Delineating the angiogenic gene expression profile before pulmonary vascular remodeling in a lamb model of congenital heart disease

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PULMONARY ARTERIAL HYPERTENSION (PAH) complicates the course of many children and adults with congenital heart diseases (CHD) (8). A wide range of cardiac defects cause increases in pulmonary blood flow. Over time, the increased flow through the pulmonary circulation leads to increased vascular reactivity and morphological changes (remodeling) in the pulmonary vasculature, which result in increases in pulmonary vascular resistance (PVR) and ultimately PAH (45). The pulmonary arterial remodeling secondary to CHD shares similarities with other etiologies of PAH, which are characterized by intimal hyperplasia, medium thickening, and muscularization of the precapillary arteries (50, 62). The mechanisms that are responsible for the initiation of the pulmonary vasculopathy are not completely understood. Generally, it is believed that increases in pulmonary blood flow exert abnormal shear stress and circumferential stretch on pulmonary artery endothelial cells (PAEC) and result in endothelial dysfunction and vascular remodeling (6). It has been shown that the endothelin, nitric oxide (NO), and prostacyclin pathways are involved in this process (6). Decreased production of prostacyclin and NO and increased production of endothelin-1 jointly promote vasoconstriction and proliferation of smooth muscle and endothelial cells in the pulmonary arteries (6).

Despite advances in surgical repair and the current anti-PAH therapy in the pre- and postoperative periods, pulmonary hypertension still carries a significant mortality and morbidity in patients with CHD (8). Surgical repair of systemic-to-pulmonary shunts during childhood has been shown to reverse the development of pulmonary vascular remodeling in some patients, but not all (7). Meanwhile, the current anti-PAH therapies including prostacyclin analogs, inhaled NO, and endothelin receptor antagonists have been shown to improve symptoms and prolong survival in some patients but have not been confirmed to change ultimate survival (22, 44), and some patients do not respond to the current “vasodilator” therapy (52). One reason for the limited success of current anti-PAH therapy is that these drugs fail to reverse the pulmonary vascular remodeling process. Thus an urgent need exists to address alternative mechanisms intrinsic to the pulmonary vascular remodeling in PAH.

Angiogenesis is the growth of new vessels on preexisting ones (26). Angiogenesis can play both beneficial roles and detrimental roles in human disease. For example, the development of collateral vessels through angiogenesis under ischemic conditions is desirable. Conversely, angiogenesis in tumors greatly expedites tumor growth and invasion (46). Recent studies indicate that angiogenesis is also involved in the pulmonary vascular remodeling in pulmonary hypertension (63). It has been shown that angiogenic biomarkers are increased in the serum of children with CHD (19). Moreover, expression of angiogenesis-related molecules has been detected in the plexiform lesions in severe pulmonary hypertension, indicating a disordered angiogenesis process (61). We previously detected...
a transient burst in angiogenesis concomitant with pulmonary vascular remodeling in an ovine model of CHD with increased pulmonary blood flow (40, 51). This Shunt model utilizes an in utero placement of vascular graft between the aorta and the main pulmonary artery, resulting in increased pulmonary blood flow after birth (51). The pulmonary vascular remodeling in the Shunt model resembles the pathological changes seen in children with PAH secondary to a ventral septal defect (VSD): dilation of the proximal pulmonary arteries, medial wall thickening of the small muscular arteries, and abnormal extension of muscle into peripheral pulmonary arteries (51). The “angiogenesis burst” we have observed in the Shunt lambs occurs between 1 and 4 wk of age. At 1 wk of age there was a trend toward increase in the intra-acinar pulmonary arteriole numbers per 100 alveoli units in Shunt lambs, and by 4 wk of age the intra-acinar pulmonary arteriole numbers per 100 alveoli units are twofold those of matched control lambs (40). The mechanisms underlying this angiogenesis process have not been well elucidated. This is important, as it could provide new insights in our understanding of how pulmonary vascular remodeling occurs in the disease process. We hypothesized that a proangiogenic gene expression profile is triggered by the increased pulmonary blood flow in the Shunt lambs and that this subsequently leads to pulmonary angiogenesis and vascular remodeling. Thus the purpose of this study was to identify new candidate genes responsible for the angiogenesis burst, with an overall goal of discovering new genes and signaling pathways that may play important roles in early onset of pulmonary vascular remodeling secondary to CHD.

MATERIALS AND METHODS

Ovine model of pulmonary hypertension with increased pulmonary blood flow. As we have previously described (51), pregnant ewes underwent in utero surgery (9 ± 6 days before term) to anastomose an 8.0-mm GORE-TEX vascular graft (~2-mm length: W. L. Gore, Milpitas, CA) between the ascending aorta and main pulmonary artery of the fetus. The incisions in the uterus and the abdomen were closed, and the sheep were allowed to deliver normally. Three days after delivery, lambs were anesthetized with ketamine hydrochloride (0.3 mg·kg⁻¹·min⁻¹), intubated, and a midsternotomy incision was performed. Measurements (including body weight, heart rate, blood pressures) and systemic arterial blood gases and pH were performed, and a midsternotomy incision was performed. Measurements of the hemodynamic variables (pulmonary and systemic arterial pressure, heart rate, left pulmonary blood flow, left and right atrial pressures) and systemic arterial blood gases and pH were performed, and blood and peripheral lung biopsies were obtained. Subsequently all lambs were killed with a lethal injection of potassium chloride (40 ml KCl 7.45%). All protocols and procedures were approved by the Committees on Animal Research of the government of Upper Bavaria, Germany, the University of California, San Francisco, and the Medical College of Georgia.

