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Diversity of Cells and Signals in the Cardiovascular System

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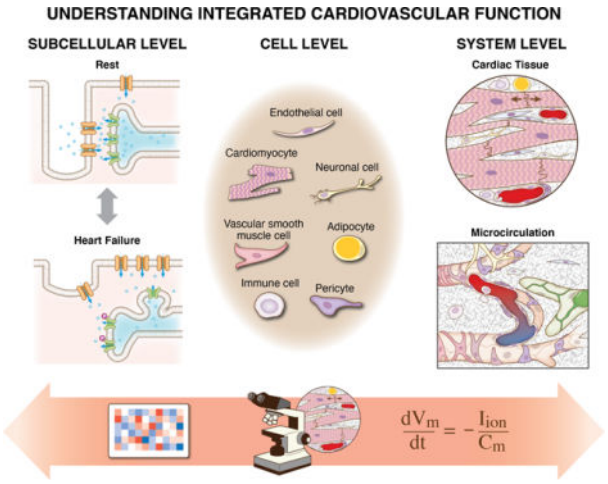
Abstract

This white paper is the outcome of the seventh UC Davis Cardiovascular Research Symposium on Systems Approach to Understanding Cardiovascular Disease and Arrhythmia. This biannual meeting aims to bring together leading experts in subfields of cardiovascular biomedicine to focus on topics of importance to the field. The theme of the 2022 symposium was "Cell Diversity in the Cardiovascular System, cell-autonomous and cell-cell signaling". Experts in the field contributed their experimental and mathematical modelling perspectives and discussed emerging questions, controversies, and challenges in examining cell and signal diversity, coordination, and interrelationships involved in cardiovascular function. This paper originates from the topics of formal presentations and informal discussions from the symposium, which aimed to develop a holistic view of how the multiple cell types in the cardiovascular system integrate to influence cardiovascular function, disease progression, and therapeutic strategies. The first section describes the major cell types (e.g., cardiomyocytes, vascular smooth muscle and endothelial cells, fibroblasts, neurons, immune cells, etc.) and the signals involved in cardiovascular function. The second section emphasizes the complexity at the subcellular, cellular, and system levels in the context of cardiovascular development, aging, and disease. Finally, the third section surveys the technological innovation that allows interrogating this diversity and advancing our understanding of the integrated cardiovascular function and dysfunction.

Graphical Abstract



The white paper discusses the cell diversity, coordination, and interaction patterns that are critical for robust cardiovascular function. We identify the major cell types and signals involved in cardiovascular function and emphasize the complexity at the subcellular, cellular, and system levels that motivate both challenges and opportunities for researchers. A survey of recent advances enabled by innovative experimental and computational modeling approaches serve to guide researchers moving forward.

Keywords

Omics; ion channels; ion transporters; animal models; signal transduction; modelling

Motivation for Understanding Cell Diversity and Dynamics Involved in Cardiovascular Function

The cardiovascular system is a highly organized, closed, and efficient circuit consisting of a pump (i.e., the heart) and pipes (i.e., the blood vessels). The heart pumps oxygen-rich blood and nutrients to the rest of the body via blood vessels, which also deliver deoxygenated blood to the lungs. This relatively straightforward process of pumping, delivering, and return of blood throughout the body belies the hidden complexity of an incredibly diverse array of molecular-cellular elements of the heart and the vasculature (Figure 1). These components coordinate via a complex network of signals to sustain roughly 1 billion heartbeats over a lifetime (Levine, 1997).

Given the interdependence of heart muscle and vascular function, it is important to appreciate the common and integrated cell dynamics between cardiac tissue and microcirculation (Figure 1). Often, cell dynamics are considered in isolation. While reductionistic, mechanistic studies have provided a foundation for our physiological understanding, emerging evidence of overlapping cellular phenotypes, common cell function mechanisms, structural interactions, and paracrine interactions motivate the conceptual and experimental integration of the diverse cell dynamics. What are the cell types involved in cardiovascular function in health and disease? Do the diverse cell populations highlight common cell lineages? Do cell contributions vary temporally? How do we dissect and interpret specific cell functions in a diverse tissue scenario? Based on the recent UC Davis Cardiovascular Research Symposium on Systems Approach to Understanding Cardiovascular Disease and Arrhythmia, the purpose of this white paper is to emphasize the cell diversity, coordination, and interaction patterns critical for robust cardiovascular function. By identifying the major cell types and signals, the article will highlight the complexity across cell and system levels that motivate both challenges and opportunities for researchers. A foundation for future research and related knowledge gaps will be framed in the context of alterations during development, aging, and pathological scenarios. The complexity will be further highlighted by considering how specific cell functions are influenced by energetic costs, cell topology, and sub-cellular mechanisms. Finally, examples of recent advances made possible by innovative experimental and computational modeling approaches will serve to guide researchers moving forward. Rather than providing a comprehensive compilation of specific cell functions, this article integrates

multiple research areas spanning cardiovascular physiology, immunology, stem cell biology, microvascular physiology, imaging, and computational modeling to generate new important yet underrecognized questions.

1. Cell Types and Signals in Cardiovascular Function

Appreciation of the multiple cell types across the cardiac and vascular systems is realized by considering system-level dynamics in cardiac tissue and the embedded microcirculation (Figure 2). The major cell types in the heart include cardiomyocytes (CMs), endothelial cells (ECs), vascular smooth muscle cells (VSMCs), and fibroblasts (FBs). Neuronal and immune cells, adipocytes, and pericytes are also found in the heart (Doll *et al.*, 2017; Litvinukova *et al.*, 2020).

The relative abundance of these various cell types is controversial and may depend on technical differences among studies (based on transcriptomic approaches and spectroscopy-based proteomics) (Doll et al., 2017; Litvinukova et al., 2020). Species differences are also important for the relative prevalence of the various cell types (e.g., rodents have no substantial adipose tissue, and FBs are less abundant in mouse vs. human).

Cardiac tissue level dynamics resulting from integrated cell interactions include muscle contraction, matrix remodeling, and neuronal control. Contributing to the complexity, cardiac tissue function is influenced by microvascular level dynamics such as additional neuronal control, vasoregulation, leukocyte trafficking, and permeability dynamics. Given their large size, CMs occupy most of the heart volume. Importantly, transcriptional differences between atrial and ventricular CM populations indicate the potential for different developmental origins, distinctive hemodynamic forces, and specialized functions in cardiac chambers. Also, the cellular composition may differ locally in different heart muscle regions (i.e., specific chambers) and between male and female hearts (Litvinukova *et al.*, 2020).

The major classes of cells along the microvasculature include ECs and mural cells (i.e., VSMCs and pericytes). The ECs face the lumen of the vessel, and mural cells cover the vascular endothelium to different degrees depending on the vessel and localization along the arterial tree. Accordingly, vascular smooth muscle wraps around the artery and arteriole endothelium, whereas pericytes have distinct levels of coverage in small arterioles and the capillary endothelial network. Importantly, vascular smooth muscle contains the contractile machinery to control arterial/arteriole diameter. Emerging evidence also suggests that pericytes may be contractile cells that help modulate capillary diameter (Gonzales *et al.*, 2020). Extracellular matrix (ECM), perivascular macrophages, and perivascular FB-like cells are also present and/or reside along the vascular wall. These cells help to facilitate vascular function by providing stability to the blood vessels, and contribute to (inter)cellular communication that is essential for the modulation of vessel diameter and, therefore, control of arterial tone, peripheral resistance, and tissue blood flow. Intriguingly, all major classes of vascular cells show remarkable heterogeneity in terms of gene expression, morphology, and even function, among other properties. Like CM heterogeneity, vascular

cell heterogeneity may result from developmental origins, structural requirements, shear stress, microenvironmental factors, and/or adaptation to specific functions or pathological conditions, such as hypertension, diabetes, and aneurism, within the vascular network.

CMs are the basic cellular units of cardiac excitability and contractility. Their structure/function characteristics are reviewed first and compared with that of VSMC. CMs are electrically and mechanically coupled with each other and communicate with other cell types via both direct physical interaction and paracrine and autocrine signaling. Other cell types are required to provide structural support and blood supply for efficient contraction and long-term survival and are described in subsequent paragraphs.

To appreciate the diversity and, at the same time, the perplexing interrelationships between the major cardiac tissue cell types and microvascular cell types, this section will focus on key known cell functions that substantiate their contributions. Importantly, excitation-contraction coupling (EC-coupling) in CMs and VSMCs is detailed to showcase both the divergent and overlapping mechanisms. The key roles of the other cell types are highlighted to introduce their underappreciated importance.

1.1 Excitation-Contraction Coupling in Cardiomyocytes vs. Vascular Smooth Muscle Cells

Nobel laureate Otto Loewi made the infamous statement: “Ja Kalzium, das ist alles” - “Yes calcium, that is all”. The universal nature of Ca^{2+} signaling is well established, including in CMs and VSMCs (Amberg & Navedo, 2013; Parekh, 2016). One key biological function that is tightly regulated by Ca^{2+} is muscle contraction. Ringer showed that heart contraction depends on Ca^{2+} (Ringer, 1883), and these foundational findings brought Ca^{2+} signaling research to the forefront for many years to follow. It is evident that all muscle types (smooth, cardiac, skeletal) contract in a Ca^{2+}-dependent fashion through a process that is widely referred to as EC-coupling. Understanding cardiac tissue function requires consideration of both the common and distinct Ca^{2+} signaling processes observed in CM and smooth muscle cells.

Cardiomyocytes (CMs)—Membrane depolarization during an action potential (AP) activates voltage-dependent L-type Ca^{2+} channels (LTCCs) in the sarcolemma, allowing for Ca^{2+} entry into the cell. This Ca^{2+} influx activates Ca^{2+} release from ryanodine receptor type-2 channels (RyR2s) in the membrane of the sarcoplasmic reticulum (SR) in a process termed Ca^{2+}-induced Ca^{2+} release (CICR). RyR2 activation and release across each CM provide enough cytosolic Ca^{2+} to trigger contraction upon binding to troponin C (Bers, 2002). The location of these Ca^{2+} channels (LTCCs and RyR2s) is critical for CM function, as the Ca^{2+} entering the cell through LTCCs rapidly diffuses in the cytoplasm, and thus $[Ca^{2+}]_i$ rapidly decreases with distance from the entry point. These intracellular Ca^{2+} gradients ensure that RyR2s are closed during diastolic periods, and RyR2s located in the vicinity of the LTCCs are activated during cardiac EC-coupling. The RyR2s are clustered in Ca^{2+}-release units, i.e., domains including the LTCC and the SR membrane with RyR2s which openings can be visualized with fluorescent dyes and confocal microscopy as localized, rapid, and brief elevations in $[Ca^{2+}]_i$ termed Ca^{2+} sparks (Cheng & Lederer,

2008). The $[Ca^{2+}]_i$ transient produced by the CICR results from the spatial and temporal summation of Ca^{2+} sparks. In ventricular CMs, the sarcolemma periodically invaginates at intervals roughly coincident with the sarcomeric Z-lines, forming a network of transverse t-tubules that plunge into the depth of these wide (~100–150 μm), thick (~5–15 μm) cells, serving to juxtapose the LTCC-containing sarcolemma within 12–15 nm of the junctional SR at specialized membrane contact sites called dyads. Ca^{2+}-release units are maintained and supported by junctional SR-anchored junctophilin-2, which tethers t-tubules to the junctional SR, traversing the dyadic cleft and interacting with sarcolemmal phospholipids at the t-tubule side. Junctophilin-2 binds to both LTCCs and RyR2 Ca^{2+} channels (Lehnart & Wehrens, 2022), and its alteration has been implicated in cardiac pathology (Guo *et al.*, 2018; Yin *et al.*, 2021). Because of this architecture, ventricular CMs respond to the AP with a fast and homogenous $[Ca^{2+}]_i$ transient. Atrial CMs possess few or no t-tubules, depending on the species. In those atrial CMs without t-tubules, Ca^{2+} release originates on the surface, where RyR2s are close to LTCCs, and then propagates to the central RyR2s from the periphery (Blatter *et al.*, 2003). However, recent work has revealed that atrial CMs have axial tubules of sarcolemma in the center of the cell, with a hypersensitive RyR2 phosphorylation state, which counteracts the scarcity of t-tubules (Brandenburg *et al.*, 2022; Zhang *et al.*, 2022a).

Mathematical models of CM electrophysiology, consisting of coupled, nonlinear differential equations, have been developed with increasing biophysical detail and complexity for more than 50 years (Fink *et al.*, 2011) and have inarguably increased our understanding of cellular electrophysiology and Ca^{2+} signaling in health and disease, including arrhythmia syndromes (Rudy & Silva, 2006; Roberts *et al.*, 2012; Winslow *et al.*, 2016). Computational and theoretical approaches have been central to establishing what we know of the fundamental structure-function relationships in EC-coupling. Mathematical models have attempted to reconcile measurable single protein function and membrane structure with observable changes in cytosolic and SR $[Ca^{2+}]$ that occur in larger surrounding volumes. These efforts generated a large family of models that include an increasingly sophisticated representation of the gradients in intracellular Ca^{2+} concentration occurring during a normal cardiac cycle (Greenstein & Winslow, 2002; Shannon *et al.*, 2004; Restrepo *et al.*, 2008; Winslow *et al.*, 2016). Models in which Ca^{2+} dynamics are governed by the underlying cell ultrastructure (Nivala *et al.*, 2015; Colman *et al.*, 2017; Shiferaw *et al.*, 2020; Zhang *et al.*, 2022a; Zhang *et al.*, 2022b) further inform how the fundamental EC-coupling relationships are altered in atrial vs. ventricular CMs or in CMs with varying t-tubule density and organization. These models have developed interactively with experimental techniques to establish the current state of knowledge describing how nanoscale Ca^{2+} signaling determines macroscopic (cell-scale) EC-coupling in the heart.

Atrial and ventricular CMs are excitable cells that respond with an AP to an electrical impulse that propagates via gap junctions and initiates heart contraction (Bers, 2002). The primary pacemaker cells located in the sinoatrial node (SAN) exhibit automaticity and originate this electrical impulse, which is then transmitted through the atria and into the atrioventricular node (AVN), with AVN myocytes also being automatic but firing APs at a lower intrinsic rate than SAN myocytes. The AVN can induce ventricular contraction when the SAN fails or slow ventricular rate with fibrillating atria. From the AVN,

the electrical impulse then propagates to the ventricles via the conducting myocytes of the His and Purkinje fibers. As discussed at the symposium, nodal cell automaticity is governed by a complex coupled system of cellular "clocks" that integrates ion channels and transporters on the cell membrane surface ("membrane clock") with subcellular Ca^{2+}-handling machinery (referred to as an intracellular "Ca^{2+} clock") (Rubenstein & Lipsius, 1989; Lakatta & DiFrancesco, 2009). While lacking t-tubules, caveolae may provide these cells with structural support for automaticity. Sophisticated nodal cell models have contributed to our understanding of how the different types of ion channels and ion channel isoforms act together with subcellular and global Ca^{2+}-handling mechanisms to produce stable pacemaker activity (Kharche *et al.*, 2011; Maltsev *et al.*, 2011; Yaniv *et al.*, 2013). Nodal cell firing is driven by gradual changes in the membrane potential (V_m) (namely, diastolic depolarization) that are mediated by the concomitant action of these clocks: nodal-specific hyperpolarization-activated current (I_f) and a low voltage-activated T-type Ca^{2+} current ($I_{Ca,T}$) contribute to early diastolic depolarization, while SR Ca^{2+} release via RyR2s occurs late during the pacemaker potential (Rubenstein & Lipsius, 1989; Lakatta & DiFrancesco, 2009). This Ca^{2+} release is spontaneous and/or synchronized by the L-type $Ca_V1.3$ channels that have an activation range more negative than the predominant $Ca_V1.2$ channels (Zhang *et al.*, 2002; Torrente *et al.*, 2016). These local Ca^{2+} releases activate an inward Na^+/Ca^{2+} exchange (NCX) carried current (I_{NCX}), contributing to the late phase of diastolic depolarization and subsequent activation of L-type Ca^{2+} current ($I_{Ca,L}$; $Ca_V1.2$ as in ventricular CMs, and $Ca_V1.3$), which initiates the rapid AP upstroke and global CICR, with $[Ca^{2+}]_i$ transients originating from the periphery and propagating towards the center as in atrial CMs (Bers, 2002).

