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Proteomics in Heart Failure: Top-down or Bottom-up?

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Summary

The pathophysiology of heart failure (HF) is diverse, owing to multiple etiologies and aberrations in a number of cellular processes. Therefore, it is essential to understand how defects in the molecular pathways that mediate cellular responses to internal and external stressors function as a system to drive the HF phenotype. Mass spectrometry (MS)-based proteomics strategies have great potential for advancing our understanding of disease mechanisms at the systems level because proteins are the effector molecules for all cell functions and, thus, are directly responsible for determining cell phenotype. Two MS-based proteomics strategies exist: peptide-based bottom-up and protein-based top-down proteomics—each with its own unique strengths and weaknesses for interrogating the proteome. In this review, we will discuss the advantages and disadvantages of bottom-up and top-down MS for protein identification, quantification, and the analysis of post-translational modifications, as well as highlight how both of these strategies have contributed to our understanding of the molecular and cellular mechanisms underlying HF. Additionally, the challenges associated with both proteomics approaches will be discussed and insights will be offered regarding the future of MS-based proteomics in HF research.

Keywords

Heart failure; proteomics; mass spectrometry; post-translational modifications; systems biology

Introduction

Heart failure (HF) afflicts an estimated 23 million people worldwide and is the single most common cause of hospital admissions for people over the age of 65 (8, 32). As a consequence of the fact that many factors, including myocardial infarction (MI), hypertension, cardiomyopathies, and atrial fibrillation (AF), contribute to or are associated

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with HF, the etiology of this syndrome can vary significantly from patient to patient—precluding a clear understanding of the cellular and molecular mechanisms underlying HF (8, 22). Nevertheless, studies employing reductionist approaches have identified HF-associated alterations in a number of critical cellular processes including Ca^{2+} handling, energy metabolism, and contractile function (35); however, how these processes, and the molecular pathways that drive and regulate them, function together to promote HF pathophysiology remains unclear. Therefore, systems biology, which is a holistic approach for describing complex multifaceted biological systems (12), holds great potential for the elucidation of HF-associated disease mechanisms.

At the core of systems biology is the notion that the thorough identification of all of the biomolecular constituents of a given system is not sufficient to understand how the system operates; instead, in order to truly understand how a system functions, it is imperative to understand how the constituents of that system interact with each other over a range of perturbations (24). Thus, proteomics, which is concerned with the comprehensive characterization of all facets of protein biology, including the determination of protein localization, modifications, interactions, activities, and, ultimately, their function (17), is particularly well-suited for advancing our understanding of complex disease mechanisms at the systems level (15). Unfortunately, unlike the genome, which is considered to be largely static, the proteome is highly dynamic owing to a large dynamic range of protein expression as well as considerable complexity that can be attributed to a myriad protein forms, or “proteoforms” (48), arising from mutations, truncations, alternative splicing events, and the addition of post-translational modifications (PTMs), which makes analysis of the proteome technically challenging.

While early proteomics studies employed two-dimensional gel electrophoresis (2DGE) to identify disease-associated changes in the proteome, mass spectrometry (MS) has quickly become the method of choice for proteomics studies due to the wealth of information that can be obtained regarding both the identity and quantity of proteins in a sample as well as their PTMs (2). In this review, we will discuss examples of studies using either bottom-up or top-down proteomics to highlight how the use of these strategies has advanced our understanding of the molecular underpinnings of HF, as well as to showcase the unique abilities of these approaches for the identification of proteins and protein interacting partners, detection of protein modifications, and quantification of changes in protein expression or PTM occupancy. Furthermore, we will discuss challenges associated with both approaches and offer insights regarding the future of MS-based proteomics in HF research.

Proteomics strategies: bottom-up or top-down

Mass spectrometers consist of three essential components: an ion source, a mass analyzer, and a detector (2). In the ion source, proteins or peptides are converted into gas phase ions, which are then separated according to their mass-to-charge ratio (m/z) in the mass analyzer, and recorded by the detector. The large biomolecules need be ionized using either matrix-assisted laser desorption/ionization (MALDI) or electrospray ionization (ESI)—so called “soft ionization” techniques that can preserve the structure of proteins and peptides as they are transferred from the condensed phase into the gas phase (33, 52). Until recently,

proteomics researchers relied on mass spectrometers with ion trap or time-of-flight (TOF) mass analyzers, which resulted in only moderate confidence in protein identification because of the low mass accuracy afforded by these instruments (29). However, in the past several years, a number of commercially available hybrid mass spectrometers with high-resolution mass analyzers such as the LTQ/FT, LTQ-Orbitrap and quadrupole-TOF (Q-TOF) have become available, which have substantially increased the number of MS-based proteomics applications in biological research (30, 58, 60).

