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Phenotyping patient-derived cells for translational studies in cardiovascular disease

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Introduction: Poised at the threshold of a genetic revolution

Studies into the genetics of cardiovascular disease and its risk factors have identified many chromosomal loci that contribute to cardiovascular traits. When combined with findings from basic, hypothesis-driven research, we now have a much clearer outline of the genes that contribute to cardiovascular traits (the “parts list”), including many that were unsuspected based on candidate gene approaches. The discovery of so many disease loci promises to remake our understanding of disease mechanisms and susceptibility. Particularly exciting is the prospect that new biomarkers of cardiovascular risk can be identified that are orthogonal to traditional cardiac risk factors; that is, they confer risk through mechanisms that are largely independent of traditional risk factors. The existence of such orthogonal risk factors is suggested by the observation that 15–20% of patients presenting with coronary disease have no traditional risk factors.[1]

However, the clinical translation of these discoveries faces several challenges. First, the cellular and pathophysiologic significance of most susceptibility alleles is unknown, especially for those at previously unannotated loci. Furthermore, it is not obvious how to integrate an individual's genotype at multiple disease loci with their environmental exposures to arrive at an assessment of individual disease risk; doing so requires not only clinical studies, but also basic investigations into how disease genes are organized into pathways and networks. In the therapeutic realm, studies are just beginning to examine how genotypic information can inform the choice and dosing of medications. More broadly, generalizable approaches are needed to identify genes and pathways that can best be targeted for therapeutic effect.

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Addressing these translational roadblocks requires patient-focused investigations, as well as experimental systems where the effects of disease alleles can be studied under their native physiologic regulatory mechanisms. Patient-derived cells may be well suited to address these challenges: these cells are increasingly amenable to phenotyping in the context of clinical studies, and are also being used as experimental model systems for mechanistic studies of disease alleles. Because patient cells are intact biological systems subject to physiologic regulation, they are a potentially powerful system for functional and mechanistic studies, including experiments that measure response to specific perturbations (e.g., by small molecule drugs or inhibitory RNAs). In addition, using patient cell samples as a model system permits the study of human disease alleles within their native genetic context, a complementary approach to the study of engineered alleles (overexpression or knockout) in cellular or animal models. Patient-derived cells may yield novel subphenotypes that integrate multiple genetic and environmental influences, help define genotype-phenotype correlations, and provide biomarkers for disease activity and susceptibility. (Figure 1)

In this review, we discuss the use of patient cells as cellular disease models and a source of novel phenotypes in cardiovascular disease, using current examples such as platelet function assays, endothelial progenitor cell phenotypes, induced pluripotent stem cells (iPSCs), and gene expression profiling of peripheral monocytes. We also discuss emerging technologies that can enable robust and sophisticated cell phenotyping on small numbers of patient-derived cells. A discussion of the use of patient-derived cells for regenerative therapies (such as induced pluripotent stem cells or autologous progenitors) is beyond the scope of this review, but has been reviewed elsewhere.[2–6]

Platelet reactivity assays: proof-of-concept for relating genetics, functional assays and therapy in individuals

Platelets, cell fragments derived from megakaryocytes, are critical pathogenic factors in acute coronary syndromes and the targets of several drugs. “Resistance” to anti-platelet therapy, or persistent platelet aggregation despite anti-platelet therapy, has emerged as a potential risk factor for secondary acute coronary events.[7] A recent meta-analysis of 20 prospective studies including 2930 patients with cardiovascular disease estimated the prevalence of aspirin “resistance” at 28%, and linked aspirin-resistance with an approximately four-fold increased risk of death and major adverse cardiovascular events.[8] Clopidogrel resistance has been reported to have a prevalence of 4–30%.[7]

Platelet aggregation and aspirin responsiveness are strongly heritable, with heritability estimates of 0.48–0.62 and 0.266–0.762, respectively.[9, 10] However, several genetic, physiologic, and environmental factors can influence response to anti-platelet therapy, such as metabolizing enzymes, genetic polymorphisms of platelet targets, drug bioavailability, high platelet turnover, drug-drug interactions, and medication non-adherence.

Functional assessments of platelet activity are attractive as a means of integrating these genetic and environmental variables at the level of the individual patient. Available platelet function assays provide quantitative measurements of platelet activation, adhesion, and aggregation (reviewed in[11]). Several assays measure glycoprotein IIb/IIIa-dependent platelet aggregation induced by an agonist such as ADP (e.g., light transmittance and impedance aggregometry: VerifyNow (Accumetrics), or Plateletworks (Helena Laboratories)). Others measure shear-induced platelet adhesion and aggregation (e.g., Impact (DiaMed) and PFA-100 (Siemens)). Flow cytometry-based methods measure a variety of species associated with platelet activation, such as phosphorylated vasodilator-stimulated phosphoprotein (VASP assay (BioCytex)), activated GP IIb/IIIa, and P-selectin.

