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What Endothelial Cells from Patient iPSCs Can Tell Us about Aortic Valve Disease

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In a recent issue of *Cell*, Theodoris et al. (2015) used a complex systems biology approach to model vascular aortic calcification caused by mutations in the NOTCH1 gene. Comparison of endothelial cells from patient hiPSCs and genetically matched controls under fluid flow revealed novel mechanisms underlying the disease.

Until recently, it was widely thought that human diseases were caused by alterations in DNA. However, largely as a result of cancer research, epigenetic changes, such as DNA methylation and histone modifications, are also widely appreciated as major contributing factors. Cardiovascular diseases are among the conditions now considered to have an epigenetic component, as recent "epidemics" associated with aging, stress, and lifestyle cannot be explained by genomic changes only. Recent studies on atherosclerosis or type 2 diabetes, for example, indicate that underlying cardiovascular risk can develop in utero through epigenetic mechanisms (DeRuiter et al., 2008; Johnstone and Baylin, 2010). Nevertheless, studying mechanisms underlying transcriptional and epigenetic changes associated with cardiovascular disease remains difficult despite its high incidence because affected cardiomyocytes and endothelial cells (ECs) can rarely be obtained in the large numbers required for laboratory experiments. At most, small biopsies are available from patients.

Srivastava's group now elegantly demonstrates the value of human induced pluripotent stem cells (hiPSCs) in addressing this challenge. They generated a genetic model of calcified aortic valve disease (CAVD) based on ECs from patient hiPSCs with NOTCH1 mutations (Figure 1) (Theodoris et al., 2015). Aortic valve calcification is a leading cause of heart disease. A major risk factor is "bicuspid aortic valve," where two "leaflets" form during development rather than the usual three. Leaflets are made of fibrous tissue lined with ECs; they ensure that blood flows from the left ventricle of the heart to the aorta. Currently, the only treatment for CAVD is valve replacement because calcification cannot be reversed, partly because the underlying mechanisms are poorly understood. Disturbed blood flow through the aortic valve is the major cause of later calcification (Weinberg et al., 2010). The leaflets on the ventricular side of the valve experience a spike of shear force when blood enters the artery from the left ventricle. The aortic side of the valve, by contrast, undergoes much less shear and is more prone to calcification. Srivastava's group showed previously that mutations in the NOTCH1 gene are associated with familial and sporadic bicuspid and calcified aortic valves (Garg et al., 2005). Since NOTCH signaling is higher on the ventricular side, it is considered protective for calcification (Wirrig and Yutzey, 2011).

NOTCH is part of a highly conserved signaling pathway that regulates many different cellular functions, from self-renewal to differentiation, and NOTCH-regulated transcriptional responses differ remarkably in each cell type (Andersson et al., 2011). NOTCH signaling is highly sensitive to the gene dose, so haploinsufficiency for NOTCH receptors or ligands caused by mutations often results in aberrant cell behavior, and this is the case for CAVD. In addition, deletion of the NOTCH ligand Jagged1 also results in aortic valve calcification, confirming the critical role of this pathway in CAVD (Hofmann et al., 2012).

Theodoris et al. therefore focused on characterization of NOTCH1-dependent transcriptional and epigenetic mechanisms in ECs. This cellular context has not been examined before and is likely deregulated in the disease state. Isogenic controls were created when Theodoris et al. "repaired" the NOTCH1 mutations in patient hiPSCs by transcription activator-like effector nucleases (TALENs). The authors then used an efficient EC differentiation protocol previously developed in their group to generate large numbers of ECs from hiPSCs (White et al., 2013). First, transcriptional and epigenetic changes during EC differentiation were examined. Since NOTCH1 regulates many steps in early development, this experiment allowed identification of the set of genes affected by NOTCH1 haploinsufficiency to be narrowed down to those specifically expressed in ECs. Next, fluid flow-mediated responses were investigated in hiPSC-derived ECs. For this, the authors cultured ECs in either a microfluidic or custom-built parallel flow chamber to study the effect of laminar shear stress that is normally observed in arteries (12,5 dyn/cm^2) on gene expression or histone modifications by chromatin immunoprecipitation sequencing (CHIP-seq). Interestingly, shear stress resulted in activation of anti-osteogenic molecules and repression of pro-inflammatory signatures in hiPSC-derived ECs that correlated with the occupancy of their enhancers by repressive or active chromatin marks. By contrast, the inflammatory proteins STAT6, NFKB2, and IRF9 were enriched under static conditions, in addition to key predictive shear stress genes like SMAD6. SMAD6 inhibits BMP signaling, which induces calcification. Loss of Smad6 in mice results in aortic calcification (Galvin et al., 2000). Interestingly, these transcriptional changes also correlated with changes in the epigenetic state of the cells. Analysis of histone

