Induced Pluripotent Stem Cell Model of Pulmonary Arterial Hypertension Reveals Novel Gene Expression and Patient Specificity

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Abstract

Rationale: Idiopathic or heritable pulmonary arterial hypertension is characterized by loss and obliteration of lung vasculature. Endothelial cell dysfunction is pivotal to the pathophysiology, but different causal mechanisms may reflect a need for patient-tailored therapies.

Objectives: Endothelial cells differentiated from induced pluripotent stem cells were compared with pulmonary arterial endothelial cells from the same patients with idiopathic or heritable pulmonary arterial hypertension, to determine whether they shared functional abnormalities and altered gene expression patterns that differed from those in unused donor cells. We then investigated whether endothelial cells differentiated from pluripotent cells could serve as surrogates to test emerging therapies.

Methods: Functional changes assessed included adhesion, migration, tube formation, and propensity to apoptosis. Expression of bone morphogenetic protein receptor type 2 (*BMPR2*) and its target, collagen IV, signaling of the phosphorylated form of the mothers against decapentaplegic proteins (pSMAD1/5), and transcriptomic profiles were also analyzed.

Measurements and Main Results: Native pulmonary arterial and induced pluripotent stem cell–derived endothelial cells from patients with idiopathic and heritable pulmonary arterial hypertension compared with control subjects showed a similar reduction in adhesion, migration, survival, and tube formation, and decreased *BMPR2* and downstream signaling and collagen IV expression. Transcriptomic profiling revealed high kisspeptin 1 (*KISS1*) related to reduced migration and low carboxylesterase 1 (*CES1*), to impaired survival in patient cells. A beneficial angiogenic response to potential therapies, FK506 and Elafin, was related to reduced slit guidance ligand 3 (*SLIT3*), an antimigratory factor.

Conclusions: Despite the site of disease in the lung, our study indicates that induced pluripotent stem cell–derived endothelial cells are useful surrogates to uncover novel features related to disease mechanisms and to better match patients to therapies.

Keywords: endothelial cells; *SLIT3*; *CES1*; *KISS1*; precision medicine

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Author Contributions: S.S. and M.G. designed and performed experiments and analyzed data. J.C. performed RNA-seq analyses with assistance of N.-Y.S. D.L. and K.A.T.E. performed some Western blot analyses. M.A. and Y.M. reprogrammed induced pluripotent stem cells (iPSC) from fibroblasts. F.G. prepared libraries for RNA-seq. A.C. helped differentiate iPSC to endothelial cell (EC). C.G.L., R.F., and L.N. performed comet assays. S.T. helped in writing the manuscript. J.C.W. was responsible for studies related to reprogramming of fibroblasts to iPSC, M.P.S. for sequencing studies, and both had substantial intellectual contributions. M.R. designed the studies, oversaw the EC differentiation and pulmonary arterial endothelial cells and iPSC-EC functional assays, data acquisition and analysis, and manuscript preparation and editing.

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At a Glance Commentary

Scientific Knowledge on the

Subject: This study was carried out to determine whether, in patients with idiopathic and heritable pulmonary arterial hypertension (IPAH/HPAH), endothelial cells differentiated from induced pluripotent stem cells (iPSC-EC) could be surrogates for native pulmonary arterial endothelial cells (PAEC) in disease modeling and drug screening.

What This Study Adds to the

Field: iPSC-EC derived from skin fibroblasts and PAEC harvested from the explanted lung of patients with IPAH/HPAH showed similar impairment in adhesion, migration, tube formation, vulnerability to apoptosis, and reduced bone morphogenetic protein receptor type 2 signaling when compared with iPSC-EC and PAEC from unused donors as controls. Comparing iPSC-EC and PAEC from IPAH/HPAH versus control by RNA-seq, we found changes in gene expression related to reduced migration and survival: high kisspeptin 1 (*KISS1*) and low carboxylesterase 1 (*CES1*), respectively. We also observed patient-specific responses to potential IPAH/HPAH therapies related in part to the antimigratory factor split guidance ligand 3 (*SLIT3*). This suggests that iPSC-EC from patients with IPAH/HPAH show promise in relating abnormalities in gene expression to pathophysiology and in developing personalized therapies. Studying gene expression in iPSC-EC is leading to the discovery of new pathophysiologic mechanisms and ultimately to testing potential biomarkers that are linked to differences in drug responsiveness.

Pulmonary arterial hypertension (PAH) is a potentially lethal disease in which small peripheral arteries are lost or obliterated and more proximal vessels narrow and stiffen, progressively increasing resistance to flow and eventually leading to right

heart failure (1, 2). Both in humans with PAH and in experimental pulmonary hypertension (PH), there is pulmonary arterial (PA) endothelial cell (EC) dysfunction characterized by susceptibility to apoptosis associated with loss of peripheral arteries (3), reduced adhesion related to impaired regeneration (4), decreased migration (5) and tube formation in angiogenesis assays (3), as well as heightened glycolysis, and emergence of apoptosis-resistant cells (6).

