Utility of induced pluripotent stem cell derived endothelial cells as pulmonary arterial hypertension models

Ryan A. Fong

Intel Science Talent Search 2015
Sheldon High School, Sacramento, CA 95829
Stanford University, Stanford, CA 94305

ryanfong@stanford.edu | ryan.a.fong@gmail.com

Abstract: Pulmonary arterial hypertension (PAH) is an incurable, lethal, and life-limiting disease in which progressively increasing pulmonary artery pressure leads to right ventricular heart failure. There remains a demonstrated need for better understanding of the cellular and molecular mechanisms underlying the disease, and for novel treatments that can improve grave patient prognosis. Endothelial cell (EC) dysfunction plays a key role in the pathology of PAH and other cardiopulmonary diseases. It has been established that native pulmonary artery ECs from idiopathic PAH (IPAH) patients have significant defects as evaluated through the angiogenesis (tube formation) and single cell gel electrophoresis (comet) assays. Recently, cell types of interest derived from induced pluripotent stem cells (iPSCs) have been shown to recapitulate disease phenotypes *in vitro*. Therefore, we investigated if ECs derived from iPSCs (iPSC-ECs) could prove useful as PAH models. Angiogenesis and comet assays were used to evaluate the extent to which iPSC-ECs derived from IPAH patients recapitulated impaired features established in native pulmonary artery ECs. IPAH patient iPSC-ECs demonstrated significantly impaired angiogenic function and had higher levels of unrepaired DNA damage versus control iPSC-ECs. These findings suggest that iPSC-ECs can be effective disease models and drug screens for novel drug candidates and personalized therapy.

PERSONAL STATEMENT

Research is very special for me. It is unequivocal that it has irrevocably changed my life, and it is my hope that by sharing my story, I might be able to demonstrate its transformative power.

As a 10th grader, I couldn't see past the street culture that my school community was awash with. Scientific research was just something in a textbook – drugs, gangbanging, fights, and pregnancies were the demanding reality in front of me. However, I had historically been a good student – I liked exploring far away ideas in my mind and exerting some measure of control over my life. Things were coming to a head, though, and events dictated that this year was to be a crossroads.

It was with the encouragement and support of a science teacher that year, Mr. Jason Brennan, that I entered the UC Davis Teen Biotech Challenge. He saw something in me that I doubt anyone else could have at that point in time. Through TBC, I landed an internship with the UC Davis stem cell program.

That first summer in research, I was dunked total immersion in a world I had never known existed. Working with genetic reprogramming of human dermal fibroblasts to induced pluripotent stem cells (iPSCs), I found an unprecedented level of fulfillment in the lab. I was a part of something larger than myself, doing my small part to effect positive change on countless lives. I was a member of a friendly, profoundly intelligent and even more extraordinarily compassionate community of scientists. I was being pushed harder than I'd ever experienced in my life, but I was growing so much as a result. Never before had I felt so free and independent, and my eyes were opened to a brand new world. I was able to learn at a pace that surprised myself, tackling material filled with jargon and ridiculously multisyllabic words, because I knew just how critical understanding that material was in order to make an effective difference. And not to mention, the science fiction nature of the work I was doing was a whole new level of awesome than anything I'd ever done.

I knew I had found something extremely special, and I begged the lab director, Dr. Jan Nolta, to let me stay on during my 11th grade year. Over the next few months, I had the amazing opportunity to work with pre-clinical mice trials of genetically engineered hematopoietic stem cells for anti-HIV gene therapy, which can be read about here: http://www.ncbi.nlm.nih.gov/pubmed/25524029.

The work I submitted to Intel was conducted in the summer before my senior year at Stanford University. I had emailed about 40 Stanford professors and gotten only 5 replies, which turned into 3 interview opportunities. This project will always be precious to me because of its incredible trial and re-affirmation. I was asked to dedicate myself to something at a level I had never done before, and learned what it really means to have a passion. Even though I was volunteering for up to 12 hour days and spending hours in the dark microscopy room at a time, all I felt was the hunger to keep grinding until I had solid results. When I was able to produce data and complete a written report, all of that work felt so worth it. Crucially, many of the questions I'd had about if I saw myself pursuing a career in this field for real were answered.

What my mentors have always told me and what I've quickly learned is that research requires both insatiable curiosity and rugged tenacity. Being able to ask the important questions that are the basis of science only results in a contribution to society's progress if one is able to relentlessly pursue it. Failure is a large part of the scientific process, and needs to be welcomed like an old friend – an old friend we sit down and learn from, so that we might be guided in the correct direction on our journey. To persist in the face of adversity, with heady optimism, is a good general life skill and one incredibly important in research and many high-level careers.

Because of my unconventional start and background, it's as crucial to me as research itself to be involved in youth STEM outreach/education. I know first-hand how these initiatives can change lives. I plan to do everything I can at the college level when I start at Stanford this Fall 2015, and hope in the future I can start an initiative of my own – whether it's opening my lab up to high school students, or founding some sort of organization.

I strongly advise high school students interested in STEM to pursue research. So many doors are opened because of it, both in terms of college but also in a personal sense. You learn to think at a higher level than ever before and will benefit personally from the fulfillment of the process. I highly recommend summer programs – many of which especially recruit low-income or underrepresented minorities, and/or are funded to be free of charge or even offer a paid stipend. If there isn't a program accessible to you,

create your own opportunity by emailing professors at your local college/university. If you don't have one of those, work out of your room or garage. The great thing about research is that it does correlate with effort. Every hour you put in will get some sort of result, so work as hard as you can and you won't fail. Be respectful of the enormity of the work you're doing, but never be intimidated into not believing in yourself.

I will be entering undergrad this Fall as a pre-med hoping to be a strong candidate for combined MD/PhD programs, and eventually have my own lab as a clinical/translational stem cell researcher working to realize all of the field's potential to redefine what is deemed "medically possible."

Please contact me at the email addresses on the title page if you have any questions at all, or even if you just need someone to talk to, and I will do everything I can to help.

Good luck, and as many Nobel laureates have said, never forget to have FUN with research! I can't wait to see all of the things you will accomplish.

INTRODUCTION

Pulmonary arterial hypertension (PAH) is a severe disease of the cardiopulmonary system in which the pulmonary arteries narrow and stiffen, progressively increasing blood pressure that strains the heart and leads eventually to right ventricular heart failure [1, 2]. The clinical definition of PAH is a resting mean pulmonary artery pressure of >25 mmHg, versus a healthy pressure of 15 mmHg [3]. PAH is classified by the World Health Organization according to the 2008 Dana Point classification system as Category 1 pulmonary hypertension, under the subcategories of idiopathic PAH (IPAH); familial PAH; or PAH associated with other diseases including human immunodeficiency viral infection (HIV), from recreational drug use (methamphetamine and cocaine), and exposure to toxins [1, 2, 4].

PAH is incident nearly two times more in women than men, occurring in the prime of life (30-50 years of age) [5-9]. Modern treatments have been able to extend the five year survival rate from 34% in 1980 to just 50% in the 21st century [5, 10]. Current treatments primarily consist of vasodilators, which do not address underlying occlusive vascular remodeling, which continues unabated [11-12], leaving whole lung transplantation as the only treatment option for patients who do not respond to other treatments or who

have progressed to end-stage disease [13]. However, transplant recipients only trade a chronic disease for another life-long medical condition because of comorbidities associated with anti-rejection immunosuppressant treatment; post-transplantation median survival was found to be only 5.3 years [13]. As such, there is a demonstrated need for novel treatments that address the underlying cellular mechanisms in the pathophysiology of PAH, to improve prognosis for a debilitating and lethal disease that affects some of the most vulnerable demographic groups.

