# Pulmonary Artery Endothelial Cell Phenotypic Alterations in a Large Animal Model of Pulmonary Arteriovenous Malformations After the Glenn Shunt

CONGENITAL HEART

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*Background.* **Longevity of the superior cavopulmonary connection (SCPC) is limited by the development of pulmonary arteriovenous malformations (PAVM). The goal of this study was to determine whether phenotypic changes in pulmonary artery endothelial cells (PAEC) that favor angiogenesis occur with PAVM formation.**

*Methods.* **A superior vena cava to right pulmonary artery connection was constructed in 5 pigs. Pulmonary arteries were harvested at 6 to 8 weeks after surgery to establish cultures of PAEC and smooth muscle cells, to determine cell proliferation, gene expression, and tubule formation. Abundance of proteins related to angiogenesis was measured in lung tissue.**

*Results.* **Contrast echocardiography revealed right-to-left shunting, consistent with PAVM formation. While the proliferation of smooth muscle cells from the right pulmonary artery (shunted side) and left pulmonary artery (nonshunted side) were similar, right PAEC proliferation was significantly higher. Expression profiles of genes encoding cellular signaling proteins were higher in PAECs from the right pulmonary artery versus left pulmonary artery. Protein abundance of angiopoietin-1, and Tie-2 (angiopoietin receptor) were increased in the right lung (both $p < 0.05$). Tubule formation was increased in endothelial cells from the right pulmonary artery compared with the left pulmonary artery (404 ± 16 versus 199 ± 71 tubules/mm$^2$, respectively; $p < 0.05$).**

*Conclusions.* **These findings demonstrate that PAVMs developed in a clinically relevant animal model of SCPC concomitantly with differential changes in PAEC proliferative ability and phenotype. Moreover, there was a significant increase in the angiopoietin/Tie-2 complex in the right lung, which may provide novel therapeutic targets to attenuate PAVM formation after a SCPC.**

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The superior cavopulmonary connection (SCPC) has been shown to reduce mortality in infants with single ventricle disease before the Fontan procedure [1]. Importantly, some infants who remain palliated by a SCPC fare better than after a Fontan [2], which often demonstrates significant late failures [3]. However, the durability of the SCPC is significantly limited owing to progressive cyanosis observed secondary to the development of pulmonary arteriovenous malformations (PAVM) [4]. Therefore, further understanding of factors that contribute to PAVM development may lead to therapies that improve outcomes for these infants.

PAVMs develop because of abnormalities in angiogenesis secondary to changes in blood flow patterns and alterations in the circulating milieu of proangiogenic and antiangiogenic factors [5]. The SCPC involves directing venous return from the superior vena cava to one (classic Glenn shunt) or both (bidirectional SCPC) lungs. This results in a loss of pulsatile flow through the pulmonary arteries (PA), and a change in shear stress and pressure loading conditions on the pulmonary vascular endothelium [6], which may alter the cellular phenotype of pulmonary artery endothelial cells (PAEC) [7]. In addition, the redirection of venous return with a SCPC is associated with a loss of hepatic effluent to the lung, which has been proposed as a mechanism for PAVM formation after SCPC [4]. Hepatic effluent

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**Abbreviations and Acronyms**

| | |
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| AVM | = arteriovenous malformations |
| Ct | = cycle threshold |
| EC | = endothelial cell |
| ECM | = extracellular matrix |
| HIF | = hypoxia inducible factor |
| LPA | = left pulmonary artery |
| PA | = pulmonary artery |
| PAEC | = pulmonary artery endothelial cell |
| PAVM | = pulmonary arteriovenous malformation |
| RPA | = right pulmonary artery |
| SCPC | = superior cavopulmonary connection |
| SMC | = smooth muscle cell |
| VEGF | = vascular endothelial growth factor |

contains a number of proteins that can promote or inhibit angiogenesis, or both [8]. Hepatic stellate cells secrete angiopoietins, which bind to specific receptors on endothelial cells (EC) and promote angiogenesis [9]. Conversely, plasminogen, produced by hepatocytes, is the precursor of angiostatin, a potent inhibitor of angiogenesis [10]. However, it remains unknown whether and to what degree these alterations in blood flow patterns or loss of hepatic effluent contribute to PAVM development.

