Combining induced pluripotent stem cell with next generation sequencing technology to gain new insights into pathobiology and treatment of pulmonary arterial hypertension

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It was an honor to give the Giles F. Filley Lecture. Dr. Filley was cofounder of the Aspen Lung Conference and a master clinician and scientist with major contributions to the field of acid base balance. He developed “caribicarb,” a combination of calcium carbonate and sodium bicarbonate. He also contributed to our understanding of the pathophysiology of chronic obstructive pulmonary disease and emphysema through his many publications including one in which he described the elastic properties of the different lobes of the human lung.^[1]

This manuscript describes using induced pluripotent stem cells (iPSCs) combined with next generation sequencing to gain new information about the pathobiology of pulmonary arterial hypertension (PAH) and to discover ways in which future therapies for this disorder might be personalized. The studies we propose represent a team effort between our laboratory, that of Dr. Joseph Wu in the Department of Medicine at Stanford University who pioneered the derivation and characterization of endothelial cells from iPSCs,^[2] and that of Dr. Michael Snyder in the Department of Genetics, who developed next generation sequencing approaches for personalized medicine.^[3]

Our plan is to harvest pulmonary artery endothelial cells (PAECs) from idiopathic PAH (IPAH) patients and unused donor controls whose lungs come to explant through the Pulmonary Hypertension Breakthrough Initiative (PHBI) Network and to compare these cells to ECs differentiated from iPSCs derived either from fibroblasts obtained from the skin incision at the time of transplant or from the PAECs as the starting cell

type (Fig. 1). The skin biopsies were provided to us by the Network investigators under a separate IRB agreement independent of PHBI. We plan to compare the three cell lines to each other and in patients versus controls in terms of gene variants using a combination of exome and low coverage whole genome sequencing analysis^[3] and then we will assess methylation changes by Methyl-Seq^[4] and gene expression changes by RNA-Seq.^[5] Differences will then be related to function as assessed by angiogenesis and signaling using a novel high throughput methodology that uses isotope labeling of antibodies to phosphoproteins and cell surface antigens.^[6]

Our hypotheses are that ECs derived from fibroblast iPSCs will reveal functional abnormalities related to gene variants in IPAH patients and that ECs derived from PAEC-iPSCs will be informative of methylation changes that are maintained as memory from the tissue that is the site of the pathology. We predict that the native PAECs will show further methylation and gene expression changes that are reflective of end-stage disease.

The next phase of the study involves comparison of all three cell types from IPAH patients after correction of a gene variant using the zinc finger nuclease technology^[7] to determine the extent to which this restores normal gene expression as assessed by RNA-Seq and normal function, as reflected in normal properties of angiogenesis and signaling. We will also compare the three cell types in terms

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of their abilities to respond to novel therapies by correction of gene expression and function.

Signaling studies are carried out according to a new methodology that involves labeling antibodies to phosphorylated or other proteins with isotopes instead of fluorescent probes, because this allows mass spectrometry detection of numerous molecules, not just those limited by the fluorescence spectra utilized by conventional flow cytometry. Mass spectrometry can thus be used to interrogate changes in signaling molecules in response to different agonists in single cells and so heat maps can be established, or 2D plots of a surface molecule and a signaling molecule, or spade analysis of a hierarchy of cells. The fidelity of the analysis is exceptionally good as previously reported and compares favorably with fluorescence flow cytometry, but the capacity of assessing more molecules and better defining signaling networks related to cell type is much greater.^[6]

Our initial studies are focused on IPAH and on ECs, although we may incorporate APAH and differentiation of iPSCs to other vascular cell types in future studies. We chose to begin with ECs as the differentiated cells because of experience in differentiating these cells from iPSCs, but mostly because ECs from PAH patients have functional abnormalities associated with pulmonary vascular pathology. These include propensity to apoptosis that leads to loss of small distal microvessels^[8] as well as later apoptosis resistance in plexogenic lesions,^[9] aberrant activation of matrix elastase and other proteinases that cause release of paracrine growth factors that contribute to the exuberant proliferation of the underlying cells of the vessel wall that culminate in occlusion of the lumen.^[10] In addition, EC dysfunction promotes inflammation through production of chemokines such as IL-6.^[11] Moreover, PAECs from PAH patients do not form normal tubes in culture and are highly glycolytic.^[9] Pulmonary arterial endothelial cells from a normal patient form a well-established network of interconnecting tubes when grown on a collagen gel as compared to the poor formation of a tubular network using PAECs from IPAH patients^[9] (Fig. 2).

Reprogramming to iPSC involves transfection of the four transcription factors described by Yamanaka (Oct 4, Sox 2 KLF 4 and c-myc) either by lentivirus or by minicircle.^[12] The procedure takes up to four weeks. Pluripotency is assessed both by teratoma formation and by specific markers of pluripotency like NANOG. Differentiation of ECs is accomplished following embryoid body formation by a combination of activin, bone morphogenetic protein (BMP), and vascular endothelial growth factor (VEGF).^[2] The ECs are evident at the edge of the embryoid body and are then expanded and harvested either by FACS or by CD31 (PECAM) antibody-coated immunobeads or by a combination of

Figure 1: Schema of proposed harvest of pulmonary artery (PA) endothelial cells and skin fibroblasts, generation of induced pluripotent stem cells (iPSC), transformation of both into endothelial cells and subsequent studies.

IPAH PAECs show impaired angiogenesis

3D Collagen based Angiogenesis Assay

Figure 2: Representative frame of collagen gel assay shows that pulmonary arterial endothelial cells from representative idiopathic pulmonary arterial hypertension patient (IPAH PAECs) show impaired tubular network formation compared to PAECs from a donor control lung

sorting with CD144 (VE-cadherin) and CD31 antibodies. Expression profiling shows that embryonic stem cell-derived and fibroblast-derived ECs have a similar expression profile by microarray analysis when compared to human umbilical vein endothelial cells that is markedly in contrast to the fibroblast.^[2] Challenges in this field remain the propensity of the differentiated ECs to dedifferentiate or to senesce.

As we are accumulating sets of PAECs, fibroblasts, iPSC-ECs differentiated from fibroblasts, and endothelial cells, we have been carrying out RNA-Seq analyses on PAECs to determine which changes in gene expression might be

of interest when interrogating the iPSC-ECs. RNA-seq involved extraction of RNA conversion to cDNA and random fragmentation of DNA to prepare libraries with fragments ligated by specific adapters.^[5] The fragments are incubated on a flowcell that is covered by a lawn of primers that attach to the adapters ligated to the DNA fragments. Polymerase chain reaction (PCR) is used to replicate the fragments, and fluorescently labeled nucleotides are added in each cycle. Sequencing records the fluorescence at each cycle and then sequences are aligned to a reference so that SNPs deletions and insertions can be identified. Sequencing depth refers to the number of times a base or region has been sequenced. The depth of reads can then be related to the length of the sequence to give an assessment of expression level.^[5] We found that comparison of two runs gave a very similar distribution of genes expressed at high frequency and gene expressed at low frequency. Then we compared each RNA-Seq for genes that were expressed by a greater than one-fold increase or decrease in expression and were able to correlate our findings with quantitative reverse transcription (qRT)-PCR quite faithfully. Preliminary studies suggest that some of these genes are related to expression of bone morphogenetic protein receptor (BMPR2), the gene mutated in the overwhelming majority of hereditary PAH.

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