These assays have been used in clinical trials to demonstrate a correlation between elevated on-treatment, or residual platelet reactivity (RPR), and adverse cardiovascular outcomes.[12, 13] For instance, Breet *et al.* compared five platelet function assays in 1069 consecutive patients undergoing elective coronary stenting and treated with aspirin and clopidogrel for at least one year. Elevated RPR as determined by three aggregation assays (light transmittance aggregometry, VerifyNow, and Plateletworks) was significantly, but modestly, associated with the primary composite endpoint of all-cause mortality, nonfatal myocardial infarction (MI), stent thrombosis, and ischemic stroke.[14] The results are consistent with several smaller studies linking elevated RPR with increased risk of drug-eluting stent (DES) thrombosis,[15, 16] major adverse cardiac events following coronary intervention for acute coronary syndromes (ACS),[17, 18] or major adverse cardiac events following DES placement for unprotected left main disease.[19] Collectively these data suggest that platelet function assays can inform risk assessment during anti-platelet therapy.

An actively studied question is whether functional platelet assays can guide individual treatment decisions, particularly when genotypic data are available. In a study of 411 patients with non-ST elevation ACS, additional clopidogrel loading doses guided by the VASP platelet function assay achieved effective platelet inhibition in the vast majority of subjects. This held true even in patients bearing the CYP2C19*2 polymorphism, which confers reduced clopidogrel metabolism and lower levels of the active metabolite. Patients with VASP index > 50% received an additional clopidogrel dose followed by a repeat VASP assay, up to a total of 4 clopidogrel loading doses. Using this protocol, 88% of patients bearing at least one copy of the CYP2C19*2 polymorphism achieved a VASP index < 50%.[20] (Figure 2)

In the GRAVITAS trial, 2,214 patients with elevated RPR based on the VerifyNow assay (measured 12–24 hours after DES placement) were randomized to high-dose clopidogrel (600mg followed by 150mg daily) vs. standard dosing (no additional loading followed by 75 mg daily) for 6 months.[21] There was no difference in the primary composite end point of cardiovascular death, nonfatal MI, or stent thrombosis at 6 months in high-dose vs. standard clopidogrel dosing. The null result may be due to several factors. First, the overall event rate for the primary composite end point (2.3% in both groups) was relatively low. Second, the arm randomized to high-dose clopidogrel only showed a modest decrease in the incidence of high RPR at 30 days, raising the possibility that more intensive clopidogrel adjustments or an alternative thienopyridine such as prasugrel may have fared better. Subsequent studies may clarify several questions raised by this trial: whether dosing changes triggered by lower RPR thresholds or repeated platelet function assays improve outcomes; whether individuals who experience an adverse event have higher RPR than those who do not; whether RPR could guide the selection of an alternative thienopyridine, such as prasugrel; and whether RPR-guided therapy can improve outcomes in selected high-risk patients. Genetic substudies of GRAVITAS are also pending.[22]

Overall, platelet function assays have gained traction as tools with both investigational and clinical significance. Several assays are available as point-of-care tests, though caution is still warranted with regard to their widespread use, due to the lack of universal standards for sample handling and defining thresholds for elevated RPR. Nonetheless, clinical data convincingly link functional phenotypes (i.e., elevated RPR) to adverse clinical outcomes. Detailed outcomes trials are underway to determine whether platelet function assays can yield clinically actionable information, particularly in the presence of CYP2C19*2 and other genetic variants that affect clopidogrel activation.

The potential of patient cell-based phenotypes

The promising results of platelet function assays raise the possibility that functional cell-based assays may have broader relevance to the study and treatment of cardiovascular disease. By analogy, oncology has benefited tremendously from routine sampling of malignant cells in the course of usual clinical care. Ready access to cancer samples has resulted in large collections of cancer cell lines that are widely studied across institutions (e.g., the NCI-60 cell lines); primary biopsy specimens are also intensively studied for genetic or phenotypic markers. Patient-derived cancer samples have led to the discovery of novel oncogenes associated with common cancers, the identification of novel disease sub-types (including those with very different prognoses), and the development of new therapies. At several institutions, patient tumor samples are genetically analyzed to identify individuals who are candidates for new molecularly targeted therapies. In sum, studies of patient-derived cancer samples have enabled profound advances in the understanding of disease mechanisms, patient diagnosis and prognosis, development of new therapies, and individualized selection of therapy.

Despite these successes in oncology, however, the broad adoption of patient cell-based studies to cardiovascular disease is not straightforward. To a first approximation, certain carcinogenic mechanisms are driven by somatic mutations that lead to dysregulated cellular proliferation in a cell-autonomous manner. As a result, studying the primary tumor can yield insights into causative genetic aberrations and critical disease mechanisms, even as other approaches are needed to define the role of host mechanisms, germline genotype, and tumor microenvironment. In contrast, cardiovascular traits commonly involve interactions between multiple cell types and organs, communicating through hormones and circulating cells. Study of any individual cell-type may thus convey only a partial view of disease biology. The effects of individual disease alleles are also likely to be more modest.

In addition, access to primary patient samples of many disease-relevant cells, such as endothelial, vascular smooth muscle, or myocardial cells, is minimal during usual clinical care. While samples may be obtained in association with invasive procedures (e.g., percutaneous vascular intervention, carotid endarterectomy, heart explantation, LVAD implantation, and coronary artery bypass grafting), specimens from end-stage disease may be less informative for earlier pathogenic processes. While a limited number of relevant cell lines are used, much research uses primary isolates that are physiologic but difficult to obtain and have short life-spans in culture.