Microarray hybridization procedures. Because of the lack of commercially available ovine gene chips when this research was conducted, we utilized the Affymetrix bovine genome chips to hybridize with ovine RNA. Our previous studies have confirmed the feasibility of this approach (59). Total RNA was isolated from the peripheral lung tissues of a 3-day shunt model of CHD and age-matched sham-operated lambs, labeled, and then hybridized to the bovine genome chips. Eight gene chips were employed in each group. The microarray protocol was as follows: RNA was isolated with the Qiashredder column and RNeasy Mini kit (Qiagen, Valencia, CA). All RNA extracted was analyzed for quantity and quality with the Agilent 2100 Bioanalyzer system (Agilent Technologies, Palo Alto, CA). Gene expression profiling was performed with the bovine genome chips. An aliquot of 1 μg of total RNA was converted into double-stranded cDNA (ds-cDNA) with the SuperScript Choice System (GIBCO BRL Life Technologies, Carlsbad, CA) with an oligo(dt) primer containing a T7 RNA polymerase promoter (Genset, San Diego, CA). After second-strand synthesis, the reaction mixture was extracted with phenol-chloroform-isooamy alcohol, and ds-cDNA was recovered by ethanol precipitation. In vitro transcription was performed on the ds-cDNA with the Enzo RNA transcript Labeling kit. Biotin-labeled cRNA was purified with an RNeasy affinity column (Qiagen) and fragmented randomly to sizes ranging from 35 to 200 bases by incubation at 94°C for 35 min. The hybridization solutions contained 100 mM 2-(N-morpholino)ethanesulfonic acid (MES), 1 M NaH₂PO₄, 20 mM EDTA, and 0.01% Tween 20. The final concentration of fragmented cRNA was 0.05 μg/μl in hybridization solution. A target for hybridization was prepared by combining 40 μl of fragmented transcript with sonicated herring sperm DNA (0.1 mg/ml), bovine serum albumin, and 5 nM control oligonucleotide in a buffer containing 1.0 M NaCl, 10 mM Tris·HCl (pH 7.6), and 0.005% Triton X-100. The target was hybridized for 16 h at 45°C to the array chips (Affymetrix). Arrays were then washed at 50°C with stringent solution and then again at 30°C with nonstringent washes. Arrays were then stained with streptavidin-phycocerythrin (Invitrogen). DNA chips were read at a resolution of 3 μm with a Hewlett-Packard GeneArray Scanner and were analyzed with GENECHIP software (Affymetrix GCOS 1.1). Both the CEL and DAT files for each hybridization have been uploaded to our server running GeneTraffic v3.2 (Iobion Informatics, La Jolla, CA).

Microarray data analysis. Microarray data were processed and normalized with RMA (11). Normalized data were subsequently analyzed with the LIMMA (56) package in R. A P value <0.05 was considered statistically significant. The false discovery rate (FDR) was calculated with the pFDR of Storey (57) and the q value package in R. Gene annotation of all 24,129 genes/expressed sequence tags (ESTs) present on the microarrays was obtained by linking them to the Gene Ontology (GO) database (http://www.geneontology.org). The identities of genes of interest were further confirmed by the NetAffx Query database provided by Affymetrix.

Real-time RT-PCR analysis. Real-time RT-PCR was employed to verify the regulation of genes of interest. Primers were designed by Primer3. Table 1 shows all the primer sets utilized. Real time RT-PCR was carried out in two steps. First, total RNA was extracted from lung tissues with the RNeasy kit (Qiagen, Valencia), and 1 μg total RNA

Table 1. Primer pairs for real-time RT-PCR analysis

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Sense Primer</th>
<th>Antisense Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine FGF2</td>
<td>5'-AGTCGCAGGGTCATCTTGCCTGACTG-3'</td>
<td>5'-AGTCGCAGGGTCATCTTGCCTGACTG-3'</td>
</tr>
<tr>
<td>Ovine Angpt2</td>
<td>5'-CCAGGCTTATAGCTTCCAGGCTGG-3'</td>
<td>5'-CATGTTGTCGTTTTCCTTGGAGGA-3'</td>
</tr>
<tr>
<td>Bovine Birc5</td>
<td>5'-GGCTGAGCCCTAGCTCTGCCTG-3'</td>
<td>5'-GGCTGAGCCCTAGCTCTGCCTG-3'</td>
</tr>
<tr>
<td>Bovine ccl2</td>
<td>5'-CTCCTAGAGCTGCTCTGCTG-3'</td>
<td>5'-CTCCTAGAGCTGCTCTGCTG-3'</td>
</tr>
</tbody>
</table>

Angpt2, Angiopoietin2.
was reverse-transcribed with the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany) in a total volume of 20 µL. Quantitative real-time PCR was conducted on Mx4000 (Stratagene) with 2 µL of RT product, 12.5 µL of QuantiTect SYBR Green PCR Master Mix (Qiagen, Hilden), and primers (400 nM) in a total volume of 25 µL. The following thermocycling conditions were employed: 95°C for 10 min, followed by 95°C for 30 s, 55°C for 60 s, and 72°C 30 s for 45 cycles. Each set of primers were tested to ensure a single melting peak and an efficiency of the PCR reaction between 90% and 110%. The threshold cycles (Ct) of a serially diluted control sample were plotted to generate a standard curve. Concentration of each sample was calculated by interpolating its Ct on the standard curve and then normalized to β-actin (housekeeping gene) mRNA levels.

