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## Unraveling the complexities of cardiac remodeling and hypertrophy — High-content screening and computational modeling

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Heart weight directly correlates with body weight in most mammals under homeostatic conditions [1]. However, numerous stimuli and stresses have been found to elicit myocardial growth, increase heart size, and remodel ventricular shape. Physiological provocations, such as pregnancy and exercise, result in an increase in heart size accompanied by enhanced cardiac output that is completely reversible and not related to adverse events later in life. On the other hand, it has been appreciated for some time that numerous molecular signals elicit hypertrophy and remodeling that initially could be considered a positive response to cope with an acute insult, but with persistent signaling become maladaptive. Numerous stressors have been implicated in pathological cardiac hypertrophy/remodeling including: aortic valve stenosis, severe mitral valve regurgitation, genetic mutations, hypertension and myocardial infarction, where the remote myocardium must compensate for the lost region of cardiomyocytes [2].

The signaling pathways that lead to cardiomyocyte hypertrophy and remodeling have been extensively studied, resulting in complex molecular pathways from the level of ligand/receptor signaling through a host of second messengers, to post-translational pathways converging on transcriptional programs. Using a reductionist approach, often incorporating pharmacologic agents and gain-/loss-of-function experimental systems, countless studies have contributed to the design of a cardiac remodeling pathway tree of enormous proportions that has been the subject of recent reviews [3–5]. However, our traditionally linear experimental approaches, while expansive and redundant in scope, have largely failed to define the exact signaling patterns that distinguish physiological from pathological remodeling [6,7]. Further, it remains unknown how physiological hypertrophy is completely

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None.

reversible without adverse effects, while pathologic hypertrophy rarely resolves and often results in heart failure or sudden cardiac death. Perhaps most damning to the utility of traditional research methodologies is the meager translation of the identified ‘key’ pathways and targets to combat the complex etiologies of clinical heart disease [5].

A major limitation of a reductionist experimental design is that often only a single stimulus is employed, such as using a single receptor ligand to stimulate cardiomyocytes and the interrogation of a known signal transduction pathway to see where a given target ‘fits’ into the canonical scheme. This remains the modus operandi even though it is recognized that many different stimuli simultaneously activate numerous pathways contributing to the overall cellular response. To widen the experimental net and unmask previously unknown associations in hypertrophic signaling a recent study by Ryall et al., “*Phenotypic screen quantifying differential regulation of cardiac myocyte hypertrophy identifies CITED4 regulation of myocyte elongation*”, published in the Journal of Molecular and Cellular Cardiology, utilized high-content imaging coupled with cluster analysis and mathematical modeling [8]. The approach they took was to expose neonatal rat cardiomyocytes (NRCMs) to 15 different receptor agonists independently at three different concentrations to stimulate cardiomyocyte hypertrophy (Fig. 1). Next, employing automated imaging cardiomyocytes were measured for four different shape features: area, elongation, perimeter and form factor as well as fluorescence intensity (Troponin T promoter driving GFP). At the end of the experiment, 48 h after ligand delivery, RNA was isolated and the expression of 12 different genes was measured by qPCR. The genes were selected for analysis based on their associations with various cardiomyocyte phenotypes with a design purpose of examining a breadth of pathways. To holistically examine how hypertrophic signaling pathways interrelate the authors performed clustering analyses and mathematical modeling to expose previously unknown relationships.

The main observation made was that while the 15 ligands employed have been well documented to induce hypertrophy, here they all displayed very unique profiles, with distinct changes in shape and gene expression. The authors computationally delineated these differences by clustering shape and gene changes into normalized input and output modules, building a theoretical model of the hypertrophic signaling network to generate novel hypotheses. Surprisingly, most of the ligands used didn’t actually change myocyte area, the hallmark measure of hypertrophy, but instead the biggest phenotypic effect was alterations in form factor, a measure of cellular circularity. The finding that only four of the agonists elicited a change in cardiomyocyte area is somewhat confounding and could be a limitation of methodology, perhaps due to the fact that freshly isolated cells were initially cultured in 15% serum which contains many growth factors that could obfuscate a potential hypertrophic effect. Regardless, the more interesting aspect here is that while historically most studies have used cross-sectional area as the cellular readout of hypertrophic signaling, this paper suggests that examining additional parameters might distinguish between pathways and perhaps even types of hypertrophy. This is especially relevant given the clear clinical difference between physiological and pathological hypertrophy.

