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Seeing the Forest for the Trees

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Summary

Although tens, or even hundreds, of molecular or pharmacological interventions have been shown to impact the course of heart failure in animal models, few have translated to clinical therapies. This likely stems from the fact that biological robustness and flexibility are conferred by the evolution of redundant or adaptive signaling and gene expression pathways, which lead to not one, but multiple “master” control nodes in the network, making it difficult to pinpoint the most effective single target intervention. Systems biology tools and approaches are helping to define how the heart is remodeled during stress and to define important control points, even as they further highlight the scope of our ignorance. Applying these analyses to time-dependent changes in heart failure is essential to understand critical thresholds for aberrant non-linear cellular responses that lead to emergent events such as sudden cardiac death.

Trying to understand the mechanisms behind heart failure often brings to mind the analogy of science as “a hungry furnace that must be fed logs from the forests of ignorance that surrounds us. In the process, the clearing we call knowledge expands, but the more it expands, the longer its perimeter and the more ignorance comes into view”¹. Extending this further, we scientists often act as independent woodcutters in our own little clearings, either focusing our reductionist minds on taking the tree down like a leaf-cutter ant rather than by chainsaw, or attempting to frame our favorite target as the master key that unlocks the secret to curing the disease (a must to enchant those study section reviewers). From the literature, it seems that there are many such master keys that prevent or reverse cardiac dysfunction related to HF in animal models – fixing various defects in SR Ca²⁺ uptake, ryanodine receptor leak, repolarization reserve, oxidative stress, nitric oxide, cGMP signaling, β -receptor desensitization, CaM kinase, anaplerosis, oxidative phosphorylation, signaling through TGF β , MAP kinase, GSK3 β , etc., all have been shown to be effective in one animal model or another. So it seems that heart failure should be cured by now. Unfortunately, the

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best medical treatment still only offers a delay in contractile failure, extension of the time to morbidity and mortality, and a backstop against sudden death by means of implantable defibrillators. No doubt, these advances have made a major impact, yet individual outcomes are still highly unpredictable.

Systems Biology approaches attempt to harness the efforts of all of the lumberjacks to fell those resistant stands of forest between the clearings. This has to occur at multiple levels. Elucidating how the subsystems of the cell (e.g., ions, energy, contraction, signal transduction, gene/protein expression/modification) interact must fit in to the larger picture of the heart as a syncytium with myocyte and non-myocyte components, and at a still larger scale, how the cardiovascular system is regulated by neural, hormonal, metabolic, immune and volume regulatory elements of the organism. Predicting emergent phenomena like sudden death requires a better understanding of the system from top-to-bottom. Significant challenges to this approach exist, including: i) the large number of free parameters to deal with and a poor understanding of their interactions, ii) dynamic remodeling of the system as the pathophysiology progresses, iii) acute events/stresses that occur locally that may or may not scale to the cell/organ/organism level.

Harnessing technological developments to tease apart the system has contributed in a deep way to understanding the multifactorial nature of heart failure, yet interpretation is often clouded by nature's robust and flexible design philosophy. Knockout of one gene, or inhibition of one pathway, does not necessarily illuminate its role in the physiological or pathological process owing to redundancy and adaptation. Redundancy in signal transduction² and gene expression³ is well documented, and can occur through activation of paralogous pathways or recruitment of different components performing similar or overlapping tasks to achieve functional plasticity, referred to as degeneracy⁴. Adaptive plasticity also explains why knockout of processes assumed to be vital, such as the cardiac sarcolemmal Na⁺-Ca²⁺ exchanger⁵ or the mitochondrial Ca²⁺ uniporter⁶, often show a mild phenotype, or one that is revealed only under stress. In such cases, the question arises as to whether one is learning about the normal role of the targeted protein or the behavior of the system in its adapted state. Unfortunately, essential information to enable assessment of the alterations in the transcriptome, proteome, or metabolome of the adapted state is rarely provided if the main 'hypothesis' of the study was more or less supported, sometimes requiring impressive feats of logical contortion. This problem will not diminish as it becomes easier and less expensive to make global mutations through new technologies like CRISPR/Cas9 to knockout genes or mutate posttranslational modification sites. Conditional or inducible genetic manipulations partially mitigate these concerns, decreasing the chances of long-term adaptation, but do not eliminate short-term workarounds through adaptive posttranslational or epigenetic mechanisms. In all cases, the key is to apply available technologies to get a sense of how the baseline is changed to better evaluate the impact of a gene on the system prior to applying the disease-inducing stimulus (pressure-overload, ischemia-reperfusion injury, etc.). Does the gene knockout increase expression of a different isoform? Is there evidence of a hormetic response? Is the effect on the heart secondary to the impact of the genetic mutation on a different organ (liver, pancreas, kidney, brain)? These types of questions are typically pursued only if the results can't be shoehorned into the

presumed model or if no obvious phenotype is observed – we would argue that they are no less important when the results fit neatly into the proposed hypothesis.