Two MS-based proteomics approaches exist, namely, bottom-up and top-down proteomics, each with its own advantages and disadvantages for the identification of proteins and protein interacting partners, protein quantification, and the analysis of PTMs (Table 1). In bottom-up proteomics, proteins are first digested and information obtained through MS analysis of the resulting peptides is used to infer the identity, quantity, and modification status of the proteins present in the sample (Figure 1). In a traditional bottom-up MS workflow, proteins extracted from a cell or tissue are either subjected to in-solution proteolytic digestion using an enzyme such as trypsin or are first separated via SDS-PAGE followed by in-gel digestion (2). In the former case, the peptides are frequently separated by two-dimensional liquid chromatography (LC) on the basis of hydrophobicity (reverse phase chromatography) and charge (ion exchange chromatography) and, in the latter case, gel bands are excised, digested, and the resulting peptides are separated using reverse phase LC (Figure 1) (50). The separated peptides are then ionized, introduced into the mass spectrometer, and the number of ions at each m/z value is registered by the detector. After a full scan of all m/z values, targeted ions are isolated and fragmented using tandem MS (MS/MS) techniques, which provide information related to protein sequence and the location of PTMs (Figure 1) (2, 58). Also, in addition to protein identification, a number of methods are currently available that allow for both the relative and absolute quantification of proteins using bottom-up MS (discussed below).

In top-down proteomics, intact proteins are analyzed, rather than peptides, which decreases sample complexity (in contrast to bottom-up MS in which sample complexity is increased as a consequence of proteolytic digestion) and preserves all information related to the status of the intact protein, including PTMs and sequence variations arising from mutations, truncations, and alternative splicing events (Figure 1) (47, 60). Unfortunately, as the physiochemical diversity of intact proteins is much greater than that of peptides, large-scale separation of intact proteins is challenging and, thus, traditional top-down studies have primarily focused on the analysis of a single or small number of proteins, typically obtained via affinity purification (AP) (5, 14, 44, 57, 59, 61). As in bottom-up proteomics (with the exception that intact proteins are analyzed in top-down MS), following MS analysis, specific proteoforms of interest can be isolated and fragmented using MS/MS techniques (Figure 1).

A variety MS/MS techniques have been developed for both bottom-up and top-down proteomics, which can be divided into two groups: energetic dissociation and non-ergodic dissociation. While invaluable for protein sequence characterization, energetic dissociation methods, such as collision-induced dissociation (CID) (45), high-energy collision dissociation (HCD) (37), post-source decay (PSD), infrared multiphoton dissociation (IRMPD) (27), and the more recently utilized UV photodissociation (UVPD) (46),

preferentially cleave bonds of the lowest activation energy, resulting in the loss of labile PTMs—thereby precluding their localization. On the other hand, the non-ergodic fragmentation techniques, which include electron capture dissociation (ECD) (63) and electron transfer dissociation (ETD) (51), are particularly valuable for the localization of labile PTMs such as phosphorylation because cleavage is primarily localized along the protein backbone and, thus, labile PTMs are retained (20, 60).

Protein identification

In order to understand how complex biological systems such as cells, tissues, or organs are altered in disease, it is imperative to not only identify the constituents of the system but also to know how those constituents interact with each other to bring about functional outcomes (15, 41). Consequently, MS-based identification of proteins and protein interacting partners is an essential first step on the path to understanding complex clinical syndromes such as HF.

Although 2DGE offers easy visualization of protein spots for quantification (discussed below), as a separation method, 2DGE suffers from limited resolution and high sensitivity to dynamic range (43). Thus, multi-dimensional LC (MDLC) has been increasingly utilized in large-scale proteomics due to the high dynamic range afforded by this separation method, which allows for the detection of low abundance proteins, as well as the fact that MDLC can be coupled directly to the mass spectrometer for on-line protein identification by LC/MS/MS—making MDLC interfaced with MS a more robust platform for protein identification than the traditional 2DGE/MALDI/MS approach. Bousette *et al.* identified 1,918 proteins from the ventricular tissue of transgenic mice overexpressing a constitutively active form of calcineurin A using MDLC in combination with an LTQ mass spectrometer (7). Using this approach the authors identified α -crystallin B as a downstream effector of calcineurin-associated cardioprotection, bringing light to a previously unrecognized mechanism opposing endoplasmic reticulum stress-associated apoptosis in the heart (7). Although MDLC in combination with LTQ MS represents a powerful platform for protein identification, the recent pairing of MDLC with the high-resolution and high sensitivity of the Orbitrap mass spectrometer has further expanded the robustness of this platform as well as increased confidence in protein identification. Tu *et al.* paired an MDLC method employing strong cation exchange and reverse phase chromatography with an Orbitrap mass spectrometer to globally identify proteins in the swine plasma proteome (54). Using this approach, they were able to identify a total of 3,421 swine serum proteins spanning a concentration range of 9–10 orders of magnitude (54). This study represents a critical first step towards the identification of serum biomarkers for cardiac injury and disease in large animal models, which can speed the translation of basic scientific discoveries to clinical applications (13).

