Early Determinants of Pulmonary Vascular Remodeling in Animal Models of Complex Congenital Heart Disease

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In this article, we review the current state of knowledge about early changes in the pulmonary vasculature that result from persistent systemic-to-pulmonary arterial shunting in newborn lambs. Data generated in this model system may be important in children with various forms of congenital heart disease (CHD), but perhaps most so in those with single-ventricle anomalies. Children born with a functional single ventricle (eg, hypoplastic left heart syndrome, tricuspid atresia, or pulmonary atresia) represent a subgroup of patients with CHD with the poorest outcome, with 5-year mortality rates up to 50%. Although functionally single-ventricle heart disease comprises many structural variants, a shared physiological feature is that both systemic and pulmonary circulations are supplied in parallel by a functionally single pumping chamber. Immediately after birth, the pulmonary vasculature of these infants is exposed to abnormal conditions, such as increased flow or pressure. Over time, if these abnormal forces are not modified, they can lead to progressive functional and morphological abnormalities in the pulmonary vasculature that are characterized by altered reactivity, increased resistance, and structural alterations (remodeling). Clinically, these abnormalities can have an important impact on surgical options and perioperative outcome. The current approach to neonates with a functional single ventricle is to establish a controlled, low-pressure source of pulmonary blood flow that facilitates systemic oxygen delivery sufficient to permit somatic growth and development without excessive ventricular volume loading, typically by means of a surgical systemic-to-pulmonary artery or ventricle-to-pulmonary artery conduit or by restricting the main or branch pulmonary arteries. The pulmonary-to-systemic blood flow balance can be difficult to achieve, and the infant runs the gauntlet between pulmonary blood flow that is too low (resulting in chronic hypoxemia and the potential consequences thereof) or too high (potentially leading to chronic heart failure, compromised systemic blood flow, and abnormal pulmonary vascular remodeling). Because subsequent stages of surgical palliation (bidirectional or partial cavopulmonary connection, followed by Fontan-type surgery or total cavopulmonary connection) result in passive pulmonary blood flow in the absence of a pumping ventricle, a normal low pulmonary vascular resistance is critical for maintenance of low central venous pressure, overall health, and long-term survival. The underlying mechanisms by which some patients with single-ventricle physiology experience increased pulmonary vascular resistance that results in failed surgical palliation whereas others do not remain unclear. We speculate that these differences in outcome are related to early, clinically undetectable pulmonary vascular abnormalities. For example, age at surgery correlates with increased transpulmonary gradient postoperatively in infants with a functional single ventricle undergoing and surviving partial cavopulmonary connection (Figure 1). This may be because of the longer duration over which the pulmonary vasculature was exposed to increased flow. Figure 1 suggests that a relatively large surgical shunt with relatively high pulmonary blood flow (thereby allowing for an older age at partial cavopulmonary connection) may lead to a subtly higher transpulmonary gradient (which suggests higher pulmonary vascular resistance) that may be detrimental in the long-term. This suggestion remains speculative because it is unknown whether subtly increased resistance leads to demonstrably worse outcome. However, it is likely that there are subtle differences in signaling pathways or gene-expression variations between patients. These signaling pathways, acted on by the increases in pulmonary blood flow, can then lead to subtle differences in pulmonary vascular resistance that dramatically affect the surgical outcome. The underlying pathways in these newborns with single-ventricle heart disease are only just beginning to be resolved. In this review, we will focus on the work over the last decade that has begun to elucidate the molecular pathways that appear to be important in the initial pulmonary
vascular remodeling in newborns with single-ventricle heart disease.

