Endothelial Biology of PAH and HHT: A “Genotype”-Phenotype Assessment

Duncan J. Stewart

NIH Workshop – Hereditary Hemorrhagic Telangiectasia: Vascular Biology and Pathophysiology
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Disclosure: DJS is the founding scientist and CSO of Northern Therapeutics
Molecular Basis for PAH and HHT

van den Driesche et al, 2002
How are mutations of BMPR2 related to the development of iPAH?

- Bone Morphogenetic Protein Receptor type II is a member of the transforming growth factor B receptor family.
- BMPR II is a receptor for a group of secreted growth factors called BMPs.
- In general, the BMPR-II pathway plays a role in inhibiting cell growth (SMCs).
- BMPs can have pro- or anti-apoptotic actions depending on cell-type and conditions.
BMPs induce apoptosis in human pulmonary artery SMCs

BMPRII mutations lead to dysregulated SMC growth

Loss of inhibitory BMP signaling

- increased SMC survival/proliferation

  → Intimal and medial hyperplasia of arterioles

  → PAH
BMPR2 is mainly localized to pulmonary vascular ECs

Effect of BMP-2 on TNFα-induced HPAEC apoptosis (TUNEL)
BMPRII gene silencing by siRNA

Circulation Research 2005
BMPRII mutations: “Double Jeopardy” for PAH?

- upregulated SMC growth
- Intimal and medial hyperplasia of arterioles

- EC apoptosis
- Regression and loss of precapillary pulmonary arterioles

SMCs

ECs

PAH
Pulmonary Microvasculature in the Rat
Monocrotaline model of PAH: 21 Days post MCT

Normal

MCT (3 wks)

FMA

SMA

Caspase 3 IHC
The Pre-Capillary Arteriole: the Achilles Heel of Pulmonary Microvasculature?

Precapillary arteriolar discontinuity
Central Role of EC Apoptosis in PAH?

Genetic susceptibility
- BMPR2 mutation
- Tie2
- other

Environmental trigger
- Toxin
- Anorexogins
- HIV
- Shear stress

Normal lung

EC injury

Endothelial dysfunction

Vasoconstriction

SMC hyperplasia

EC apoptosis

Microvascular dropout

Increased PVR

PAH

Vascular repair

EC proliferation

Emergence of apoptosis-resistant hyperproliferative EC clones

Plexiform arteriopathy

EPCs
Role of EPCs in pulmonary vascular regeneration?
Isolation and characterization of EPCs

- Bone marrow was harvested from tibia and femur of syngeneic Fisher 344 rats
- Mononuclear cells were isolated by Ficoll gradient centrifugation
- Differential culture in EBM-2 medium supplemented with endothelial growth factors for 7-10 days
Heterogenous response to BMP-2 in EPCs from patients with iPAH

Circulation Research 2005
Engraftment of EPCs into lung microcirculation and re-endothelialization of distal arterioles

15 minutes

1 week post MCT
Right Ventricular Systolic Pressure (RVSP)

Effect of EPC transplant on lung microvascular structure: 21 Days post MCT

Right Ventricular Systolic Pressure (RVSP)

Survival analysis of eNOS EPC treatment in reversal PAH model

Safety study (18 patients)
- 1º EP: tolerability of cell transplantation in patients with PAH refractory to all standard therapies

Cell delivery
- eNOS transfected autologous EPCs
- Delivery via SG catheter
  - Pacing port (i.e. RV delivery)
    - allows continuous monitoring of PA pressure
    - exclude intra-cardiac shunting (echo bubble study)
- Cell dose: extrapolation from rat and porcine models
  - Dose ranging up to $150 \times 10^6$ eNOS transfected cells given over 3 days in divided doses
Hypothesis

- Do mutations in the TGF-β receptor superfamily effect progenitor cell number and function?
- Do the specific mutations associated with PAH and HHT produce distinct disease-specific abnormalities in EPC phenotype?
Circulating “EPCs”

Flow Cytometry

% of MNCs Positive for Marker

CD34+ CD133+

+ - + - + - + - +

Controls (n=10) IPAH (n=14) HHT (n=14)