Cardiac EC-coupling is physiologically regulated by the autonomic nervous system. Under stress conditions, catecholamines are secreted from sympathetic neurons to cause β-adrenoceptor (β-AR) stimulation that activates adenylyl cyclase, producing cAMP, and initiates a signaling cascade allowing the heart to increase its output by increasing the beating rate (positive chronotropy), accelerating conduction (positive dromotropy), increasing strength of contraction (positive inotropy), and ensuring appropriate filling of the chambers by accelerating relaxation (positive lusitropy). The parasympathetic nerves secrete acetylcholine to cause opposite effects, by activation of the muscarinic receptors that suppress adenylyl cyclase activity, thus limiting cAMP levels. Modulation of cAMP levels has several downstream signaling components and functional effects depending on the cell type. In CMs, the main cAMP effect is through activation of the protein kinase A (PKA) (Bers *et al.*, 2019), which phosphorylates several proteins that are key to EC-coupling. The LTCC regulation by β-ARs is mediated by the small GTP-binding protein Rad (Ahern *et al.*, 2019; Liu *et al.*, 2020) and results in a gain of $I_{Ca,L}$ function and subsequent increase in Ca^{2+} content (Liu *et al.*, 2020), along with phosphorylation of phospholamban, which hastens Ca^{2+} reuptake in the SR, and accelerates relaxation (positive lusitropic effect) (Bers, 2002). These effects are also achieved by phosphorylation of the contractile myofibrils by altering cross-bridge cycling and modulating Ca^{2+} affinity. RyR2 is also phosphorylated by PKA, which increases channel open probability. Various sites of RyR2 phosphorylation by PKA have been identified (e.g. S2808 and S2030), though the functional roles of this modulation are debated, especially regarding their importance concerning CaMKII phosphorylation

(Dobrev & Wehrens, 2014). Indeed, the increased $[Ca^{2+}]_i$ facilitated by PKA activation downstream of β-AR stimulation binds to calmodulin (CaM) and activates CaMKII, which phosphorylates several of the same targets as PKA (at different sites), and is itself further activated by Epac - another cAMP effector. Nodal cell AP firing rate acceleration occurs via a direct effect of cAMP on the HCN channels (i.e. via a shift of the I_f activation curve to more negative potentials (Wainger *et al.*, 2001)), PKA phosphorylation of I_f-carrying hyperpolarization-activated cyclic nucleotide-gated channels (Liao *et al.*, 2010), and effects on several Ca^{2+} handling proteins that increase the frequency of diastolic SR Ca^{2+} releases (Vinogradova & Lakatta, 2009).

To probe the functional interactive consequences of protein phosphorylation in an integrative cellular environment, many computational modeling studies incorporate steady-state effects of β-AR activation on the various ion channels and Ca^{2+}-handling proteins (Winslow *et al.*, 2016). Saucerman et al. were the first to develop and validate a functionally integrated system coupling a biochemically detailed model of β-AR signaling with models of Ca^{2+} handling and electrophysiology (Saucerman *et al.*, 2003). This framework was later expanded to study the synergy between PKA and CaMKII signaling and adopted in several contemporary descriptions of EC-coupling in various species (Soltis & Saucerman, 2010; Morotti *et al.*, 2021) and cardiac regions, providing quantitative insights into the individual and combined roles of these kinases in regulating EC-coupling (Winslow *et al.*, 2016).

Vascular Smooth Muscle Cells (VSMCs)—Spindle-shaped VSMCs play an important role in determining vascular diameter and, therefore, control of blood flow and blood pressure. Arteries and arterioles are surrounded by multiple layers or a single layer of SMCs, respectively. In distinction from CMs, each VSMC has only one central nucleus. Importantly, VSMCs allow blood vessels to constrict and dilate and therefore play crucial roles in controlling blood flow, peripheral resistance, and blood pressure.

The mechanisms underlying VSMC contraction are uniquely distinct from those in other muscle types. Canonically, SMC contraction is triggered by a physiological stimulus that increases intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). Different stimuli achieve this outcome through various mechanisms. For instance, an increase in intravascular pressure elevates the vascular wall stress, depolarizing the smooth muscle plasma membrane. VSMC membrane potential (V_M) depolarization is mediated by transient receptor potential (TRP) channels and subsequently leads to the graded opening of voltage-gated Ca^{2+} channels at the VSMC plasma membrane (Knot & Nelson, 1998; Welsh *et al.*, 2002; Inoue *et al.*, 2009). A steady-state change in V_M and Ca^{2+} influx from the extracellular space through voltage-gated Ca^{2+} channels establish the vascular tone. The LTCC is the primary voltage-activated Ca^{2+} influx pathway in VSMCs (Knot & Nelson, 1998). Pharmacological inhibition of LTCCs lowers peripheral resistance in the vasculature and therefore is widely used therapeutically to treat hypertension.

Like CMs, the LTCCs in VSMCs are the target of post-translational modifications that alter their function (Keef *et al.*, 2001). Intriguingly, $Ca_V1.2$ activity can be modified by β-AR/PKA signaling, but the functional implications are unclear. More recently, it was found that elevations in extracellular glucose (e.g. hyperglycemia) potentiate LTCC current

density by direct PKA-mediated phosphorylation of the $Ca_V1.2$ pore-forming subunit α_{1C} at serine 1928, which led to increased vasoconstriction (Nystoriak *et al.*, 2017; Prada *et al.*, 2019; Nieves-Cintron *et al.*, 2021).

Recent data highlight a clear difference in how PKA regulates $Ca_V1.2$ channels in the heart versus the vasculature. Accordingly, PKA-dependent regulation of $Ca_V1.2$ channels in the heart proceeds via Rad proteins, while in the vasculature, β-AR regulation of LTCC occurs via direct phosphorylation of the channel (Liu *et al.*, 2020; Nieves-Cintron *et al.*, 2021). Intriguingly, the cardiac $Ca_V1.2$ pore-forming subunit can still be directly phosphorylated by PKA, although the precise functional implications of this pos-translational modification are still unclear (De Jongh *et al.*, 1996; Hulme *et al.*, 2006; Lemke *et al.*, 2008; Katchman *et al.*, 2017). These observations highlight clear tissue-specific differences in $Ca_V1.2$ regulation. Yet, the detailed mechanisms are unclear, thus representing an area for further exploration.

Other physiological stimuli that evoke VSMC contraction and vasoconstriction include hormone or neurotransmitter release. Several vasoactive substances (e.g., vasoconstrictors such as norepinephrine) are released from sympathetic nerves near the vascular wall and act through G-protein coupled receptors in VSMCs. Receptor agonists that transduce through G_q-coupled receptors ultimately elevate cytosolic $[Ca^{2+}]_i$, due to the generation of inositol 1,4,5-trisphosphate (IP_3) and the downstream activation of IP_3-receptors which facilitate Ca^{2+} release from the SR. Alternatively, the formation of second messengers such as diacylglycerol upon vasoactive receptor activation can stimulate TRP channels and increase $[Ca^{2+}]_i$ due to direct Ca^{2+} influx or due to vascular SMC depolarization and subsequent Ca^{2+} influx (Earley & Brayden, 2015).

When a stimulus elevates cytosolic $[Ca^{2+}]_i$ in VSMCs, the "*contraction*" component of EC-coupling ensues. Unlike CMs, where Ca^{2+} binds to troponin C, Ca^{2+} binds to CaM in VSMCs, activating light chain kinase and phosphoryl transfer to myosin (Eisner *et al.*, 2017). Phosphorylation of myosin triggers myosin/actin binding and cross-bridge cycling and ultimately leads to SMC contraction. Notably, the actin-to-myosin ratio is higher in smooth muscle averaging 15:1, compared to 6:1 in CMs (Murakami & Uchida, 1985). Another distinction is that VSMCs have no intercalated disks or z-disks like those found in CMs. Rather, SMC dense bodies are considered to be analogous to z-disks (Cole & Welsh, 2011; Eisner *et al.*, 2017). Activation of myosin light chain phosphatase (e.g., via β_2-AR stimulation) directly dephosphorylates myosin regulatory light chain and drives the reaction away from cross-bridge cycling, leading to smooth muscle relaxation (Cole & Welsh, 2011; Eisner *et al.*, 2017). An additional key difference between VSMCs and CMs is how RyRs are activated, the role of $Ca_V1.2$ channels in controlling the RyR-mediated Ca^{2+} sparks, and their functional role in controlling EC-coupling (discussed below).

As in the cardiac field, detailed mathematical models have been developed that integrate plasma membrane electrophysiology and Ca^{2+} dynamics in VSMCs to investigate EC-coupling responses to various stimuli (Kapela *et al.*, 2008; Karlin, 2015). These models, validated against broad experimental data sets, have provided mechanistic and quantitative

insights about how different ion channels and distinct signaling pathways interact to alter VSMC electrophysiology and global $[Ca^{2+}]_i$, in health, in response to stress and in disease (Morotti *et al.*, 2017; Syed *et al.*, 2019).

The further refinement of VSMC computational models to include, for example, detailed signaling and regulatory processes, as well as their use to discover new mechanisms that may lay the foundation for the formulation of new hypotheses, represent areas of opportunity.

1.2 Other Cell Types in the Cardiovascular System

The cardiovascular system is highly dependent on intercellular communication for cardiac and vascular development, normal cardiovascular function, as well as in the evolution of disease-causing cardiovascular remodeling. Both myocytes and non-myocytes in the heart and vasculature respond to physiological and pathological stress, and maladaptive changes in non-myocytes, such as cardiac fibrosis or reduced capillary density, are key elements in the pathogenesis of cardiovascular disease. The role of myocytes in normal and pathological heart and vessel function is well documented. Despite the importance of non-myocytes in cardiovascular health and disease, we are only just starting to dissect the interaction patterns between these various cell types and to understand their physiological and pathophysiological significance. In this section, we review the main non-myocyte cell types in the heart and the vasculature (Figure 2) and discuss how the non-myocytes could influence cardiovascular function in health and disease (Figure 3).

Fibroblasts (FBs)—FBs are non-excitable cells that may originate from primary mesenchymal cells, local epithelial-mesenchymal transition, or bone marrow-derived precursors (Stenmark *et al.*, 2013; Saljic *et al.*, 2022). FBs are genetically, morphologically, and functionally distinct from smooth muscle, myo-FBs, and pericytes (Di Carlo & Peduto, 2018; Muhl *et al.*, 2020; Rajan *et al.*, 2020; Lendahl *et al.*, 2022). The key function of FBs is to produce constituents of the extracellular matrix (ECM). Intriguingly, FBs show both common and distinctive signature markers in different organs and pathological conditions (Muhl *et al.*, 2020), thus highlighting their heterogeneity and ability to produce diverse ECM in various tissues. In the heart, FBs are interspersed between CMs throughout the myocardium and interconnected by connexin junctions to form a sheet-like cellular layer that supports cardiac structures (reviewed in (Ongstad & Kohl, 2016)). In addition, FBs reside proximal to blood vessels, where they contribute to vessel structure, formation, and stabilization (Newman *et al.*, 2011; Rajan *et al.*, 2020). FBs possess a large surface area with sheet-like extensions, elongated cytoplasmic processes, and irregular folds that allow for long-distance signaling.

In the heart, most cardiac FBs originate from the epithelial cells of the proepicardium through epithelial-mesenchymal transition. Cardiac FBs may also originate from the epicardium before migrating into atria and ventricles and differentiating into cardiac FBs. In the vasculature, single-cell transcriptomic analysis and lineage studies suggest that FBs most likely arise from cells of mesenchymal origin in different animal models (Muhl *et al.*, 2020;

Rajan *et al.*, 2020). Endothelial-mesenchymal transformation has also been described, along with other less frequent origins, such as bone marrow-derived cells, hematopoietic cells, and mesangioblasts.

FBs in cardiac and vascular tissue respond to a wide range of (patho)physiological stimuli. Various interactions between CMs, vascular cells, and FBs have been described in the heart, blood vessels, and lung vasculature and include biochemical, electrical, and/or biomechanical signals (Stenmark *et al.*, 2013; Pellman *et al.*, 2016). Paracrine mediators allow for indirect communication as they are secreted from one cell type and diffuse onto the other cell types. Activation of paracrine TGF-β1 and angiotensin II signaling have been widely studied and shown to affect CM-FB communication in the context of cardiac fibrosis, hypertrophy, arrhythmias, hypertension, atherosclerosis, and vascular injury (Stenmark *et al.*, 2013). Recent data suggest that FB Nox2 signaling plays a role in the development of vascular remodeling and hypertension, and this may be driven by paracrine signaling between FBs and VSMCs (Harrison *et al.*, 2021). The impact of these signals on fibrotic and arrhythmogenic pathways might differ in atria, and this may involve both effects on CM-FB paracrine interaction and direct effects on CMs. Likewise, given the role of FBs in different vascular beds, regulatory signals, such as TGF-β1 and angiotensin II, may have a distinct impact on vascular SMC excitability, migration, proliferation, and growth (Stenmark *et al.*, 2013). Additionally, interleukins have more recently been involved in CM-FB communication in the context of cardiac hypertrophy, and Wnt signaling has been implicated in fibrosis and cardiac repair. Interleukins also influence FB-VSMC communication to promote vascular smooth muscle and ECM remodeling via non-coding RNA modifications, thus contributing to hypertension (Melton & Qiu, 2021). CMs and FBs can also be directly coupled via gap junctions (De Maziere *et al.*, 1992) and membrane nanotubes (He *et al.*, 2011), which provide support for electrical communication. While FBs are unable to generate APs, they can alter myocardial conduction via an electrotonic load through effectively increasing the cardiac cell capacitance and altering the resting membrane potential.

Computational models linking CMs and FBs, initially represented as electrically passive and eventually incorporating active experimentally-determined ion currents (MacCannell *et al.*, 2007), have been useful for examining the role of cardiac FBs in electrical conduction and tissue remodeling in the heart (reviewed in (Zeigler *et al.*, 2016)). FBs may alter vascular tone or vascular reactivity by modulating the contractility of VSMCs or remodeling the structure of the vessel wall (Melton & Qiu, 2021). Moreover, FB activation in response to pathological stress may lead to excess production of ECM components (e.g. collagen and fibronectin) and secretion of enzymes that degrade ECM components (e.g. matrix metalloproteinases, MMPs, and tissue inhibitors of MMPs), which can be sensed by CMs and VSMCs, thus altering their electrophysiology, organization and contractile state (Melton & Qiu, 2021). Several multi-scale computational models that incorporate FB-vascular cell interactions have been developed and have been used to acquire insight into mechanisms underlying atherosclerosis, restenosis, and vascular remodeling (Corti *et al.*, 2021).

The exact molecular pathways controlling FB activation, proliferation/differentiation, and those regulating transcription of ECM proteins remain largely unknown. To address these

unresolved questions, large-scale computational models of cardiac FB signaling have been developed, accounting for several context-specific regulators of fibrosis (Zeigler *et al.*, 2016) and virtual drug screening (Zeigler *et al.*, 2021). New modeling approaches are required to deepen our understanding of how FB-VSMC interaction patterns alter vascular reactivity in health and disease. Moreover, a key question that remains to be addressed is the extent to which FB-myocyte electrical coupling alters the properties of native cardiac and vascular tissue (Ongstad & Kohl, 2016).

Endothelial Cells (ECs)—ECs originate from the mesoderm, and their renewal and replacement are mediated by resident cells in the local environment (Aquino *et al.*, 2021). ECs are critical regulators of the cardiovascular system. In the vasculature, the influence of ECs in the control of vascular function is well documented (King *et al.*, 2022). These cells line the lumen of arteries and arterioles and are the principal cell type forming capillaries. EC expression profile may be distinct depending on the vascular bed, which may lead to diverse functions. For example, ECs in the central nervous system have low expression of leukocyte adhesion molecules that may limit the movement of immune cells into the brain (Rossler *et al.*, 1992). Another example is the unique ability of capillary ECs, compared with arterial/arteriolar ECs, to initiate retrograde hyperpolarization leading to vasodilation and increased cerebral blood flow, with arteries and arterioles presumably exhibiting faster signal attenuation (Longden *et al.*, 2017). ECs communicate with underlying VSMC via diffusible molecules (e.g. nitric oxide, ATP, K^+) and/or electrical signals via gap junctions in myoendothelial projections. These endothelial-derived signals act on VSMCs to alter their contractile state, thus influencing vascular reactivity, tissue perfusion, and blood pressure in health and disease. Capillaries are critical for gas and nutrient exchange and, more recently, have been proposed as a sensory web for the regulation of neurovascular coupling and cerebral blood flow (Longden *et al.*, 2017; Alvarado *et al.*, 2021). The communication between ECs and VSMCs was examined in further detail in a recent comprehensive review (King *et al.*, 2022). Biophysically detailed models of EC plasma membrane electrophysiology allowed analyzing the interplay between V_m and intracellular Ca^{2+} dynamics (Silva *et al.*, 2007). This integrative model substantially improved our understanding of the vascular EC-VSMC interaction and how this communication affects vasoreactivity (Kapela *et al.*, 2010).