Fortunately, certain cell types are both accessible from patients and clearly involved in disease pathogenesis, such as monocytes and lymphocytes. Furthermore, accessible cells, even if not conventionally regarded as key participants in disease, may share signaling pathways with canonical disease tissues, and thus be useful as surrogate model systems. In perhaps the most prominent historical example of the use of accessible, surrogate cell models, Brown and Goldstein demonstrated regulation of HMG-CoA-reductase by LDL not in liver cells, but in primary skin fibroblasts.[23] They also demonstrated LDL-receptor activity *in vivo* for the first time using circulating blood lymphocytes.[24]

In the following sections, we highlight recent research that utilizes patient-derived cells to study disease mechanisms or patient populations, and may illustrate paradigms for how these cells could be powerful tools for translational studies. The cells discussed are accessible in the context of patient care or clinical research cohorts, and are appealing for several reasons: they are accessible by routine phlebotomy or skin biopsy (in the case of induced pluripotent stem cells (iPS)); they include inflammatory and immune cells such as monocytes, or iPS-derived cells that directly contribute to cardiovascular pathogenesis;

certain cell populations can be purified based on surface marker expression; and peripherally circulating cells are in direct contact with the endothelium, cytokines, and other hormones, thereby providing an integrated readout of cellular responses to environmental factors.

Peripheral Blood Mononuclear Cell Phenotypes

Circulating inflammatory and immune cells, such as peripheral blood mononuclear cells (PBMCs) and T-cells, play important roles in atherosclerosis, remodeling after myocardial infarction, heart failure, and transplant rejection.[25–27] In an early example of a cell-based biomarker, the white blood cell count was long ago established as a predictor of myocardial infarction.[28] More recently, leukocytosis has been shown to predict CAD burden and adverse outcome after MI.[29, 30] Subsequent research has correlated increasing monocyte count with adverse risk or outcomes in cardiovascular disease, particularly acute MI,[31] in-stent restenosis,[32] subclinical carotid atherosclerosis,[33] and post-MI LV dysfunction and aneurysm.[34]

Several early efforts to use PBMCs as surrogate cell models employed genome-wide gene expression analysis. Differentially regulated genes, many involved in macrophage activation and differentiation, were identified in multiple forms of cardiovascular disease, including atherosclerosis,[35, 36] carotid disease,[37] ischemic stroke,[38] and diabetes.[39]

In the most clinically advanced application to date of PBMC analysis, gene expression profiling has been used to detect rejection following heart transplantation. In transplant patients undergoing endomyocardial biopsy, Cappola and colleagues identified an expression signature in whole blood that differentiated control patients without rejection from those with stage 3A rejection or higher.[40] Recently, Pham *et al*. randomized cardiac transplant patients to rejection monitoring by routine endomyocardial biopsy vs. a PBMC gene expression signature score, previously shown to have a 99.6% negative predictive value for histologic rejection.[41, 42] Over a median follow-up period of 19 months, there was no difference using a non-inferiority comparison between the two groups in the primary composite endpoint of first occurrence of rejection with hemodynamic compromise, graft dysfunction due to other causes, death, or retransplantation. Overall death at two years was also comparable. Notably, gene expression monitoring significantly decreased the number of biopsies performed (0.5 vs. 3.0, $P < 0.001$). This result awaits further confirmation in larger populations, but constitutes an exciting example of a cell-based phenotype that conveys diagnostic information and can impact patient management.

A growing body of work has identified monocyte subpopulations with distinct functions in normal and pathological states, particularly in atherosclerosis and healing after MI (reviewed in[43, 44]). In mice, monocytes expressing high levels of the lymphocyte antigen complex Ly6-C (Ly6-C^{hi}) secrete numerous inflammatory and proteolytic mediators, increase in response to hypercholesterolemia, home to sites of injury, and differentiate into foam cells.[45] Ly6-C^{lo} monocytes, in contrast, have an immunomodulatory role, and mediate granulation and wound-healing. Moreover, LV healing after myocardial infarction involves sequential mobilization of Ly6-C^{hi} monocytes followed by Ly6-C^{lo} monocytes.[46]

Although humans lack direct equivalents of the murine Ly6-C^{hi} and Ly6-C^{lo} monocytes, functional monocyte subsets also contribute to human cardiovascular disease and post-MI LV healing.[43, 44, 47] Increased CD14^{+}CD16^{+} monocytes are associated with coronary artery disease and carotid intima-media thickness.[48, 49] After anterior STEMI, CD14^{+}CD16^{-} and CD14^{+}CD16^{+} monocytes sequentially accumulate in infarcted tissue, analogous to the murine observations; the magnitude of the early CD14^{+}CD16^{-} monocyte peak was negatively correlated with myocardial salvage.[50, 51] These studies demonstrate strong biologic links between monocyte subset dynamics and cardiovascular disease. Further

clinical studies and functional assays for monocyte subsets (that complement FACS-based counts of cell number) may illuminate the role of these subsets in disease, and lead to the use of monocyte phenotypes as biomarkers of CAD risk or adverse LV remodeling after MI.