**Lamb Model of Neonatal Increased Pulmonary Blood Flow**

Although many animal models have been used to study increased blood flow or pressure on the pulmonary vasculature, most models use older or adult animals. Therefore, these models neglect the initial postnatal effects of increased blood flow or pressure on the developing pulmonary vasculature during the natural course of postnatal transition from fetal to adult pulmonary blood flow. A model to study the initial onset of pulmonary vascular remodeling associated with single-ventricle physiology needs to have a large aorto-pulmonary shunt from birth so that the natural course of postnatal transition from fetal to adult pulmonary blood flow can be considered in the pathophysiology. To the best of our knowledge, the only existing animal model that can be used to study the initial onset of neonatal pulmonary vascular remodeling associated with single-ventricle physiology is the neonatal lamb model with an aortopulmonary shunt placed in utero (Figure 2). This model does have its limitations as a true mimic of single-ventricle physiology. The shunt model allows studies into the effects of increased pulmonary blood flow and pressure on pulmonary vascular remodeling observed in neonates with single-ventricle physiology. Nevertheless, it is the best model available for these types of investigations, and over the last decade, this model has been used extensively to study the early sequential evolution of pulmonary vascular remodeling mimicking the pathophysiologically relevant elements of neonatal pulmonary blood flow in single-ventricle heart disease. In the following sections, we will briefly describe the current knowledge regarding the well-described nitric oxide (NO) and endothelin-1 (ET-1) signaling pathways, as well as newly emerging pathways that may have potential for therapeutic intervention.

**Established Signaling Pathways**

**The NO-cGMP Signaling Cascade**

NO production by the vascular endothelium is integral to the maintenance of the low-resistance state of the pulmonary vasculature, and dynamic alterations in NO production modulate vascular relaxation and constriction in response to various stimuli. Once formed, NO diffuses into vascular smooth muscle cells, where it activates soluble guanylate

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**Figure 1.** Retrospective analysis of 69 consecutive patients younger than 15 months (subgroup from study by Cleuziou et al4) with single-ventricle physiology who underwent and survived partial cavopulmonary connection (PCPC). The x-axis depicts age at PCPC, in months; y-axis depicts transpulmonary gradient, defined as left atrial pressure minus pulmonary artery pressure during heart catheterization before total cavopulmonary connection (TCPC) surgery.

**Figure 2.** Representative hematoxylin-and-eosin stains of peripheral lung fixed in 4% paraformaldehyde and sectioned at 7 μm from 4-week control (left) and shunt (right) lambs. Note the increase in medial thickness in the shunt lambs. Magnification ×20.
gas18 and therapies aimed at enhancing NO signaling have
also been found in children with CHD and increased
polymorphism has been associated with decreased NO
metabolites.7,13 A decreased NO and ET-1 signaling in
newborns with CHD is temporally related to severe
vascular remodeling.9,10 Decreases in ET-1 levels have
also been found in children with advanced forms of
pulmonary vascular remodeling.20,31 However, the fact that
ETB receptors have returned to control levels, but its
localization is altered, being predominantly on the smooth
muscle cells, where it then mediates vasoconstriction.
Furthermore, pulmonary blood pressure and not flow is
associated with net ET-1 production in patients with CHD
and normal pulmonary vascular resistance.29,30 As with the
NO-cGMP signaling pathway, shunt lambs exhibit temporal
alterations in the ET-1 cascade. As shown in Table 1, within
1 week of life, there is an enhanced ET-1-mediated signaling in
shunt lambs, whereas at 4 weeks of age, the enhanced
circulating levels of ET-1 lead to increased vasoconstriction
through increases in the expression of ETB receptor and
decreases in ETB receptor. By 2 months of age, expression of
the ETB receptor has returned to control levels, but its
localization is altered, being predominantly on the smooth
muscle cells, where it then mediates vasoconstriction.
Therapies aimed at decreasing the vasoconstrictor and prolifera-
tive effects of ET-1 are being pursued for newborns with
increased pulmonary resistance.20,31 However, the fact that
there are changes in the expression and localization of ET
receptors in shunt lambs during the temporal progression of
both endothelial dysfunction and vascular remodeling means
that the correct receptor antagonist strategy (ETA versus ETB
versus dual) is still controversial. As Table 1 suggests, the
best therapeutic option may depend on where the patient is in
the disease process.