It is not entirely clear whether the geometric shape of the heart in response to a pathological stimulus is solely defined by differences in molecular signaling between patients, or more a

reflection of disease progression. Arguing in favor of a molecular switch driving variations in hypertrophic phenotypes, numerous mutant mouse models have been shown to respond differently to a given hypertrophic stimulus [5,9]. Some mouse models develop clear concentric hypertrophic remodeling in response to pressure overload, while others immediately progress to either eccentric remodeling or left ventricular dysfunction. While these genetic models have proven very valuable in identifying key targets and pathways they have fallen short of distinguishing the complex molecular interplay responsible for the variation in hypertrophic phenotypes that is elicited by a wide-range of stimuli. However ambiguous, the importance of differences in left ventricular shape should not be understated as LV shape alterations have been directly correlated to mortality [10]. The current study by Saucerman and colleagues builds upon previous work by the group, now incorporating a more complex screen design and bioinformatics analyses to discover connections between different pathways that could be important to distinguish different types left ventricular remodeling [11,12].

One of the interesting pathways uncovered by the Saucerman lead study is a link between the fibrosis-associated gene, connective tissue growth factor (CTGF), and the pro-death gene Bcl2-associated X protein (BAX). While it is easy to speculate that apoptotic and fibrotic gene programs may possess commonality in origin, the correlation identified by cluster analysis suggested a potential causal relationship. The agonists Angiotensin II and Endothelin-1 both drove increases in CTGF and BAX expression. Subsequent loss-of-function experiments found that CTGF was required for Ang II mediated increases in BAX expression, placing a key fibrotic component directly in the apoptotic-signaling pathway.

It is well known that CTGF becomes upregulated in the fibrotic heart and is induced by many of the ligands examined. Further, pathological hypertrophy is often associated with fibrosis and cell loss adding meaningful context to the link between these pathways [13,14]. While CTGF is viewed as a strong fibrotic cue, to what extent CTGF signals independently of transforming growth factor- $\beta$  (TGF $\beta$ ) is not entirely clear. It's interesting to note that the current study also found TGF $\beta$  to have a positive correlation with CTGF expression, although it did not show an association with BAX expression. To what degree CTGF modulates BAX-dependent cell death in an in vivo model system remains to be tested. A superficial assessment allows for the obvious conclusion that increasing CTGF signaling would promote pathology in the heart by driving fibrosis and simultaneously increasing pro-death signaling, however, recent in vivo gain-of-function experiments (transgenic overexpression of CTGF) have found increased levels of CTGF to be associated with a decrease in disease indices. In a myocardial infarction model CTGF transgenic mice were found to display preserved LV function and decreased hypertrophy [15]. Also, in an Ang II osmotic pump model it was reported that mice overexpressing CTGF displayed preserved LV function compared to non-transgenic controls [16]. Interestingly, in neither study did increasing CTGF expression in the heart overtly alter fibrosis. However, it is noteworthy that in the same study CTGF overexpressors developed LV dilation and decreased LV function at 7 months of age, suggesting that another possible mechanism outside fibrosis was at play. In addition, a few reports have stated that CTGF is sufficient to induce apoptosis although a mechanism of action was not explored [17, 18]. In light of the current study it's intriguing to speculate that CTGF signaling via BAX may be responsible for some

of these counterintuitive results. Further experimental data is needed to resolve the molecular interplay between CTGF and BAX in the hypertrophic response and its biological relevance.

A second interesting correlation revealed by the current study is the potential link between myocyte elongation and Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 4 (Cited4) expression. To test this relationship the authors utilized neuregulin-1 (Nrg1) and leukemia inhibitory factor (LIF), which both induced elongation but had differing effects on Cited4 expression. Knockdown of Cited4 increased Nrg1-induced elongation, indicating a negative regulatory role. Mathematical modeling suggested that Nrg1 stimulates both elongation and Cited4 expression; with Cited4 acting as the brake on eccentric growth only once a given threshold of elongation is reached. This computational model was supported by the observation that the loss of Cited4 did not effect elongation under control conditions and that adenoviral overexpression of Cited4 in vivo did not effect myocyte elongation but did increase myocyte area and markers of proliferation.