Endothelial Progenitor Cells

Cells termed “endothelial progenitor cells” (EPCs) have been intensively studied as a potential biomarker and source of cells for cardiovascular regenerative therapy. EPCs, broadly defined, refer to circulating bone-marrow derived cells that exhibit endothelial properties *in vitro*, and promote neovascularization and endothelial repair *in vivo*.[52] Asahara *et al*. first described cells with these properties, and identified them as circulating cells expressing CD34 -- as well as varying degrees of Flk1 (VEGF receptor 2), CD45, and CD31.[53]

Several methods have been used in the literature to isolate EPCs from the PBMC fraction, including FACS and *in vitro* culture assays. FACS-based definitions typically include combinations of CD34, VEGF receptor 2 (VEGFR-2, or KDR) or the hematopoietic stem cell marker AC133. (These markers may not be required for EPC activity, however; a murine subpopulation of $lin^{-}/c\text{-}Kit^{-}/Sca\text{-}1^{-}$ cells has been reported that differentiates into endothelial-like cells *in vitro* with high replicative potential.[54]) Culture-based approaches include a colony-forming-unit (CFU) assay,[55] and an early outgrowth EPC assay for uptake of acetylated LDL (AcLDL) and *Ulex europaeus* agglutinin I.[56]

In the absence of a specific marker for EPCs, none of these assays isolates a homogeneous cell population, and the different assays identify distinct functional cell subsets, each of which likely correlates with different biological activities. Furthermore, many lines of evidence suggest that (a) vasculogenic properties of EPCs likely arise from paracrine effects as opposed to true progenitor cell activity, and (b) commonly used EPC definitions identify cells of hematopoietic and not endothelial origin, including varying proportions of monocytes, lymphocytes, and platelets.[57–60] For example, in early outgrowth EPC protocols that lack a pre-plating step, platelet contamination may lead to uptake of platelet microparticles by adherent mononuclear cells.[58, 61] Contaminating platelets and platelet microparticles can confer binding to *Ulex europaeus* agglutinin I and transfer of platelet proteins to the mononuclear cells,[62] resulting in apparent endothelial patterns of staining which are in fact due to platelet-derived proteins. Platelet microparticles can also alter the functional phenotype of early outgrowth EPCs, increasing expression of proangiogenic paracrine factors, and enhancing EPC adhesion to injured vascular endothelium with accelerated reendothelialization *in vivo*.[62] In light of the difficulty of isolating a homogeneous cell population that confers EPC-like properties, unbiased approaches such as genome-wide gene expression may provide empiric signatures of circulating cells important for vascular repair and neovascularization.[63]

Yoder and colleagues have identified an endothelial colony-forming cell (ECFC; also called late outgrowth endothelial cells) that forms *in vitro* colonies with a typical cobblestone appearance and shows a robust *in vitro* capacity for proliferation, self-renewal, and tube formation.[64, 65] ECFCs are most abundant in umbilical cord blood and are present in peripheral circulation at very low concentrations (~1 colony per 10^8 mononuclear cells plated). Thus ECFCs could conceivably be isolated from cord blood and used for mechanistic studies and genotype-phenotype correlations. Their scarcity in peripheral blood, however, may render their use in adult clinical studies impractical, barring improved methods for their detection.

Multiple studies in selected patient populations have reported that EPCs are inversely associated with overall cardiovascular risk, clinical outcomes, traditional cardiovascular risk

factors, and markers of endothelial function.[55, 56, 66–68] In two large community cohorts, EPCs showed only sparse associations with traditional cardiovascular risk factors.[69, 70] Despite this, in the community-based Framingham Heart Study, the EPC CFU phenotype was inversely associated with the Framingham risk score and subclinical phenotypes of cardiovascular disease, such as coronary and aortic calcification.[71] Furthermore, a genome-wide association study revealed that SNPs at the SLC22A3-LPAL2-LPA locus on chromosome 6, a locus previously associated with myocardial infarction,[72] were highly associated with CFU ($P=4.9\times10^{-7}$) and risk of MI ($P=1.1\times10^{-4}$). Specifically, SNPs associated with lower CFU numbers were associated with increased risk of MI, and vice versa.[70] These data provide genetic support for the notion that EPCs mediate risk of cardiovascular disease and MI at least in part through mechanisms orthogonal to traditional risk factors. Going forward, patient-derived EPCs and ECFCs may be useful as biomarkers of cardiovascular risk, or model systems to study vascular repair. However, further efforts are needed to clarify which cell subsets contribute to observed EPC phenotypes *in vitro*, which *in vivo* biological processes are mediated by EPCs, and whether simply measuring number, rather than function, of EPCs is a faithful surrogate for *in vivo* activity.

