Endothelial Biology of PAH and HHT: *A "Genotype"-Phenotype Assessment*

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NIH Workshop – Hereditary Hemorrhagic Telangiectasia: Vascular Biology and Pathophysiology

Hyatt Regency, Bethesda, MD

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Disclosure: DJS is the founding scientist and CSO of Northern Therapeutics

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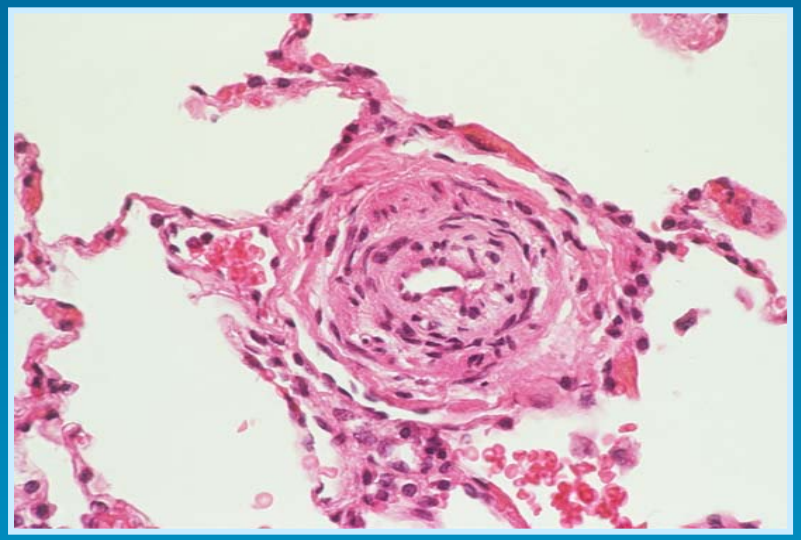
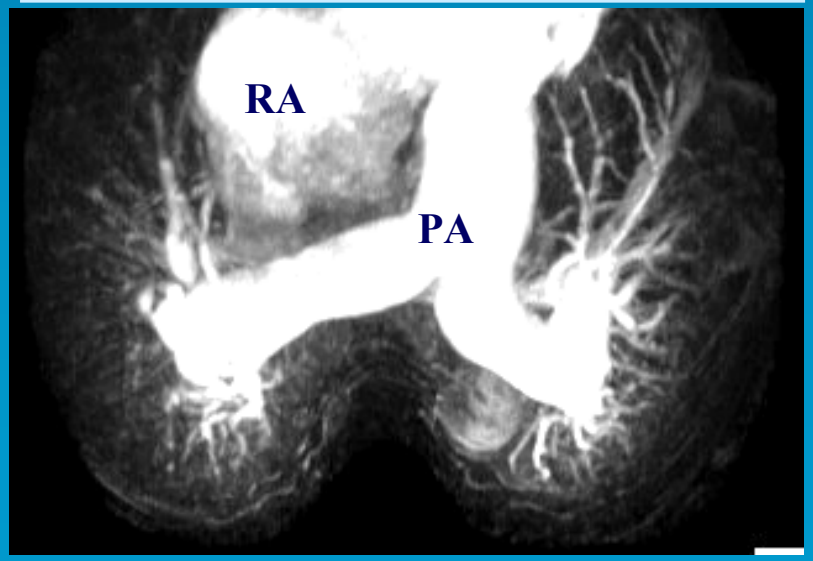
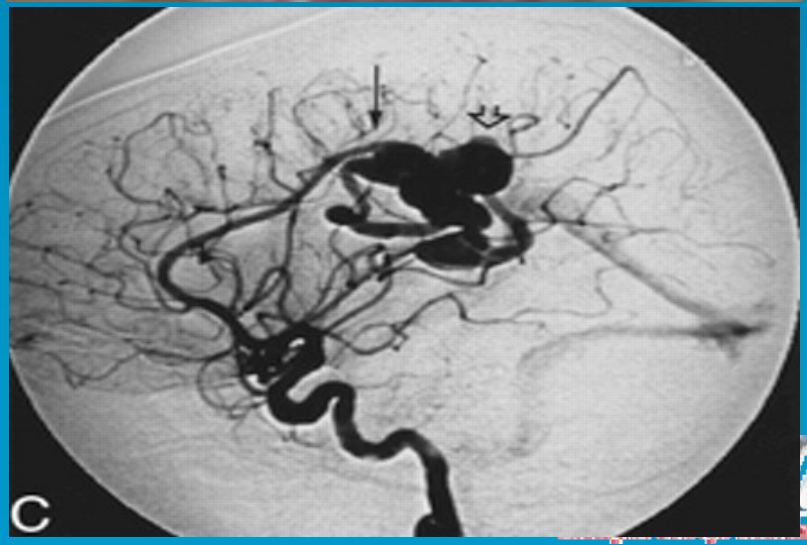
PAH

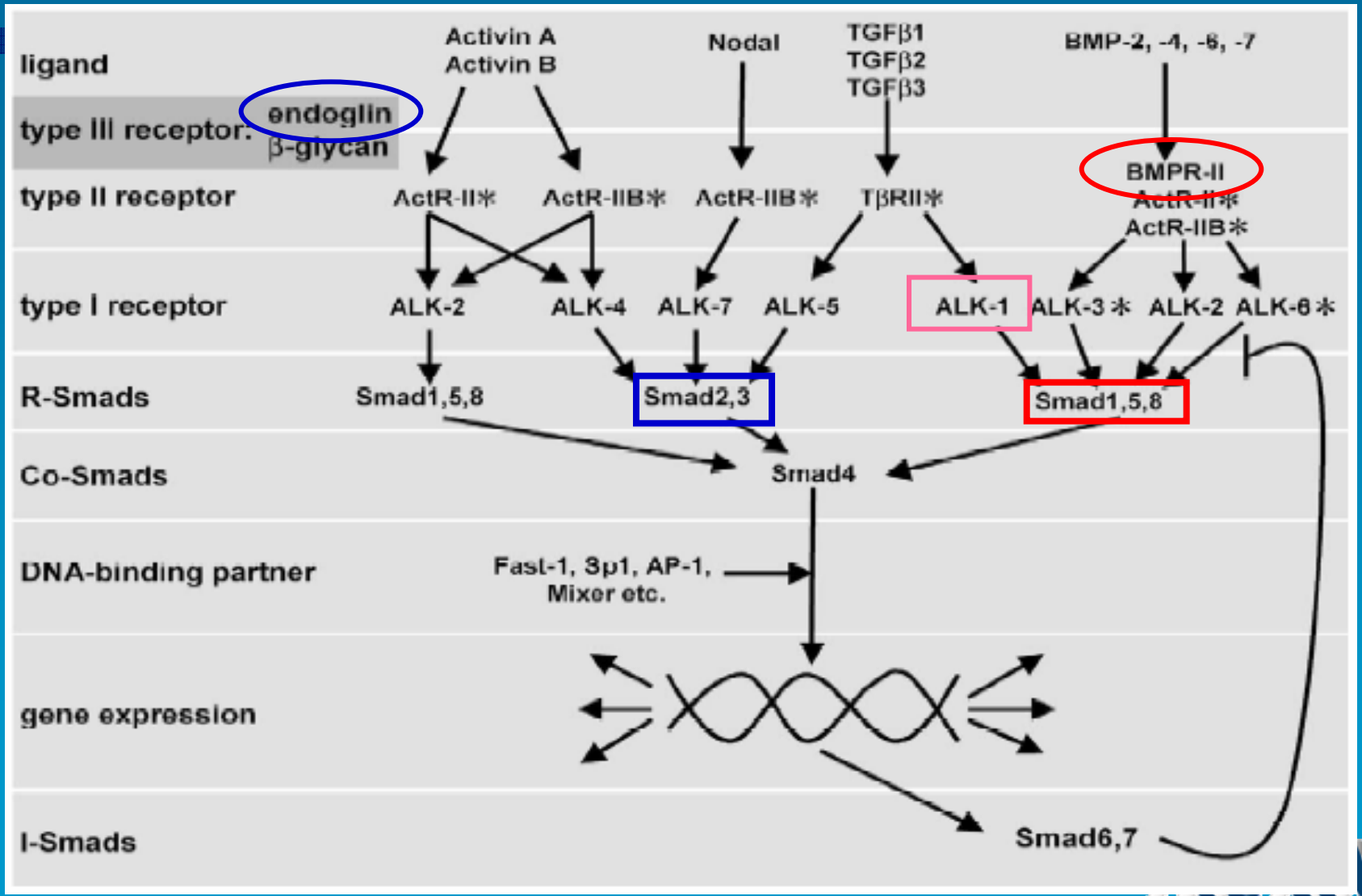




HHT

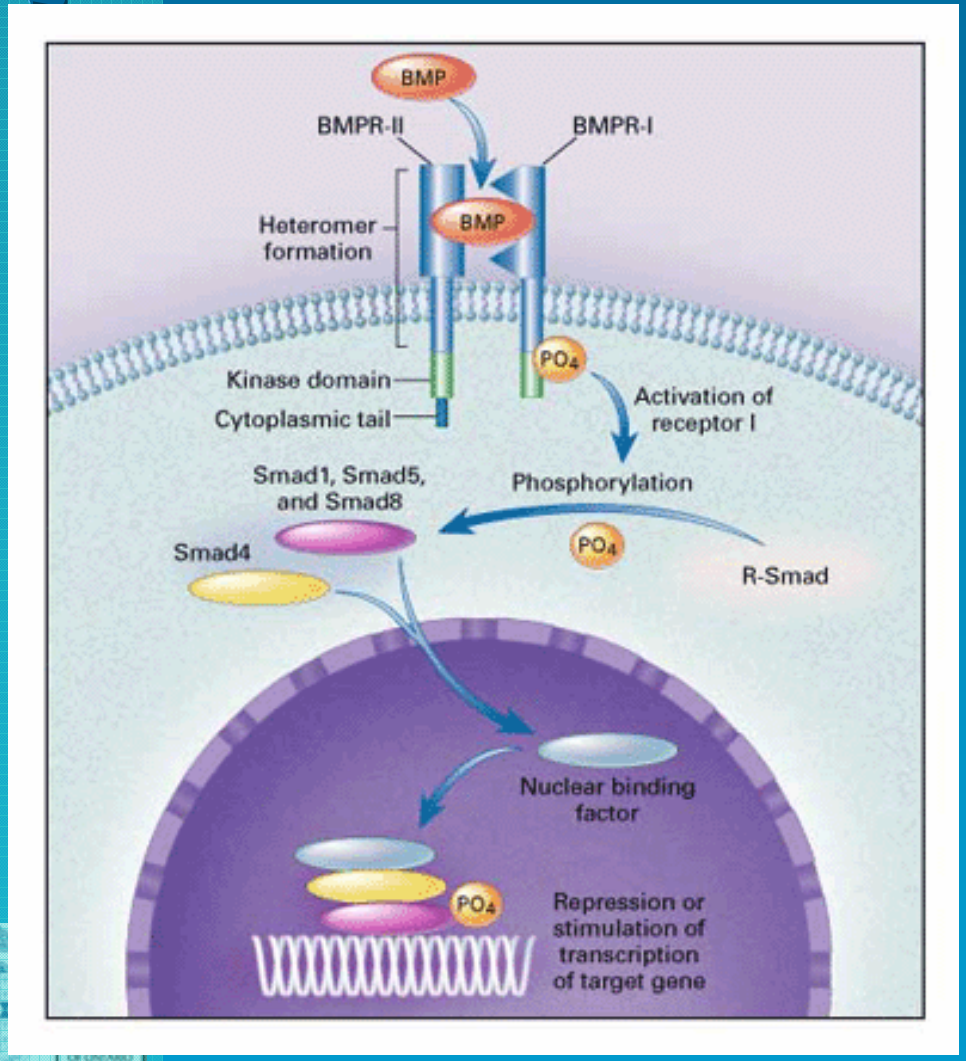


Molecular Basis for PAH and HHT



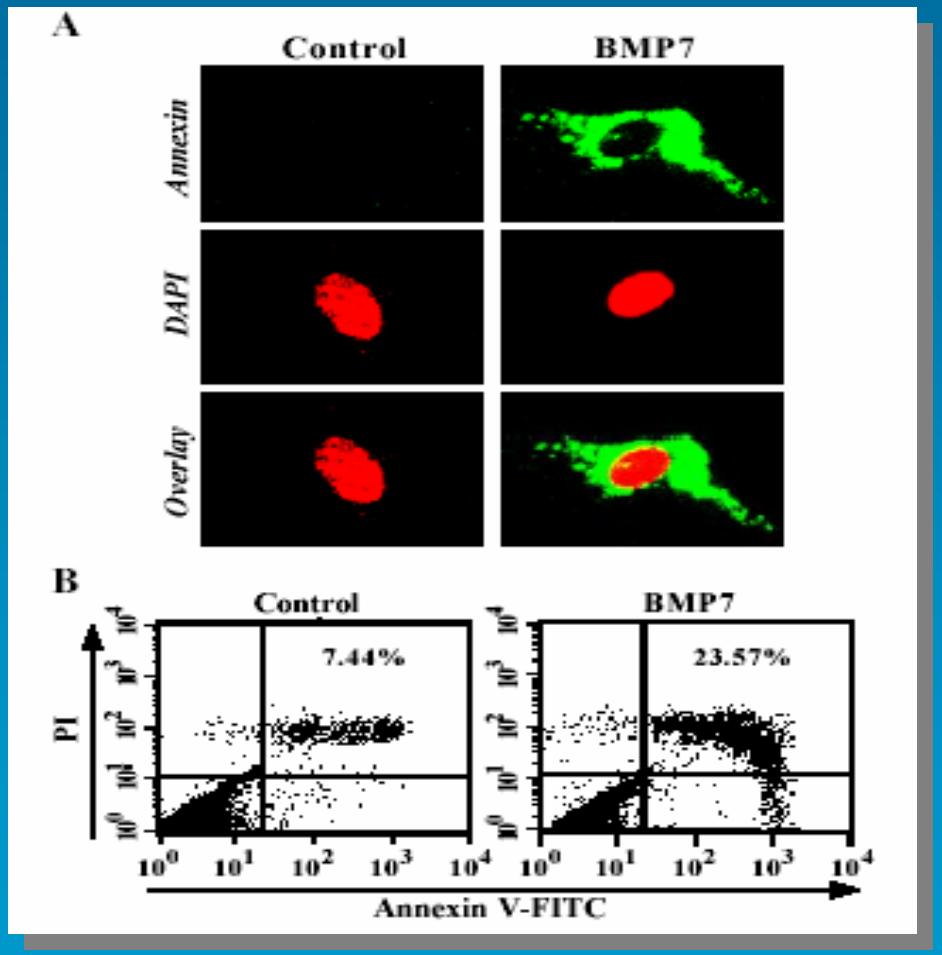
van den Driesche et al, 2002

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



Zhang S, AmJ Physiol Lung Cell Mol Physiol, 2003

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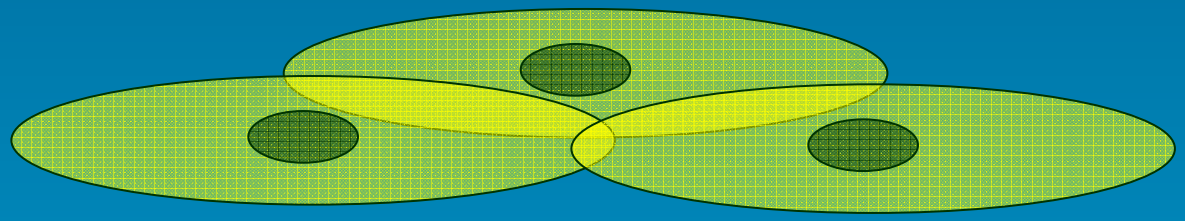
BMPRII mutations lead to dysregulated SMC growth

Loss of inhibitory BMP signaling



- increased SMC survival/proliferation

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Intimal and medial hyperplasia of arterioles

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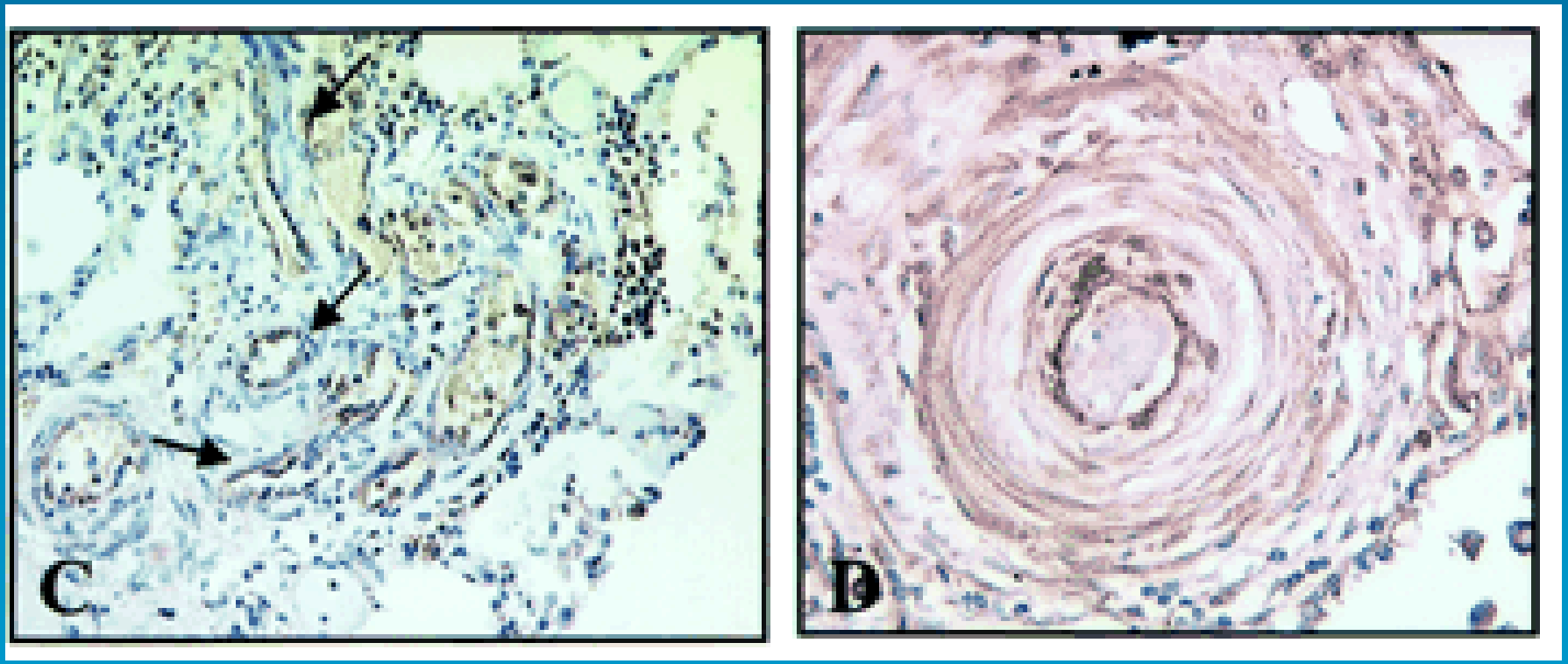
PAH

BMPR2 is mainly localized to pulmonary vascular ECs



Atkinson et al. Circulation 105:1672,2002

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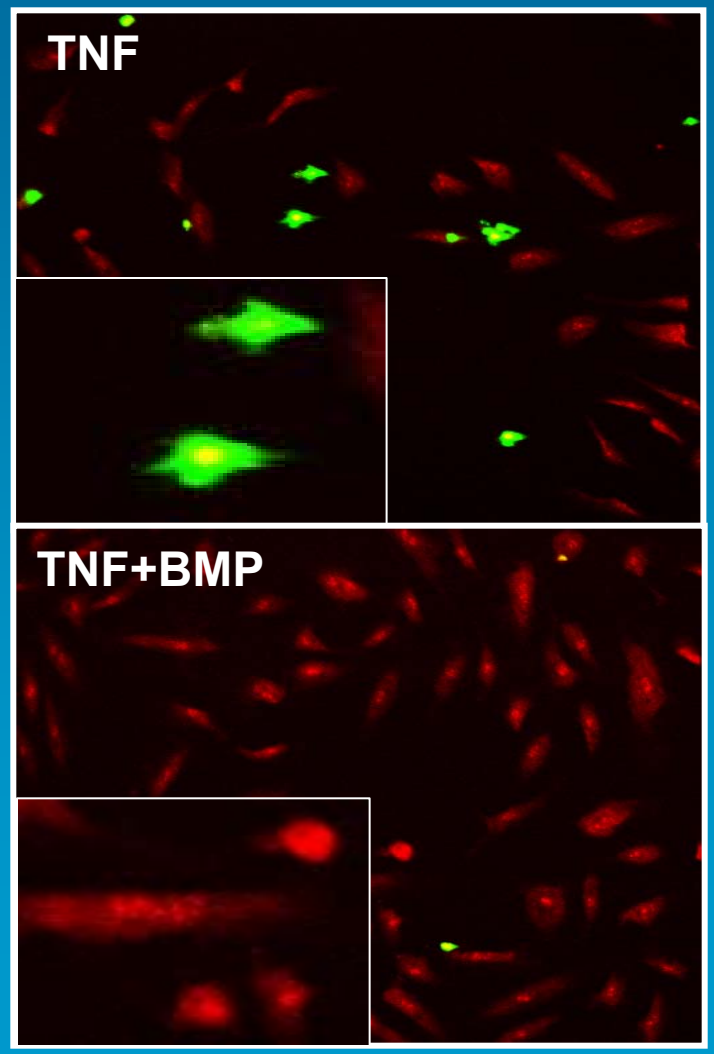
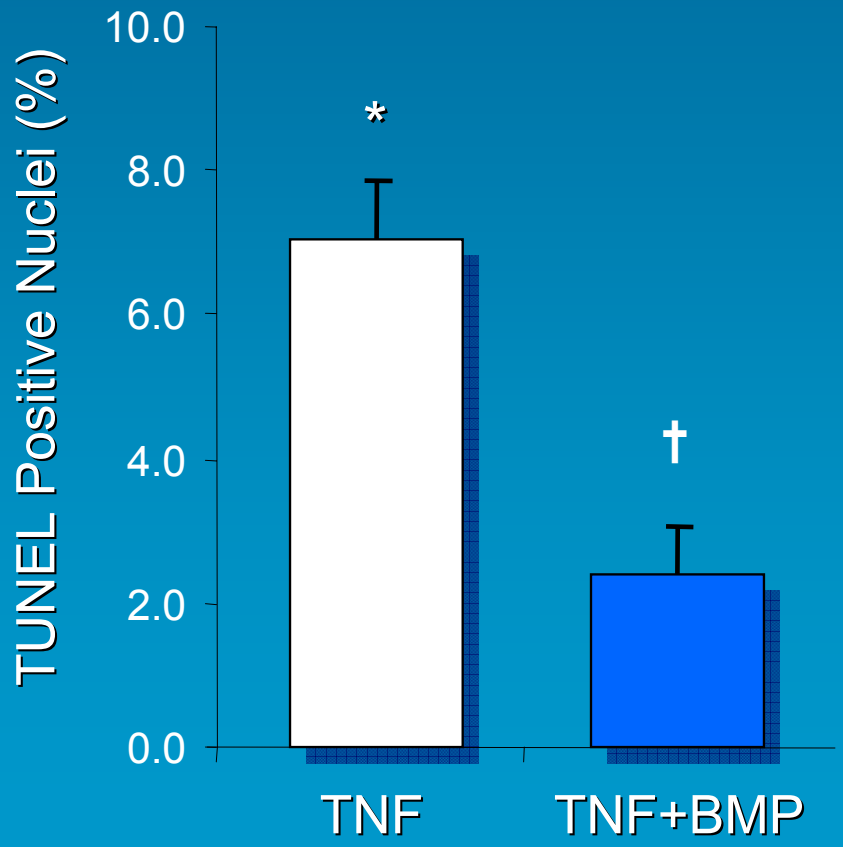
Effect of BMP-2 on TNFα-induced HPAEC apoptosis (TUNEL)





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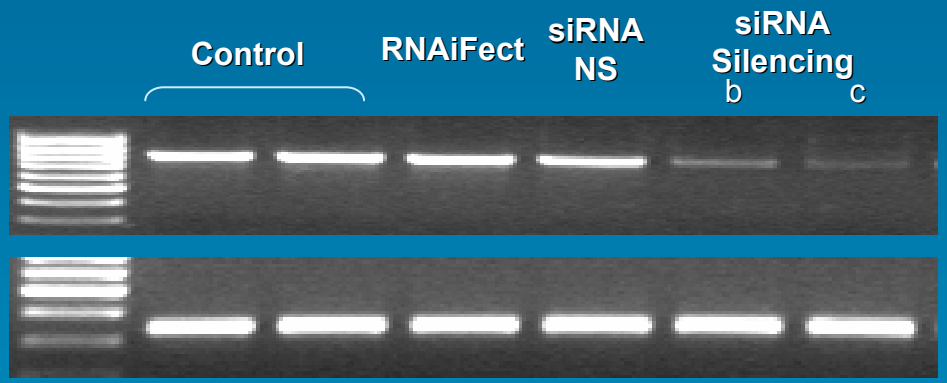
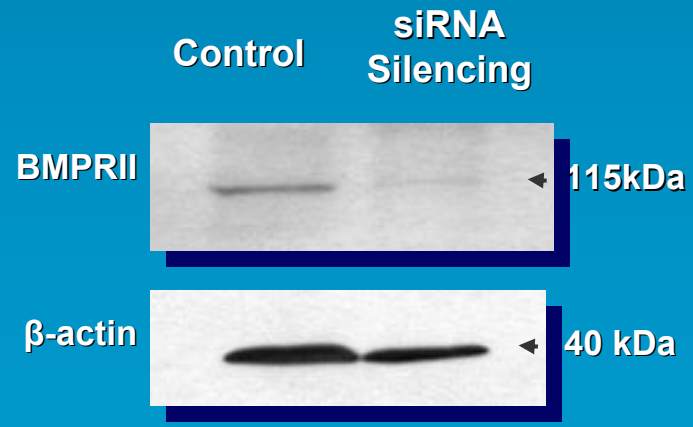
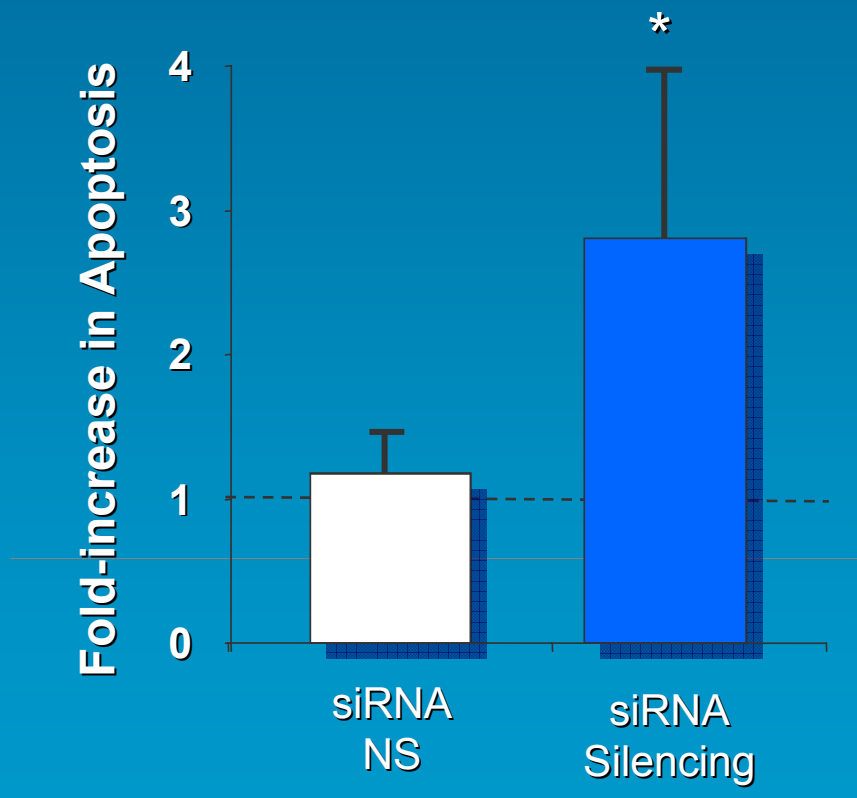
BMPRII gene silencing by siRNA







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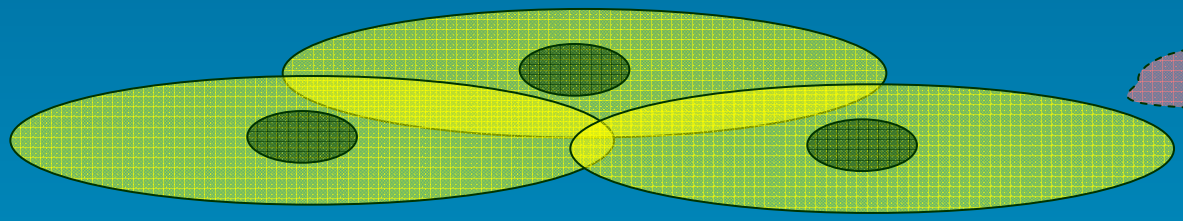
BMPRII mutations: "Double Jeopardy" for PAH?

SMCs



- upregulated SMC growth

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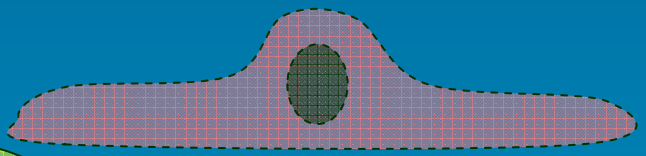
Intimal and medial hyperplasia of arterioles

ECs



- EC apoptosis

↓

Regression and loss of precapillary pulmonary arterioles

PAH

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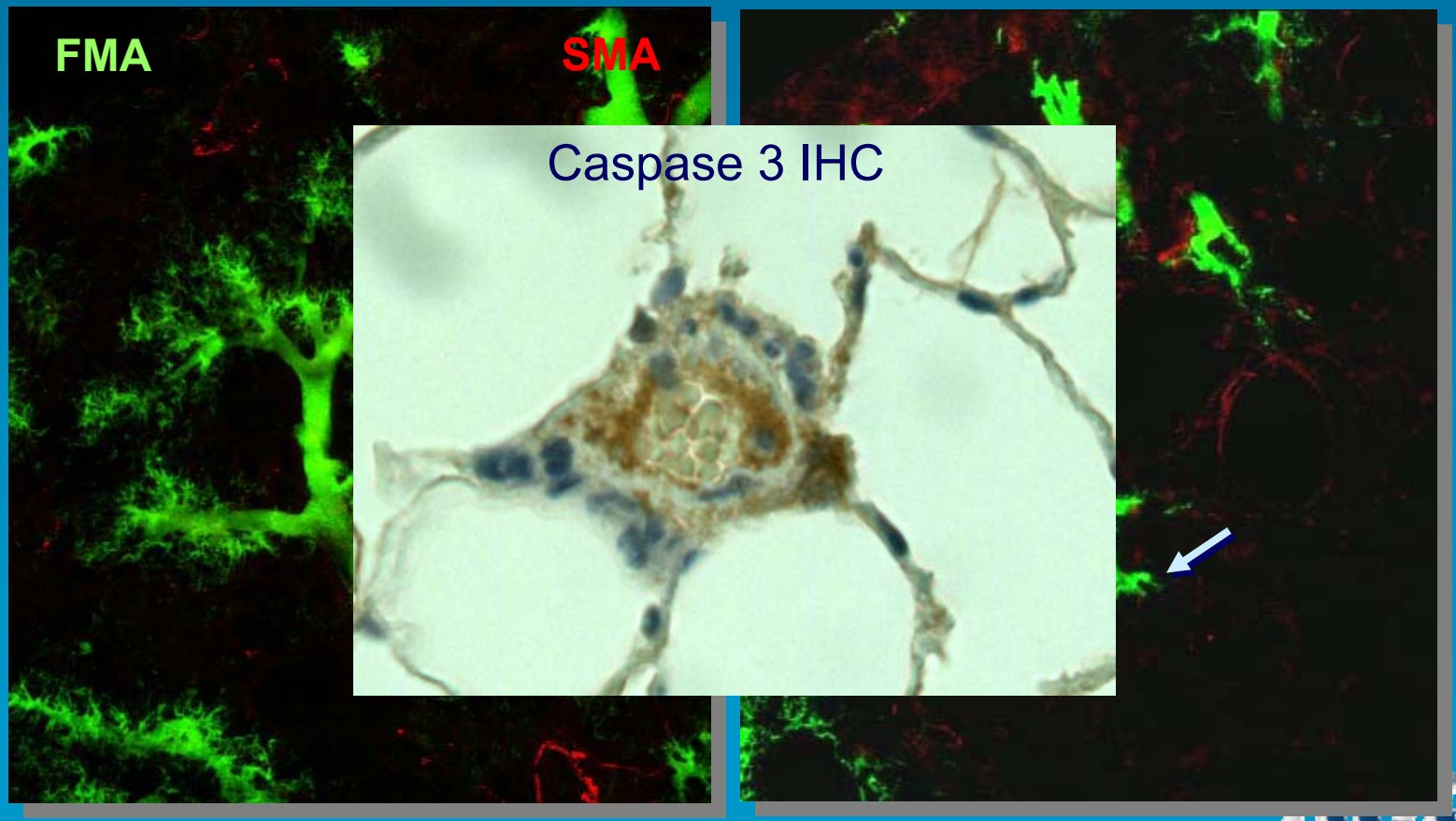
Pulmonary Microvasculature in the Rat Monocrotaline model of PAH: *21 Days post MCT*

Normal

MCT (3 wks)



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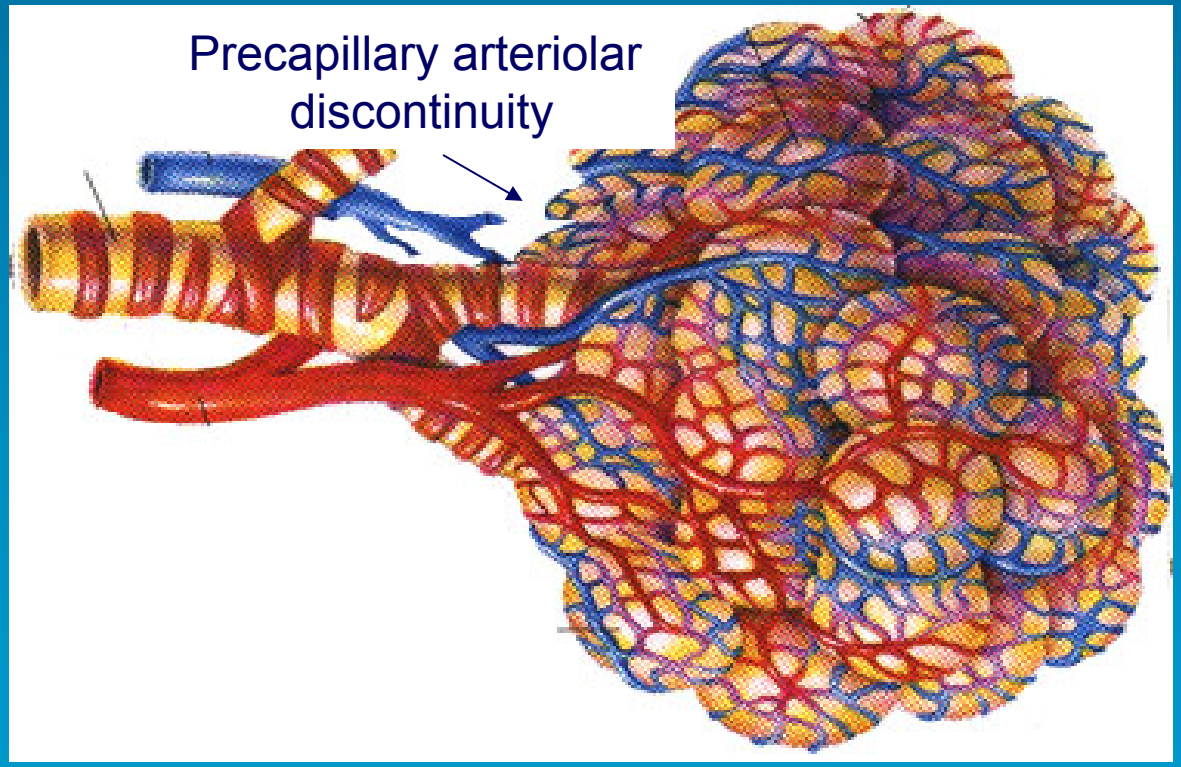
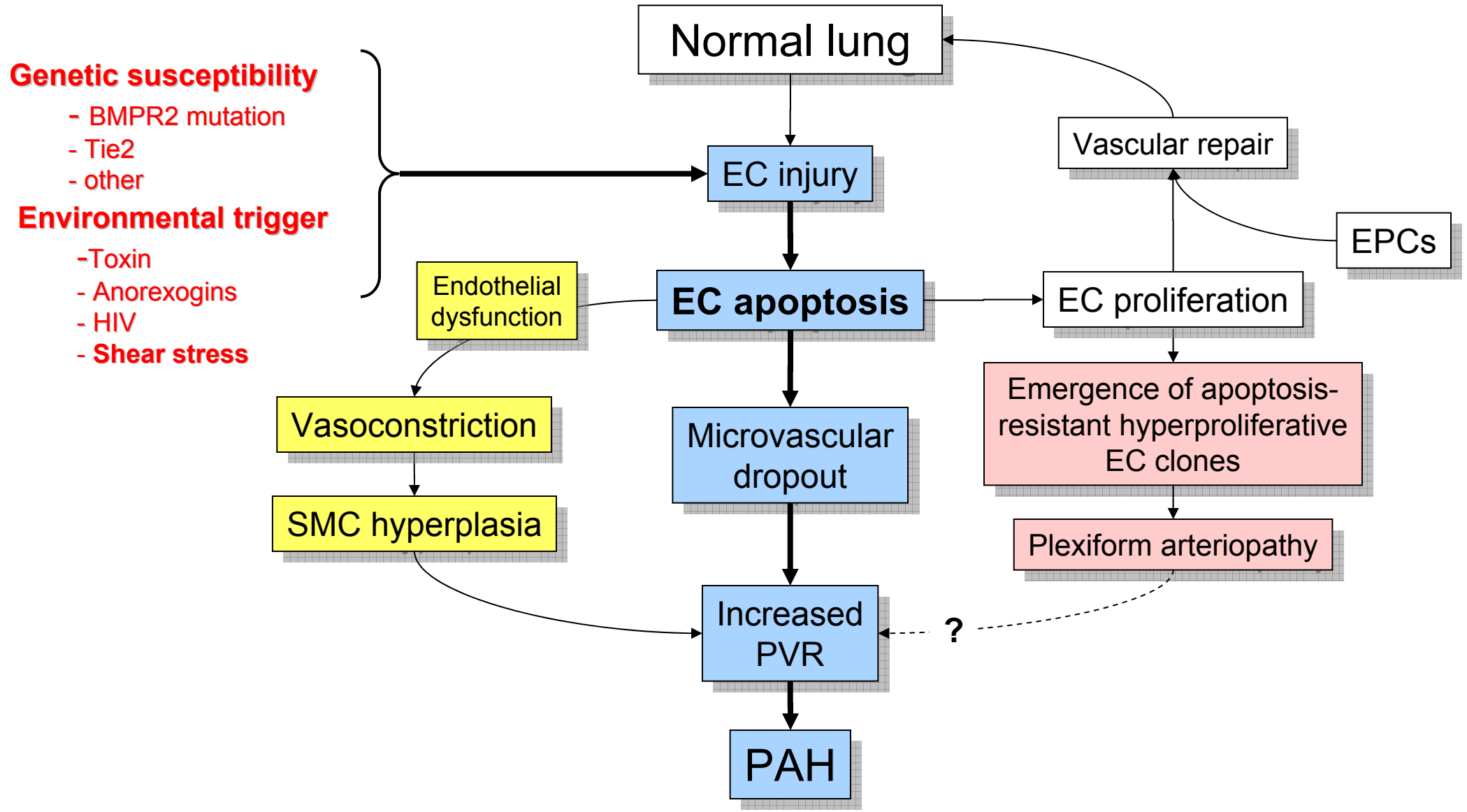
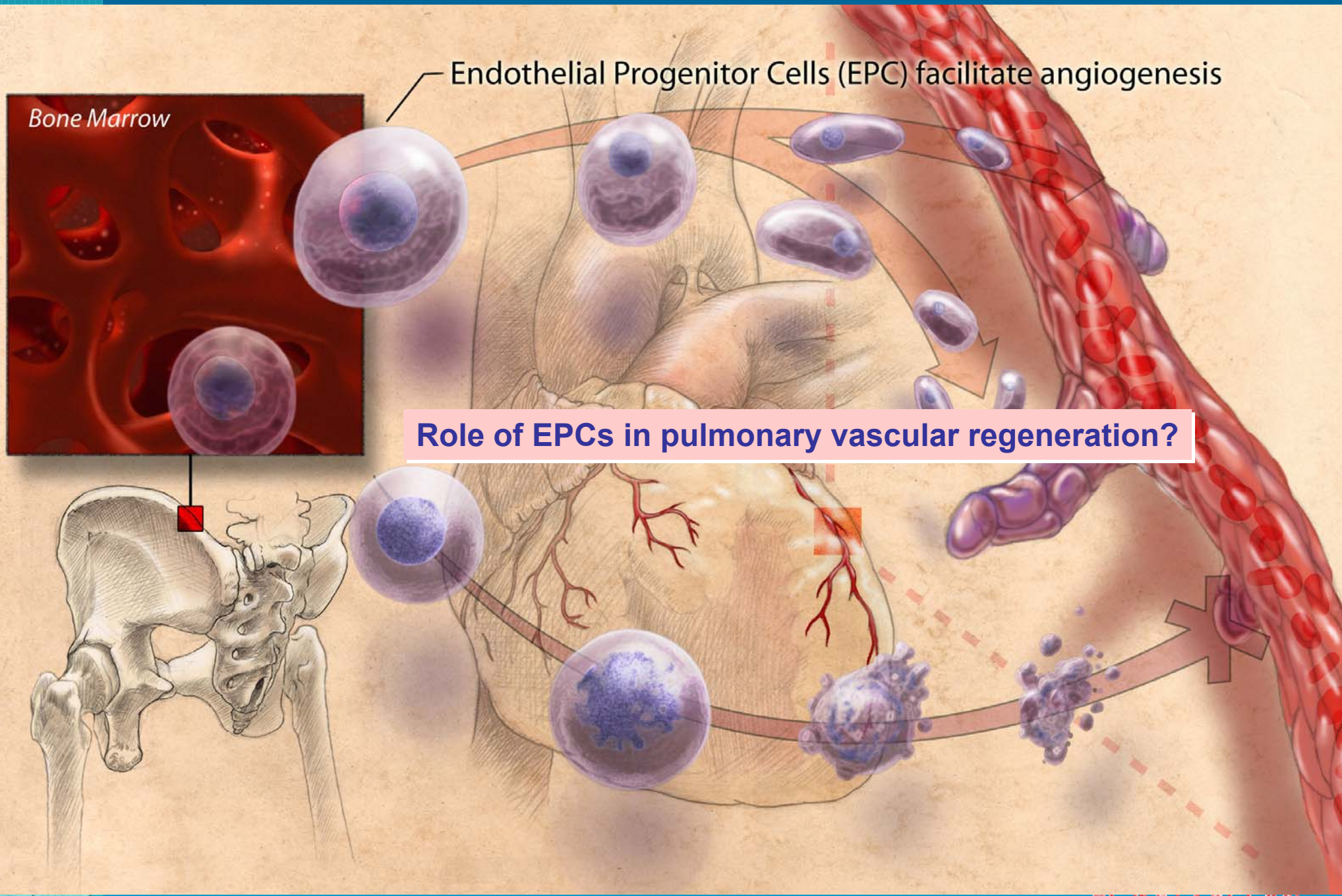
The Pre-Capillary Arteriole: the Achilles Heel of Pulmonary Microvasculature?