Decreased expression of bone morphogenetic protein receptor type 2 (*BMPR2*) is associated with all forms of PAH, and a mutation in this receptor is seen in 70% of patients with the heritable form of PAH (HPAH), and in 20% of sporadic cases of idiopathic PAH (IPAH). Reduced *BMPR2* is associated with impaired adhesion and migration of PA endothelial cells (PAEC) related to decreased expression of downstream targets collagen (*COL*) 4A1 and A2 and Ephrin A1 (5), heightened susceptibility to inflammation (7), and defects in cytoskeletal protein phosphorylation (8). In addition, decreased *BMPR2* suppresses downstream activation of mothers against decapentaplegic homolog proteins (*SMAD*)1/5 and transcription of the early response gene inhibitor of DNA binding 1 (*ID1*) (9) that regulates angiogenesis (10), and *SMAD*1/5 independent signaling (3, 9).

Features of PAEC dysfunction in PAH have been modeled in cultured cells and in animal models of PH. However, the nature and severity of PAEC dysfunction and the underlying abnormalities in gene expression and the response to specific therapies may vary among individuals. There is no source of patient-specific PAEC that can be studied at the onset of PAH, and little potential for continuous renewal of these cells to test novel therapies as they develop. Furthermore, current animal models of PH only capture some of the features of the human pathology.

Induced pluripotent stem cells (iPSC) reprogrammed from somatic cells could provide a new resource to understand the personal and the common genetic underpinnings related to the pathogenesis of IPAH/HPAH. iPSC can be propagated indefinitely, differentiated into EC and smooth muscle cells (11), and used for vascular

disease modeling (12) and drug screening (13), as shown with iPSC-derived cardiomyocytes (14, 15).

In this study, we compared IPAH/HPAH PAEC, harvested and cultured from lungs removed at the time of transplantation, with IPAH/HPAH EC differentiated from iPSC (iPSC-EC) that had been reprogrammed from skin fibroblasts obtained from a small biopsy taken at the incision site of the same patients. Control fibroblasts and PAEC were obtained from donors of unused lungs. We determined whether abnormalities in IPAH/HPAH PAEC function could be recapitulated in iPSC-EC from patients with IPAH/HPAH versus control subjects or whether EC dysfunction is PA-specific. We then investigated whether (1) there was a common pattern of altered gene expression related to EC dysfunction in IPAH/HPAH PAEC and iPSC-EC, (2) IPAH/HPAH patient-specific iPSC-EC could be surrogates for native PAEC in drug testing, and (3) transcriptomic profiling could uncover a potential drug-responder signature.

Methods

Extended description of the materials and methods is provided in the online supplement.

PAEC Culture

PAEC were obtained from explanted lungs through the Pulmonary Hypertension Breakthrough Initiative Network by scraping the endothelial layer of PAs (Tables 1 and 2) before culture in EC medium according to previous protocols (5). Procurement of the tissues from human subjects is approved by the Administrative Panel on Human Subjects in Medical Research at Stanford University (IRB #350, Panel 6). Written informed consent was received from participants before inclusion in the study.

Derivation of Human iPSC

Dermal fibroblasts were derived from a skin biopsy. Integration-free iPSC clones were generated from dermal fibroblasts using the CytoTune-iPS Sendai Reprogramming Kit (ThermoFisher Scientific, Waltham, MA).

Table 1. Characteristics of Patients with PAH

Patient ID	PAEC Type	Assay	Age (yr)-Sex	Race/Ethnicity	Diagnosis	PAP* (s/d/m)	PVR† (WU)	6MW‡ (m)	PAH Medications [§]	Known Mutation
PAH-1	Large	Adhesion, angiogenesis, caspase, migration, RT-PCR, comet, growth curve, JC-1, RNA-seq, siKISS1, siSLIT3	25-M	White/Hispanic	IPAH	65/15/36	NA	511	Epoprostenol, sildenafil, treprostinil	—
PAH-2	Large	Adhesion, angiogenesis, caspase, migration, RT-PCR, comet, growth curve, JC-1, RNA-seq, siSLIT3, OCT4 immunostaining	56-F	White/non-Hispanic	IPAH	83/39/57	11.41	137	Sildenafil, ambrisentan, treprostinil	—
PAH-3	Small	Adhesion, angiogenesis, RT-PCR, comet	40-F	White/non-Hispanic	IPAH	105/63/55	9.84	472	Sildenafil, bosentan	—
PAH-4	Small	Adhesion, angiogenesis, caspase, migration, RT-PCR, growth curve, JC-1, RNA-seq, siKISS1, OCT4 immunostaining	40-M	White/Hispanic	IPAH	89/46/64	16.8	420	Sildenafil, ambrisentan, treprostinil	—
PAH-5	Small	Adhesion, angiogenesis, caspase, migration, RT-PCR, Western blot, comet, growth curve, JC-1, RNA-seq, flow cytometry	39-F	Other/Hispanic	IPAH	112/45/69	14.97	262	Sildenafil, bosentan, iloprost, epoprostenol	—
PAH-6	Small	Adhesion, angiogenesis, caspase, migration, RT-PCR, Western blot, comet, growth curve, JC-1, RNA-seq, OCT4 immunostaining	37-M	White/non-Hispanic	HPAH	119/51/77	14.22	309	Sildenafil, sitaxentan, ambrisentan, epoprostenol, imatinib (invest. medication)	BMPR2 c.1471C>T p.R491W
PAH-7	Large	Adhesion, angiogenesis, caspase, migration, RT-PCR, Western blot, comet, growth curve, JC-1, RNA-seq, siKISS1, siSLIT3, OCT4 immunostaining	33-F	Black or African American/non-Hispanic	HPAH	75/33/48	15.57	326	Epoprostenol, bosentan, sildenafil, treprostinil	BMPR2 c.1471C>T p.R491W
PAH-8	Small	Angiogenesis, migration, flow cytometry	16-F	White/non-Hispanic	IPAH	NA/NA/95	NA	102	Sildenafil, treprostinil	—
PAH-9	Small	Angiogenesis, migration, flow cytometry	53-M	White/non-Hispanic	IPAH	56/15/33	3.86	78	Sildenafil, tadalafil, macitentan, epoprostenol	—