ECs have been described as the most abundant non-myocyte population in the heart (Pinto *et al.*, 2016). Most ECs in the heart are in the microcirculation, where they form the capillaries and serve a structural role as a barrier between blood and the myocardial tissue. ECs also communicate with adjacent CMs by producing paracrine factors that play essential roles in cardiovascular homeostasis, including modulation of CM contractility, growth, and survival (Lim *et al.*, 2015). In the heart, nitric oxide, endothelin-1, neuregulin, and apelin produced by ECs have been shown to regulate the functions of neighboring CMs, and disturbances in these pathways have been shown to contribute to the development of heart failure (Lim *et al.*, 2015). VEGFR2-ErbB signaling has recently emerged as a key pathway for the regulation of cardiac growth and homeostasis, whereby activation of VEGFR2 signaling

in cardiac ECs induces angiogenesis and release of ErbB receptor ligands, which in turn activate growth signaling in CMs (Kivela *et al.*, 2019).

Neuronal cells—Neuronal control of the cardiovascular system is well appreciated. Both heart and vascular tissue are surrounded by neurons to control their function. Neuronal activity may also influence the structural organization of the tissue, including vascular networks (Lacoste *et al.*, 2014). The intrinsic cardiac nervous system includes all neurons located in the heart within the pericardium and those in the ganglionated plexi primarily located on the posterior atrial wall and fat pads. The intrinsic cardiac nervous system contains complex networks of neurons and interconnecting nerves that include afferent neurons (i.e., sensory), efferent neurons (i.e., parasympathetic and sympathetic), and interconnecting local circuit neurons (Hanna *et al.*, 2017). Likewise, blood vessels are innervated by afferent (e.g., sensory fibers) and efferent (e.g., autonomic sympathetic fibers) fibers to control vascular function (Westcott & Segal, 2013; Shoemaker *et al.*, 2015). Intriguingly, autonomic innervation increases with a decrease in the size of blood vessels, with arterioles being highly innervated by these fibers. Afferent neurons are mechano- and/or chemoreceptors that sense cardiovascular function on a beat-to-beat basis (Hadaya & Ardell, 2020). These signals are transmitted locally as well as to higher centers, which in turn modulates efferent outflow. Even minor alterations in R-R interval and/or activation sequence (as occurs during premature ventricular contractions) increase afferent activity and efferent outflow, leading to electrical instability on subsequent beats (Hamon *et al.*, 2017). During ischemia, the release of metabolites, including bradykinin, prostaglandins, and protons, stimulates afferent neurons and leads to reflex increases in sympathetic outflow (Longhurst *et al.*, 2001). Afferent signaling also plays a role in chronic disease remodeling, as ablation of sensory neurons improves cardiac function after myocardial infarction (MI) and slows the progression of heart failure (Wang *et al.*, 2014), as well as leads to a reduction in blood pressure in spontaneously hypertensive rats (Li *et al.*, 2022).

Efferent fibers communicate with CMs and blood vessels via neurotransmitters (primarily norepinephrine for sympathetic and acetylcholine for parasympathetic neurons). Norepinephrine produces prototypical cardiac fight-or-flight responses (e.g., CM and VSMC contractility leading to increased inotropy and vasoconstriction), and acetylcholine has the opposite effects (e.g., VSMC relaxation leading to vasodilation). Sympathetic neuron-tissue signaling is likely to occur in a quasi-synaptic fashion, with relatively limited diffusion of norepinephrine outside the junctional cleft, thus facilitating the stimulation of only those β-ARs on cells that are in close contact with the neuron (Prando *et al.*, 2018). Efferent sympathetic neurons can modulate CM size via regulation of cellular proteolytic machinery, with increased neuron density correlating with increased CM size (Pianca *et al.*, 2019). Moreover, sympathetic neuronal activity distinctively influences different vascular beds, with effects in smaller arteries leading to increased vascular resistance and alterations in blood flow, and in larger arteries resulting in alterations in arterial stiffness (Bruno *et al.*, 2012). Sex differences may also play a key role in how sympathetic neuronal activity modulate cardiovascular function (Hissen & Taylor, 2020). In addition to the primary neurotransmitters, efferent neurons also release abundant co-transmitters, including neuropeptide Y, galanin, and ATP for sympathetic neurons, and vasoactive intestinal peptide,

nitric oxide, and ATP for parasympathetic neurons (Burnstock, 2009). Co-transmitter releases affect many cell types, including CMs, VSMCs, ECs, and also feedback to the neurons themselves to impact subsequent neurotransmitter release (Tan *et al.*, 2018).

Immune cells—Macrophages are essential components of the immune system and play a role in the normal functioning of the cardiovascular system (Moskalik *et al.*, 2022). Macrophages may derive from embryonic precursors, blood monocytes, or hemogenic endocardium and increase in number by *in situ* proliferation (Moskalik *et al.*, 2022). Macrophages are in close association with the vasculature, where they can serve many roles as part of the immune response to injury or pathological state, including in vascular repair and regeneration and modulation of vascular reactivity leading to changes in blood flow and tissue perfusion (Corliss *et al.*, 2016; Hong & Tian, 2020; Barkaway *et al.*, 2022). In the adult heart, resident cardiac macrophages represent ~6–8% of the non-myocyte population (Litvinukova *et al.*, 2020), with their proportion changing with aging, cardiovascular disease state and progression, and depending on sex. The various functions of macrophage populations in the cardiovascular tissue depend on their origin and phenotype.

Macrophages in the cardiovascular system maintain tissue homeostasis by defending the body from pathogens and eliminating apoptotic cells. Macrophages also help to regulate extracellular matrix turnover, remove debris shed by local cells, and adapt to altered and increased tissue stress and strain. It has been suggested (but not yet tested) that macrophages may therefore play an important role in physiological hypertrophy that occurs with exercise or pregnancy (Swirski & Nahrendorf, 2018). According to a recent report, resident macrophages located in the atrioventricular node directly modulate electrical properties of CMs owing to macrophage coupling with CMs via Cx43-containing gap junctions (Hulsmans *et al.*, 2017). Specifically, macrophages depolarize the resting membrane potential of CMs and, according to computational modeling, accelerate their repolarization, thereby potentially leading to arrhythmias (Hulsmans *et al.*, 2017). Likewise, macrophages can modulate vascular function, blood flow, and tissue perfusion by altering the contractile state of VSMCs and pericytes via the release of vasoactive molecules (Barkaway *et al.*, 2022). Mechanisms by which macrophages could modulate the contractility of mural cells include changes in intracellular Ca^{2+}, ROS signaling, reduced nitric oxide bioavailability, and inhibition or hyperactivation of ion channels.

Direct electrical coupling between VSMCs and macrophages, and the *in vivo* significance of electrical coupling of CMs and macrophages remain to be determined and thus represent areas for promising research.

Within minutes of ischemia, the leukocyte population of the cardiac and vascular network changes dramatically, with neutrophils infiltrating first and peaking within ~24 hours, followed by the influx of monocyte-derived macrophages (Frodermann & Nahrendorf, 2018; Hong & Tian, 2020). This process is critical for proper wound healing, vascular repair, and angiogenesis (Shirai *et al.*, 2015). However, dysregulated cardiac and vascular inflammation may play a role in arrhythmias (e.g., atrial fibrillation (Dobrev *et al.*, 2022)) and heart failure progression (Dick & Epelman, 2016), as well as atherosclerosis and vasculitis (Shirai *et*

al., 2015). Indeed, recent studies suggest that infiltrating neutrophils following MI increase arrhythmia burden via ROS production, whereas infiltrating macrophages reduce arrhythmia incidence by removing dead or damaged cells and by supporting CM mitochondrial function (Grune *et al.*, 2022). Infiltrating macrophages post-MI, after inflammatory condition or pathology may also secrete a variety of pro-inflammatory cytokines, including TNF-α, IL-1β, and IL-6, which may have deleterious effects on CM and mural cell function (Francis Stuart *et al.*, 2016; Heijman *et al.*, 2020; Barkaway *et al.*, 2022; Dobrev *et al.*, 2022). In this issue of the Journal of Physiology, Chowkwale et al. developed a computational model of diverse immune cell types (neutrophils, monocytes, macrophages, FBs) and cytokine dynamics post-MI. The model predicted that neutrophil-IL-1β positive feedback recruited/activated monocytes/macrophages, which amplified TGFβ-mediated fibrosis (Chowkwale *et al.*, 2022). The authors termed this cascade inflammation-fibrosis coupling, drawing parallels with the regulatory structure of excitation-contraction coupling. Further, the model predicted that the ultrasensitive amplification of fibrosis by inflammation was driven primarily by FB proliferation rather than collagen expression per cell (Chowkwale *et al.*, 2022).

Adipocytes—Adipose tissue regulates cardiovascular health by producing and releasing several active molecules that may impact cardiovascular function. Adipose tissue that directly surrounds the heart is known as epicardial adipose tissue. Adipocytes originate from adult cardiac progenitor cells in the epicardium that can undergo epithelial-to-mesenchymal transition. Epicardial adipose tissue is present in both healthy and diseased individuals, and its volume increases during the first 40 years of life, as well as in the presence of inflammation or cardiomyopathy. Its main function is to protect the heart from mechanical deformation and provide storage for free fatty acids (Ernault *et al.*, 2021). Likewise, most blood vessels are surrounded by adipose tissue, known as perivascular adipose tissue (Chang *et al.*, 2020). The developmental origins of adipocytes of the perivascular adipose tissue are unclear but seem to vary depending on the location of the blood vessel and could even share similar precursor cells as those leading to vascular smooth muscle in the particular vascular bed.

Adipocytes secrete numerous adipokines, which modulate (myo)FB and myocyte physiology (Chang *et al.*, 2020; Ernault *et al.*, 2021). These adipokines may be protective in healthy hearts, preventing inflammation and fibrosis. However, adipokines secreted from adipocytes may switch to pro-inflammatory and pro-fibrotic, leading to reactive oxygen species generation. Pro-fibrotic adipokines stimulate myoFB differentiation, causing pronounced fibrosis in the epicardial adipose tissue and the myocardium and promoting arrhythmogenic cardiac remodeling (Gawalko *et al.*, 2022). Inflamed perivascular adipose tissue in response to pathological conditions may also influence neointima formation by triggering phenotypic switching of VSMCs from contractile to synthetic (Chang *et al.*, 2020).

Adipose tissue may also influence cardiac and vascular electrophysiology via adipokines and/or through electrotonic interactions, which could have an impact on cardiac function

and vascular tone. However, whether adipocytes in can electrically couple to CMs or VSMCs remains unknown, and thus an area for further exploration.

Pericytes—Pericytes are emerging as significant regulators of the cardiovascular system. They may originate from mesenchymal and/or neural crest cells, depending on their location in the body, but may also be derived from bone marrow cells during tumor-induced angiogenesis (Kelly-Goss *et al.*, 2014; Stapor *et al.*, 2014; Trost *et al.*, 2016). The genetic profile of pericytes seems to be heterogenous between different tissues but less variable within the same tissue (Trost *et al.*, 2016). In the vasculature, pericytes wrap around ECs. Pericyte morphology is diverse, with recent examples showing their characteristic “bump on a log” shape with differences in extension and projection as the vessel transition from the post-arteriole to the higher-order capillary regions (Gonzales *et al.*, 2020; Hariharan *et al.*, 2020). Pericytes communicate with ECs via gap junction and more recently was shown that they could even communicate among themselves via interpericyte tunneling nanotubes (Alarcon-Martinez *et al.*, 2020). Although the functional role of pericytes is still under intense investigation, they have been clearly shown to contribute to the stabilization of blood vessels, angiogenesis, regulation of EC function, and maintenance of the blood-brain barrier (Stapor *et al.*, 2014; Trost *et al.*, 2016; Hartmann *et al.*, 2022). Recent data also suggest that pericytes contribute to the regulation of blood flow by contracting in capillary junctions to direct flow (Gonzales *et al.*, 2020).

In addition to their well-established association with ECs, pericytes play an essential role in cardiac physiology and pathology by interacting with other adjacent cell types, such as CMs, FBs, and adipocytes (Su *et al.*, 2021). Several lines of evidence suggest that cardiac pericytes are critical to sustaining cardiac function during myocardial injury, as reviewed in (Yin *et al.*, 2021). Specifically, VEGF and miR-132 from pericytes were found to induce survival responses in CMs, and through secreting stromal cell-derived factor-1, pericytes were shown to activate the mobilization of stem cells, thus benefiting cardiomyogenesis. Furthermore, some pericytes sharing the same origin of epicardium with CMs exhibit myogenic capacity and were found to function as (and replace) CMs. In addition, some pericytes can differentiate into immature CM-like cells.

In many ways, cell diversity in cardiac tissue is confounded by overlapping and interconnected cell behaviors (Figure 3). The complexity and emerging knowledge gaps are highlighted by the overlapping cell phenotypes and the potential for direct cell coupling. CMs, FBs, pericytes, macrophages, ECs, and neuronal cells share phenotypical and potential lineage characteristics. Future studies are needed to elucidate the implications of common cell origins and how the differentiated cell populations interact. Throughout this section, we have highlighted several unresolved questions, which focus on the local cardiac tissue microenvironment, investigation of multi-cell interactions, and understanding how these interactions are temporally and spatially regulated.

2. Contribution of Cell Diversity to Cardiovascular Remodeling Associated with Development, Disease, and Aging

The diverse cell types and signals described in the previous sections distinctly contribute to the development of disease-promoting cardiovascular remodeling with molecular, cellular, and interstitial changes manifesting clinically as alterations in the structure and function of the heart and vessels after injury. The remodeling process involves myocyte growth and death, fibrosis, inflammation, and electrophysiological alterations, which are the result of subcell-, cell- and tissue-level responses to pathophysiological stimuli. In the following paragraphs, we describe the multi-level aspects of cardiovascular remodeling during development, aging, and diseased states, as well as the contribution of different cell populations and signals.

2.1 Cellular Energetic Costs of Cardiac Function

Due to the immense energetic requirements of mechanical pump function, the heart is a major contributor to whole body oxygen consumption (Rolfe & Brown, 1997; Knaapen *et al.*, 2007). At an organ level, cardiac consumption rates for oxygen and catabolic substrates are dynamic and determined largely by the systolic parameters of contractile force generation, wall stress, and heart rate. With each contractile cycle, CMs expend ATP in biochemical reactions that maintain transmembrane ionic gradients and drive actin-myosin cross-bridge cycling, thereby orchestrating AP generation and repolarization, CICR, contraction, and relaxation. Recent work using single-cell RNA sequencing (scRNA-seq) has indicated subpopulations of CMs in adult human myocardium that are enriched in nuclear-encoded mitochondrial genes (*NDUFB11*, *NDUFA4*, *COX7C*, and *COX5B*), suggesting regional heterogeneities in cellular workload capacities (Litvinukova *et al.*, 2020).

Whether specialized subgroups of CMs represent stable cell populations or are modified in number or spatial distribution throughout development or in response to environmental or pathological stimuli is still unclear. If so, modulation of regional CM heterogeneities may drive large-scale changes in cardiac metabolic requirements during development and in homeostasis and disease progression.

Cell types in the heart other than CMs contribute directly and indirectly to balancing oxygen and nutrient delivery, ATP generation, and CM contractility. While the oxygen and substrate input needed to support cellular functions in VSMCs and ECs, FBs, and immune cells are low relative to that in CMs (Wagner *et al.*, 2011), these cell populations influence CM workloads and, thus, energetic demand. For instance, cardiac FBs proliferate and activate to a contractile myoFB state in response to mechanical tension or injury-associated stimuli (cytokines and chemokines) (Frangogiannis, 2019). Once activated, cardiac FBs remodel the structure and composition of the ECM. This ECM remodeling can occur within days of ischemic injury to replace dead or dying CMs to prevent wall rupture or can develop more slowly in the interstitium and perivascular spaces to provide structural support as an adaptation to hemodynamic stress. Regardless of the stimulus, ECM replacement,

collagen deposition, and structural modifications (i.e., crosslinking) can alter myocardial wall compliance and influence the relationship between energy expenditure and cardiac output (Lopaschuk *et al.*, 2021). Thus, ECM-dependent changes in wall stress contribute to CM remodeling that ultimately impacts metabolic pathway flux and may contribute to energetic deficits in the failing heart.

The immune system also exerts profound effects on myocardial function and metabolism. Macrophage populations include those of embryonic origin (tissue resident) and monocyte-derived, which differ with respect to inflammatory patterns and metabolic profiles (Patel *et al.*, 2018; Dick *et al.*, 2019). Differential consumption of nutrient metabolites by distinct populations of macrophages may regulate the local availability of substrates for other cell types (Kedia-Mehta & Finlay, 2019). Tissue-resident macrophages have also been shown to eliminate dysfunctional mitochondria that are expelled by otherwise healthy proximal CMs (Nicolas-Avila *et al.*, 2020). Conversely, proinflammatory macrophages may play a key role in altered redox conditions that are linked to age-associated disease states. In advanced age, accumulated senescent cells, via secretion of cytokines, can promote the expansion of $CD38^+$ M1-like macrophages and reductions in tissue NAD levels (Covarrubias *et al.*, 2020), as well as CM activation leading to the production of IL-1β and consequent arrhythmia (Heijman *et al.*, 2020). Activation of inflammatory pathways and secreted inflammatory cytokines have also been shown to impact repolarizing currents and cause Ca^{2+}-mediated voltage instabilities, such as delayed afterdepolarizations, in CMs (Heijman *et al.*, 2020), which may produce a substrate for arrhythmia and remodeling of the conduction system (Lazzerini *et al.*, 2022).