Lymphoblast Cell Lines

Lymphoblast cell lines (LCLs), created by the transformation of human B-lymphocytes by Ebstein-Barr virus, were originally devised as a renewable source of DNA for genetic studies. For instance, with the collection of 270 LCLs genotyped by the International HapMap Project[73] (representing individuals of Northern European, African, and Asian ancestry), investigators can leverage publicly available datasets of genome-wide SNP genotyping and gene expression for their studies. LCLs possess certain limitations that must be addressed or mitigated for individual studies, including a restricted repertoire of expressed genes related to their B-lymphocyte lineage, variable growth rates, and the potential for confounding effects of EBV infection.[74]

Nonetheless, the availability of LCLs in association with many phenotyped patient cohorts, coupled with their capacity for *in vitro* expansion, make patient-derived LCLs an appealing model system for functional studies. LCLs have been widely used to study the genetic determinants of variation in human gene expression.[75] LCLs have also been used successfully as experimental models in the study of congenital hyperinsulinism/hyperammonemia,[76] pharmacogenomic studies,[77–79] ion channel disorders,[80] and Huntington's disease.[81] In Huntington's disease, LCLs are much more accessible than primary neuronal tissue and display disease-relevant phenotypes. For instance, the ratio of cellular [ATP]/[ADP] shows an inverse correlation with the length of the unstable triplet repeat in both human LCLs and striatal neurons from mice expressing mutant huntingtin (with 111 triplet repeats).[81] This surrogate phenotype led to the discovery of a defect in mitochondrial energy metabolism in Huntington's disease and is also correlated with other Huntington's subphenotypes.[82]

More broadly, the existence of genotyped LCLs from clinical cohorts has opened new avenues for assigning function to disease alleles. LCLs have been used to elucidate the function of susceptibility alleles identified through genome-wide association studies (GWAS) for systemic lupus erythematosus (SLE)[83] and childhood asthma.[84] Importantly, the capacity of LCLs for *in vitro* expansion enables their use in high-throughput perturbational screening. For instance, a chemical-genetic interaction screen was performed in LCLs from related individuals with a monogenic form of diabetes (Maturity Onset Diabetes of the Young type 1, or MODY1), caused by mutation in the orphan nuclear hormone receptor HNF4α.[85] LCLs were treated with approximately 4,000 marketed drugs and other compounds with characterized mechanisms. By analyzing screening data for LCLs

from 10 MODY1 individuals and from 8 wild-type related controls, classes of compounds were identified that induced distinct effects between mutant and wild-type LCLs (i.e., a chemical-gene interaction). Many of these functional interactions were preserved in pancreatic β-cells, a canonical cell model for diabetes. Furthermore, several of these compounds favorably modulated a key disease phenotype, insulin secretion from pancreatic β-cells.[85] Thus LCLs can serve as a surrogate cell system for functional studies of disease alleles. The use of LCLs in perturbational screens (by small molecules or inhibitory RNA, for instance) allows the logic of synthetic genetic interaction screens to be applied to human cells, in order to elucidate networks of genes that interact with disease alleles, and identify proteins or pathways that can be modulated for therapeutic effects. (Figure 3)

Induced pluripotent stem cells (iPSCs)

iPSCs have recently emerged as a transformative technology for patient-specific cellular disease models (in addition to their potential as a source of cells for regenerative therapies).[86–88] Adult somatic cells, such as skin fibroblasts, are reprogrammed into pluripotent stem cells with defined factors, and may then be differentiated into specific cell lineages. iPS-derived cardiomyocyte models have been developed for long QT syndrome type 1 (caused by mutation in KCNQ1)[89] and type 2 (caused by mutation in KCNH2).[90] In both cases, cardiomyocytes display altered activity of the expected current (I_{Ks} and I_{Kr}, respectively), prolonged action potential duration, and enhanced arrhythmogenicity.

iPS-derived cells can also model cardiac manifestations of complex multi-organ syndromes. For instance, iPSC were derived from patients with LEOPARD syndrome bearing mutation in the PTPN11 gene (encoding the SHP2 phosphatase).[91] LEOPARD iPS-derived cardiomyocytes show increased size, sarcomeric organization and NFATC4 nuclear localization consistent with hypertrophic cardiomyopathy (commonly observed in LEOPARD patients). In addition, LEOPARD iPSCs recapitulate the expected abnormalities in RAS-MAPK signaling characteristic of the syndrome. iPSC have also been derived from patients with Timothy syndrome (due to mutation in the L-type calcium channel Ca(V)1.2, and associated with a long QT syndrome);[92] differentiated ventricle-like cells display abnormal Ca^{+2} transients, prolonged action potentials, and irregular electrical activity and contraction. In Hutchison Gilford Progeria (HGP, caused by mutation in lamin A, and associated with premature atherosclerosis), iPS-derived vascular smooth muscle and mesenchymal stem cells have decreased viability in response to stress (such as hypoxia); these defects may underlie the impaired ability of HGP iPS-derived mesenchymal stem cells to rescue hind limb ischemia *in vivo*.[93]

iPS-derived cellular models may also facilitate the discovery of new therapies. For instance, iPS-derived cardiomyocyte models of long QT syndrome were successfully used to evaluate the effects of established and novel drugs on action potential duration and arrhythmic susceptibility.[89, 90] These and other iPS-derived cardiomyocyte models may provide a platform to screen drug candidates for their propensity to prolong the QT interval, an adverse effect of great concern in drug development. More generally, iPS-derived cellular models may facilitate drug discovery by providing a ready source of traditionally non-accessible human cells (e.g., cardiomyocytes) that harbor clinically relevant disease mutations in their native genetic context.