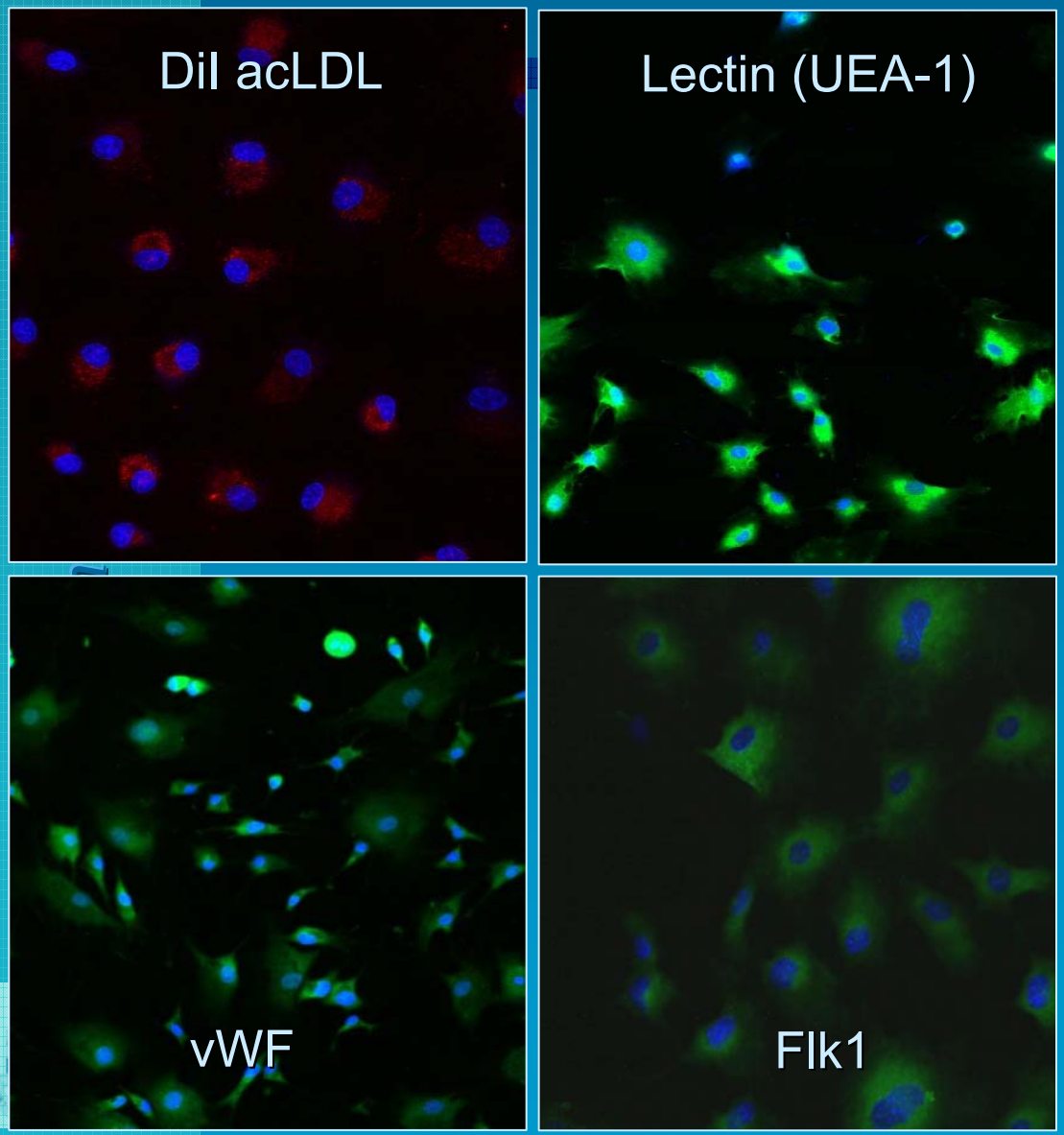


Central Role of EC Apoptosis in PAH?



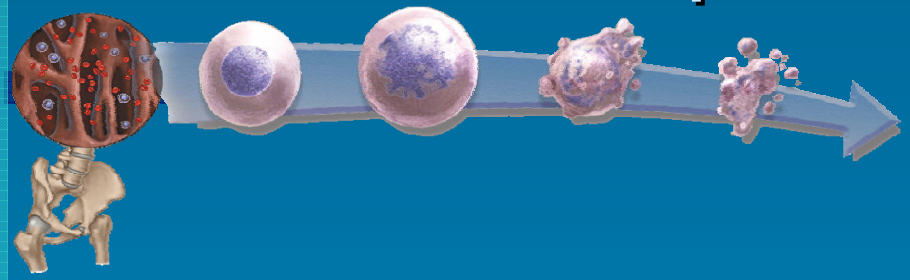

Endothelial Progenitor Cells (EPC) facilitate angiogenesis
Bone Marrow
Role of EPCs in pulmonary vascular regeneration?

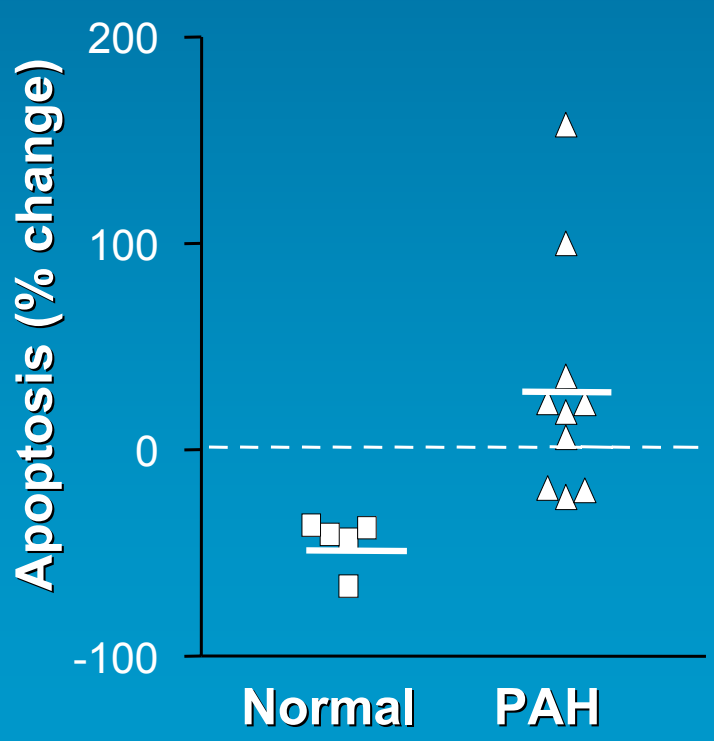
Isolation and characterization of EPCs

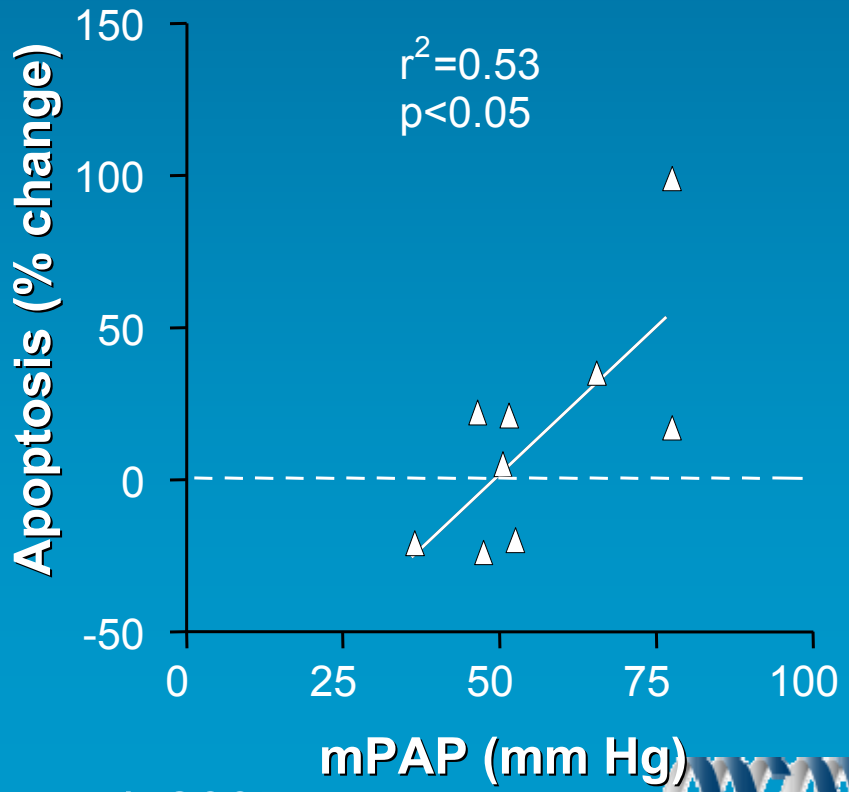


- Bone marrow was harvested from tibia and femur of syngeneic Fisher 344 rats
- Mononuclear cells were isolated by Ficoll gradient centrifugation
- Differential culture in EBM-2 medium supplemented with endothelial growth factors for 7-10 days

Heterogenous response to BMP-2 in EPCs from patients with iPAH







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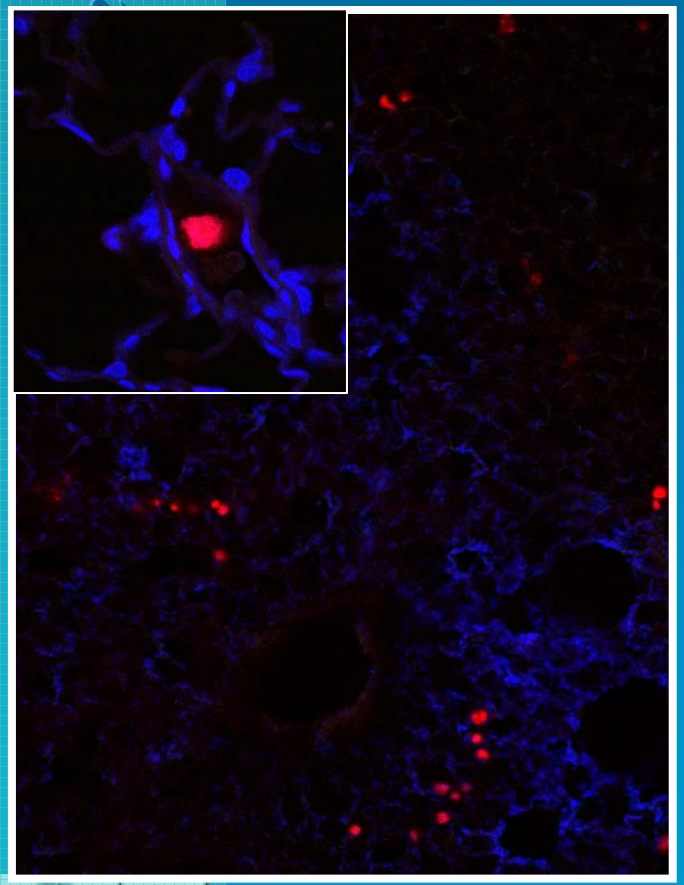
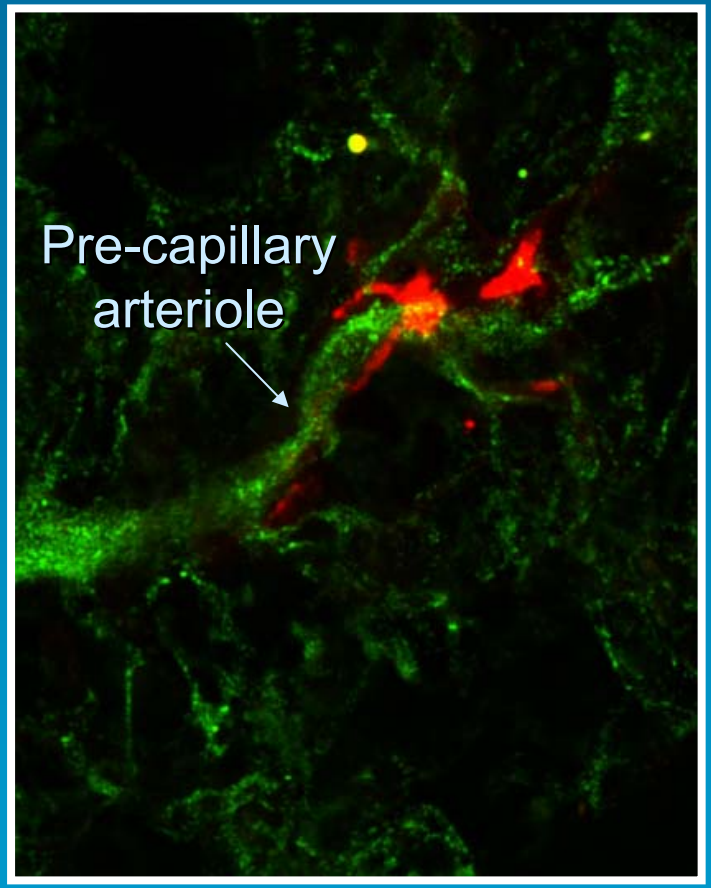
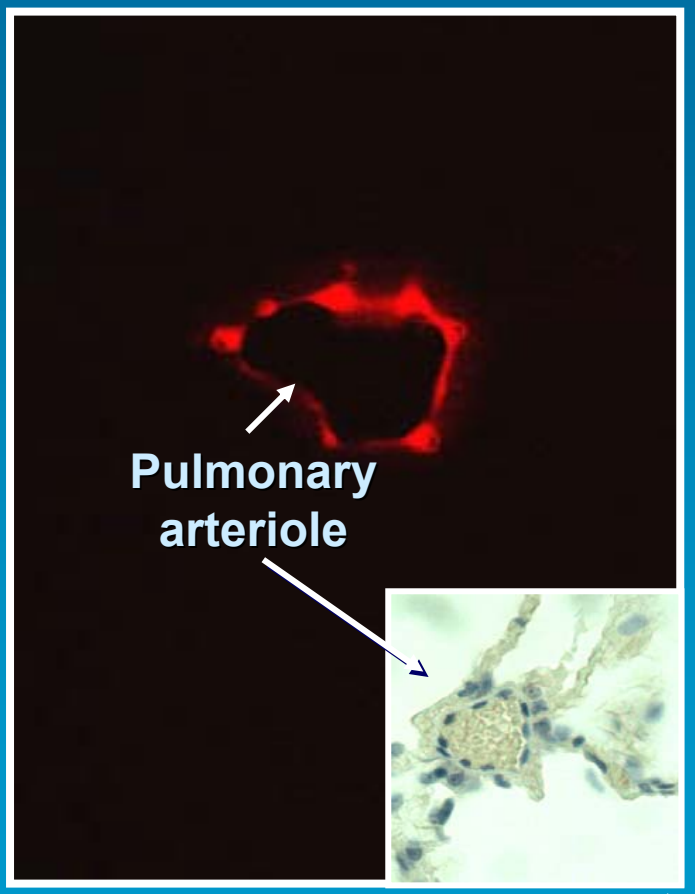
Engraftment of EPCs into lung microcirculation and re-endothelialization of distal arterioles



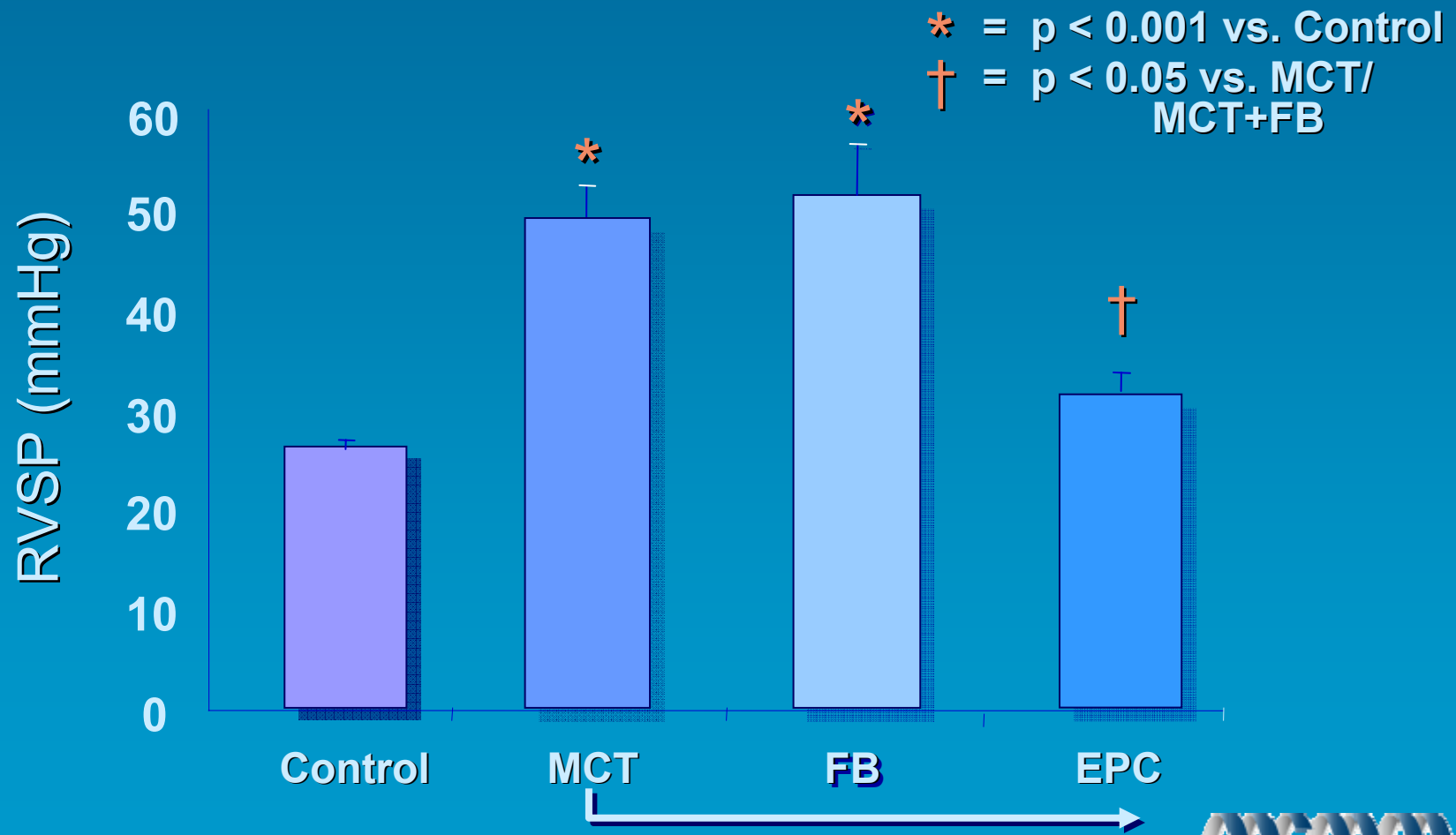
15 minutes





1 week post MCT

Right Ventricular Systolic Pressure (RVSP)



Zhao et al. Circ Res. 2005; 96(4):442-50

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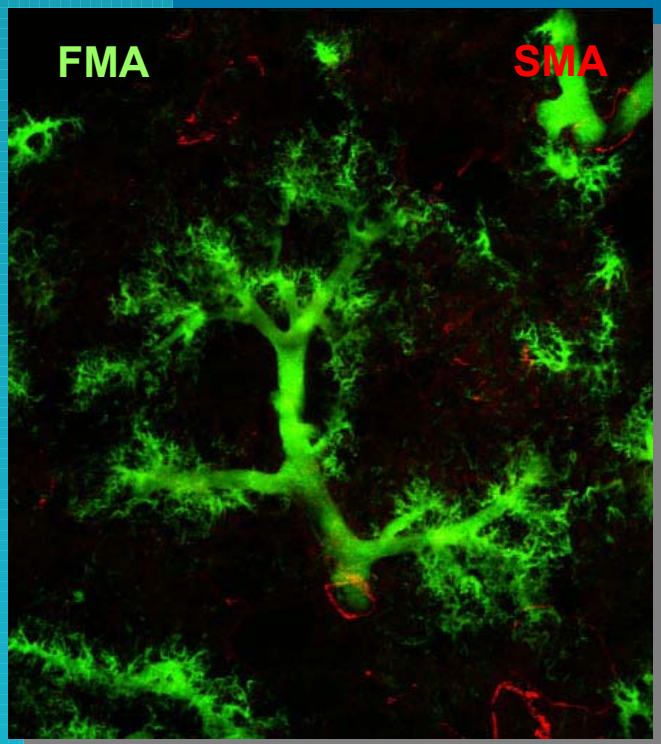
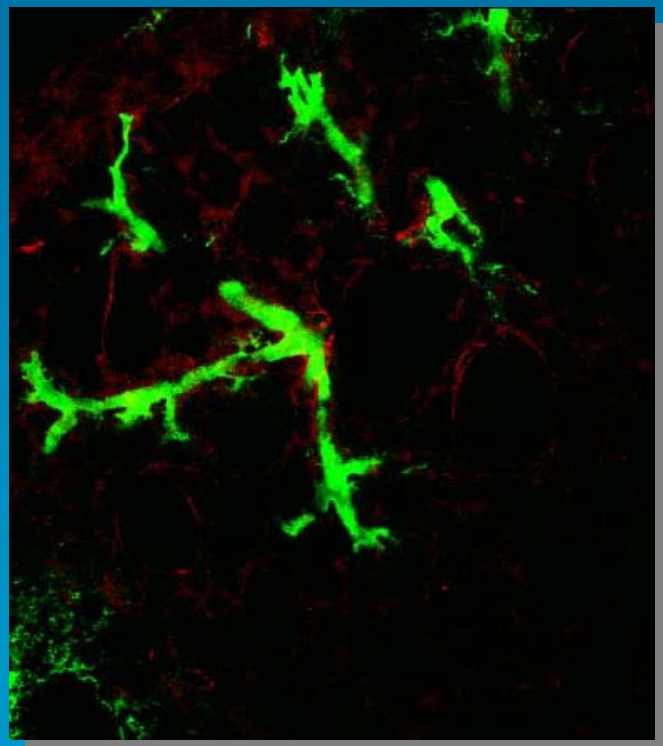
Effect of EPC transplant on lung microvascular structure: *21 Days post MCT*

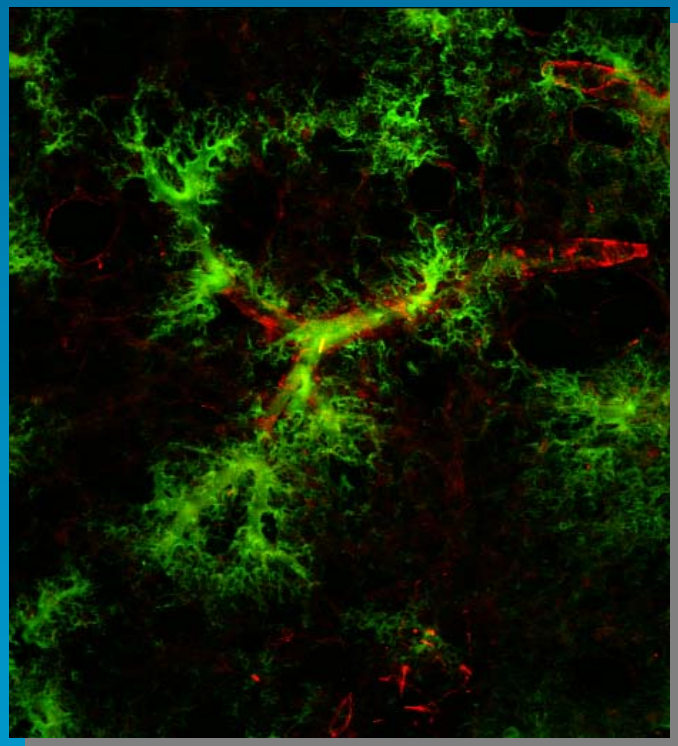
Control

MCT-FB

MCT-EPC







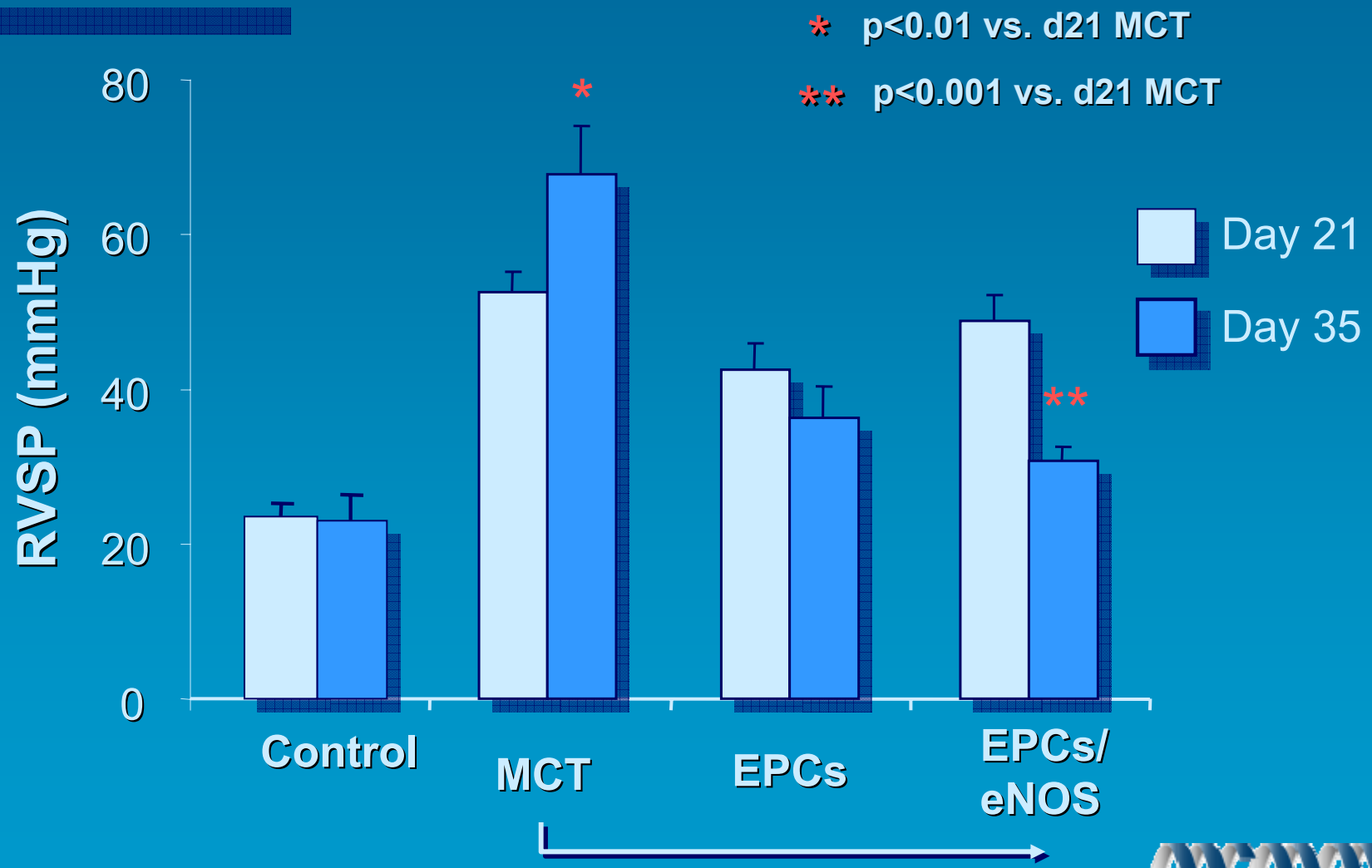
100 x

100 x

100 x

Zhao et al. Circ Res. 2005; 96(4):442-50

Right Ventricular Systolic Pressure (RVSP)



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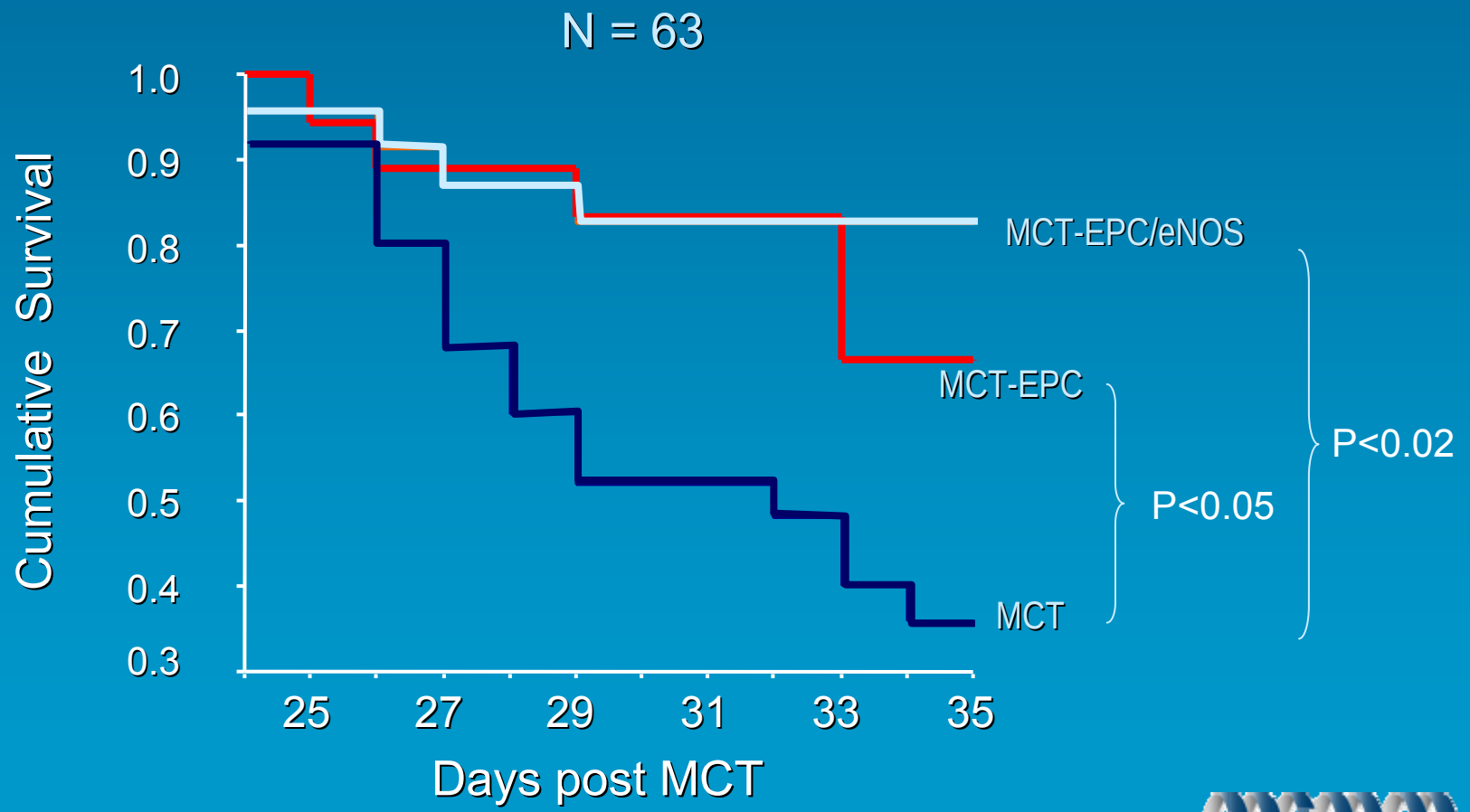
Survival analysis of eNOS EPC treatment in reversal PAH model



Zhao et al. Circ Res. 2005; 96(4):442-50

Pulmonary Hypertension And Cell Therapy (PHACeT) Trial

- Safety study (18 patients)
  - I^{o} 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 x 10^{6} eNOS transfected cells given over 3 days in divided doses

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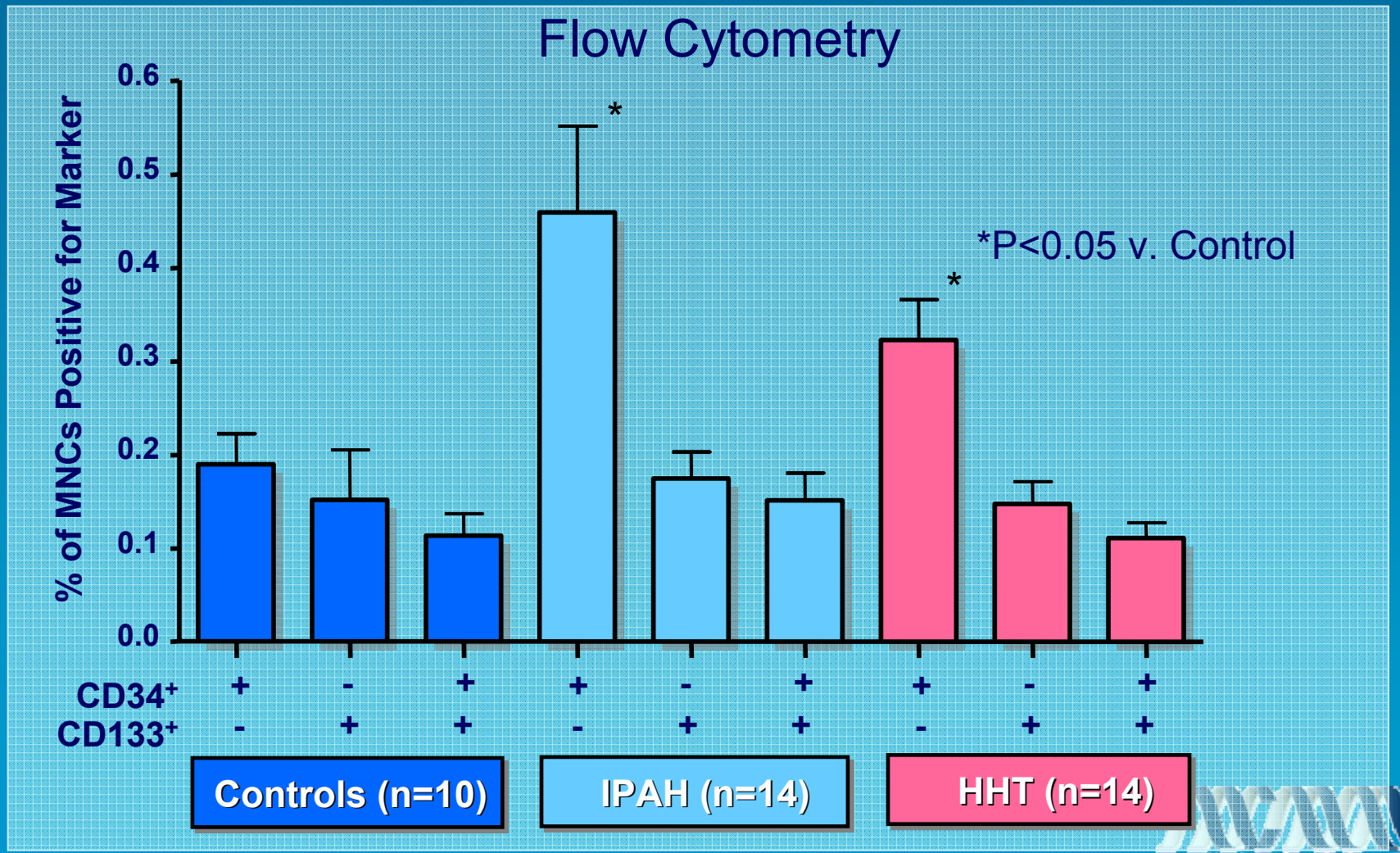
Hypothesis

- Do mutations in the TGF-β receptor super-family 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"



Liana Zucco, PhD Candidate

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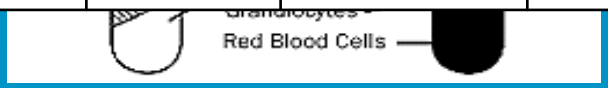
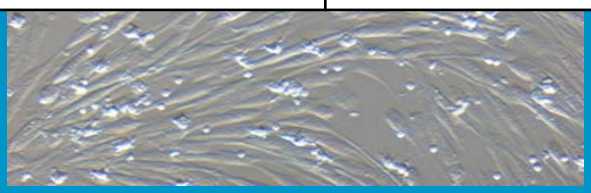
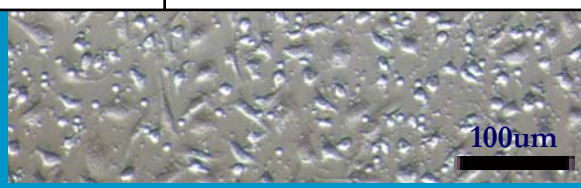
EPC Isolation and Culture

