyond [45, 49–52], the potential role of thrombospondin-1 in normal or disrupted human postcanalicular lung remodeling has not previously been studied. In the present study, we identified robust more than 5-fold up-regulation of thrombospondin-1 mRNA expression in ventilated preterm lungs. Thrombospondin-1 protein was immunolocalized to platelets and platelet-fibrin aggregates within the pulmonary microvasculature and, to much lesser extent, to alveolar macrophages and interstitial dendritic cells. Although anucleate and devoid of nuclear DNA, platelets retain megakaryocyte-derived mRNAs and are capable of protein biosynthesis from cytoplasmic mRNA [53, 54]. Previous studies have demonstrated that platelets have the capacity to synthesize and assemble the subunits of thrombospondin [53, 55]. Although platelets are considered the primary source of thrombospondin-1 [42], previous studies have described the presence of thrombospondin-1 mRNA and/or protein in alveolar macrophages [50, 51] and dendritic cells [56]. In contradistinction with other studies [45, 49–51], we found no localization of thrombospondin-1 protein in alveolar or bronchial epithelial cells.

The molecular mechanisms underlying increased pulmonary thrombospondin-1 mRNA levels in ventilated preterm lungs remain unclear. Based on the abundance and intense immunoreactivity of thrombospondin-1 protein in platelets and intravascular fibrin aggregates, it is plausible that both increased numbers of pulmonary platelets and increased platelet activation may have contributed to the increased thrombospondin-1 mRNA levels in lung homogenates of ventilated preterm infants. By analogy, megakaryocyte and platelet homeostasis were reportedly altered in diffuse alveolar damage [57]. It remains to be determined whether platelet expression of thrombospondin-1 in preterm lungs is regulated by any of the candidate factors implicated in the pathogenesis of BPD, such as infection/inflammation, oxygen toxicity, hyperoxia/hypoxia, and mechanical stretch/strain [1]. Several lines of evidence suggest that megakaryocyte and/or platelet homeostasis and activation may be modulated by oxygen tension, hypoxia and/or hyperoxia, at least in some conditions [58–61]. Interestingly, a recent report described up-regulation of thrombospondin-1 mRNA expression in ovine fetal lungs subjected to stretch following tracheal occlusion [52].

Several limitations of this study need to be addressed. First, the microarray analysis was performed using postmortem material. Human postmortem preterm lungs represent an invaluable and unique resource that cannot be replaced by in vitro or animal models and is essential for closing the gap between basic science research and applied clinical medicine. However, any study using autopsy material is inherently limited by variable degrees of autolysis and protein/RNA degradation. Fortunately, lungs—especially those of preterm infants—appear to be relatively resistant to autolysis [17]. Nevertheless, it needs to be recognized that altered expression of potentially relevant genes scored as 'absent' or 'not significantly changed' may have been missed due to differential gene degradation rates. Second, the sample sizes in this study were relatively small. Third, microarray analysis was performed in whole lung lysates, which comprise vascular, epithelial, interstitial, and alveolar compartments.

Altered gene expression in minor cell populations may have been overlooked by this approach. Fourth, we used a focused microarray approach that allows the interrogation of 112 angiogenesis-related genes. Although easier to interpret, focused microarray analyses may miss differential expression patterns of unexpected yet important genes. Genome-wide studies of transcriptomic changes in endothelial cells or vascular and/or oxygen-delivering organs such as placenta and thyroid have demonstrated that regulated genes are not restricted to traditional 'endothelial-associated' genes [62–64]. Finally, this study was performed in postmortem tissues, which represent the most severe—lethal—end of the BPD spectrum. It is unclear to what extent our results can be extrapolated to less severe, survivable degrees of neonatal lung injury.

In conclusion, microarray analysis of postmortem preterm lungs detected differential expression of 13 angiogenesis-related genes in ventilated lungs compared with controls. The overall tendency toward up-regulation of angiostatic genes and down-regulation of proangiogenic genes is suggestive of an angiogenic shift underlying the dysmorphic microvascular phenotype of ventilated preterm lungs. The marked up-regulation of thrombospondin-1, immunolocalized predominantly to platelets and intravascular fibrin aggregates, highlights the potential importance of the platelet-thrombospondin-microvascular axis in BPD. The pathophysiology of BPD may thus be even more complex and multifactorial than currently recognized. Indeed, in addition to dysregulated interactions between 'intrinsic' lung players (epithelium, endothelium, and mesenchyme), it is plausible that 'extrinsic' passenger elements such as platelets may have important angiogenic contributions as well.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

REFERENCES

[1] Jobe AH, Bancalari E: Bronchopulmonary dysplasia. Am J Respir Crit Care Med. 2001;163:1723–1729.

[2] Husain AN, Siddiqui NH, Stocker JT: Pathology of arrested acinar development in postsurfactant bronchopulmonary dysplasia. Hum Pathol. 1998;29:710–717.