Using a porcine model of unidirectional SCPC, we tested the hypothesis that PAVM formation occurs coincident with changes in the phenotypic and functional characteristics of PAEC or smooth muscle cells (SMC) or both, with the emergence of a proangiogenic signal or the loss of a preexisting antiangiogenic signal.

## Material and Methods

A unidirectional SCPC was constructed in 5 female pigs (weight 27 kg to 31 kg) through a right thoracotomy. The superior vena cava and right pulmonary artery (RPA) were mobilized and the RPA was temporarily clamped and then completely divided at the bifurcation. The proximal end was sutured close and the distal end anastomosed to the superior vena cava using a 10 mm to 12 mm Gore-Tex (Gore Medical, Flagstaff, AZ) interposition graft (necessary due to unique porcine PA anatomy). This model results in a lack of pulsatile flow to the RPA and antegrade pulsatile flow to the left pulmonary artery (LPA). The left lung and LPA served as contralateral controls for the right lung and RPA. The lungs and PAs from 2 age-matched pigs were harvested and used as referent control specimens, which were used to account for changes in unknown factors that could influence the phenotype of PAECs from the LPA. Hemodynamic measurements including oxygen saturation was measured at baseline and during the terminal procedure. Animals were treated and cared for in accordance with the National Institutes of Health "Guide for the Care and Use of Laboratory Animals" (National Research Council, Washington, 1996), and the Institutional Animal Care and Use Committee approved the protocol.

At 6 to 8 weeks after SCPC, contrast echocardiography and angiography were performed to ascertain PAVM formation. Right and left lung biopsies were obtained at each terminal experiment (n = 7, 5 pigs with a SCPC and 2 referent controls), and stored in formalin for immunohistochemistry or flash frozen.

The PAEC and SMC were isolated from the RPA (RPA/SCPC side; n = 2 PAEC, n = 3 SMC) and left (LPA/non-shunted side; n = 3 PAEC, n = 3 SMC) from the SCPC pigs. In addition, PAEC and SMC were isolated from the RPA (n = 2 PAEC, n = 2 SMC) and LPA (n = 2 PAEC, n = 2 SMC) from the referent controls. Each cell line was assayed in triplicate and values for each pig were averaged to form a single data point [11]. Briefly, the PAs were opened and the luminal surface scraped into porcine EC growth media (Cell Applications, San Diego, CA). The SMC from the PAs were cultured using the outgrowth technique [12]. The PAs were minced into 2 mm × 2 mm pieces and cultured in Smooth Muscle Growth Media 2 (Promocell, Heidelberg, Germany) containing 20% FBS (Life Technologies, Grand Island, NY). The cells were incubated at 37°C, 5% $CO_2$ (21% $O_2$) and allowed to propagate until time of studies. All cell cultures were analyzed in early passages three through eight.

### *Cell Proliferation Assay*

Cell proliferation was quantified utilizing the CyQuant Assay (Life Technologies). The PAEC and SMC were seeded in a 96-well plate (Corning Inc, Corning, NY) at a density of $5 \times 10^3$ cells per well. Cells were then stained with 0.004% CyQuant Direct nucleic stain in phosphate-buffered saline and a direct background suppressor (0.02%) for 1 hour at 37°C with 5%$CO_2$. Fluorescence (excitation/emission: 480/535 nm), which reflected the number of cells, was measured at standard timepoints of 24, 30, 44, 54, and 68 hours after seeding and expressed as a change in cell number from the 24-hour time point [13].

### *Tubule Formation Assay*

Tubule formation assay was performed to determine a functional phenotype for the EC [11]. The PAEC ($1.2 \times 10^5$ cells per well) were added to 24-well plates coated with basement membrane matrix (298 μL Matrigel, 9.2 mg/ml per well [BD Biosciences, San Jose, CA]). Cells were incubated at 37°C for 3 hours and imaged (2.5× objective; Zeiss Axiovert, Gottingen, Germany). Number of tubules, length of the tubules, and nodes at which the tubules originated were identified using a tracking algorithm and quantified by image analysis (SigmaScan Pro 5; Systat Software, San Jose, CA).

### *Quantitative Polymerase Chain Reaction*

The PAEC from the LPA and RPA from SCPC animals and controls were denatured to extract RNA, which was reverse transcribed to generate cDNA ($RT^2$ First Strand Kit; Qiagen, Hilden, Germany). The cDNA was stored

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