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

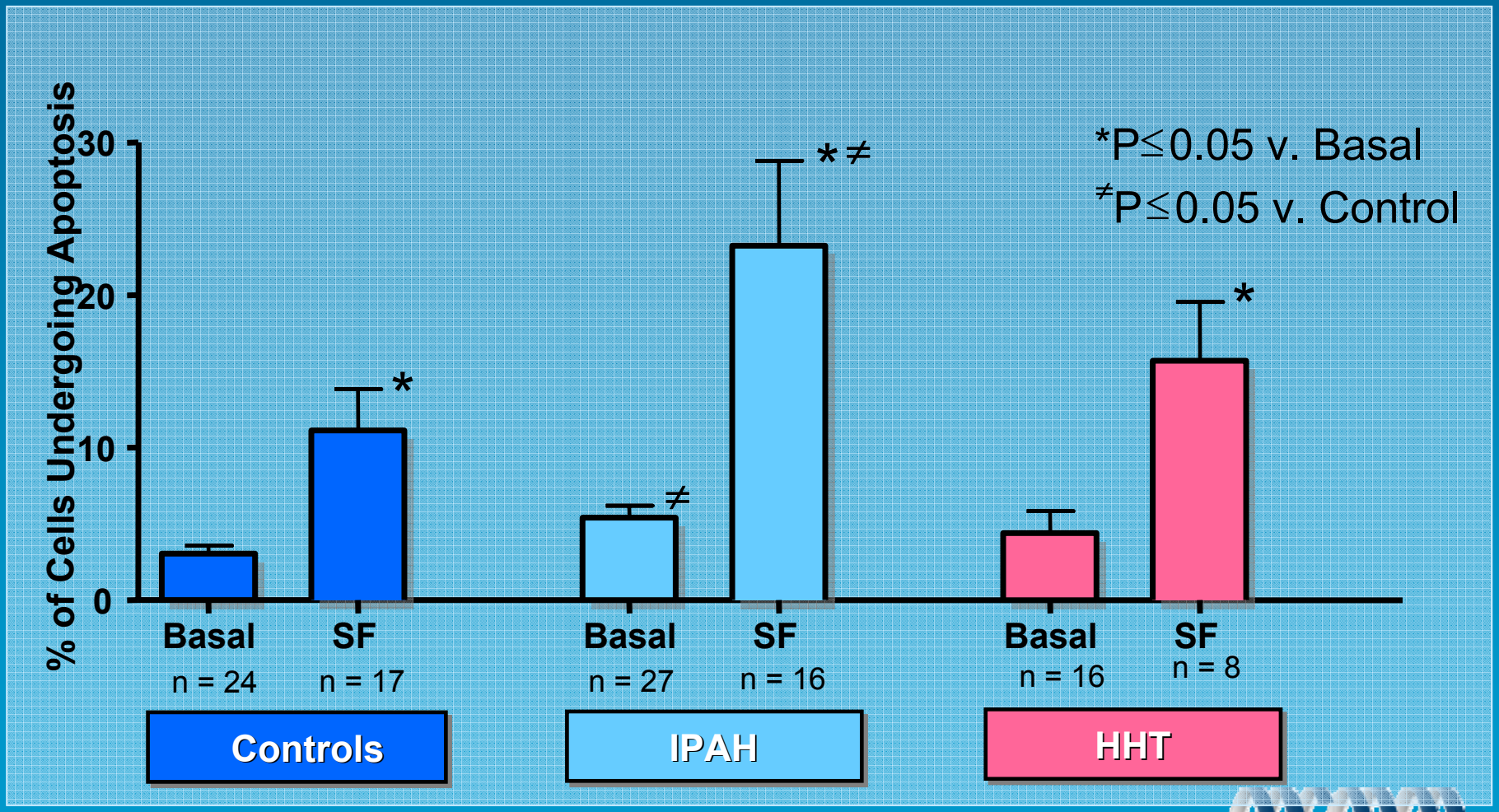




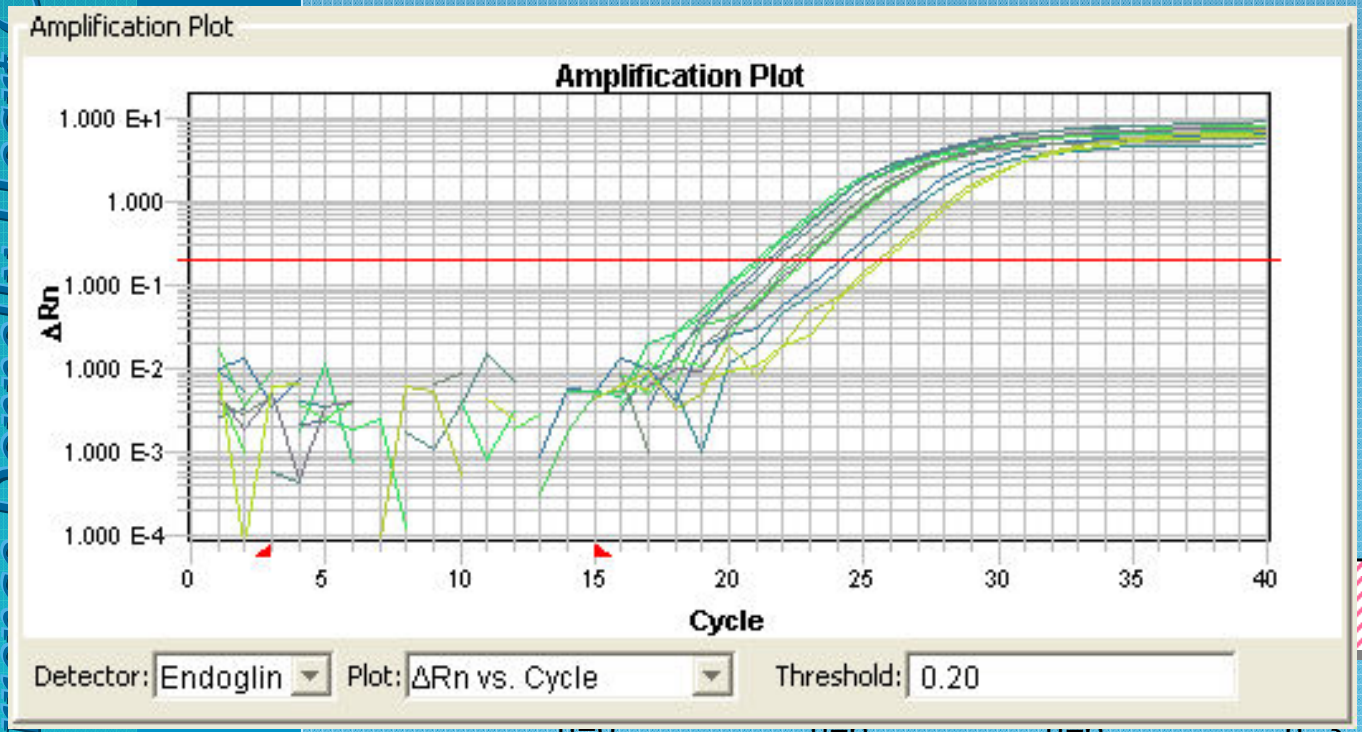


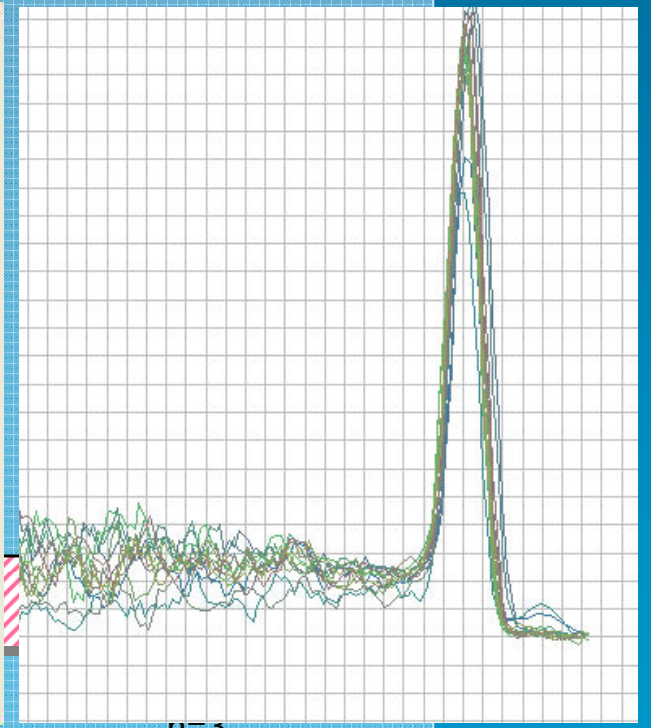
Liana Zucco, PhD Candidate

Effect on Apoptosis



Effect on Gene Expression





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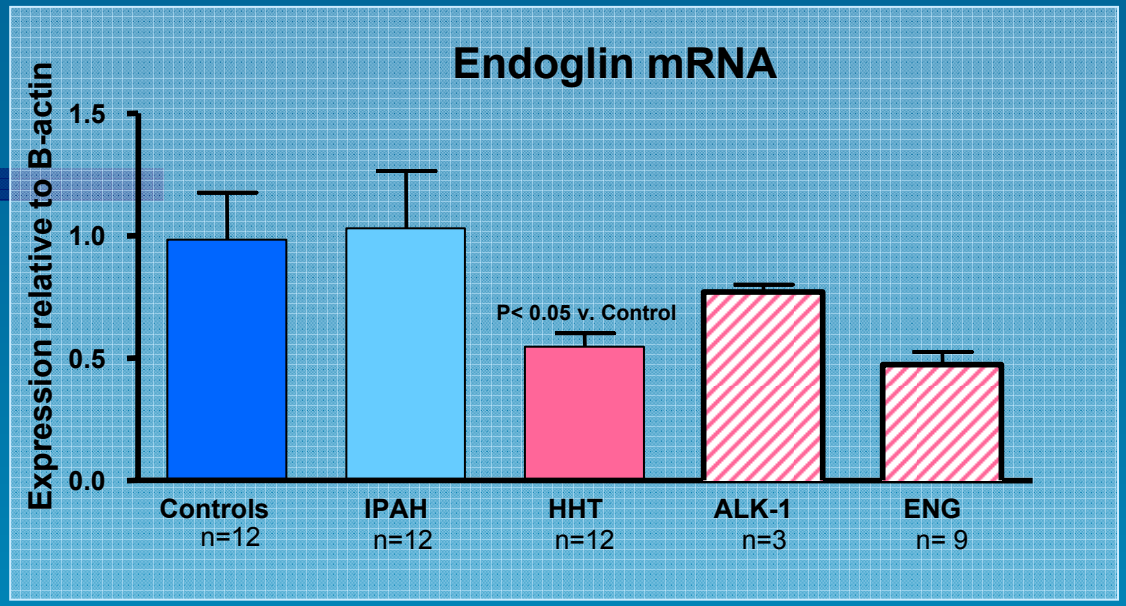
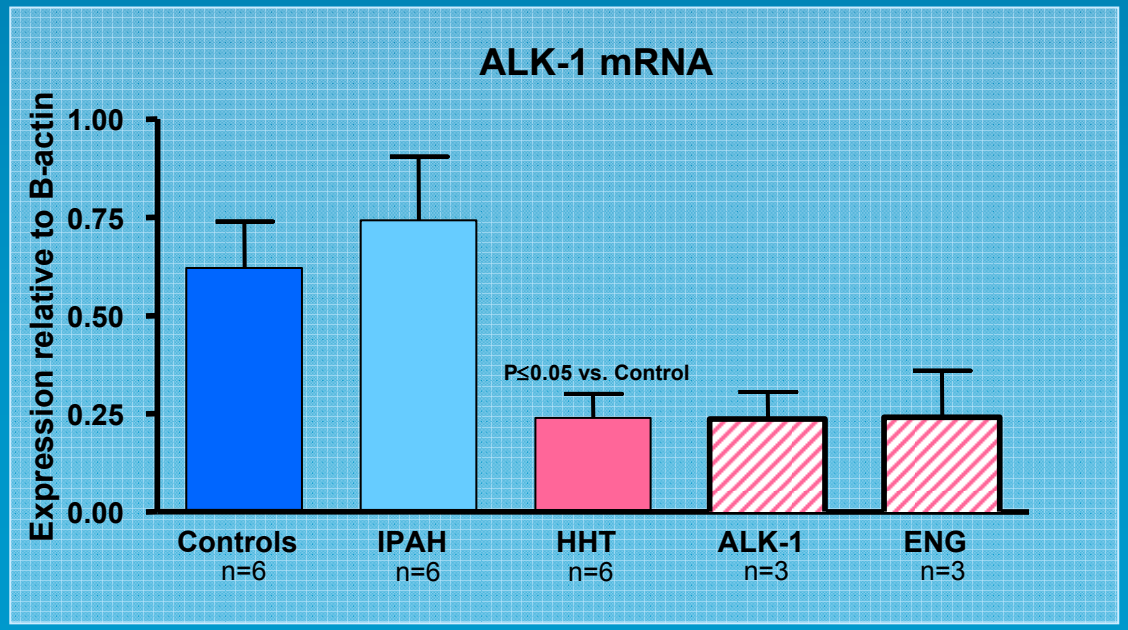
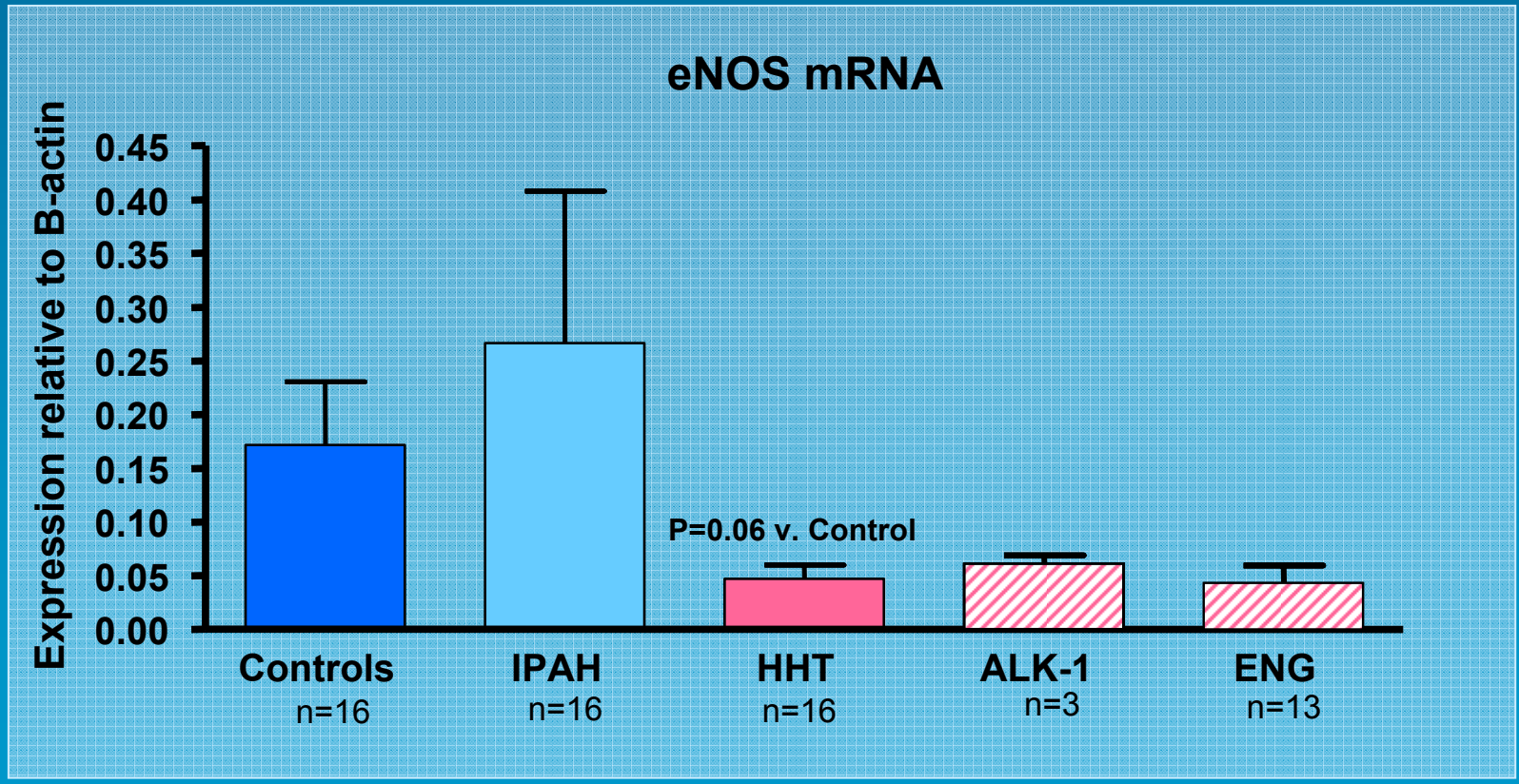
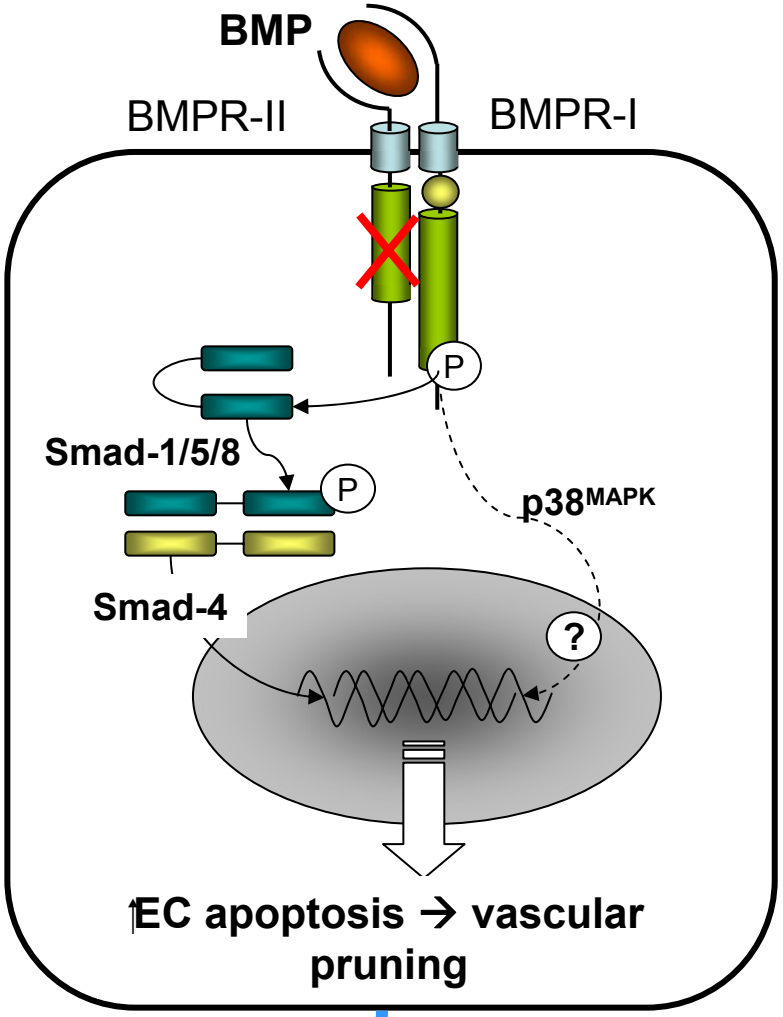


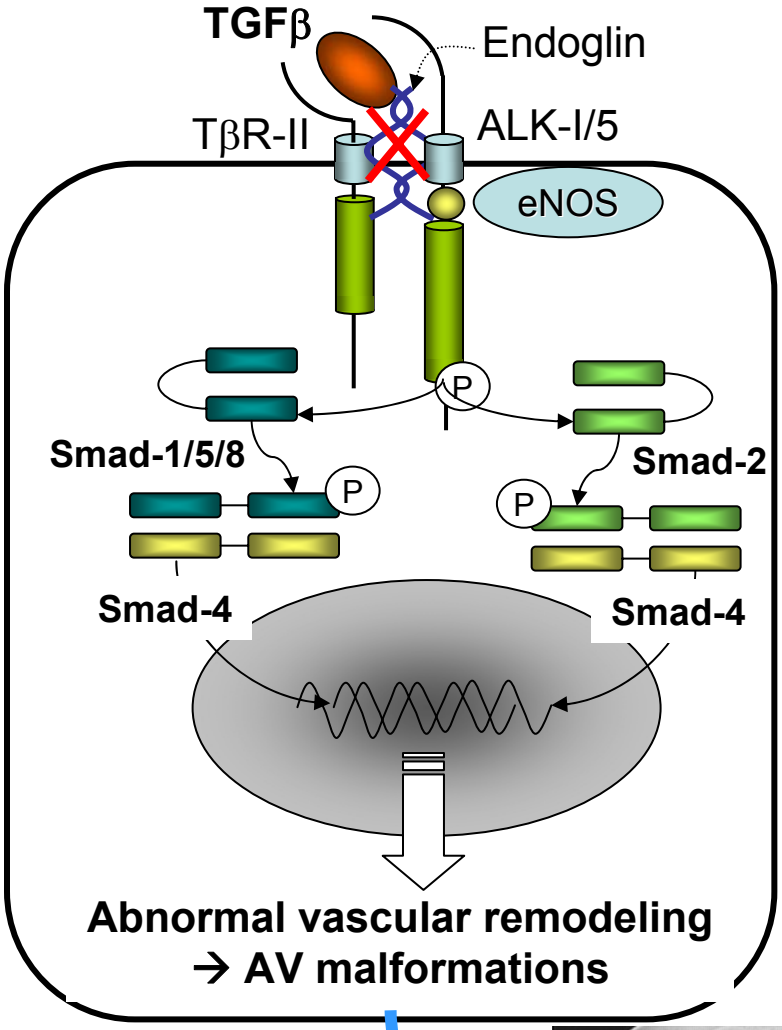


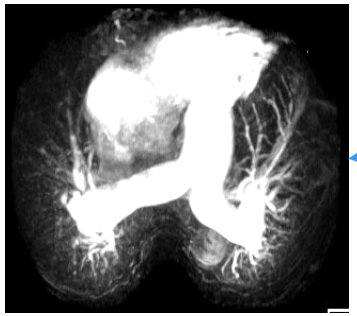
Effect on Gene Expression



Endothelial Cells/EPCs

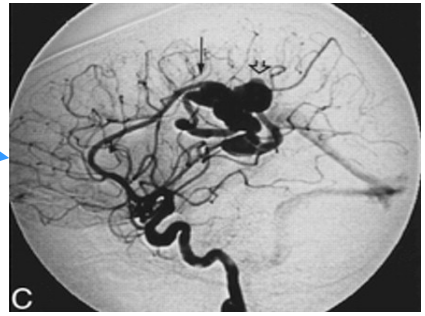






PAH

HHT



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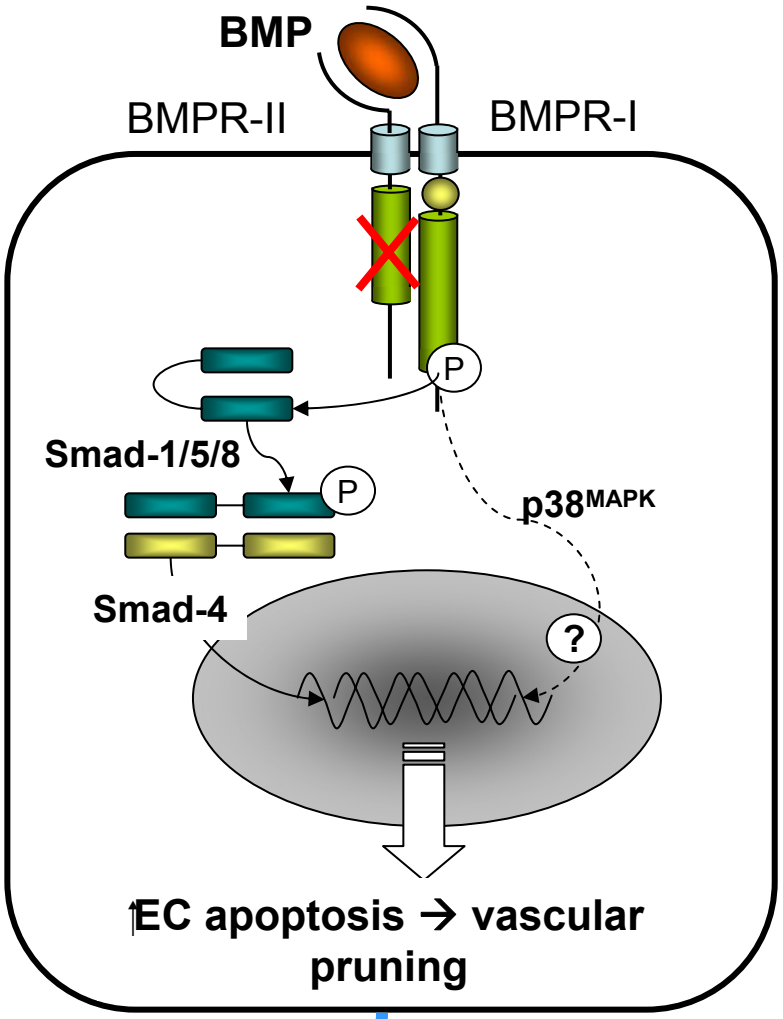
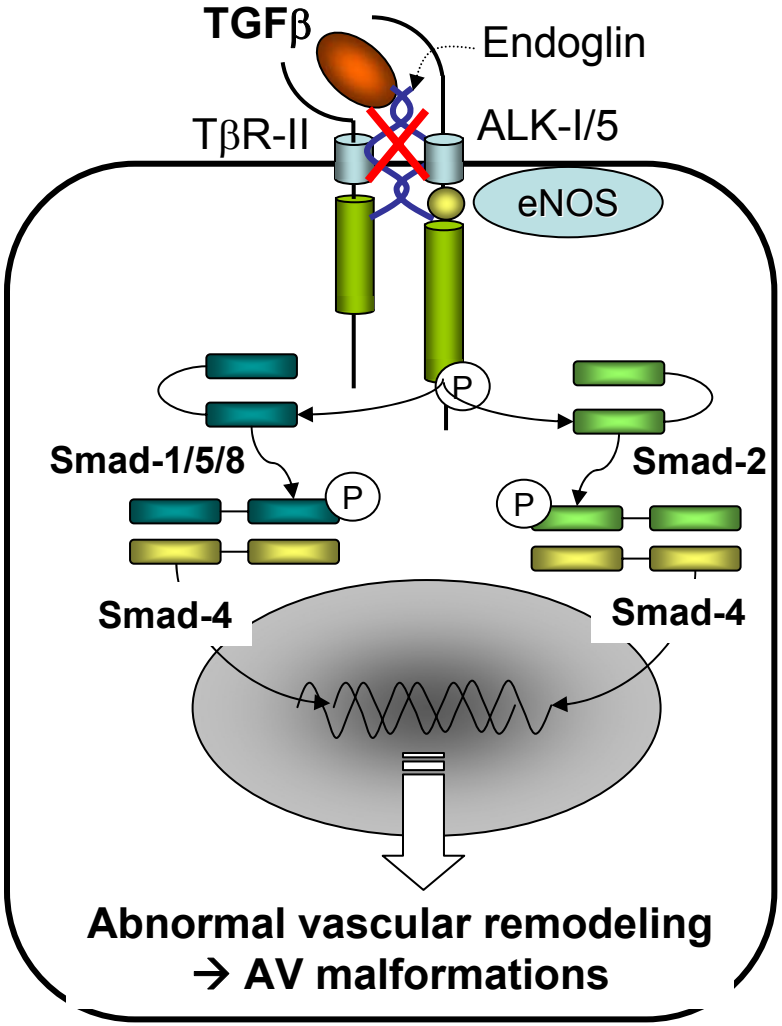
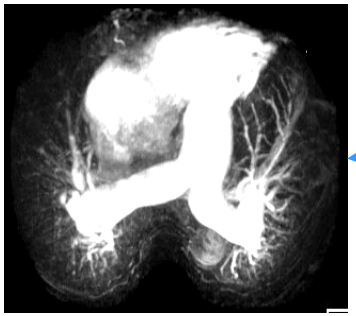
  - Mike Ward
  - Marie Faughnan
  - NicK Morrell

Supported by Northern Therapeutics

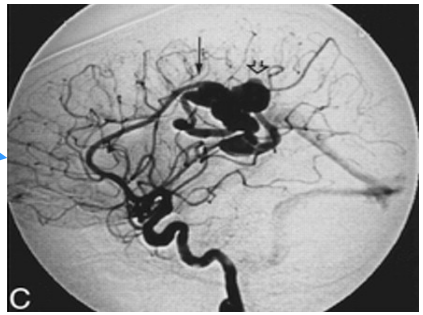

BMP
BMPR-II
BMPR-I
P
Smad-1/5/8
P
p38MAPK
Smad-4
?
↑EC apoptosis → vascular pruning


TGFβ
Endoglin
TβR-II
ALK-I/5
eNOS
P
Smad-1/5/8
P
Smad-2
P
Smad-4
Smad-4
Abnormal vascular remodeling → AV malformations

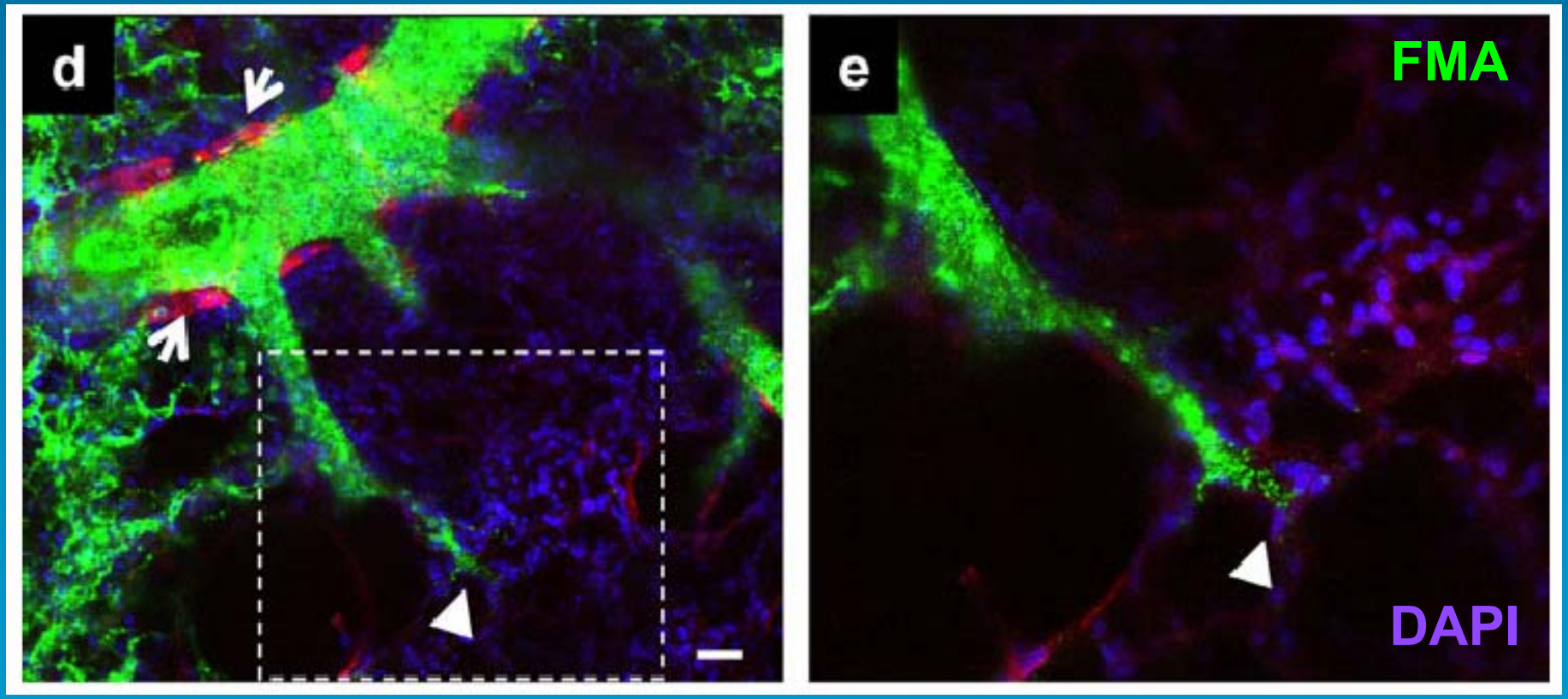


PAH

HHT


C

Arteriolar “discontinuity” in MCT-induced PAH



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

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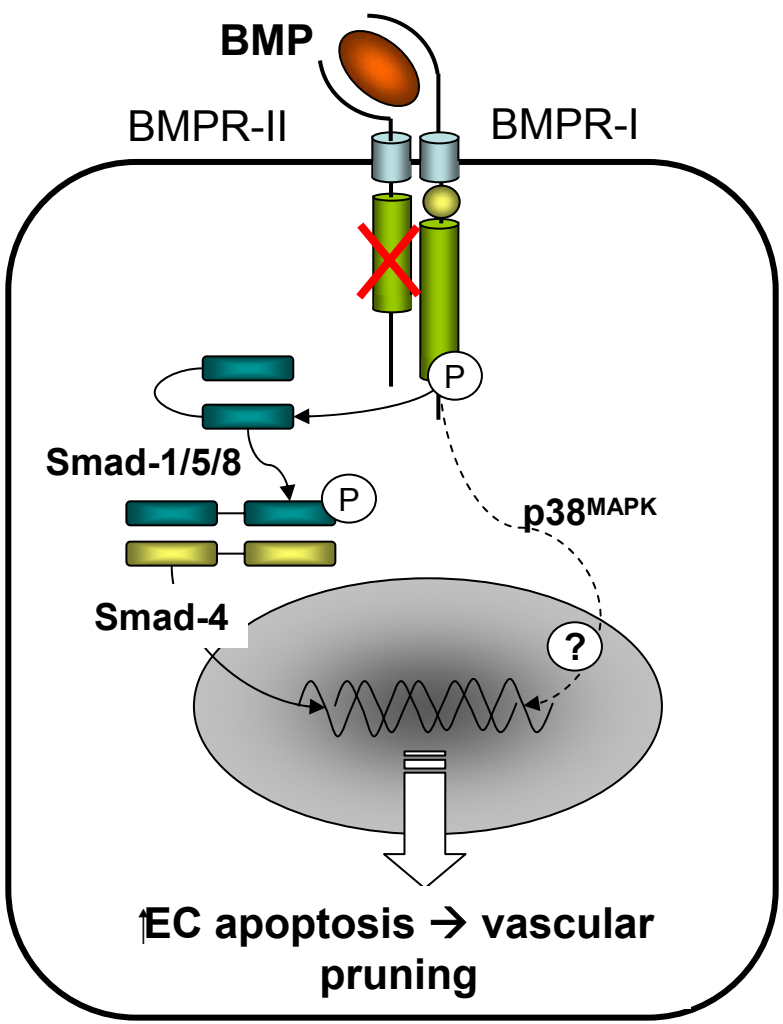
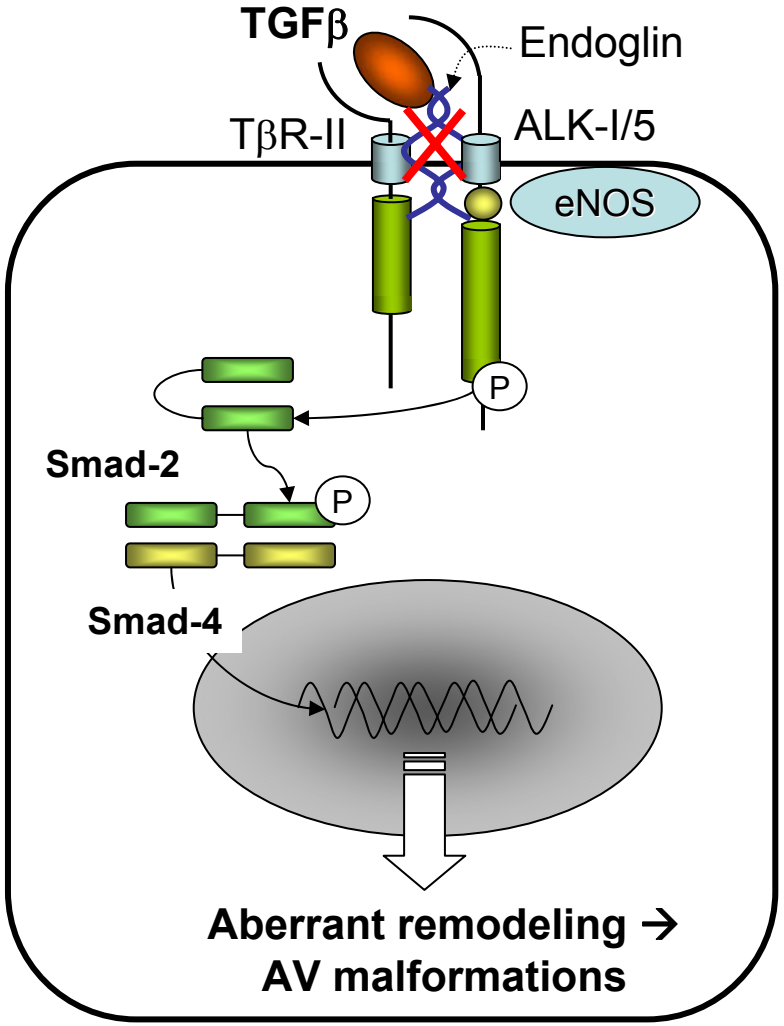
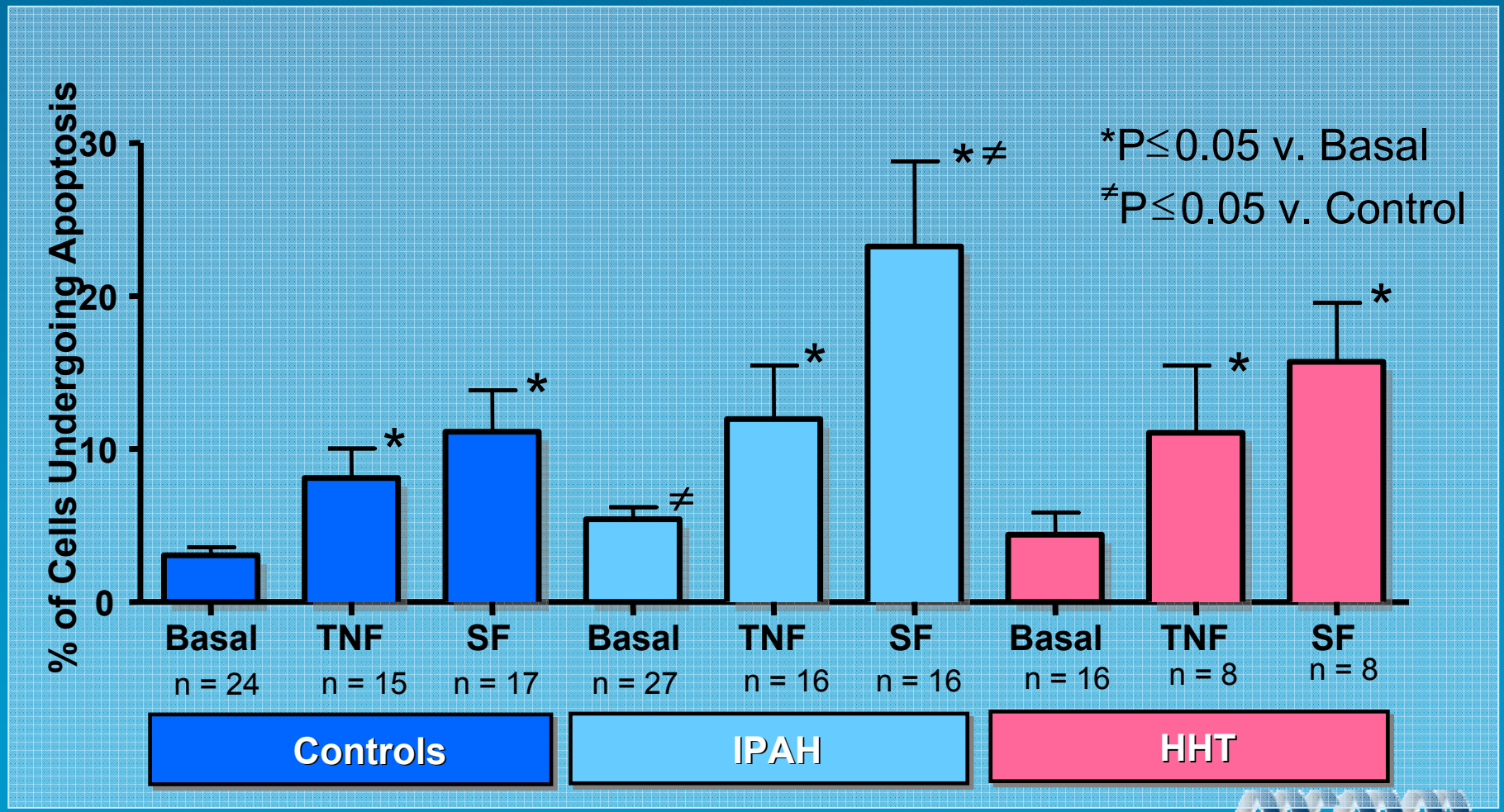

BMP
BMPR-II
BMPR-I
P
Smad-1/5/8
P
p38MAPK
Smad-4
?
↑EC apoptosis → vascular pruning


TGFβ
Endoglin
TβR-II
ALK-I/5
eNOS
P
Smad-2
P
Smad-4
Aberrant remodeling → AV malformations

Effect on Apoptosis



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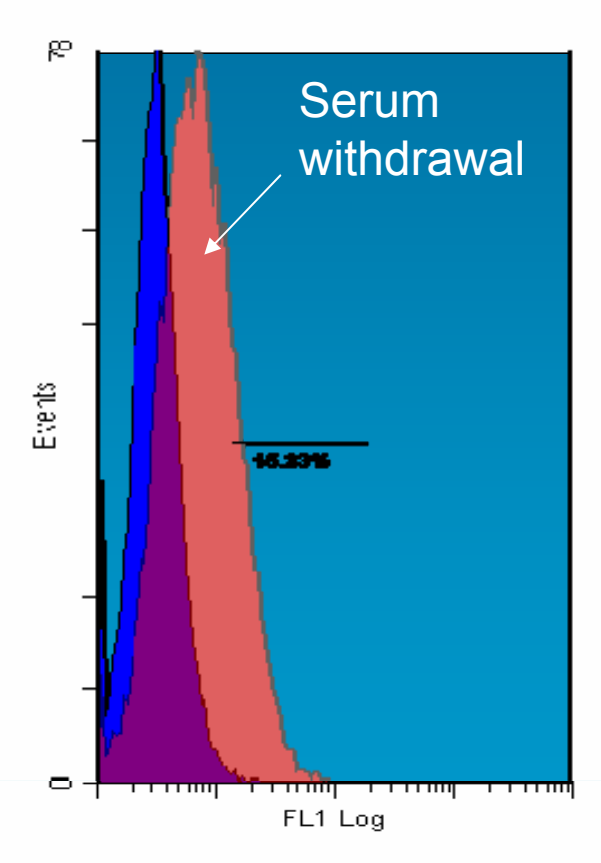
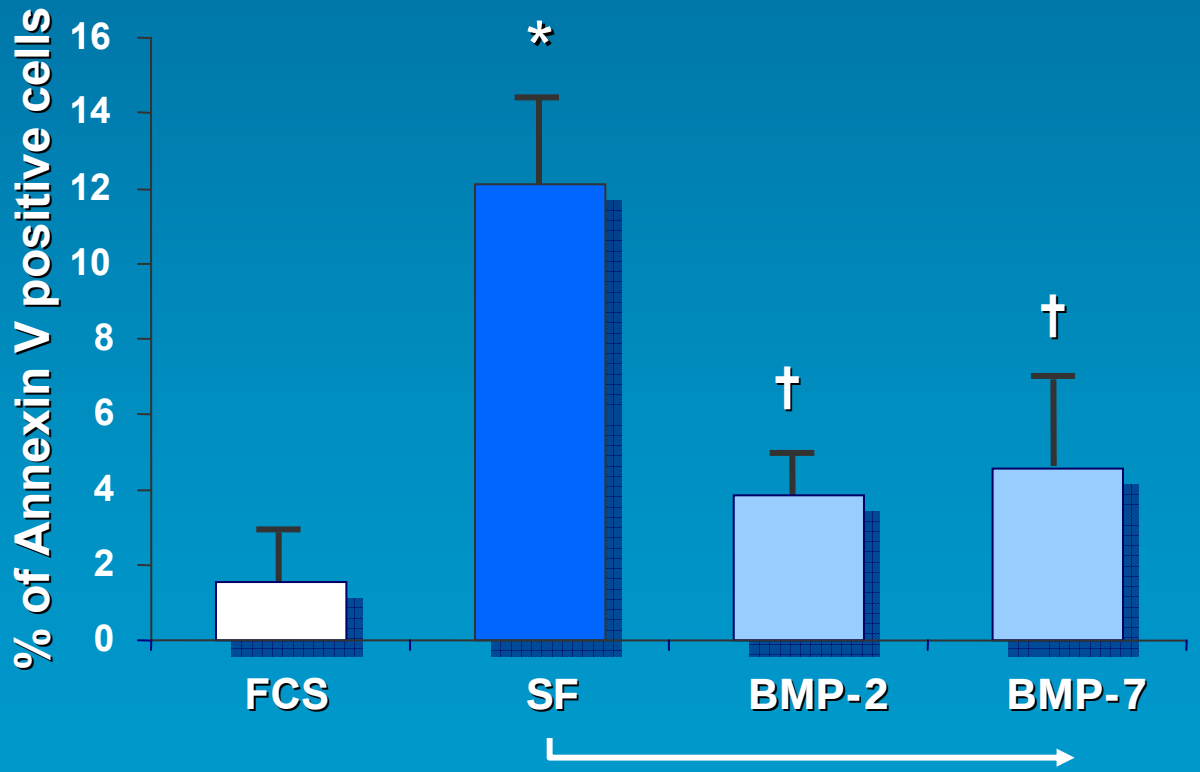
BMPs Inhibits Apoptosis in human PAEC post serum withdrawal





Circulation Research 2005

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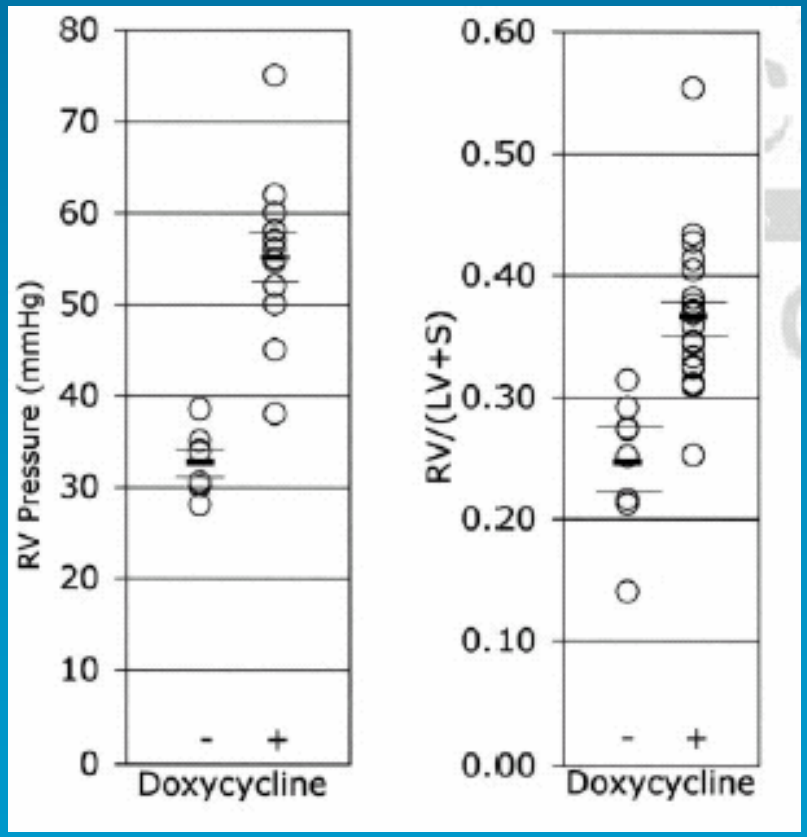
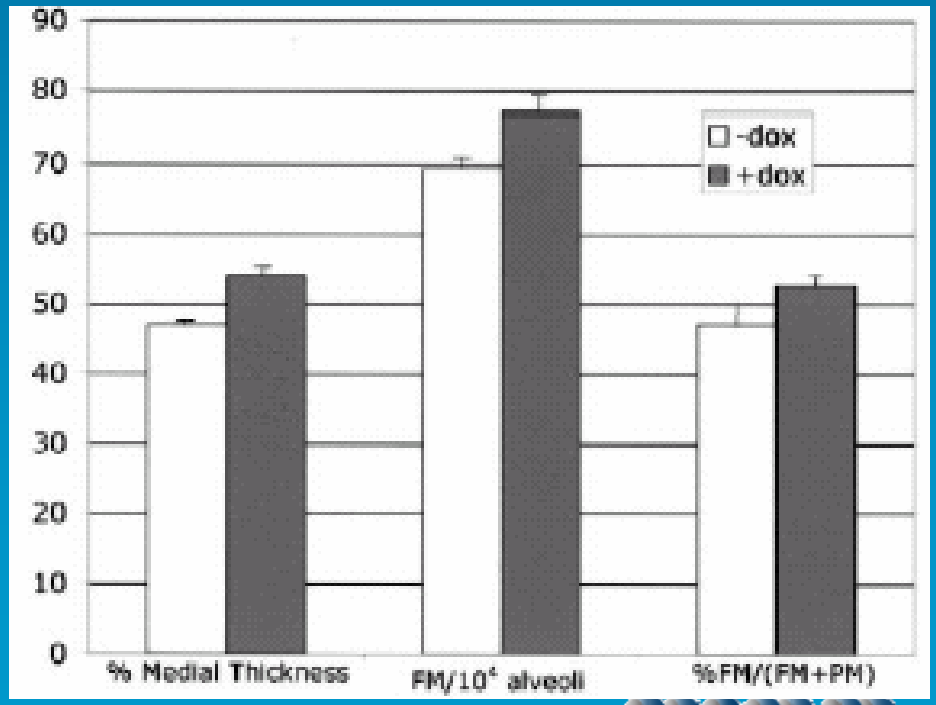
PAH in transgenic mice overexpressing dominant negative BMRPR2



…but lack of marked arteriolar remodeling?