**Western blot analysis.** Lung tissues were homogenized in Triton X-100 lysis buffer [20 mM Tris-HCl (pH 7.6), 0.5% Triton X-100, 20% glycerol] supplemented with protease inhibitors (100 µg/ml PMSF, 1 µg/ml leupeptin and aprotonin) and clarified by centrifugation at 20,000 g for 20 min at 4°C, and the supernatant was stored at −80°C until use. Protein concentrations were determined with the Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL). Proteins were then run on a 4–20% gradient SDS-PAGE gel (NuSep), transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA), and blotted with the appropriate primary antibody overnight at 4°C, followed by incubation with the horseradish peroxidase-conjugated secondary antibody (Pierce) for 1 h at room temperature. After the membrane was exposed to Supersignal West Femto Maximum Sensitivity Substrate (Pierce), proteins were detected and quantified on a Kodak Image Station 440.

**Cell culture and shear stress studies.** Ovine PAEC isolated from fetal lambs, harvested as previously described (38, 66), were grown in 10% FBS-DMEM in a humidified chamber of 5% CO2 at 37°C to confluence and then exposed to unidirectional laminar shear of 20 or 35 dyn/cm² for 3 h with a cone-and-plate viscometer as we described previously (66).

**Matrigel tube formation analysis.** Subsequent to exposure to shear stress, PAEC were trypsinized and resuspended in 10% FBS-DMEM. Equal numbers of cells were then seeded onto 24-well Matrigel (BD Biosciences)-coated plates and incubated for 6 h. Tube formation was inspected under a microscope and documented by photography. Tube length was calculated with Image-Pro Plus 5.0 software. To assess the effects of blocking FGF-2, Angiopoietin2 (Angpt2), or Birc5 signaling on tube formation in certain experiments, neutralizing antibodies to FGF2 (4 µg/ml, Santa Cruz), Angpt2 (0.4 µg/ml, Cell Signaling), and Birc5 (8 µg/ml Cell Signaling) or a control rabbit IgG (10 µg/ml, Cell Signaling) were added to the culture media after shear stress.

**Small interfering RNA-mediated knockdown.** PAEC were transfected individually with 80 nM ccl2, FGF2, Angpt2, or Birc5 small interfering RNA (siRNA) (Qiagen) with the use of HiPerFect transfection reagent (Qiagen) according to the manufacturer’s instructions. A scrambled siRNA with no known homology to any known human gene was used as a control. Validation of the gene silencing effect in PAEC was carried out by real-time RT-PCR or Western blot 48 h after transfection.

**Statistical analysis.** Statistical calculations were performed with GraphPad Prism v. 4.01 software. Means ± SD or SE were calculated for all samples, and significance was determined by either the t-test (for 2 groups) or ANOVA (for >2 groups) with a Newman-Keuls post hoc test. A value of P < 0.05 was considered significant.

**RESULTS**

**Hemodynamics in Shunt and control lambs.** The hemodynamic data for sham-operated control and Shunt lambs at 3 days of age are shown in Table 2. There was a trend toward increase in pulmonary arterial pressure, although the difference did not reach statistical significance (Table 2). The left atrial pressure, left pulmonary blood flow, pulmonary-to-systemic blood flow ratio, and pulmonary arterial P02 were significantly higher in Shunt compared with sham-operated control lambs (Table 2). The left pulmonary vascular resistance was significantly lower in Shunt compared with sham-operated control lambs (Table 2).

**Angiogenesis-related genes are differentially expressed in Shunt and control lambs.** Analysis of gene expression changes in 3-day Shunt lambs compared with age-matched sham-operated control lambs revealed significant differences in expression of 115 angiogenesis-related genes by >1.2-fold change (P < 0.05). Among these, 89 genes were upregulated and 26 genes were downregulated in Shunt lambs. The majority of the upregulated angiogenesis-related genes were proangiogenic (81/89; Table 3). The downregulated angiogenesis-related genes comprised both antiangiogenic genes (9/26; Table 4) and proangiogenic genes (17/26; Table 4).

**Validation of candidate genes by quantitative real-time RT-PCR and Western blot analysis.** Four genes were chosen for further validation. Ccl2 is the top upregulated gene on the microarray list and an important chemokine in the inflammatory processes that are increasingly recognized as major pathogenic components of pulmonary vascular remodeling. Angpt2 is the fourth most upregulated gene on the microarray list and plays important roles in angiogenesis and vascular remodeling. Fgf2 is a key player in angiogenesis and ranked eleventh on the upregulated gene list. Moreover, we found previously that FGF2 is upregulated in the Shunt lambs (10). Birc5 is an antiapoptotic protein and plays important roles in pulmonary hypertension as well as cancer angiogenesis. However, whether flow regulates the expression of this gene has not been identified. Utilizing quantitative real-time RT-PCR (qRT-PCR) we confirmed the upregulation at the mRNA level of FGF2 (2.1-fold; Fig. 1A), Angpt2 (2-fold; Fig. 1B), Birc5 (1.7-fold; Fig. 1C), and ccl2 (4.4-fold; Fig. 1D) in the Shunt lambs. Similarly, we confirmed that the protein levels of FGF2 (4.9-fold; Fig. 2A), Angpt2 (3.3-fold; Fig. 2B), and Birc5 (6.7-fold; Fig. 2C) were all significantly increased in the Shunt lambs. However, we were unable to detect a band for ccl2 protein, possibly because of a lack of cross-species reactivity.