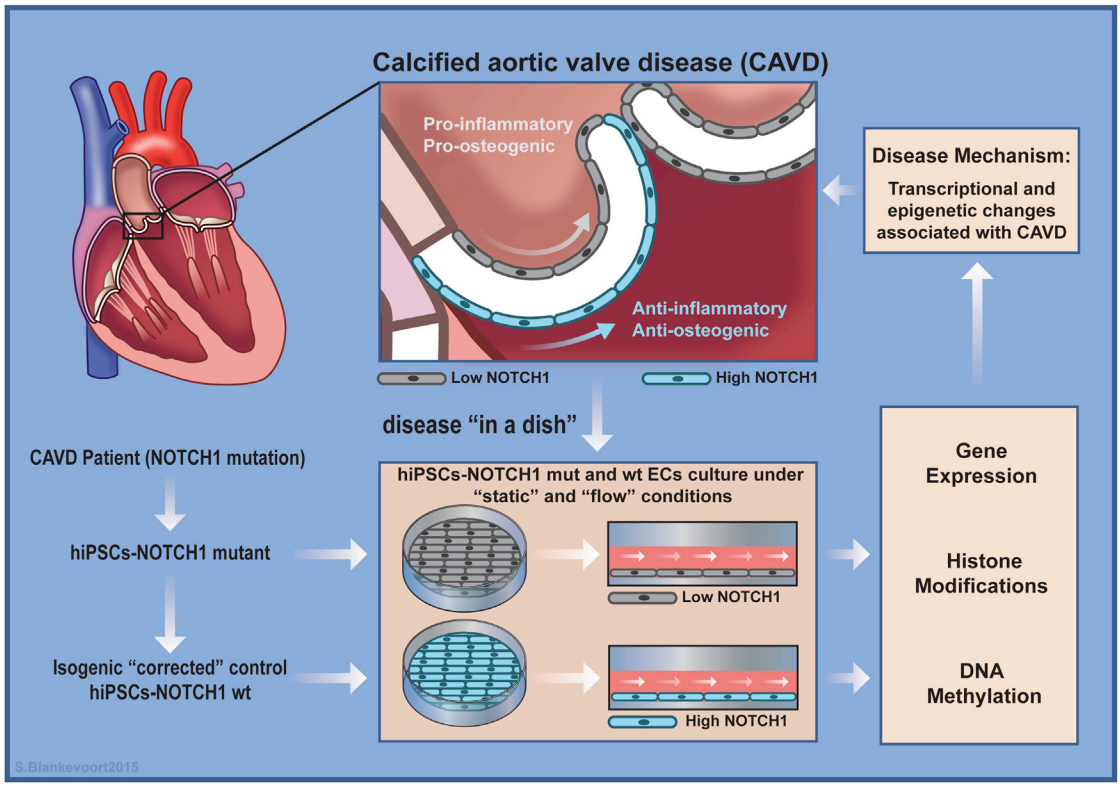

Figure 1. Model of Calcified Aortic Valve Disease "In a Dish"
Endothelial cells (ECs) were derived from patient hiPSCs with a NOTCH1 mutation (hiPSCs-NOTCH1 mutant) and isogenic "corrected" controls (hiPSC-NOTCH1 wt). Gene expression, histone modifications, and DNA methylation was compared under "static" and fluid "flow" culture conditions. This revealed a molecular mechanism that explained valve calcification in hiPSC-NOTCH1 mutant ECs specifically on the ventricular side of the valve where NOTCH1 activity is decreased by differential blood flow. The illustration is courtesy of Bas Blankevoort.

modification patterns revealed enrichment of pro-inflammatory genes (STAT and IRF) in active enhancers marked by H3K27ac. Moreover, shear stress caused preferential activation of anti-inflammatory enhancers, anti-oxidants, and TGF-β inhibitory signaling. Furthermore, Smad2/3/4 motifs co-existed with repressive H3K27me3 signature. There was thus a clear relationship between shear stress, inflammation, and genetic/epigenetic status in hiPSC-derived ECs.