West et al. Circ Res 94: ●●●, 2004

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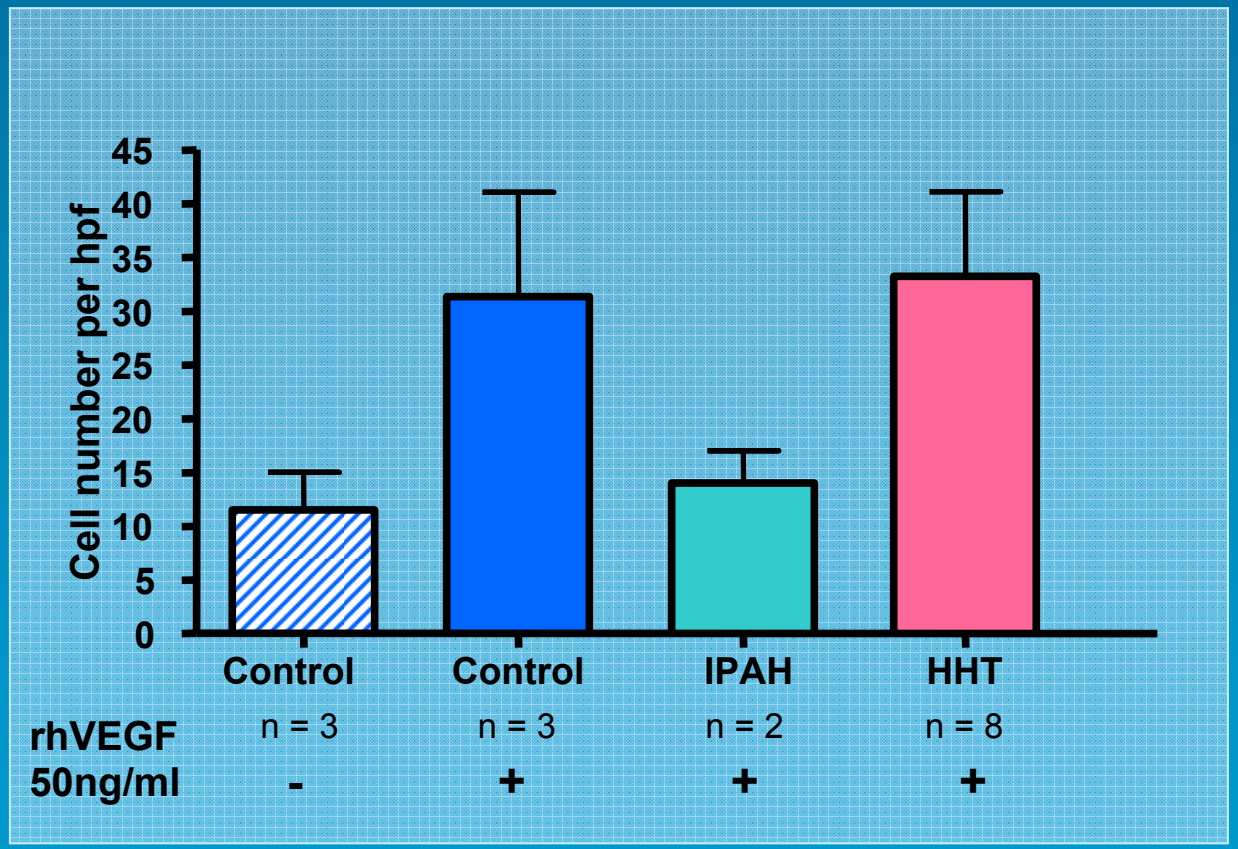
Effect of "Genotype" of EPC Migration



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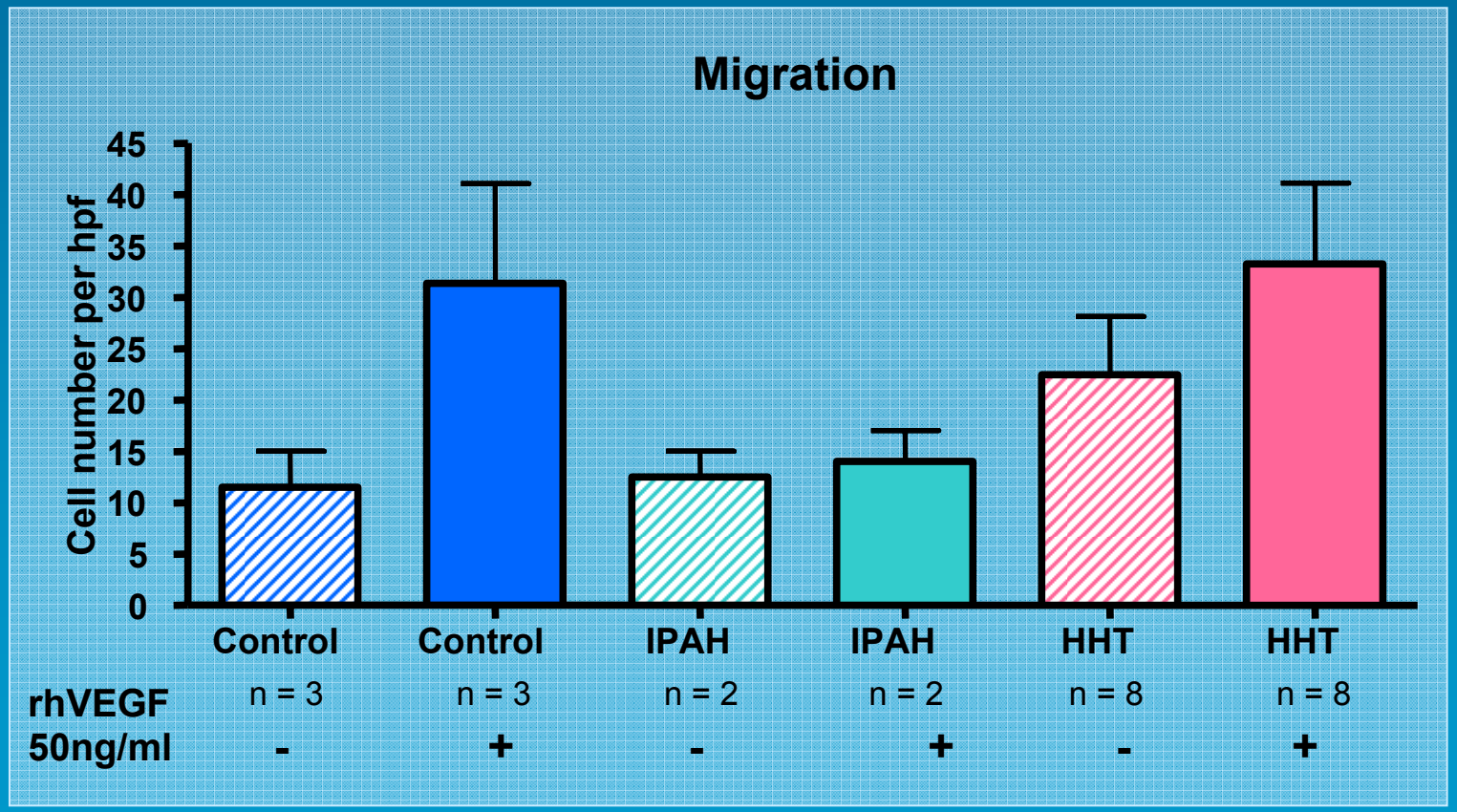
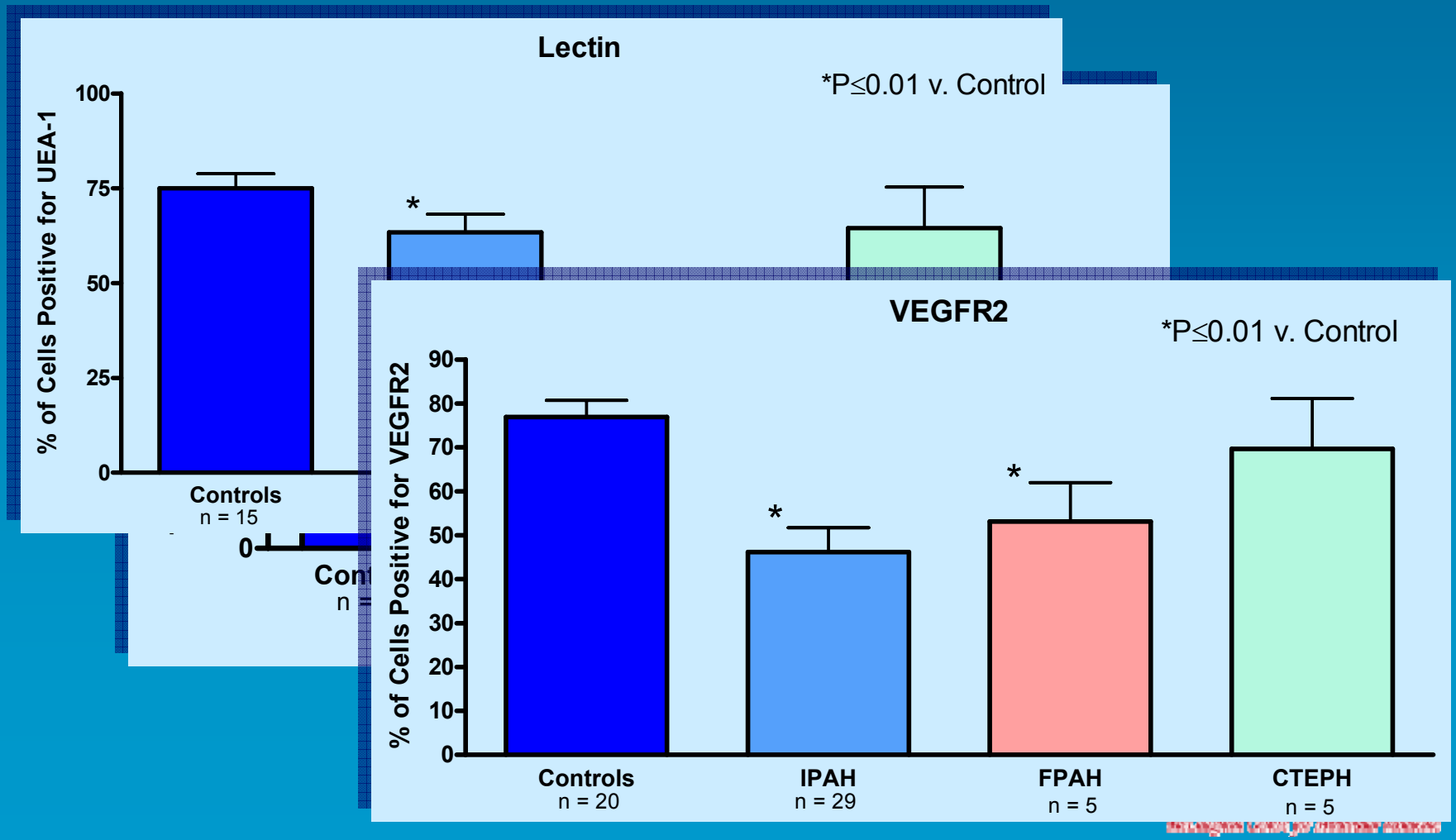
Effect of "Genotype" of EPC Migration



EPC Differentiation



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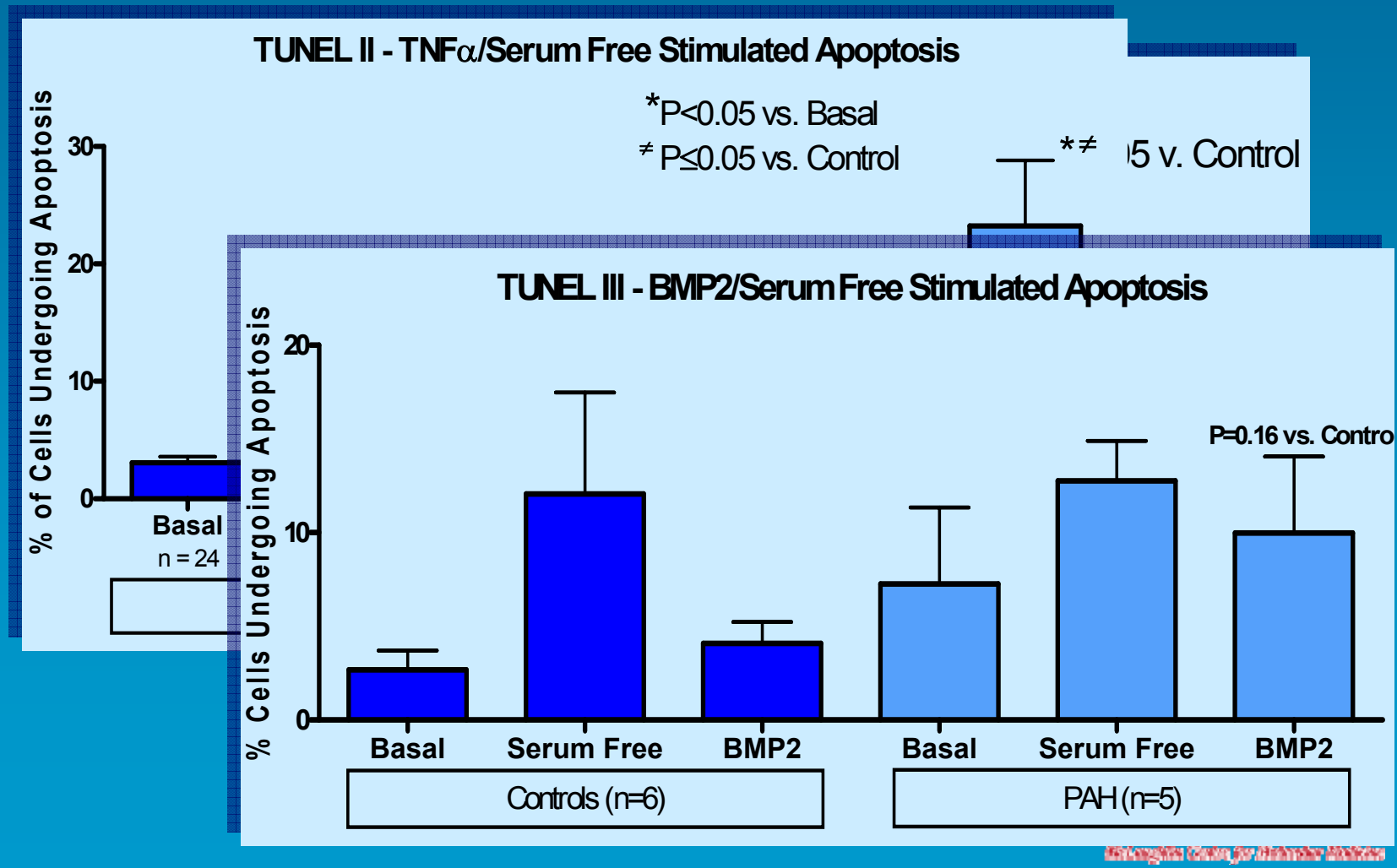
Effect of "genotype" on EPC apoptosis



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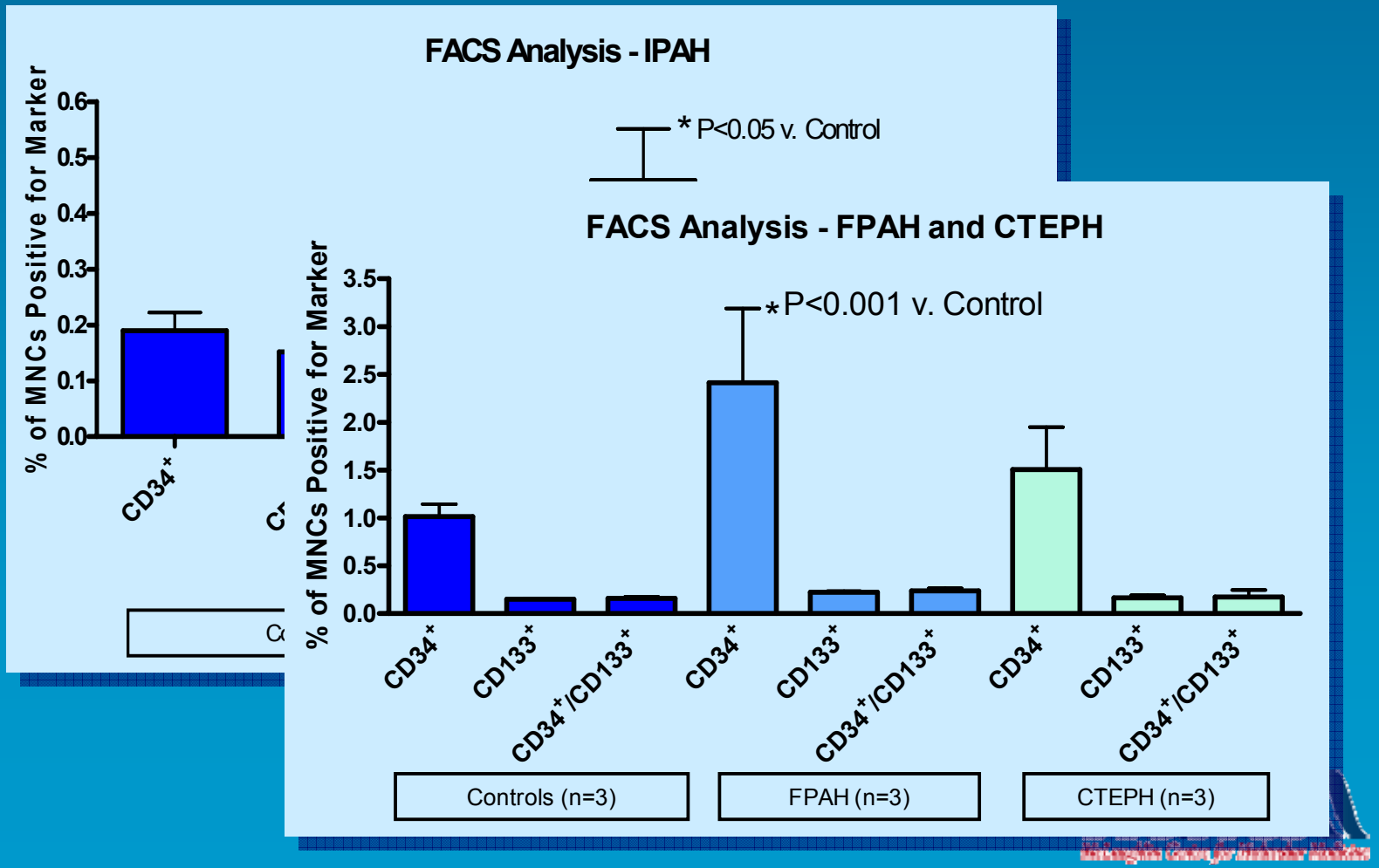
Circulating "EPCs"



MRI perfusion imagining of the pulmonary vasculature

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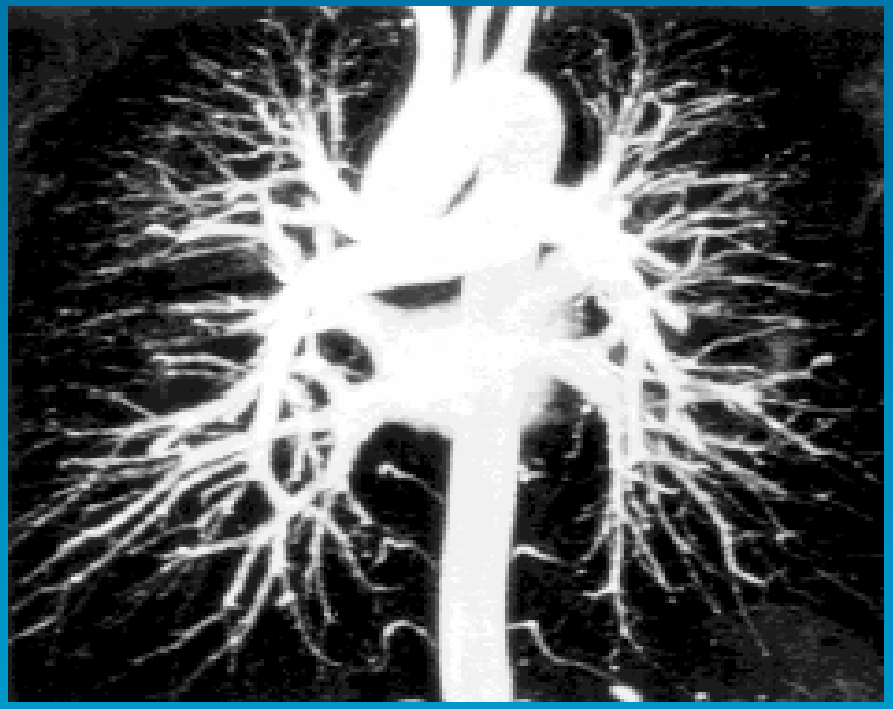
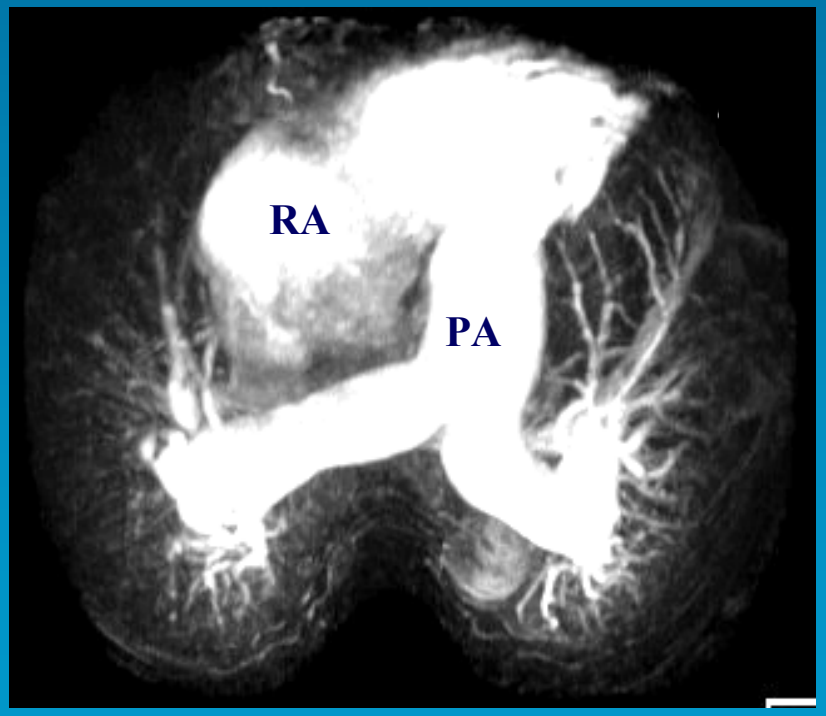
Normal

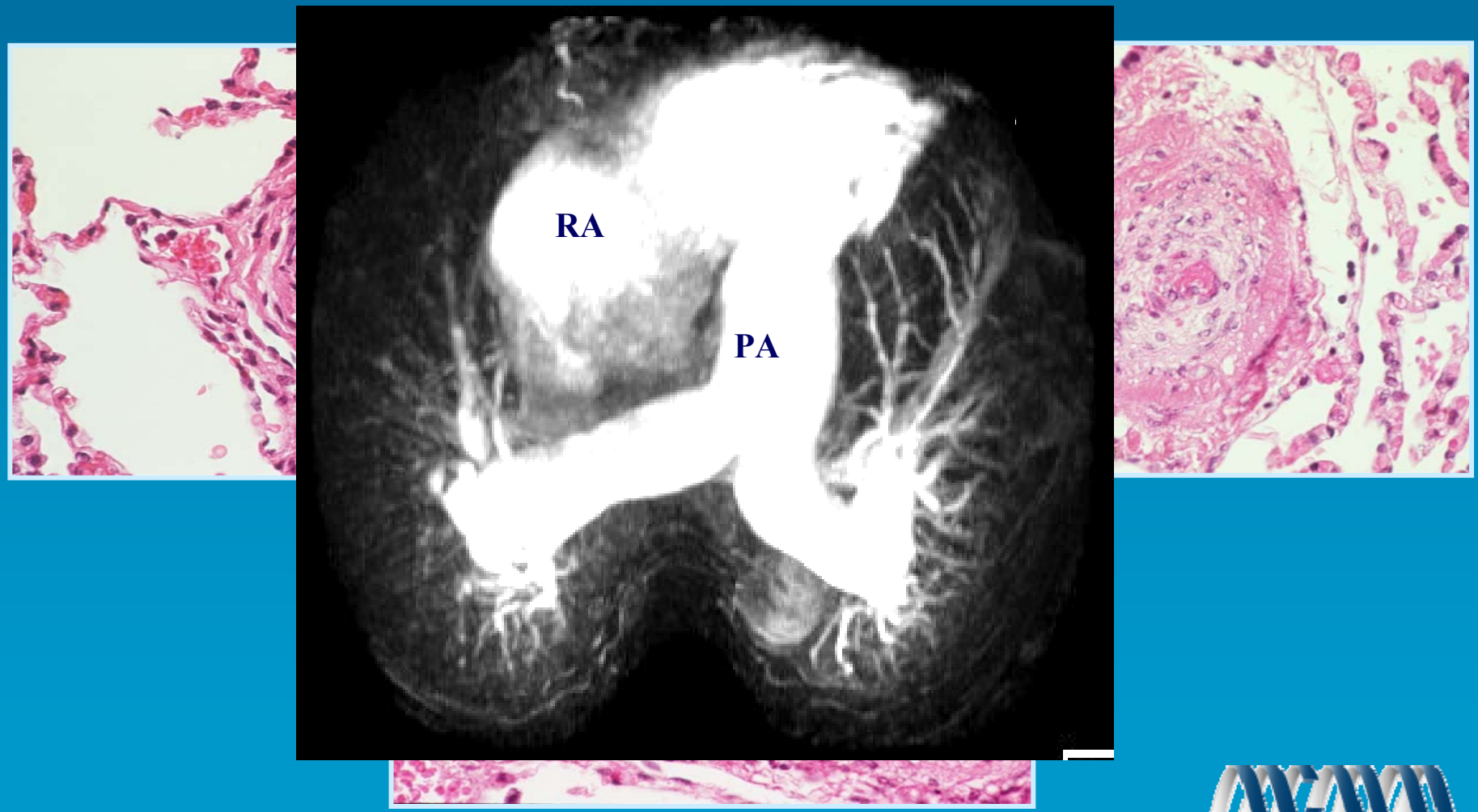


PAH



Courtesy of Evangelos Michelakis, U of Alberta

The problem – PAH through the looking glass



Archer and Rich: Circulation 102:2781, 2000

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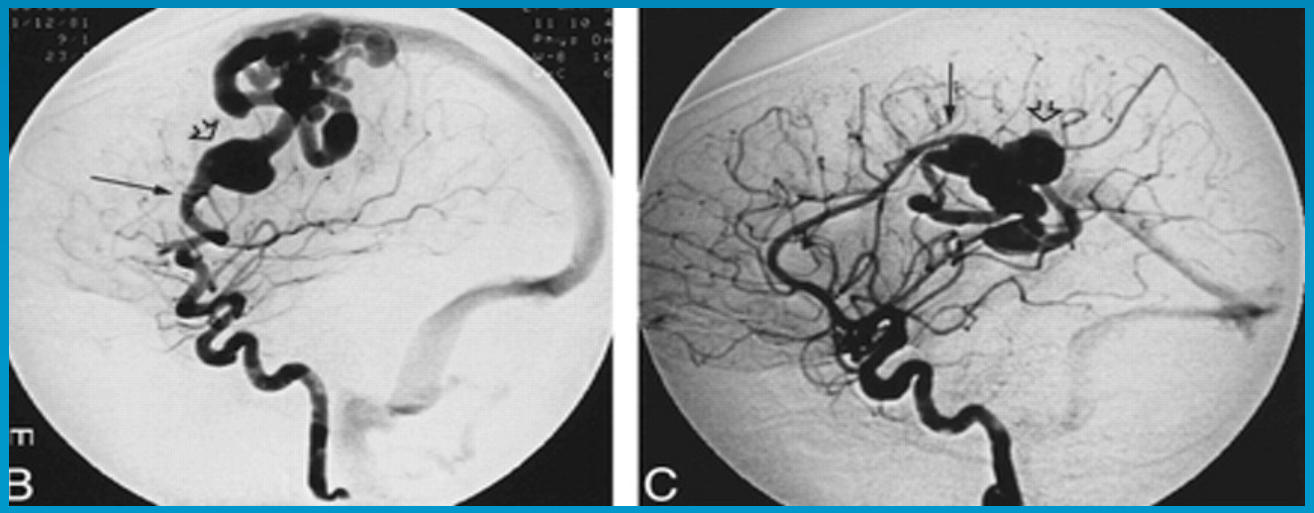
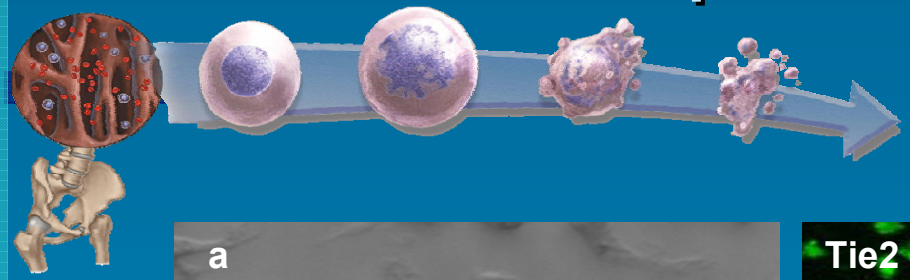
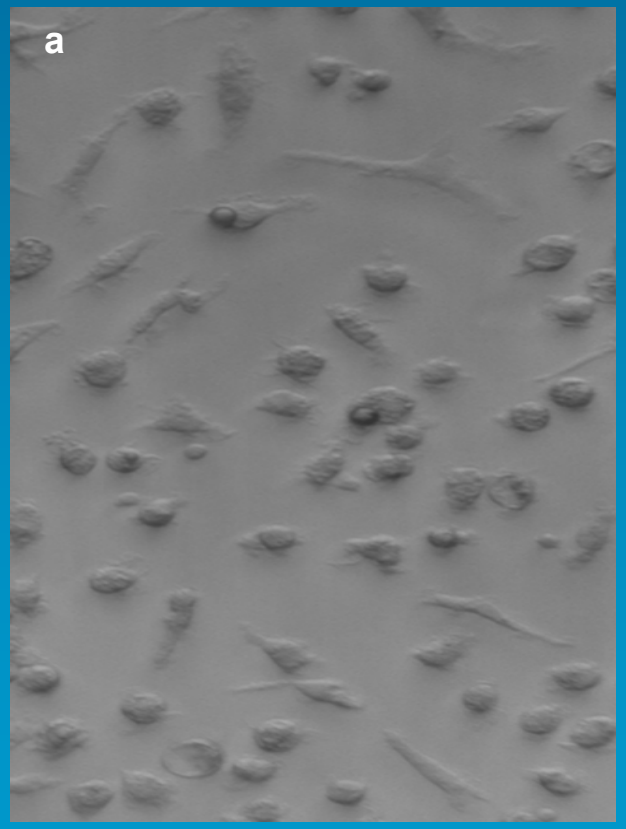
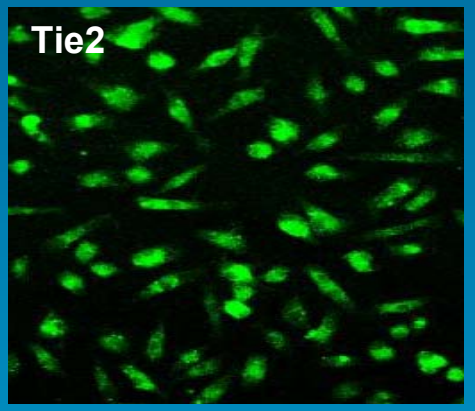
Thy lesions of HHT

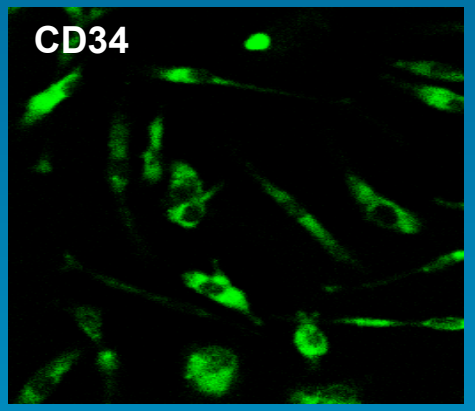


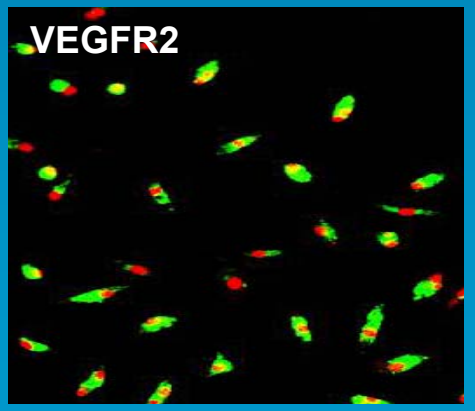
Heterogenous response to BMP-2 in EPCs from patients with iPAH

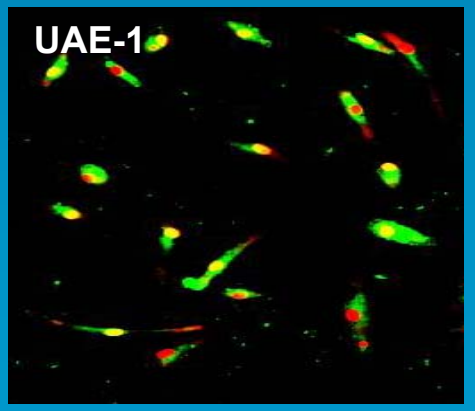












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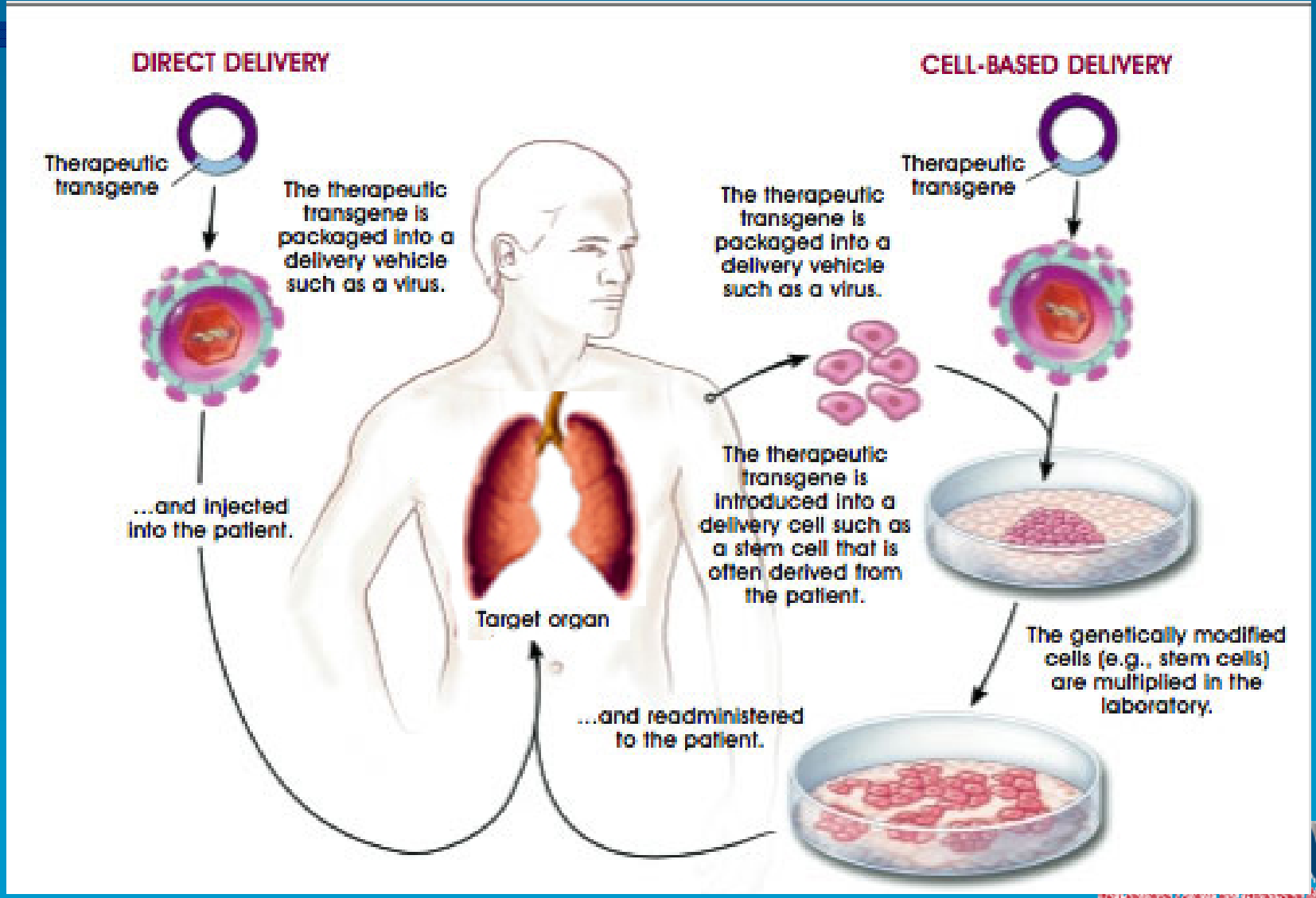
Cell-based gene therapy



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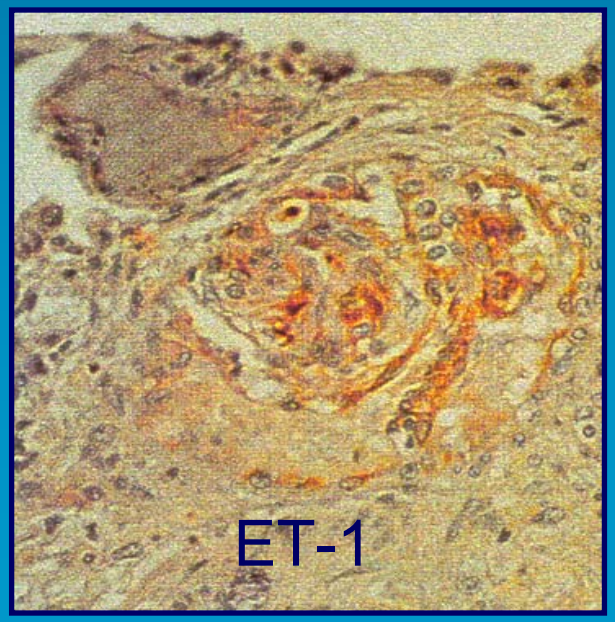
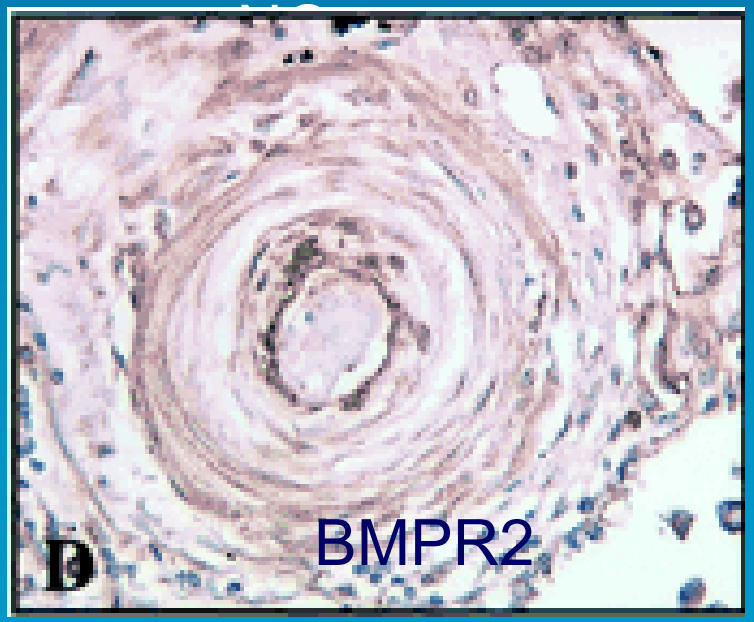
Imbalance in growth regulation?

↑ Vascular growth factors



Giaid … Stewart NEJM, 1993

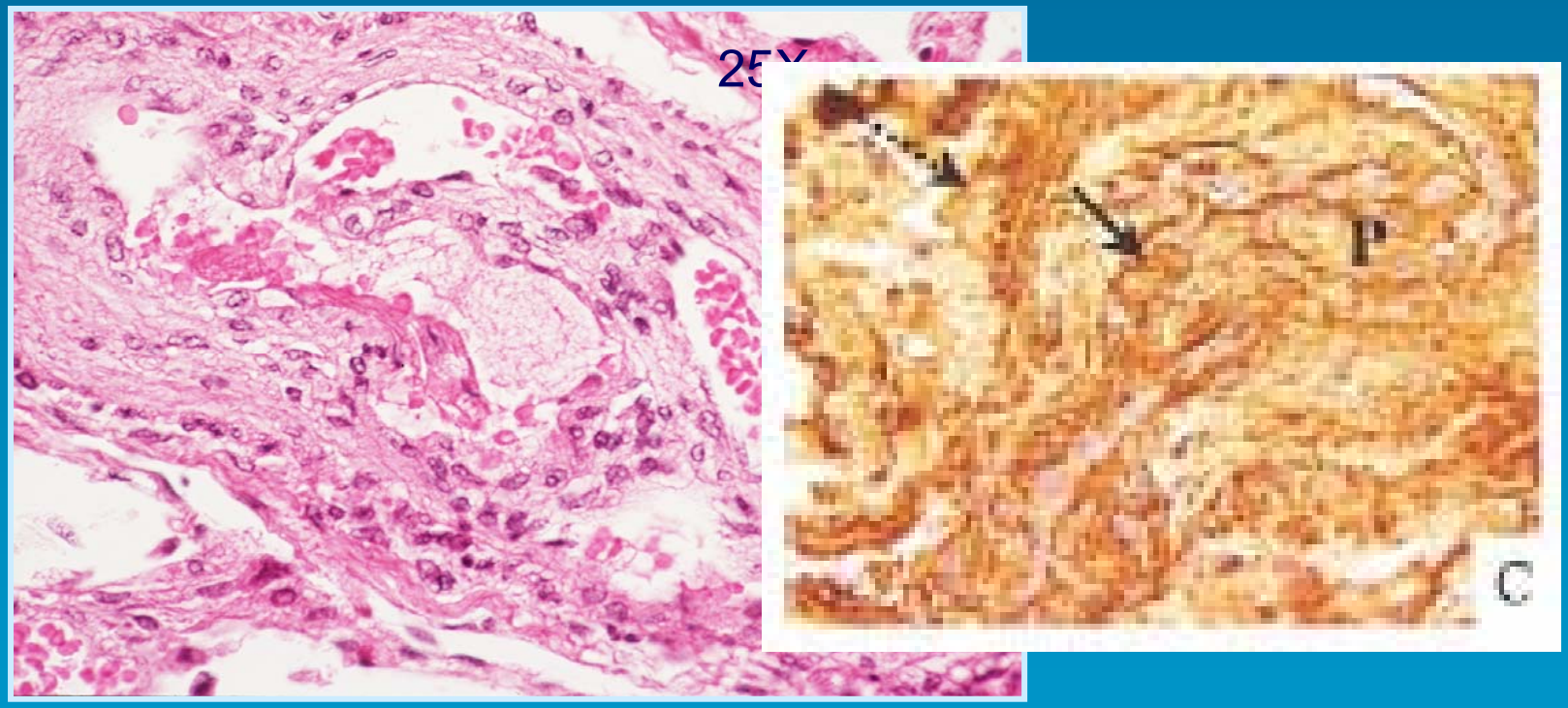
↓ Growth inhibitory pathways



Atkinson et al. Circulation 105:1672,2002

The plexiform lesion: role of angiogenesis?