**Table 2. Hemodynamics data for 3-day sham-operated control and shunted lambs**

<table>
<thead>
<tr>
<th></th>
<th>3-day Control</th>
<th>3-day Shunt</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAP mean, mmHg</td>
<td>17 ± 3</td>
<td>20 ± 4</td>
</tr>
<tr>
<td>LAP mean, mmHg</td>
<td>1 ± 3</td>
<td>3 ± 1*</td>
</tr>
<tr>
<td>Systolic BP, mmHg</td>
<td>64 ± 18</td>
<td>59 ± 18</td>
</tr>
<tr>
<td>Diastolic BP, mmHg</td>
<td>26 ± 8</td>
<td>19 ± 6</td>
</tr>
<tr>
<td>Mean BP, mmHg</td>
<td>37 ± 9</td>
<td>32 ± 6</td>
</tr>
<tr>
<td>Flow, mg·min⁻¹·kg⁻¹</td>
<td>64 ± 23</td>
<td>133 ± 35*</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>156 ± 16</td>
<td>148 ± 8</td>
</tr>
<tr>
<td>QCO₂, ml/min</td>
<td>1.0 ± 0.2</td>
<td>3.0 ± 1.2*</td>
</tr>
<tr>
<td>LPVR, mmHg·m⁻¹·min⁻¹·kg⁻¹</td>
<td>0.3 ± 0.1</td>
<td>0.1 ± 0.1*</td>
</tr>
<tr>
<td>pH</td>
<td>7.28 ± 0.07</td>
<td>7.36 ± 0.05</td>
</tr>
<tr>
<td>Paco₂, Torr</td>
<td>41 ± 7</td>
<td>36 ± 7</td>
</tr>
<tr>
<td>Paco₂, Torr</td>
<td>38 ± 11</td>
<td>74 ± 12*</td>
</tr>
</tbody>
</table>

Values are means ± SD. PAP, pulmonary arterial pressure; LAP, left atrial pressure; RAP, right atrial pressure; BP, systemic blood pressure; Qp/Qs, pulmonary-to-systemic blood flow ratio; LPVR, left pulmonary vascular resistance; Paco₂, arterial Pco₂; Paco₂, arterial Pco₂. *P < 0.05 compared with sham-operated control lambs.
### Table 3. Upregulated angiogenesis-related genes in 3-day Shunt lambs

<table>
<thead>
<tr>
<th>Probe Set ID</th>
<th>Gene Symbol</th>
<th>Fold Change (Shunt/Control)</th>
<th>P Value</th>
<th>FDR</th>
<th>Gene Name</th>
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<tbody>
<tr>
<td>Bt.2408.1.S1_at</td>
<td>CCL2</td>
<td>2.05</td>
<td>0.0066</td>
<td>0.23</td>
<td>Chemokine (C-C motif) ligand 2</td>
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<tr>
<td>Bt.2408.1.S2_at</td>
<td>CCL2</td>
<td>1.68</td>
<td>0.0140</td>
<td>0.27</td>
<td>Chemokine (C-C motif) ligand 2</td>
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<tr>
<td>Bt.149.1.S1_at</td>
<td>IGFBP2</td>
<td>1.63</td>
<td>0.0001</td>
<td>0.06</td>
<td>Insulin-like growth factor binding protein 2</td>
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<td>Bt.2717.1.A1_at</td>
<td>FAP</td>
<td>1.59</td>
<td>0.0121</td>
<td>0.26</td>
<td>Fibroblast activation protein, α</td>
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<td>Bt.137.1.S1_at</td>
<td>ANGPT2</td>
<td>1.58</td>
<td>7.61E-05</td>
<td>0.06</td>
<td>Angiopoietin2</td>
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<td>Bt.4844.1.S1_at</td>
<td>PTN</td>
<td>1.56</td>
<td>0.0025</td>
<td>0.20</td>
<td>Pleiotrophin</td>
</tr>
<tr>
<td>Bt.1926.1.S1_at</td>
<td>SOX18</td>
<td>1.50</td>
<td>0.0025</td>
<td>0.20</td>
<td>SRY (sex determining region Y)-box 18</td>
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<td>Bt.2177.1.A1_at</td>
<td>ELTD1</td>
<td>1.46</td>
<td>1.36E-05</td>
<td>0.03</td>
<td>EGF, latrophilin and seven transmembrane domain containing 1</td>
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<td>Bt.11942.1.S1_at</td>
<td>COL18A1</td>
<td>1.46</td>
<td>0.0005</td>
<td>0.12</td>
<td>COL18A1 collagen, type XVIII, α1</td>
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<tr>
<td>Bt.2799.1.S1_at</td>
<td>HMRM</td>
<td>1.45</td>
<td>0.0033</td>
<td>0.21</td>
<td>Hyaluronan-mediated motility receptor</td>
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<td>Bt.27854.1.S1_at</td>
<td>ADAM12</td>
<td>1.41</td>
<td>0.0130</td>
<td>0.26</td>
<td>ADAM metallopeptidase domain 12</td>
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<td>Bt.20348.1.A1_at</td>
<td>HEYL</td>
<td>1.41</td>
<td>0.0003</td>
<td>0.11</td>
<td>Hairy/enhancer-of-split related with YRPW motif-like</td>
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<td>FLI1</td>
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<td>4.58E-05</td>
<td>0.06</td>
<td>Friend leukemia virus integration 1</td>
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<td>Bt.3014.1.A1_at</td>
<td>PDGFC</td>
<td>1.31</td>
<td>0.0003</td>
<td>0.11</td>
<td>Platelet-derived growth factor C</td>
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<td>NOV</td>
<td>1.32</td>
<td>0.0004</td>
<td>0.12</td>
<td>Nephroblastoma overexpressed</td>
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<td>Bt.15707.1.S1_at</td>
<td>ITGA5</td>
<td>1.33</td>
<td>0.0002</td>
<td>0.08</td>
<td>Integrin, αV (vitronectin receptor, α polypeptide, antigen CD51)</td>
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<td>ITGAV</td>
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<td>Gap junction protein, γL</td>
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<td>0.0002</td>
<td>0.08</td>
<td>Axial skeleton protein, α</td>
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<td>AXL</td>
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<td>0.0001</td>
<td>0.08</td>
<td>AXL receptor tyrosine kinase</td>
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<td>Bt.272.1.S1_at</td>
<td>PIM1</td>
<td>1.34</td>
<td>0.0103</td>
<td>0.26</td>
<td>pim-1 oncogene</td>
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<tr>
<td>Bt.279.1.A1_at</td>
<td>CD14</td>
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<td>0.26</td>
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<td>0.12</td>
<td>Elastin</td>
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<td>Hyaluronan-mediated motility receptor</td>
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<td>0.06</td>
<td>WAP four-disulfide core domain 1</td>
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<tr>
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<td>MYC</td>
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<td>0.26</td>
<td>v-myc myelocytomatosis viral oncogene</td>
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<td>0.35</td>
<td>v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog</td>
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<td>0.0001</td>
<td>0.07</td>
<td>Fibroblast growth factor 2 (basic)</td>
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<td>MSR1</td>
<td>1.41</td>
<td>0.0029</td>
<td>0.21</td>
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<td>Bt.2799.1.S1_at</td>
<td>HMRM</td>
<td>1.45</td>
<td>0.0033</td>
<td>0.21</td>
<td>Hyaluronan-mediated motility receptor</td>
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<td>Bt.272.1.S1_at</td>
<td>PIM1</td>
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<td>Bt.17865.1.A1_at</td>
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<td>0.0293</td>
<td>0.32</td>
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Table 3.—Continued