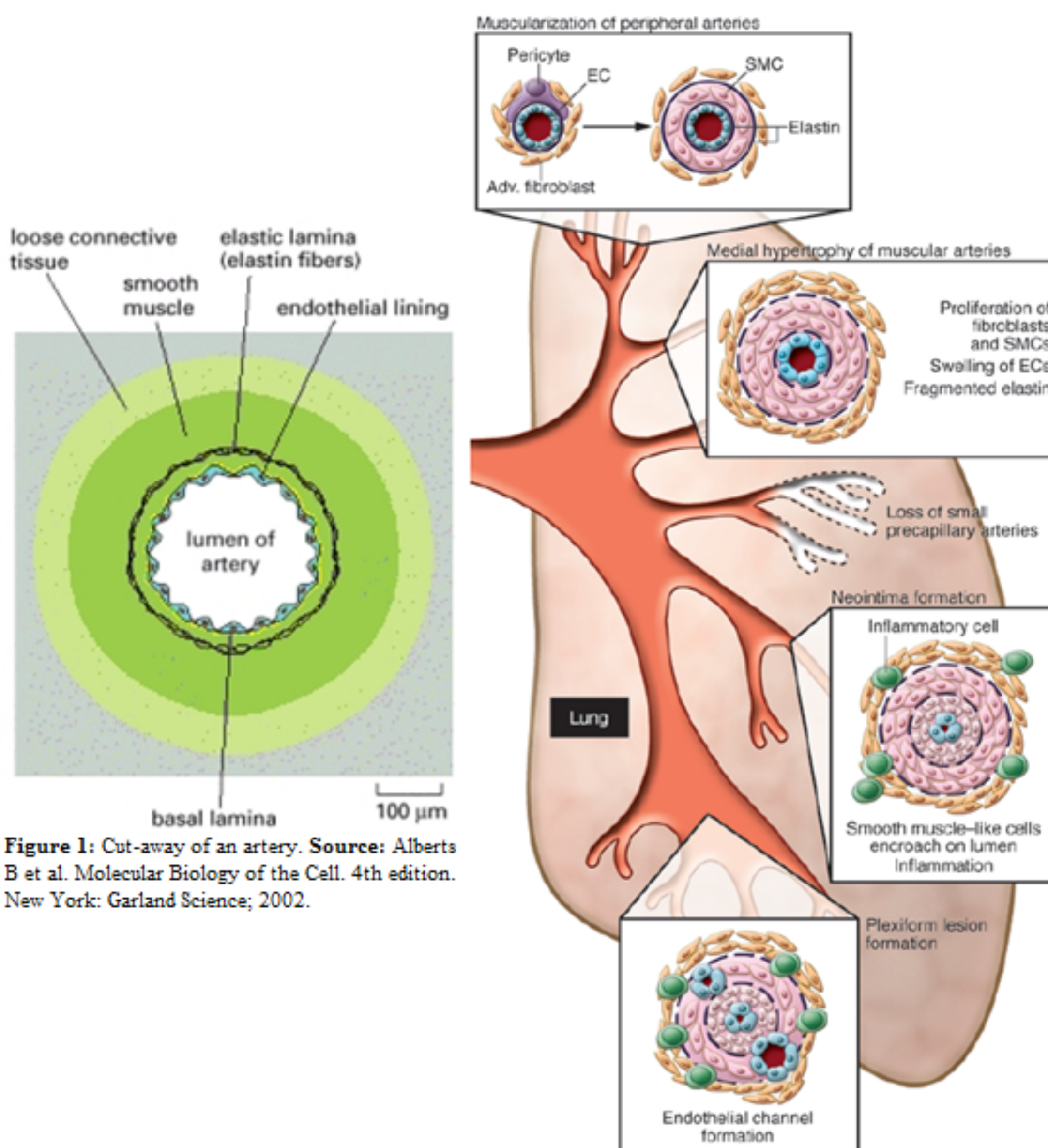

Figure 1: Cut-away of an artery. **Source:** Alberts B et al. Molecular Biology of the Cell. 4th edition. New York: Garland Science; 2002.

Figure 2: Pathophysiological features of PAH in the pulmonary vasculature. **Source:** Rabinovitch M. Molecular pathogenesis of pulmonary arterial hypertension. *J Clin Invest.* 2012;122(12):4306-13.

Endothelial cells (ECs) are a fundamental part of the pulmonary vasculature. ECs form the lining of all blood vessels (endothelium), covering vessel walls of connective tissue and smooth muscle cells (SMCs) (Figure 1); ECs control the extension and remodeling of blood vessel networks for tissue growth and repair [14]. Occlusive vascular remodeling, loss of vessels and subsequent fail of ECs to replenish them, and stiffening muscularization of the pulmonary arteries occur in part because of EC dysfunction in PAH [15]. The pulmonary artery endothelial cells (PAECs) in PAH patients stimulate and fail to suppress SMC proliferation via paracrine signaling, causing the vessel wall to thicken and muscularize [4]. Dysregulated EC and SMC proliferation obliterate the lumen of pulmonary arteries, and plexiform lesions form from aberrant, tumor-like clonal apoptosis-resistant EC proliferation [1, 4, 16-17]. There is a known genetic component in PAH, with ~80% of familial PAH and 20% of IPAH patients having a germline mutation that causes loss of bone morphogenetic protein receptor type II (BMPR2) function [1-2, 4, 11, 17-21], which is an important regulatory protein of growth and differentiation with impairment linked to vessel loss [18]. Even PAH patients without a mutation display reduced BMPR2 expression [15, 18]. Many of these advances in the understanding of the molecular and cellular basis of PAH have occurred only recently, and these paradigms remain to be thoroughly investigated and understood.

Induced pluripotent stem cells (iPSCs) are reprogrammed somatic cells with the same capability as embryonic stem cells (ESCs) to indefinitely self-renew and to differentiate into any of the three germ layers that give rise to every cell type in the adult body [22-25]. Methods to differentiate endothelial cells from iPSCs (iPSC-ECs) consistently and accurately have been well established [26-30]. iPSCs differentiated into relevant cell types have been shown to recapitulate pathological features of diseases with genetic bases *in vitro*, providing a renewable source of cells that can provide new insights into a disease [23-24, 31]; use of iPSCs from patients to screen novel drug candidates *in vitro* stand to improve the drug development process by saving time and money [32]. iPSCs are patient-specific, meaning that they could be genetically corrected and then transplanted back into the same patient without risk of immune rejection; patient-specific iPSCs can also be used to personalize medicine, screening potential drug compounds for efficacy and safety based on individual-level differences in a patient's pharmacodynamic profile [32].

Native pulmonary artery ECs (PAECs) from IPAH patients have been shown to have several impaired features *in vitro* that could be tested in iPSC-ECs to evaluate novel potential as disease models – a variety of cell types differentiated from iPSCs have been shown to recapitulate disease models, but no precedent exists in literature using endothelial cells. iPSC-EC disease models could impact current understanding and future treatment of a range of cardiopulmonary diseases, including PAH, atherosclerosis, scleroderma, bronchopulmonary dysplasia, Kawasaki disease, and chronic obstructive pulmonary disease [33-37].