Emerging technology platforms for phenotyping of patient-derived cells

With the exception of LCLs and iPSCs, the patient-derived cells mentioned above have limited *in vitro* proliferative capacity. As a result, the number of cells available for study is principally determined by the sample size feasibly obtained from subjects, such as the volume of peripheral blood. These limitations can be mitigated by the use of sensitive

detection methodologies (often with amplification steps as in gene expression studies) or the use of relatively simple phenotypes, such as cell counts based on FACS measurements or CFU assays. However, functional studies would benefit from technology platforms that enable more detailed phenotyping on limited samples. Ideally, such platforms would facilitate (i) isolation of potentially rare cells from peripheral blood at a reasonable yield and purity, (ii) phenotypic analysis of small numbers (i.e., hundreds to thousands) of cells with minimal subculturing, and (iii) preserved cell viability. Finally, these platforms should be sufficiently inexpensive and robust to be applied to patient samples.

One approach to decreasing cell requirements is to measure cell phenotypes in formats denser than 384-well assay plates. For instance, cellular microarrays consist of small molecules, or lentiviruses encoding cDNA or shRNA, printed at high density on glass slides. When cell suspensions are incubated on the slides, cells attach and are perturbed by the compounds, cDNA, or shRNA.[94–96] Cellular phenotypes arising from these perturbations can then be quantified using automated microscopy. This format allows perturbational screens to be performed on ~100 cells per spot (a decrease of approximately one order of magnitude compared to screening in 384-well plates), with approximately 5,000 spots per glass slide.[96] (Figure 4)

Microfluidic chips combine nanotechnology with microfluidic large-scale integration, resulting in devices that manipulate nanoliter fluid volumes across thousands of integrated, miniaturized components for detailed cellular processing, functional assays and phenotyping.[97–100] The miniaturization of these devices allows significant economies of scale for cells, reagents, and labor (such as pipetting). Devices are typically fabricated from a biocompatible substrate (such as polydimethylsiloxane, or PDMS), with controllable valves that enable sample loading and mixing of fluid streams. Cell subpopulations may be presorted before loading onto the chip, or potentially sorted on-chip. Integrated fluidic circuits can accomplish a wide variety of manipulations, including sample processing, incubation with small molecules, and parallel handling of multiple samples. Phenotyping can be performed on the level of single cells or cell populations using such readouts as fluorescent microscopy or quantitative RT-PCR.[101–104]

Microfluidic applications have phenotyped circulating cells from patients. For instance, a microfluidic device can capture neutrophils from a drop of whole blood, generate chemoattractant gradients, and monitor chemotaxis at the level of the individual cell.[105, 106] Another promising application detects and characterizes circulating tumor cells (CTCs) in patients with epithelial cancers,[107] particularly prostate cancer.[108] Cells are captured by immobilized antibodies against a cell surface marker (e.g., EpCAM), fluorescently labeled with antibodies against prostate-specific antigen (PSA), and then subjected to semi-automated image capture and analysis. This device allows the monitoring of CTC numbers after primary tumor resection, and extraction of DNA or RNA for analysis. Nanopatterning of the PDMS surface has been used to enhance the efficiency of CTC capture. For instance, three-dimensional nanotexturing increases the surface area for capture,[109] and nanochannels etched in a herringbone pattern induce microvortices, chaotic mixing, and increased encounters between flowing CTCs and immobilized capture antibodies.[110] In another example, a microfluidic device was combined with a nuclear magnetic resonance nanosensor array and targeted magnetic nanoparticles, allowing highly sensitive detection of cancer cells bearing specific surface markers.[111]

The integration of microfluidics with sophisticated measurements of gene expression, proteins,[112] and epigenetic marks[113] may yield devices that can interrogate pathway activity in patient-derived cells. For instance, Tay *et al*. combined fluorescence microscopy and gene expression to characterize transduction of TNF-α signals into NF-κB-dependent

transcription at the resolution of single cells.[114] Robust microfluidic platforms for quantitative RT-PCR gene expression analyses are now commercially available, and may be useful to study patient samples.[115] (Figure 5)

Conclusions

The notion of cell-based phenotypes in cardiovascular disease is not new to this moment. As mentioned above, patient-derived cell models figured prominently in the work of Brown and Goldstein almost 40 years ago. The advantages of studying patient cells were also recognized in the early days of peripheral blood gene expression analysis for atherosclerosis.[116]

In light of several recent developments, patient-derived cells increasingly represent a powerful tool for both patient-focused and basic science studies in cardiovascular disease. The great success of human genetic studies has identified dozens of novel genes that contribute to cardiovascular traits. For instance, 95 loci were recently associated with serum lipids, which together explain 10–12% of the observed variation.[117] These discoveries created two opportunities: to understand the functional significance of novel disease loci and how they contribute to disease; and to translate these findings into improved clinical care, through novel biomarkers or therapies. The large number of disease loci found to contribute to cardiovascular traits makes both of these goals challenging, and highlights the need for new approaches that can be generalized to many loci and diseases.