*Liana Zucco, PhD Candidate*
## EPC Isolation and Culture

### Table

<table>
<thead>
<tr>
<th>Group</th>
<th>Males</th>
<th>Females</th>
<th>Average Age (M)</th>
<th>Average Age (F)</th>
<th>Mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (30)</td>
<td>9</td>
<td>21</td>
<td>35.8 ± 6.5</td>
<td>40.7 ± 8.3</td>
<td>None</td>
</tr>
<tr>
<td>IPAH (30)</td>
<td>5</td>
<td>25</td>
<td>48.5 ± 5.58</td>
<td>45.2 ± 12.3</td>
<td>N/A (Possibly 2 Familial)</td>
</tr>
<tr>
<td>FPAH (5)</td>
<td>1</td>
<td>4</td>
<td>N/A</td>
<td>46.3</td>
<td>5</td>
</tr>
<tr>
<td>CTEPH (6)</td>
<td>3</td>
<td>3</td>
<td>47.0</td>
<td>74.0</td>
<td>None</td>
</tr>
<tr>
<td>HHT (32)</td>
<td>9</td>
<td>23</td>
<td>49.4 ± 22.2</td>
<td>44.5 ± 14.4</td>
<td>Endoglin: n=23 ALK-1: n = 8 Smad4: n = 1</td>
</tr>
</tbody>
</table>

Liana Zucco, PhD Candidate
Effect on Apoptosis

% of Cells Undergoing Apoptosis

Basal
n = 24

SF
n = 17

Basal
n = 27

SF
n = 16

Basal
n = 16

SF
n = 8

Controls

IPAH

HHT

*P≤0.05 v. Basal

*P≤0.05 v. Control
Effect on Gene Expression

**VEGF mRNA**

* P=0.1 v. Control

n=6

**ALK-1 ENG**
n=3

Expression relative to B-actin
Endoglin mRNA

Expression relative to B-actin

P < 0.05 v. Control

n=12 n=12 n=12

ALK-1 ENG

n=3 n= 9

Expression relative to B-actin

ALK-1 mRNA

Expression relative to B-actin

P ≤ 0.05 vs. Control

n=6 n=6 n=6

Controls n=6 IPAH n=6 HHT n=6 ALK-1 n=3 ENG n=3
Effect on Gene Expression

- eNOS mRNA

Expression relative to B-actin

- Controls: n=16
- IPAH: n=16
- HHT: n=16
- ALK-1: n=3
- ENG: n=13

P=0.06 v. Control
Regulation of EC growth, ECM and vessel structure?

Endothelial Cells/EPCs

EC apoptosis → vascular pruning

Abnormal vascular remodeling → AV malformations

PAH

HHT
Acknowledgements

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- Jan Mitchell

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- John Granton
- Mike Kutryk
- Mike Ward
- Marie Faughnan
- NicK Morrell

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EC survival signaling

microvascular homeostasis

p38MAPK

Regulation of EC growth, ECM and Motility?

ALK-1/5T

βR-II

P

Endoglin

β

BMP TGF

β

Smad-2

P

Smad-4

Smad-1/5/8

EC apoptosis  vascular pruning

EC apoptosis  vascular pruning

Abnormal vascular remodeling  AV malformations

PAH

HHT
Arteriolar “discontinuity” in MCT-induced PAH

Han et al. Am J Respir Cell Mol Biol 35, 2006; in press
EC survival signaling

microvascular homeostasis

p38MAPK

BMPR-IBMPR-II

EC apoptosis → vascular pruning

Smad-1/5/8

p38MAPK

Smad-4

BMP TGFβ

βR-II

ALK-I/5

Endoglin

eNOS

β

Smad-2

Smad-4

Aberrant remodeling → AV malformations
Effect on Apoptosis

% of Cells Undergoing Apoptosis

- Basal
- TNF
- SF
- Basal
- TNF
- SF
- Basal
- TNF
- SF

Controls
IPAH
HHT

*P≤0.05 v. Basal
*P≤0.05 v. Control
BMPs Inhibits Apoptosis in human PAEC post serum withdrawal

Serum withdrawal

Circulation Research 2005
PAH in transgenic mice overexpressing dominant negative BMRPR2

...but lack of marked arteriolar remodeling?