In this light, omics technologies are rapidly advancing to a point where system-wide changes, induced by genetic engineering or disease induction, can be assessed fairly comprehensively. Coupled with advanced bioinformatics, strong inferences can be made about causality in terms of pathways and transcription factors active during adaptive or pathophysiological responses. We have recently applied a triple omic strategy (transcriptome, proteome, metabolomic) to investigate the ventricular adaptations during the compensatory hypertrophy and failure in a guinea pig HF/SCD model⁷. This non-ischemic model of HF displays acquired long QT, decreased repolarizing K⁺ currents, impaired Ca²⁺ handling and spontaneous arrhythmogenic sudden death, providing the opportunity to study how time-dependent cardiac remodeling might contribute to a lowered threshold for acute events to bring about fatal consequences⁸. We learned several lessons from this approach and also recognized important limitations. First, as others have previously reported⁹, there was a significant degree of discordance between the transcriptome and proteome. Hypertrophic growth was accompanied by increased transcripts of fibrosis-inducing and acute phase immune response pathways (FGF, TGFβ, TNFα) while few quantitative changes in the proteome were observed in the compensated state. However, further degradation of contractile function to heart failure was accompanied by strong downregulation of metabolic, antioxidant defense and hypertrophic growth pathways, most convincingly revealed by network analysis of the proteomic data. Specific deficiencies in enzymes involved in fatty acid activation, transport and oxidation were consistent with changes observed in the metabolome, which also revealed the accumulation of an unexpected, potentially toxic, metabolite (2-methylcitrate). The overall picture pointed to vulnerability of mitochondrial energy supply and redox balance, consistent with functional observations in failing cardiac myocytes⁸. Each methodology provided a unique window into the process of cardiac decompensation, which was distinct from the hypertrophic growth phase, and each had its own limitations. The mature technology of transcriptome analysis had the highest coverage of expressed genes, but did not necessarily predict the protein profile, while proteomics provided a direct assessment of the actual functional molecules in the cell, but with incomplete coverage of low abundance or membrane-localized proteins. Moreover, a true system-wide analysis requires quantitation of all proteoforms¹⁰ generated by the many post-translational modification mechanisms, localization of each proteoform, and determination of its binding partners (interactome)¹¹. Thus far, this has only been pursued for a few selected modifications and protein complexes. Metabolomics gives a readout of changes in the intracellular milieu but presents the challenge of trying to infer enzyme fluxes from pathway intermediates (unless tracer methods are used) and could benefit from further improvements in coverage and sensitivity.

A fully integrated and complete omic analysis incorporating all available technologies is unrealistic in most cases. Nevertheless, important insights can be gained from limited datasets by coupling the output with bioinformatics tools that harness cumulative knowledge bases to identify pathways and factors underlying system remodeling. Keeping in mind the caveats mentioned above regarding single genes or master keys, statistical methods such as causal network analysis¹² can be used to build hierarchies of potential control nodes in the

cellular protein expression network. In our case, the transition from compensated hypertrophy to heart failure clearly pointed to defects in PGC1 α , retinoic acid, and mitochondrial transcription/replication pathways. At some point, the nuclear integration of cytoplasmic second messengers (e.g., Ca, ROS, cyclic nucleotides, nitric oxide) or other signal transducers fails to maintain the machinery required for mass-energy transformation. We believe that this results in a threshold-lowering effect for disruptive emergent events. For example, a decline in metabo-redox coupling, that is, a decrease in the ability of the heart to provide reducing equivalents for ATP production (NADH) and ROS scavenging (NADPH) at sufficient rates to meet metabolic/oxidative demand lowers the threshold for nonlinear positive feedback loops to occur, resulting in cell-wide ROS overload, collapse of the mitochondrial membrane potential, uncontrolled Ca²⁺ release, and arrhythmias. This combination of flux imbalances under stress and vulnerability of the myocardial substrate accounts for an increase in the probability of a seemingly unpredictable event, such as cell death, spontaneous Ca²⁺ release, or a cellular arrhythmia to scale to the level of the whole heart.

The bridge between altered cytoplasmic signals and nuclear metabo-redox gene expression, and how it might fail, is still unknown. However, based on previous work on transcriptional control of PGC1 α , as well as a high representation of cyclic AMP response elements (CREs) and/or PPAR γ transcriptional elements in the promoter regions of the downregulated proteins, we propose that failed CREB/CREB-binding protein transcriptional signaling could be one factor⁷. Of course, given the redundancy argument, it is likely that CREB would not be the only nuclear integrator regulating metabolic and antioxidant pathways. For example, the proteomic analysis also revealed that retinoic acid and estrogen receptor signaling are also impaired in the HF/SCD model, and there is substantial target overlap in these pathways, particularly with respect to lipid transport, β -oxidation, and ion transport. Indeed, it has been elegantly argued that the accumulation of multiple master regulators for critical cellular processes occurs naturally during evolution via the accrual of neutral (non-adaptive) gains of regulatory connections³. If that is the case, we should probably give up on the notion of a single master key to heart failure; instead, we should define the groups of master regulators that positively impact outcomes and figure out how their inputs or interconnections fail. Much additional work needs to be done to confirm these hypotheses and conceive of ways to correct possible defects for therapeutic benefit. In particular, it will be important to apply available omics technologies to better define how the system is changing in time, and how functional perturbations are handled as the heart chronically remodels subsequent to load, oxidative- or ischemia-induced stress.

In summary, we must remain vigilant to avoid falling into the trap of single target cures for heart failure. Nevertheless, detailed investigation of the control properties of the system may indeed identify those processes that could be targeted effectively without being circumvented by pathophysiological redundancy or causing further maladaptation. The concept of a variable “emergence threshold” as chronic proteome remodeling occurs is worthy of further investigation to explain the increased propensity for arrhythmias, increased rates of cell dropout, or sudden death associated with heart failure.

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