[3] Coalson JJ: Pathology of chronic lung disease in early infancy. In: Bland RD, Coalson JJ, eds. Chronic Lung Disease in Early Infancy. New York: M. Dekker; 2000:85–124

[4] Bhatt AJ, Pryhuber GS, Huyck H, Watkins RH, Metlay LA, Maniscalco WM: Disrupted pulmonary vasculature and decreased vascular endothelial growth factor, Flt-1, and TIE-2 in human infants dying with bronchopulmonary dysplasia. Am J Respir Crit Care Med. 2001;164:1971–1980.

[5] De Paepe ME, Mao Q, Powell J, Rubin SE, DeKoninck P, Appel N, Dixon M, Gundogan F: Growth of pulmonary microvasculature in ventilated preterm infants. Am J Respir Crit Care Med. 2006;173:204–211.

[6] Coalson JJ, Winter VT, Siler-Khodr T, Yoder BA: Neonatal chronic lung disease in extremely immature baboons. Am J Respir Crit Care Med. 1999;160:1333–1346.

[7] Maniscalco WM, Watkins RH, Pryhuber GS, Bhatt A, Shea C, Huyck H: Angiogenic factors and alveolar vasculature: development and alterations by injury in very premature baboons. Am J Physiol Lung Cell Mol Physiol. 2002;282:L811–L823.

[8] Jakkula M, Le Cras TD, Gebb S, Hirth KP, Tuder RM, Voelkel NF, Abman SH: Inhibition of angiogenesis decreases alveolarization in the developing rat lung. Am J Physiol Lung Cell Mol Physiol. 2000;279:L600–L607.

[9] Le Cras TD, Markham NE, Tuder RM, Voelkel NF, Abman SH: Treatment of newborn rats with a VEGF receptor inhibitor causes pulmonary hypertension and abnormal lung structure. Am J Physiol Lung Cell Mol Physiol. 2002;283:L555–L562.

[10] Abman SH: Bronchopulmonary dysplasia: "a vascular hypothesis." Am J Respir Crit Care Med. 2001;164:1755–1756.

[11] Thebaud B, Abman SH: Bronchopulmonary dysplasia: where have all the vessels gone? Roles of angiogenic growth factors in chronic lung disease. Am J Respir Crit Care Med. 2007;175:978–985.

[12] Carmeliet P: Mechanisms of angiogenesis and arteriogenesis. Nat Med. 2000;6:389–395.

[13] Adams RH, Alitalo K: Molecular regulation of angiogenesis and lymphangiogenesis. Nat Rev Mol Cell Biol. 2007;8:464–478.

[14] De Paepe ME, Patel C, Tsai A, Gundavarapu S, Mao Q: Endoglin (CD105) up-regulation in pulmonary microvasculature of ventilated preterm infants. Am J Respir Crit Care Med. 2008;178:180–187.

[15] De Paepe ME: Lung growth and development. In: Churg AM, Myers JL, Tazelaar HD, Wright JL, eds. Thurlbeck's Pathology of the Lung. New York: Thieme Medical Publishers; 2005; pp. 39–84.

[16] De Paepe ME, Friedman RM, Gundogan F, Pinar H: Postmortem lung weight/body weight standards for term and preterm infants. Pediatr Pulmonol. 2005;40:445–448.

[17] De Paepe ME, Mao Q, Huang C, Zhu D, Jackson CL, Hansen K: Postmortem RNA and protein stability in perinatal human lungs. Diagn Mol Pathol. 2002;11:170–176.

[18] Livak KJ, Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001;25:402–408.

[19] Geijtenbeek TB, Kwon DS, Torensma R, van Vliet SJ, van Duijnhoven GC, Middel J, Cornelissen IL, Nottet HS, KewalRamani VN, Littman DR, Figdor CG, van Kooyk Y: DC-SIGN, a dendritic cell-specific HIV-1-binding protein that enhances trans-infection of T cells. Cell. 2000;100:587–597.

[20] Stahlman MT, Orth DN, Gray ME: Immunocytochemical localization of epidermal growth factor in the developing human respiratory system and in acute and chronic lung disease in the neonate. Lab Invest. 1989;60:539–547.

[21] Strandjord TP, Clark JG, Guralnick DE, Madtes DK: Immunolocalization of transforming growth factor-alpha, epidermal growth factor (EGF), and EGF-receptor in normal and injured developing human lung. Pediatr Res. 1995;38:851–856.

[22] Gougos A, Letarte M: Identification of a human endothelial cell antigen with monoclonal antibody 44G4 produced against a pre-B leukemic cell line. J Immunol. 1988;141:1925–1933.