These observations fit the recent paradigm proposed by the Spiegelman group, suggesting Cited4 is upregulated with exercise (negatively regulated by C/EBP $\beta$ ) and promotes physiological hypertrophy [19]. How these new findings relate to the previous work remains to be determined. There have been many studies that have attempted to define the differences in signal transduction pathways to discriminate between eccentric and concentric remodeling. Clinically, this is of great importance since during pathological stress elongation is a hallmark of deleterious remodeling (dilation) and deterioration of function (decompensation). The most important determinant of whether the heart displays concentric or eccentric hypertrophy is the type of growth the individual cardiomyocytes display. When a cardiomyocyte hypertrophies, it can add sarcomeres either in series (eccentric hypertrophy) or in parallel (concentric) [20,21]. However, merely defining hypertrophic growth as concentric or eccentric is a gross over simplification. For example, hypertension does not always induce concentric hypertrophy. Often, there is a mix of concentric and eccentric, or even asymmetric growth [22,23]. The signaling pathways governing these different types of growth are not well understood, but the ERK branch of the MAPK signaling pathway has been shown to be a key determinant of the switch between concentric and eccentric hypertrophy, where loss of Erk1/2 resulted in lengthening of cardiomyocytes and eccentric heart growth [24]. In light of the current work, an in-depth study to delineate the molecular interplay of C/EBP $\beta$ , Cited4, and MAPK signaling is warranted.

More work is clearly needed to determine whether there are indeed different types of cardiac hypertrophy that can be distinguished based on shape changes in cell culture models and gene expression data, but the Saucerman group has presented us with an exciting screening approach that may shed light on the multifaceted signaling that drives hypertrophic growth. One can envision further development of this methodology, increasing the breadth of input and output modules and defining to what extent these data can be translated to adult cardiomyocytes, and in vivo models of disease. This innovative approach may help clarify the intricacies of hypertrophic signaling and lead to a more comprehensive understanding of the interconnectedness of known molecular pathways. Potentially, such an approach could identify signaling nodal points to develop targeted therapies to prevent the detrimental

consequences of cardiac hypertrophy. With the continued evolution of high-throughput methodologies we may yet solve the mystery that is cardiomyocyte growth.

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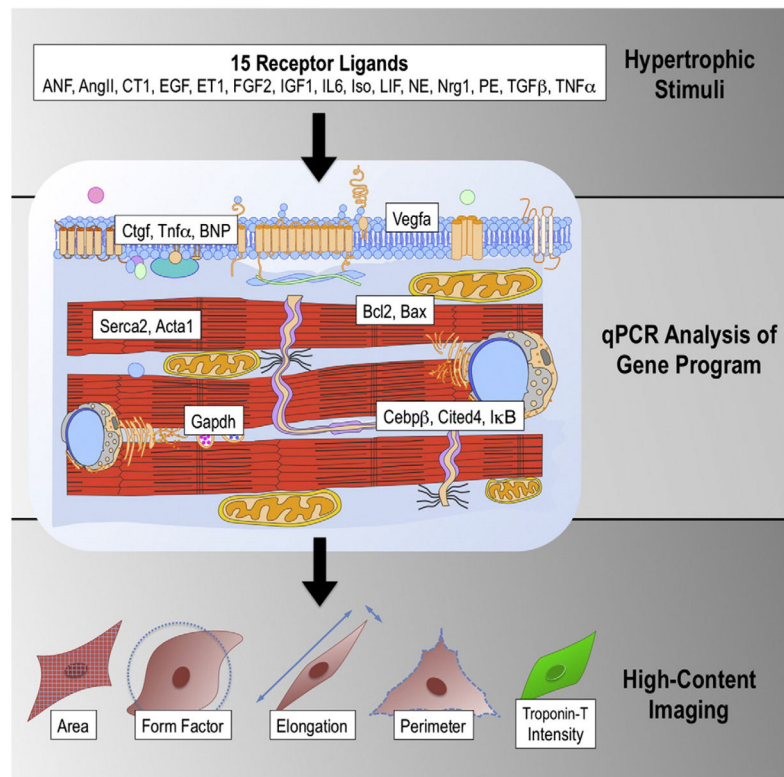
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**Fig. 1.** Screening approach to identify new regulatory pathways governing diverse types of cardiomyocyte hypertrophy. Neonatal rat cardiomyocytes (NRCM) were isolated, plated in 96-well plates and transfected with a troponin-T GFP reporter. Two days after transfection, 15 different hypertrophic agonists, at three different doses, were delivered individually to individual wells. 48 h after stimulation, automated microscopy was performed and individual NRCMs were analyzed for a transformation in shape. Cells were measured for changes in area, form factor (a measurement of circularity), elongation, perimeter, and also for GFP fluorescence intensity. Immediately following imaging, RNA was isolated and the expression of 12 different genes was examined by qPCR. Next, computational analysis was performed and the data was clustered into input and output modules. Using this approach the authors were able to discover novel pathways for hypothesis-based experimentation.