Patient-derived cells are uniquely positioned to address these unmet needs, because they can be obtained directly from phenotyped patients, and also serve as model systems for *in vitro* studies. As clinical phenotypes and outcomes become mapped onto sophisticated cell-based measurements, patient cells will increasingly help draw connections between disease alleles, clinical phenotypes, circulating biomarkers, cellular pathways, and therapeutic hypotheses. An important translational goal is the development of improved biomarkers that can impact clinical decisions; to date, using genetic scores and serum biomarkers to improve cardiovascular risk classification over that conferred by traditional risk factors has proven surprisingly difficult.[118–123] Cell-based phenotypes can integrate the effects of multiple genetic and environmental factors, and represent a new dimension for circulating biomarkers. Overall, patient cell phenotyping is likely to become an important tool to study genetically complex cardiovascular traits, providing a complementary approach to genetic, physiologic, proteomic, and metabolomic methods.

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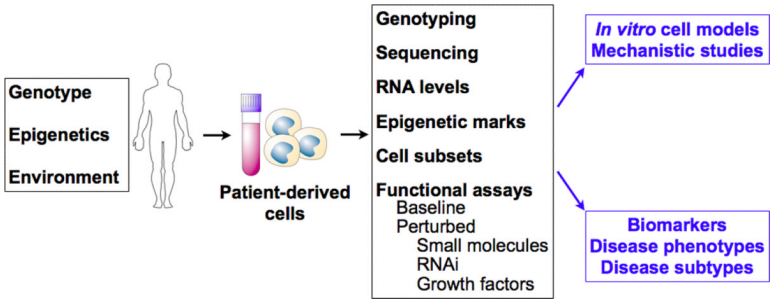
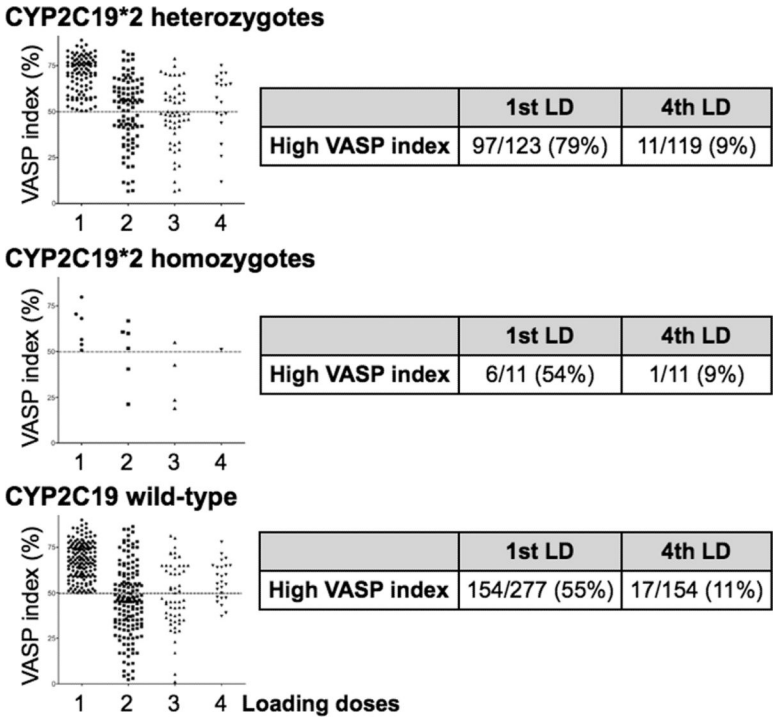


Fig. 1.

Phenotypes of patient-derived cells reflect an integration of genetic, epigenetic and environmental influences. Summary of potential utility of patient-derived cells as a source of functional phenotypes, *in vitro* model systems to study disease biology, and novel biomarkers.



	1st LD	4th LD
High VASP index	97/123 (79%)	11/119 (9%)

	1st LD	4th LD
High VASP index	6/11 (54%)	1/11 (9%)

	1st LD	4th LD
High VASP index	154/277 (55%)	17/154 (11%)

Fig. 2.

Clopidogrel dosing guided by platelet function assays helps achieve platelet inhibition in carriers of the CYP2C19*2 allele. Graphs show individuals who received additional clopidogrel loading doses (LD) if their post-clopidogrel platelet function remained elevated (according to the VASP assay), up to a total of 4 loading doses. Charts summarize the percentage of patients with elevated VASP index after the 1st and 4th loading doses (if needed). Adapted from[20].