**Table 1. Developmental Changes in NO and ET-1 Signaling in Shunt Lambs**

<table>
<thead>
<tr>
<th></th>
<th>1 Week</th>
<th>2 Weeks</th>
<th>4 Weeks</th>
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<td>↑↑</td>
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<tr>
<td>Relative NOS activity</td>
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<td>↓↓↓</td>
<td>↓↓↓</td>
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<tr>
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<td>↑</td>
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<tr>
<td>Nitrated eNOS</td>
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<td>↑</td>
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<td>Plasma ET-1</td>
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<td>ND</td>
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<tr>
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<td>ECE-1 mRNA</td>
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<tr>
<td>ECE-1 protein levels</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
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<tr>
<td>ETα receptor protein levels</td>
<td>←←</td>
<td>←←</td>
<td>↑</td>
<td>↑</td>
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<tr>
<td>ETβ receptor protein levels</td>
<td>↓↓↓</td>
<td>ND</td>
<td>↓↓↓</td>
<td>↓↓↓</td>
</tr>
</tbody>
</table>

NOS indicates NO synthase; NOx, nitrogen oxide; ND, not determined; Hsp90, 90-kD heat shock protein; sGC, soluble guanylate cyclase; PDE5, phosphodi-
esterase type 5; BNP, brain natriuretic peptide; ET, endothelin; ECE-1, endothelin-converting enzyme-1; ←←, no change; ↓↓↓, mildly decreased; ↓↓↓, moderately decreased; ↓↓↓, severely decreased; ↑↑, mildly increased; ↑↑↑, moderately increased; and ↑↑↑↑, severely increased.

cyclase, which leads to increases in the second messenger,
cGMP. The resulting activation of cGMP-dependent protein
kinase in the pulmonary vascular smooth muscle layer leads
to a decrease in intracellular calcium and relaxation of the
vessel.8 Extensive temporal investigations in the shunt model
over the first 2 months of life have shown that there is a
complex pattern of regulation in the NO-cGMP signaling
cascade that occurs both before and after overt alterations
in endothelial function or vascular remodeling. As shown in
Table 1, over the first 2 months of life, there is a
progressive loss of NO signaling. Loss of NO signaling is
also found in humans with advanced forms of pulmonary
vascular remodeling.9,10 Decreases in NO metabolites have
also been found in children with CHD and increased
pulmonary blood flow,11,12 as well as after cardiopulmo-
nary bypass.13,14 Interestingly, a genetic polymorphism
(894G→T) has also been identified with CHD.15 This
polymorphism has been associated with decreased NO
generation in humans.16,17 Accordingly, inhalation of NO
gas18 and therapies aimed at enhancing NO signaling have
already become a common treatment strategy for newborns
with increased pulmonary resistance.19,20

**The Endothelin System**
The hemodynamic effects of ET-1 are mediated by at least 2
distinctive receptor populations, ETα and ETβ, the densities
of which are different depending on the vascular bed studied.
ETα receptors are located on vascular smooth muscle cells
and mediate vasoconstriction, whereas ETβ receptors are
located on endothelial cells and mediate vasodilation.7,21,22 In
addition, a second subpopulation of ETβ Receptors is located
on smooth muscle cells and mediates vasoconstriction.23 The
vasodilating effects of ET-1 are thought to be associated with
the release of NO and potassium channel activation.22,24,25
The vasoconstricting effects of ET-1 are associated with
phospholipase activation; the hydrolysis of phosphoinositol
to inositol 1,4,5-triphosphate, and diacylglycerol; and the
subsequent release of Ca2+.26 In addition to its vasoactive
properties, ET-1 is mitogenic for pulmonary arterial smooth
muscle cells via ETα receptor–mediated superoxide generation
and therefore may participate in vascular remodeling.27
Patients with advanced forms of pulmonary vascular remodel-
ing have increased lung and circulating ET-1 levels.28
Furthermore, pulmonary blood pressure and not flow is
associated with net ET-1 production in patients with CHD
and normal pulmonary vascular resistance.29,30 As with the
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best therapeutic option may depend on where the patient is in
the disease process.

**Emerging Signaling Pathways**
Recent studies in shunt lambs have identified 5 signaling
pathways that may represent therapeutic targets: Peroxisome
proliferator–activated receptor (PPAR)-γ, arginine metabo-
lism, oxidative and nitrosative stress, thrombin, and carnitine.
Furthermore, microarray analyses have identified new classes
of genes that appear to be associated with early pulmonary
vascular remodeling and may prove to be amenable to
therapeutic interventions.