[23] Sanchez-Elsner T, Botella LM, Velasco B, Langa C, Bernabeu C: Endoglin expression is regulated by transcriptional

cooperation between the hypoxia and transforming growth factor-beta pathways. J Biol Chem. 2002;277:43799–43808.
[24] Li DY, Sorensen LK, Brooke BS, Urness LD, Davis EC, Taylor DG, Boak BB, Wendel DP: Defective angiogenesis in mice lacking endoglin. Science. 1999;284:1534–1537.
[25] Arthur HM, Ure J, Smith AJ, Renforth G, Wilson DI, Torsney E, Charlton R, Parums DV, Jowett T, Marchuk DA, Burn J, Diamond AG: Endoglin, an ancillary TGFbeta receptor, is required for extraembryonic angiogenesis and plays a key role in heart development. Dev Biol. 2000;217:42–53.
[26] Adams JC: Thrombospondin-1. Int J Biochem Cell Biol. 1997;29:861–865.
[27] O'Reilly MS, Boehm T, Shing Y, Fukai N, Vasios G, Lane WS, Flynn E, Birkhead JR, Olsen BR, Folkman J: Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. Cell. 1997;88:277–285.
[28] Abdollahi A, Hahnfeldt P, Maercker C, Grone HJ, Debus J, Ansorge W, Folkman J, Hlatky L, Huber PE: Endostatin's antiangiogenic signaling network. Mol Cell. 2004;13:649–663.
[29] Ryu J, Vicencio AG, Yeager ME, Kashgarian M, Haddad GG, Eickelberg O: Differential expression of matrix metalloproteinases and their inhibitors in human and mouse lung development. Thromb Haemost. 2005;94:175–183.
[30] Fett JW, Strydom DJ, Lobb RR, Alderman EM, Bethune JL, Riordan JF, Vallee BL: Isolation and characterization of angiogenin, an angiogenic protein from human carcinoma cells. Biochemistry. 1985;24:5480–5486.
[31] Pavlov N, Badet J: [Angiogenin: involvement in angiogenesis and tumour growth]. Bull Cancer. 2001;88:725–732.
[32] Kadomatsu K, Tomomura M, Muramatsu T: cDNA cloning and sequencing of a new gene intensely expressed in early differentiation stages of embryonal carcinoma cells and in midgestation period of mouse embryogenesis. Biochem Biophys Res Commun. 1988;151:1312–1318.
[33] Choudhuri R, Zhang HT, Donnini S, Ziche M, Bicknell R: An angiogenic role for the neurokines midkine and pleiotrophin in tumorigenesis. Cancer Res. 1997;57:1814–1819.
[34] Muramatsu H, Muramatsu T: Purification of recombinant midkine and examination of its biological activities: functional comparison of new heparin binding factors. Biochem Biophys Res Commun. 1991;177:652–658.
[35] Takada T, Toriyama K, Muramatsu H, Song XJ, Torii S, Muramatsu T: Midkine, a retinoic acid-inducible heparin-binding cytokine in inflammatory responses: chemotactic activity to neutrophils and association with inflammatory synovitis. J Biochem. 1997;122:453–458.
[36] Reynolds PR, Mucenski ML, Whitsett JA: Thyroid transcription factor (TTF)-1 regulates the expression of midkine (MK) during lung morphogenesis. Dev Dyn. 2003;227:227–237.
[37] Reynolds PR, Mucenski ML, Le Cras TD, Nichols WC, Whitsett JA: Midkine is regulated by hypoxia and causes pulmonary vascular remodeling. J Biol Chem. 2004;279:37124–37132.
[38] Baier RJ, Majid A, Parupia H, Loggins J, Kruger TE: CC chemokine concentrations increase in respiratory distress syndrome and correlate with development of bronchopulmonary dysplasia. Pediatr Pulmonol. 2004;37:137–148.
[39] Adams JC, Lawler J: The thrombospondins. Int J Biochem Cell Biol. 2004;36:961–968.
[40] Lawler J: The functions of thrombospondin-1 and -2. Curr Opin Cell Biol. 2000;12:634–640.
[41] Good DJ, Polverini PJ, Rastinejad F, Le Beau MM, Lemons RS, Frazier WA, Bouck NP: A tumor suppressor-dependent inhibitor of angiogenesis is immunologically and functionally indistinguishable from a fragment of thrombospondin. Proc Natl Acad Sci U S A. 1990;87:6624–6628.
[42] Baenziger NL, Brodie GN, Majerus PW: Isolation and properties of a thrombin-sensitive protein of human platelets. J Biol Chem. 1972;247:2723–2731.
[43] Corless CL, Mendoza A, Collins T, Lawler J: Colocalization of thrombospondin and syndecan during murine development. Dev Dyn. 1992;193:346–358.
[44] Iruela-Arispe ML, Liska DJ, Sage EH, Bornstein P: Differential expression of thrombospondin 1, 2, and 3 during murine development. Dev Dyn. 1993;197:40–56.
[45] O'Shea KS, Dixit VM: Unique distribution of the extracellular matrix component thrombospondin in the developing mouse embryo. J Cell Biol. 1988;107:2737–2748.
[46] Patel MK, Lymn JS, Clunn GF, Hughes AD: Thrombospondin-1 is a potent mitogen and chemoattractant for human vascular smooth muscle cells. Arterioscler Thromb Vasc Biol. 1997;17:2107–2114.
[47] Isenberg JS, Wink DA, Roberts DD: Thrombospondin-1 antagonizes nitric oxide-stimulated vascular smooth muscle cell responses. Cardiovasc Res. 2006;71:785–793.
[48] Ren B, Yee KO, Lawler J, Khosravi-Far R: Regulation of tumor angiogenesis by thrombospondin-1. Biochim Biophys Acta. 2006;1765:178–188.
[49] Wight TN, Raugi GJ, Mumby SM, Bornstein P: Light microscopic immunolocation of thrombospondin in human tissues. J Histochem Cytochem. 1985;33:295–302.
[50] Sage H, Farin FM, Striker GE, Fisher AB: Granular pneumocytes in primary culture secrete several major components of the extracellular matrix. Biochemistry. 1983;22:2148–2155.
[51] Yehualaeshet T, O'Connor R, Green-Johnson J, Mai S, Silverstein R, Murphy-Ullrich JE, Khalil N: Activation of rat alveolar macrophage-derived latent transforming growth factor beta-1 by plasmin requires interaction with thrombospondin-1 and its cell surface receptor, CD36. Am J Pathol. 1999;155:841–851.
[52] Sozo F, Hooper SB, Wallace MJ: Thrombospondin-1 expression and localization in the developing ovine lung. J Physiol. 2007;584:625–635.
[53] Kieffer N, Guichard J, Farcet JP, Vainchenker W, Breton-Gorius J: Biosynthesis of major platelet proteins in human blood platelets. Eur J Biochem. 1987;164:189–195.
[54] Weyrich AS, Schwertz H, Kraiss LW, Zimmerman GA: Protein synthesis by platelets: historical and new perspectives. J Thromb Haemost. 2009;7:241–246.
[55] Belloc F, Heilmann E, Combrie R, Boisseau MR, Nurden AT: Protein synthesis and storage in human platelets: a defective storage of fibrinogen in platelets in Glanzmann's thrombasthenia. Biochim Biophys Acta. 1987;925:218–225.
[56] Doyen V, Rubio M, Braun D, Nakajima T, Abe J, Saito H, Delespesse G, Sarfati M: Thrombospondin 1 is an autocrine negative regulator of human dendritic cell activation. J Exp Med. 2003;198:1277–1283.
[57] Mandal RV, Mark EJ, Kradin RL: Megakaryocytes and platelet homeostasis in diffuse alveolar damage. Exp Mol Pathol. 2007;83:327–331.
[58] Mostafa SS, Miller WM, Papoutsakis ET: Oxygen tension influences the differentiation, maturation and apoptosis of human megakaryocytes. Br J Haematol. 2000;111:879–889.
[59] Segall M, Goetzman B: Hypoxic pulmonary hypertension: changes in platelet size and number. Am J Perinatol. 1991;8:300–303.
[60] Barazzone C, Tacchini-Cottier F, Vesin C, Rochat AF, Piguet PF: Hyperoxia induces platelet activation and lung sequestration: an event dependent on tumor necrosis factor-alpha and CD11a. Am J Respir Cell Mol Biol. 1996;15:107–114.