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Angiogenesis-related genes were subclassified into pro- and antiangiogenic genes based on the PubMed database. For each gene, the Affymetrix probe set ID, gene symbol, fold change, P value, false discovery rate (FDR), and gene name are shown.

**Shear stress increases FGF2, Angpt2, Birc5, and ccl2 expression in pulmonary arterial endothelial cells.** The shear stress to which the vessel wall is exposed is proportional to the flow velocity (Q) according to the Hagen-Poiseuille law: wall shear stress (τ) = 4ρQ/πR², where ρ is viscosity and R is vessel radius (37). The PAEC in Shunt lambs are exposed to increased pulmonary blood flow, and therefore they are exposed to elevated shear stress (23). To determine whether elevated shear stress induced greater angiogenic gene expression we exposed PAEC to both physiological-level shear stress (20 dyn/cm²) and elevated shear stress (35 dyn/cm²) for 3 h and determined how this altered the expression of FGF2, Angpt2, Birc5, and ccl2. We found that elevated shear stress significantly increased FGF2 mRNA (1.9-fold; Fig. 3A) and protein (3-fold; Fig. 4A). We found a similar pattern for Angpt2 mRNA (1.5-fold; Fig. 3B) and protein (2.2-fold; Fig. 4B) and Birc5 mRNA (2.6-fold; Fig. 3C) and protein (1.8-fold; Fig. 4C) as well as ccl2 mRNA (2.2-fold; Fig. 3D).

Exposure of pulmonary arterial endothelial cells to elevated shear stress initiates proangiogenic phenotype. Next we determined whether PAEC exposed to the elevated shear stress displayed a proangiogenic phenotype by examining tube formation in Matrigel. In keeping with the increased production of proangiogenic factors (ccl2, FGF2, Angpt2, and Birc5), we found that PAEC preexposed to the elevated shear stress displayed significantly greater tube formation (2.75-fold; Fig. 5). Finally, we determined whether ccl2, FGF2, Angpt2, and Birc5 could be potential therapeutic targets in reversing the angiogenesis process. PAEC preexposed to 35 dyn/cm² shear were seeded onto Matrigel in the presence or absence of neutralizing antibodies to FGF2, Angpt2, or Birc5, and the effect on shear stress-mediated tube formation was determined. While a control IgG had no significant effect on tube formation (Fig. 6, A, B, and F), the antibodies against FGF2 (Fig. 6, C and F), Angpt2 (Fig. 6, D and F), and Birc5 (Fig. 6, E and F) all significantly inhibited tube formation. Furthermore, we utilized an siRNA approach to silence ccl2, FGF2, Angpt2, or Birc5 expression in PAEC (Fig. 7, A–D). Utilizing this approach we also observed an attenuation in tube formation in PAEC exposed to elevated shear stress (Fig. 7, E–J). Together, these data suggest that ccl2, FGF2, Angpt2, and Birc5 are potential therapeutic targets for preventing and/or reversing the enhanced angiogenesis process in vivo.