Effect of “Genotype” of EPC Migration

- Control: n = 3 (rhVEGF 50ng/ml -)
- Control: n = 3 (rhVEGF 50ng/ml +)
- IPAH: n = 2 (rhVEGF 50ng/ml +)
- HHT: n = 8 (rhVEGF 50ng/ml +)

Cell number per hpf
Effect of “Genotype” of EPC Migration

![Bar chart showing the effect of rhVEGF on cell migration in different conditions.](image-url)

**rhVEGF 50ng/ml**
- Control (n = 3, -)
- Control (n = 3, +)
- IPAH (n = 2, -)
- IPAH (n = 2, +)
- HHT (n = 8, -)
- HHT (n = 8, +)
EPC Differentiation

**Lectin**

*P ≤ 0.01 v. Control

**VEGFR2**

*P ≤ 0.01 v. Control

### Total Cell Number

<table>
<thead>
<tr>
<th>Group</th>
<th>Controls IPAH</th>
<th>FPAH</th>
<th>CTEPH</th>
</tr>
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<tbody>
<tr>
<td>Total</td>
<td>0</td>
<td>100</td>
<td>200</td>
</tr>
<tr>
<td>Cell No.</td>
<td>n = 19</td>
<td>n = 28</td>
<td>n = 5</td>
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### Total Cell Number/mm²

<table>
<thead>
<tr>
<th>Group</th>
<th>Controls IPAH</th>
<th>FPAH</th>
<th>CTEPH</th>
</tr>
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<tbody>
<tr>
<td>Lectin</td>
<td>0</td>
<td>25</td>
<td>50</td>
</tr>
<tr>
<td>Cell No.</td>
<td>n = 15</td>
<td>n = 25</td>
<td>n = 5</td>
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### % of Cells Positive for UEA-1

<table>
<thead>
<tr>
<th>Group</th>
<th>Controls IPAH</th>
<th>FPAH</th>
<th>CTEPH</th>
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</thead>
<tbody>
<tr>
<td>% Cells</td>
<td>0</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>Positive</td>
<td>n = 20</td>
<td>n = 29</td>
<td>n = 5</td>
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</table>

### % of Cells Positive for VEGFR2

<table>
<thead>
<tr>
<th>Group</th>
<th>Controls IPAH</th>
<th>FPAH</th>
<th>CTEPH</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Cells</td>
<td>0</td>
<td>40</td>
<td>60</td>
</tr>
<tr>
<td>Positive</td>
<td>n = 20</td>
<td>n = 29</td>
<td>n = 5</td>
</tr>
</tbody>
</table>

*Correlation significance: *P ≤ 0.01 v. Control*
Effect of “genotype” on EPC apoptosis

TUNEL II - TNFα/Serum Free Stimulated Apoptosis

*P<0.05 vs. Basal
≠ P≤0.05 vs. Control
** 15 v. Control

TUNEL III - BMP2/Serum Free Stimulated Apoptosis

P=0.16 vs. Control
Circulating “EPCs”

FACS Analysis - IPAH

* P<0.05 v. Control

FACS Analysis - FPAH and CTEPH

* P<0.001 v. Control

Controls (n=3)  FPAH (n=3)  CTEPH (n=3)
MRI perfusion imaging of the pulmonary vasculature

Normal

PAH

Courtesy of Evangelos Michelakis, U of Alberta
The problem – PAH through the looking glass


RA

PA
Thy lesions of HHT
Heterogenous response to BMP-2 in EPCs from patients with iPAH