Much attention has focused on how cardiac metabolism adapts under physiologic and pathologic states and how altered metabolic states influence overall function. The heart is thought of as a metabolic omnivore in that it is capable of processing multiple substrates to produce ATP. While the healthy heart relies on β-oxidation of fatty acids for the majority of ATP generated, it can also derive energy from other sources, including glucose, pyruvate, lactate, branched-chain amino acids, and ketone bodies (Lopaschuk & Ussher, 2016). The balance of substrate utilization becomes altered toward greater reliance on glucose in the setting of heart failure and is associated with decreased mechanical efficiency and adverse outcomes (Lopaschuk *et al.*, 2021). Moreover, advanced age is associated with a reduction in the mitochondrial machinery required for fatty acid oxidation and an increase in glycolytic enzymes and intermediates in CMs (Lesnefsky *et al.*, 2016).

The upstream mechanisms that drive metabolic remodeling are poorly understood. Whether these changes occur because of direct signaling at the level of CMs or from external factors such as vascular dysfunction and insufficient oxygen delivery relative to demand remains to be clarified. Nonetheless, changes in vascular structure or function may be a key contributor (Anversa *et al.*, 1994). Oxygen delivery via the blood supply is inherently linked to the metabolic demand of the myocardium (Feigl, 1983). Changes in vascular structure and function with age may suppress perfusion with the consequence of ischemia-related remodeling and contractile dysfunction (Heusch, 2022). Further work to

determine the underlying causes of disease-related metabolic and functional remodeling is warranted.

Metabolic adaptations are also important for postnatal heart development. Fetal and neonatal cardiac physiology is fundamentally different from that of an adult, and a number of modifications are required to transition from the intra- to extrauterine environment (Hew & Keller, 2003; Morton & Brodsky, 2016; Prada *et al.*, 2020). This dynamic process is most evident at the cellular level, as CMs undergo a series of morphological and metabolic changes during their transformation into mature CMs (Chang, 1988; Kannan & Kwon, 2020; Karbassi *et al.*, 2020). To adapt to increased workload and energy demand after birth, immature CMs undergo physiological hypertrophy, which is accompanied by a withdrawal from the cell cycle and loss of proliferative capacity (Li *et al.*, 1996; Soonpaa & Field, 1998). Hypertrophic growth transforms these small, circular cells into large rod-shaped CMs with a massive 10-fold increase in cell volume (Mollova *et al.*, 2013; Pohjoismaki *et al.*, 2013; Velayutham *et al.*, 2020). Developing CMs also undergo a metabolic switch, wherein cells become less dependent on glucose and more dependent upon fatty acid oxidation for efficient energy production (Lopaschuk & Jaswal, 2010). To accommodate this switch, mitochondria increase in number and size - occupying nearly 20–40% of the total cell volume in adult CMs (Barth *et al.*, 1992). As mitochondria mature, densely organized cristae are also formed and the mitochondrial network aligns with myofibrils to efficiently supply ATP for contractile function and ion pump activity (Kaasik *et al.*, 2001; Saks *et al.*, 2001; Andrienko *et al.*, 2003; Bleck *et al.*, 2018). The overall increase in cell size is accompanied by a large-scale expansion and alignment of myofibrils (Bishop *et al.*, 1990; Lundy *et al.*, 2013), and maturation of sarcomeric proteins from fetal to adult isoforms (e.g., troponin, titin, myosin heavy chain, myosin light chain; (Opitz *et al.*, 2004; Talman *et al.*, 2018)).

2.2 Cellular Topologies and Impact on Cardiovascular Function in Health and Disease

Maintenance of optimal cardiac function depends on complex heterocellular signaling paradigms that serve to maintain homeostasis, as discussed above. Likewise, aberrant communication among CMs and interstitial cells is a central player in the development and progression of declining cardiac function in disease. A primary example is the regulation of cardiac FB activation by CMs and macrophages that contributes to ECM remodeling. In the setting of acute myocardial injury due to ischemia, death of CMs triggers leukocyte infiltration and an acute inflammatory response (Prabhu & Frangogiannis, 2016). Resultant elevation of macrophage-derived inflammatory cytokines (e.g., TGF-β, IL-6, IL-4, and IL-1) promotes the proliferation and activation of cardiac FBs, transforming them into a profibrotic phenotype (Guo *et al.*, 2012; Kanellakis *et al.*, 2012; Saxena *et al.*, 2013; Khalil *et al.*, 2017). Activated FBs degrade existing ECM and deposit altered matrix proteins that ultimately result in scar formation in the infarct region that prevents wall rupture but also increases wall stiffness and reduces compliance (Nielsen *et al.*, 2019). Changes in wall tension may also alter the availability of latent ECM-localized growth factors and cytokines to CMs (Bowers *et al.*, 2022), thus promoting hypertrophic growth and, in turn, altering wall mechanics and CM-derived proteins to the ECM that could influence immune cell and FB activation states. Even in the absence of ischemia, prolonged activation of

several molecular processes and increased FB activation contributes to age-related collagen accumulation, interstitial and perivascular fibrotic remodeling, and functional decline (Biernacka & Frangogiannis, 2011). Further research that elucidates molecular interactions between cells of the heart and the ECM is needed to advance the development of more effective antifibrotic therapies.

Recent work has also advanced our knowledge of the local intercellular signaling that underlies local control of oxygen delivery to the myocardium. Zhao et al. investigated the molecular processes that mediate coupling between CM metabolic demand and local and regional blood flow, which have remained elusive for decades. The authors provided evidence that a population of capillary ECs is electrically coupled to CMs, such that hyperpolarizing signals may be transmitted from the myocardium to the vasculature (Zhao *et al.*, 2020). In the case of declining ATP in CMs, activation in ATP-sensitive K^+ channels could thereby be transmitted to the local capillary network to induce upstream arteriolar vasodilation and increase oxygen delivery to the affected CMs. At the level of coronary arteries and arterioles, other work has shown that acute increases in CM workload and oxygen demand are transmitted by an unknown mechanism to proximal VSMCs, culminating in altered vascular redox pairs (Dwenger *et al.*, 2022). As discussed at the symposium, acute elevation of cytosolic NADH levels in VSMCs as a result of enhanced myocardial oxygen demand is, in turn, sensed by smooth muscle Kv1 channels, which are differentially regulated by cellular redox via associating heteromeric β subunit assemblies (Dwenger *et al.*, 2018; Ohanyan *et al.*, 2021).

To date, the mechanisms of local blood flow regulation have been identified/investigated under homeostatic conditions. Further work is necessary to determine how altered myocardial metabolism in aging and specific cardiovascular disease states may disrupt the balance of oxygen supply-demand balance via newly identified heterocellular signaling axes.

In a similar manner, biochemical cues including oxygen levels, glucose/fatty acid availability, glucocorticoid or thyroid hormone signaling (Burridge *et al.*, 2014; Neary *et al.*, 2014; Yang *et al.*, 2014b; Rog-Zielinska *et al.*, 2015; Parikh *et al.*, 2017; Yang *et al.*, 2019) and biophysical cues including electromechanical conditioning, micropatterning, or substrate stiffness also help to guide CM development (Bakunts *et al.*, 2008; Heidi Au *et al.*, 2009; Bhana *et al.*, 2010; Kim *et al.*, 2010; Feaster *et al.*, 2015; Ronaldson-Bouchard *et al.*, 2018; Herron *et al.*, 2016). As an example, hypoxia signaling has been identified as a key regulator of CM maturation. Fetal CMs are well adapted to the intrauterine hypoxic environment, but with birth, the change in ambient oxygen levels initiates a reduction in hypoxia signaling (e.g., oxygen-sensitive transcription factors HIF1α and HAND1), which is accompanied by subsequent adaptations in cardiac bioenergetics and mitochondrial remodeling (Breckenridge *et al.*, 2013; Neary *et al.*, 2014). Of interest, chronic hypoxia can extend the window of CM immaturity, as evident by smaller CM size, continued CM proliferation, increased HIF1α signaling, reduced mitochondrial cristae density, and metabolism – while hyperoxemia promotes oxygen-dependent mitochondrial metabolism and cell cycle arrest (Puente *et al.*, 2014). Hypoxic signaling is being explored as a potential

therapeutic target, which could allow CMs to reenter the cell cycle, promote cardiac regeneration, and improve myocardial recovery following injury (e.g., myocardial infarction (Jopling *et al.*, 2012; Kimura *et al.*, 2016; Kimura *et al.*, 2017; Nakada *et al.*, 2017; Savla *et al.*, 2018).

CM maturation is also guided through interactions with non-CM cells and the tissue microenvironment. When co-cultured with adult FBs (but not neonatal FBs), neonatal CMs display multiple markers of maturation, including withdrawal from the cell cycle, aligned myofibrils, formation of t-tubules, and increased expression of the gap junctional protein connexin-43 (Wang *et al.*, 2020). Similarly, human stem cell derived-CMs (hiPSC-CM) hypertrophy and develop well-organized myofibrils when engrafted into an adult rat heart, while the maturation rate is significantly slowed when engrafted into younger neonatal hearts (Kadota *et al.*, 2017). Co-culturing hiPSC-CMs with epicardial cells (precursor to cardiac FBs and ECs) or ECs alone also produces a more mature electrophysiological phenotype (Dunn *et al.*, 2019), improved Ca^{2+} handling and contractility, and promotes vascularization when implanted (Bargehr *et al.*, 2019). Similarly, when 3D multicell-type microtissues are constructed, intercellular interactions with FBs and ECs promote the maturation of CMs – with the development of sarcomeric structures, enhanced contractility, increased oxidative metabolism, and a more mature electrophysiological phenotype (Giacomelli *et al.*, 2020). Taken together, these findings indicate that CMs are highly responsive to cell-cell interactions with non-myocytes and that both FBs and ECs play a supportive role in guiding the CM maturation process.

2.3 Subcellular Structural and Functional Heterogeneity in Cardiovascular in Health and Disease

While we have so far focused on the diversity of cell types that govern cardiovascular function, it is important to note that heterogeneity also exists within any given cell type, including molecular structural and functional diversity that play important roles in normal function and can importantly contribute to disease induction and progression. Ion channel remodeling is a hallmark of cardiovascular development, disease, and aging. The number and functional availability of ion channels at the sarcolemma limit the magnitude ion fluxes across it. In the heart and vasculature, these fluxes impact contraction and relaxation of the muscles. The abundance of ion channels at the sarcolemma is dictated by the lifetime of the channels and the balance between channel insertion, removal, and degradation. A further regulatory influence over ion flux across the plasma membrane is the clustering state of the channels. The idea that the nanoscale arrangement of ion channels in the plasma membrane can impact their function is an emerging concept in several fields, including cardiac and vascular smooth muscle physiology (Del Villar *et al.*, 2021; Dixon *et al.*, 2022). At the symposium, it was discussed that this concept that the clustering state of ion channels affects their activity also extends to SR-localized RyR2, where heterogeneity of cluster sizes has been found to support arrhythmogenic Ca^{2+}-wave propagation (Galice *et al.*, 2018; Xie *et al.*, 2019). Control of transmembrane ion fluxes is also determined by the expression of distinct pore-forming and accessory subunit isoform heterogeneity, which may evolve during maturation to achieve optimal fine-tuning of cardiovascular signaling and function and can be dysregulated in disease. We discuss the state of knowledge for each of these

channel types below, considering how maturation, aging, and disease processes, including heart failure and hypertension, affect channel arrangements with functional implications and highlighting open questions, controversies, and challenges in the field.

Trafficking, recycling, and nanostructural rearrangement of L-Type Ca^{2+} Channels (LTCCs)—The first important question to answer is how can clustering of LTCCs affect their activity. Work from multiple labs has revealed that physical proximity between adjacent Ca_V channels in clusters facilitates their dynamic, reciprocal, and allosteric interactions (recently reviewed in (Dixon *et al.*, 2022)). Groups of channels within clusters communicate with each other to coordinate their gating behavior so that the opening of one channel can drive the opening of other adjoined channels. The interactions are initiated by the binding of incoming Ca^{2+} to CaM and proceed through Ca^{2+}/CaM binding to the $Ca_V1.2$ pre-IQ domain (Dixon *et al.*, 2015; Moreno *et al.*, 2016). This 'functional coupling' or 'cooperative gating' facilitates and amplifies Ca^{2+} influx because physically interacting channels within clusters adopt the open probability of the most active channel, as demonstrated in optogenetic experiments in which stimulated interactions between $Ca_V1.2$ channel C-termini resulted in enhanced whole-cell $I_{Ca,L}$ and EC-coupling in ventricular CMs (Dixon *et al.*, 2012). Experimental (Dixon *et al.*, 2012) and computational approaches (Sato *et al.*, 2018) have further demonstrated that excessive $Ca_V1.2$ channel cooperativity can become pathogenic and promote cardiac arrhythmias, including alternans.

In the heart, at least two physiological signaling cascades have been demonstrated to trigger nanoscale rearrangements of $Ca_V1.2$ channels, affecting their clustering and activity. Acute (2 – 8 min long) activation of β-ARs with isoprenaline (ISO) leads to $Ca_V1.2$ channel cluster enlargement in mouse ventricular CMs (Ito *et al.*, 2019; Del Villar *et al.*, 2021) while more prolonged (30 min) activation of type 1 angiotensin receptors (AT_1R) with angiotensin II favors channel endocytosis (Hermosilla *et al.*, 2017), thereby reducing whole-cell $I_{Ca,L}$. In the case of the β-AR stimulated cluster enlargement, the mechanism involves mobilization of an endosomal reservoir of $Ca_V1.2$ channels to the t-tubule sarcolemma via Rab4-dependent fast and Rab11-dependent slow recycling pathways (Del Villar *et al.*, 2021; Westhoff & Dixon, 2021). The enhanced channel recycling and insertion reinforces the $Ca_V1.2$ population at the sarcolemma, favoring enhanced EC-coupling and the positive inotropic response associated with fight-or-flight (Figure 4).

Whether $Ca_V1.2$ clustering is altered in heart failure and/or in aging remains to be fully elucidated. In failing hearts, nanostructural rearrangements of $Ca_V1.2$ have been described with channel localization shifting away from the t-tubules toward the sarcolemmal crest (Bryant *et al.*, 2015; Sanchez-Alonso *et al.*, 2016; Sanchez-Alonso *et al.*, 2020) (Figure 4). As yet, there have been no reports of how these rearrangements impact channel clustering, although constitutive phosphorylation of $Ca_V1.2$ by PKA reportedly enhances cooperative gating behavior of t-tubules localized channels in failing right ventricular CMs in an ischemic cardiomyopathy model inviting speculation that $Ca_V1.2$ channel cluster size may be augmented in those cells (Medvedev *et al.*, 2021).

Hypertension (Navedo *et al.*, 2010a), diabetes, and hyperglycemia (Navedo *et al.*, 2010b; Nystoriak *et al.*, 2017; Prada *et al.*, 2019; Syed *et al.*, 2019; Prada *et al.*, 2020) have been linked with alterations in $Ca_V1.2$ channel function that favor enhanced cooperative gating behavior in VSMCs. Given that enhanced proximity between $Ca_V1.2$ channels facilitates cooperative gating, these studies hint that these pathophysiological stimuli may also be driven by $Ca_V1.2$ channel cluster area augmentation, but this hypothesis remains to be experimentally explored.

Trafficking and recycling of $Ca_V1.2$ channels in VSMCs is a largely understudied area, although it is known that Rab25-dependent recycling is important for $Ca_V1.2$ expression at the sarcolemma of VSMCs (Bannister et al., 2016). Future work should explore whether VSMCs have an endosomal reservoir of pre-formed $Ca_V1.2$ channels analogous to that seen in CMs, and if so, whether $Ca_V1.2$ cargo can be dispatched to the sarcolemma to reinforce LTCC clusters or sequestered to endosomes for storage during various signaling pathways.