Tuder et al J Pathol 195:367, 2001



Archer and Rich Circulation 102:2781, 2000

- Abnormal growth of vascular endothelial cells
- Upregulation of VEGF

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Experimental studies: inhibition of growth factor signaling

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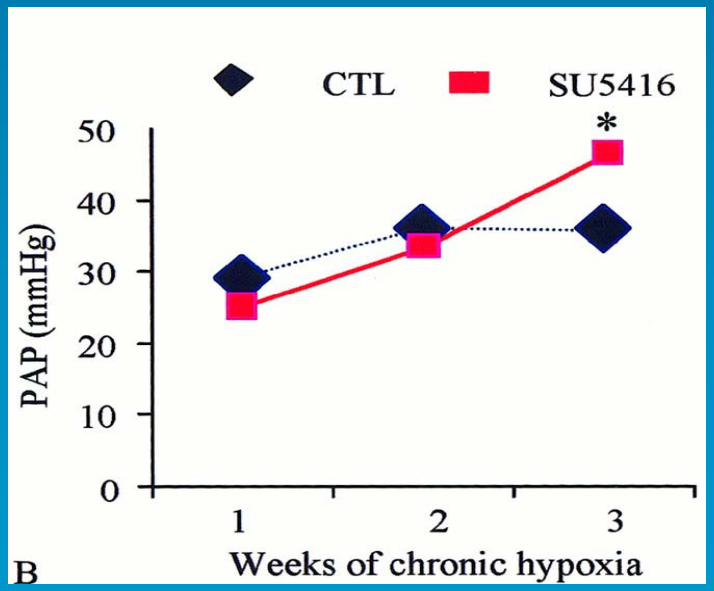
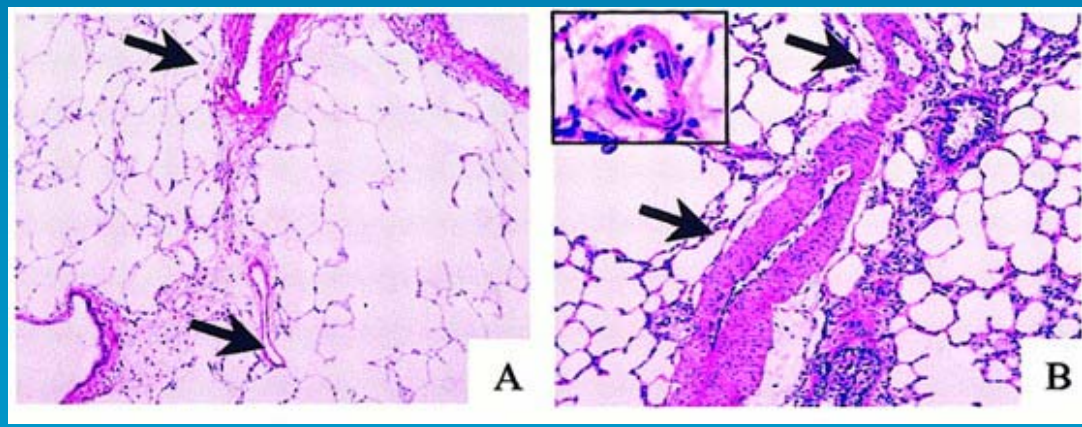
Effect of VEGF receptor antagonist (SU5416)



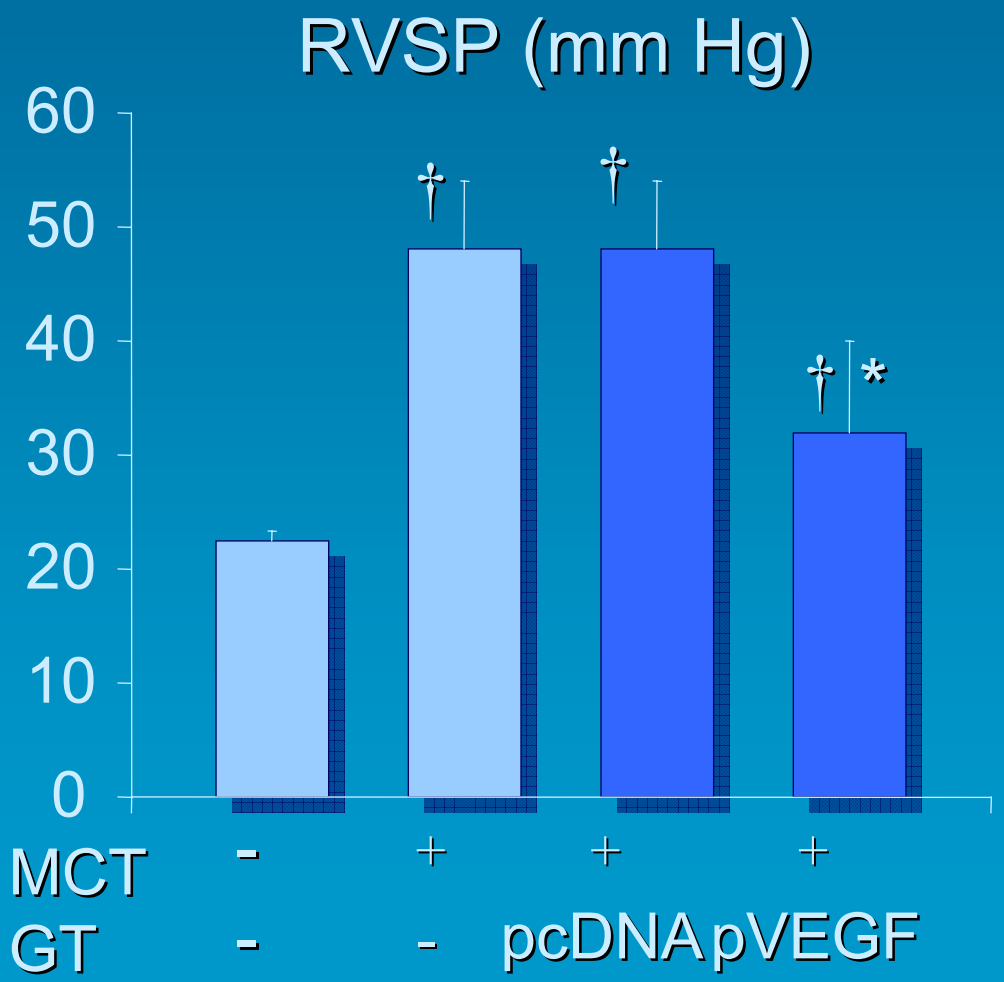
Hypoxia **Hypox & SU5416**

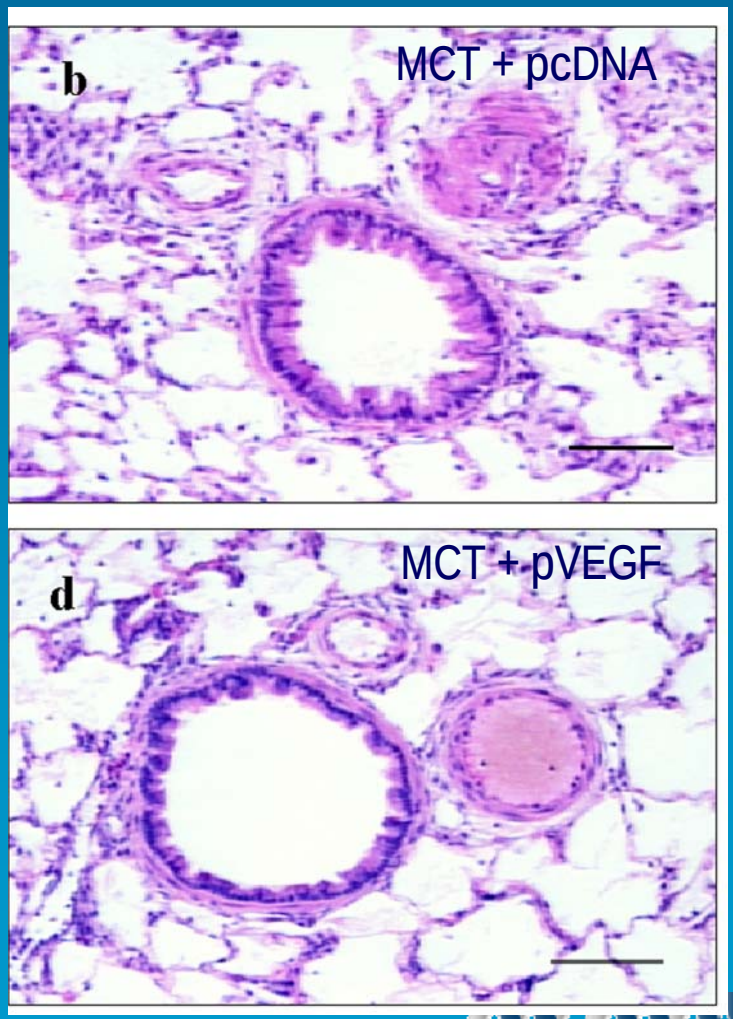


Effects of SU5416 reversed by z-ASP

Taraseviciene-Stewart et al. FASEB J. 15:427,2001

Effect of VEGF gene transfer in MCT model of PAH



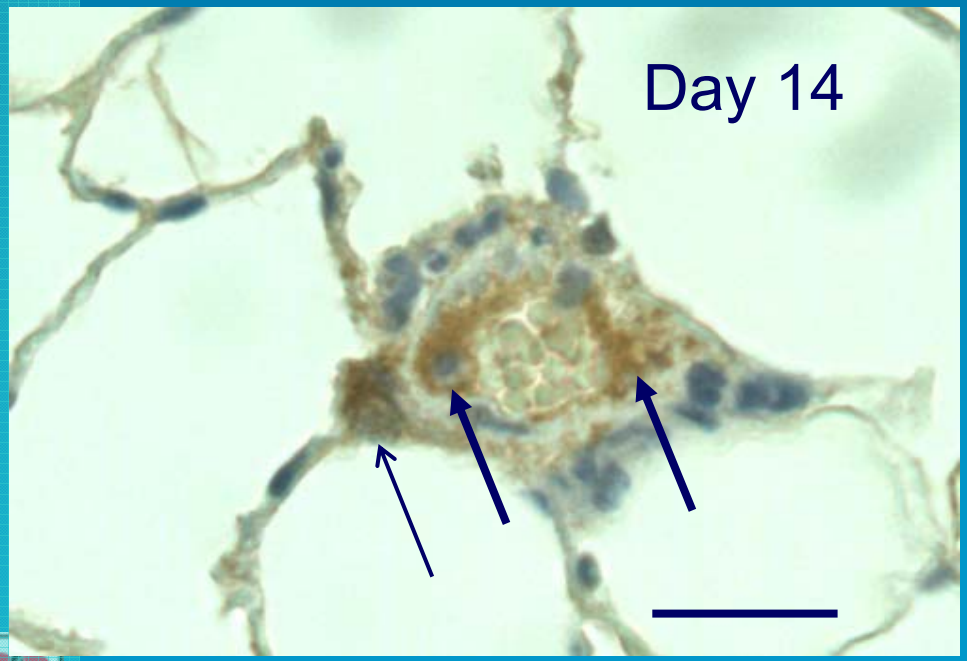


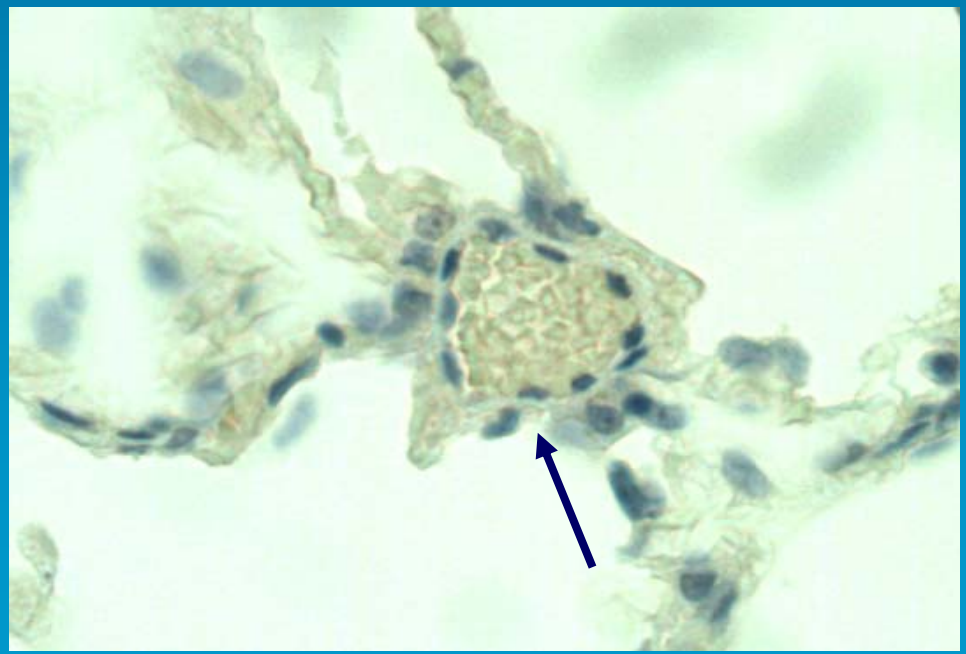
Campbell et al. Circulation 2001;104:2242-2248

VEGF inhibits MCT-induced microvascular EC apoptosis

MCT alone

MCT and pVEGF

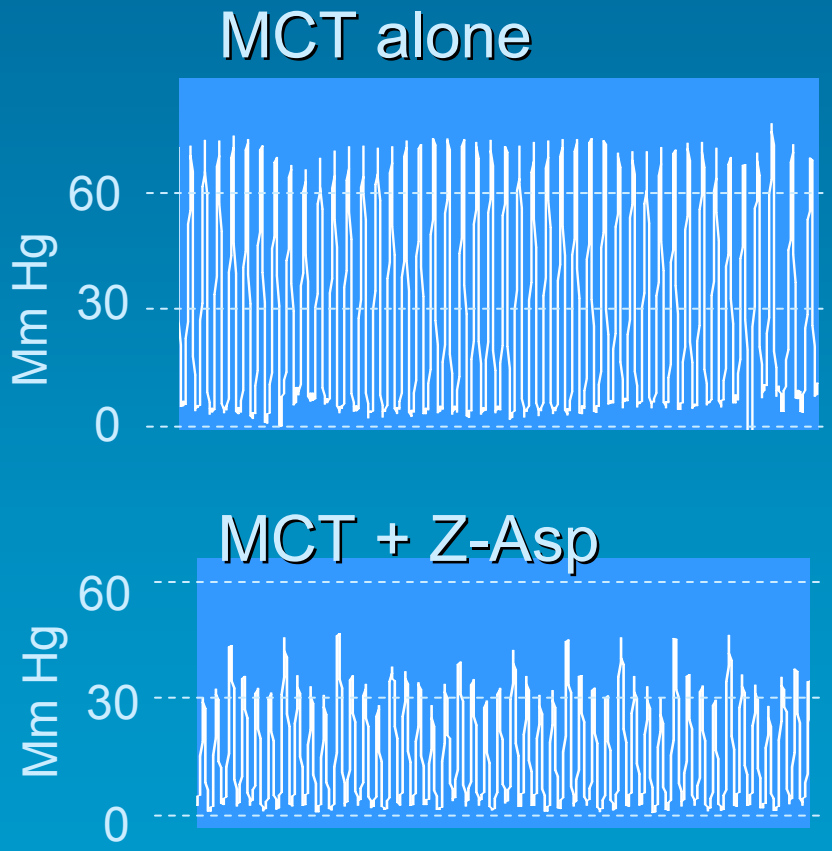


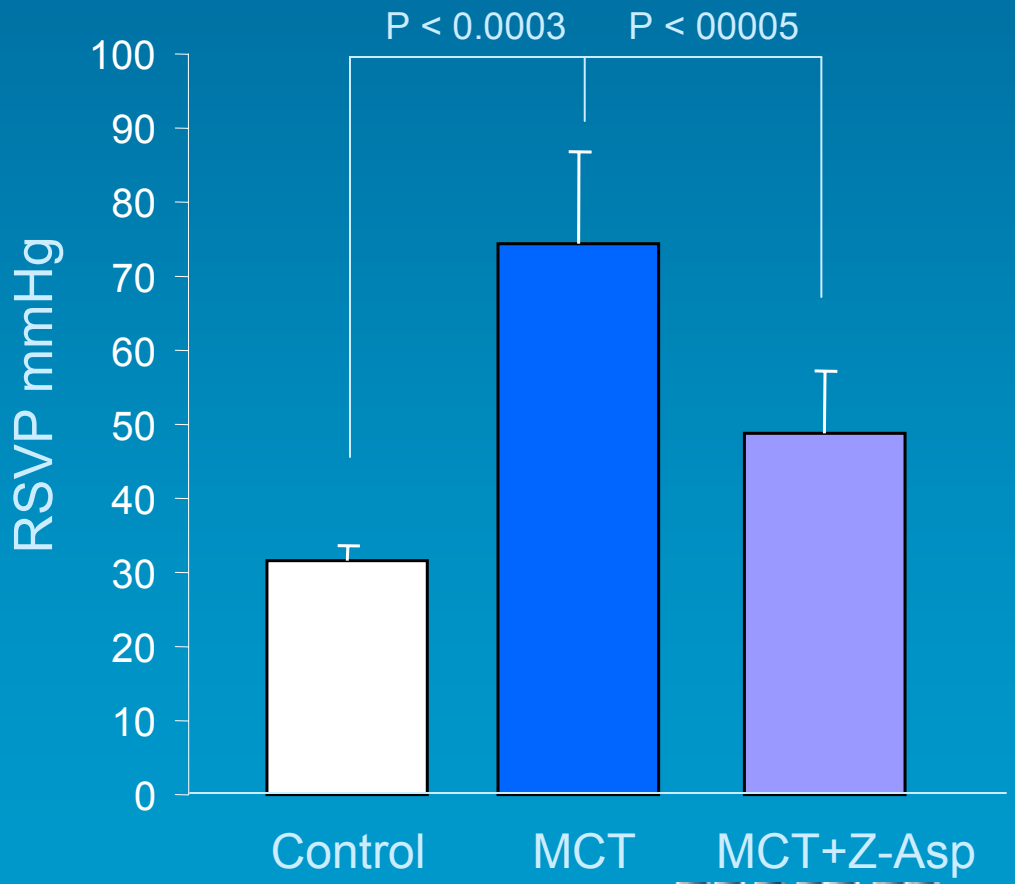


Campbell et al. Circulation 2001;104:2242-2248

Effect of Z-Asp on RVSP at day 21

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





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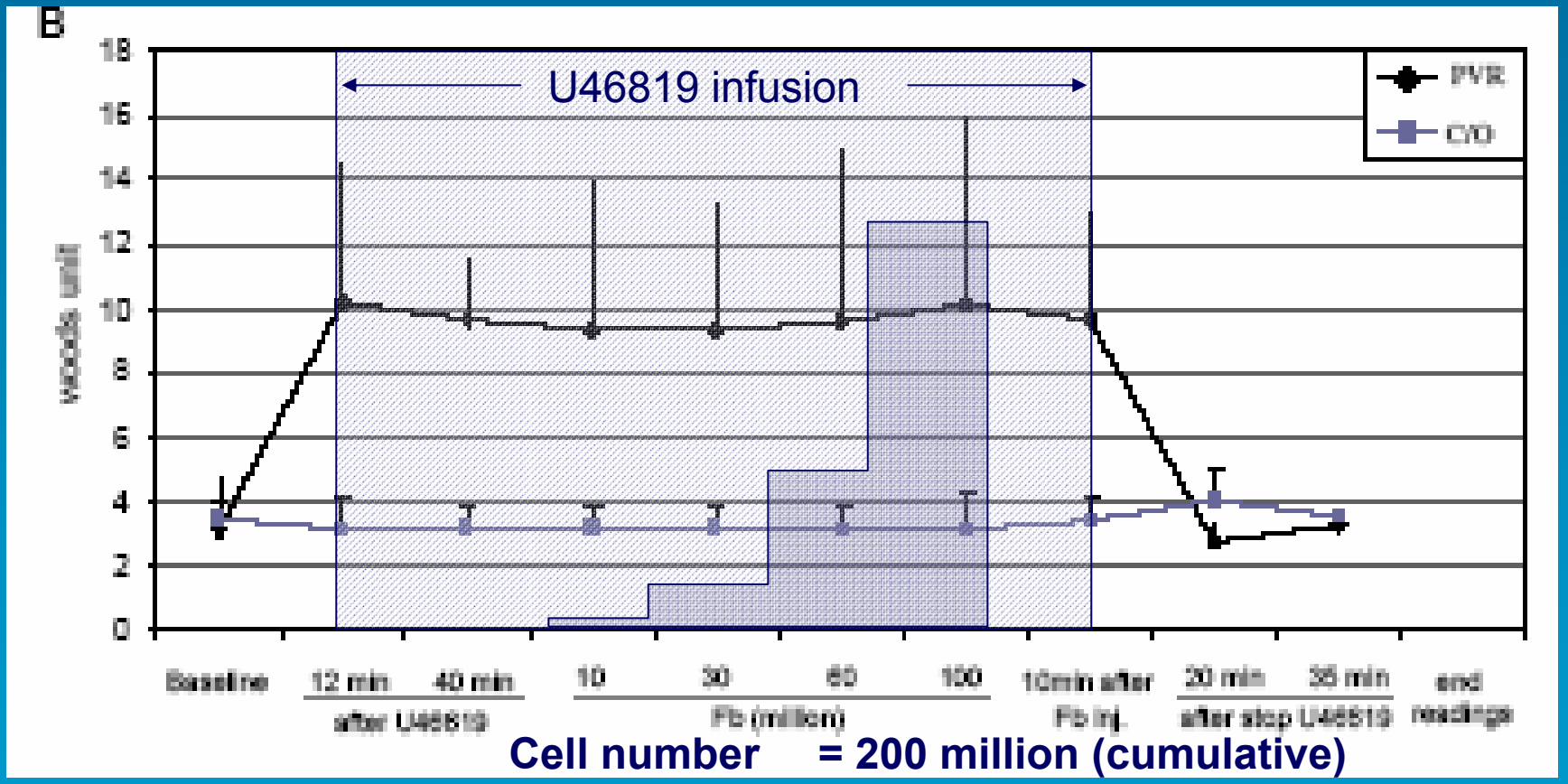
Stopping Criteria for Dose Escalation

- 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)

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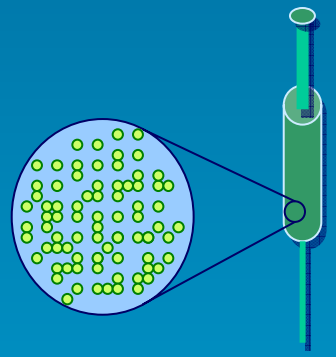
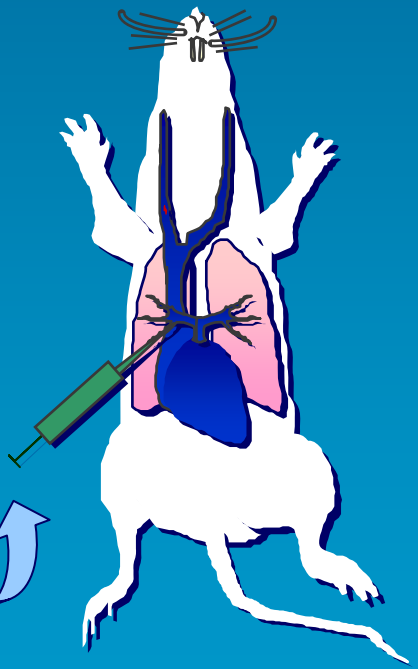
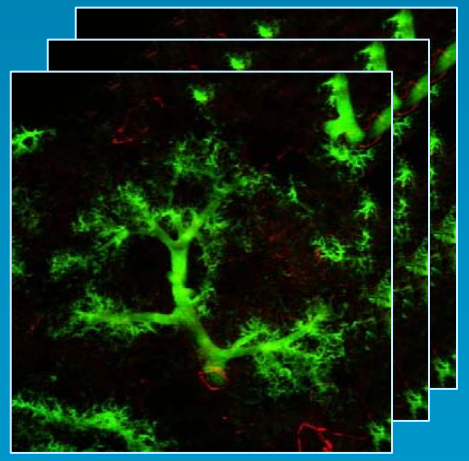
Fluorescent 3-dimensional microangiography



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





Confocal optical sectioning of thick (100 – 200μm) sections of lung

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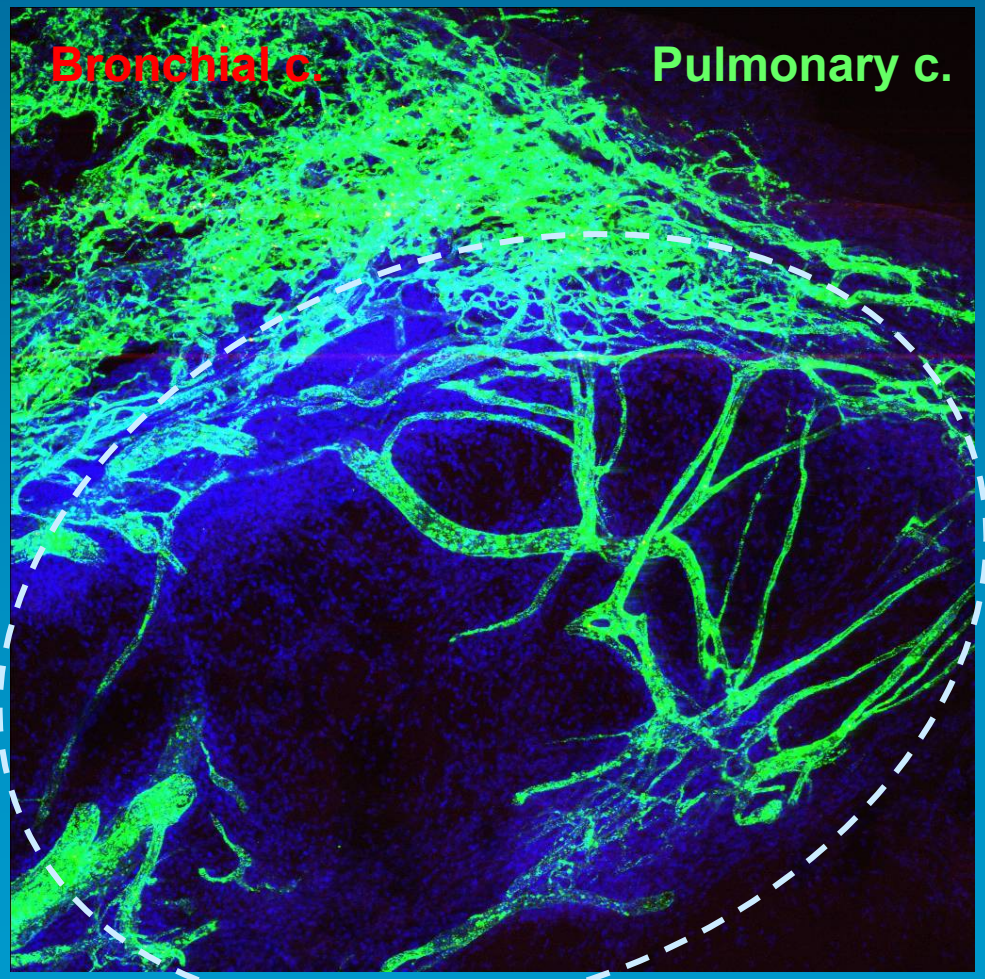
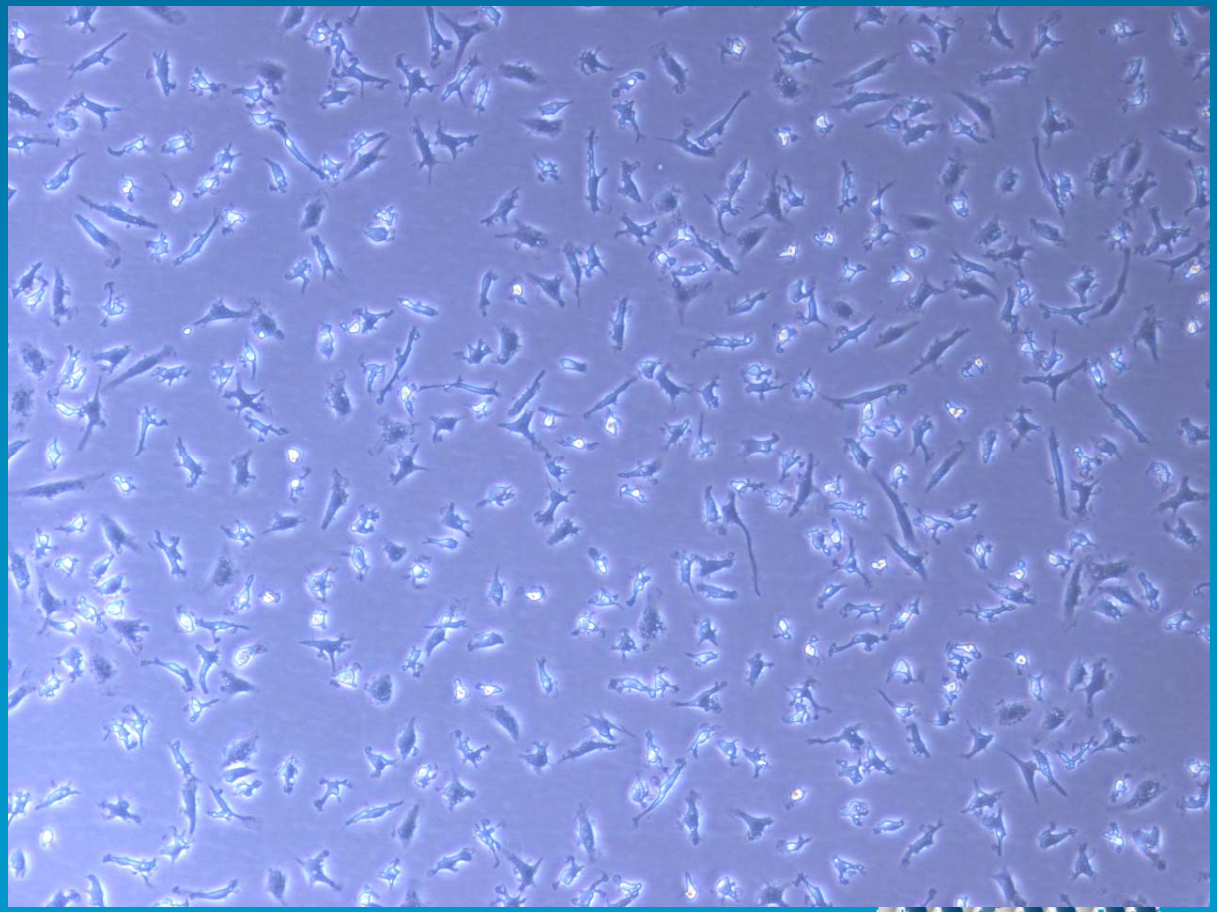
De novo angiogenesis from the pulmonary microvasculature



Avascular Matrigel plug

The final product!

2.5 x10^6 heNOS-Tx EPCs/ml



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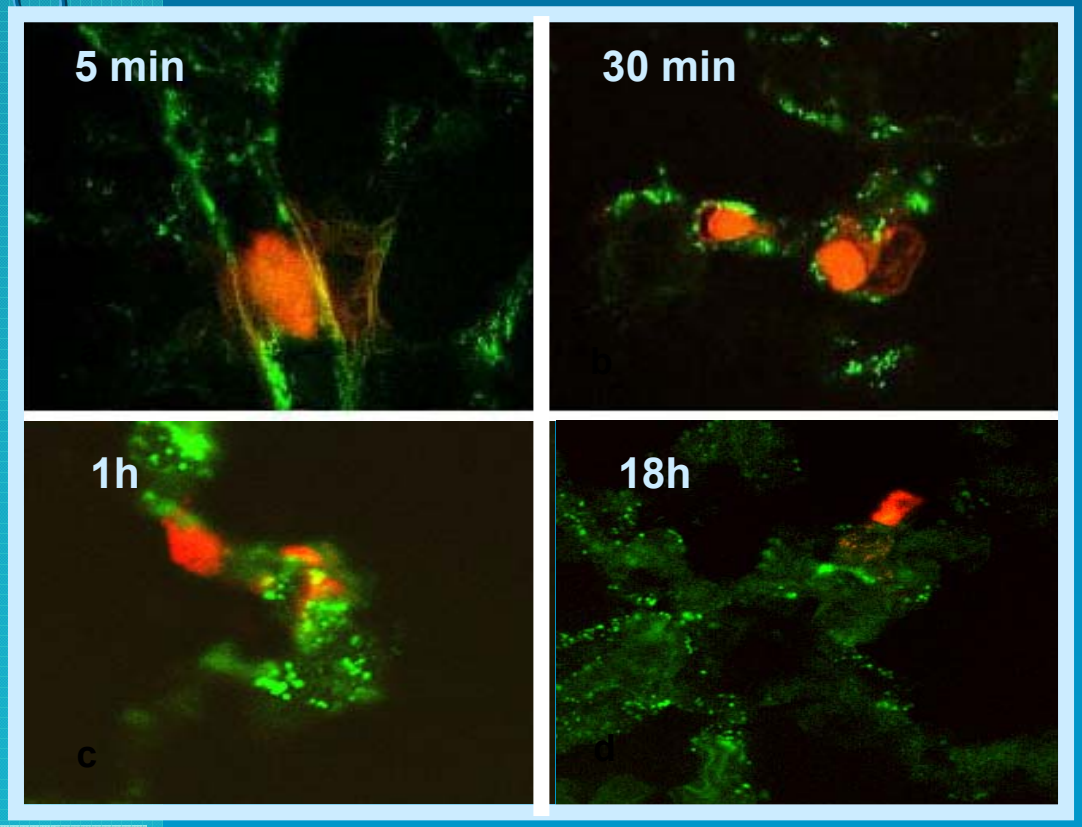
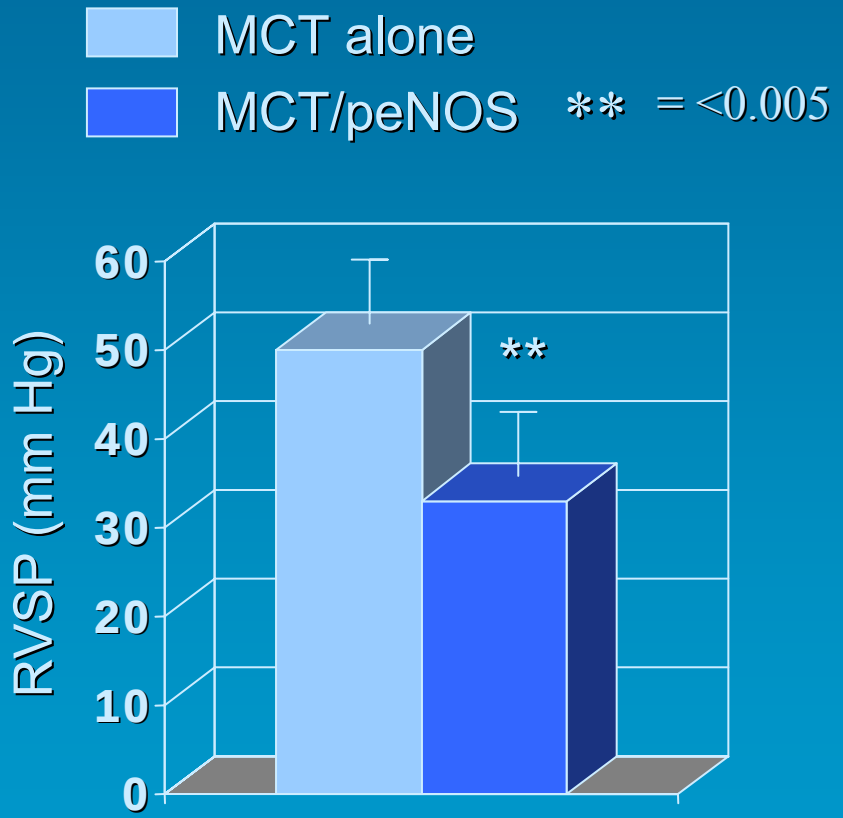
CMTMR-labeled SMCs

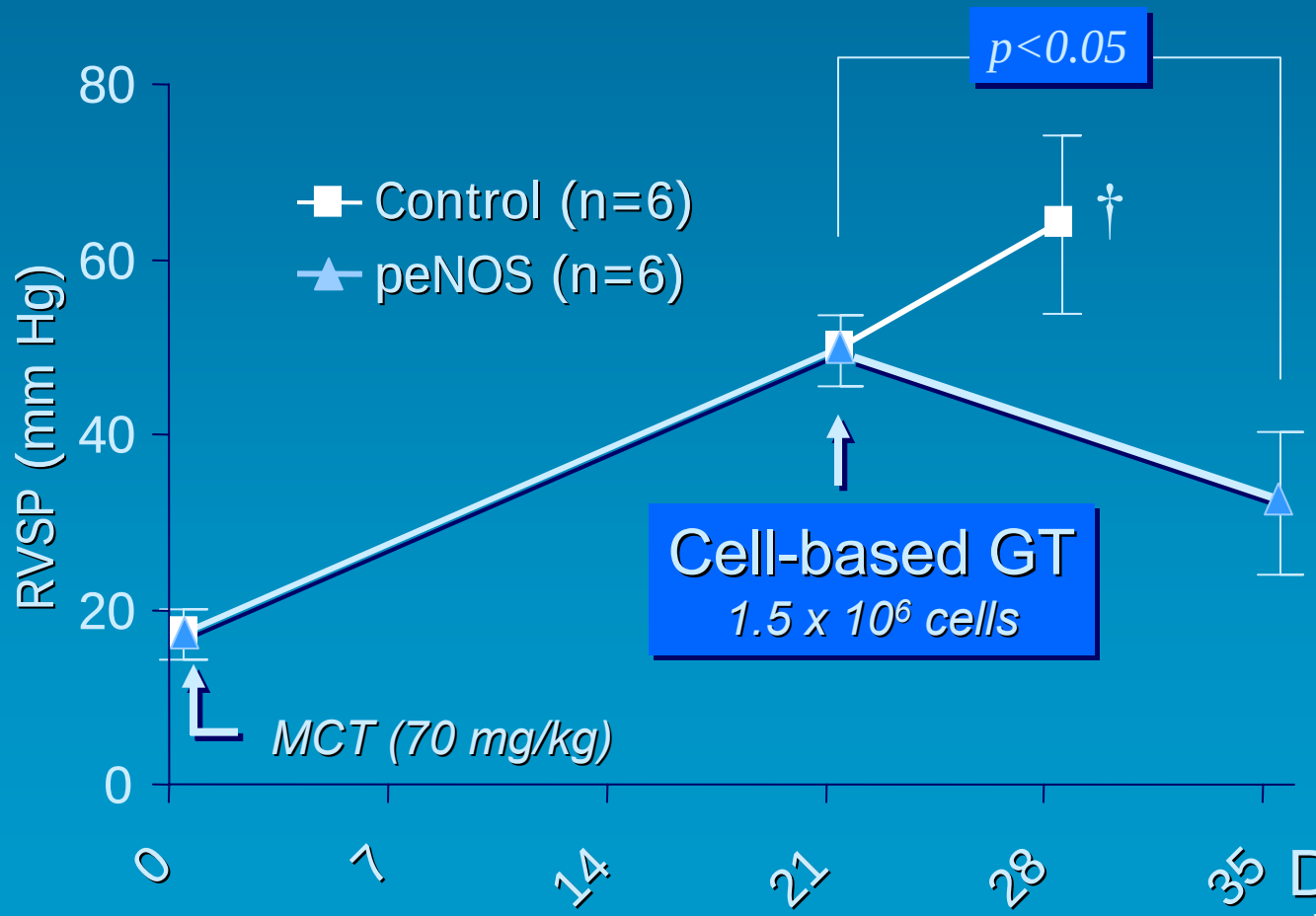
Transmigration through arteriolar endothelium





Campbell et al. Circulation 2001;104:2242-2248

Reversal of PH by eNOS gene transfer

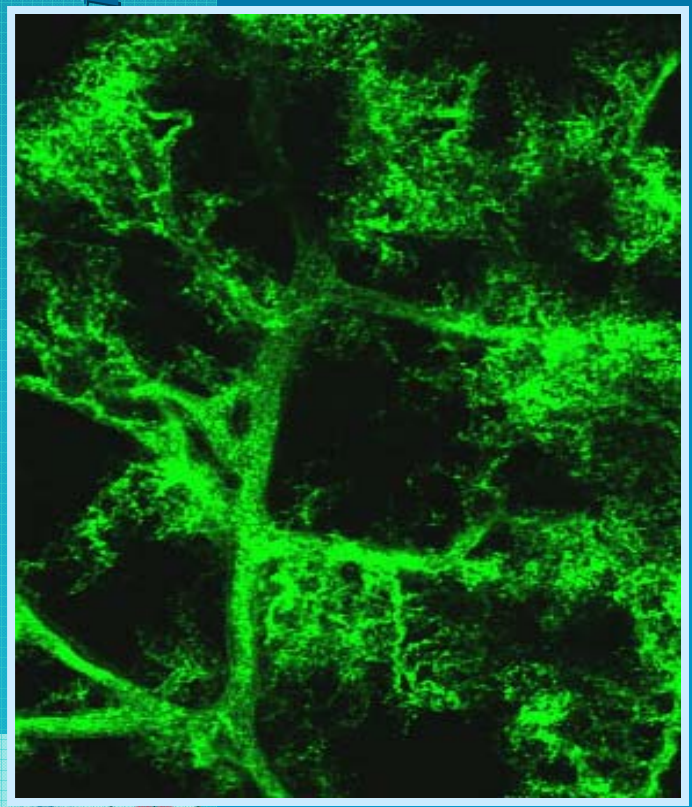


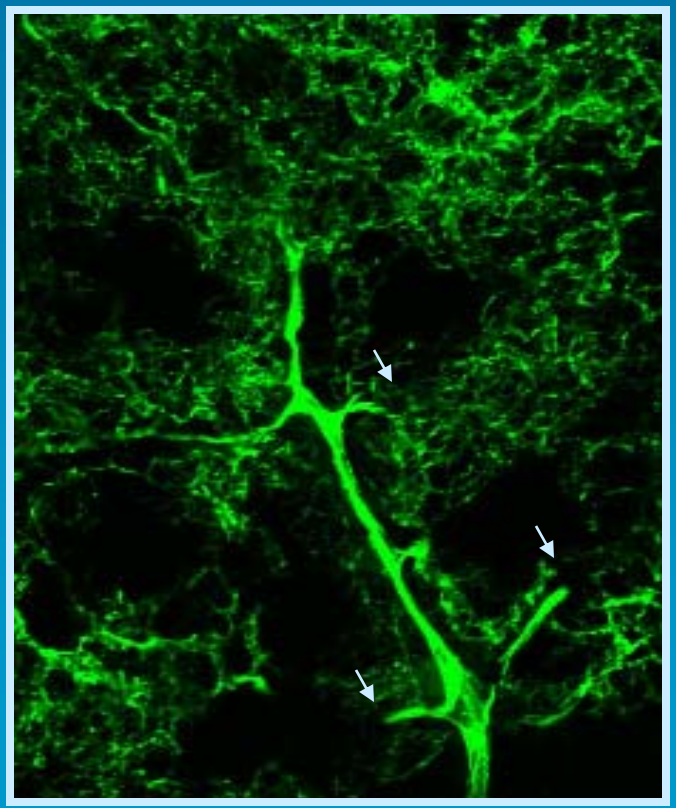
Reversal of PAH: *evidence for pulmonary vascular regeneration?*

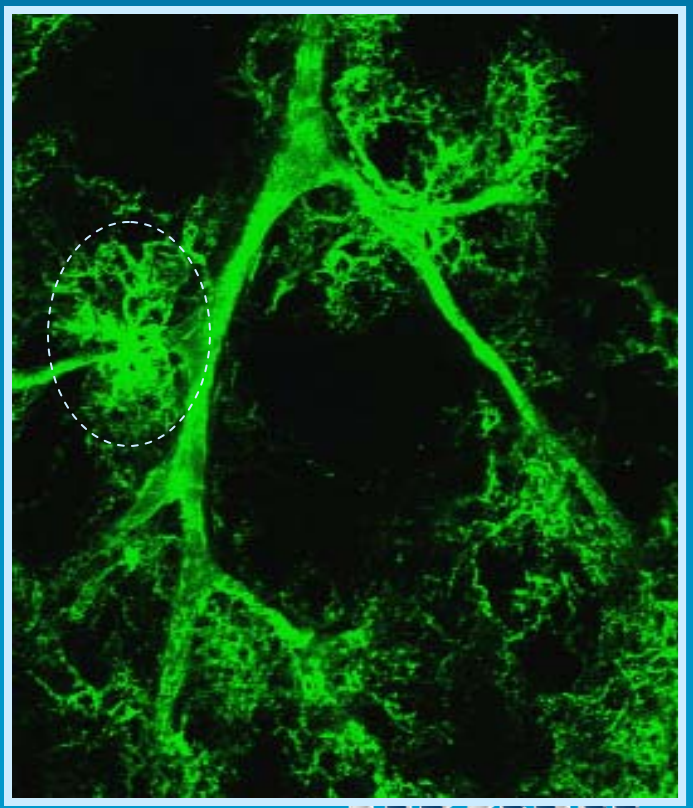
Normal

MCT (3 wks)

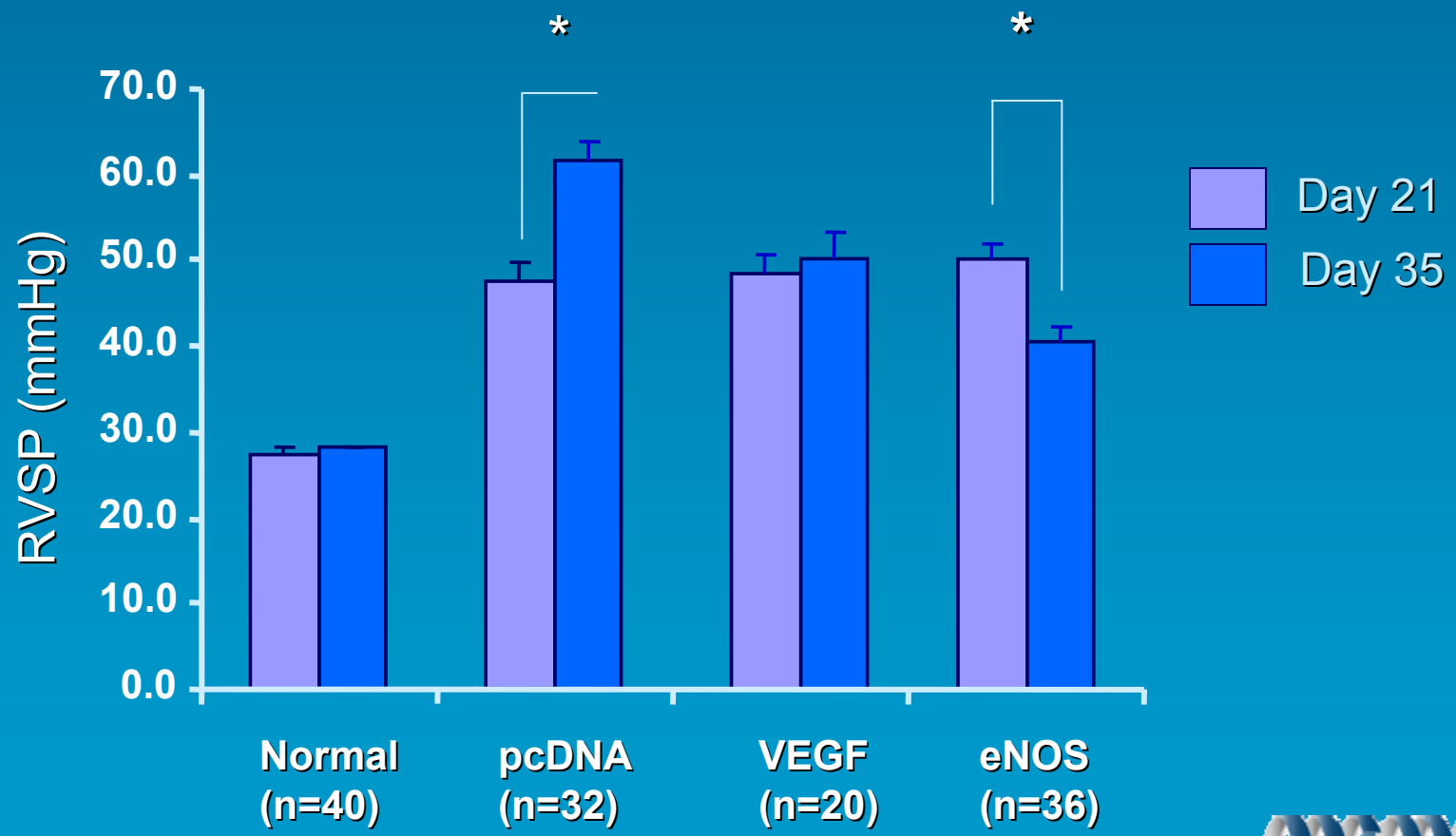
MCT (5 wks)/
eNOS (3 wks)