Definition of abbreviations: 6MW = 6-minute-walk test; BMPR2 = bone morphogenetic protein receptor type 2; HPAH = heritable PAH; IPAH = idiopathic PAH; JC-1 = mitochondrial membrane potential assay; NA = not available; OCT4 = octamer-binding transcription factor 4; PAEC = pulmonary artery endothelial cell; PAH = pulmonary arterial hypertension; PAP = pulmonary arterial pressure; PVR = pulmonary artery resistance; RT-PCR = reverse-transcriptase polymerase chain reaction; siKISS1 = knockdown of kisspeptin 1 (KISS1); siSLIT3 = knockdown of slit guidance ligand 3 (SLIT3); WU = Wood Units.

*PAP, systolic (s) diastolic (d) and mean (m) catheterization study performed closest to transplantation.

†PVR from catheterization study performed closest to transplantation.

‡6MW distance walked (meters) in 6 minutes, from study performed closest to transplantation with cardiac output calculated by Fick oxygen consumption.

§PAH medications listed according to total drug exposure during treatment period up to transplantation, not necessarily in combination.

Table 2. Characteristics of Control Subjects (Unused Donor Lungs)

Patient ID	PAEC Type	Assay	Age (yr)-Sex	Race/Ethnicity	Cause of Death
CON-1	Small	Adhesion, migration, RT-PCR, comet, growth curve	60-F	White/non-Hispanic	Intracranial hemorrhage
CON-2	Small	Adhesion, angiogenesis, caspase, RT-PCR, Comet, JC-1	13-M	White/non-Hispanic	Head trauma, following motor vehicle accident
CON-3	Small	Adhesion, angiogenesis, caspase, migration, Western blot, comet, growth curve, RNA-seq, flow cytometry, OCT4 immunostaining	33-F	White/non-Hispanic	Head trauma, blunt injury
CON-4	Small	Adhesion, angiogenesis, caspase, migration, RT-PCR, Western blot, comet, growth curve, JC-1, RNA-seq, siCES1, flow cytometry	1-M	White/non-Hispanic	Anoxia/drowning
CON-5	Small	RT-PCR	46-M	Asian/non-Hispanic	Cerebrovascular/stroke
CON-6	Small	Caspase, migration, comet, growth curve, JC-1, RNA-seq, OCT4 immunostaining	47-M	White/non-Hispanic	Intracranial hemorrhage
CON-7	Small	Adhesion, angiogenesis, caspase, RT-PCR, comet, flow cytometry, OCT4 immunostaining	47-M	White/non-Hispanic	Motor vehicle accident
CON-8	Small	Adhesion, angiogenesis, RT-PCR, JC-1, RNA-seq	45-M	White/non-Hispanic	Cerebrovascular/stroke
CON-9	Large	Adhesion, angiogenesis, caspase, migration, Western blot, comet, growth curve, JC-1, RNA-seq, siCES1, OCT4 immunostaining	43-F	White/unknown	Anoxia
CON-10	Large	RNA-seq, siCES1, OCT4 immunostaining	57-F	White/non-Hispanic	Cerebrovascular/stroke
					Acute myocardial infarction

Definition of abbreviations: CON = control; JC-1 = mitochondrial membrane potential assay; OCT4 = octamer-binding transcription factor 4; PAEC = pulmonary artery endothelial cell; RT-PCR = reverse-transcriptase polymerase chain reaction; siCES1 = knock down of carboxylesterase 1 (CES1).

In Vitro Monolayer Endothelial Differentiation of Human iPSC

EC were differentiated from iPSC by a modified published protocol (16). The cultured iPSC monolayer was placed in differentiation medium (RPMI and B-27 supplement minus insulin) with 6 μ M of the GSK-3 inhibitor CHIR-99021 (CHIR) for 2 days, followed by differentiation medium with 3 μ M CHIR for 2 days. The medium was then changed to differentiation medium with 50 ng/ml vascular endothelial growth factor, 10 ng/ml fibroblast growth factor basic for 5 days. On Day 9, iPSC-ECs were purified by magnetic-activated cell sorting for cluster of differentiation 144 (CD144)⁺ and cultured in EGM-2 medium.

Cell Adhesion

Cell adhesion assays were performed as previously described (4).