Therefore, we set out to evaluate the utility of iPSC-ECs as a model for PAH. We hypothesized that iPSC-ECs derived from PAH patients will recapitulate a disease phenotype of native PAH patient EC dysfunction, and evaluated our hypothesis through the following aims:

1. Characterization of endothelial cells differentiated from iPSCs as having a native endothelial cell phenotype.
    I. Evaluated morphology for endothelial cell phenotype, typified as long and tube-like with a cobblestone appearance.
    II. Functional assay for low density lipoprotein (LDL) uptake, which is an attribute of endothelial cells and macrophages [38].
    III. Immunostaining for vWF antigen and VE-cadherin/CD144 cell surface marker expression. Von Willebrand Factor (vWF) is produced constitutively in the endothelium [39-40] and VE-cadherin/CD144 is a strict EC-specific surface molecule [41].
2. Evaluation of the extent to which iPSC-ECs derived from IPAH patients recapitulated impaired features demonstrated in native PAECs from IPAH patients.
    I. Endothelial cells will spontaneously form hollow capillary-like tube networks on certain cell matrices, in a process known as angiogenesis [10]. Native IPAH PAECs have been established to have significantly impaired angiogenic capability on Matrigel [11]. The extent to which iPSC-ECs from IPAH patients formed tube networks on Matrigel in the

angiogenesis assay was evaluated versus control iPSC-ECs derived from unused lung donors.

II. Unpublished work in our lab has established that native PAECs from IPAH patients have significantly more unrepaired DNA damage versus controls, as analyzed through the single cell gel electrophoresis (comet) assay which visualizes DNA with single or double-stranded breaks that migrate out of the nucleoid, forming a "comet tail" [42-43]. Unrepaired DNA damage causes mutations that can lead to disease [44]. The amount of unrepaired DNA damage in iPSC-ECs from IPAH patients was evaluated versus control iPSC-ECs derived from unused lung donors.

MATERIALS AND METHODS

Lung tissue collection and skin biopsies from IPAH patients and non-PAH unused lung donors. Lung tissue was collected from explanted lungs from IPAH patients enrolled through the Cardiovascular Medical Education and Research Fund's Pulmonary Hypertension Breakthrough Initiative (PHBI), which is a nation-wide network for pulmonary hypertension study. Lung tissue was also collected from non-PAH unused lung donors, whose lungs are not used for transplantation but are otherwise healthy. Skin biopsies were collected at the same time as lung tissue collection.

Cell isolation from lung tissue and skin biopsy. PAECs were isolated from dissected and digested distal pulmonary arteries (PAs) from the lung tissue collection. Fibroblasts were isolated from skin biopsy [45].

Generation and cell culture of iPSCs. iPSCs were obtained from a partner lab, which were derived from the skin fibroblasts and reprogrammed through lentiviral transduction of the four pluripotency transcription factors *Oct-4*, *Sox-2*, *Klf-4*, and *c-Myc* as previously described [23].

iPSCs were cultured under chemically defined feeder-free conditions in Essential 8 medium (Life Technologies) on Matrigel-coated 6-well plates (BD Biosciences) and subcultured at a 1:3 ratio using Accutase (Sigma) as previously described [46-47]. Rho kinase (ROCK) activity was inhibited to improve recovery and growth from cryopreservation and passage as previously described [48].

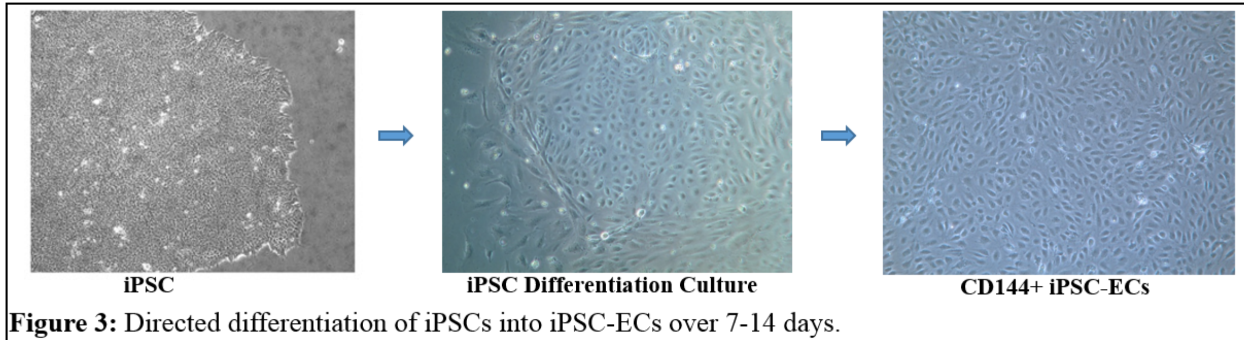

Figure 3: Directed differentiation of iPSCs into iPSC-ECs over 7-14 days.

Directed differentiation of iPSC-ECs. At 90% confluence, iPSC medium was changed to differentiation medium consisting of RPMI+B27 supplement (Life Technologies) with 6 μM CHIR (R&D Systems). CHIR is a small-molecule that inhibits glycogen synthase kinase-3β, and has been shown to improve the efficiency of directed differentiation [49]. On the third day, and every two days following, medium was changed to freshly made differentiation medium consisting of RPMI+B27 with 25 ng/mL vascular endothelial growth factor (VEGF) (R&D Systems) and 10 ng/mL basic fibroblast growth factor (FBF-β) (R&D Systems) following precedent [26-30]. When good endothelial cell morphology could be observed, characterized by "cobble-stone" cells sprouting from iPSC colonies (Figure 3) [26-30], iPSC-ECs were sorted using magnetic-activated cell sorting (MACS) (Miltenyi Biotec) for CD144 expression. Sorted cells were seeded on gelatin-coated 6-well plates (Sigma, BD Biosciences) at 400,000 cells/well. Additional MACS sorting was performed if necessary to purify the population of iPSC-ECs.

Cell culture of iPSC-ECs and PAECs. Human PAECs were cultured in 5% FBS ECM media (Life Technologies) in gelatin or collagen IV-coated 6-well plates (BD Biosciences). PAECs were subcultured at a 1:2 ratio**.** iPSC-ECs were grown in 5% FBS EGM-2MV medium (Lonza) on collagen IV-coated 6-well plates (BD Biosciences). iPSC-ECs were subcultured using Accutase (Sigma) at a 1:2 ratio.

Characterization of iPSC-ECs. iPSC-ECs were characterized through low density lipoprotein (LDL) uptake and immunostaining for von Willebrand Factor (vWF) and VE-cadherin/CD144 using native PAECs as a positive control. PAECs and iPSC-ECs were incubated for 45 minutes at 37°C with 1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate (dil, a fluorescent dye)-conjugated

acetylated LDL (Dil-Ac-LDL) (Invitrogen) at a 1:200 dilution in 5% FBS ECM (Life Technologies). After washing twice with phosphate-buffered saline (PBS), LDL uptake was visualized with fluorescent microscopy as previously described at 200x magnification [26]. For immunostaining, iPSC-ECs were fixed with 2% paraformaldehyde (Sigma) in PBS for 10 minutes at room temperature and permeated [50] in 0.2% Triton 100X in PBS for 2 minutes. Non-specific binding was blocked for 20 minutes in 5% bovine serum albumin (BSA) (Sigma). Cells were then incubated overnight at 4°C in the dark with primary antibodies anti-vWF (Abcam, ab6994) and anti-VE Cadherin/CD144 (Abcam, ab33168). The cells were then incubated for 2 hours with Alexa 488-conjugated donkey anti-rabbit secondary antibody (Invitrogen) and counterstained with 4,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories). Pictures were taken at 200x magnification.

Angiogenesis (tube formation) assay. iPSC-ECs were starved overnight in 0.2% FBS ECM medium (Life Technologies) as previously described [11]. iPSC-ECs were seeded at 20,000 cells/well in a Matrigel-coated 24-well plate (BD Biosciences). After incubation for four hours at 37°C, 9 pictures were taken at 100x magnification. EC tube networks were manually quantified by tube length and number using ImageJ (National Institutes of Health). Tube length was measured by length in pixels between two nodes. Angiogenesis assays were performed by mentor, and student completed a round of quantification.