**Peroxisome Proliferator–Activated Receptors**
PPARs are ligand-activated transcription factors that belong
to the nuclear hormone receptor family. Recent evidence has
established a role for altered PPAR signaling in the develop-
ment of both systemic and pulmonary vascular disease.32
Recent experimental studies suggest that the PPAR-γ isoform may have a role in advanced pulmonary hypertension. PPAR-γ signaling is attenuated in shunt lambs. A microarray analysis has been performed in ovine pulmonary endothelial cells exposed to the PPAR-γ inhibitor GW9662 to mimic the loss of PPAR-γ signaling. This analysis identified more than 100 genes that were either upregulated or downregulated (Table 2). The upregulated genes are broadly classified into 4 categories: Cell cycle–related genes, angiogenesis-related genes, ubiquitin-related genes, and zinc finger proteins. Furthermore, these in vitro results were confirmed in the shunt lamb by Western blot analysis. Therefore, potential targets that may be good candidates for therapeutic intervention in patients with early neonatal pulmonary vascular remodeling associated with single-ventricle physiology may soon be identified.

### Arginine Metabolism

L-Arginine bioavailability plays a key role in the generation of NO in the pulmonary vasculature. L-Arginine is actively transported into endothelial cells through the cationic amino acid transporter-1, where it is then used as a substrate by endothelial NO synthase (eNOS) to form NO and L-citrulline or metabolized by arginase to form urea and ornithine. Thus, eNOS and arginase are in direct competition for L-arginine. Furthermore, these in vitro results were confirmed in the shunt lamb by Western blot analysis. Therefore, potential targets that may be good candidates for therapeutic intervention in patients with early neonatal pulmonary vascular remodeling associated with single-ventricle physiology may soon be identified.

### Oxidative and Nitrosative Stress

Excessive production of reactive oxygen species (ROS), outstripping endogenous antioxidant defense mechanisms, has been implicated in many disease processes of the vasculature. Decreases in bioavailable NO can be related to increases in oxidative stress. In shunt lambs, oxidative stress occurs secondary to both increased production of ROS and decreases in scavenging. The source of the increased superoxide is multifactorial, involving the upregulation of vascular xanthine oxidase and NADPH oxidase and an uncoupling of eNOS. The mechanisms underlying the uncoupling of eNOS (ie, the consumption of NADPH becomes uncoupled from NO synthesis) are also complex and involve decreases in L-arginine and alterations in biopterin metabolism. Superoxide and other ROS have also been shown to have a signaling function in vascular cells that promotes hypertrophy, proliferation, migration, matrix remodeling, and even the formation of new vessels. The activation of NADPH oxidases with subsequent ROS production has been associated with increased proliferation of pulmonary artery smooth muscle cells isolated from both sheep and humans.

Both NO and superoxide possess unpaired electrons and react rapidly to form the reactive nitrogen species peroxynitrite (ONOO−), which decreases NO bioavailability. In addition, ONOO− can exert effects through the nitration of protein tyrosine residues. Tyrosine nitration, a covalent modification that adds a nitro group (−NO2) to the ortho carbon of the phenolic ring, yields 3-nitrotyrosine. The nitration of tyrosine residues to form 3-nitrotyrosine is widely used as a marker of ONOO− formation, and recent studies have shown that there is a temporal increase in protein nitration in the lungs of shunt lambs. Furthermore, eNOS itself is susceptible to nitration, and mass spectroscopy studies have demonstrated that these nitrated residues are in regions of the protein that are important to its function. Thus, the potential of antioxidant therapies to reduce both ROS and reactive nitrogen species formation has been postulated for the treatment of early neonatal pulmonary vascular remodeling associated with single-ventricle physiology. However, these studies highlight the limitation of current investigations: The lack of easily available means to identify modified proteins and residues. This is a roadblock in understanding the potential mechanistic contribution of these modifications, and it is imperative that there be a greater focus on identifying the individual tyrosine residues targeted by nitration and the effect these nitration events have on the structure-function relationship of the protein. Only when these goals have been met will it be possible to develop directed therapies.