Migration Assay

Migration assays were modified from a previous publication (17). Cells were seeded into a 24-well plate overnight to form confluent cell monolayers. Then, a linear scratch ("wound") was generated in the monolayer with a sterile 20- μ l plastic pipette tip. Representative images were converted to black and white by Adobe Photoshop from each well, to enhance clarity of the figure.

Caspase and Angiogenesis Assays

Caspase and angiogenesis assays were modified from a previous publication (3). For angiogenesis assays, PAEC and iPSC-EC were seeded in a 24-well plate in growth factor reduced Matrigel (Corning, Corning, NY). Tube length and number of tubes were measured after 4 hours in three random microscopic fields. For clarity of the figure, representative images were converted to black and white by Adobe Photoshop (San Jose, CA). The 4-hour time point was chosen as optimizing the difference between all control and IPA/HPAH PAEC and iPSC-EC. Control cells were not treated with Elafin or FK506 because no differences were observed in a previous study (3).

Quantitative Reverse-Transcriptase Polymerase Chain Reaction

Quantitative polymerase chain reaction was performed as previous published (5).

Western Immunoblotting

Western immunoblotting was performed based on a previous publication (5).

RNA-seq of PAEC and iPSC-EC

RNA was extracted and libraries were prepared and subjected to sequencing with an Illumina HiSeq2500 instrument (Stanford Personalized

Medicine Sequencing Core, San Diego, CA).

Small Interfering RNA Transfection

Small interfering RNA (siRNA) transfections were performed according to a modification of a protocol previously published by our group (5).

Statistical Analysis

Histograms represent the arithmetic mean $\pm$ SEM. Statistical significance was determined by two-sided unpaired Student's *t* test for comparison of two groups or by one-way analysis of variance, with Bonferroni *post hoc* testing (for three or more groups). Analyses were performed using GraphPad Prism 6.0 (La Jolla, CA).

Results

iPSC-EC have Typical Morphology and Functional Characteristics of PAEC

PAEC were cultured as previously described (5) from explanted lungs of patients with IPA/HPAH, or HPAH with a *BMP2* mutation, undergoing a lung transplant, and from unused donor lungs as control subjects. Information related to demographics and hemodynamics is found in Tables 1 and 2. From the same patients and control subjects, we obtained skin fibroblasts from

the midline incision site, reprogrammed these to iPSC, and differentiated the iPSC to EC as previously published (16, 18). There was marked similarity between iPSC-EC and native PAEC judged by the typical EC cobblestone morphology, acetylated low-density lipoprotein uptake, immunolocalization of vascular endothelial cadherin (CD144) (Figure 1A), and the expression of cluster of differentiation 31 (CD31) and vascular endothelial growth factor receptor 2 (see Figure E1 in the online supplement), commonly used identifiers of EC. Compared with human umbilical vein endothelial cells (HUVEC), control and IPA/HPAH PAEC and iPSC-EC expressed significantly reduced markers of venous EC as judged by mRNA and protein levels of ephrin type-B receptor 4 (*EPHB4*) and nuclear receptor subfamily 2 (*NR2F2*) (see Figures E2A, E2B, and E2E). In contrast, expression of arterial EC gene ephrin-B2 (*EFNB2*) tended to be higher in control and IPA/HPAH PAEC and iPSC-EC than in HUVEC but the difference was not significant (see Figure E2C). IPA/HPAH PAEC had significantly greater expression of the arterial EC gene activin receptor-like kinase 1 (*ALK1*) than HUVEC, whereas control PAEC and control and IPA/HPAH iPSC-EC expressed a trend in that direction when compared with HUVEC. IPA/HPAH PAEC expressed higher *ALK1* than control PAEC and IPA/HPAH iPSC-EC (see Figure E2D). There were no significant differences in the expression of the other arterial or venous EC genes tested, comparing PAEC and iPSC-EC (see Figure E2).

IPA/HPAH PAEC and iPSC-EC compared with those from control lungs showed reduced adhesion (Figure 1B), decreased migration detected by a wound closure scratch assay (Figure 1C), increased vulnerability to apoptosis with serum withdrawal analyzed by caspase activity (Figure 1D), and impaired angiogenesis judged by the inability to form capillary-like tubes when seeded onto growth factor reduced Matrigel (Figure 1E). iPSC-EC from two iPSC clones (clone A and clone B) derived from each of two patients (responder PAH-4 and nonresponder PAH-2) were comparable in all the above functional assays, confirming that there was no batch to batch difference between the iPSC-EC because of reprogramming or differentiation (see Figure E3).