Single cell gel electrophoresis (comet assay). Confluent iPSC-ECs and PAECs were suspended at 300,000 cells/mL in PBS. 50 µL cell solution was mixed thoroughly in 500 µL molten LMagarose (Trevigen). 50 µL iPSC-EC or PAEC agarose solution was then dispensed on each of two wells on glass comet assay microscope slides (Trevigen), with 1 cell line per glass slide. The agarose-cell solution was allowed to solidify at 4°C in the dark for 1 hour, then immersed in cell lysis solution (Trevigen) at 4°C overnight in the dark (approx. 12 hours). Alkaline rinse and electrophoresis solutions were prepared, consisting of NaOH pellets (Mallinckrodt), ethylenediaminetetraacetic acid (EDTA) (Trevigen, Ambion), and deionized water (dH_2O) as previously established [44]. Slides were immersed in alkaline rinse solution for 1 hour at room temperature in the dark. Gel electrophoresis was then run at constant 30 volts and 0.300 amperes for 30 minutes in alkaline electrophoresis solution at 4°C in the dark. Slides were washed

in dH_2O and 70% ethanol (Decon Laboratories, Inc.) and then dried at 45° C. Slides were then stained with SYBR Green (Invitrogen) diluted at 1 μL/10 mL EDTA at pH 8, and allowed to dry at room temperature. Alkaline conditions were used to visualize both single- and double-stranded breaks [42-43]. 20-40 microscope pictures per cell line were taken under fluorescent light at 200x magnification the same day using an upright microscope. Pictures were converted to monochrome to minimize background noise and optimize detection of fluorescent intensity. 150 comets per cell line were then quantified using ImageJ (National Institutes of Health), using a "Comet Assay" plugin (University of North Carolina at Chapel Hill) [51]. The plugin quantifies tail length by averaging all of the x and y coordinates in the comet head to define a "centroid," and defining a "center of mass" of the tail by taking the average of all x and y coordinates weighted for brightness. The Pythagorean distance between two points, the centroid of the comet head to the center of mass of the tail, defines tail length. Quantification was performed blind to the status of a cell line's status as patient or control to remove bias.

Statistical analysis. The number of samples studied per experiment is indicated in the figure legends. Values from multiple experiments are expressed as mean±SEM. Statistical significance was determined using an unpaired t-test with a 95% confidence interval, with a P value <0.05 regarded as statistically significant.

RESULTS

Endothelial cells differentiated from IPAH iPSCs exhibit a native endothelial cell phenotype. ECs differentiated from iPSCs and purified for $CD144^+$ expression displayed indistinguishable morphology versus native PAECs, were shown to uptake LDL, and stained positive for von Willebrand Factor and VE-cadherin/CD144 (Figure 4, 5).

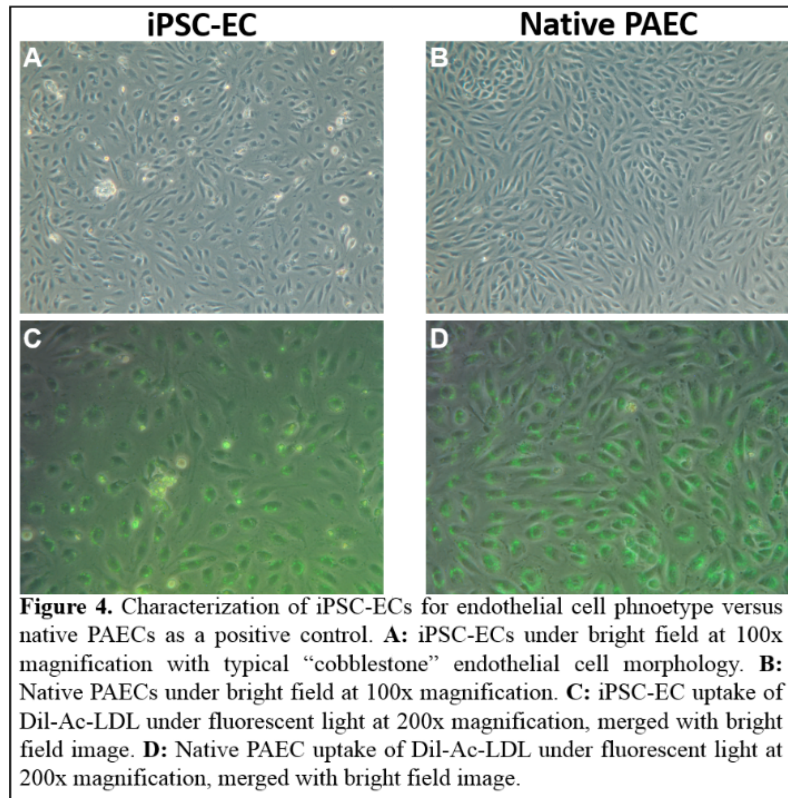

Figure 4. Characterization of iPSC-ECs for endothelial cell phnoetype versus native PAECs as a positive control. **A:** iPSC-ECs under bright field at 100x magnification with typical "cobblestone" endothelial cell morphology. **B:** Native PAECs under bright field at 100x magnification. **C:** iPSC-EC uptake of Dil-Ac-LDL under fluorescent light at 200x magnification, merged with bright field image. **D:** Native PAEC uptake of Dil-Ac-LDL under fluorescent light at 200x magnification, merged with bright field image.

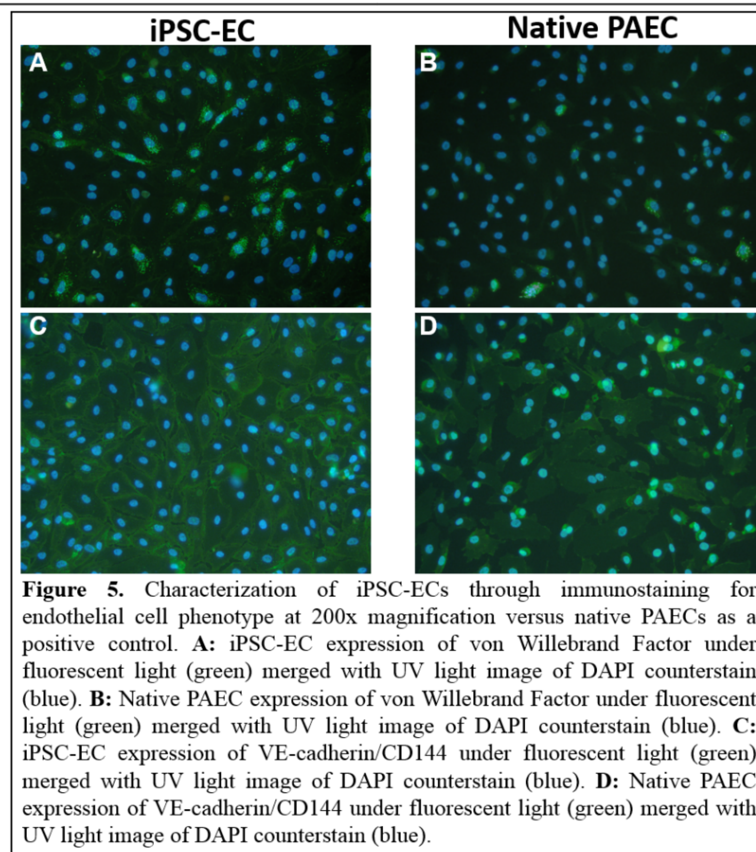

Figure 5. Characterization of iPSC-ECs through immunostaining for endothelial cell phenotype at 200x magnification versus native PAECs as a positive control. **A:** iPSC-EC expression of von Willebrand Factor under fluorescent light (green) merged with UV light image of DAPI counterstain (blue). **B:** Native PAEC expression of von Willebrand Factor under fluorescent light (green) merged with UV light image of DAPI counterstain (blue). **C:** iPSC-EC expression of VE-cadherin/CD144 under fluorescent light (green) merged with UV light image of DAPI counterstain (blue). **D:** Native PAEC expression of VE-cadherin/CD144 under fluorescent light (green) merged with UV light image of DAPI counterstain (blue).

