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Why is it important to analyze the cardiac sarcomere subproteome?

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“Cardiac sarcomeres represent a highly ordered and multifunctional protein network, significant not only as the molecular machines responsible for ejection of blood, but also as critical elements in signal reception and transduction.”

Diseases of the heart are epidemic and there is an urgent need for better diagnosis, prevention and treatment of these disorders. We focus here on proteomic analysis of cardiac sarcomeres, which house the molecular machines and major energy consumers for cardiac contraction. Established and emerging evidence [1] on the significance of an altered sarcomere subproteome in acquired and genetic disorders of the heart forms a significant rationale for our focus on the sarcomeric subproteome. Moreover, the relatively large abundance of sarcomeric proteins in heart muscle cells makes them excellent candidates for developing and refining analytical methods for the detection of important modifications in less abundant subproteomes.

Cardiac sarcomeres represent a highly ordered and multifunctional protein network, significant not only as the molecular machines responsible for ejection of blood, but also as critical elements in signal reception and transduction [2]. Moreover, proteins of the Z-disk and M-band, which communicate with each other through the giant protein, titin, and with the cytoskeletal network form a powerful cellular tool for mechano-signaling.

A major signal is cellular Ca^{2+} , which binds to the sarcomere receptor troponin C and triggers systole by setting into motion a complex set of protein–protein interactions, promoting the reaction of myosin cross-bridges (the molecular motors) with actin [3]. Membrane channels, exchangers, and transporters control release and reuptake of cellular Ca^{2+} sensed by the sarcomeres [4], but there is strong evidence that systole is maintained by mechanisms intrinsic to the sarcomeres involving cooperative processes which spread activation along the thin filaments independently of bound Ca^{2+} [3]. Thus, in conjunction with the activity of these membrane proteins, sarcomeric proteins play a major role in the control of cardiac dynamics and in tuning the heartbeat to the prevailing heart rate.

“There is ample evidence that acquired stresses induce specific post-translational modifications in sarcomeric proteins...”

Physiological stresses, such as exercise, evoke beat-to-beat alterations in the form of post-translational modifications (PTMs), which alter the function of the sarcomeres permitting adaptation to the stress. Chronic stress, such as prolonged exercise training, promotes the

growth of terminally differentiated heart cells by increasing myocyte sarcomeric mass and inducing myocardial remodeling [1]. With acquired pathological stresses, such as in chronic hypertension, reduced coronary blood flow, and myocardial infarction, beat-to-beat and chronic alterations in the post-translational state and the abundance of sarcomeric proteins become maladaptive. Heart failure follows as characterized by depressed and inefficient force generation, dynamics and power, and a propensity toward arrhythmias, the common terminal event.

There is ample evidence that acquired stresses induce specific PTMs in sarcomeric proteins, which are linked to the transition from adaptive responses to maladaptive stress responses leading to heart failure [1,5]. In the case of genetically determined cardiomyopathies, linkage to mutations in the sarcomeric proteins is without question [6]. Prevalent and penetrant mutations in sarcomeric proteins form the major links to both hypertrophic and dilated cardiomyopathies. In these disorders, heart cells undergo maladaptive growth as well as cellular and subcellular disarray, triggered by modifications in sarcomeric proteins. PTMs of sarcomeric proteins, including phosphorylation [7], acetylation [8], methylation [9], oxidation [10] and nitration [11], represent potent mechanisms for altering sarcomere function. Yet apart from phosphorylation [7], the effects and presence of these other PTMs remain poorly understood. Even in the case of phosphorylation of sarcomeric proteins, which has been studied extensively, there is controversy regarding the significance and/or presence of this PTM in common disorders of the heart [12,13].

“Biologically significant changes in the sarcomere could ... be selectively targeted and quantified using multiple reaction monitoring in a label-free manner, allowing for sensitive and high-throughput comparisons of proteomic changes...”

The relationship between a particular mutation and molecular and cellular phenotype has led to drug targeting for potential rescue of the phenotype. For example, many of the hypertrophic cardiomyopathy (HCM)-linked mutations in sarcomeric proteins induce an enhanced sensitivity of myofilament force development to Ca^{2+} , whereas dilated cardiomyopathy (DCM)-linked mutations induce a decreased sensitivity to Ca^{2+} [14]. Agents that sensitize myofilaments to Ca^{2+} have demonstrated an ability to rescue the DCM phenotype [15], whereas agents that desensitize the myofilaments to Ca^{2+} rescue the HCM phenotype [16]. This is a significant aspect of the need for proteomic information for animal models and humans. Treatment may depend on the extent of expression of the protein and its localization in sarcomeric or nonsarcomeric compartments of the myocytes. There is also evidence that phosphorylation of a nearby neighbor of the mutant proteins may amplify or expose its effect on sarcomeric function [17]. Other PTMs may also modify the functional effect of a particular mutation, but this has not been systematically investigated.