Ryanodine Receptor Type-2 (RyR2) clustering—As discussed at the symposium, there is an accumulating body of experimental and *in silico* evidence suggesting that RyR2 cluster size can impact the magnitude of SR Ca^{2+} release and muscle contraction in CMs. The larger clusters of RyR2 in CMs increase frequency and size of Ca^{2+} sparks, and cause greater Ca^{2+} transients and stronger contractions, while smaller RyR2 clusters have the opposite effect (Sobie *et al.*, 2002; Galice *et al.*, 2018). Like $Ca_V1.2$ channels, RyR2 clustering plasticity has been reported in response to activation of physiological signaling pathways with their acute phosphorylation downstream of β-AR stimulation or treatment with a phosphorylation-promoting cocktail linked to cluster enlargement (Fu *et al.*, 2016; Asghari *et al.*, 2020). Given the association between RyR2 cluster size and functional output, these cluster enlargements would be predicted to favor enhanced contractility, supporting the positive inotropic response observed during fight-or-flight. Recent work has revealed that more prolonged β-AR stimulation favors RyR2 cluster fragmentation after ~60 mins of stimulation with ISO (Shen *et al.*, 2022) (Figure 4). This fragmentation was accompanied by decreased Ca^{2+}-transient amplitude and was dependent on CaMKII and PKA-mediated RyR2 phosphorylation. Prolonged β-AR stimulation is a feature of heart failure and indeed, several investigators have reported similar RyR2 cluster or Ca^{2+} release unit (a functionally grouped collection of RyR2 clusters) fragmentation/dispersal in models of heart failure (Kolstad *et al.*, 2018; Sheard *et al.*, 2019), and in AF (MacQuaide *et al.*, 2015) (Figure 4). Notably, similar RyR2 cluster fragmentation and smaller Ca^{2+} transients were observed in CMs isolated from patient samples with idiopathic dilated cardiomyopathy compared to healthy controls (Hou *et al.*, 2021). However, the phenomenon was not observed in atrial CMs from AF patients suggesting that dispersion of RyR2 is not an essential feature of that pathology (Munro *et al.*, 2021). Careful analysis of RyR2 cluster size may reveal new insight moving forward since the report that a heterogeneous mixture of large and small RyR2 clusters can facilitate Ca^{2+} wave propagation and associated arrhythmias (Xie *et al.*, 2019).

RyR2 are also present in VSMCs where Ca^{2+} sparks are not coupled to a contractile process, but instead promote hyperpolarization and vasodilation (relaxation) due to their proximity and functional coupling to large conductance Ca^{2+}-activated BK channels. There is evidence however, that RyR2 clustering plasticity also affects this physiological process with RyR2 cluster enlargement favoring enhanced BK channel activity and reduced cerebral artery tone and vascular dysfunction in Duchenne muscular dystrophy *mdx* mice compared to WT controls (Pritchard *et al.*, 2018). Thus, it appears nanostructural RyR2 arrangement also impacts smooth muscle function.

While RyR2 cluster enlargement and dispersal have been observed in various cell types, the mechanisms of trafficking and recycling of RyR2 that contribute the to their dynamic clustering responses are poorly understood.

Heterogeneity and Overlap of K^+ Channel Activity in Cardiovascular Health and Disease—K^+ channel activity universally mediates repolarization in cardiovascular tissues. Accordingly, K^+ channel distribution and expression are tightly controlled to coordinate chamber and tissue-specific electrical activity. Several mechanisms have been identified that help to ensure the appropriate balance of repolarizing (K^+) and depolarizing (Na^+ and Ca^{2+}) channels at the surface membrane. For example, the inward rectifier $K_{ir}2.1$ expression is reciprocal to $Na_V1.5$ (Milstein *et al.*, 2012). The $K_{ir}2.1/Na_V1.5$ interaction ensures appropriate conduction velocity when $K_{ir}2.1$ is upregulated (Varghese, 2016), as while I_{K1} upregulation is expected to lower excitability and conduction velocity, reciprocal modulation leads to I_{Na} enhancement and consequent increase in conduction velocity. Similarly, $K_V11.1$ subunit transcripts are co-translated at the ribosome with $Na_V1.5$-encoding transcripts (Eichel *et al.*, 2019). How either of these mechanisms is affected in cases of cardiac disease has yet to be studied directly.

AF is associated with complex remodeling in several K^+ currents (Dobrev & Ravens, 2003; Gaborit *et al.*, 2005; Heijman *et al.*, 2014). Chronic AF in human tissue down was shown to downregulate I_{to}, upregulate I_{K1}, I_{KACh}, I_{Ks}, and I_{K2P}, and has mixed or no effects on I_{Kr} and I_{Kur} (Dobrev *et al.*, 2001; Workman *et al.*, 2001; Dobrev *et al.*, 2005; Caballero *et al.*, 2010; Schmidt *et al.*, 2015). However, works in animal models have shown that the effects on K^+ currents in the context of AF are dependent on the duration of AF and the underlying cardiac disease (Li *et al.*, 2000; Gaborit *et al.*, 2005; Sridhar *et al.*, 2009). The identification of the specific triggers of K^+-channel dysregulation in AF is still onging. Most recently Aromolaran et al. demonstrated using a rat lipotoxicity model of AF that mTOR signaling increases I_{Kr} magnitude by selectively promoting surface expression of the $K_V11.1$ channel subunit, $K_V11.1b$ (Martinez-Mateu *et al.*, 2019; Aromolaran *et al.*, 2022).

In the failing ventricle, the major K^+ currents I_{to}, I_{Kr}, and I_{Ks}, and I_{K1} are downregulated and cause homeostatic responses that lead to arrhythmogenesis (Michael *et al.*, 2009). The specific molecular mechanisms pertaining to each current continue to be dissected. In healthy canine heart, the I_{Kr} conducting $K_V11.1$ channel is distributed within the lateral membrane along the Z-lines, with particular enrichment within the t-tubules (Jones *et al.*, 2004). As t-tubule organization is altered in heart failure (Lipsett *et al.*, 2019), $K_V11.1$

organization could be similarly disrupted. Indeed, $K_V11.1$ organization is highly sporadic in stem cell-derived CMs, which lack any discernable t-tubule structure (Jones *et al.*, 2014). The E3 ubiquitin ligase Nedd4–2 was recently shown to be upregulated in the failing heart (Wang *et al.*, 2021). Nedd4–2 mediates $K_V11.1$ degradation and could contribute to reduced $K_V11.1$ in the failing heart (Guo *et al.*, 2012). Holzem et al. used human tissue to demonstrate that while I_{Kr} is downregulated in the failing human heart, $K_V11.1$ subunit composition is also shifted towards a more fetal-like composition (Crotti *et al.*, 2013; Holzem *et al.*, 2015). The mechanism by which this shift occurred remains unclear. A cis-regulatory element was recently identified as a subunit-specific regulator in mouse (van den Boogaard *et al.*, 2019), but its role in the failing heart has not been explored.

Reduced I_{to} contributes to the diminished contractility of the failing heart, where the reduced phase 1 repolarization leads to diminished $I_{Ca,L}$ and reduced Ca^{2+} transient. It was shown in failing human CMs that the fast component of I_{to}, I_{toF}, is selectively downregulated, whereas the slow component (I_{toS}) is unaffected (Johnson *et al.*, 2018b). Nassal et al. demonstrated that KChIP2 function is disrupted in the failing heart, triggering increased miR-34 activity that then disrupts the production of the I_{toF} conducting channel, Kv4.3 (Nassal *et al.*, 2017). $K_V4.3$ was also shown to promote $K_V11.1$ expression in HEK293 cells by upregulating hsp70 (Zhao *et al.*, 2017). This finding highlights the complex crosstalk between distinct K^+ channel subtypes in cardiac tissue.

Accessory proteins (i.e., minK, miRP1–4) were originally thought to be relatively exclusive to a single family of pore-forming K^+ channel subunits. The recently established knowledge that accessory subunits interact with multiple ion channel groups with distinct effects provides the potential for targeted therapies that could benefit multiple K^+ currents. The accessory K^+-channel protein minK is upregulated in heart failure. Xu et al showed that phosphorylation at S102 drives $K_V7.1$/minK channel internalization during sustained PKC activity (Xu Parks *et al.*, 2020). Because minK interacts with $K_V11.1$, minK could represent a singular target to restore I_{Ks} and I_{Kr} activity in the failing heart. Phosphatidylinositol 4,5 bisphosphate (PIP2) represents an additional target for improving K^+-channel function in failing CMs. PIP2 is decreased in spontaneously hypertensive rats (Zhou *et al.*, 2021). PIP2 depletion has been known for years to diminish I_{Ks}, I_{Kr}, and I_{K1}. A recent study in stem cell-derived CMs demonstrated that PIP2 depletion using HIV-Tat reduced I_{Kr} and I_{Ks} in tandem (Es-Salah-Lamoureux *et al.*, 2016). Identifying the networks of interactions between ion channel proteins across distinct cardiovascular tissues will help to identify future targets for broad therapeutic effects.

Ion channel remodeling also occurs throughout development, which helps to produce a more mature electrophysiological and contractile phenotype. Adult ventricular CMs are quiescent, while immature CMs display pronounced automaticity (Sun *et al.*, 2017) that can be attributed to a less negative resting membrane potential with reduced expression of the inwardly rectifying K^+ channel (Wahler, 1992; Masuda & Sperelakis, 1993; Vaidyanathan *et al.*, 2016; Goversen *et al.*, 2018), increased pacemaker current (Cerbai *et al.*, 1999; Giannetti *et al.*, 2021), and/or spontaneous SR Ca^{2+} leak from the sarcoplasmic reticulum that can trigger depolarizations via NCX activation (Kim *et al.*, 2015; Carmeliet, 2019). The shape of the cardiac AP also differs between immature and adult CMs, albeit the

details of these differences are species dependent (Wang *et al.*, 1996; Mummery *et al.*, 2003; Hamaguchi *et al.*, 2013; Swift *et al.*, 2020; Cooper *et al.*, 2021). Likewise, EC-coupling becomes more efficient in mature CMs (Escobar *et al.*, 2004; Lundy *et al.*, 2013; Louch *et al.*, 2015), as these large rod-shaped cells contain t-tubules or sarcolemmal invaginations that allow an AP to quickly reach the interior of the myocyte. This structural adaptation improves CICR in adult CMs (Haddock *et al.*, 1999; Hamaguchi *et al.*, 2013) by juxtaposing LTCCs in the plasma membrane with RyR2s in the SR (Seki *et al.*, 2003; Ziman *et al.*, 2010). In comparison, neonatal CMs are less dependent on LTCC-mediated CICR, and instead rely more heavily on reverse-mode NCX for Ca^{2+} influx and EC-coupling (Wetzel *et al.*, 1995; Huang *et al.*, 2005). Developmental maturation also includes the formation of specialized intercellular junctions called intercalated discs (Peters *et al.*, 1994; Angst *et al.*, 1997), which facilitate electrical and mechanical communication between neighboring CMs (Angst *et al.*, 1997; Vreeker *et al.*, 2014). Structurally, the intercalated discs connect the actin cytoskeleton and intermediate filament network between CMs and also establish intercellular gap junctions, which permit the cell-cell transmission of ions and metabolites. Several ion channels have also been found to reside within the intercalated disc (e.g., K_V, K_{ir}, Na_V; (Vermij *et al.*, 2017)). Collectively, these structures allow an AP to quickly propagate through the myocardium syncytium to coordinate contraction.

K^+ channels are also critical in VSMC regulation. Accordingly, K^+ channels, including K_V, K_{ATP}, BK and K_{ir}, control VSMC membrane potential and, therefore, LTCC activity, $[Ca^{2+}]_i$, myocyte contractility, myogenic tone, and vascular reactivity (Tykocki *et al.*, 2017). The expression of VSMC K^+ channel subunits is heterogeneous and depends on the vessel location (Tykocki *et al.*, 2017). K^+ channel subunits have a tremendous influence on channel activity and cellular function (Tykocki *et al.*, 2017). For example, $K_V\beta$ subunits can interact with the K_V1 channels to link excitability and metabolic state to channel activity and VSMC contractile state (Ohanyan *et al.*, 2021). This mechanism can help modulate myocardial blood flow. Another well-known example is BK β1, which contributes to BK channel voltage and Ca^{2+} sensitivity and modulates vascular tone (Tykocki *et al.*, 2017). The expression of VSMC K^+ channel subunits can also be modified during pathological conditions, such as diabetes and hypertension (Eichhorn & Dobrev, 2007; Tykocki *et al.*, 2017; Nieves-Cintron *et al.*, 2018; Nieves-Cintron *et al.*, 2021). For example, $K_V2.1$ and BK β1 subunits are downregulated in diabetic and hypertensive VSMC (Nieves-Cintron *et al.*, 2018; Nieves-Cintron *et al.*, 2021). This $K_V2.1$ and BK β1 downregulation is mediated by activation of the transcription factor NFATc3 (Nystoriak *et al.*, 2014; Nieves-Cintron *et al.*, 2015). Downregulation of these subunits was correlated with an increase in VSMC contractility, myogenic tone, and mean arterial pressure. VSMC K^+ channel functional expression is also modulated by the dynamic trafficking of different K^+ channel subunits (Leo & Jaggar, 2016). This dynamic trafficking of VSMC K^+ channel subunits such as $K_V1.5$ and BK β1 has been shown to be influenced by membrane potential, stimulation of angiotensin II signaling, and pathological conditions such as hypertension to control VSMC contractility and vascular reactivity (Leo & Jaggar, 2016). Because of the diversity of K^+ channel subunits, the impact of each of the subunits is unclear and thus represents an area for future research.

Action potential and ionic current variability—The concerted action of several ionic channels and transporters shapes the cardiac AP (see section 1). There are large species differences in ion channel expression patterns, especially considering K^+ channels that govern the repolarization dynamics (Grandi *et al.*, 2017). Small animal models of cardiovascular diseases can inform about changes in ventricular I_{to}, I_{K1} and I_{NaL}, and the signaling mechanisms involved. However, they provide little or no information about I_{Kr} and I_{Ks} (the physiological role of I_{Kr} and I_{Ks} is unclear in small animals) and phase 3 repolarization dynamics (small animals have different AP morphology). Therefore, large animal models with ion channel expression patterns and AP morphology resembling the human heart have a pivotal role in understanding exact arrhythmia mechanisms relevant to human. Guinea pig has been a highly useful small animal model in which cardiac repolarization mechanisms are more alike those in larger mammals. However, the use of guinea pig has decreased due to the rise of much more sophisticated tools for genetic manipulation of mice.

A potential alternative for interspecies translation of electrophysiology and Ca^{2+} handling from mouse to human is the use of a powerful computational framework as recently established (Morotti *et al.*, 2021), and discussed below in section 3.3. It is also still unknown whether a central genetic or epigenetic mechanism(s) responsible for pathogenic ionic remodeling exists and whether this central mechanism is cell-specific, all shared among different cell lines (CM-FB-EC), and emerging areas for future research.

In traditional voltage-clamp experiments, only one ionic current can be reliably measured at a time. Using novel approaches such as sequential dissection of ionic currents under AP-clamp using selective ion channel inhibitors (Hegyi *et al.*, 2018) or complex voltage protocols optimized by a genetic algorithm (Clark *et al.*, 2022) may inform about multiple ionic current magnitudes in CMs yielding rich data from a limited number of diseased cells/animals/patients. These same approaches could be extended to examine ionic conductances in VSMCs. Moreover, these methods may help to better understand the co-expression and co-regulation of multiple ion channels within the same cell and their variability among cells, including VSMCs. The presence of significant cell-cell variability in ionic current densities, even in the same cardiac region in the very same heart, has been long recognized. Moreover, similar AP duration can be achieved by various combinations of ionic current densities (see “good enough solutions” (Weiss *et al.*, 2012)). Increasing data show that the large variability in each ionic current density/expression is not random but that there is a strong correlation between distinct ion channels in their expression, trafficking, membrane targeting, functional coherent translation, and regulation. Such coordinated interactions were demonstrated between I_{K1} and I_{Na} (Milstein *et al.*, 2012; Ponce-Balbuena *et al.*, 2018), I_{Kr} and I_{CaL} (Ballouz *et al.*, 2021; Horvath *et al.*, 2021), and I_{Na} and I_{Kr} (Eichel *et al.*, 2019; Hegyi *et al.*, 2020). Interestingly, this mutual regulation between ionic currents may also be altered in disease (Hegyi *et al.*, 2020), which may represent a new avenue of understanding disease mechanisms and discovering novel therapeutic targets.

There exist many sources of cell-cell variability in cardiovascular physiology, which encompass multiple spatial and temporal scales, and may play important roles in the development and progression of cardiovascular diseases and could impact the outcome of therapeutic interventions. We have highlighted here some critical sources of natural physiological diversity, and in the next section, we will overview novel technologies that allow for interrogating this diversity.

3. Technical innovations to assess biological diversity

There is a long history of new technologies leading to breakthroughs in scientific discovery. Here, we review selected technologies that are providing new insights into biological diversity. We highlight new technical developments in animal models, omics, mathematical modeling, and subcellular imaging. All four of these areas have enabled substantial discoveries in biological discovery yet realizing their full benefit towards biological diversity will require a closer interaction between biologists, electrophysiologists, and engineers. Technical innovations work together in an iterative cycle to bring greater and deeper insight into cardiovascular diversity (Figure 5).