Comparison of eNOS vs VEGF gene therapy for reversal of MCT PH



* = P<0.05

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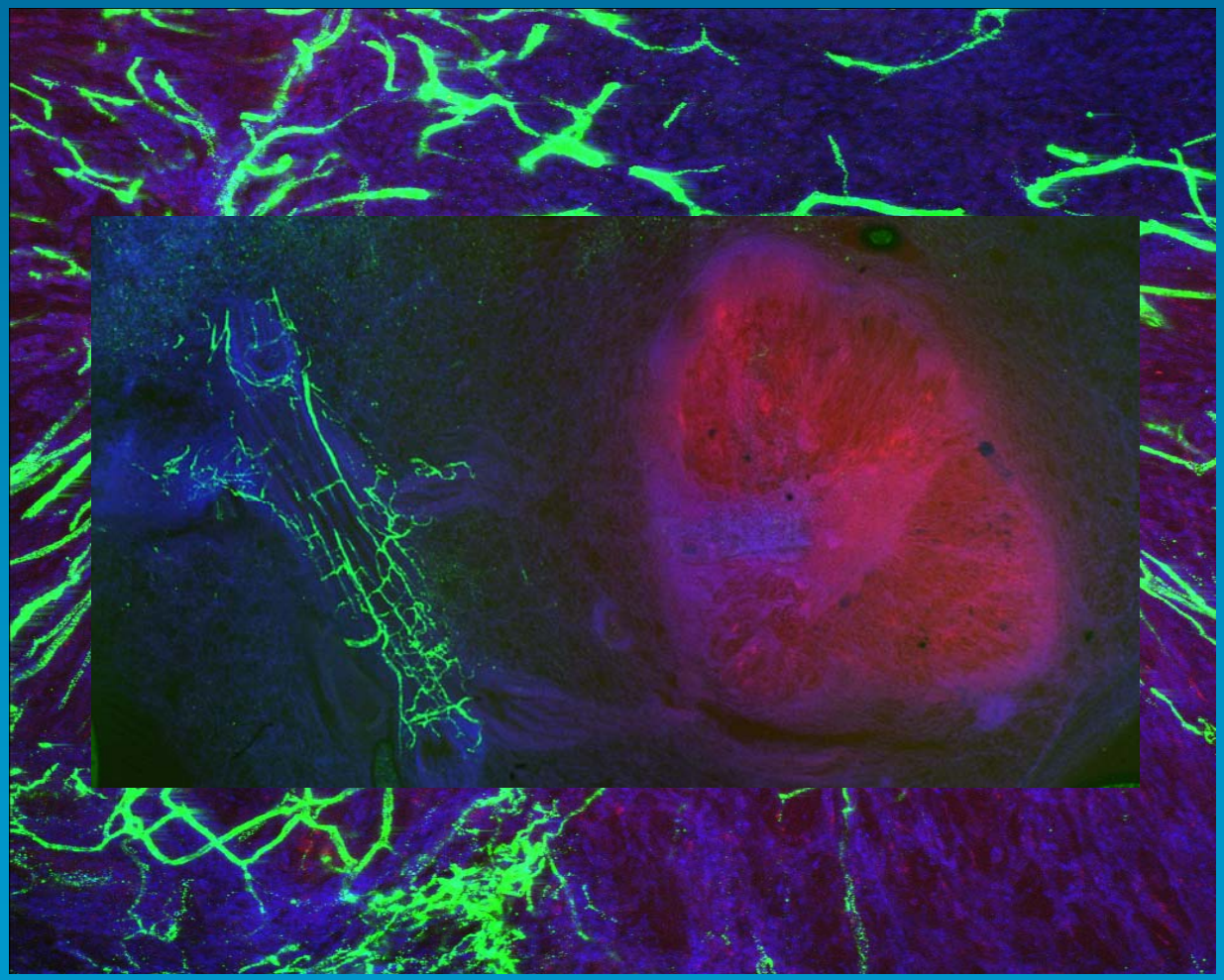
De novo angiogenesis from the pulmonary microvasculature



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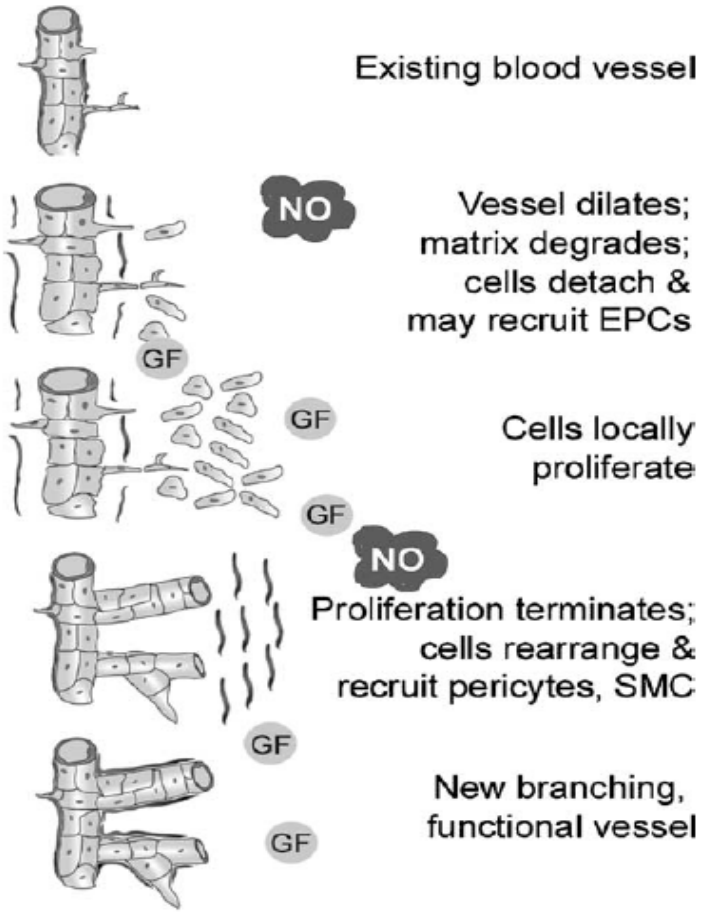
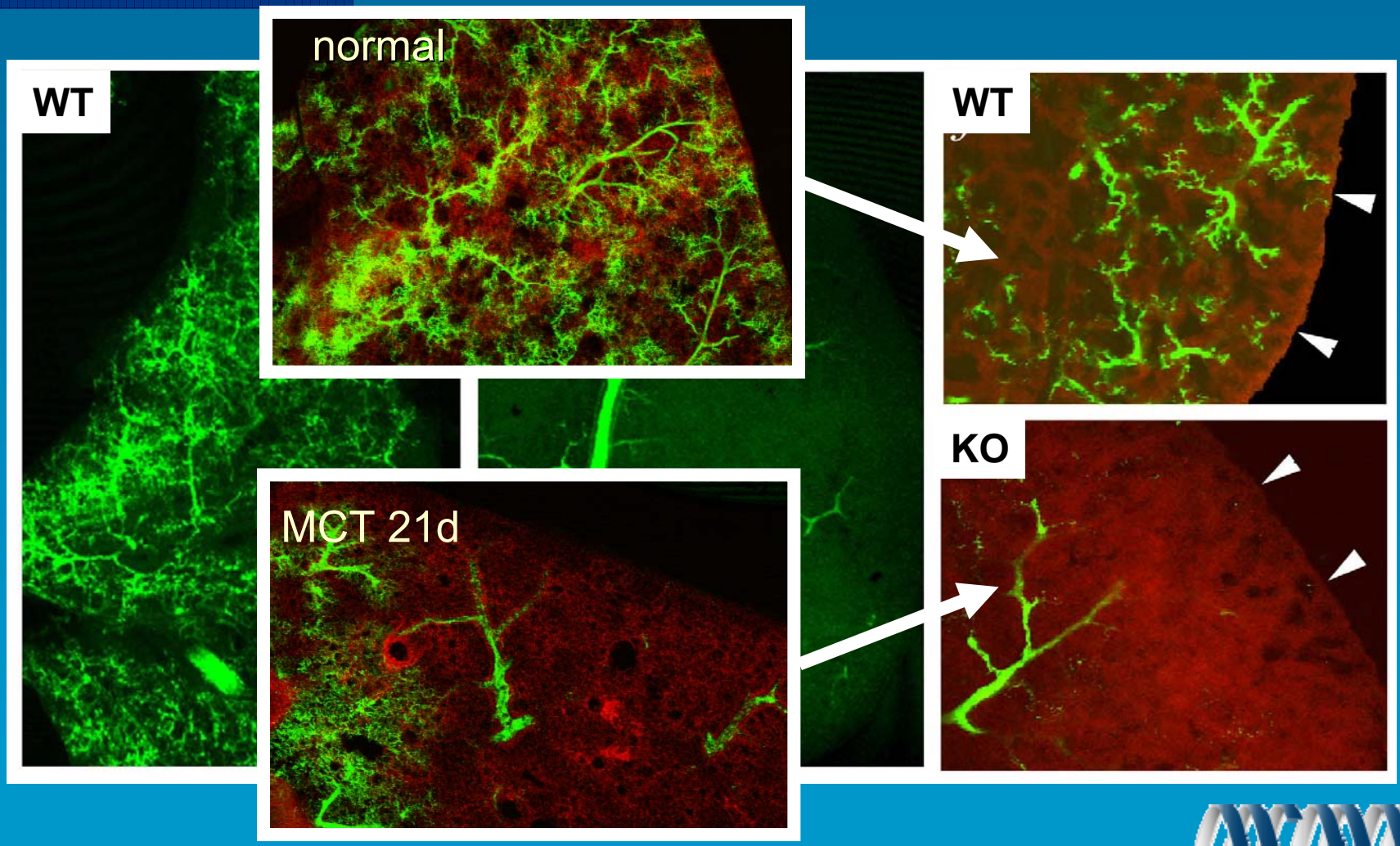
NO and Neovascularization



- 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

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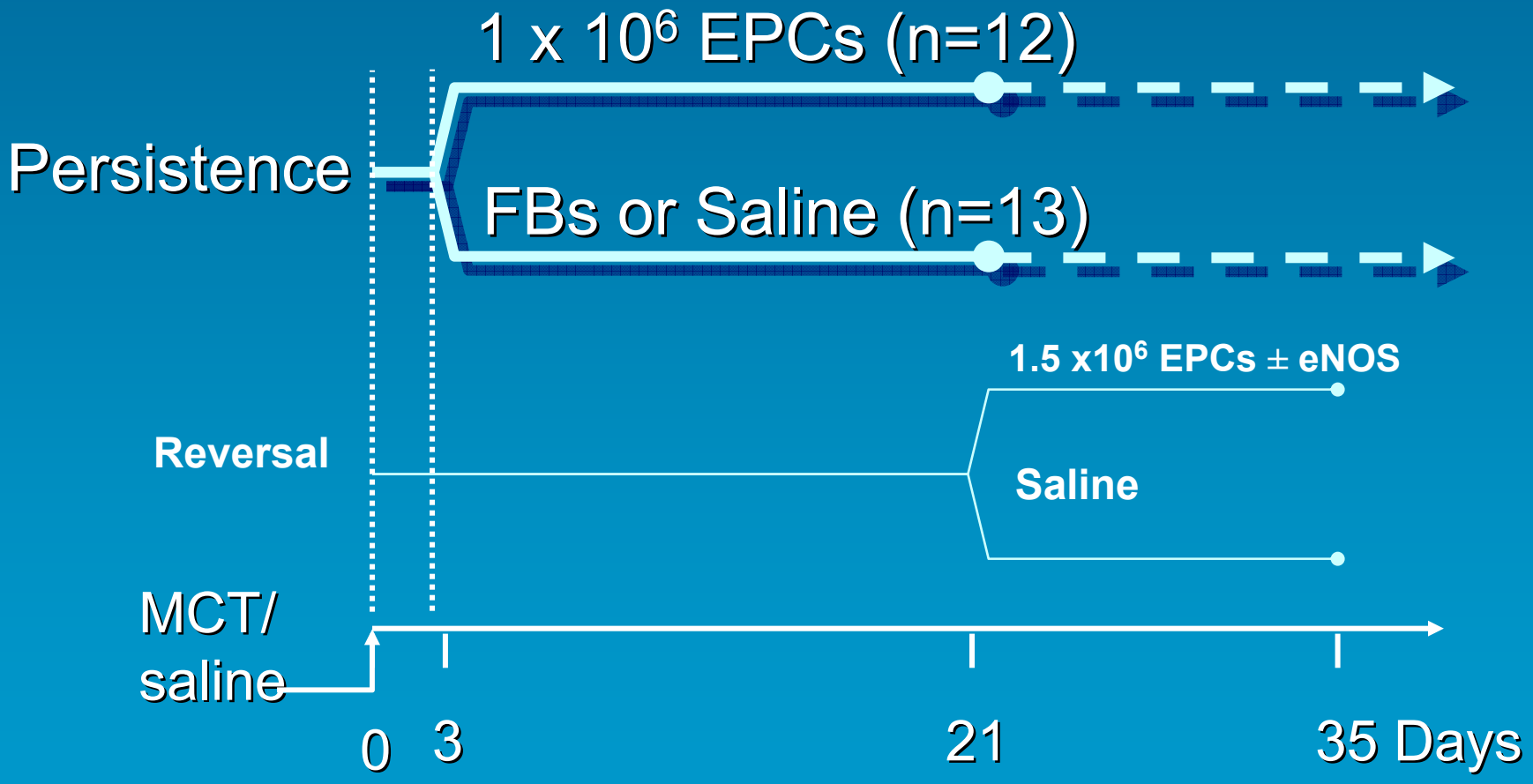
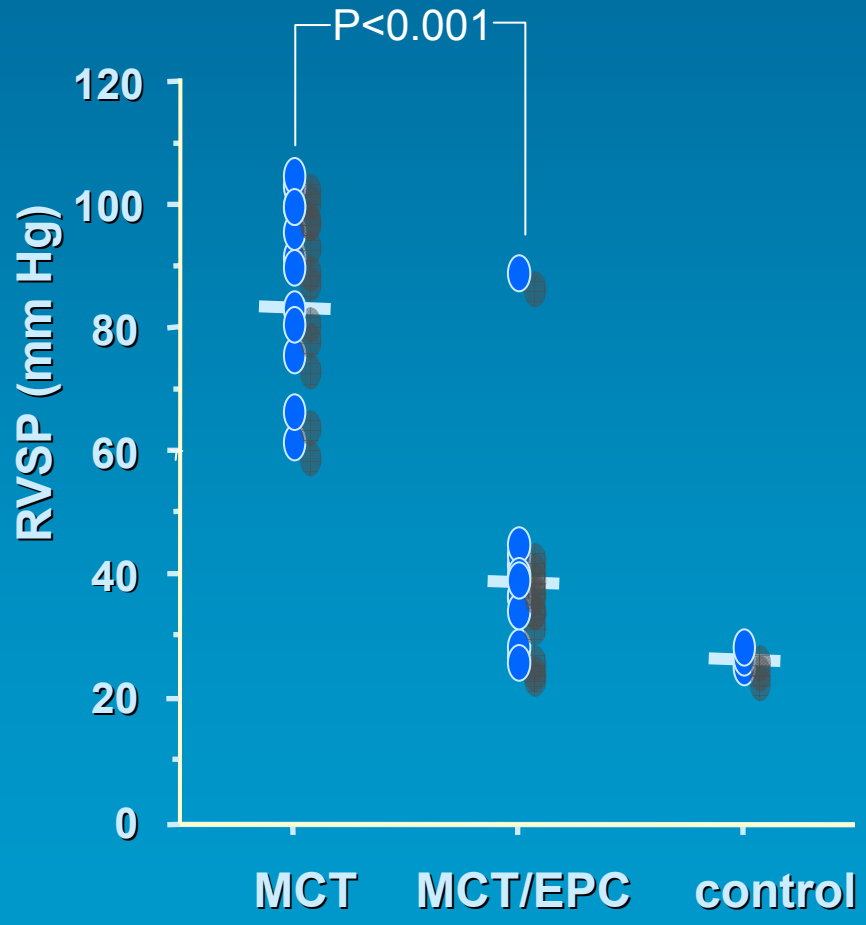
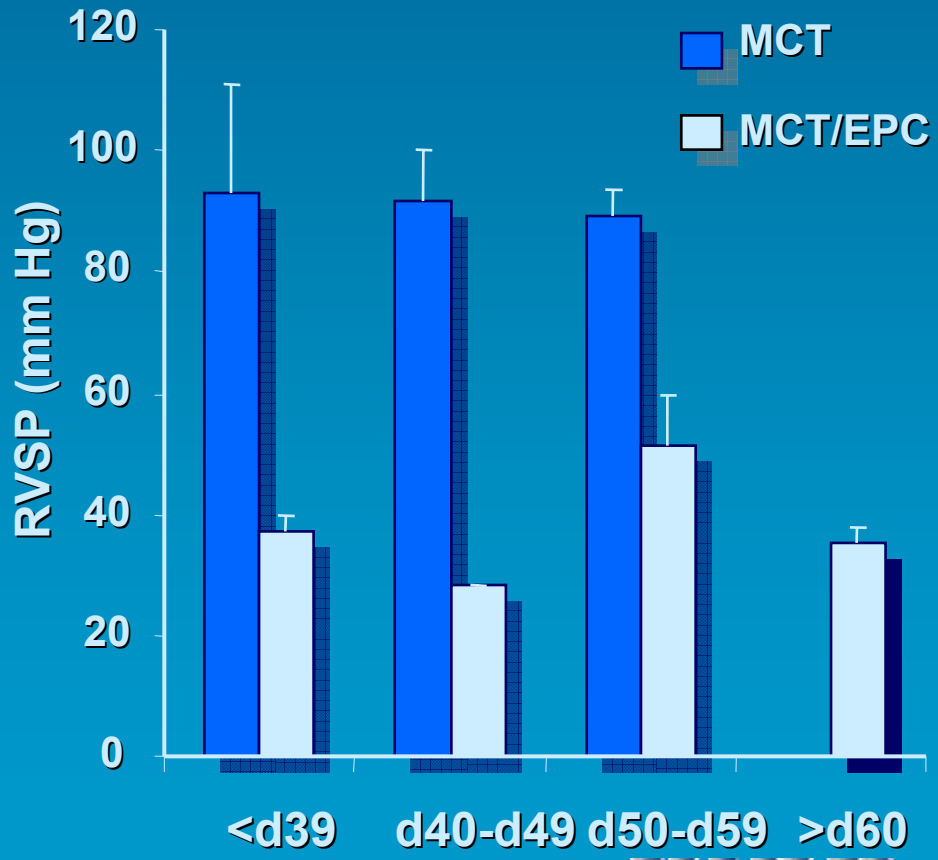
Experimental Plan: persistence of beneficial effects in the prevention model



Persistence of the effect EPCs treatment on RVSP >60 days





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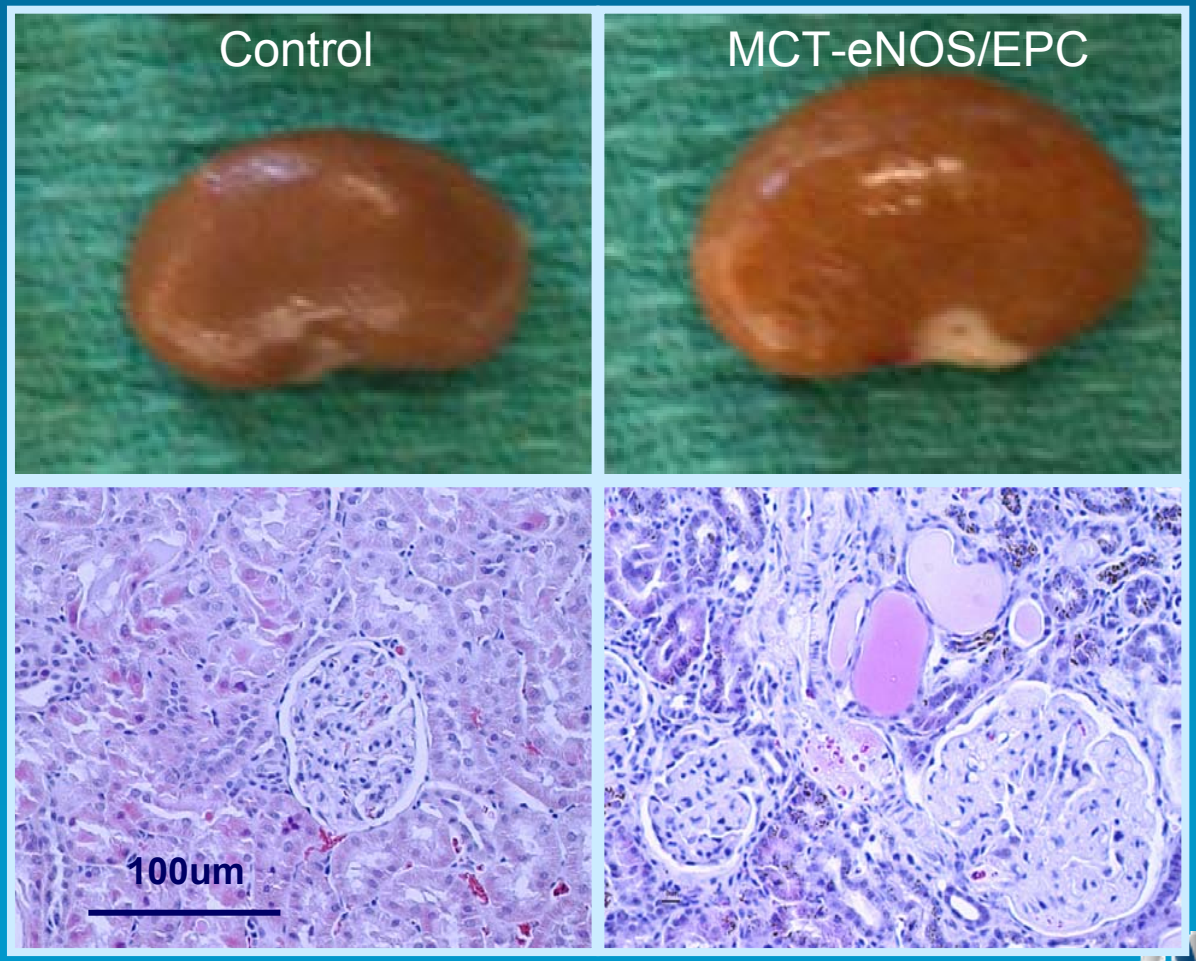
Histological Changes of kidney from MCT treated rats



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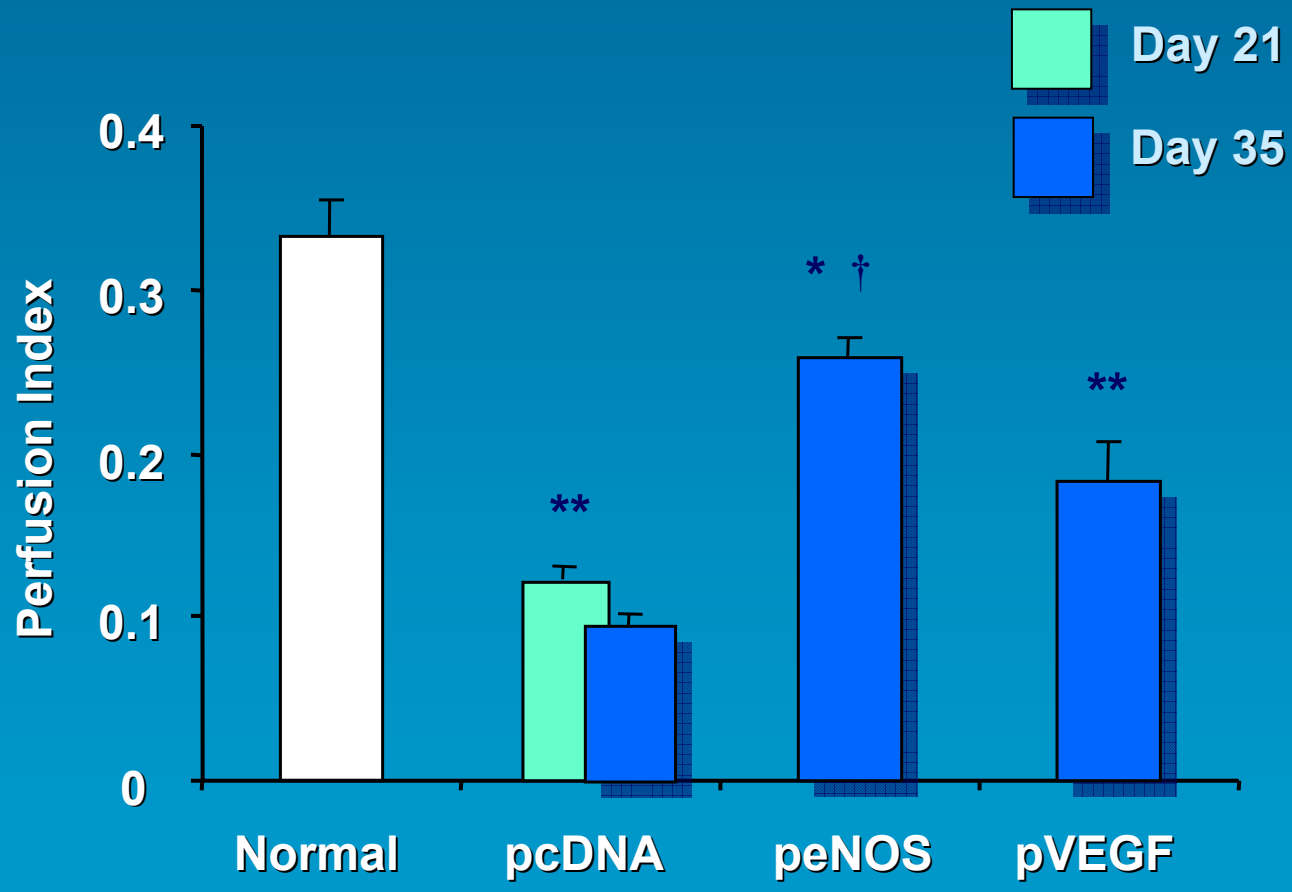
Comparison of eNOS vs VEGF gene therapy for reversal of MCT PH



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

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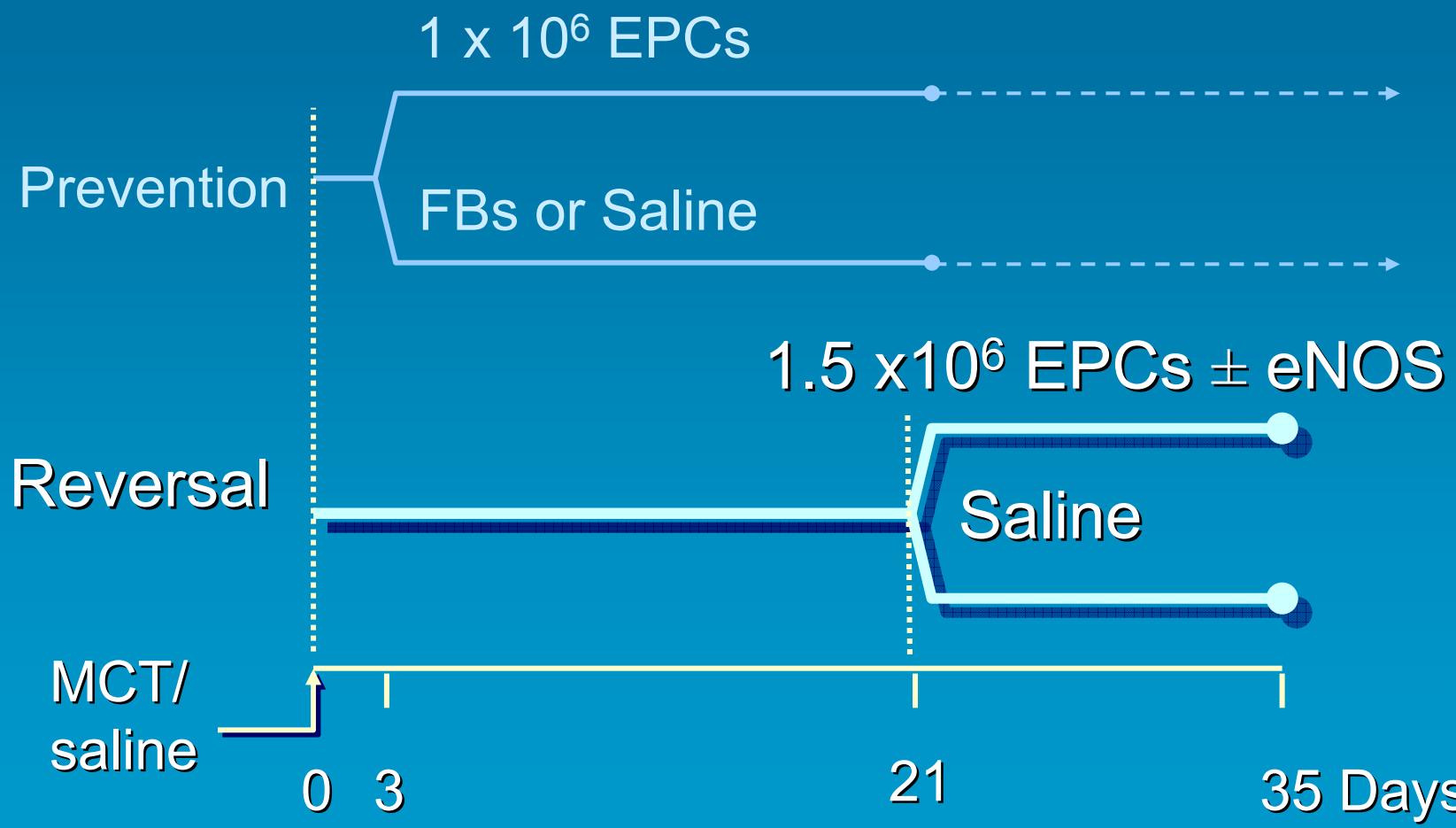
Reversal Protocol



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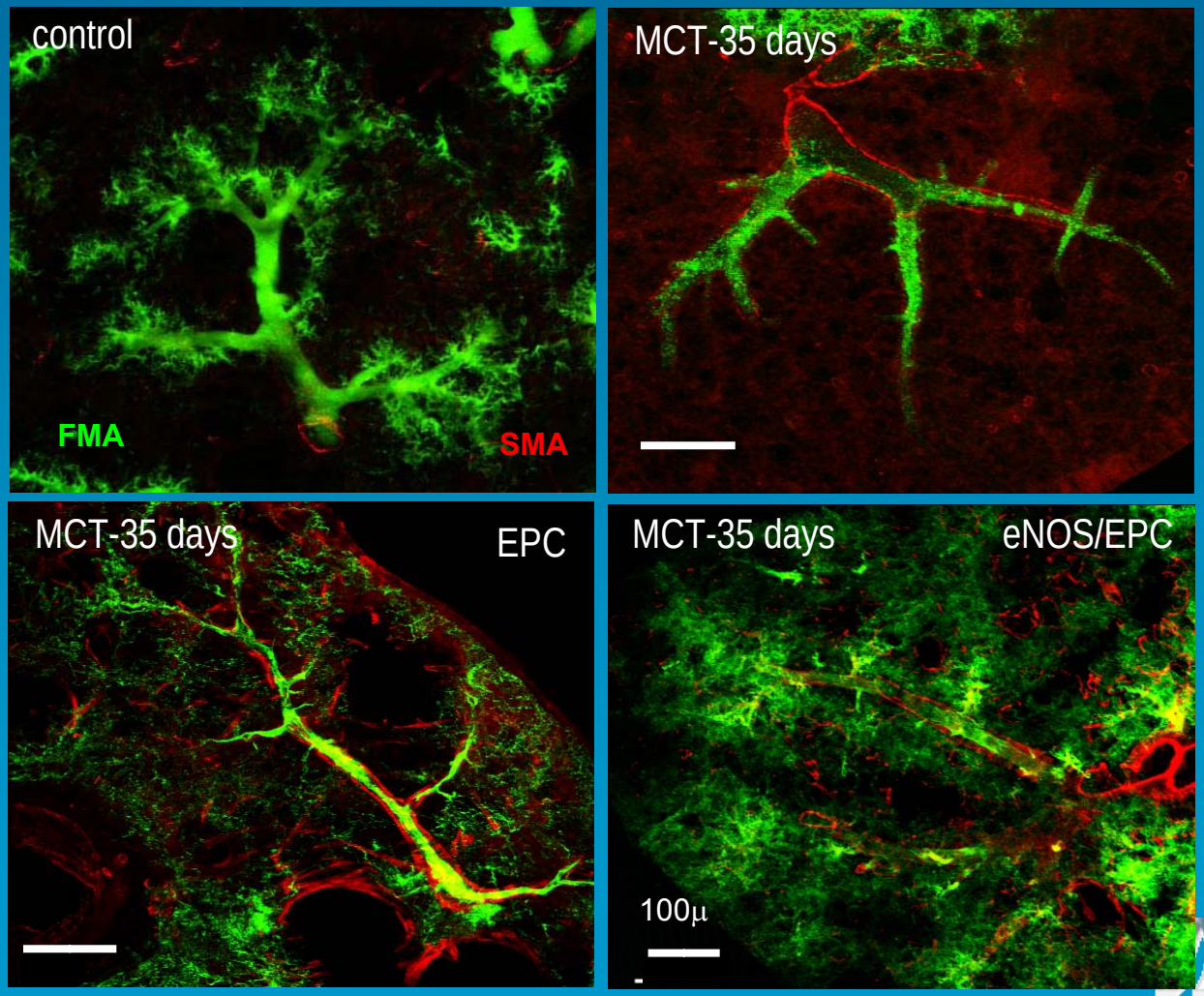
Fluorescent microangiography



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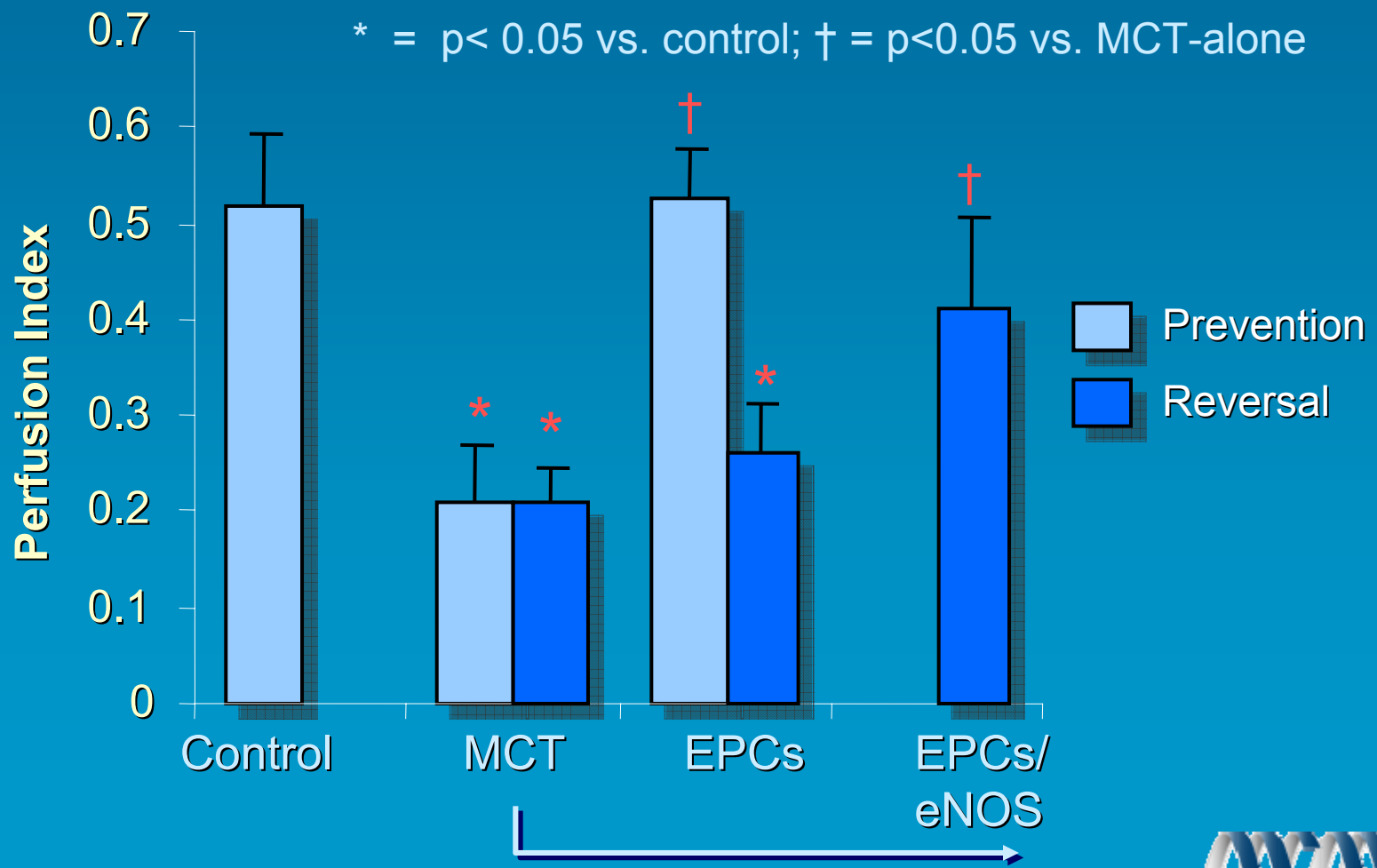
FMA - perfusion index (PI):



Zhao et al. Circ Res. 2005 (in press)

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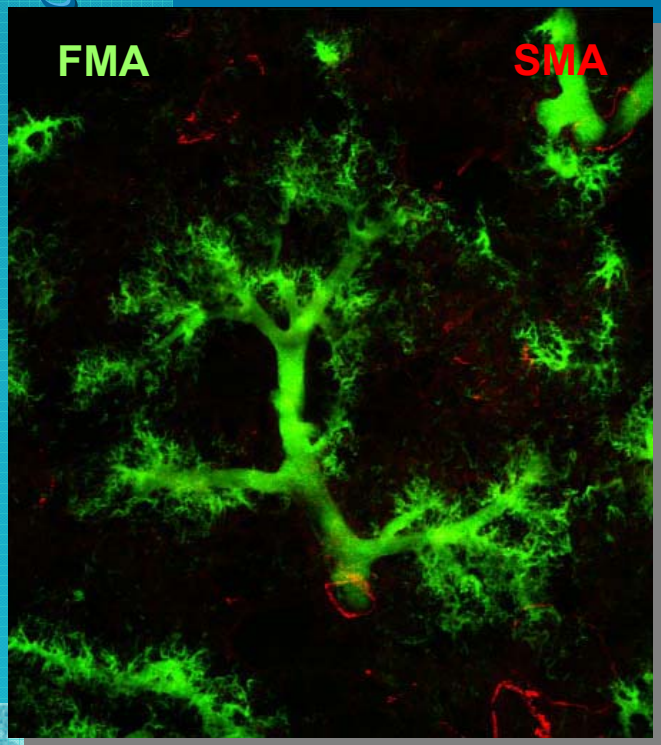
Summary and Conclusions

- 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

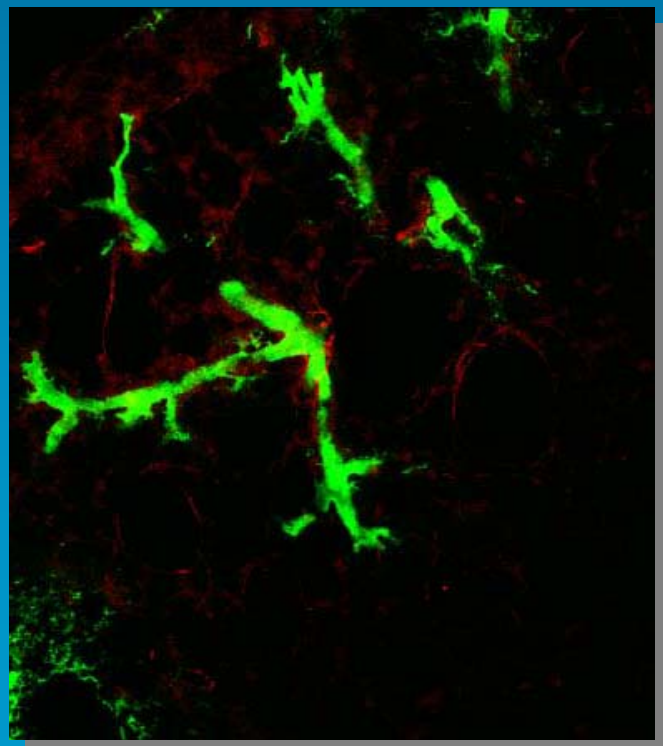
Effect of EPC transplant on lung microvascular structure: *21 Days post MCT*

