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Appendix from Zhao et al., "Pulmonary vascular changes 22 years after single lung transplantation for pulmonary arterial hypertension: a case report with molecular and pathological analysis" (Pulm. Circ., vol. 5, no. 4, p. 000)

Supplementary methods

Immunoblotting

Protein concentrations were determined using the bicinchoninic acid protein assay (Pierce, Rockford, IL). Equal amounts of the protein lysates were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes. The membranes were incubated overnight at 4°C with the following antibodies from Abcam: anti-BMPRII (1 : 1,000, Santa Cruz), anti-RhoA (1 : 2,000, Santa Cruz), anti-PCNA (1 : 1,000, Abcam), anti-caspase 3 and 8 (1 : 2,000, Cell Signaling), and anti-cPARP (1 : 1,000, Abcam). Housekeeping proteins used were GAPDH (1 : 40,000, Santa Cruz) and actin (1 : 5,000, Sigma). After wash with Tris-buffered saline–Tween, the blots were incubated for 60 min at room temperature with horseradish peroxidase-conjugated antibodies, respectively: anti-rabbit or mouse antibodies (1 : 15,000; Sigma-Aldrich, St. Louis). Signals from immunoreactive bands were visualized by fluorography using an enhanced chemiluminescence reagent (Pierce).

Immunohistochemistry

The sections of both PAH and control lung tissue were fixed for 4 hours at room temperature with phosphate-buffered saline (PBS) made of 4% formaldehyde, permeabilized for 30 min in Triton X-100 (0.5% in PBS), and incubated with 5% nonfat skim milk in PBS for 90 min. Sections were incubated for 180 min at room temperature with antibodies for anti-caspase 3 (1 : 1000, Cell Signaling), anti-CD31 (1 : 100, Abcam), anti-Ki67 1 : 1000, Millipore), and anti-smooth muscle actin (SMC, 1 : 2000, Sigma). The sections were then incubated with fluorescent-conjugated secondary antibodies. The sections were visualized with the Zeiss LSM 510 confocal microscope.

Transcription analysis

The mRNA samples from the control, transplanted PH, and native PAH lungs were isolated as described.[13] Molecular related profiles were compared between a control group, transplanted PH lung, and native (original) idiopathic PAH. Briefly, the total RNA analysis in lung tissues was performed using Trizol extraction according to the manufacturer's instructions. The real-time PCR was performed using the primers in Table S1.

Table S1. Real-time polymerase chain reaction primers

Oligo name	Oligo sequence (5′-3′)	Oligo nucleotides
HuGapDH-F	GGTGAAGGTCGGAGTCAACG	20
HuGapDH-R	GAGTTAAAAGCAGCCCTGGTGA	22
HuTP53-F	CGACATAGTGTGGTGGTGCC	20
HuTP53-R	TCAAAGCTGTTCCGTCCCA	19
HuPARP-F	CGGAAGCTGGAGGAGTGACA	20
HuPARP-R	CCAATTCCATCCTGGCCTTT	20
HuBMPRII-F	TGGCTACCATGGACCATCCT	20
HuBMPRII-R	GCCGTTCTTGATTCTGCGAA	20
HuP21-F	TCTACCACTCCAAACGCCG	19
HuP21-R	AGGACTGCAGGCTTCCTGTG	20
HuPCNA-F	TGAGGGCTTCGACACCTACC	19
HuPCNA-R	CCGGCGCATTTTAGTATTTTG	20
HuBcl2-F	GGATCCAGGATAACGGAGGC	20
HuBcl2-R	GGGCCGTACAGTTCCACAAA	20
HuRhoA-F	TGTGGCAGATATCGAGGTGG	20
HuRhoA-R	ATCTTCCTGCCCAGCTGTGT	20

References Cited Only in the Appendix

13. Zhao Y, Peng J, Lu C, Hsin M, Mura M, Wu L, Chu L, et al. Metabolomic heterogeneity of pulmonary arterial hypertension. PLoS ONE 2014; 9(2):e88727.