Figure 1. Idiopathic and heritable pulmonary arterial hypertension (IPA/HPAH) pulmonary arterial endothelial cells (PAEC) and endothelial cells differentiated from induced pluripotent stem cells (iPSC-EC) show similar functional abnormalities. (A) PAEC isolated and cultured from explanted donor lungs and iPSC-EC differentiated from skin fibroblast-derived iPSC have the similar cobblestone morphology, incorporate acetylated low-density lipoprotein (Ac-LDL, green), and express the endothelial marker cluster of differentiation 144 (CD144) (green; blue = 4',6-diamidino-2-phenylindole, dihydrochloride [DAPI]). (B) Adherent cells per $\times 10$ field were counted 30 minutes after

Figure 1. (Continued). seeding on 10 $\mu\text{g}/\text{cm}^2$ laminin ($n = 7$). (C) A scratch was applied to cell monolayers, and migration of the cells toward the wound was recorded by photomicrographs at 6 hours ($n = 5$ Control, and 6 IPAH/HPAH; 4 IPAH and 2 HPAH). Representative images of wound closure on top. Related quantitative data are below. (D) Apoptosis was evaluated by the caspase-3/7 assay 12 hours after serum withdrawal in Control and IPAH/HPAH PAEC and iPSC-EC ($n = 6$ Control, and 6 IPAH/HPAH; 4 IPAH and 2 HPAH). (E) Angiogenesis assays: representative images of tube formation (top), and quantitative analysis (bottom) indicates the number of tubes formed and average tube length 4 hours after seeding cells in Matrigel ($n = 6$ control and 7 IPAH/HPAH; 5 IPAH and 2 HPAH). Bars represent mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$, unpaired Student's t test IPAH/HPAH versus control. High and low values for patients and control subjects are designated by identifiers in the tables (see Figures E1–E5). RLU = relative light unit.

Two abnormalities in IPAH/HPAH PAEC were not significantly different in iPSC-EC. An increase in unrepaired DNA damage judged by the “comet tails” of nuclear DNA following alkali digestion in IPAH/HPAH PAEC, but not iPSC-EC (see Figure E4A), may in part be masked by a baseline increase in comet tails even in the parent fibroblasts (see Figure E4A). In IPAH/HPAH PAEC, but not iPSC-EC, the mitochondrial membrane potential measured by the JC-1 assay after serum starvation was significantly higher compared with control subjects (see Figure E4B). Neither IPAH/HPAH PAEC nor iPSC-EC showed a difference in cell growth when compared with control cells as judged by the WST-8 assay over 7 days in full-serum media (see Figure E5). As in our previous study changes in mitochondrial membrane potential could be detected without a change in proliferation (19).

***BMPR2* and *COL4A2*, and *BMPR2* Signaling in PAEC and iPSC-EC**

Our previous studies showed reduced expression and signaling function of *BMPR2* in IPAH/HPAH PAEC compared with control subjects (9). Previous RNA-seq analyses of human microvascular ECs revealed that 10 genes were differentially expressed in IPAH/HPAH versus control subjects, and six were reproduced in control PAEC by reducing levels of *BMPR2* by siRNA (5). Consistent with this study, we detected a reduction in *BMPR2* mRNA in both PAEC and iPSC-EC from patients with IPAH/HPAH versus control subjects. *COL4A2*, although overall higher in iPSC-EC than in PAEC, was significantly decreased in both IPAH/HPAH cell types as assessed by quantitative real-time polymerase chain reaction (Figures 2A and 2B). However, five differentially expressed genes previously reported in IPAH/HPAH PAEC did not show significant changes. This could be the result of having used microvascular PAEC in the previous study, whereas the PAEC in the present study came from small and large PA. In response to stimulation with the *BMPR2* ligand BMP9, *BMPR2*, pSMAD1/5, and ID1 protein levels were significantly reduced in IPAH/HPAH PAEC and iPSC-EC compared with respective control cells (Figure 2C).

Figure 2. Reduced expression of bone morphogenetic protein receptor type 2 (*BMPR2*) and collagen type IV and alpha 2 chain (*COL4A2*); and reduced *BMPR2* signaling in idiopathic and heritable pulmonary arterial hypertension (IPAH/HPAH) pulmonary arterial endothelial cells (PAEC) and endothelial cells differentiated from induced pluripotent stem cells (iPSC-EC). (A) *BMPR2* and (B) *COL4A2* mRNA were measured by quantitative reverse-transcriptase polymerase chain reaction. $n = 6$ control and $n = 7$ IPAH/HPAH (5 IPAH and 2 HPAH). * $P < 0.05$ versus control, unpaired Student's t test. (C) Representative Western immunoblot of *BMPR2*, pSMAD1/5, ID1, and glyceraldehyde phosphate dehydrogenase (top) with BMP9 (10 ng/ml) or vehicle (phosphate-buffered saline) treatment in PAH-4, with densitometry on the bottom ($n = 3$; two IPAH and one HPAH). Bars represent mean $\pm$ SEM. * $P < 0.05$ versus untreated # $P < 0.05$ versus genotype, two-way analysis of variance with Bonferroni test. High and low values for patients and control subjects are designated by identifiers in the tables. BMP9 = bone morphogenetic protein 9; GAPDH = glyceraldehyde phosphate dehydrogenase; ID1 = inhibitor of DNA binding 1; pSMAD1/5 = phosphorylated form of the mothers against decapentaplegic proteins; Veh = vehicle.