3.1 Experimental models of cardiovascular disease

Duality in animal models of cardiovascular diseases: Disease as outcome vs. etiology—Although cardiovascular diseases are frequently named under an umbrella term, e.g., hypertension and heart failure, which reflect critical functional changes in the cardiovascular system, these diseases have many different etiologies. In turn, current clinical guidelines recommend therapies for a disease and not the pathophysiology (e.g., treatment recommendations for heart failure do not differentiate between ischemic and non-ischemic origin, sex, or genetic background). With the emergence of personalized medicine and patient-specific therapies, there is hope that patient-specific pathophysiology and genetics can be targeted in the future. At the same time, basic cardiovascular research that uses animal models for cardiovascular diseases is inherently pathophysiology-based. When creating an animal model of a disease, researchers usually start with a healthy animal and make an intervention (e.g., surgery, treatment, or genetic manipulation) that is associated with only one cardiovascular risk factor among the many that contribute to the clinical evolution of disease in human patients.

Complementary use of small and large animals in cardiovascular research provides important insights about impaired signaling mechanisms in disease and may identify and immediately test novel therapeutic targets. The use of small animals (mouse, rat) has important advantages, including easy genetic manipulations, faster disease progression and reproductive times, and lower costs, but their hemodynamics, as well as protein expression and metabolic profiles, may markedly differ from the human (Dobrev & Wehrens, 2018; Riehle & Bauersachs, 2019). Larger animals provide more closely translatable results, but the exponentially higher cost, long disease progression times, and difficult genetic manipulations limit their use. See recent reviews comparing small vs. large animal models of hypertension (Lerman *et al.*, 2019) and heart failure with reduced ejection fraction (Pilz *et al.*, 2022). Moreover, naturally occurring diseases in animals and data from

veterinary practice can also provide additional insights, e.g., due to frequent hypertrophic cardiomyopathy occurrence in cats (Luis Fuentes *et al.*, 2020), and degenerative mitral valve disease and dilated cardiomyopathy in dogs (Keene *et al.*, 2019).

In the past decade, the exponential increase in the use of hiPSC-CMs has revolutionized cardiovascular research. The clear advantages of hiPSC-CMs are that these cells carry the patients-specific genetics and have a virtually unlimited source that is more easily applicable in high-throughput screenings for drugs. While hiPSC-CMs are still limited in their usually immature cellular phenotype, as noted in section 2.2 above, notable progress towards a more adult-like phenotype has been achieved by maturation approaches, including hormonal stimulation (Parikh *et al.*, 2017), providing oxidative metabolic substrates (Feyen *et al.*, 2020), and co-culturing with ECs and FBs in 3-D microtissues with biophysical stimulation (Giacomelli *et al.*, 2020). Engineered heart tissues (Zimmermann *et al.*, 2002) and cardiac organoids (Kupfer *et al.*, 2020) can further improve cell maturation and physiological cell function. Conditional immortalization of human atrial CMs constitutes a novel methodology to generate functional cell lines for the development of *in vitro* models of human disease (Harlaar *et al.*, 2022).

Heart failure with preserved ejection fraction: a truly heterogeneous disease—Heart failure with preserved ejection fraction (HFpEF) is set to become the leading form of heart failure due to the rapid populational growth of disease-associated conditions (e.g., aging) and co-morbidities, including obesity, diabetes, hypertension, kidney disease, depression, etc. Patients with HFpEF represent a highly heterogeneous group with variable presence and severity of different co-morbidities. Animal models of HFpEF were initially focused only on one major aspect of the disease but fell short of recapitulating the clinical characteristics and complex phenotype of HFpEF patients. Recently, two- or multi-hit models that better recapitulate human HFpEF have emerged (Withaar *et al.*, 2021). These small and large animal models of HFpEF are reviewed and compared by Roh et al. (Roh *et al.*, 2022). However, none of the more translational animal models is expected to cover the whole spectrum of HFpEF characteristics. Thus, creating and investigating multiple different disease models that recapitulate specific sub-phenogroups of HFpEF patients, with distinct phenotypical characteristics and underlying pathological mechanisms, may deepen our understanding of HFpEF biology and improve therapeutic testing. Assessing extracardiac functional impairments (lungs, kidney, skeletal muscle, etc.) and their contributions to HFpEF are key elements in developing animal models for HFpEF. HFpEF is considered a cardio-immuno-metabolic disease (Schiattarella *et al.*, 2022) in which the function of different cell types in the heart must be carefully evaluated. There is an expectation that a better understanding of the various cell-to-cell and subcellular signaling networks by omics approaches may identify specific patient subgroups and may uncover novel therapeutic targets. A detailed cardiac transcriptomic analysis (Hahn *et al.*, 2021) matched with clinical characteristics in human HFpEF identified three patient subgroups using a non-negative matrix factorization (NMF) (Gaujoux & Seoighe, 2010) and weighted gene co-expression network analysis (WGCNA) (Langfelder & Horvath, 2008). Such approaches can be of value when comparing different HFpEF animal models and potentially translating the findings toward human HFpEF subgroups. The disease pathophysiology is likely similarly

diverse and includes oxidative and nitrosative stresses, inflammation, volume or high salt-sensitive hypertension, obesity, diabetes, and other metabolic derangements, etc. (Mishra & Kass, 2021).

3.2 Omics technologies to assess molecular and cellular diversity

Genomics—Some of the most transformative biotechnologies are those for comprehensive molecular profiling, collectively known as omics. The development of high-throughput DNA sequencers enabled in 2001 the sequencing of the human genome (Venter *et al.*, 2001), which turned a $3.8 billion investment by the U.S. government into a genome-enabled industry with $796 billion economic impact by 2011 (Tripp & Grueber, 2011). However, this initial genome sequence contained only euchromatin, with the remaining heterochromatin regions sequenced only recently (Nurk *et al.*, 2022). Further technological advances enabled the decreasing cost of sequencing at a rate even exceeding Moore's law. For example, the UK Biobank has developed a database containing genomes and deep phenotyping from 500,000 individuals from the United Kingdom, 22,000 of which reported ethnic backgrounds originating outside of Europe (Bycroft *et al.*, 2018). Such resources and studies enable the quantification of ancestry diversity at greater scale (Prive, 2022).

Measures of genomic diversity are now being combined with phenotypic data to provide important insights into the genetic basis of cardiac disease. Accordingly, a genome-wide association study (GWAS) for features derived from cardiac magnetic resonance imaging in 36K UK Biobank participants for genetic variants found a number of loci near genes well-established for Mendelian cardiomyopathies, as well as 45 new loci that enabled a polygenic risk score for dilated cardiomyopathy (Pirruccello *et al.*, 2020). In a follow-up study, deep learning approaches were implemented using the UK Biobank participants with magnetic resonance imaging to characterize features of the right heart (Pirruccello *et al.*, 2022). Results revealed key genes known in congenital heart disease (e.g. NKX2–5, TBX5) and also a powerful new polygenic predictor of right heart function.

Transcriptomics—Comprehensive measurements of the transcriptome have also had a major impact on our ability to assess biomolecular diversity. One of the most influential methods is RNA sequencing (RNA-seq) (Wang *et al.*, 2009). RNA-seq has had an important impact on many cardiovascular studies, such as profiling the transcriptome in response to G_q-overexpression (Matkovich *et al.*, 2010) or discovering noncoding RNAs in failing human hearts. Transcriptome measurements capture a much broader, more diverse range of transcripts than would be possible with RT-qPCR (Yang *et al.*, 2014a). However, a limitation of RNA-seq in bulk samples, such as a tissue, is that it does not capture the cellular diversity of gene expression within a sample.

Because the heart and vasculature contain many cell types and states, profiling bulk tissue may not adequately capture a complex disease. To study congenital heart disease in human hearts at the single-cell level, Reichart et al. performed single-nucleus RNA-sequencing of patients with cardiomyopathies, with or without known genetic causes (Reichart *et al.*, 2022). They found important differences in intercellular signaling between genotypes, such as endothelin signaling in Lamin A/C gene (LMNA)-related dilated cardiomyopathy and

TNFα in plakophilin-2 gene (PKP2)-related cardiomyopathy. Network models demonstrated genotype-specific heart failure pathways. Such insights are leading toward precision medicine approaches.

Single-cell RNA-seq is also having an impact on fundamental cardiac biology. The process of cardiac differentiation from a stem cell involves transition through multiple states, and the mechanisms for robust differentiation are unclear. As presented at the UC Davis Cardiovascular Research Symposium, the gene *Brm* was critical for cardiac differentiation (Hota *et al.*, 2022). Single-cell RNA-seq revealed that *Brm*-deleted cells instead became neural precursors, which were rescued with BMP4. Mathematical modeling of the single-cell RNA-seq data showed how *Brm* deletion disrupted hysteresis, a pair of saddle-node bifurcations needed for a robust cardiac differentiation (Hota *et al.*, 2022). Audience members drew parallels to bifurcations in other cardiovascular processes, such as arrhythmia.

A variety of new methods are being developed to not only provide single-cell transcriptomes but also provide spatial resolution to contextualize cell niches or tissues (Moffitt *et al.*, 2022). These spatial omics methods may be particularly valuable for investigating structural remodeling in development or disease. An impressive example was recently reported of spatial omics of the human heart after myocardial infarction (Kuppe *et al.*, 2022). Accordingly, a profile of the post-MI heart with single-cell transcriptomes and chromatin accessibility with a resolution of ~100 μm revealed the spatial relationships of disease-specific cell states, including a continuum of states of CMs and myeloid cells. Such spatially detailed datasets will be invaluable for later multi-scale computational models that combine molecular and cellular heterogeneity. In addition, the roles of non-coding RNA including microRNAs and long non-coding RNAs in the regulation of cardiac excitability are well recognized (Johnson *et al.*, 2018a; Yang *et al.*, 2022).

Proteomics—Global measures of protein abundance and modifications (proteomics) provide a complementary view to that of transcriptomics, with advantages including proximity to cellular function and the utility of proteins as clinical biomarkers. A variety of proteomics technologies have been applied for heart failure proteomics, including mass spectrometry, protein microarray, aptamers, and proximity extension arrays, each of which has its advantages (Michelhaugh & Januzzi, 2020). In a wide survey of the field, an American Heart Association consensus statement noted the broad potential of proteomics to establish cause-and-effect mechanisms and signaling networks of cardiovascular disease (Lindsey *et al.*, 2015).

Proteomics methods have been effectively used to identify the diverse protein responses to distinct stimuli. With their previous studies implicating MMP-12 in caspase-3 activity and cardiac wound healing, Chalise et al. asked what proteomic responses may mediate this effect (Chalise *et al.*, 2022). They subjected neutrophils to MMP-12, IL-1β, or IL-4 and measured the intracellular signaling state via a 304-plex antibody array and 28 secreted cytokines. They found that these stimuli induced diverse proteomic responses, with a specific signature of MMP-12 signaling including increased FOXO1, apoptotic proteins, and

decreased Wnt signaling with MMP-12. This provides a mechanistic basis for how MMP-12 modulates neutrophil polarization and apoptosis.

Clinical proteomics has demonstrated a substantial ability to identify new biomarkers and, when paired with other approaches, mechanisms of disease. As part of the TOPMed Consortium, Katz et al. used aptamer methods to profile 1301 plasma proteins in 1852 adults of African ancestry (Katz *et al.*, 2022), who have considerable genetic diversity (Genomes Project *et al.*, 2015). Paired proteomic and genomic profiling revealed 114 novel locus-protein relationships, including a locus at the HPX gene, associations between APOE, ZAP70 and MMP-3, and association between ATTR amyloidosis and RBP4 expression. Importantly, these results were validated with complementary proteomics methods and replicated in other studies such as the Multi-Ethnic Study of Atherosclerosis (Katz *et al.*, 2022).

Multi-omics and other omics—While we have focused on genomics, transcriptomics, and proteomics, there are a number of additional profiling technologies that assess other molecular states. Some notable examples include metabolomics (Li *et al.*, 2011; McGarrah *et al.*, 2018), lipidomics (Tabassum & Ripatti, 2021), and epigenomics (Rosa-Garrido *et al.*, 2018). All of these methods have made substantial contributions to understanding cardiovascular disease. One of the major frontiers is integrating multiple omics technologies (Joshi *et al.*, 2021). As one recent example, a single-nucleus RNA sequencing from human hearts with multiple congenital heart diseases found diverse, disease-specific CM and FB states (Hill *et al.*, 2022). Applying imaging mass cytometry, a spatial proteomics approach, Hill *et al.* identified a distinct immunosuppressed perivascular microenvironment, which was further characterized by immune cell profiling. mRNA expression, ligand-receptor analysis, and plasma protein profiling all reinforced a role for THBS1 in CM pathology of congenital heart disease (Hill *et al.*, 2022). The battery of different approaches in this study reinforced and validated their findings. With the ability to perform such multi-modal experiments, a rising challenge is to develop computational methods to integrate these datasets rigorously (Leon-Mimila *et al.*, 2019; Joshi *et al.*, 2021).

Omics technologies have provided nearly comprehensive profiling of molecular states. However, it is also important to recognize these methods' limitations. In particular, molecular profiles may correlate with developmental or disease states, but are not sufficient to characterize underlying mechanisms or predict the effect of new perturbations. Omics also do not elucidate the causal relationships between molecular components, cellular states, and biological function. The high throughput omics technologies are outstanding at characterizing molecular diversity, but complementary techniques are needed to understand how molecular/cellular diversity arises and how it can be harnessed for therapeutic purposes. As described in the next section, mathematical modeling has significant potential for complementing omics technologies toward these goals.

Current methods for omic data analyses focus on statistical methods to identify patterns. New computational modeling frameworks are needed to integrate and distill mechanistic insight from omic data and integrate it with prior knowledge.

3.3 Mathematical modeling of population heterogeneity

In recent years, increased focus on the significance of cellular heterogeneity and diversity, especially among CMs, has prompted an evolution of in silico modeling from single cells to heterogeneous populations. One approach to such modeling is to generate a large set of models by randomly sampling values of select parameters and subsequently discard models that generate non-physiological output. For example, in pioneering this approach for CM electrophysiology modeling, Britton et al. constructed a population of 10,000 candidate models through Latin hypercube sampling of values for 11 different ionic current conductances and kinetics parameters (Britton *et al.*, 2013). Candidate models whose simulated behavior was not consistent with experimental AP recordings were eliminated, reducing the population down to 213 models. When simulating I_{Kr} block in this calibrated population, the range of variability in AP prolongation seen in the models agreed with that measured experimentally with the I_{Kr} blocker dofetilide.

In general, this approach can be helpful in determining why some cells/organs/individuals are more prone to pathological outcomes than others and what quantitative feature(s) of a subpopulation is associated with specific disease patterns (Sarkar & Sobie, 2011; Passini *et al.*, 2017; Ni *et al.*, 2018). In a recent study, a model was constructed with four subpopulations of ventricular CMs based on sex and disease state (male/female, healthy/heart failure) and found that the female heart failure group was the most susceptible to proarrhythmic events when simulating effects of drugs proposed for COVID-19 treatment (Varshneya *et al.*, 2021). These results suggested that women with heart disease are particularly vulnerable to cardiac arrhythmias if treated with the drugs examined in the study. In addition to this subgroup-specific prediction, logistic regression was used to identify three specific ionic currents (I_{Kr}, I_{CaL}, I_{NCX}) as most important for arrhythmogenesis, thus helping determine the relative roles of individual parameters in identifying ionic mechanisms underlying arrhythmogenesis. The population-of-models approach has also been used in making quantitative predictions on the role of combined inhibition of the atrial-dominant currents I_{K2P}, I_{KCa}, and I_{Kur} in different chronic AF subpopulations, demonstrating the advantage of computer power in assessing a number of drug combinations across multiple subpopulations (Ni *et al.*, 2020).

Outside of the heart, heterogeneity in the density of inward rectifier K^+ channels among capillary ECs has been included to simulate electrical responses in microvascular networks, specifically to predict the amount of hyperpolarization in response to local K^+ stimulation as part of the neurovascular coupling (Moshkforoush *et al.*, 2020). Population-based methods have significant potential for biochemical networks and mechanical modeling as well.

Cell-type translation—Simulated data from a population of models can form the basis for statistical or machine learning-based analyses allowing translation of the behavior in one cell type into quantitative predictions of outcomes in a different cell type. This approach has been applied to derive predictions of drug effects on electrophysiological metrics in adult human CMs based on measurements or simulations in hiPSC-CMs or animal CMs (Gong & Sobie, 2018; Aghasafari *et al.*, 2021; Morotti *et al.*, 2021). When subsequently testing predictions of human responses to different ion-channel blockers based

on animal experiments, translators were found to closely reproduce drug-induced changes seen in human data (Morotti *et al.*, 2021). While these approaches have focused mainly on translating across cell differences that limit the usefulness of experimental cells as models of adult human physiology, this methodology is directly applicable to investigate differences along disease axes (e.g. from healthy state to heart failure (Gong & Sobie, 2018; Morotti *et al.*, 2021)), or between different non-CM cell types.