### Thrombin

Recent studies have elucidated a novel positive-feedback loop in which thrombin increases ROS levels via the consecutive activation of protease activated receptor 1 (PAR1), Rac1, and p21-activated kinase. This also results in activation of the transcription factor nuclear factor-κB, which in turn leads to activation of hypoxia-inducible factor-1α. Under these conditions, hypoxia-inducible factor-1 target genes, such as plasminogen activator inhibitor-1 or vascular...
endothelial growth factor, are upregulated. The upregulation of plasminogen activator inhibitor-1, which is the primary physiological inhibitor of tissue plasminogen activator and urokinase plasminogen activator, can then lead to reductions in fibrin clearance. In addition, increases in plasminogen activator inhibitor-1 can promote pulmonary artery smooth muscle cell proliferation. Together, these antifibrinolytic and growth-modulating activities have an immediate impact on pulmonary vascular remodeling, and p21-activated kinase levels are elevated in remodeled pulmonary vessels in patients with CHD and pulmonary vasculopathy. There appears to be a secondary feed-forward loop activated by hypoxia-inducible factor-1 that leads to further increases in the expression of Rac1 and p21-activated kinase, producing enhanced cytoskeletal remodeling and proliferation of pulmonary artery smooth muscle cells, which further contributes to medial hypertrophy and vascular remodeling. As part of this feed-forward mechanism, ROS levels are stimulated because of the increased abundance of Rac1 and p21-activated kinase, which produces sustained activation of the hypoxia-inducible factor-1 pathway and increased pulmonary vascular remodeling. This novel pathway may play an important role in the early neonatal pulmonary vascular remodeling associated with single-ventricle physiology, and therapeutic treatment strategies targeting thrombin blockade in these patients may have clinical utility.

**Carnitine Metabolism and Mitochondrial Dysfunction**

L-carnitine is a trimethylated amino acid that is involved in the transport of long-chain fatty acids across the inner mitochondrial membrane (Figure 3). Carnitine is present in the form of either free carnitine (nonesterified molecule) or acylcarnitines (esterified form). Acylcarnitines are products of the reaction in which acyl moieties are transferred to carnitine from acyl-CoA (acyl-coenzyme A). This reaction is catalyzed by acyltransferases. Thus, the acylcarnitine/free carnitine ratio is a measure of acylated carnitines versus free carnitines. A low acylcarnitine/free carnitine ratio indicates a healthy mitochondrion, and a high acylcarnitine/free carnitine ratio indicates mitochondria with a reduced capacity for ATP production. Data in shunt lambs indicate that there is a disruption in carnitine metabolism that involves decreased expression of carnitine palmitoyltransferase-1 and -2, as well as carnitine acetyltransferase. In addition, reactive nitrogen species appear to be involved in the disruption of carnitine homeostasis, because nitrated carnitine acetyltransferase is elevated in shunt lambs, whereas the exposure of purified carnitine acetyltransferase to authentic ONOO− decreases its activity. Together these alterations result in mitochondrial dysfunction, as demonstrated by decreased superoxide dismutase 2 expression, increased uncoupling protein-2 expression, and an increased lactate/pyruvate ratio. Decreased expression of superoxide dismutase 2 has also been demonstrated recently in smooth muscle cells isolated from patients with pulmonary arterial hypertension. It is now becoming apparent that mitochondria play a key role in the development of various cardiovascular diseases, including heart disease, diabetes mellitus, and atherosclerosis. Studies have shown that mitochondria-targeted antioxidants have a beneficial effect in stroke-prone rats, sepsis-induced cardiac dysfunction, and cardiac ischemia-reperfusion injury, and there is increasing interest in the potential of mitochondria-targeted antioxidants in the treatment of cardiovascular disease. It remains to be seen whether this is a potential therapeutic strategy for children with single-ventricle physiology.