Control



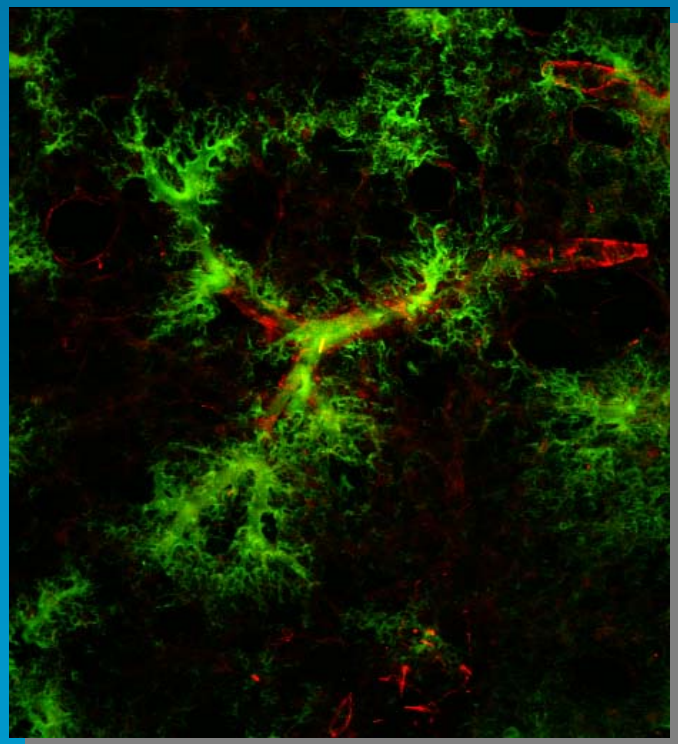
40 x

MCT-FB



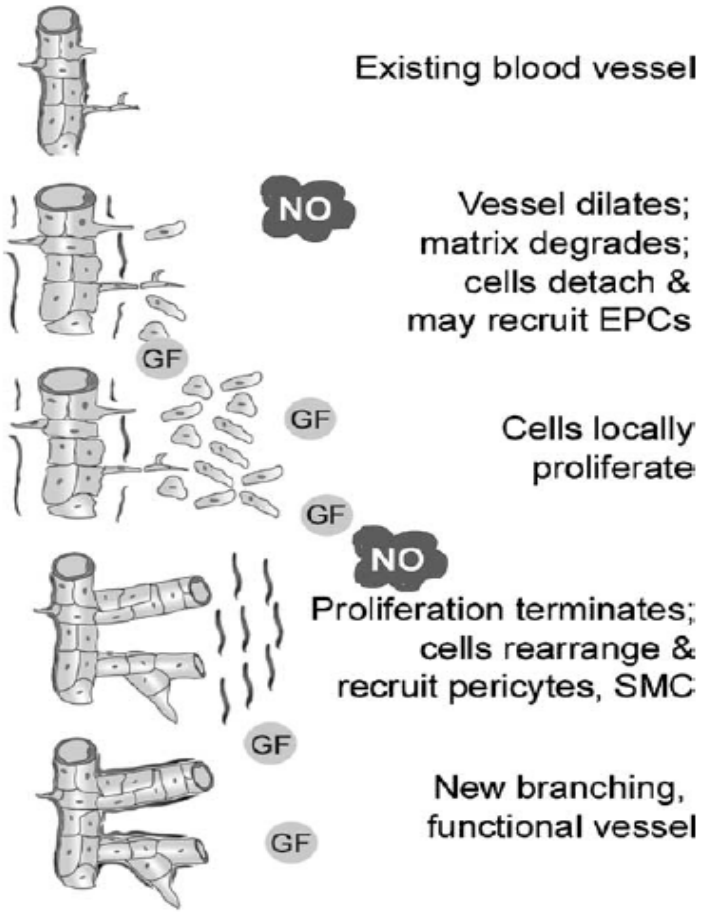
40 x

MCT-EPC



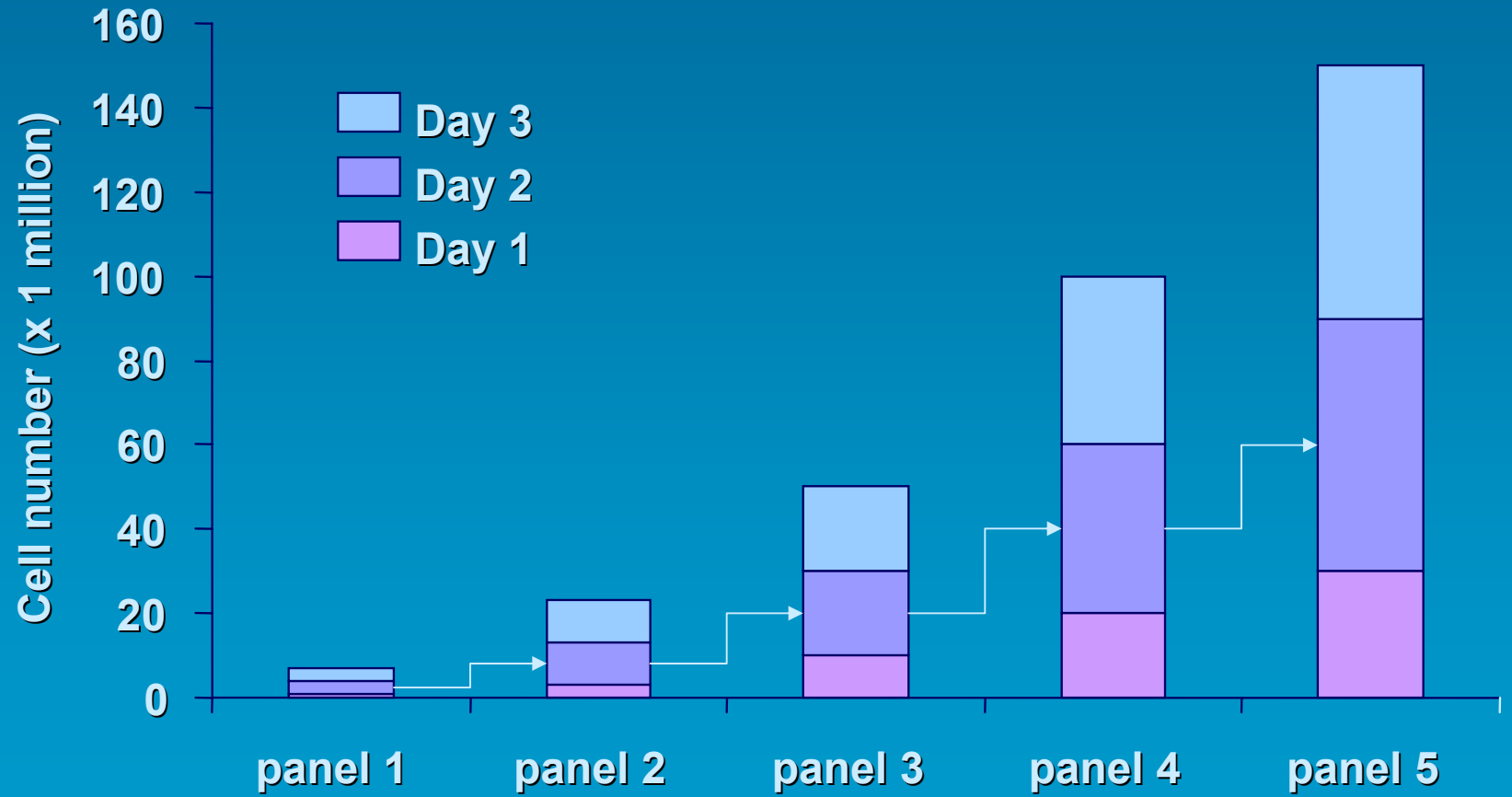
40 x

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



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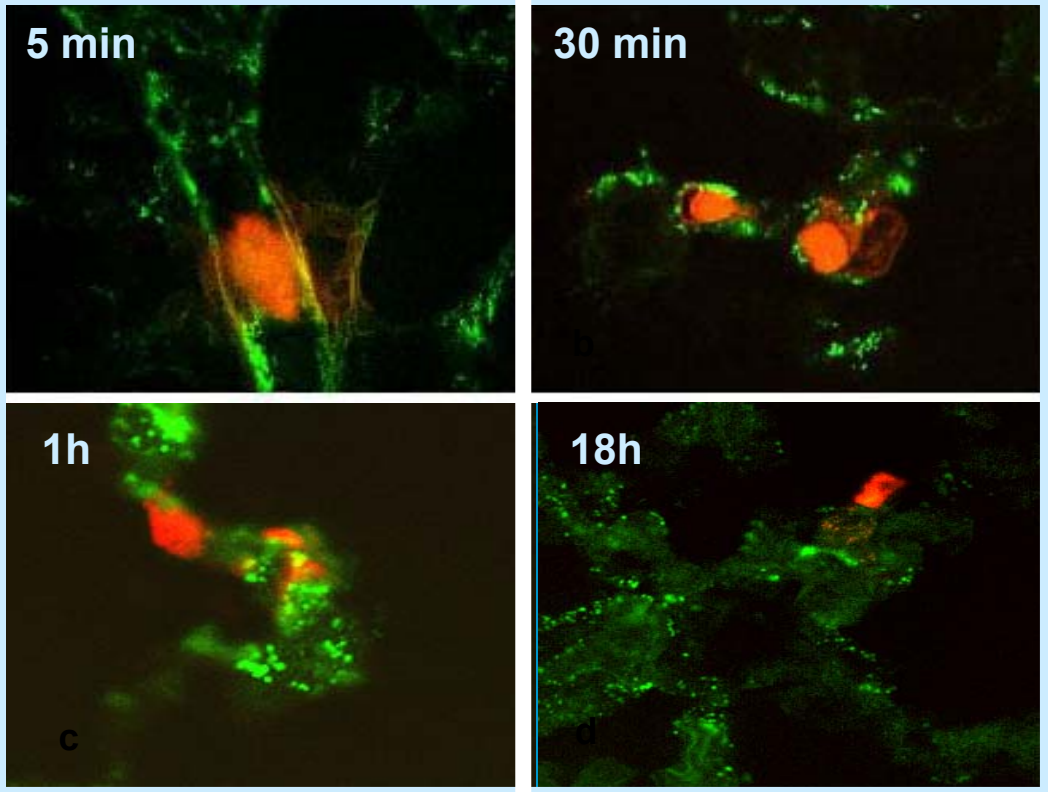
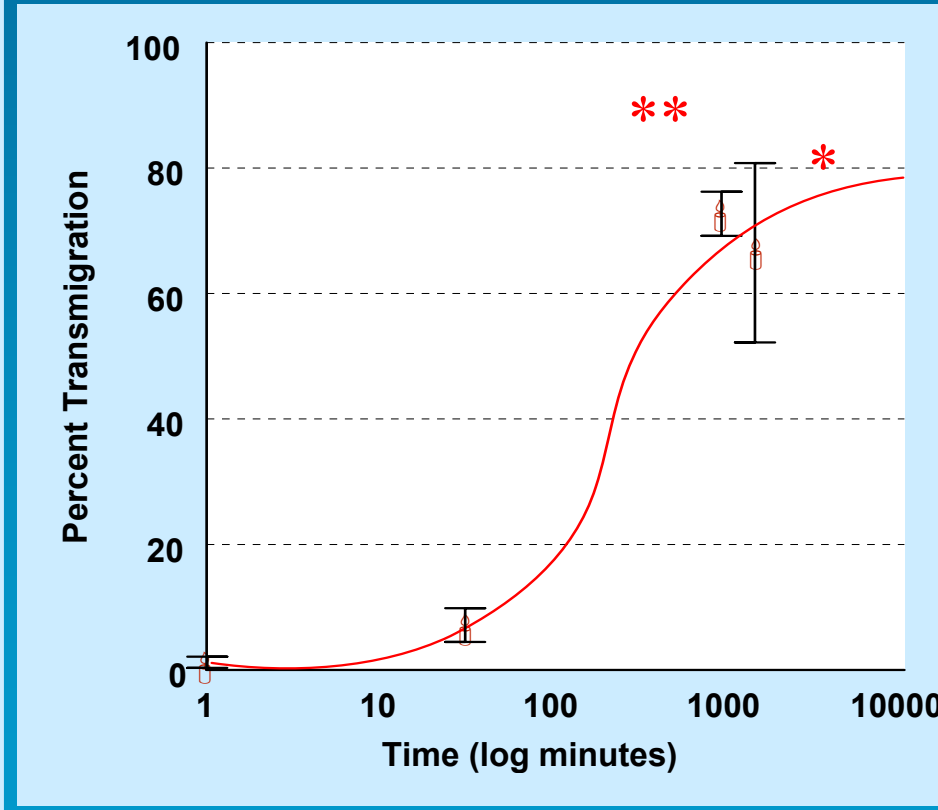
CMTMR-labelled SMCs

Transmigration through arteriolar endothelium

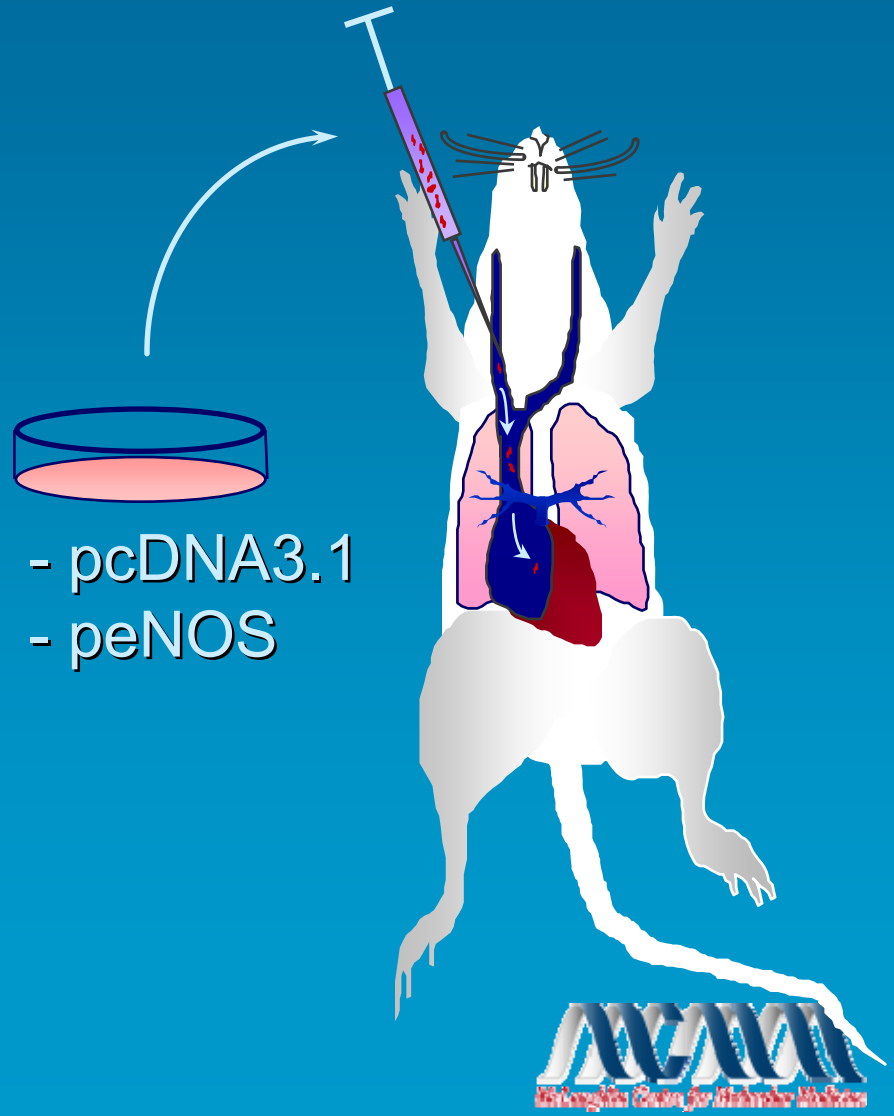




Campbell et al. Circulation 2001;104:2242-2248

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



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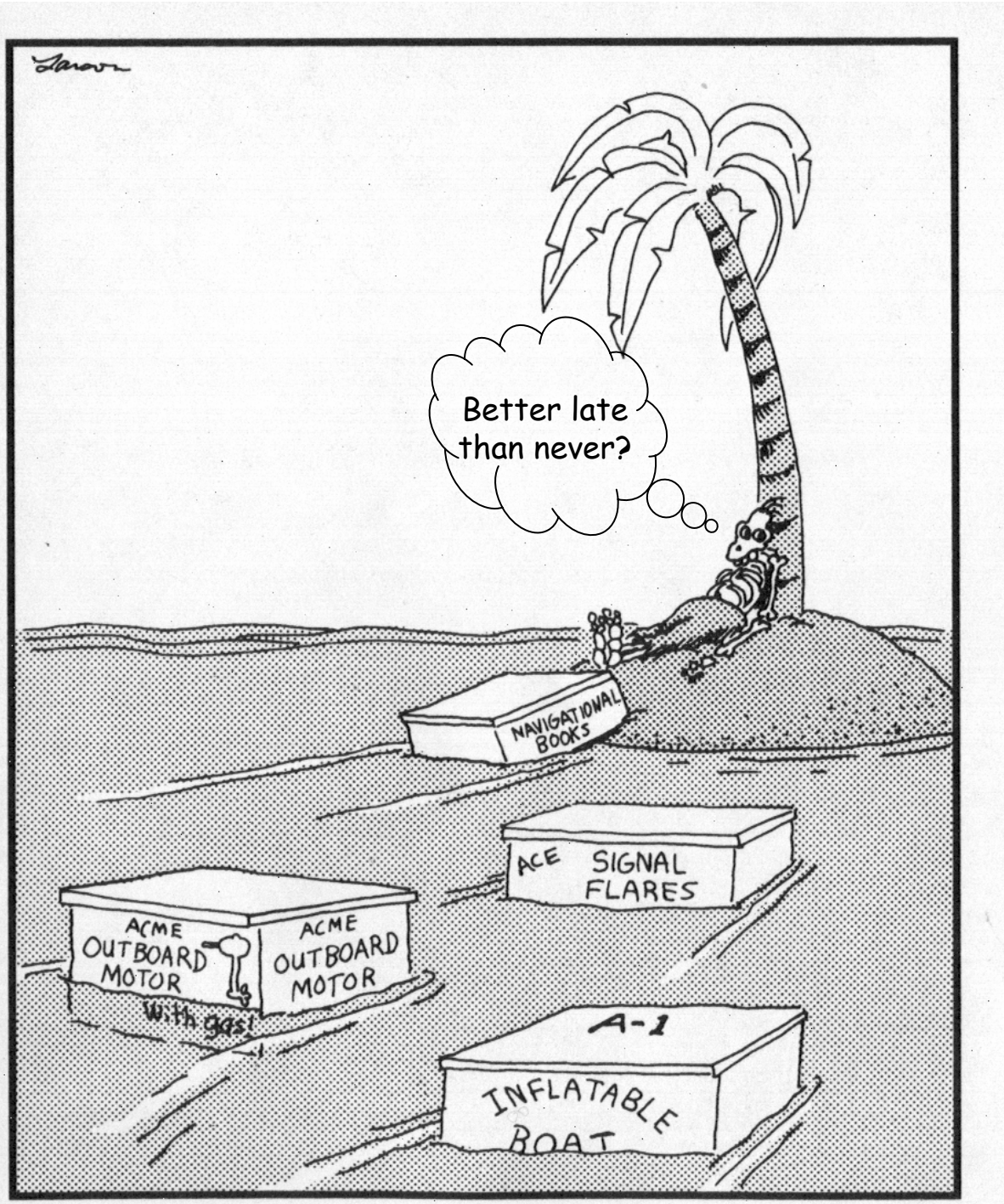
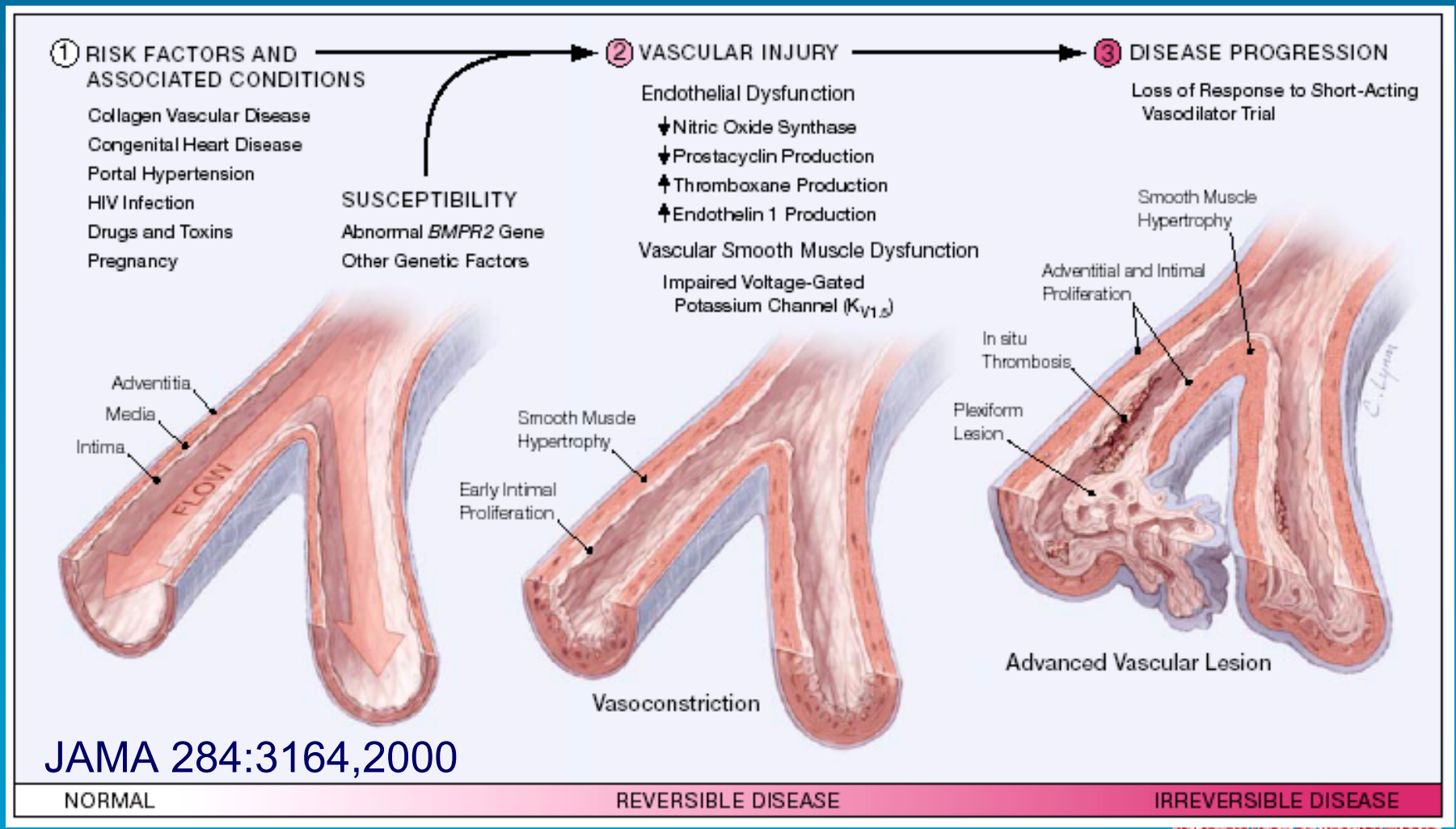


The future – reversing "irreversible" PAH?



JAMA 284:3164,2000

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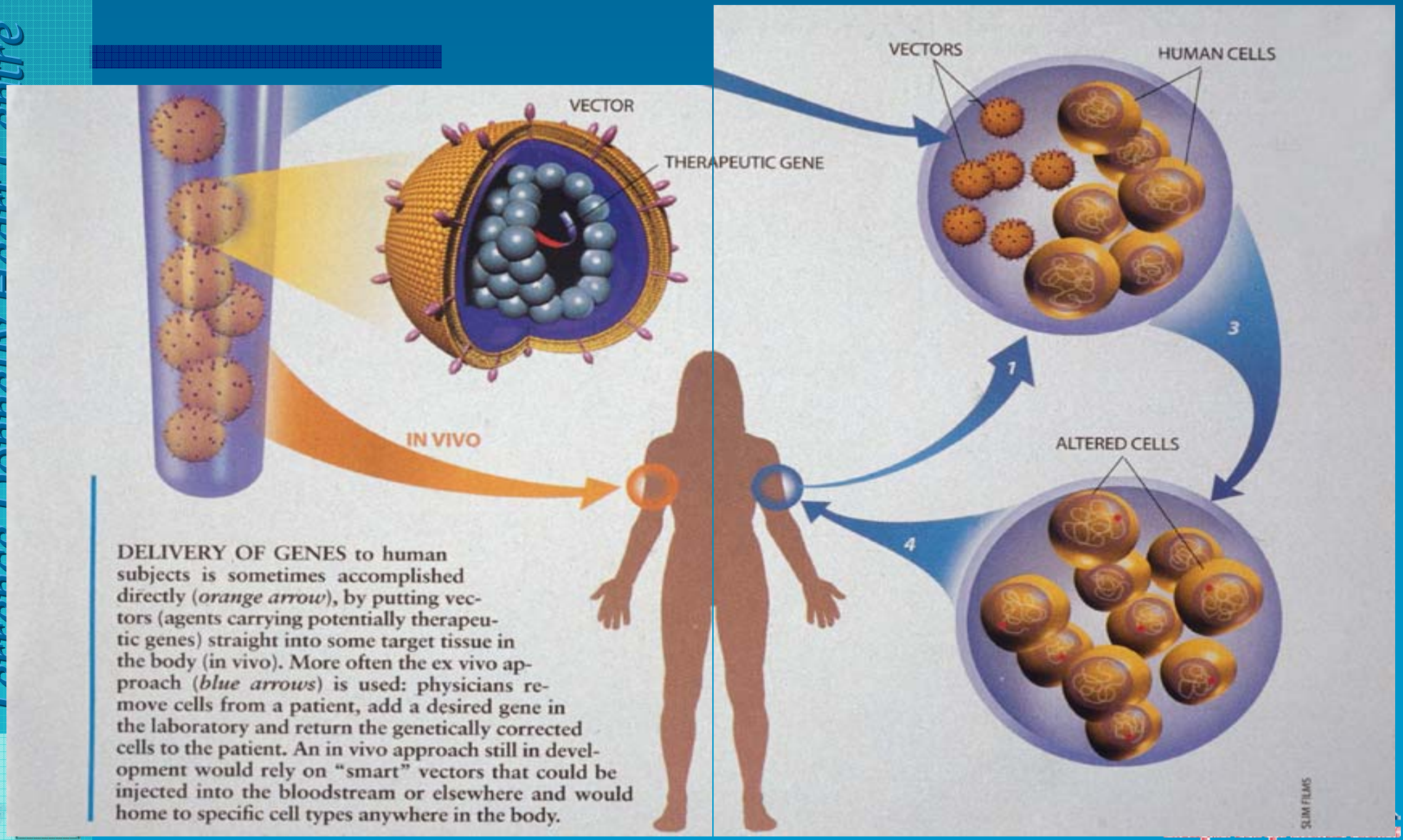
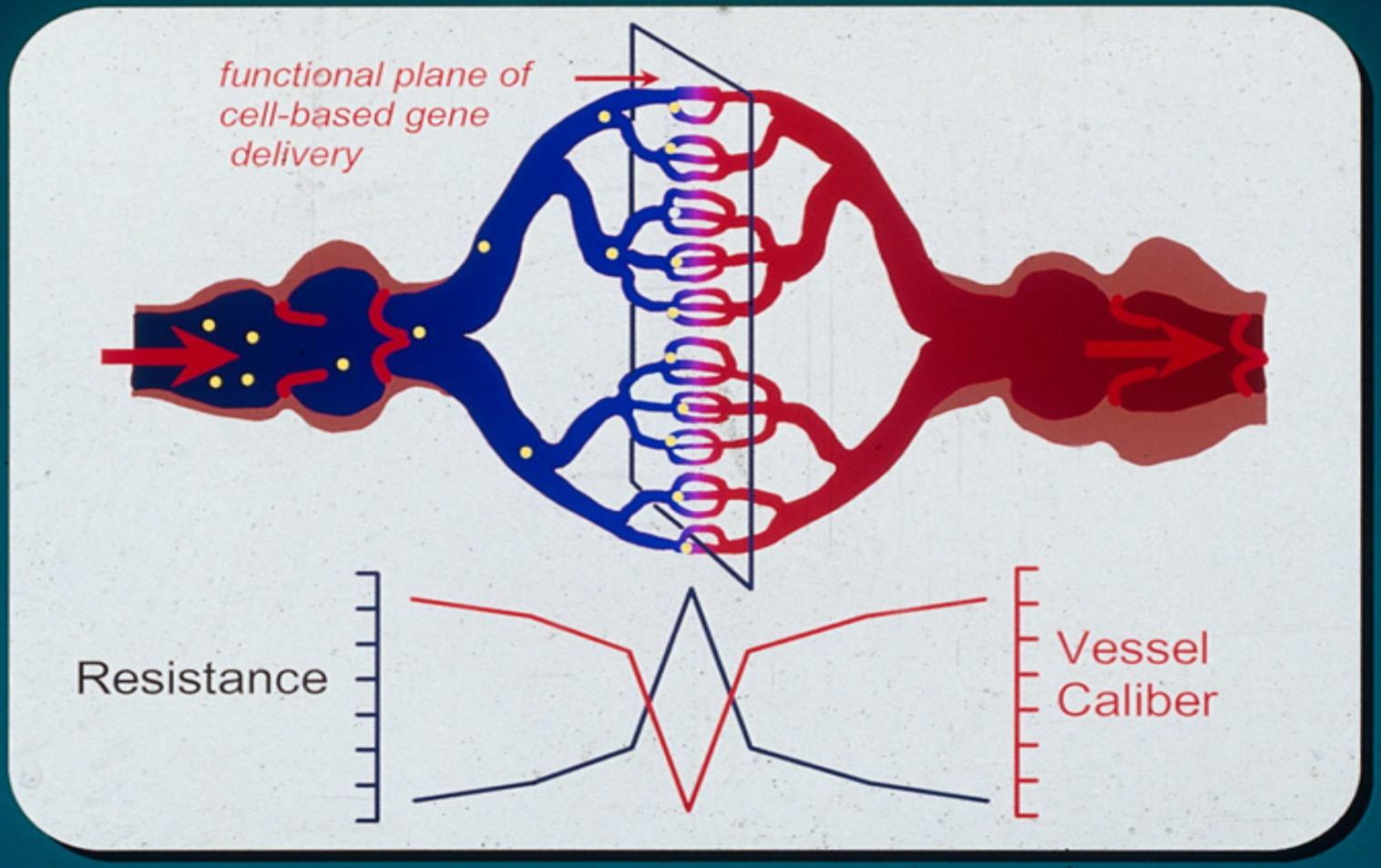
Cell-based gene therapy



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



The Pulmonary Vasculature

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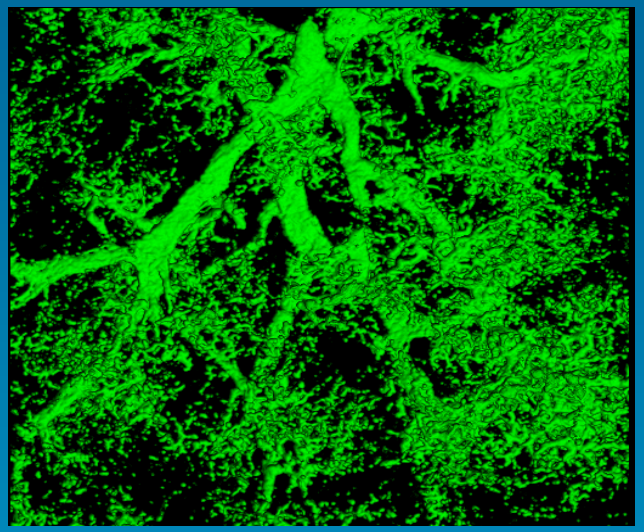
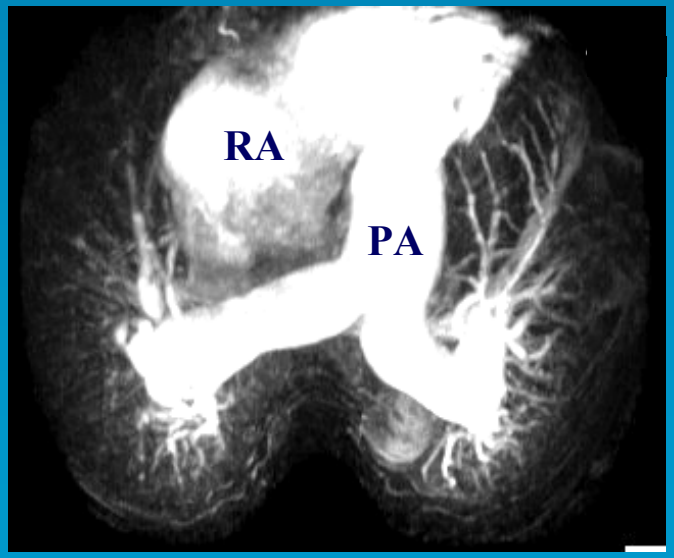
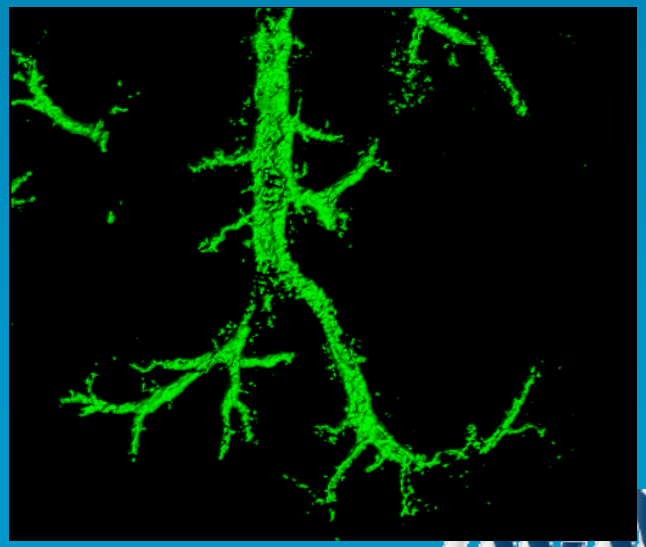
	Human	Rat MCT model
Normal		
PAH		

Courtesy of Evangelos Michelakis, U of Alberta

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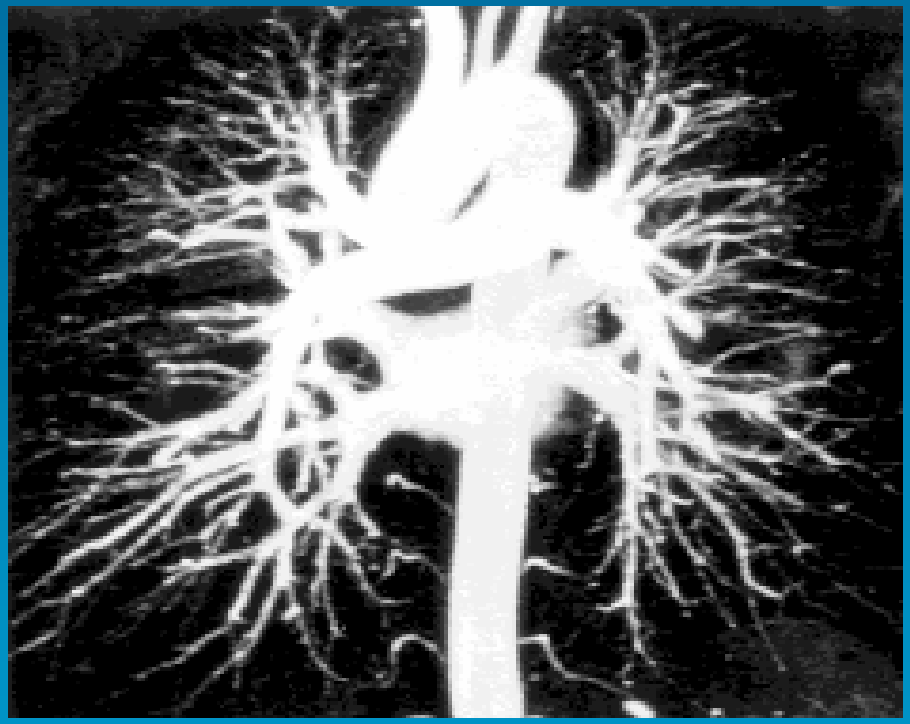
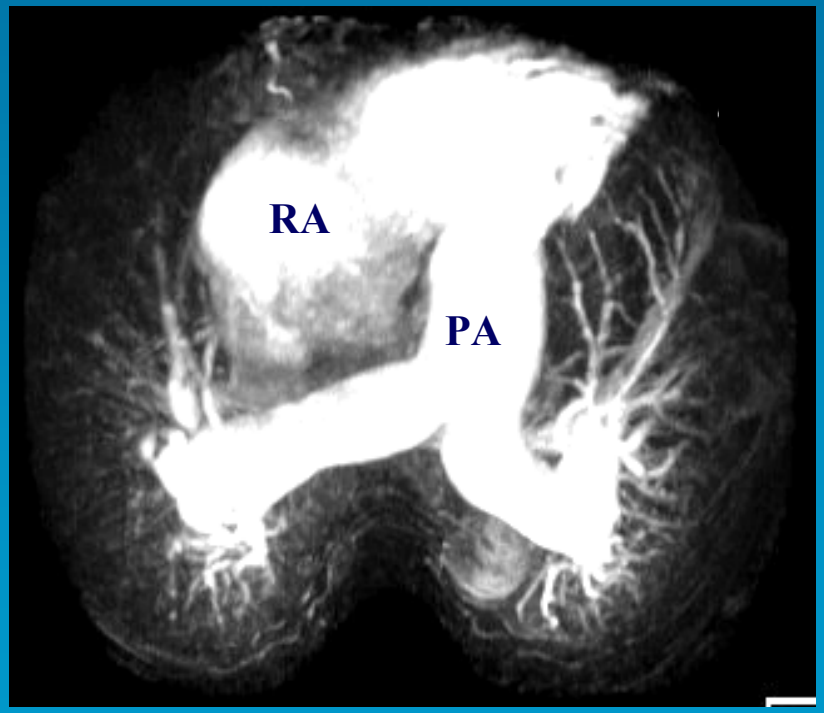
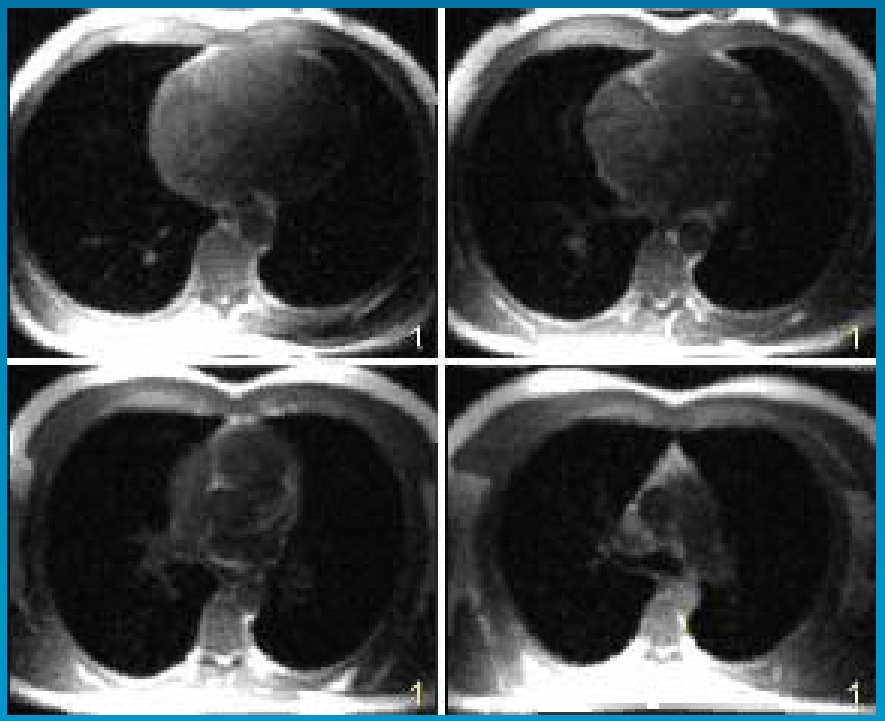
The Normal Pulmonary Vasculature



Courtesy of Evangelos Michelakis, U of Alberta

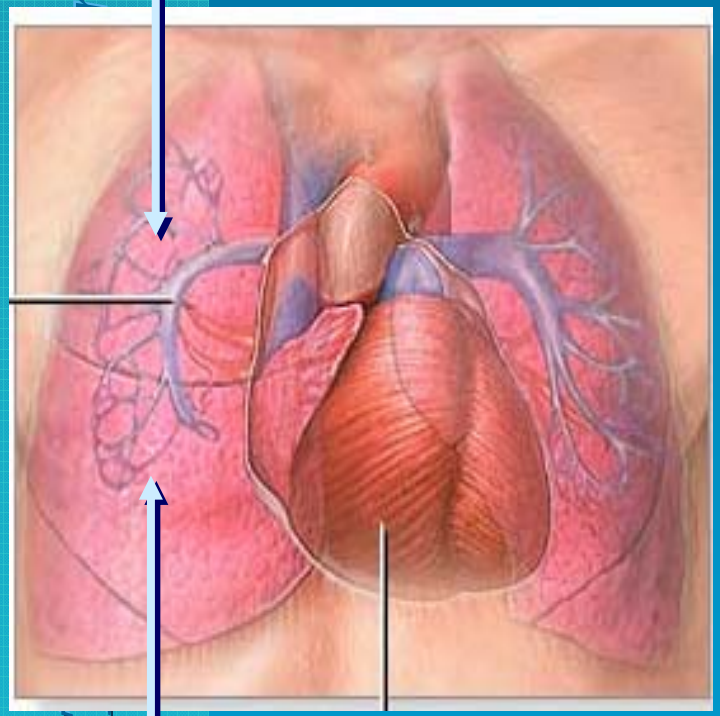
Pulmonary Vascularity in PAH





Courtesy of Evangelos Michelakis, U of Alberta

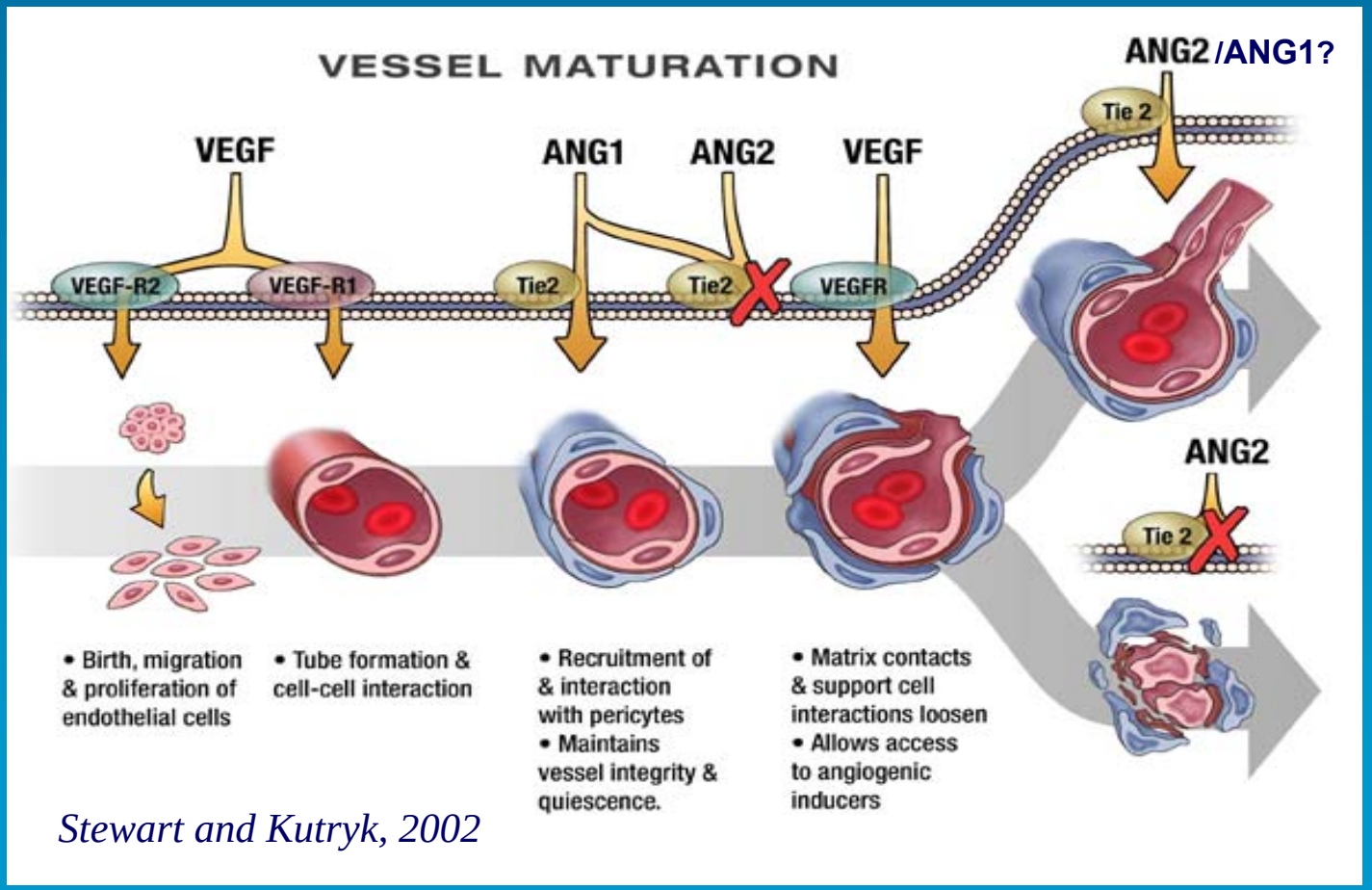
Pulmonary Arterial Hypertension (PAH)



- 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



Stewart and Kutryk, 2002

Angiogenesis/ arteriognesis

Vascular regression

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Ang1 gene transfer inhibits apoptosis in rat model of MCT-induced PH

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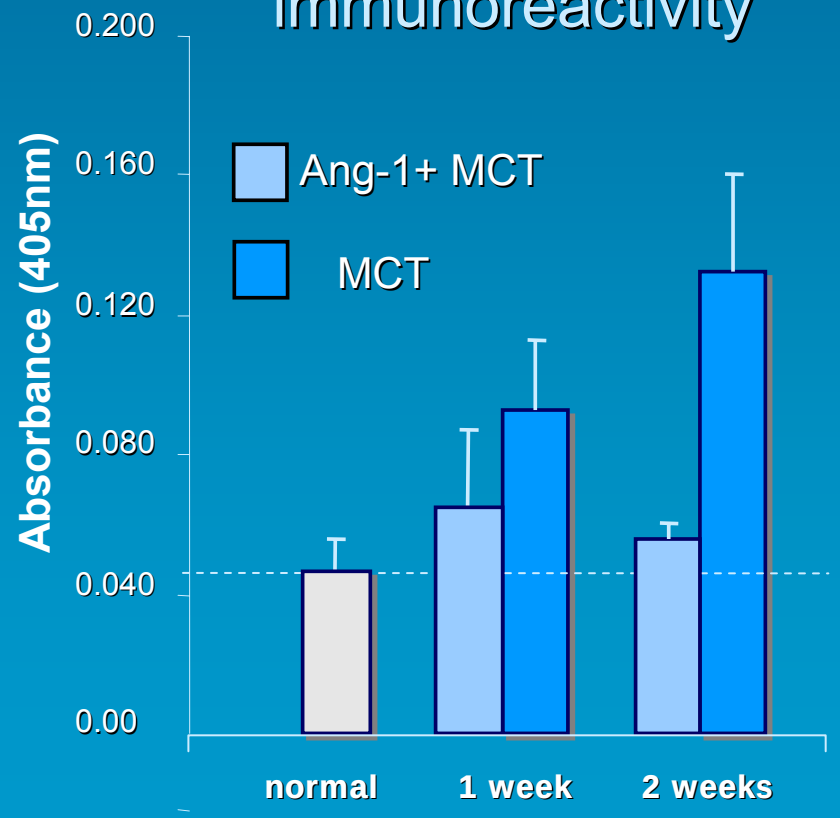
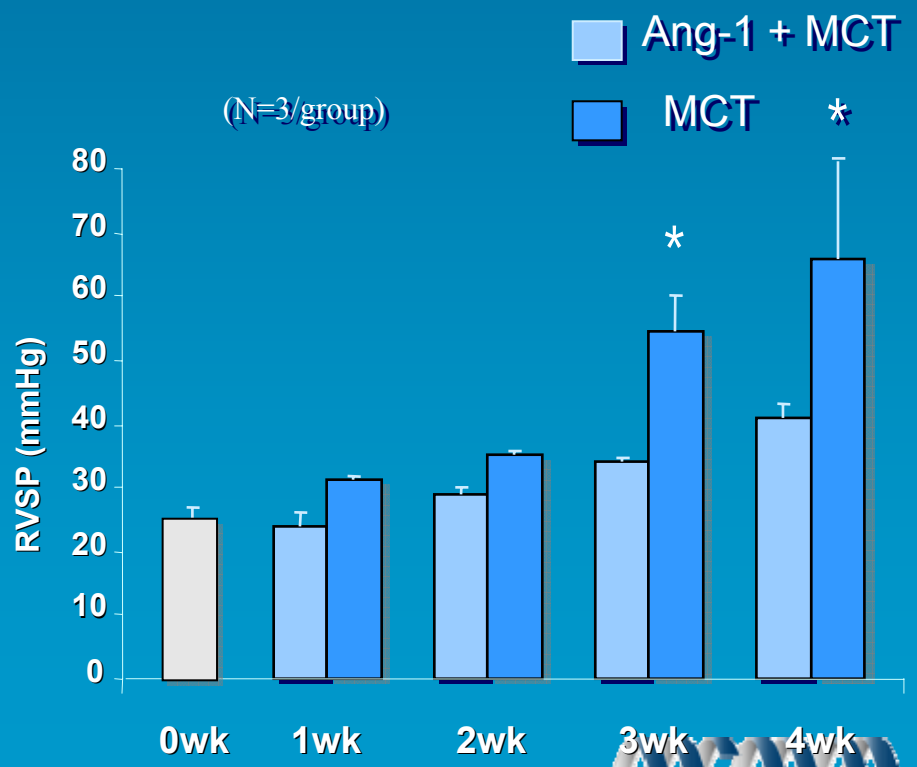
Activated caspase-3 immunoreactivity

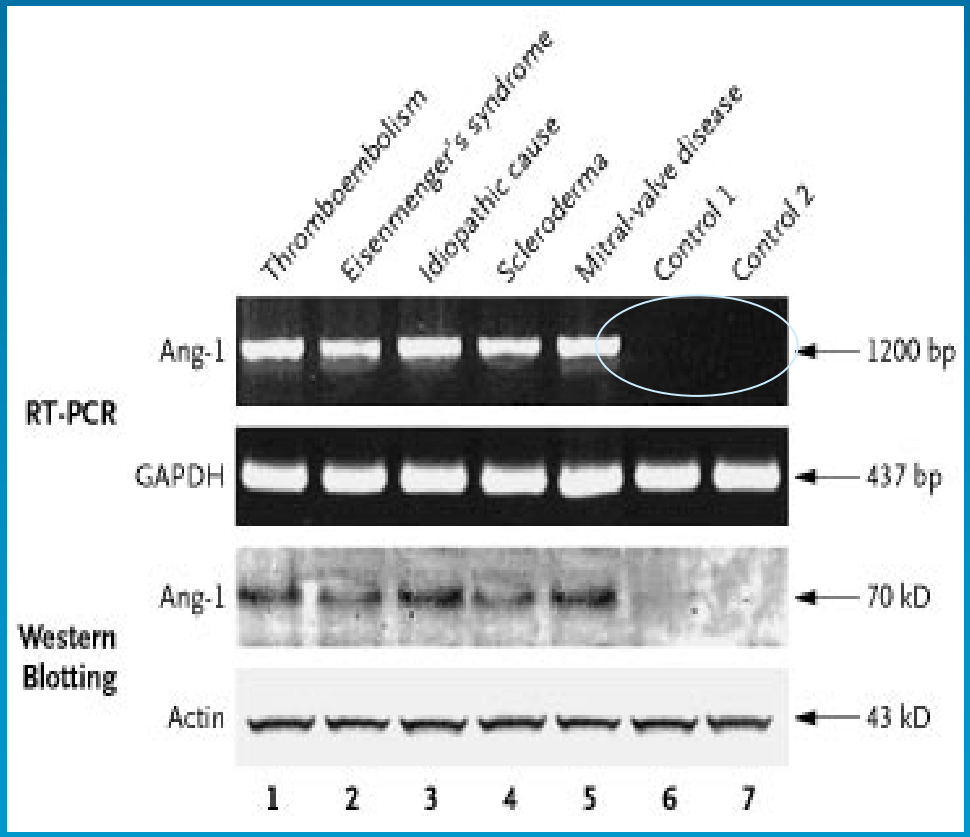


RV systolic pressure



Zhao et al Circulation Research 92:984, 2003

Is Angiopoietin-1 a mediator of PAH?