**Image Description:**
- **Panel a:** Light microscopy image showing a culture of cells, likely EPCs, with varying cell types and morphologies.
- **Tie2:** Immunofluorescence staining highlighting Tie2 protein expression, with green fluorescence indicating the presence of the protein.
- **CD34:** Staining for CD34, a marker for hematopoietic progenitor cells, showing green fluorescence.
- **VEGFR2:** Staining for VEGFR2, a receptor for VEGF, with green fluorescence indicating receptor expression.
- **UAE-1:** Staining for UAE-1, likely a marker for a specific subpopulation of cells, with red fluorescence.
Cell-based gene therapy

**DIRECT DELIVERY**

- Therapeutic transgene
- The therapeutic transgene is packaged into a delivery vehicle such as a virus.
- ...and injected into the patient.
- Target organ

**CELL-BASED DELIVERY**

- Therapeutic transgene
- The therapeutic transgene is introduced into a delivery cell such as a stem cell that is often derived from the patient.
- The genetically modified cells (e.g., stem cells) are multiplied in the laboratory.
- ...and readministered to the patient.
Imbalance in growth regulation?

↑ Vascular growth factors

↓ Growth inhibitory pathways

ET-1
Giaid ... Stewart NEJM, 1993

BMPR2
The plexiform lesion: role of angiogenesis?

- Abnormal growth of vascular endothelial cells
- Upregulation of VEGF


Archer and Rich Circulation 102:2781, 2000
Experimental studies: inhibition of growth factor signaling

Effect of VEGF receptor antagonist (SU5416)

Effects of SU5416 reversed by z-ASPF

Effect of VEGF gene transfer in MCT model of PAH

RVSP (mm Hg)

<table>
<thead>
<tr>
<th>MCT GT</th>
<th>-</th>
<th>+</th>
<th>+</th>
<th>+</th>
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<tbody>
<tr>
<td>pcDNA</td>
<td>††</td>
<td>††</td>
<td>††</td>
<td>††</td>
</tr>
<tr>
<td>pVEGF</td>
<td>††</td>
<td>††</td>
<td>††</td>
<td>††*</td>
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</tbody>
</table>

Campbell et al. Circulation 2001;104:2242-2248
VEGF inhibits MCT-induced microvascular EC apoptosis

MCT alone

MCT and pVEGF

Day 14

Campbell et al. Circulation 2001;104:2242-2248
Effect of Z-Asp on RVSP at day 21

Z-Asp (2.5 mg/kg i.p.) administered 3-times/week

MCT alone

MCT + Z-Asp

P < 0.0003

P < 0.0005
Stopping Criteria for Dose Escalation

- 1 pt with SAE “definitely related”* to cell delivery
- 2 pts with SAE “probably related”* to cell delivery
- 3 pts with SAEs “possibly related”* to cell delivery
  ➔ Go back to lower dose and complete enrol and additional 3 pts to complete the trial

* Definition arrived at in consultation with the Safety Committee (D. Langleben, Mtl; S. Mehta, London)
Safety study in acute porcine PAH model – PVR (Wood’s units)

Cell number = 200 million (cumulative)
Fluorescent 3-dimensional microangiography

Microangiography with fluorescent microspheres (0.2 μm) suspended in agarose solution

Confocal optical sectioning of thick (100 – 200 μm) sections of lung
De novo angiogenesis from the pulmonary microvasculature

Avascular Matrigel plug
The final product!

2.5 x10^6 heNOS-Tx EPCs/ml
CMTMR-labeled SMCs
Transmigration through arteriolar endothelium

5 min
30 min
1h
18h

MCT alone
MCT/peNOS ** = <0.005

RVSP (mm Hg)

Campbell et al. Circulation 2001;104:2242-2248
Reversal of PH by eNOS gene transfer

Cell-based GT
1.5 x 10^6 cells

RVSP (mm Hg)

Control (n=6)

peNOS (n=6)

MCT (70 mg/kg)

Days

†
p<0.05
Reversal of PAH: evidence for pulmonary vascular regeneration?
Comparison of eNOS vs VEGF gene therapy for reversal of MCT PH

RVSP (mmHg)

* = P<0.05

- Normal (n=40)
- pcDNA (n=32)
- VEGF (n=20)
- eNOS (n=36)
De novo angiogenesis from the pulmonary microvasculature
NO and Neovascularization