IPAH iPSC-ECs recapitulate significantly impaired angiogenesis. A defining characteristic of healthy ECs is spontaneous angiogenesis, the *in vitro* formation of elaborate, interconnected hollow capillary-like tube structures which is related to ECs' fundamental function as an adaptable blood-supply life support system [10]. It has been established that native PAECs from PAH patients have a significant angiogenic defect on Matrigel, which is related to the progressive loss of small vessel and impaired EC-mediated regeneration of those vessels [14].

To assess whether this defect would be recapitulated in iPSC-ECs, 5 control iPSC-EC lines derived from the unused lung donors versus 5 iPSC-EC cell lines from IPAH patients were statistically analyzed with an unpaired t-test. The mean tube length and number of 5 IPAH patient iPSC-ECs were significantly reduced (P value<0.05) versus the mean tube length and number of 5 control iPSC-EC lines from unused lung donors (Figure 6A, 6B), strikingly failing to form tube networks on Matrigel (Figure 6C, 6D). These results confirm that iPSC-ECs from IPAH patients recapitulate an angiogenic defect.

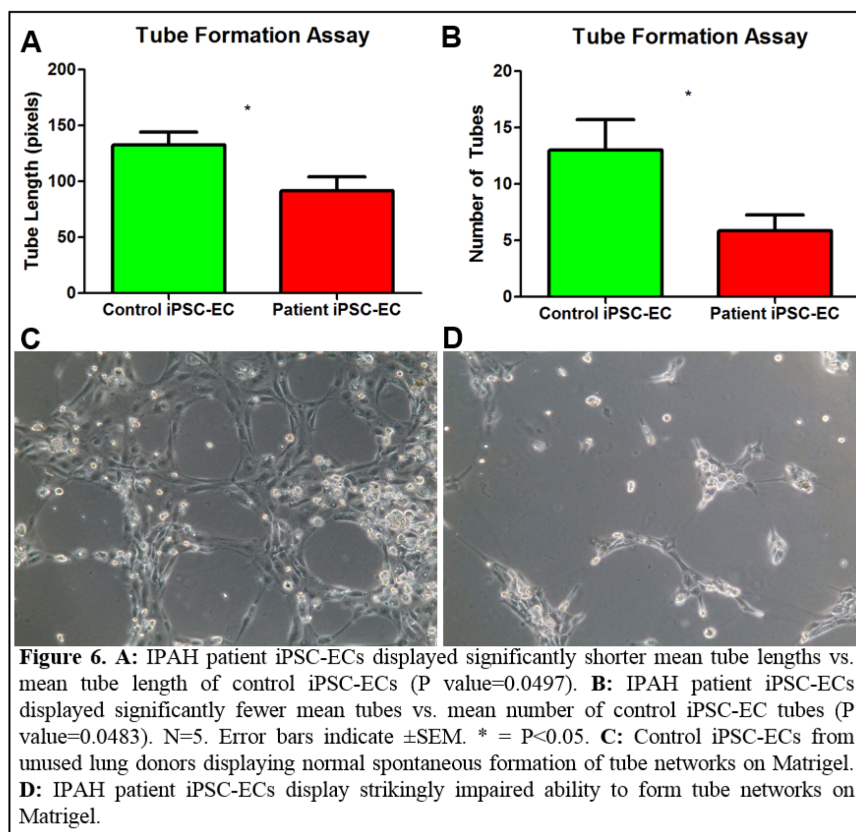

Figure 6. A: IPAH patient iPSC-ECs displayed significantly shorter mean tube lengths vs. mean tube length of control iPSC-ECs (P value=0.0497). **B:** IPAH patient iPSC-ECs displayed significantly fewer mean tubes vs. mean number of control iPSC-EC tubes (P value=0.0483). N=5. Error bars indicate ±SEM. * = P<0.05. **C:** Control iPSC-ECs from unused lung donors displaying normal spontaneous formation of tube networks on Matrigel. **D:** IPAH patient iPSC-ECs display strikingly impaired ability to form tube networks on Matrigel.

IPAH iPSC-ECs have higher levels of unrepaired DNA damage. Significantly higher amounts of unrepaired DNA damage were established in native PAECs from IPAH patients versus PAECs from unused donors by our lab (unpublished data). The amount of unrepaired DNA damage was assessed through the single cell gel electrophoresis (comet) assay by statistically analyzing the mean comet tail length of 5 lines of iPSC-ECs from IPAH patients with an unpaired t-test versus the mean comet tail length of 5 lines of iPSC-ECs from unused lung donors, finding non-significant (P value>0.05) but higher levels of unrepaired DNA as visualized by longer mean comet tail lengths in IPAH iPSC-ECs (Figure 7). Unrepaired DNA damage was particularly striking in two IPAH patients, patients 3 and 5 (Figure 7B).

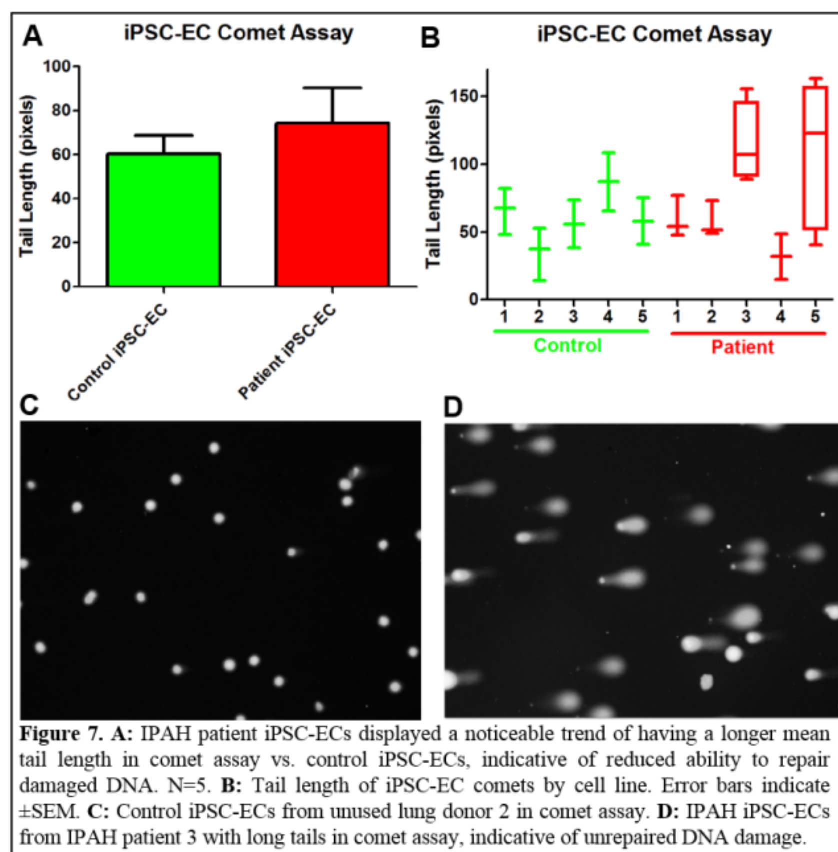

Figure 7. A: IPAH patient iPSC-ECs displayed a noticeable trend of having a longer mean tail length in comet assay vs. control iPSC-ECs, indicative of reduced ability to repair damaged DNA. N=5. **B:** Tail length of iPSC-EC comets by cell line. Error bars indicate ±SEM. **C:** Control iPSC-ECs from unused lung donor 2 in comet assay. **D:** IPAH iPSC-ECs from IPAH patient 3 with long tails in comet assay, indicative of unrepaired DNA damage.