PAH PAEC and iPSC-EC Respond Similarly to Elafin and FK506

Given the functional similarities in IPAH/HPAH PAEC and iPSC-EC, we determined whether iPSC-EC could be used as surrogates for native PAEC in drug screening studies, using new promising treatments for PAH, FK506, and Elafin. FK506 is an immunosuppressant identified as an activator of *BMPR2* signaling in a high throughput screen of Food and Drug Administration–approved drugs. FK506 dissociates FKBP12 from the *BMPR2* coreceptor, permitting its phosphorylation and downstream signaling. FK506 reverses severe PH in rats (9), is in compassionate use for patients with PAH (20), and is in preparation for a pivotal clinical trial. Elafin is a neutrophil elastase inhibitor that reverses severe PH in rats and improves angiogenesis in IPAH/HPAH PAEC by enhancing *BMPR2* signaling in a caveolin-1-dependent manner (3). Elafin has been used in patients without PAH as a single-dose intravenous therapy (21) and is being developed for chronic subcutaneous administration in patients with PAH. In PAEC and iPSC-EC from two patients with IPAH/HPAH, Elafin and FK506 improved angiogenesis as judged by number of tubes and tube length, whereas in the other five patients, there was no change in either cell type (Figures 3A and 3B). iPSC-ECs from one patient with IPAH/HPAH only showed improved number of tubes, but PAEC from the same individual showed improvement in both number of tubes and tube length. The function that best predicted the iPSC-EC and PAEC response to FK506 and Elafin was migration (Figure 3C).

RNA-seq Analysis of PAEC and iPSC-EC from Patients with IPAH/HPAH

Transcriptomic profiling was performed on PAEC and iPSC-EC from control subjects and patients with IPAH/HPAH ($n = 6$ per group) (Tables 1 and 2) to find novel gene expression changes that could explain the similar functional abnormalities observed in both cell types. Principal component analysis of the resulting transcriptomes demonstrated that iPSC-EC are similar to PAEC, and are separated from undifferentiated iPSC and fibroblasts (Figure 4A). The *Nanog* homeobox (*Nanog*), sex determining region Y-box 2 (*SOX2*), and octamer-binding transcription factor 4 (*OCT4*) gene expression by

Figure 3. Proangiogenic responders to Elafin and FK506 in idiopathic and heritable pulmonary arterial hypertension (IPAH/HPAH) pulmonary arterial endothelial cells (PAEC) and endothelial cells differentiated from induced pluripotent stem cells (iPSC-EC). (A) Representative images of angiogenesis assay in patient IPAH-5 PAEC and iPSC-EC following treatment for 4 hours with vehicle (0.9% sodium chloride for Elafin; dimethyl sulfoxide for FK506), Elafin (1 μ g/ml), or FK506 (15 μ g/ml) show improved angiogenesis with both agents. (B) Elafin and FK506 versus vehicle, assessed by the number of tubes or tube length (pixels) in eight matched IPAH/HPAH PAEC and iPSC-EC. Patients IPAH, 8, 9, 1, 2, 4, 5, and HPAH 6, 7 (Table 1) are represented on the figure. Bars represent mean $\pm$ SEM. $^{**}P < 0.005$, $^{***}P < 0.0005$ versus vehicle by one-way analysis of variance, followed by Bonferroni test. (C) Data from wound closure assay show that migration may be predictive of angiogenic response to Elafin and FK506.

Figure 4. RNA-seq analysis of pulmonary arterial endothelial cells (PAEC) and endothelial cells differentiated from induced pluripotent stem cells (iPSC-EC) from control versus idiopathic and heritable pulmonary arterial hypertension (IPAH/HPAH), and drug responders versus nonresponders, reveals differences in gene expression. (A) Principal component analysis plot showing that the first and second principal components separate PAEC and iPSC-EC from undifferentiated iPSC and fibroblasts (control $n = 6$, CON-3, 4, 6, 8, 9, and 10; IPAH/HPAH $n = 6$, PAH-1, 2, 4, 5, 6, and 7). (B) Venn diagram shows differentially expressed genes (DEG) with a maximum false discovery rate of 0.1. On the *top* are genes up-regulated in control cells, and on the *bottom* are

RNA-seq normalized read counts, and immunofluorescent staining of OCT4 protein revealed no significant differences between PAEC and iPSC-EC (see Figure E6). OCT4 protein expression in PAEC and iPSC-EC was significantly lower than those of iPSC (see Figures E6D and E6E), indicating that the iPSC-EC had exited their pluripotent state.

An overlapping set of six up-regulated and 10 down-regulated genes was found in both control PAEC and iPSC-EC versus IPA/HPAH counterparts (Figures 4B and 4C; see Figure E7A). To relate changes in gene expression to function, we investigated two differentially expressed transcripts. Kisspeptin 1 (*KISS1*), is up-regulated in PAH PAEC and iPSC-EC. Kisspeptin-10, a *KISS1*-derived decapeptide, inhibits EC migration and angiogenesis (22, 23). Consistent with this function, both IPA/HPAH PAEC and iPSC-EC transfected with *KISS1* siRNA versus scrambled siRNA show enhanced migration (Figure 4D; see Figure E6B). Carboxylesterase 1 (*CES1*), a metabolizing enzyme suppressed by cancer therapy in association with induction of apoptosis (24), was down-regulated in IPA/HPAH PAEC and iPSC-EC. *CES1* siRNA in control cells reproduced the propensity to apoptosis seen in I/HPAH PAEC and iPSC-EC (Figure 4E; see Figure E7C).