Analysis of the sarcomeric subproteome has been carried out by top-down [18–21] and bottom-up approaches [22–24]. Top-down approaches have provided significant and novel information on the extent and type of PTMs; however, they are as yet not practical for application to small samples, such as a single mouse heart or a biopsy. Currently, study of the entire sarcomeric fraction, rather than selected proteins of interest, requires a bottom-up approach. In our workflows we have employed subcellular fractionation to increase our detection limit of the sarcomeric subproteome. In conjunction with the core technology of high-level mass spectrometry (MS), we have employed a number of widely used approaches, such as anti-phospho-specific antibodies, 2D gels coupled with differential in gel electrophoresis [2D-DIGE], and O^{18} labeling of post-tryptic peptides [22–24]. An important recent approach in our analysis has been a gel-free method employing tandem OFFGEL electrophoresis-high-performance liquid chromatography (HPLC) for in solution separation of functionally distinct charge variants of intact sarcomeric proteins prior to digestion and LC tandem MS (LC-MS/MS) analysis, [25,26]. This workflow permitted the

preparation of microgram amounts of intact sarcomere proteins, notably myosin regulatory light chain (RLC), sufficient to identify for the first time a novel arrangement of sites of RLC phosphorylation linked to cardiac function [22]. The salient advantages of this approach included the avoidance of difficulties with in-gel enzymatic digestion and extraction, and the ability to enrich for the specific population of RLC containing the phosphorylation thus substantially increasing the identification potential by LC-MS/MS. In addition to intact protein separation, the development of OFFGEL electrophoresis has proven largely successful for peptide separation by isoelectric point in the liquid phase prior to LC-MS/MS [26]. We [27] and others [28,29] have recently demonstrated that the use of peptide OFFGEL separation coupled to iTRAQ labeling allows for multiplexing and relative quantifying of the sarcomeric subproteome. OFFGEL electrophoresis compares favorably with the classic MudPIT approach [30], especially when coupled with iTRAQ labeling [30].

Multiplexing of clinical samples through the application of iTRAQ labeling coupled to OFFGEL peptide separation has proven successful for biomarker discovery and quantification by making comparisons less variable and MS instrument and analysis time more efficient [28,29]. Our future direction will be to incorporate sarcomeric subproteomic fractionation, OFFGEL and iTRAQ as a discovery-based workflow to determine significant changes in protein expression and PTMs of the sarcomere. Biologically significant changes in the sarcomere could then be selectively targeted and quantified using multiple reaction monitoring (MRM) in a label-free manner, allowing for sensitive and high-throughput comparisons of proteomic changes occurring in both human heart failure and animal models of disease. A component of the sarcomeric master regulatory troponin complex, cardiac troponin T, has been quantified using MRM for marking myocardial injury [31]. In addition, methods have been developed employing the specific and sensitive nature of MRM to identify sites of phosphorylation. This is termed MRM-initiated detection and sequencing [32]. With the rapid development and application of both instrumentation and technologies, as mentioned earlier, incorporation of an MRM workflow to quickly and quantitatively determine changes in protein abundance and some selected PTMs of interest in several key sarcomeric proteins all in one assay will be a valuable tool.

The need for highly sensitive analysis of PTMs in the cardiac subproteome is exemplified in our recent studies with a transgenic mouse model expressing cardiac specific and pseudo-phosphorylated mutants of the critical thin filament protein, troponin I [33]. In these studies, extensive proteomic analysis demonstrated replacement of the endogenous cTnI with only $7.2 \pm 0.5\%$ of the mutant [33]. Even with this modest alteration in cTnI, there was a significant induction of reduced myocardial pressure development and dynamics unrelated to changes the intracellular Ca^{2+} transients [33]. Similar effects have been noted with this level of expression of mutant troponin T linked to HCM [6]. Moreover, ablating sites of phosphorylation on the N-terminal region of the thick filament RLC evoked substantial alterations in cardiac ventricular function and phosphorylation of neighboring proteins [6]. The cardiac sarcomere is an organelle awaiting detailed exploration by proteomic approaches. As quantitative proteomic approaches become more precise and reproducible, we will be able to apply these to the sarcomere, where small changes in protein expression or PTMs elicit large effects on function.

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