**DISCUSSION**

In this study, we have identified a proangiogenic gene expression profile that occurs within 3 days of increased pulmonary blood flow. The proangiogenic gene expression profile precedes the angiogenic phenotype at 4 wk of age in our Shunt model of CHD (40, 51). Furthermore, we demonstrated that PAEC exposed to elevated shear stress displayed both angiogenic genotype and phenotype as evidenced by increased expression of FGF2, Angpt2, Birc5, and ccl2 as well as...
enhanced tube formation. Finally, we demonstrated that immunoneutralizing FGF2, Angpt2, and Birc5 or decreasing the expression of FGF2, Anagpt2, Birc5, or ccl2 with an siRNA approach significantly inhibited tube formation by PAEC in vitro, suggesting that these could be potential therapeutic targets in attenuating the pathological angiogenesis in pulmonary vascular remodeling in vivo.

PAH, regardless of the idiopathic type or secondary types (including the type secondary to CHD) is characterized by changes in pulmonary vascular tone (vasoconstriction) and pulmonary vascular remodeling (44, 52). While the former pathological change is relatively well known and is well targeted by clinical treatments, the latter is less understood and responds poorly to the current therapies (44, 52). Recent studies indicate that angiogenesis plays an important role in the pulmonary vascular remodeling of PAH secondary to CHD. Significant higher serum levels of VEGF, platelet-derived endothelial cell growth factor, and leptin have been observed in PAH patients with PAH (19).

Our aim was to delineate the gene expression profile that underlies the angiogenic phenotype in Shunt lambs. Since the angiogenic phenotype occurs between 1 and 4 wk of age in Shunt lambs (40, 51), an earlier time point (3 days) was adopted for our microarray studies. We found that 89 angiogenesis-related genes were overexpressed and 26 angiogenesis-related genes were reduced in expression in the 3-day Shunt lungs. We further subcategorized these into pro- and antiangiogenic genes based on the current PubMed database. While the preponderance of upregulated genes (91%) were proangiogenic, a significant percentage (35%) of the downregulated genes were antiangiogenic. Overall, this suggests that a proangiogenic gene expression profile is established within 3 days of exposure to increased pulmonary blood flow and precedes the proangiogenic phenotype observed at later stages (4 wk). It is also important to note that the angiogenic phenotype in the Shunt lambs is not sustained. By 8 wk of age, the vessel numbers in the lungs of the Shunt lambs are not different from those in age-matched control lambs (40). This suggests that the angiogenesis we have observed in the Shunt lambs at 4 wk of age is not well formed but rather is disorganized. It is also worth noting that one limitation of this study is the use of whole lung tissue for the microarray analysis; therefore the gene expression changes that were detected do not only reflect changes in the pulmonary vasculature. Thus it is possible that changes in expression that may occur in the lung epithelium, interstitial tissues, and the infiltrating cells could identify false positive changes in the microarray. Conversely, it is
pulsatile flow in addition to the increased laminar shear stress, nary blood flow may create eddies, oscillatory flow, and
be upregulated in vitro both by oscillatory shear stress and remodeling process (24). ccl2 has also been shown
induced by ccl2 may both contribute to the pulmonary vascular
pathogenesis of PAH. We speculate that the inflammatory
effect versus the angiogenic effect of ccl2 in
possible that we may be missing expression changes in vascu-
cells because of the noise introduced from other cell types.
We focused our interest on the proangiogenic genes and
factors in the Shunt lamb: ccl2 at the mRNA level and Angpt2,
Birc5, and FGF2 at both mRNA and protein levels. ccl2, also
known as the monocyte chemotactic protein-1 (MCP-1), is a
member of the family of C-C (or β) chemokines (16). ccl2 is
secreted by a variety of cell types including monocytes/mac-
phages and endothelial and smooth muscle cells. ccl2 regul-
ates the migration and infiltration of monocytes/macrophages
into tissues and plays important roles in the inflammatory
processes (16). ccl2 has also been shown to induce angiogen-
both in vitro and in vivo (33). Moreover, this effect seems
to be independent of its chemotactic activity and is mediated
via direct effects on the vascular endothelium (53). Previous
studies have shown that ccl2 levels are elevated in the plasma
and lung tissues of patients with idiopathic PAH (30, 54). ccl2
was also found to be overexpressed in the lungs of a rat model
of PAH induced by monocrotaline (MCT) injection (32). Our
data suggest that ccl2 may also play an important role in the
pulmonary vascular remodeling in PAH secondary to CHD. It
is also worth noting that it is difficult to weigh the importance
of the chemotactic effect versus the angiogenic effect of ccl2 in
the pathogenesis of PAH. We speculate that the inflammatory
cascade triggered by ccl2 and the pathological angiogenesis
induced by ccl2 may both contribute to the pulmonary vascular
remodeling process (24). ccl2 gene expression has been shown
to be upregulated in vitro both by oscillatory shear stress and
by high pulsatility flow (27, 36). As in vivo increased pulmo-
nary blood flow may create eddies, oscillatory flow, and
pulsatile flow in addition to the increased laminar shear stress,
these effects may combine to contribute to the ccl2 induction in
the Shunt lambs. Previous studies have demonstrated that
blocking ccl2 (with neutralizing antibodies or gene therapy)
attenuates PAH in the MCT model (29, 34). However, it is
unclear whether diminishing ccl2 will also have a positive
effect in PAH secondary to CHD or other types of PAH.
Angpt2 is a member of the angioptoin family (58). Angpt2
is generally considered as an antagonist for angiopoieta1
(angpt1), inhibiting angpt1-promoted Tie2 signaling, which is
critical for blood vessel maturation and stabilization (20, 25).
Angpt2 also modulates angiogenesis in a cooperative manner
with VEGF-A (28). It has been well established that angpt2
plays an important role in angiogenesis during the development
and growth of human cancers (28). Previous studies have also
demonstrated increased expression of angpt2 in MCT-induced
PAH (12) and in a piglet model of high pulmonary blood flow
(42). Interestingly, in the latter study 1 wk of shunt closure
casted Angpt2 expression to return to baseline, suggesting that
the gene expression changes are reversible rather than perma-
(42). It has also been reported that Angpt2 is induced by
oscillatory shear stress in human umbilical vein endothelial
cells, although there did not appear to be an effect between
low and high magnitudes of laminar shear (60). However, this
could be due to the fact that only modest levels of shear stress (5
dyn/cm² and 15 dyn/cm²) were utilized, both of which are within
the physiological range of shear stress in the major human arteries
(5–20 dyn/cm²) (18). Our results suggest that the overexpression
of angpt2 may also be involved in the pulmonary vascular remodel-
ing secondary to CHD, although the exact mechanisms and the
therapeutic potential remain to be defined.