An overlapping set of three up-regulated and eight down-regulated genes distinguished IPA/HPAH PAEC and matched iPSC-EC drug no responders from responders (Figure 4F; see Figure E7D). One of the up-regulated genes in the nonresponders slit guidance ligand 3 (*SLIT3*), was chosen for further investigation because it is known to reduce the migratory

activity of melanoma cells (25). siRNA knockdown of *SLIT3* in IPA/HPAH PAEC and iPSC-EC from nonresponders resulted in significant improvement in migration (Figure 4G; see Figure E7).

Transcriptome profiling was performed on all 12 (six control and six IPA/HPAH) PAEC, and their corresponding iPSC-EC to find differences as opposed to similarities between PAEC and iPSC-EC. EBseq was used to test for differentially expressed genes (DEG) between each individual's PAEC and their iPSC-EC, and 47 of the DEGs were identified as common to all 12 individuals (see Figures E8A–E8E, Table E1). GO and Reactome pathway database were used to analyze these 47 DEGs and find functional pathways that differentiated PAEC and iPSC-EC. Figures E8F and E8G indicate features that would be important to consider in the future use of iPSC-EC as surrogates for native PAEC. The most robust difference was related to cell–extracellular matrix interaction.

Discussion

EC dysfunction plays a key role in PAH development but the mechanism may vary in individuals, perhaps because of genetic factors and environmental exposures. This may challenge the institution of therapies that will consistently reverse the disease process (1). We reasoned that by producing patient-specific iPSC that can be differentiated into EC, the gene variants that give rise to EC dysfunction (providing it was not PA-specific) would be preserved, and the opportunity to individualize therapy would be greatly enhanced. We could also discover common differentially expressed genes that would reveal new

genetic underpinnings that might even link IPA/HPAH with PAH. Considering that PAH is a rare disease, the cohort size was limited. However, matching native PAEC and iPSC-EC from the same patients and control subjects minimized variability. DEGs and related functional pathways that distinguished PAEC and iPSC-EC were also provided and will be important to note in future investigations using iPSC-EC. Two abnormalities in IPA/HPAH versus control PAEC, unrepaired DNA damage and decreased mitochondrial membrane potential, were not recapitulated in iPSC-EC from the same patients. These features may be related to cell-specific epigenetic changes in IPA/HPAH PAEC that have been induced over time, and are not captured by a strong genetic predisposition, or are lost through the reprogramming and differentiation processes.

Some studies have suggested that PA cell-specific features are required for the development of PAH. For example, differential methylation seems to explain the cell-specific expression of the superoxide dismutase, and the endothelial nitric oxide synthase genes (26, 27). Other studies, however, have shown similarities in PAEC and systemic EC dysfunction studied in relation to loss of *BMPR2* (19, 28), and to PAH in general (29). Here, we provide evidence for the first time that IPA/HPAH PAEC and iPSC-EC from the same individuals have similar functional impairment related at least in part to reduced *BMPR2* and downstream signaling, and gene expression of *COL4A2*.

The two potential therapeutic agents tested influence *BMPR2* signaling. It was particularly interesting that both PAEC and iPSC-EC from only one of two patients

Figure 4. (Continued). genes up-regulated in IPA/HPAH cells, with the overlap indicating common genes. (C) Heatmap displaying the 16 overlapping DEG in IPA/HPAH PAEC and iPSC-EC versus control PAEC and iPSC-EC. Hierarchical clustering was performed using the average gene expression from each of the indicated groups. (D) *KISS1*, an up-regulated gene in I/HPAH, was reduced by small interfering RNA (siRNA) and wound closure assessed for improvement in both PAEC and iPSC-EC of PAH patient 1, 4, and 7. (E) *CES1*, a down-regulated gene in IPA/HPAH, was reduced by siRNA in CON 4, 9, and 10 PAEC and iPSC-EC and vulnerability to apoptosis assessed by active caspase after serum withdrawal at 96 hours. (F) Heatmap displaying 11 overlapping DEG between Elafin and FK506 responders versus nonresponders, in both PAEC and iPSC-EC (n = 3; two IPA/HPAH and one HPAH). Hierarchical clustering used the average gene expression from each of the indicated conditions. DEG revealed three up-regulated and eight down-regulated genes in nonresponders versus responders. (G) *SLIT3*, a gene up-regulated in the nonresponders, was reduced by siRNA in PAEC and iPSC-EC of IPA/HPAH nonresponders PAH-1, 2, and 7, and wound closure assessed 96 hours after transfection. Scr = scrambled siRNA. Bars represent mean ± SEM. *P < 0.05, unpaired Student's *t* test versus siScr, n = 3 (see Figure E6). *ADRB1* = adrenoceptor β1; *AFP* = α fetoprotein; *C17orf53* = chromosome 17 open reading frame 53; *CES1* = carboxylesterase 1; *CLIC3* = chloride intracellular channel 3; *CRHBP* = corticotropin releasing hormone binding protein; *CTNNA3* = catenin α3; *ESX1* = ESX homeobox 1; *GDA* = guanine deaminase; *GLIS3* = GLIS family zinc finger 3; *GPR183* = G protein-coupled receptor 183; *KCNS3* = potassium voltage-gated channel modifier subfamily S member 3; *KISS1* = kisspeptin 1; *LINC00960* = long intergenic non-protein coding RNA 960; *OLFML2A* = olfactomedin like 2A; *OPCML* = opioid binding protein/cell adhesion molecule like; *PLA1A* = phospholipase A1 member A; *PTPN20A* = protein tyrosine phosphatase, non-receptor type 20A; *PTPN20B* = protein tyrosine phosphatase, non-receptor type 20B; *RERG* = RAS-like estrogen regulated growth inhibitor; RLU = relative light unit; *SATB1* = SATB homeobox 1; *SLC2A14* = solute carrier family 2 member 14; *SLIT3* = slit guidance ligand 3; *TGFA* = transforming growth factor α; *UTS2* = urotensin 2.