- Angiogenic factors (VEGF, bFGF, TGFβ) upregulate eNOS and stimulate NO release
- VEGF-stimulated capillary formation is prevented by inhibitors of NOS in vitro and in vivo (Hood 1998 and Ziche 1997)
- eNOS knockout mice have impaired neovascularization (Murohara 1998)
- eNOS has been shown to upregulate VEGF (Dulak 2000, Jozkowicz 2001)
Abnormal vascular development in E20 eNOS-/- fetal lungs

Han et al. Circ. Res. in press, 2004
Experimental Plan: persistence of beneficial effects in the prevention model

- 1 x 10^6 EPCs (n=12)
- FBs or Saline (n=13)
- 1.5 x 10^6 EPCs ± eNOS
- Saline

Timeline:
- MCT/saline
- Persistence
- Reversal
- 0 3 21 35 Days
Persistence of the effect EPCs treatment on RVSP >60 days

P<0.001
Histological Changes of kidney from MCT treated rats
Comparison of eNOS vs VEGF gene therapy for reversal of MCT PH

** p<0.01 vs. normal; * p<0.05 vs. normal; †<0.05 vs. Day 21
Reversal Protocol

1 x $10^6$ EPCs

FBs or Saline

Prevention

1.5 x $10^6$ EPCs ± eNOS

Saline

MCT/saline

0 3 21 35 Days
Flurorescent microangiography
FMA - perfusion index (PI):

* = p<0.05 vs. control; † = p<0.05 vs. MCT-alone

Summary and Conclusions

- Cell-based gene therapy with eNOS can prevent and reverse experimental PAH at least in part by regeneration of pulmonary microvasculature.
- EPC alone given within 3 days of MCT prevent the development of PAH, but did not reverse established disease when delivered at 3 weeks.
- EPCs transfected with eNOS dramatically improved survival in established PAH suggesting that the combination of regenerative cell and gene therapy will be the most effective in the treatment of this disease.
Effect of EPC transplant on lung microvascular structure: *21 Days post MCT*
NO and Neovascularization

- Angiogenic factors (VEGF, bFGF, TGFβ) upregulate eNOS and stimulate NO release
- VEGF-stimulated capillary formation is prevented by inhibitors of NOS in vitro and in vivo  Hood 1998 and Ziche 1997
- eNOS knockout mice have impaired neovascularization Murohara 1998
- eNOS has been shown to upregulate VEGF Dulak 2000, Jozkowicz 2001
Dose escalation

Cell number (x 1 million)

- **Day 3**
- **Day 2**
- **Day 1**

Day 3
Day 2
Day 1

Panel 1
Panel 2
Panel 3
Panel 4
Panel 5
CMTMR-labelled SMCs
Transmigration through arteriolar endothelium

5 min
30 min
1h
18h

Campbell et al. Circulation 2001;104:2242-2248
Effect of cell-based eNOS gene therapy: rat MCT model of PH

- Fisher 344 rats injected with 70 mg/kg MCT s.c. and 500,000 transfected smooth muscle cells via the internal jugular vein
- RVSP measured at 28 days
- Animals sacrificed and RV/LV ratio measured, pulmonary histology examined, RNA extracted
- pcDNA3.1
- peNOS
average of 3-5 year survival after diagnosis!

Better late than never?
The future – reversing “irreversible” PAH?