We subsequently sought to investigate these unexpected results, conducting head-to-head experiments using native PAEC cell lines derived from the same individuals as the cell lines used in our iPSC-EC comet assay (Figure 8B). Native PAECs from IPAH patients had significantly higher levels of unrepaired DNA damage (Figure 8A) as analyzed with an unpaired t-test (P value<0.05), and visualized

through longer mean comet tails versus control native PAECs from unused lung donors (Figure 8C, 8D). Further analysis of unrepaired DNA damage by individual showed overall higher damage in iPSC-ECs, but variations in damage in iPSC-ECs versus native PAECs. By conducting head-to-head comparisons, it was observed that iPSC-ECs did not fully recapitulate a phenotype of significant DNA damage versus control iPSC-ECs, but it is evident that iPSC-ECs from IPAH patients had higher levels of damage.

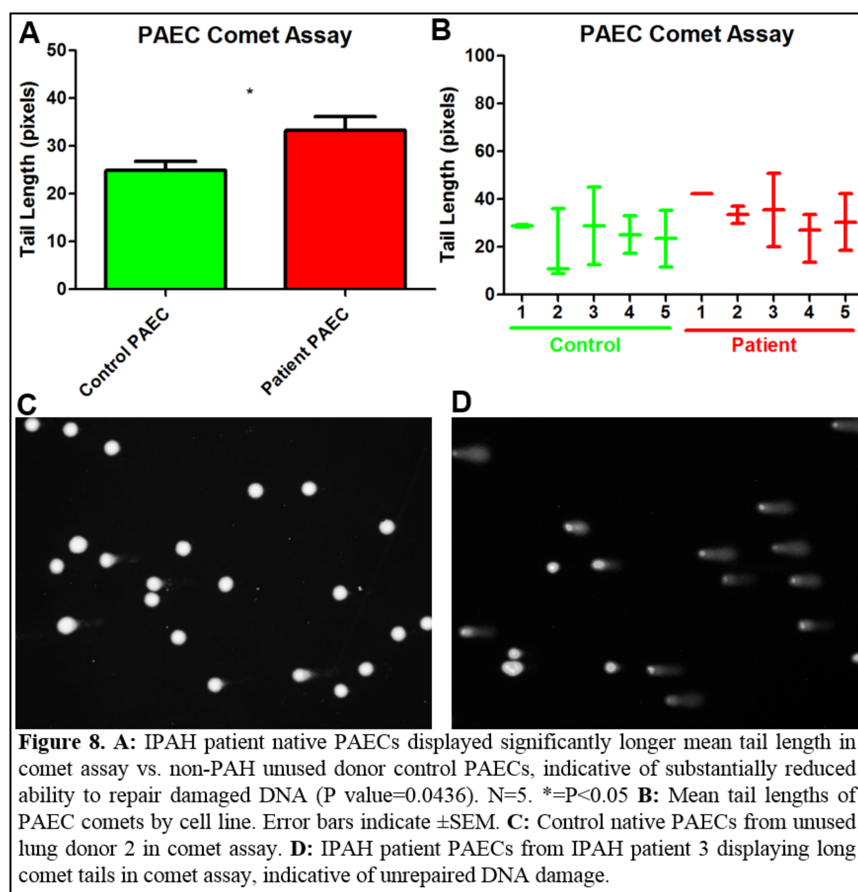

Figure 8. A: IPAH patient native PAECs displayed significantly longer mean tail length in comet assay vs. non-PAH unused donor control PAECs, indicative of substantially reduced ability to repair damaged DNA (P value=0.0436). N=5. *=P<0.05 **B:** Mean tail lengths of PAEC comets by cell line. Error bars indicate ±SEM. **C:** Control native PAECs from unused lung donor 2 in comet assay. **D:** IPAH patient PAECs from IPAH patient 3 displaying long comet tails in comet assay, indicative of unrepaired DNA damage.

Discussions, Conclusions, and Future Work

This study represents the first to study the utility of the endothelial cell type differentiated from iPSCs for disease modeling. Although many other cell types have been studied for recapitulation of disease phenotypes, no examples can be found in current literature studying iPSC-ECs. There is a demonstrated need for novel treatments for PAH, as demonstrated by persisting grave prognosis and high mortality [10], and severe impairment in the ability of patients to lead normal lifestyles [52]. Live PAECs are difficult to obtain, usually only following lung transplantation, compared to the relative ease with which

iPSCs can be reprogrammed from easily accessible somatic cells to form a self-renewing source of cells for study and for screening of novel drug candidates. Personalized medicine facilitated by patient-specific iPSCs stands as a significant way to improve treatment, especially in a disease like PAH that can have different underlying cellular and molecular mechanisms [2, 4, 17], and requires patient-specific individualized therapies [11-12, 52]. If iPSC-ECs can successfully model PAH, they can be applied towards a variety of cardiopulmonary diseases in which EC dysfunction plays a role [33-37].

Our results confirm that iPSC-ECs are promising disease models, as evidenced by recapitulation of significantly impaired angiogenic potential. In general, the higher levels of unrepaired DNA damage observed in iPSC-ECs may make it difficult to tease out a disease phenotype as established in native PAECs [11]. The genetic manipulation associated with the reprogramming process to derive iPSCs [23-24] may account for this overall higher level of DNA damage versus native PAECs, and the non-significant differences in tail length we observed. The implications of this higher amount of DNA damage is unknown, and ongoing work in our lab substituting lentiviral reprogramming for the non-integrating Sendai virus or other forms of reprogramming may improve the health of these iPSCs [53]. This potential for iPSC-ECs to be effective disease models opens possibilities in the drug development process and for personalized treatment for patients.

Investigation of additional assays that differentiate IPAH patients and controls in native PAECs may allow us to find more phenotypic similarities in iPSC-ECs. We have begun testing new therapies for PAH using iPSC-ECs known to improve EC angiogenesis, and plan to relate these findings to patients' responses to therapy. We expect that improved angiogenic ability in iPSC-ECs will be related to regeneration of lost microvessels. Two of these therapies are novel PAH drug candidates elafin, an endogenous inhibitor of vascular elastase, and FK506, an immunosuppresive, both of which have been shown to improve BMPR2 signaling and reverse pulmonary hypertension in experimental animal models (unpublished data from our lab) [15, 54].

Other assays that will be tested in IPAH vs. control iPSC-ECs include adhesion to laminin, a feature that is impaired in IPAH native PAECs [11], down-regulation of mRNA expression of genes

collagen IV and ephrin 1 (unpublished data from our lab), decreased signaling of bone morphogenetic proteins 4 and 9 (unpublished data from our lab), mitochondrial abnormalities [55], and elevated expression of cytokines at the protein level [56]. We are currently investigating if iPSC-ECs recapitulate these abnormalities found in native PAECs.

Patient-specific treatment for diseases like PAH using iPSCs stand to make cell therapy and personalized medicine a reality. Ongoing work in our lab concerns the correction of genes in iPSC-ECs from IPAH patients with BMPR2 mutation, using the highly-specific genomic editing system CRISPR/Cas9 [57]. Transplanted patient-specific iPSC-ECs would not trigger an immune response, representing an autologous transplantation [32]; improved clinically compliant iPSCs culture-conditions ensure the clinical feasibility of iPSC cell therapy [58]. An emerging but exciting observation that cells can form tunneling nanotubes to transport mitochondria and rescue damaged cells could be another possible application of iPSC-EC cell therapy [59]. Endothelial progenitor cells have been shown to rescue premature senescence in ECs [60].