with the same *BMPR2* mutation showed a good angiogenic response to these therapies. This suggests specific genetic modifiers that could be the subject of future studies with a larger cohort. Moreover, our gene expression studies and drug testing were done with a very small cohort, and the determination of responders versus nonresponders was based only on the angiogenic response of the cells. Future prospective iPSC-EC studies incorporated into clinical trials with a larger cohort of patients will ultimately determine whether angiogenesis, other functional responses to therapy, and gene expression profile predict a positive clinical outcome.

Given the small cohort size and the stringency of our analysis, it is perhaps not surprising that we identified only 16 genes differentially expressed in control versus IPA/HPAH PAEC and iPSC-EC by RNA-seq. There may be considerable patient-specific variability in the nature of altered gene expression that gives rise to the same functional abnormality. Although the functions of some of the genes differentially expressed in IPA/HPAH versus control cells are unknown, others could be directly related to the abnormal phenotype observed, and these are novel and have not been previously associated with IPA/HPAH. Up-regulation of *KISS1* impeded migration, suppression of *CES1* increased propensity to apoptosis, and up-regulation of *SLIT3* in nonresponders to therapy impeded migration. It would be interesting in future analyses of a larger cohort of iPSC-EC from patients with IPA and HPAH to determine whether expression of these or related genes are linked to functional impairment and could be used to develop a panel of biomarkers.

Other up-regulated genes of interest in responders are Catenin α 3 (*CTNNA3*) and potassium voltage-gated channel, modifier subfamily S, member 3 (*KCNK3*). *CTNNA3* is necessary for the formation

of stretch-resistant cell-cell adhesion complexes (30), and increased *CTNNA3* might improve PAEC adhesion and angiogenesis (31). *KCNK3* is significantly up-regulated in PA smooth muscle cells by BMP2 stimulation (32), related to its proapoptotic and/or antiproliferative effects, and may therefore also be related to the prosurvival function of BMP2 in PAEC (31). Not all the changes in gene expression observed were consistent with the functional abnormality. For example, α -fetoprotein, a proangiogenic factor, was surprisingly increased in PAH. Perhaps this reflects a compensatory mechanism.

Other transcripts that were increased included transforming growth factor- α , urotensin II (UTS2), guanine deaminase (GDA), and β_1 -adrenergic receptor. Transforming growth factor- α can trigger transition from epithelial to mesenchymal phenotype through epidermal growth factor receptor, and might, like high mobility group AT-hook 1 previously described, be implicated in neointimal formation in PAH (33). UTS2 was previously associated with PH and vascular diseases (34), and UTS2 expression by PAEC and smooth muscle cells was significantly enhanced in rats with PH induced by aortocaval shunting (35). However, the functional impact of increased UTS2 gene expression in PAH PAEC is unknown. GDA, an important enzyme of purine metabolism, is up-regulated in spontaneously hypertensive rats with left ventricular hypertrophy and diastolic heart failure (36). In hemolysis-associated PH, adenosine deaminase, another important enzyme of purine metabolism, is released from injured erythrocytes into plasma and can increase the risk for vasoocclusive events and PH (37). Therefore, GDA might have a role in the pathogenesis of PAH. β_1 -adrenergic receptor in the transforming growth factor β pathway is associated with PH (38), but

how its function is altered in PAH has not been elucidated.

Further analyses in a much larger cohort could determine whether there are polymorphisms or epigenetic changes, or altered regulation of transcription factors that explain aberrant expression of these and other genes differentially expressed in IPA/HPAH or that distinguish IPA from HPAH. In future prospective studies, it will be interesting to determine whether these novel genes can be used as biomarkers for PAH or response to therapy.

In conclusion, we compared native IPA/HPAH PAEC with iPSC-EC from the same individuals and identified similar functional impairment, reduced *BMPR2* signaling, and subgroup responsiveness to potential IPA/HPAH therapy, suggesting that despite the site of disease in the lung and the particular vulnerability of the PA vasculature, iPSC-EC are good surrogates for IPA/HPAH modeling and drug testing. Furthermore, the RNA-seq analysis reveals novel changes in gene expression that are fundamental to EC dysfunction related to IPA/HPAH pathogenesis. ■

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