**Table 3. Developmental Changes in Gene Expression in Shunt Lambs**

<table>
<thead>
<tr>
<th>Functional Classification</th>
<th>No. of Genes</th>
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<td>Cell cycle–related genes</td>
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<td>Cell proliferation–related genes</td>
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<td>Angiogenesis-related genes</td>
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<td>Extracellular matrix– and cell adhesion–related genes</td>
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**Figure 3.** The “carnitine shuttle” and role of carnitine in the mitochondrial oxidation of fatty acids. CPT-I indicates carnitine palmitoyltransferase-1; CPT-II, carnitine palmitoyltransferase-2; CACT, carnitine acylcarnitine translocase; CrAT, carnitine acetyltransferase; ACoA, acetyl coenzyme A; OMM, outer mitochondrial membrane; IMM, inner mitochondrial membrane; and CoASH, coenzyme A.
ATP dependent, and there is a temporal decrease in eNOS/Hsp90 interactions in shunt lambs that is correlated with a decline in mitochondrial function. Data indicate that the mitochondrial dysfunction may result from an increase in the nitration of mitochondrial proteins due to an asymmetrical dimethylarginine–mediated redistribution of eNOS to the mitochondria. Increased levels of asymmetrical dimethylarginine have been implicated in the pathogenesis of pulmonary hypertension and in a single study of children and young adults with CHD and increased pulmonary blood flow. Because studies support the view that the ratio between L-arginine and asymmetrical dimethylarginine is a key component in the regulation of eNOS activity, it is possible that L-arginine could be a therapy for children with single-ventricle physiology.

**Figure 4.** Relevance network of genes belonging to angiogenesis-related signaling pathways identified with Pathway Architect software. Yellow indicates the genes that are altered between shunt and control lambs at 3 days of age.

**Identification of New Genes Involved in Early Neonatal Pulmonary Vascular Remodeling**

Previous studies have identified a burst of pulmonary angiogenesis in shunt lambs that peaks at 4 weeks of age and is then lost; however, it is unclear how this angiogenic and apparently antiangiogenic signaling is regulated. Microarray analysis has been used to identify gene changes in shunt lambs at an early time point before the angiogenic burst (3 days of age) and at 4 weeks of age to determine whether there are differences in gene expression between the 2 ages and between the age-matched control lambs. The data indicated that there are a large number of gene categories that are regulated between the shunt lambs and the age-matched controls at both 3 days and 4 weeks of age (Table 3). Furthermore, when only genes directly related to angiogene-
sis and the extracellular matrix are examined, the data indicate that these genes are predominantly upregulated at 3 days of age and downregulated at 4 weeks of age (Table 3). However, relevance network analyses in the 3-day-old shunt lambs reveal just how complex and interconnected these signaling pathways are (Figure 4). Thus, although these studies will likely lead to novel signaling pathways that can be investigated to produce exciting new results, they are unlikely to lead to new therapeutic strategies in the near future.

Conclusions

In summary, with the use of an animal model that enables us to study the initial onset of neonatal pulmonary vascular remodeling associated with single-ventricle physiology, new pathways for further mechanistic studies have been identified. New potential targets for therapeutic intervention have been identified and are now being tested: L-Arginine, antioxidants, thrombin, and 1-caritnine. In the longer term, we can hope that the use of newer techniques such as metabolomics, proteomics, and gene profiling may lead to new therapeutic targets to address the initial onset of neonatal pulmonary vascular remodeling associated with single-ventricle physiology.

Acknowledgments

The authors thank the rest of the members of their respective research groups, without whom this review could not have been written.

Sources of Funding

This research was generously supported by the Fondation Leducq to allow the formation of a Transatlantic Network. This work was also funded in part by grants HL61284 (to Dr Fineman), and HL086513 (to Dr Oishi), all from the National Institutes of Health; an American Heart Association Southeast Affiliate Beginning Grant-in-Aid Award (09BGIA2310050, to Dr Sharma); and a Seed Award from the Cardiovascular Discovery Institute of the Medical College of Georgia to (Dr Sharma).

Disclosures

None.

References


**Key Words**: endothelial dysfunction | endothelin | mitochondria | nitric oxide synthase | oxidative stress
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_Circulation_. 2011;123:916-923
doi: 10.1161/CIRCULATIONAHA.110.978528

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/123/8/916

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