In conclusion, this project represents an important part of an ongoing, novel study investigating the utility of the iPSC-EC cell type for disease modeling. iPSC-ECs expressed an endothelial cell phenotype and recapitulated known impaired features in IPAH ECs. The full clinical potential of iPSCs stands to revolutionize medicine [25], and our study represents the application of that potential in cardiopulmonary disease, breaking new ground through evaluation of the iPSC-EC as a disease model using PAH as a prototype disease.

ACKNOWLEDGEMENTS

Thank you to my mentors, my lab's administrative staff, and my labmates for their extraordinary kindness, patience, and generosity in hosting me as a student in their lab; to the Pulmonary Hypertension Breakthrough Initiative, through which we received lung tissue from selfless control and patient donors.

REFERENCES

1. McLaughlin VV, McGoon MD. Pulmonary arterial hypertension. *Circulation*. 2006; 114(13):1417-1431. [PubMed: 17000921]
2. Farber HW, Loscalzo J. Pulmonary arterial hypertension. *N Engl J Med*. 2004;351(16):1655-1665. [PubMed: 15483284]
3. Kovacs G, Berghold A, Scheidl S, Olschewski H. Pulmonary arterial pressure during rest and exercise in healthy subjects: a systematic review. *Eur Respir J*. 2009;34(4):888-894. [PubMed: 19324955]
4. Rabinovitch M. Molecular pathogenesis of pulmonary arterial hypertension. *J Clin Invest*. 2012;122(12):4306-13. [PubMed: 23202738]
5. Rich S, Dantzker DR, Ayres SM, Bergofsky EH, Brundage BH, Detre KM, Fishman AP, Goldring RM, Groves BM, Koerner SK. Primary pulmonary hypertension. A national prospective study. *Ann Intern Med*. 1987 Aug; 107(2):216-223. [PubMed: 3605900]
6. D'Alonzo GE, Barst RJ, Ayres SM, Bergofsky EH, Brundage BH, Detre KM, Fishman AP, Goldring RM, Groves BM, Kernis JT, et al. Survival in patients with primary pulmonary hypertension: results from a national prospective registry. *Ann Intern Med.* 1991;115:343–349. [PubMed: 1863023]
7. Hyduk A, Croft JB, Ayala C, Zheng K, Zheng ZJ, Mensah GA. Pulmonary hypertension surveillance—United States, 1980-2002. *MMWR Surveill Summ*. 2005 Nov;54(5):1-28. [PubMed: 16280974]
8. Badesch DB, Raskob GE, Elliott CG, Krichman AM, Farber HW, Frost AE, Benza RL, Liou TG, Turner M, Giles S, Feldkircher K, Miller DP, McGoon MD. Pulmonary arterial hypertension: baseline characteristics from the REVEAL registry. *Chest*. 2010 Feb;137(2):376-387. [PubMed: 19837821]
9. George MG, Schieb LJ, Ayala C, Talwalker A, Levant S. Pulmonary hypertension surveillance: United States, 2001 to 2010. *Chest*. 2014 Aug;146(2):476-495. [PubMed: 24700091]
10. Newman JH, Fanburg BL, Archer SL, Badesch DB, Barst RJ, Garcia JGN et al. Pulmonary Arterial Hypertension—Future Directions—Report of a National Heart, Lung, and Blood Institute/Office of Rare Diseases Workshop. *Circulation*. 2004; 109:2947–2952. [PubMed:15210611]
11. de Jesus Perez VA, Yuan K, Orcholski ME, Sawada H, Zhao M, Li CG, Tojais NF, Nickel N, Rajagopalan V, Spiekerkoetter E, Wang L, Dutta R, Bernstein D, Rabinovitch M. Loss of adenomatous poliposis coli-α3 integrin interaction promotes endothelial apoptosis in mice and humans. *Circ Res*. 2012 Dec;111(12):1551-1564. [PubMed: 23011394]
12. Stacher E, Graham BB, Hunt JM, Gandjeva A, Groshong SD, McLaughlin VV, Jessup M, Grizzle WE, Aldred MA, Cool CD, Tuder RM. Modern age pathology of pulmonary arterial hypertension. *Am J Respir Crit Care Med*. 2012 Aug;186(3):261-272. [PubMed: 22679007]
13. George MP, Champion HC, Pilewski JM. Lung transplantation for pulmonary hypertension. *Pulm Circ*. 2011 Apr-Jun;1(2):182-191. [PubMed: 22034605]
14. Alberts B, Johnson A, Lewis J et al. Molecular Biology of the Cell. 4th edition. New York: Garland Science; 2002. ISBN-10: 0-8153-3218-1 [NCBI Books: NBK21054]
15. Spiekerkoetter E et al. FK506 activates BMPR2, rescues endothelial dysfunction, and reverses pulmonary hypertension. *J Clin Invest*. 2013;123(8):3600-13. [PubMed: 23867624]
16. Jonigk D et al. Plexiform lesions in pulmonary arterial hypertension. *Am J Pathol*. 2011 Jul;179(1):167-179. [PubMed: 21703400]
17. Archer SL, Weir EK, Wilkins MR. Basic science of pulmonary arterial hypertension for clinicians: new concepts and experimental therapies. *Circulation*. 2010;121:2045-2066. [PubMed: 20458021]
18. Atkinson C, Stewart S, Upton PD, Machado R, Thomson JR, Trembath RC, Morell NW. Primary pulmonary hypertension is associated with reduced pulmonary vascular expression of type II bone morphogenetic protein receptor. *Circulation*. 2002 Apr;105(14):1672-1678. [PubMed: 11940546]
19. Lane KB et al. Heterozygous germline mutations in BMPR2, encoding a TGF-beta receptor, cause familial primary pulmonary hypertension. *Nat Genet*. 2000 Sep;26(1):81-84. [PubMed: 10973254]
20. Deng Z et al. Familial primary pulmonary hypertension (gene PPH1) is caused by mutations in the bone morphogenetic protein receptor-II gene. *Am J Hum Genet*. 2000 Sep;67(3):737-744. [PubMed: 10903931]
21. Aldred MA et al. Somatic chromosome abnormalities in the lungs of patients with pulmonary arterial hypertension. *Am J Respir Crit Care Med*. 2010 Nov;182(9):1153-1160. [PubMed: 20581168]
22. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell*. 2006 Aug;126(4):663-676. [PubMed: 16904174]
23. Takahashi K et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell*. 2007 Nov;131(5):861-872. [PubMed: 18035408]
24. Yu J et al. Induced pluripotent stem cells derived from human somatic cells. *Science*. 2007 Dec;318(5858):1917-1920. [PubMed: 18029452]
25. Yamanaka S. Induced pluripotent stem cells: past, present, and future. *Cell Stem Cell*. 2012 Jun;10(6):678-684. [PubMed: 22704507]
26. Li Z, Hu S, Ghosh Z, Han Z, Wu JC. Functional characterization and expression profiling of human induced pluripotent stem cell- and embryonic stem cell-derived endothelial cells. *Stem Cells Dev*. 2011;20(10):1701-1710. [PubMed: 21235328]
27. Rufaihah AJ, Huang NF, Kim J, Herold J, Volz KS, Park TS, Lee JC, Zambidis ET, Rejio-Pera R, Cooke JP. Human induced pluripotent stem cell-derived endothelial cells exhibit functional heterogeneity. *Am J Transl Res*. 2013;5(1):21-35. [PubMed: 23390563]
28. Adams WJ, Zhang Y, Cloutier J, Kuchimanchi P, Newton G, Sehrawat S, Aird WC, Mayadas TN, Luscinskas FW, Garcia-Cardeña G. Functional vascular endothelium derived from human induced pluripotent stem cells. *Stem Cell Reports*. 2013 Jul;1(2):105-113. [PubMed: 24052946]
29. Li Z, Wilson KD, Smith B, Kraft DL, Jia F, Huang M, Xie X, Robbins RC, Gambhir SS, Weissman IL, Wu JC. Functional and transcriptional characterization of human embryonic stem cell-derived endothelial cells for treatment of myocardial infarction. *PLoS One*. 2009 Dec;4(12):e8443. [PubMed: 20046878]
30. Li Z, Wu JC, Sheikh AY, Kraft D, Cao F, Xie X, Patel M, Gambhir SS, Robbins RC, Cooke JP, Wu JC. Differentiation, survival, and function of embryonic stem cell derived endothelial cells for ischemic heart disease. *Circulation*. 2007 Sep;116(11):I46-I54. [PubMed: 17846325]
31. Wu SM, Hochedlinger K. Harnessing the potential of induced pluripotent stem cells for regenerative medicine. *Nat Cell Bio*. 2011;13(5):497-505. [PubMed: 21540845]