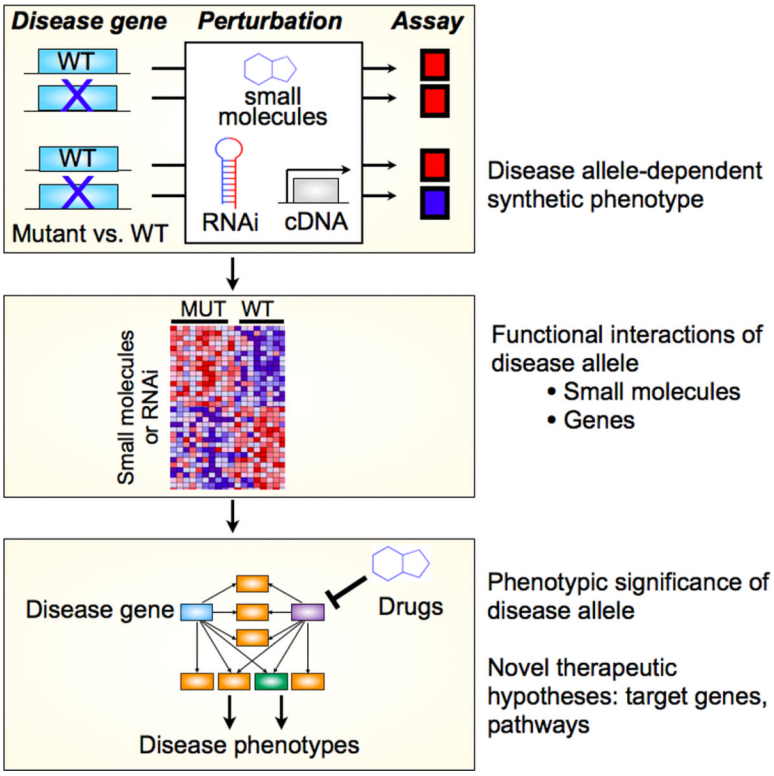


Fig. 3.

Perturbational screens on patient-derived cells can clarify the functional significance of disease alleles and lead to new therapeutic hypotheses. Using the logic of synthetic genetic interactions, patient cells can be screened for small molecules, RNAi, or cDNA that cause different phenotypes in cells that are mutant or wild-type at a disease gene. This identifies small molecules or genes that functionally interact with a disease gene, identifies pathways that may connect the disease allele to disease phenotypes, and provides clues for new therapeutic targets and hypotheses. Adapted from[85].

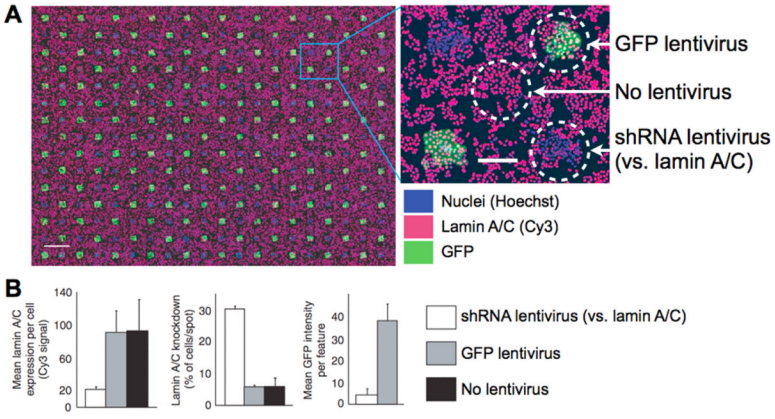


Fig. 4.

Cell microarray incubated with HeLa cells demonstrates both lentiviral gene knockdown and gene expression on printed spots. Each spot is approximately 250 μm in diameter and contains ~150 cells. **A.** Cells in contact with a GFP lentivirus spot show effective GFP expression (circle at top right); cells in contact with shRNA against lamin A/C show loss of Cy3 lamin A/C signal (lower right). The circle at center shows HeLa cells attached to the slide but not touching a lentivirus spot. **B.** Quantitation of lamin A/C knockdown and GFP expression from cell microarrays. Scale bar, 1 mm (left panel) and 200 μm (right). Adapted from[96].

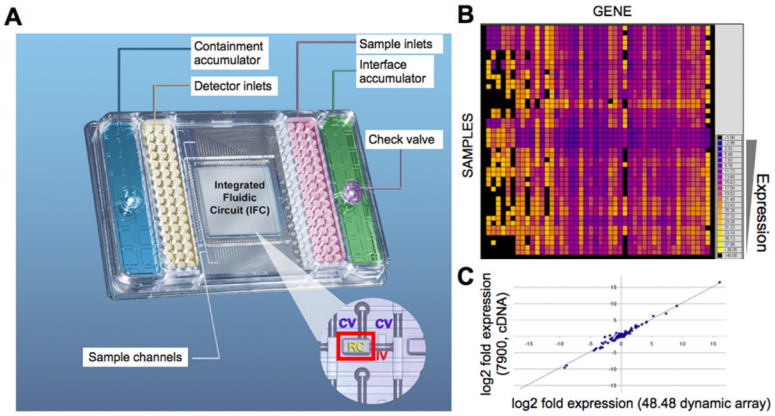


Fig. 5.

Miniaturized quantitative RT-PCR analysis enabled by microfluidic integrated fluidic circuits. **A.** Image of a 48 × 48 microfluidic chip. The integrated fluidic circuit (at center) contains 2304 reaction chambers (RC, outlined by a red box in inset), bounded by control valves (CV) and interface valves (IV). **B.** Heatmap of RT-PCR C_t values from individual reaction chambers on a single chip. **C.** Correlation between expression levels obtained by microfluidic (nanoliter scale) and conventional (microliter scale) quantitative RT-PCR. r = 0.986. Adapted from[115].