Cell- and patient-specific modeling—The populations of models derived by randomizing parameter values should be understood to be hypothetical populations, as they do not build on specific information regarding parameter values in individual cells. An alternative approach to modeling a heterogeneous population is to generate models that are calibrated to data from individual cells/organs/subjects, thereby creating cell-, organ, or subject-specific models. For CM electrophysiology models, such an approach has been demonstrated for determining ionic current conductances in guinea pig ventricular CMs (Groenendaal *et al.*, 2015) and tailored models have been developed for hiPSC-CMs (Lei *et al.*, 2017). In both cases, the reparameterized models improved predictive power over the non-tailored baseline models.

If accurately calibrated, a cell-specific model population could help identify population heterogeneity both across healthy tissue (e.g., left vs. right ventricle, atrial vs. ventricular cells) and heterogeneity resulting from pathological ionic remodeling. A cell-specific model population should also capture correlations among ionic currents observed experimentally, in particular positive correlations between inward and outward currents arising due to co-expression of ion channels or co-regulation at the translational level (Rees *et al.*, 2018; Eichel *et al.*, 2019; Hegyi *et al.*, 2020), which we have discussed in section 2.3 above. Such co-variations could also be built into the population-of-models approach as a next step for development (Ballouz *et al.*, 2021) and applied across different disease and cell-types, to potentially help to address whether there are central genetic or epigenetic mechanisms underlying ionic remodeling in CM, ECs, and VSMCs.

At the organ level, patient-specific cardiac models may be constructed based on personalized geometries, fiber orientation, infarct scars, fibrosis, and/or electrophysiology (Niederer *et al.*, 2019; Corral-Acero *et al.*, 2020). Patient-specific models are expected to contribute increasingly to clinical decision-making and have been utilized in predicting arrhythmia risk in patients with hypertrophic cardiomyopathy (O'Hara *et al.*, 2022) or myocardial infarction (Arevalo *et al.*, 2016). Still, challenges remain in developing methodologies and pipelines for personalized model creation at scale, including building frameworks for high-quality clinical data acquisition, automating image segmentation, and overcoming computational costs of model development and simulation of predictions (Costa *et al.*, 2020).

Beyond conductances—The electrophysiology modeling approaches discussed above have prioritized varying conductance parameters, which have traditionally been based on data from voltage clamp experiments. More recently, expression data have been utilized to inform conductance levels between subpopulations (female vs. male, (Yang *et al.*, 2017)) or individual patients (Smirnov *et al.*, 2020)). An open question remains as to how to go beyond conductance parameters and consider variations in kinetics, channel subunit

distribution, and signaling processes and pathways. One issue is obtaining measurements that can inform the estimation of the many parameters involved in, e.g., kinetics for multiple ion channels. Using phenomenological models with relatively few parameters rather than biophysically detailed models may therefore provide a helpful starting point for cell-specific modeling of kinetics (Cairns *et al.*, 2017).

Validation—Although the need for model validation has been highlighted in recent years and generally called for by experimentalists at the meeting, there is no existing framework for validation. The process of validation involves comparing model predictions to experimental or clinical data not used in building the model. As an example, a recent optimization of the much-used O'Hara-Rudy human ventricular CM model carefully separated validation from calibration data in a multi-objective optimization using complex data sets, including AP and ionic current recordings, Ca^{2+} transients, drug block data, rate dependencies, and conduction velocity (Tomek *et al.*, 2019).

However, a model can only be validated for a certain context of used – a general sticker of "validated model" cannot be meaningfully applied (Pathmanathan & Gray, 2018). If the intended use of a model is to gain insights into the biophysical underpinnings of an observed phenomenon, larger differences between model output and validation data would be acceptable than if the model is intended for quantitatively accurate predictions. Therefore, the selection of validation data should also be done within the context of use, with the process of validation benefitting from synergistic collaborations between experimentalists and modelers in protocol design to generate information-rich data sets for calibration and validation (Carusi *et al.*, 2012; Pathmanathan & Gray, 2018; Whittaker *et al.*, 2020).

3.4 What tools are needed for us to understand subcellular cardiovascular function in greater detail?

Over the last few decades, sub-cellular heterogeneities from nano through micro scales have emerged as key determinants of cellular and tissue scale physiology. Perhaps the most illustrative example is that of EC-coupling, where heterogeneous organization of t-tubules, SR structures, and dyadic nanodomains enables local control and governs cellular-scale behavior in ways that cannot be predicted without accounting for subcellular heterogeneities (Qu *et al.*, 2013; Winslow *et al.*, 2016; Radwanski *et al.*, 2018). Indeed, our understanding of other areas of cardiovascular physiology and biology are undergoing similar paradigm shifts, underscoring the need for experimental and computational tools to investigate subcellular heterogeneity. Here, we highlight some recently developed and emerging approaches that meet this need.

Experimental Imaging Approaches—The ability to assess subcellular structural and functional heterogeneities has been greatly enhanced by the advent of novel sample labeling and interrogation strategies. Super-resolution imaging techniques such as single molecule localization microscopy (stochastic optical reconstruction microscopy [STORM], photoactivation localization microscopy [PALM] etc) and stimulated emission depletion (STED) have pushed light microscopy to molecular-scale resolution. Although these techniques have been applied to live cells, their live-cell friendliness is limited by the

massive photon fluxes involved. Methods like structured illumination microscopy (SIM) and fluctuation-based super-resolution methods (super-resolution optical fluctuation imaging [SOFI], super-resolution radial fluctuations [SRRF], etc) enable sub-diffraction imaging in live cells with minimal phototoxicity. Also, of note here are expansion microscopy methods (Wassie *et al.*, 2019; Gallagher & Zhao, 2021), which enable sub-diffraction resolutions to be attained using simple hardware and emerging techniques like minFlux (Gwosch *et al.*, 2020) and minSTED (Weber *et al.*, 2021), which are achieving near-atomic scale resolutions comparable to electron microscopy. The impressive power of these light microscopy techniques to reveal the molecular workings of cells is further enhanced by the addition of ultrastructural context through correlative light and electron microscopy (CLEM) techniques (Dillard *et al.*, 2018; Mezache *et al.*, 2019; Heiligenstein & Lucas, 2022; Jeong & Kim, 2022; van den Dries *et al.*, 2022). For a more detailed review of current super-resolution methods, readers are referred to the excellent recent review by Jacquemet et al (Jacquemet *et al.*, 2020). As discussed at the symposium, some of these methods have been applied to cardiac cells and tissues (Agullo-Pascual *et al.*, 2013; Veeraraghavan *et al.*, 2015; Leo-Macias *et al.*, 2016; Veeraraghavan & Gourdie, 2016; Dixon *et al.*, 2017; Ghosh *et al.*, 2018; Veeraraghavan *et al.*, 2018; Mezache *et al.*, 2019), but we are still in the early days of leveraging them for cardiovascular research. It should also be noted here that the advent of scanning ion conductance microscopy (SICM) –guided “smart” patch clamp paralleled the aforementioned developments in microscopy and enabled direct interrogation of electrophysiology within sub-cellular niches (Gorelik *et al.*, 2002; Bhargava *et al.*, 2013; Schobesberger *et al.*, 2017).

Of equal importance to imaging techniques discussed above have been advances in the ability to label specific types of molecules and structures within cells. In addition to the development of nanobodies (de Beer & Giepmans, 2020; Erreni *et al.*, 2020), which lower linkage error in protein labeling, new methods have been developed to visualize other types of biomolecules and sub-cellular structures, such as single molecule fluorescence *in situ* hybridization (smFISH) to visualize mRNA (Savulescu *et al.*, 2021; Piskadlo *et al.*, 2022), OligoSTORM/OligoDNA-PAINT to image genomic DNA (Beliveau *et al.*, 2017; Nir *et al.*, 2018; Nguyen *et al.*, 2020), and single molecule Förster resonance energy transfer (smFRET) to study protein-protein interactions / protein conformational dynamics and subcellular domain function (Lerner *et al.*, 2021; Ha *et al.*, 2022). Likewise, the expanding toolbox for label-free imaging is enabling unprecedented interrogation of live cell dynamics with minimal perturbation (Ojaghi *et al.*, 2020; Borile *et al.*, 2021; Morgan *et al.*, 2021).

The investigative power of sample labeling and imaging techniques is ultimately contingent upon the ability to robustly quantify structural and/or functional properties from the resulting data, particularly when it comes to capturing heterogeneity. Thus, recent advances in artificial intelligence / machine learning –powered image segmentation (Vicar *et al.*, 2019; Durkee *et al.*, 2021; Liu *et al.*, 2021), cluster analysis for single molecule localization data (Levet *et al.*, 2015; Chamma *et al.*, 2016; Veeraraghavan & Gourdie, 2016), and spatial analysis of image data (Bogdanov *et al.*, 2021; Bao *et al.*, 2022) merit attention here.

Multiscale Modeling Approaches—Directly and indirectly based on innovations in subcellular imaging, the integration of subcellular structural detail within and between

CMs has facilitated simulations of multiscale models, enabling predictions of the role of subcellular structure on cellular- to tissue-scale function. Early multiscale modeling studies investigated the role of subcellular structure in EC-coupling and Ca^{2+} handling, representing ~10,000s distinct, coupled Ca^{2+} release units, each with stochastic gating of LTCCs and RyR2s and distinct subcellular Ca^{2+} compartments (Gaur & Rudy, 2011; Nivala & Qu, 2012). These early generation subcellular models often assumed homogeneous subcellular structure. Yet simulations predicted that even in the absence of spatial heterogeneity, stochasticity and channel refractoriness can drive spatial heterogeneity of subcellular Ca^{2+} signaling and importantly, can link local Ca^{2+}-spark properties with pro-arrhythmic whole-cell alternans behavior (Nivala & Qu, 2012). To further connect subcellular structure with disease, disease-related structural disruption required model representation of subcellular spatial heterogeneity. Multiscale simulations investigating heart failure-induced arrhythmia showed that both RyR2 redistribution and alteration of the t-tubule membrane structure could alter Ca^{2+}-release properties, which in turn can drive Ca^{2+} alternans and, in some conditions, triggered activity (Nivala *et al.*, 2015; Shiferaw *et al.*, 2018; Song *et al.*, 2018; Shiferaw *et al.*, 2020). High-resolution structural electron micrograph imaging has further been integrated into modeling frameworks to directly address the impact of membrane geometry relative to channel distribution (Colman *et al.*, 2017).

The inclusion of highly detailed 3-dimensional subcellular geometry comes at a cost, specifically a large computational cost, such that simulations extending the multiscale model to include hundreds to thousands of cells, each with ~10,000s stochastic Ca^{2+}-release units, are generally computational prohibitive. Thus, the question arises of how to directly investigate the role of subcellular structure and heterogeneity at the tissue-level? As presented at UC Davis Cardiovascular Research Symposium, one innovative solution applied to this question has been the use of phenomenological relationships. Specifically, key statistical properties of the subcellular dynamics (e.g. Ca^{2+}-spark kinetics) define a phenomenological relationship that can be simulated at the whole-cell level, which in turn is integrated into a multicellular tissue framework (Colman, 2019; Greene *et al.*, 2022). Thus, the key properties of the subcellular structure are captured and represented at the tissue level. Two excellent recent reviews have highlighted advances in subcellular Ca^{2+} modeling and the integration of image-based subcellular structure as described in section 2.3 (Colman *et al.*, 2022; Louch *et al.*, 2022).

In addition to Ca^{2+} signaling, recent imaging-driven simulation studies have suggested that subcellular structure can directly impact electrical conduction via interactions at the cell-cell junctions, specifically at the intercalated disk. Two critical structural properties governing these interactions are the localization of high-density clusters of voltage-gated Na^{+} channels at the intercalated disk, in close proximity to electrical and mechanical junctional proteins, and the narrow cleft spacing between adjacent cells, both identified with electron and super-resolution imaging (Veeraraghavan *et al.*, 2014a, b; Veeraraghavan *et al.*, 2015). Model predictions incorporating these key structural features predict additional cell-cell coupling mechanisms beyond direct electrical coupling via gap junctions, which have been termed ‘ephaptic coupling’ (i.e. interactions occurring in the narrow extracellular space between cells) (Lin & Keener, 2010; Weinberg, 2017; Nowak *et al.*, 2021b). Recent modeling studies have predicted that subcellular structural properties, including the localization and density

of Na^+ channels within the intercalated disk, can have significant effects on conduction (Kucera *et al.*, 2002; Hichri *et al.*, 2018; Ivanovic & Kucera, 2021). Further, multiscale models integrating intercalated disk structure, based on electron microscopy measurements, predict that heterogeneity *within* the intercalated disk can also impact conduction (Moise *et al.*, 2021). In addition to conduction, simulations have predicted that the intercalated disk structure can impact repolarization in arrhythmias such as long QT syndrome (Greer-Short *et al.*, 2017; Nowak *et al.*, 2021a), spontaneous ectopy (Poelzing *et al.*, 2021; Ly & Weinberg, 2022; Yu *et al.*, 2022), and reentry formation (Tsumoto *et al.*, 2020). Thus, the combination of imaging-based details of subcellular structure and multiscale modeling provides a powerful framework for investigating functional relationships. While most such studies, to our knowledge, have focused on CM subcellular or CM-CM interactions, generalization to subcellular and cell-cell interactions with other diverse cell types, including FBs, vascular cells, and immune cells, is an area for much fruitful future investigation.

4. Summary and Opportunities for Future Research

It is becoming increasingly evident that a complex interplay of signaling events among various cell types plays key roles in cardiovascular function and that abnormal cell-autonomous and cell-cell signaling is involved in the development and progression of cardiovascular disease. In this white paper, stemming from the seventh UC Davis Cardiovascular Research Symposium, we illustrated how biological diversity in structure-function (electrical, Ca^{2+}, and regulatory signaling) within myocytes and non-myocytes (e.g., FBs, ECs, neuronal and immune cells, etc.) integrate to influence normal cardiovascular function, and disease development and progression. Moreover, we highlight the unmet need to quantitatively characterize and model additional cell types (e.g., stem cells, neutrophils, T cells) and systems (e.g. lymphatics) that have had lesser attention to date. Technical innovations in animal models, omics, mathematical modeling, and subcellular imaging are discussed as tools to illuminate and provide insight into the diversity of subcellular, cellular, and tissue-level processes. Integration of these diverse technologies and data types is challenging but offers unique opportunities to interpret the rich datasets and translate findings to human health and disease, with the ultimate goal to better our mechanistic understanding and gain a holistic view of disease progression and therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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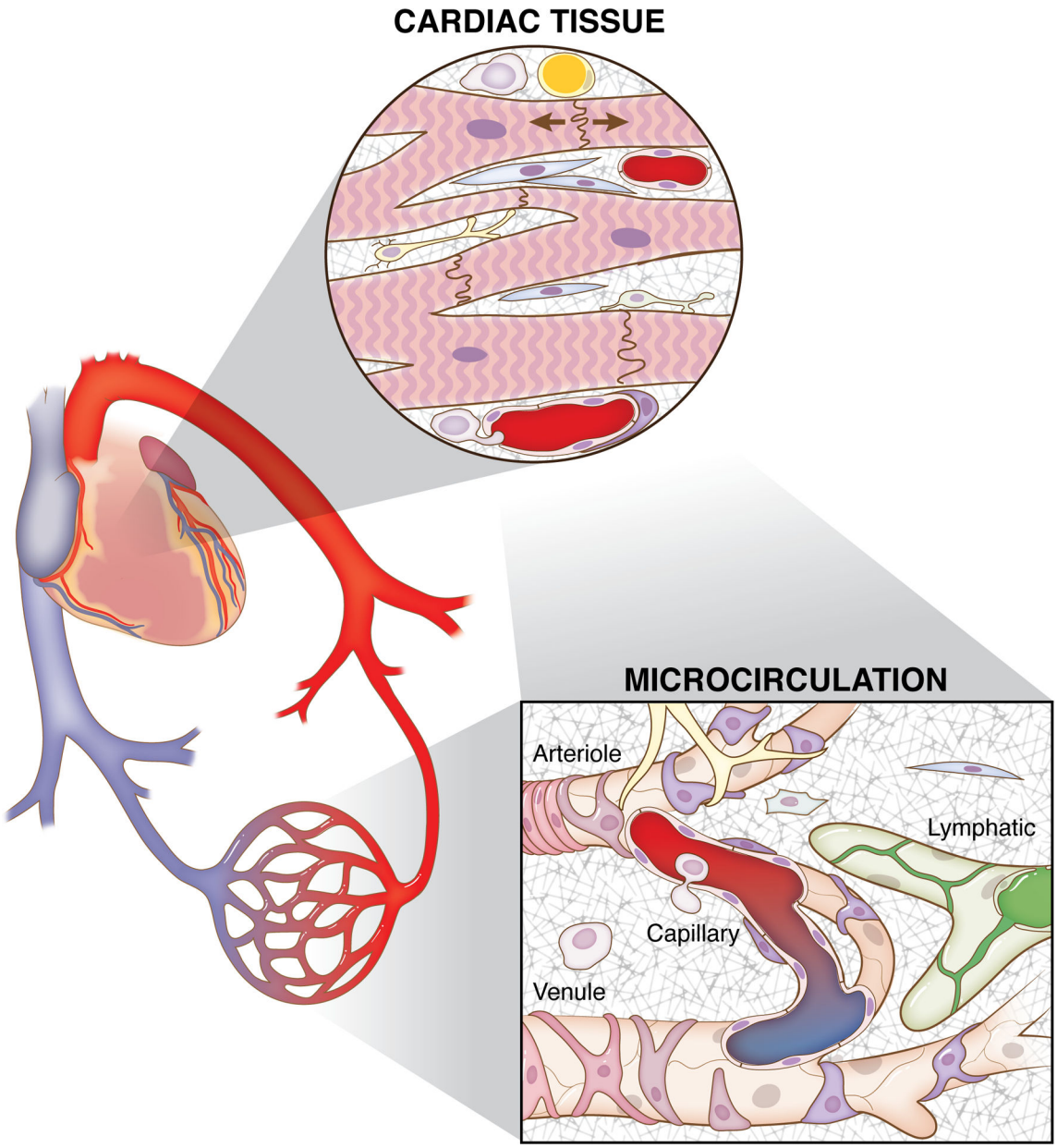

Figure 1:
Cardiovascular function depends on integrated cell dynamics across scales. The purpose of this review is to emphasize the involved cellular diversity, coordination, and interrelationships. The concert of interactions is appreciated through consideration of the specific cell types in cardiac tissues and the embedded microcirculation.