32. Inoue H, Yamanaka S. The use of induced pluripotent stem cells in drug development. *Clin Pharmacol Ther*. 2011;89(5):655-661. [PubMed: 21430656]
33. Libby P, Aikawa M, Jain MK. Vascular endothelium and atherosclerosis. *Handb Exp Pharmacol*. 2006;176:285-306. [PubMed: 16999230]
34. Fleming JN, Schwartz SM. The pathology of scleroderma vascular disease. *Rheum Dis Clin North Am*. 2008 Feb;34(1):41-55. [PubMed: 18329531]
35. Thébaud B, Abman SH. Bronchopulmonary dysplasia: where have all the vessels gone? Roles of angiogenic growth factors in chronic lung disease. *Am J Respir Crit Care Med*. 2007 May;175(10):978-985. [PubMed: 17272782]
36. Ghelani SJ, Singh S, Manojkumar R. Endothelial dysfunction in a cohort of North Indian children with Kawasaki disease without overt coronary artery involvement. *J Cardiol*. 2009 Apr;53(2):226-231. [PubMed: 19304127]
37. Walters EH, Reid D, Soltani A, Ward C. Angiogenesis: a potentially critical part of remodelling in chronic airway diseases? *Pharmacol Ther*. 2008 Apr;118(1):128-137. [PubMed: 18358536]
38. Voyta JC, Via DP, Butterfield CE, Zetter BR. Identification and isolation of endothelial cells based on their increased uptake of acetylated-low density lipoprotein. *J Cell Biol*. 1984 Dec;99(6):2034-2040. [PubMed: 6501412]
39. Sadler JE. Biochemistry and genetics of von Willebrand factor. *Annu Rev Biochem*. 1998;67:395-424. [PubMed: 9759493]
40. Belloni PN, Tressler RJ. Microvascular endothelial cell heterogeneity: interactions with leukocytes and tumor cells. *Cancer Metastasis Rev*. 1990 Feb;8(4):353-389. [PubMed: 2182212]
41. Vestweber D. VE-cadherin: the major endothelial adhesion molecule controlling cellular junctions and blood vessel formation. *Arterioscler Thromb Vasc Biol*. 2008 Feb;28(2):223-232. [PubMed: 18162609]
42. Olive PL, Banath JP. The comet assay: a method to measure DNA damage in individual cells. *Nat Protoc*. 2006;1(1):23-29. [PubMed: 174062028]
43. Collins AR. The comet assay for DNA damage and repair: principles, applications, and limitations. *Mol Biotechnol*. 2004 Mar;26(3):249-261. [PubMed: 15004294]
44. Clancey, S. DNA damage & repair: mechanisms for maintaining DNA integrity. *Nat Educ*. 2008;1(1):103.
45. Normand J, Karasek MA. A method for the isolation and serial propagation of keratinocytes, endothelial cells, and fibroblasts from a single punch biopsy of human skin. *In Vitro Cell Dev Biol Anim*. 1995 Jun;31(6):447-455. [PubMed: 8589888]
46. Chen G et al. Chemically defined conditions for human iPSC derivation and culture. *Nat Methods*. 2011 May;8(5):424-429. [PubMed: 21478862]
47. Stover AE, Schwartz PH. Adaptation of human pluripotent stem cells to feeder-free conditions in chemically defined medium with enzymatic single-cell passaging. *Methods Mol Biol*. 2011;767:137-146. [PubMed: 21822872]
48. Claassen DA, Desler MM, Rizzino A. ROCK inhibition enhances the recovery and growth of cryopreserved human embryonic stem cells and human induced pluripotent stem cells. *Mol Reprod Dev*. 2009 Aug;76(8):722-732. [PubMed: 19235204]
49. Lam AQ, Freedman BS, Morizane R, Lerou PH, Valerius MT, Bonventre JV. Rapid and efficient differentiation of human pluripotent stem cells into intermediate mesoderm that forms tubules expressing kidney proximal tubular markers. *J Am Soc Nephrol*. 2014 Jun;25(6):1211-1225. [PubMed: 24357672]
50. Jamur MC, Oliver C. Permeabilization of cell membranes. *Methods Mol Biol*. 2010;588:63-66. [PubMed: 20012820]
51. http://www.med.unc.edu/microscopy/resources/imagej-plugins-and-macros/comet-assay
52. NW Morrell, Archer SL, Evans S, Fiszman M, Martin T, Saulnier N, Rabinovitch M, Schermuly R, Stewart D, Truebel H, Walker G, Stenmark KR. Anticipated classes of new medications and molecular targets for pulmonary arterial hypertension. *Pulm Circ*. 2013 Jan;3(1):226-244. [PubMed: 23662201]
53. Fusaki N, ban H, Nishiyama A, Saeki K, Hasegawa M. Efficient induction of transgene-free human pluripotent stem cells using a vector based on Sendai virus, an RNA virus that does not integrate into the host genome. *Proc Jpn Acad Ser B Phys Biol Sci*. 2009;85(8):348-362. [PubMed: 19838014]
54. Shaw L, Wiedow O. Therapeutic potential of human elafin. *Biochem Soc Trans*. 2011 Oct;39(5):1450-1454. [PubMed: 21936832]
55. Bonnet S et al. An abnormal mitochondrial-hypoxia inducible factor-1alpha-Kv channel pathway disrupts oxygen sensing and triggers pulmonary arterial hypertension in fawn hooded rats: similarities to human pulmonary arterial hypertension. *Circulation*. 2006 Jun;113(22):2630-2641. [PubMed: 16735674]
56. Soon E et al. Elevated levels of inflammatory cytokines predict survival in idiopathic and familial pulmonary arterial hypertension. *Circulation*. 2010 Aug;122(9):920-927. [PubMed: 20713898]
57. Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science*. 2012 Aug;337(6096):816-821. [PubMed: 22745249]
58. Durruthy-Durruthy J et al. Rapid and efficient conversion of integration-free human induced pluripotent stem cells to GMP-grade culture conditions. *PLoS One*. 2014 Apr;9(4):e94231. [PubMed: 24718618]
59. Koyanagi M, Brandes RP, Haendeler J, Zeiher AM, Dimmeler S. *Circ Res*. 2005 May;96(10):1039-1041. [PubMed: 15879310]
60. Yasuda K, Khandare A, Burianovsky L, Maruyama S, Zhang F, Nasjletti A, Goligorsky MS. Tunneling nanotubes mediate rescue of prematurely senescent endothelial cells by endothelial progenitors: exchange of lysosome pool. *Aging*. 2011 Jun;3(6):597-608. [PubMed: 21705809]