Fig. 1. Peripheral lung mRNA levels of FGF2, Angiopoietin2, Birc5, and ccl2
in Shunt and sham-operated control lambs. Total RNA was isolated from the
peripheral lung tissues of 3-day Shunt or sham-operated control (CTL) lambs.
Messenger RNA levels for FGF2 (A), Angiopoietin2 (B), Birc5 (C), and ccl2
(D) were all significantly increased in Shunt lambs as determined by SYBR
Green real-time RT-PCR analyses. Values are means ± SE; n = 6–8. *P <
0.05 vs. sham-operated control lambs. All mRNA data were normalized to
β-actin mRNA levels.

Fig. 2. Peripheral lung protein levels of FGF2, Angiopoietin2, Birc5, and ccl2
in Shunt and sham-operated control lambs. Protein extracts (100 µg) prepared
from the peripheral lung tissues of 3-day Shunt or sham-operated control lambs
were subjected to Western blot analysis. The protein levels of FGF2, Angiopoietin2,
Birc5 (C), and ccl2 (D) were all significantly increased in Shunt lambs. A representative image is shown for each Western blot. Values are
means ± SE; n = 4–6. *P < 0.05 vs. sham-operated control lambs. All protein
levels were normalized by reprobing the membrane with an antibody to
β-actin.
The effect of shear stress on Birc5 gene expression has until now been unexplored, and our data identify Birc5 as a newly described shear stress-regulated gene. Birc5 (Survivin) is a member of the inhibitor of apoptosis protein (IAP) family. Birc5 is often found to be overexpressed in cancer tissues compared with normal tissues (2, 3). However, recent studies indicate that increased Birc5 expression may also be linked to the promotion of angiogenesis. Increased expression of Birc5

Fig. 3. Elevated shear stress increases FGF2, Angiopoietin2, Birc5, and ccl2 mRNA levels in cultured ovine pulmonary arterial endothelial cells (PAEC). PAEC were subjected to either 20 or 35 dyn/cm² of laminar shear stress for 3 h, and then total RNA was isolated and analyzed. Messenger RNA levels for FGF2 (A), Angiopoietin2 (B), Birc5 (C), and ccl2 (D) were all significantly higher in PAEC exposed to 35 compared with 20 dyn/cm² of laminar shear stress as determined by SYBR Green real-time RT-PCR analyses. Values are means ± SE; n = 6. *P < 0.05 vs. 20 dyn/cm² shear.

Fig. 4. Elevated shear stress increases FGF2, Angiopoietin2, and Birc5 protein levels in cultured ovine PAEC. PAEC were subjected to either 20 or 35 dyn/cm² of laminar shear stress for 3 h, and then total protein extracts were prepared and subjected to Western blot analysis. Twenty-five micrograms of protein was loaded to detect FGF2 and Birc5 protein expression, while 100 μg of protein was loaded for detection of Angiopoietin2 protein levels. Protein levels for FGF2 (A), Angiopoietin2 (B), and Birc5 (C) were all significantly higher in PAEC exposed to 35 compared with 20 dyn/cm² of laminar shear stress. A representative image is shown for each Western blot. Values are means ± SE; n = 4–6. *P < 0.05 vs. 20 dyn/cm² shear. All protein levels were normalized by reprobing the membrane with an antibody against β-actin.
has been found in newly formed microvessels within the injured brain of a mouse model of stroke (14). Mice with heterozygous deficiency for Birc5 also have reduced vessel density and decreased neovascularization (14). Delivery of Birc5 antisense oligonucleotides or siRNA to endothelial cells demonstrated that downregulation of Birc5 leads to decreased capillary formation (43). Previous studies have also shown abnormal expression of Birc5 in the lungs of humans with PAH as well as in the animal models of PAH (1, 41). Moreover, gene therapy selectively targeting Birc5 induces pulmonary vascular apoptosis and reverses PAH in rats (41). The efficacy of Birc5 antagonists in treating PAH is currently being tested in preliminary clinical studies (17). Corroborating these previous findings, our data show an early induction of Birc5 expression in Shunt lambs that precedes increased angiogenesis and vascular remodeling. In the future it may be worthwhile to test the efficacy of blocking Birc5 in treating the pulmonary vascular remodeling in patients with CHD.

FGF2 is a pleiotropic angiogenesis inducer belonging to the family of the heparin-binding FGF growth factors (47, 48). Increased lung and circulating FGF2 levels have been reported in both MCT-induced and human idiopathic PAH (4, 9, 31). We have previously reported persistent upregulation of FGF2 in Shunt lambs at 2 (59) and 4 (65) wk of age. FGF2 mRNA levels have also been shown to be increased to a greater extent in bovine aortic endothelial cells exposed to 36 dyn/cm² compared with 15 dyn/cm² (37). This strongly suggests that FGF2 is a key player in the pulmonary vascular remodeling process of PAH. FGF2 siRNA has shown promising effects in treating MCT-induced PAH (31), and our data suggest that FGF2 is a strong candidate for targeted therapy in PAH secondary to CHD. Furthermore, because we observe increased expression at 3 days we speculate that any interventions should be started as early as possible.