Du L. Signaling Molecules in Nonfamilial Pulmonary Hypertension. NEJM 2003;348:500-509.

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

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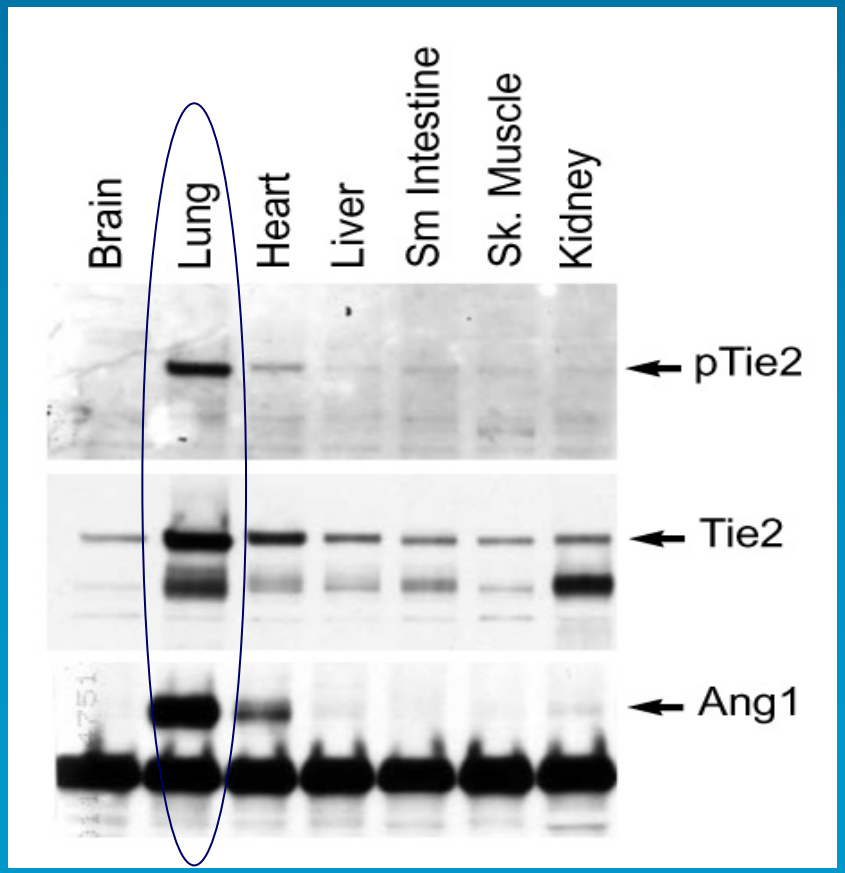
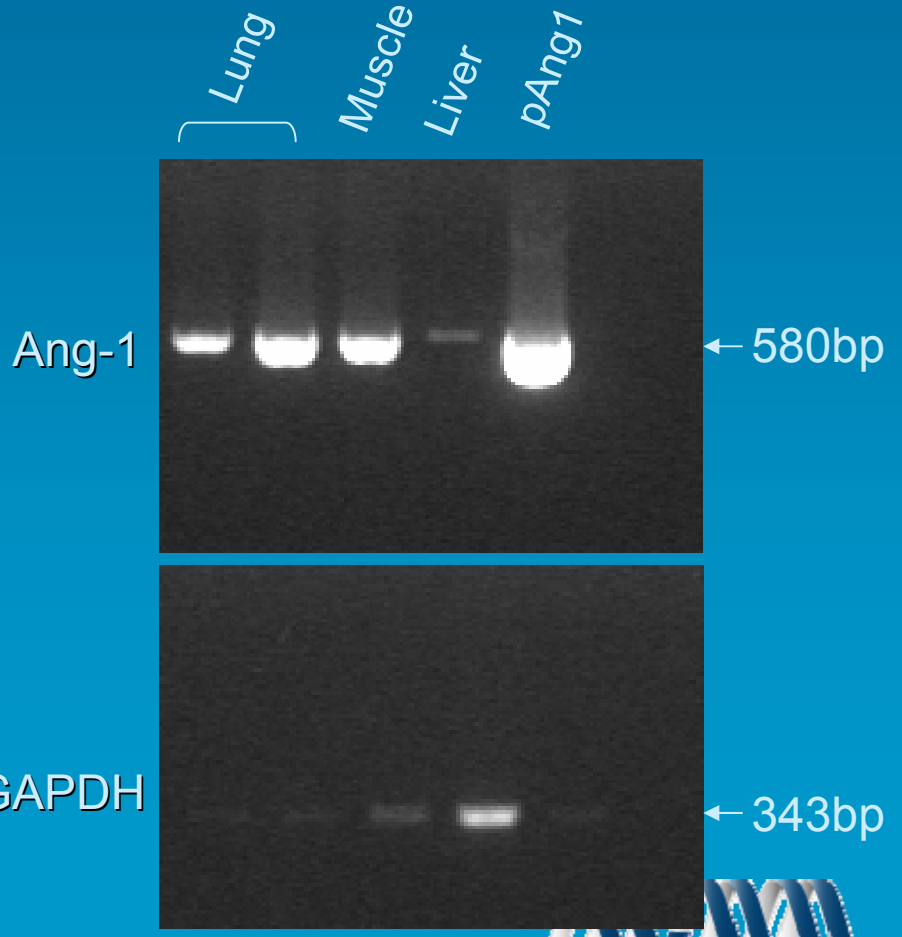
Murine

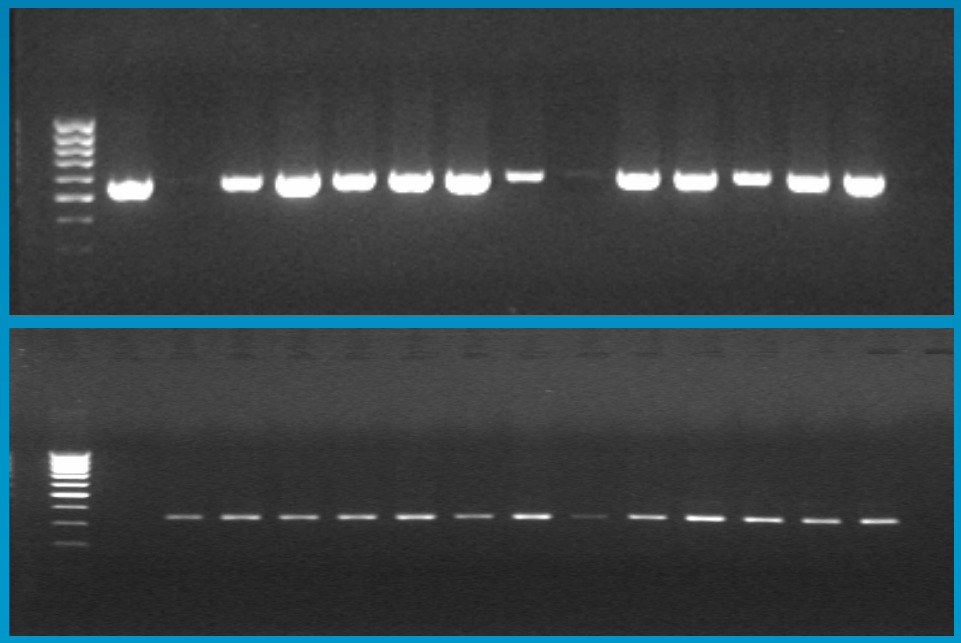


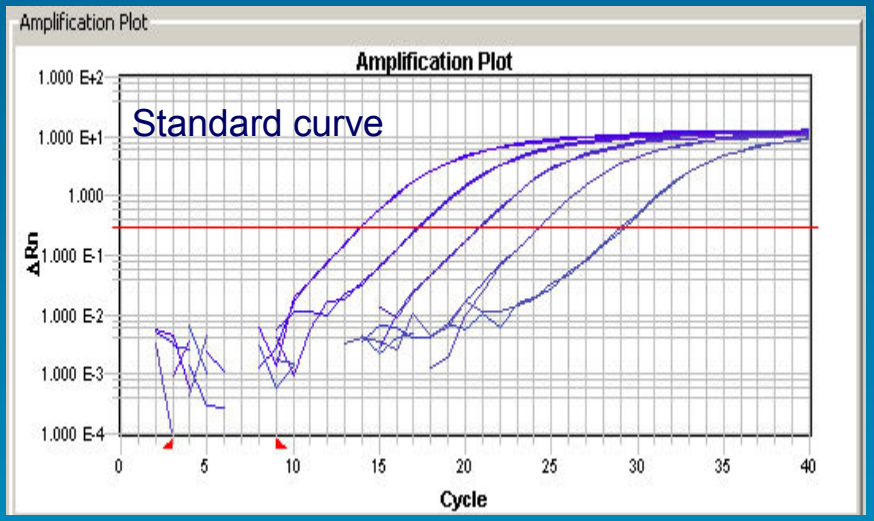
Human

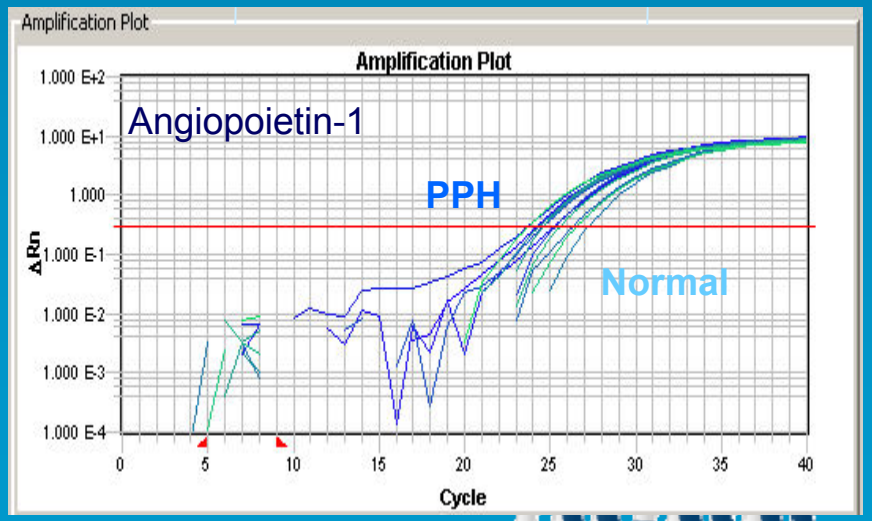


Rudge and Yancopolous, Regeneron

Ang-1 in PPH lungs







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

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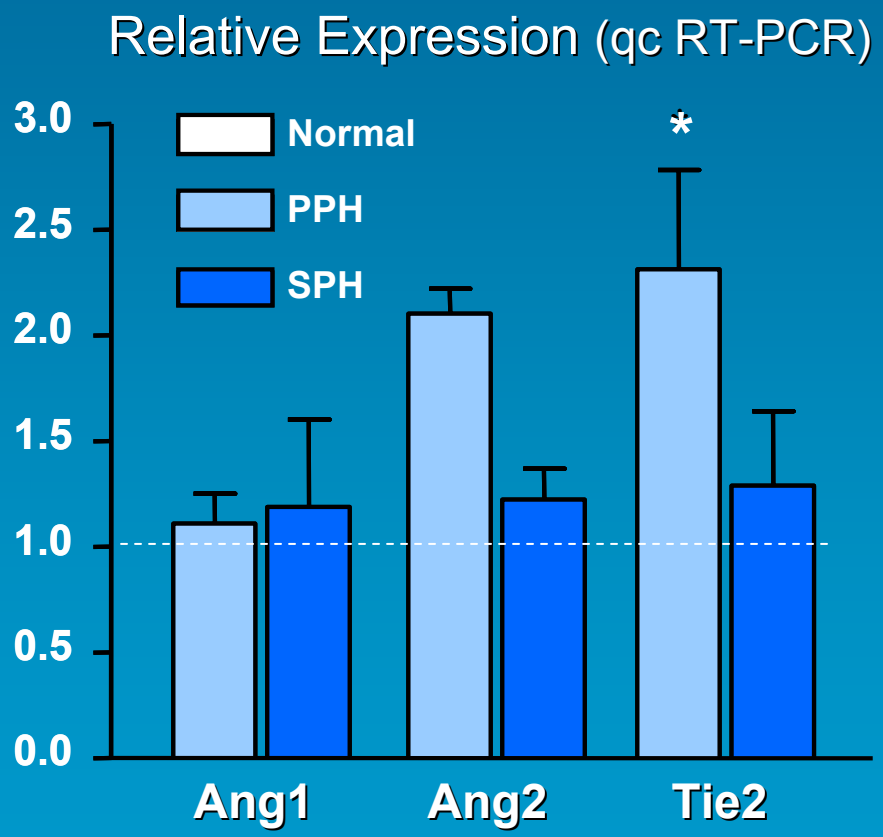
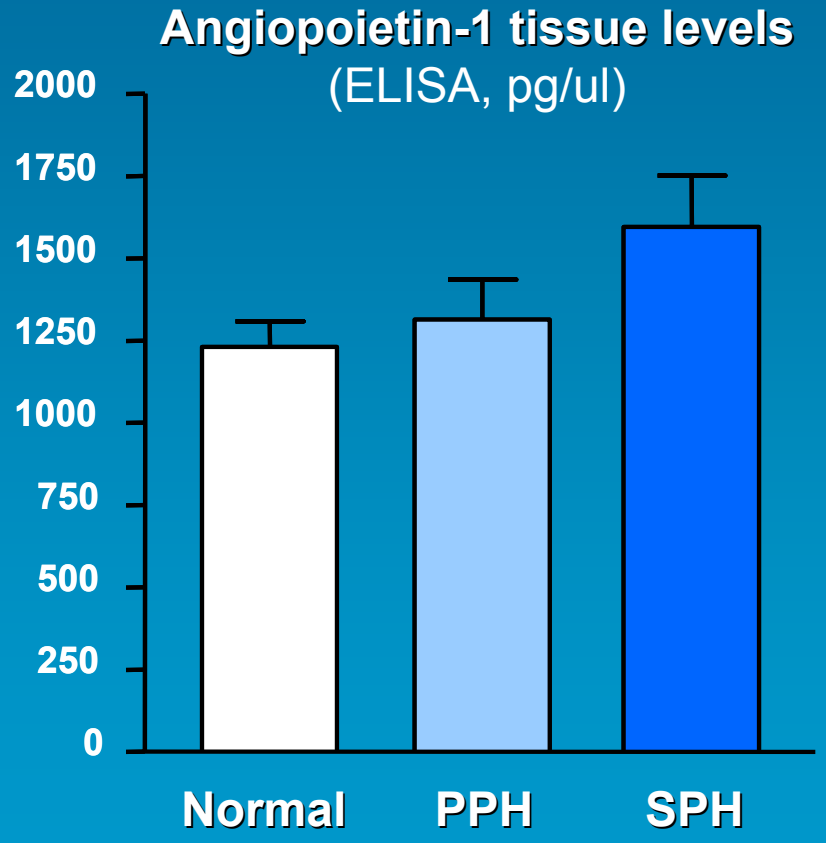
Angiopoietin expression in PAH





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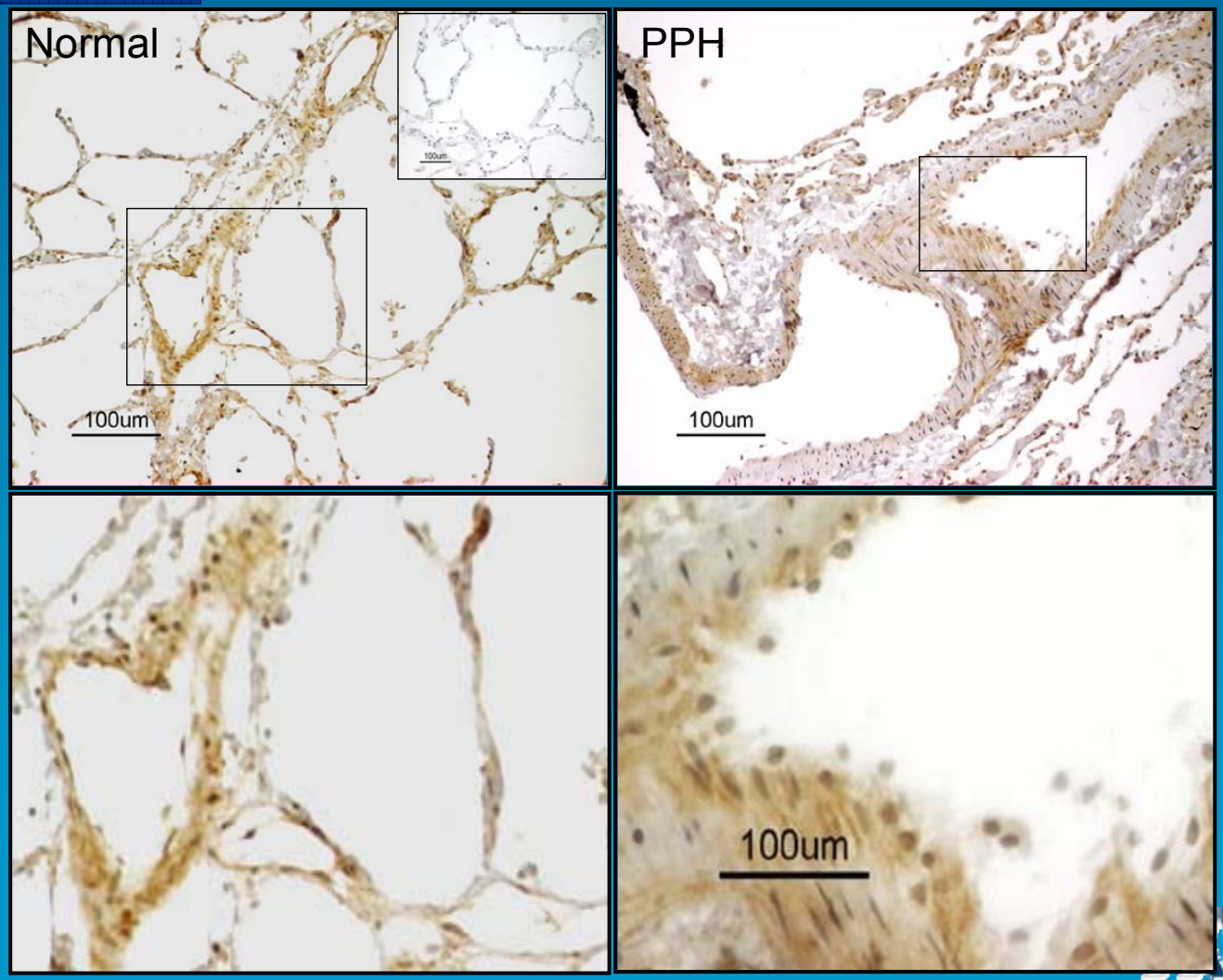
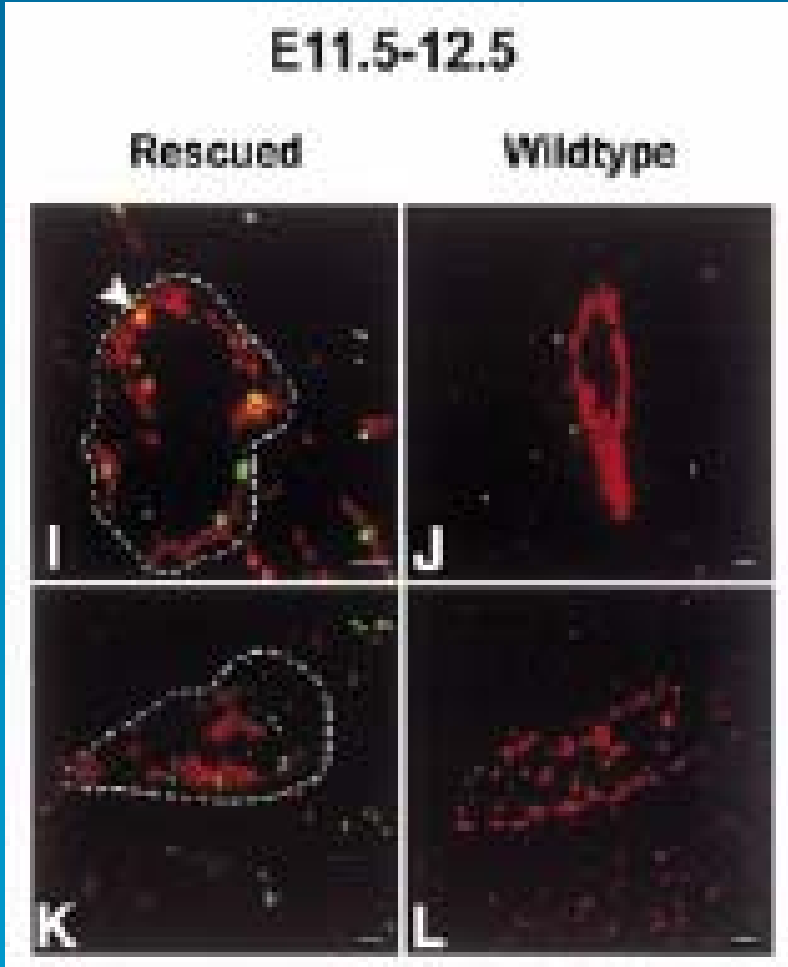


Partial rescue of Tie2-/- mice by dox-conditional targeted Tie2 expression



Jones et al. EMBO J 5:438, 2001

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Tie2 +/- mice develop spontaneous PAH

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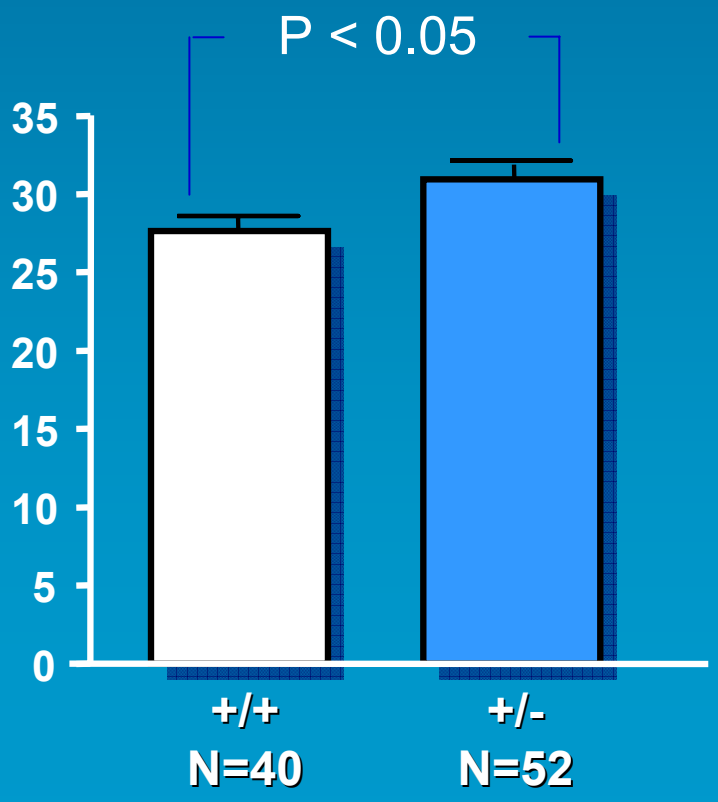
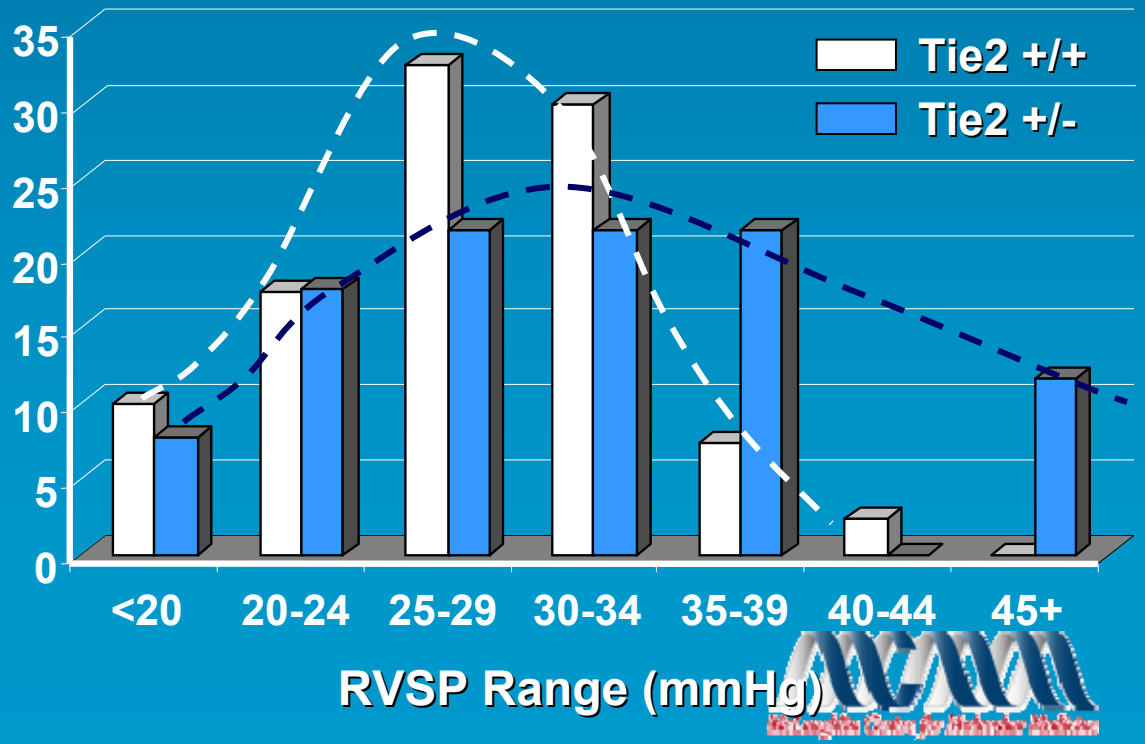
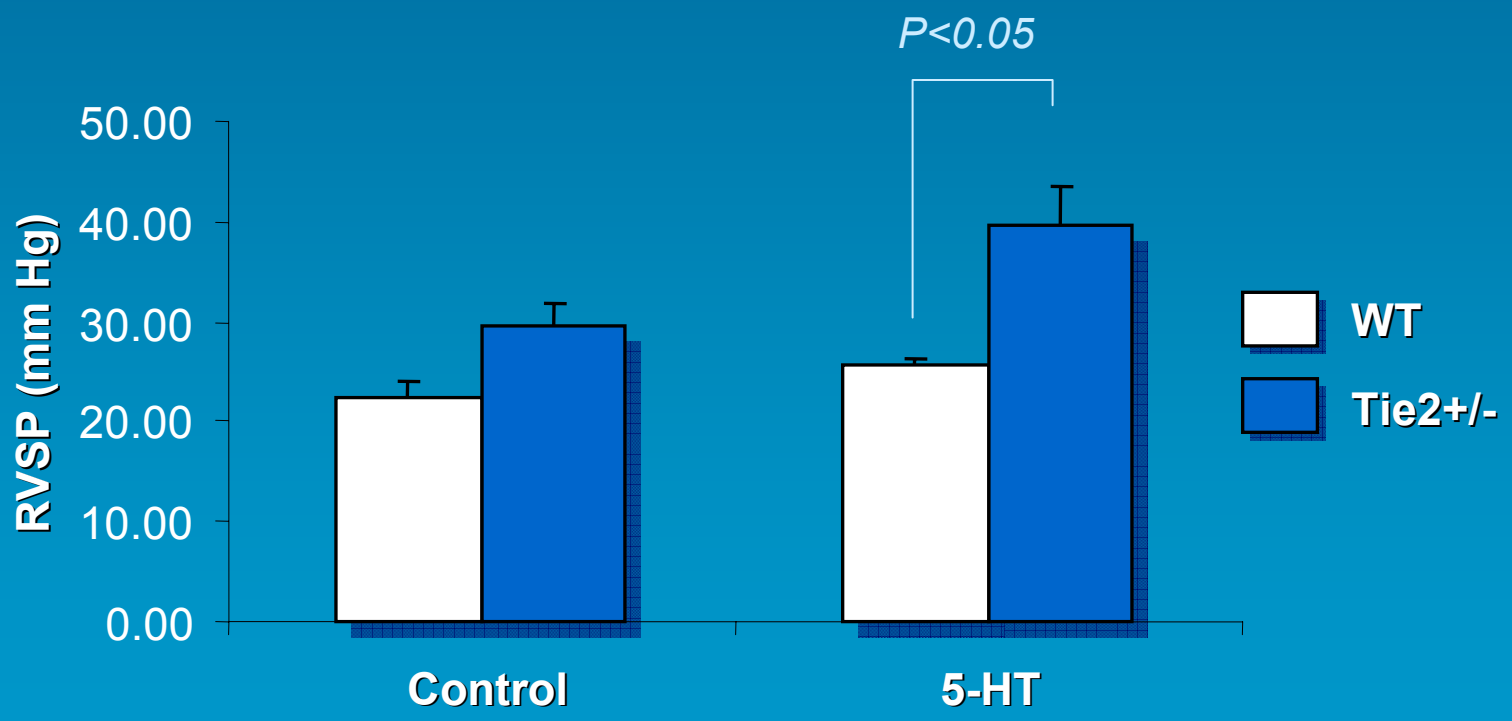
RVSP (mmHg)



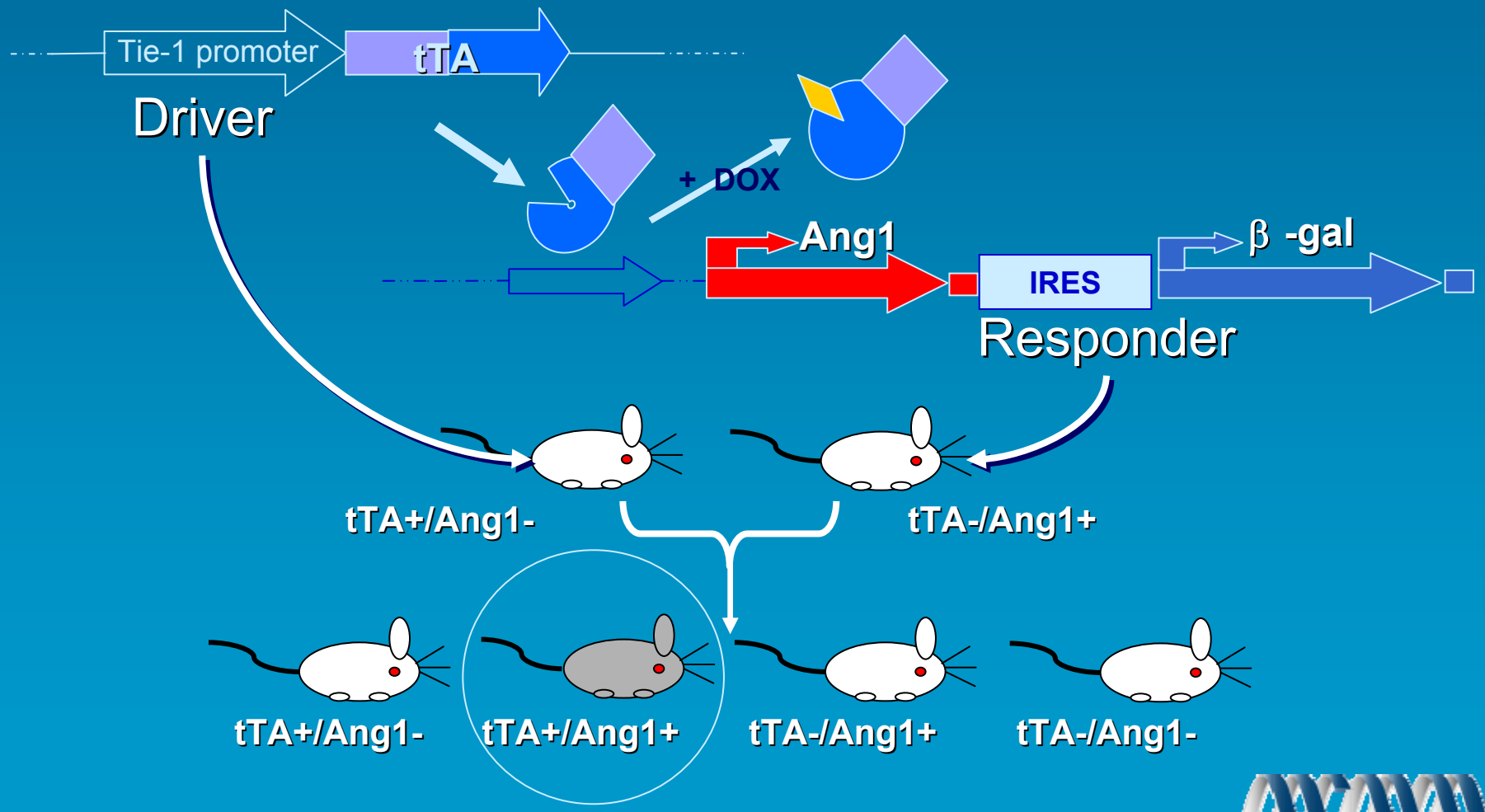
Frequency Distribution (%)



Effect of chronic Serotonin infusion



Conditional Endothelial Angiopoietin-1 Overexpression



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Conditional Endothelial Angiopoietin-1 Overexpression: protein

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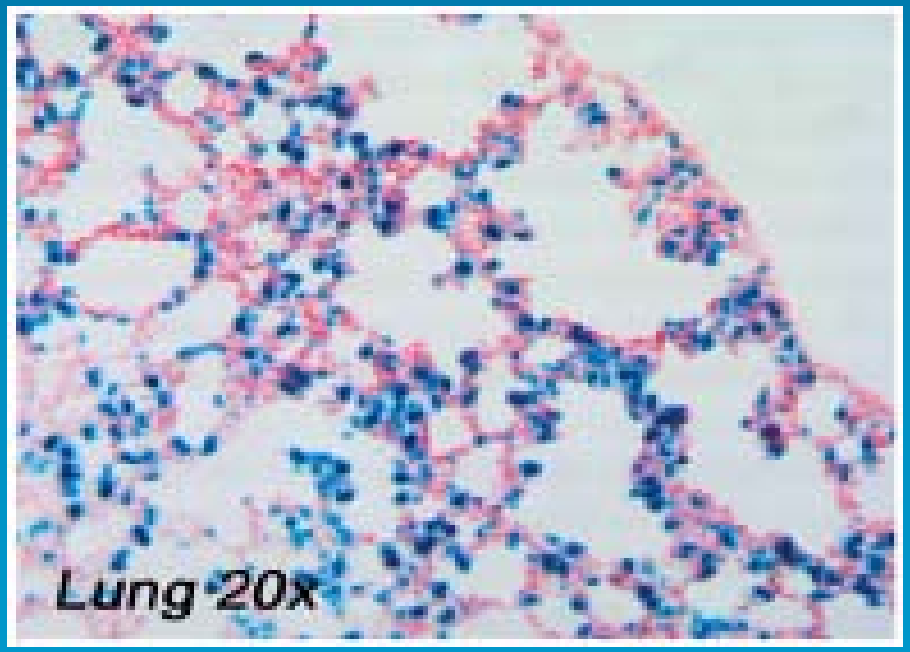
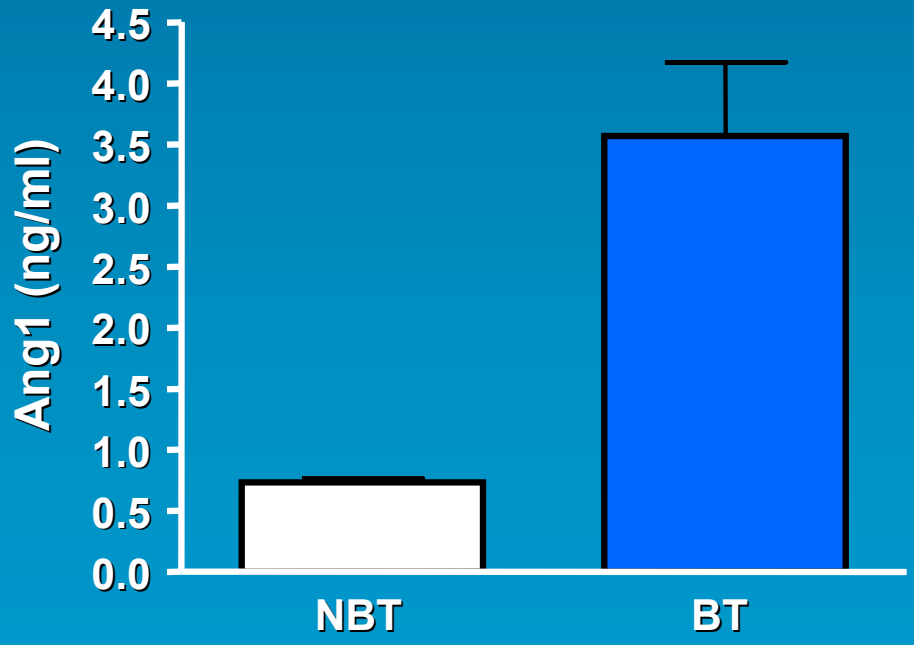
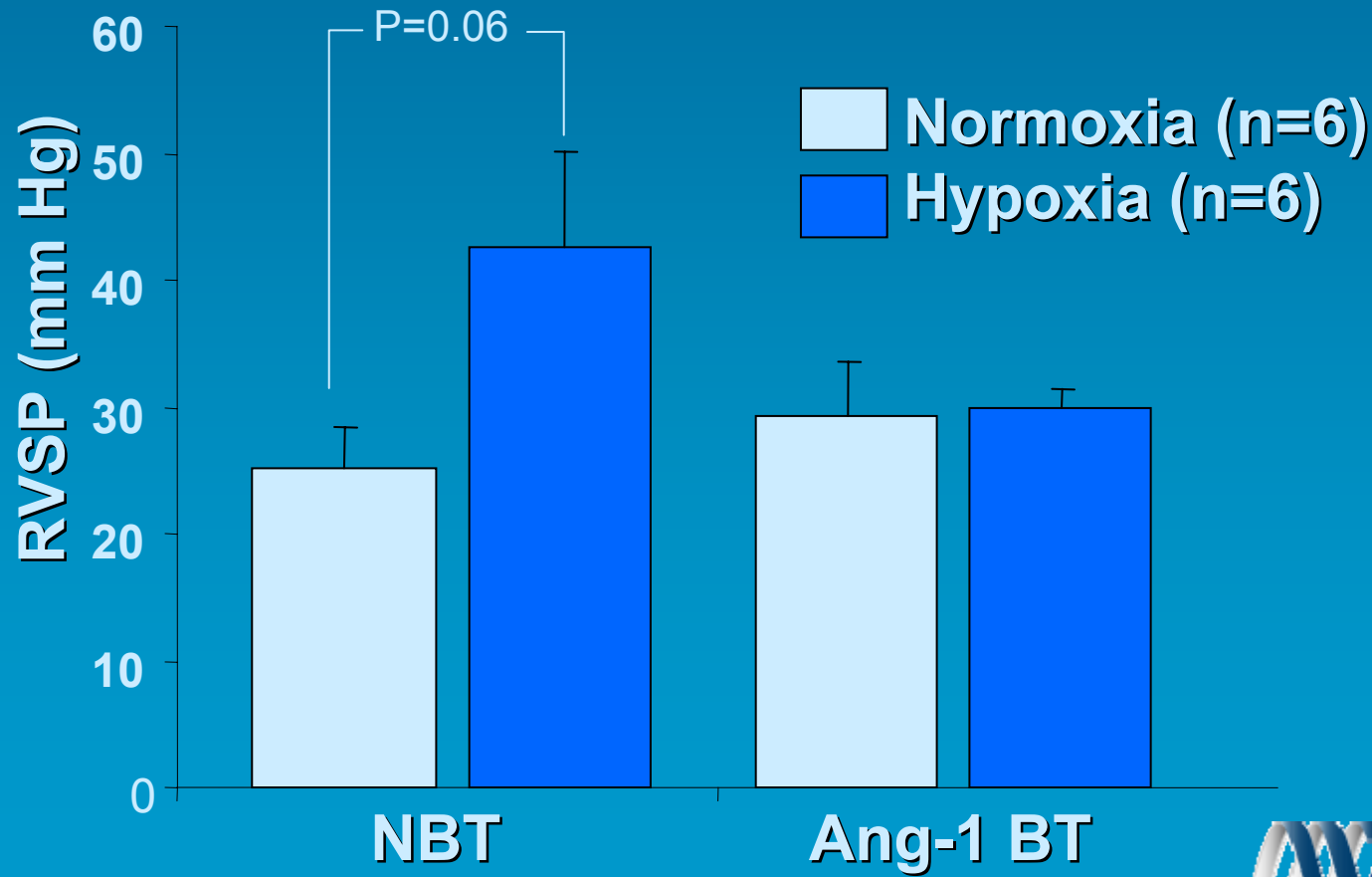
B-Gal staining



Ang-1 ELISA



Endothelial Angiopoietin-1 overexpression reduces hypoxic PAH



Lakshmi Kugathasan – Msc Candidate

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Angiopoietin-1 in pulmonary hypertension: *Cause or cure*?

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

- 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