The NOTCH1-deficient hiPSC ECs were next compared to controls under similar static and laminar flow conditions. NOTCH1-deficient ECs exhibited increased pro-inflammatory gene expression and failed to activate an anti-osteogenic program upon shear stress. This aberrant transcriptional response correlated with the distribution of active and repressive chromatin marks. Active chromatin marked by H3K27ac was increased in pro-inflammatory STAT and IRF motifs under static and shear conditions in NOTCH1-deficient ECs. Examination of repressive chromatin marks with H3K27me3 showed re-activation of pro-osteogenic BMP signaling. The inflammatory and "calcification" response of NOTCH1 mutant ECs was thus different from that of their controls under shear stress and this could be explained by differences in their epigenetic status.

Finally, the authors identified a gene regulatory network in NOTCH1-deficient ECs indicating novel targets, such as ARHGEF7, as well as key regulatory "node genes." Among the dysregulated genes, SOX7, TCF4, and SMAD1 were the most interconnected. The authors next attempted rescue of the phenotype in NOTCH1-deficient ECs with siRNA against the node genes. Modifying SOX7 and TCF4 expression with siRNA resulted in downregulated pro-inflammatory genes and upregulated anti-osteogenic factors, identifying a potential new route to treat calcification in patients at risk.

The work by Theodoris et al. sets a new standard for genetic disease modeling and drug screening using hiPSCs. Their comprehensive analysis involved characterization of all key EC differentiation steps at the transcriptional and epigenetic level. This in itself adds invaluable bioinformatics data to that already published on lineage commitment of hiPSCs. In addition, this study demonstrates that patient-specific hiPSC-derived ECs recapitulate a disease phenotype and thus that similar approaches could be used to study mechanisms of different vascular genetic disease. More specifically, diseased ECs exhibited aberrant genetic and epigenetic responses that were different under static or shear stress conditions. As in primary ECs, shear stress responses in control hiPSC-derived ECs induced transcriptional and epigenetic regulation of anti-osteogenic, anti-inflammatory, and anti-oxidant pathways, which were abrogated by NOTCH1 haploinsufficiency.

Remarkable in the study is that the exceptionally comprehensive analysis facilitated extraction of information about interconnectivity of dysregulated genes and identification of novel key regulatory node genes that might be important for aortic calcification. Thus, the work by Theodoris et al. represents a significant step forward in shedding light on the mechanism of aortic valve calcification, the epigenome and transcriptome in a human disease model of which can be altered by dose-reduction of a transcription factor, illustrating possible pathways for therapeutic intervention.

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Converting a Problem into an Opportunity: mtDNA Heteroplasmy Shift

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The transmission of mitochondrial DNA (mtDNA) disease from a mother with a heteroplasmic mtDNA mutation to her children is unpredictable. In a recent issue of *Cell*, Reddy et al. (2015) present the potential for mitochondrial-targeted nucleases to remove mutated mtDNA through the induction of heteroplasmy shift in oocytes and zygotes.

Mitochondria are the centerpiece of cellular metabolic machinery and contain multiple copies of a small genome, the mtDNA. Because an individual cell contains hundreds of mitochondria, each with its own varying genome, there is a substantial amount of mitochondrial genomic diversity, and this phenomenon is referred to as mtDNA heteroplasmy. Diseases associated with mtDNA mutations are unexpectedly common and represent a broad range of deteriorating conditions. The estimated incidence of mtDNA disease in adults is 1 in 5,000, but low levels of pathogenic mutations have been detected in 1 out of 200 births. Pathogenic mutations provoke symptoms only when mutant mtDNA is above a specific threshold, and every new mtDNA mutation creates a heteroplasmic mixture. Thus, a shift, or change in percentage of mutant alleles through replicative segregation, in mtDNA heteroplasmy is responsible for the pathogenicity of mitochondrial diseases. The mechanism by which this segregation occurs in either somatic or germ cells remains unknown (Wai et al., 2008). Importantly, mutated copies of mtDNA in the oocyte are transmitted to the embryo, but transmission of mtDNA disease from a mother with a heteroplasmic mtDNA mutation to her children is unpredictable. Preimplantation genetic diagnosis of embryos in affected mothers can reduce, but not eliminate, mitochondrial disease due to uncontrolled heteroplasmy. Therefore, affected mothers have no real choices for having healthy children except to play a form of reproductive "roulette," where they are left to choose between risking the possibility of their child having disabilities and a shortened life or terminating their pregnancy.

In a recent issue of *Cell*, Reddy and colleagues address this issue by exploiting mtDNA heteroplasmy as a therapeutic opportunity rather than an insurmountable problem (Reddy et al., 2015). Also, they take advantage of the poor capacity of the mitochondria to repair damaged mtDNA. To this end they use mitochondrial-targeted nucleases to remove mutated mtDNA in oocytes and zygotes