In addition to showing the induction of angiogenic genes in the Shunt lambs, we further confirmed in vitro that increased shear stress is a key mechanism in the upregulation of these angiogenic genes. Increased shear stress has been recognized as a major determinant in arteriogenesis (26). Increased pulmonary blood flow generates increased shear stress in the vessel wall, and we found that elevated shear stress induced more robust tube formation in vitro. This is consistent with previous reports that increased shear stress promoted angiogenesis both in vivo and in vitro (5, 49, 64). We further demonstrated that elevated shear stress dramatically increased

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**Fig. 5.** Ovine PAEC exposed to elevated shear stress exhibit an enhanced angiogenic response. PAEC were exposed to either 20 or 35 dyn/cm² of laminar shear stress for 3 h. The cells were then trypsinized, resuspended in complete media, and seeded onto 24-well Matrigel-coated plates. Capillary tube formation was documented 6 h after plating. PAEC exposed to 35 dyn/cm² shear stress displayed significantly greater tube formation compared with cells exposed to 20 dyn/cm² shear stress. Representative images are shown. Values are means ± SE; n = 4–6. *P < 0.05 vs. 20 dyn/cm² shear.

**Fig. 6.** Blockade of FGF2, Angiopoietin2, or Birc5 signaling inhibits the angiogenic response induced by elevated shear stress in cultured ovine PAEC. PAEC were exposed to 35 dyn/cm² laminar shear for 3 h. The cells were then trypsinized, resuspended in complete media, and seeded onto 24-well Matrigel-coated plates either alone (A) or simultaneously with a rabbit IgG (B) or neutralizing antibodies raised against FGF2 (C), Angpt2 (D), or Birc5 (E). Capillary tube formation was then documented 6 h after plating. Compared with the control, the rabbit IgG had no significant effect on tube formation, while the neutralizing antibodies against FGF2, Angpt2, and Birc5 all significantly inhibited tube formation (F). Representative images are shown. Values are means ± SE; n = 4–6. *P < 0.05 vs. control.
Fig. 7. Silencing ccl2, FGF2, Angiopoietin2 (Angpt2), or Birc5 gene expression attenuated the angiogenic response induced by elevated shear stress in cultured ovine PAEC. A–D: PAEC were grown to 70% confluence and then transfected with a control small interfering RNA (siRNA) or target siRNA at a concentration of 80 nM. Forty-eight hours after transfection, cells were harvested for real-time RT-PCR analysis (for ccl2) or Western blot analysis (for FGF2, Angpt2, and Birc5). Real-time RT-PCR using SYBR Green demonstrated that the ccl2 siRNA significantly decreases mRNA levels (A). Western blot analysis also confirmed the siRNA-mediated knockdown of FGF2 (B), Angpt2 (C), and Birc5 (D). Twenty-five micrograms of protein was loaded to detect FGF2 and Birc5 protein expression, while 100 μg of protein was loaded for detection of Angpt2 protein levels. A representative image is shown for each Western blot. Values are means ± SE; n = 3–6. *P < 0.05 vs. negative control siRNA. All protein levels were normalized by reprobing the membrane with an antibody against β-actin. PAEC transfected with either a control siRNA or siRNAs for ccl2, FGF2, Angpt2, or Birc5 for 48 h were then exposed to 35 dyn/cm² laminar shear for 3 h. The cells were subsequently trypsinized, resuspended in complete media, and seeded onto 24-well Matrigel-coated plates. Capillary tube formation was then documented 6 h after plating. Compared with the negative control siRNA (E), gene silencing of ccl2 (F), FGF2 (G), Angpt2 (H), or Birc5 (I) was sufficient to significantly attenuate tube formation (J). Representative images are shown for each transfection. Values are means ± SE; n = 3–6. *P < 0.05 vs. negative control siRNA.
FGF2, Angpt2, Birc5, and ccl2 gene expression. Shear stress has long been recognized as regulating the endothelial transcriptome (13, 15, 60). Promoter sequences termed shear stress response elements (SSREs) mediate the responsiveness of endothelial genes to shear stress (55). Our microarray data demonstrate that increased pulmonary blood flow induces a repertoire of gene expression changes. In addition, increasing the shear magnitude in vitro also modulated the expression of multiple genes. However, it is unclear whether these responses are mediated through a common SSRE, through individual SSREs, or through other cis elements. It is well known that multiple genes and signaling pathways are involved in the pathogenesis of PAH, and blocking only individual signaling pathways may only partially alleviate the disease. Thus it will be important in future studies to determine whether there is a common SSRE that is responsible for the multiple gene expression changes in response to the increased pulmonary blood flow. If this is the case, it opens up the possibility that blocking the activation of this SSRE could enhance the therapeutic efficiency in treating high pulmonary flow-induced PAH.

In conclusion, we have identified a “proangiogenic” gene expression profile that occurs before overt pulmonary vascular remodeling in our lamb model of PAH with increased pulmonary blood flow. The angiogenic gene profile we have identified could lead to the development of novel therapeutic targets for treating the pulmonary vascular remodeling that occurs secondary to CHD.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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