JAMA 284:3164, 2000

NORMAL

REVERSIBLE DISEASE

IRREVERSIBLE DISEASE
DELIVERY OF GENES to human subjects is sometimes accomplished directly (orange arrow), by putting vectors (agents carrying potentially therapeutic genes) straight into some target tissue in the body (in vivo). More often the ex vivo approach (blue arrows) is used: physicians remove cells from a patient, add a desired gene in the laboratory and return the genetically corrected cells to the patient. An in vivo approach still in development would rely on “smart” vectors that could be injected into the bloodstream or elsewhere and would home to specific cell types anywhere in the body.
Targeted transgene delivery to the lung

functional plane of cell-based gene delivery

Resistance
Vessel Caliber
The Pulmonary Vasculature

Human

Rat MCT model

Normal

PAH

RA

PA

Courtesy of Evangelos Michelakis, U of Alberta
The Normal Pulmonary Vasculature

Courtesy of Evangelos Michelakis, U of Alberta
Pulmonary Vascularity in PAH

Courtesy of Evangelos Michelakis, U of Alberta
Pulmonary Arterial Hypertension (PAH)

- The pulmonary vasculature bed is a high flow, low impedance system that accommodates entire cardiac output with low arterial pressures.

- In PAH, there is a persistent elevation in PA pressure due to narrowing of arterioles and loss of pulmonary microvessels.
Regulation of vascular growth and regression

Angiogenesis/arteriogenesis

Vascular regression

Stewart and Kutryk, 2002
Ang1 gene transfer inhibits apoptosis in rat model of MCT-induced PH

Activated caspase-3 immunoreactivity

RV systolic pressure

Zhao et al Circulation Research 92:984, 2003
Is Angiopoietin-1 a mediator of PAH?

Expression of Angiopoietin in Multiple Organ Blots: “Of Mice and Men”

Murine

Human

Rudge and Yancopolous, Regeneron
Ang-1 in PPH lungs

- Human pAng1
- Surgical control
- Normal donor
- Surgical Control
- PPH Lungs

A.E. Dutly et al. Robust expression of angiopoietin-1 in human lung. Poster
Angiopoietin expression in PAH

Relative Expression (qc RT-PCR)

- Normal
- PPH
- SPH

Ang1, Ang2, Tie2

Angiopoietin-1 tissue levels (ELISA, pg/ul)

- Normal
- PPH
- SPH

* indicates statistical significance.
Partial rescue of Tie2-/- mice by dox-conditional targeted Tie2 expression

Jones et al. EMBO J 5:438, 2001
Tie2 +/- mice develop spontaneous PAH

RVSP (mmHg) vs Frequency Distribution (%)

P < 0.05

+/-
N=52

+/-
N=40

RVSP Range (mmHg)

<20 20-24 25-29 30-34 35-39 40-44 45+

Tie2 +/-

Tie2 +/+
Effect of chronic Serotonin infusion

RVSP (mm Hg)

Control

5-HT

P<0.05

WT

Tie2+/--
Conditional Endothelial Angiopoietin-1 Overexpression

Tie-1 promoter

Driver

tTA

Responder

+ DOX

Ang1

IRES

β-gal

tTA+/Ang1-
tTA+/Ang1+
tTA-/Ang1+
tTA-/Ang1-

tTA+/Ang1-
tTA+/Ang1+
tTA-/Ang1+
tTA-/Ang1-

Driver

Responder

tTA+/Ang1-
tTA+/Ang1+
tTA-/Ang1+
tTA-/Ang1-

tTA+/Ang1-
tTA+/Ang1+
tTA-/Ang1+
tTA-/Ang1-

tTA+/Ang1-
tTA+/Ang1+
tTA-/Ang1+
tTA-/Ang1-
Conditional Endothelial Angiopoietin-1 Overexpression: protein

B-Gal staining

Ang1 (ng/ml)

Lung 20x

Ang-1 ELISA

NBT

BT
Endothelial Angiopoietin-1 overexpression reduces hypoxic PAH

![Graph showing RVSP (mm Hg) for Normoxia and Hypoxia]

- Normoxia (n=6)
- Hypoxia (n=6)

P = 0.06

Lakshmi Kugathasan – Msc Candidate
Angiopoietin-1 in pulmonary hypertension: *Cause or cure?*

Promotes EC survival; Does not induce mitosis

Reduces vascular permeability

Stabilizes microvessels by pericyte recruitment and *physiological* muscularization

Thought to be a key factor in the maintenance of postnatal vascular homeostasis

*Editorial comment: Rudge, Thurston and Yancopolous, Circ Res 92:947, 2003*