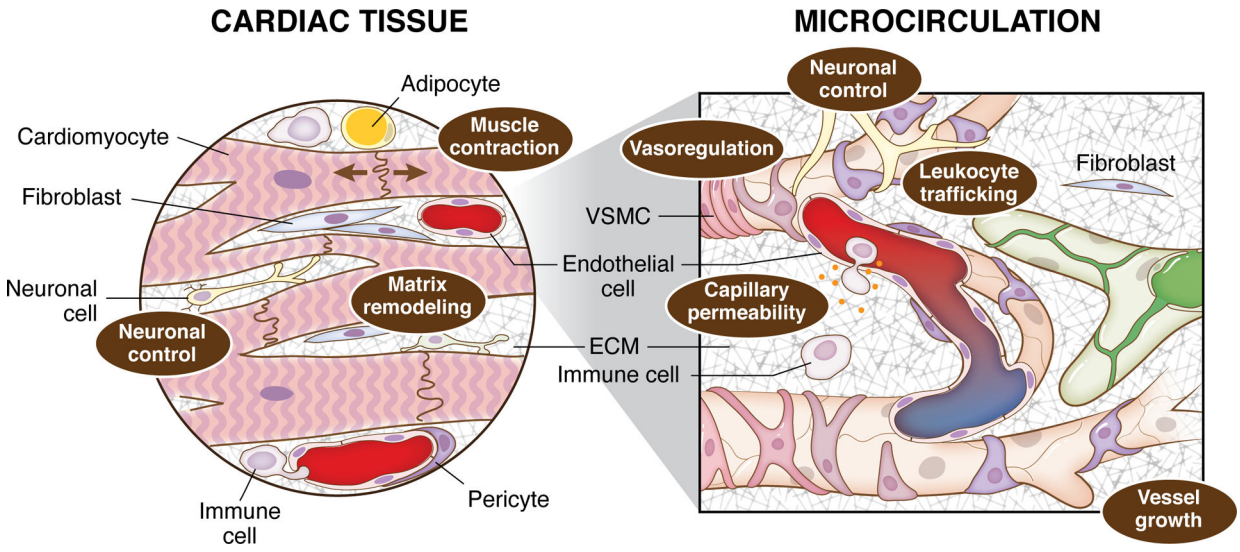

Figure 2:

Cardiac tissue function depends on multiple cell types across the cardiac and microvascular systems. The major cell types include cardiomyocytes, endothelial cells, vascular smooth muscle cells, and fibroblasts, neuronal, immune cells, adipocytes, and vascular pericytes. Interactions between the cell types influence multiple dynamics: muscle contraction, extracellular matrix (ECM) remodeling and neuronal control (cardiac tissue level) and neuronal control, vasoregulation, leukocyte trafficking and permeability dynamics (microcirculation level). The dynamics across the system level scales influence each other and contribute to the local microenvironment.

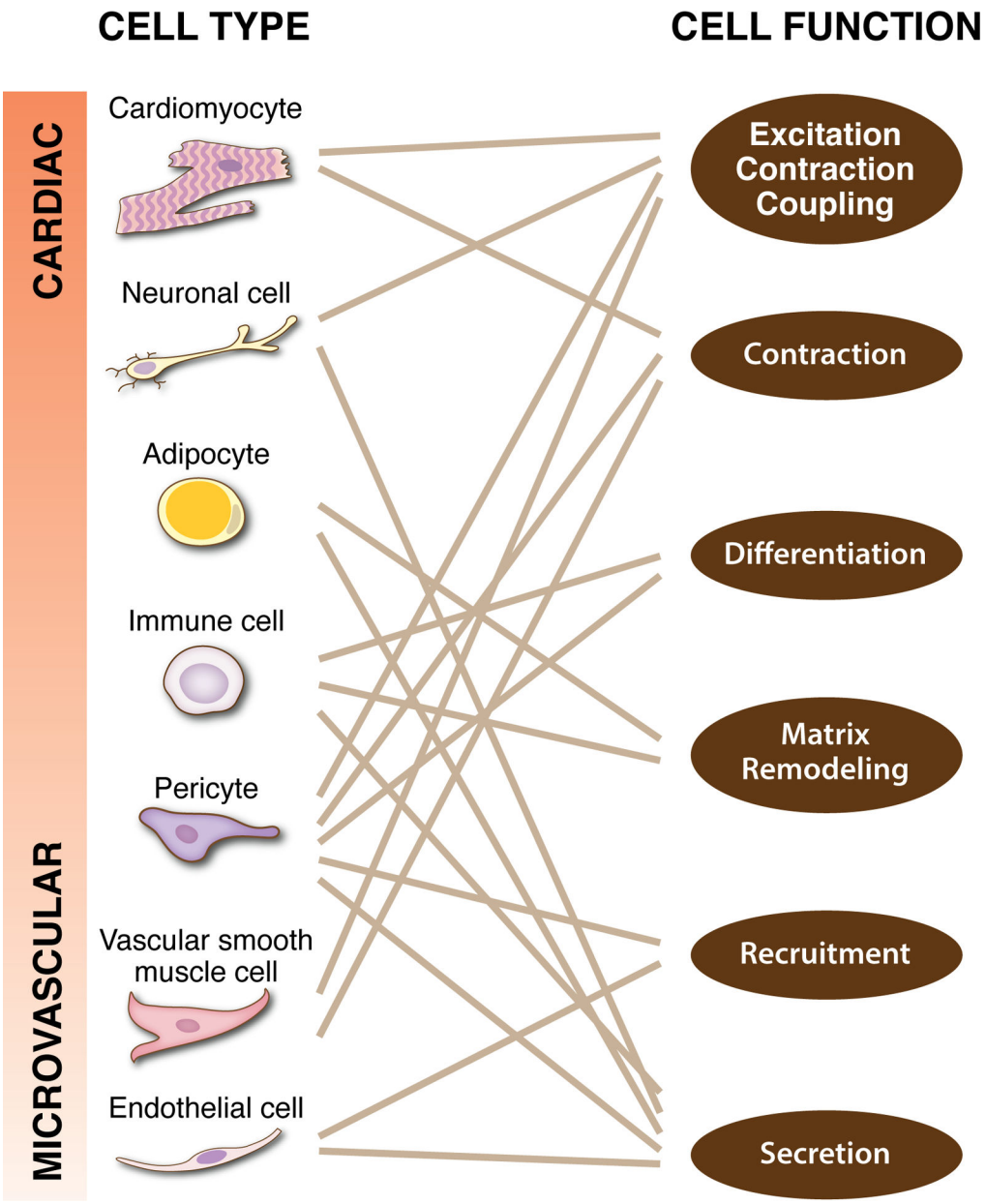

Figure 3:

Understanding cardiac tissue function is complicated by the common functions across the diverse cell types. Cell types associated with the cardiac and microvascular systems are often considered to be distinct, yet common cell functions highlight their potential overlaps along a cellular continuum. Each specific cell function can be attributed to or influenced by multiple cell types. The solid lines represent functional links and are not inclusive of all overlaps. The potential for shared mechanisms across cell types and cell-cell interactions motivates future research focused on novel models and approaches that enable multi-scale integration.

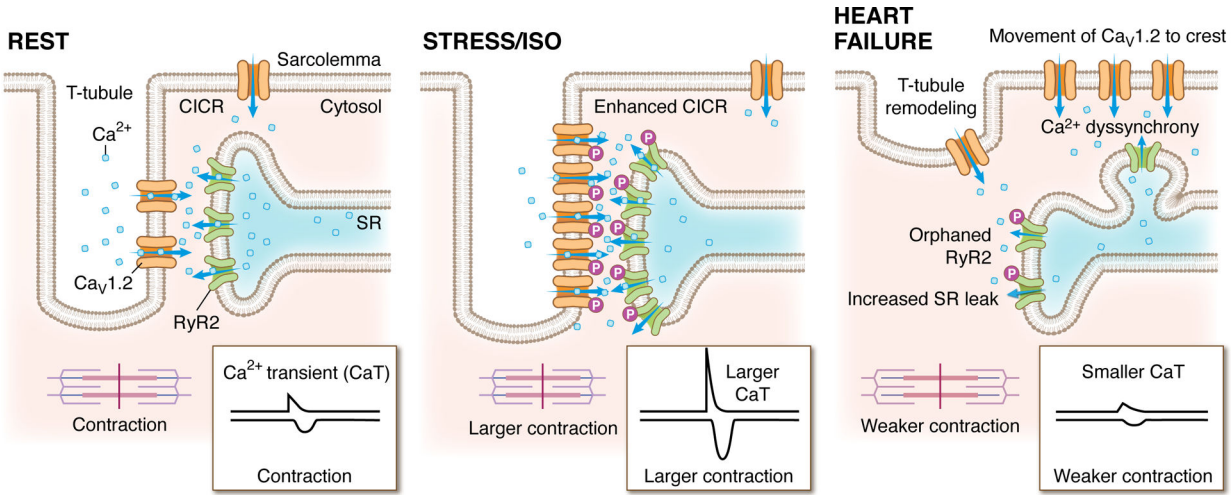

Figure 4: Nanoscale arrangement and rearrangement of dyadic calcium channels.

The localization and degree of clustering of cardiac $Ca_V1.2$ and RyR2 channels influences functional output. These cartoon depictions of the cardiac dyad, illustrate the clustering state and localization of $Ca_V1.2$ channels and juxtaposed RyR2 in resting (left), acutely stressed (middle), and heart failure conditions (right). At rest, clusters of $Ca_V1.2$ channels are located on the t-tubules opposite clusters of RyR2 on the jSR. During exercise, acute stress, or isoprenaline (ISO) application, the ensuing β-AR/G_s/adenylyl cyclase/cAMP/PKA signaling cascade results in phosphorylation of both $Ca_V1.2$ channels and RyR2, increasing their activity (P_o). In parallel, PKA stimulates enhanced recycling of $Ca_V1.2$ from an endosomal reservoir to augment $Ca_V1.2$ number and cluster size at the t-tubules. On the jSR, RyR2 clusters also undergo remodeling, becoming enlarged during stress. Altogether, the combination of enhanced channel P_o, increased levels of cooperative gating, and larger numbers of $Ca_V1.2$ channels within enlarged clusters leads to greater Ca^{2+} entry which stimulates more CICR. Larger RyR2 clusters also generate larger, more frequent sparks which summate to form larger Ca^{2+} transients and stronger contractions, creating the characteristic positive inotropic response during acute stress. Right: In heart failure, cardiomyocytes undergo architectural remodeling and ion channel cluster rearrangements. RyR2 channel cluster fragmentation/dispersal and orphaning occur as jSR elements are left behind while t-tubules recede. $Ca_V1.2$ channels migrate to the crest but the reduced proximity to RyR2 leads to an inefficient CICR that results in Ca^{2+} dyssynchrony. Hyperphosphorylation of RyR2 increases their activity leading to SR leak which elevates diastolic Ca levels in the cytosol and reduces the releasable pool in the SR that can be accessed during CICR. The result is smaller Ca^{2+} transients and weaker contractions.

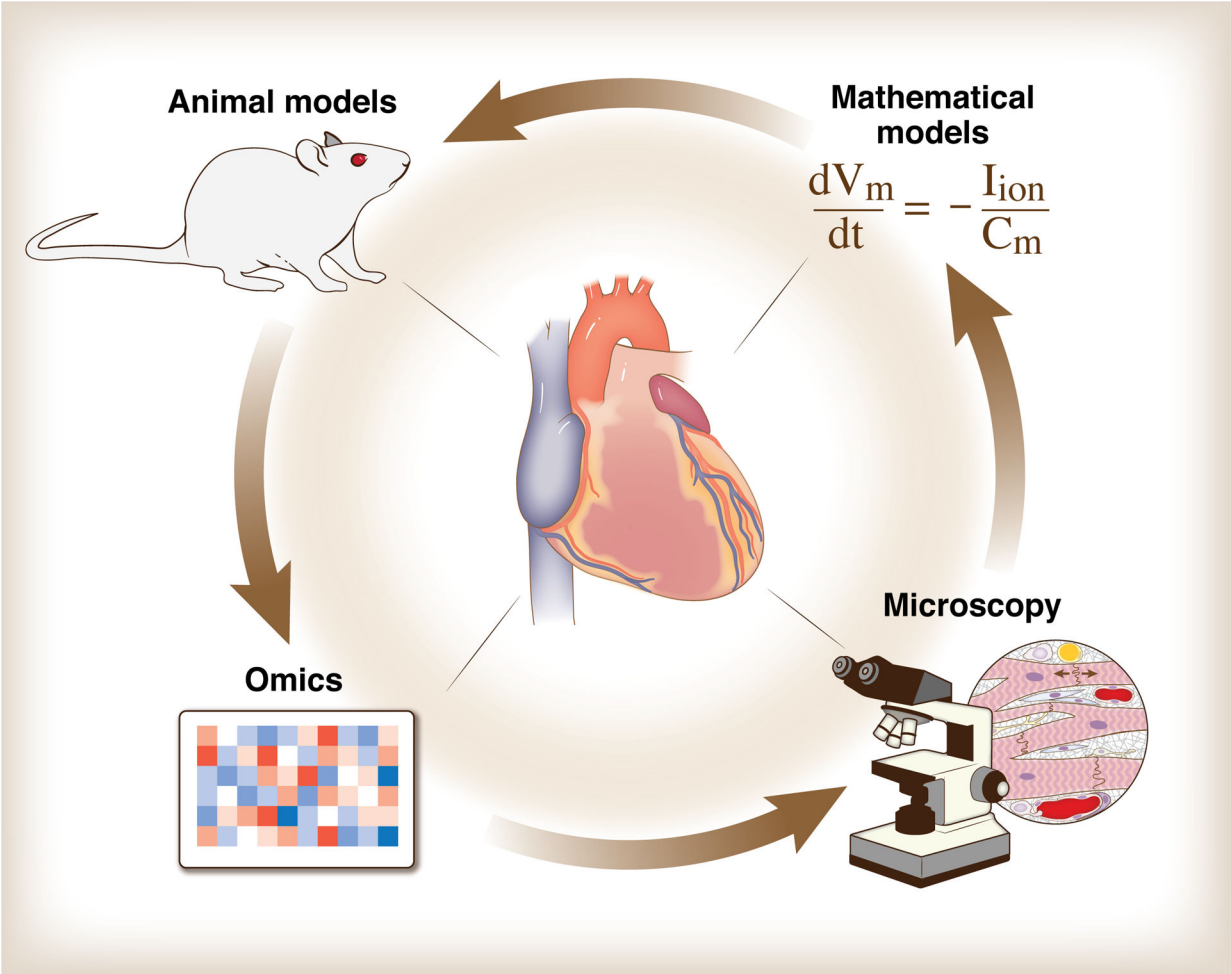

Figure 5:

Iterative cycling between technical innovations to measure and model cardiovascular function. High throughput characterization of genome, proteome, transcriptome, etc. (“omics”) and microscopy allow scientists to capture rich tapestries of cardiovascular variation in space, time, and molecule. Both animal model systems and mathematical models provide abstractions from reality that enable new insights. Used together, these and other technologies are accelerating the discovery of novel biological mechanisms and therapeutics.V_m: transmembrane voltage; I_{ion}: sum of the ionic currents; C_m: membrane capacitance.