[61] Yang J, Yang M, Xu F, Li K, Lee SK, Ng PC, Tam JS, Yuen PM, Fok TF: Effects of oxygen-induced lung damage on megakaryocytopoiesis and platelet homeostasis in a rat model. Pediatr Res. 2003;54:344–352.

[62] Javerzat S, Franco M, Herbert J, Platonova N, Peille AL, Pantesco V, De Vos J, Assou S, Bicknell R, Bikfalvi A, Hagedorn M: Correlating global gene regulation to angiogenesis in the developing chick extra-embryonic vascular system. PLoS One. 2009;4:e7856.

[63] Favre CJ, Mancuso M, Maas K, McLean JW, Baluk P, McDonald DM: Expression of genes involved in vascular development and angiogenesis in endothelial cells of adult lung. Am J Physiol Heart Circ Physiol. 2003;285:H1917–H1938.

[64] Wallgard E, Larsson E, He L, Hellstrom M, Armulik A, Nisancioglu MH, Genove G, Lindahl P, Betsholtz C: Identification of a core set of 58 gene transcripts with broad and specific expression in the microvasculature. Arterioscler Thromb Vasc Biol. 2008;28:1469–1476.

SUPPLEMENTARY MATERIAL AVAILABLE ONLINE

Supplementary Table S1