In addition to general protein identification, bottom-up MS has also been used to identify protein interacting partners. In contrast to traditional bottom-up strategies for protein identification, the identification of protein interacting partners requires the purification of intact signaling complexes from cell or tissue lysates via AP prior to digestion and MS analysis (19). Ping *et al.* identified 36 proteins, including structural, signaling, and stress-

activated proteins, which were physically associated with PKC ϵ in mouse myocardium using bottom-up AP-MS (42). Interestingly, the authors noted alterations in the composition of PKC ϵ signaling complexes in the myocardium of transgenic mice expressing low levels of constitutively active PKC ϵ , which have enhanced resistance to ischemia/reperfusion injury that is similar to preconditioning—suggesting a novel role for PKC ϵ signaling in ischemic preconditioning-associated cardioprotection (42). While these examples showcase the power of bottom-up for the identification of proteins and protein interacting partners, it should be noted that the limited sequence coverage afforded by bottom-up proteomics (which occurs because easily ionized peptides outcompete peptides that are harder to ionize for ionization and, therefore, some peptides can be detected while others will not) can preclude accurate protein identification if the sequences of the detected peptides are common to a group of proteins (e.g. conserved regions of protein isoforms resulting from alternative splicing events) (50).

Top-down MS does not suffer from this drawback, however, because the molecular weight (MW) of the intact protein is determined, which, when combined with sequence information obtained using MS/MS, allows for the reliable identification of proteins, including alternatively spliced isoforms differing only by the inclusion/exclusion of a single exon. Unfortunately, the underdevelopment of front-end protein separation strategies and back-end bioinformatics tools for top-down MS has traditionally limited the throughput of this method, making large-scale protein identification challenging. However, in recent years the throughput of top-down proteomics has advanced significantly with several groups reporting the identification of hundreds of proteins using top-down MS (4, 9, 53). For example, utilizing a four-dimensional separation platform employing solution isoelectric focusing, gel-eluted liquid fraction entrapment electrophoresis (GELFrEE), and reverse phase LC in combination with top-down MS, Tran *et al.* identified over 3,000 intact proteoforms arising from 1,043 different gene products from HeLa cell extracts (53). Furthermore, the use of this platform allowed for the identification of a wide variety of proteins including high MW proteins (up to 105 kDa) as well as membrane proteins with as many as 11 transmembrane helices, which have traditionally been difficult to analyze using top-down MS (53). This study exemplifies the progress that has been made in recent years in terms of intact protein analysis on a large-scale; and it is likely that, as the throughput of top-down MS continues to improve and more mature bioinformatics tools become available, this strategy will play a critical role in the identification of proteins in HF research.

Protein modifications

The site-specific addition of modifying groups to proteins represents an important regulatory mechanism that is involved in the control of all aspects of protein biology including activity, localization, turnover, and protein-protein interactions (28). Consequently, the study of protein PTMs is an essential step toward understanding the molecular mechanisms of disease at the systems level.

Although Western blot can be used to detect known protein modifications, MS is currently the only detection method that can identify PTMs without *a priori* knowledge—making it a powerful tool for the identification of protein PTMs as well as the study of PTM-regulated

cellular processes in health and disease (10, 28). MS can identify previously unknown PTMs because the addition of a modification such as a phosphate group to a protein results in a characteristic mass difference between the modified and unmodified forms of the protein that corresponds to the mass of the specific modification (e.g., in the case of phosphorylation, an 80 Da mass difference will be observed).

The detection of PTMs represents a significant challenge for bottom-up proteomics due to the inherent increase in sample complexity that results from proteolytic digestion. Since the success of peptide ionization and detection are inversely correlated with abundance, low abundance peptides harboring PTMs will be out-competed by their more abundant unmodified counterparts and, thus, will be difficult to detect using bottom-up MS (10). To circumvent this limitation, affinity-based enrichment strategies are employed to enrich peptides harboring specific PTMs (e.g., phosphate groups, acetyl groups, etc.) prior to MS analysis. Jia *et al.* identified a novel PKA phosphorylation site (Ser311) within the M domain of a recombinant cardiac myosin binding protein-C (cMyBP-C) using both immobilized-metal affinity chromatography (IMAC) and TiO₂ chromatography to enrich phosphopeptides (generated by *in vitro* phosphorylation of cMyBP-C) prior to MS analysis (23). However, whether or not this modification exists *in vivo* and its significance remains to be determined (23). Similarly, Huang *et al.* utilized a TiO₂-based enrichment strategy to identify phosphorylation sites in Connexin43 following *in vitro* phosphorylation by CaMKII (21). Bottom-up MS analysis of enriched phosphopeptides revealed a total of 16 CaMKII phosphorylation sites, which represents the first study of CaMKII-mediated phosphorylation of Connexin43—a protein that is involved in aberrant electrical activity in the diseased hearts (21).

In addition to strategies that have enabled the bottom-up MS analysis of phosphorylated peptides, a number of additional enrichment strategies have been employed to study different PTMs. Murray *et al.* identified 83 S-nitrosylation (SNO) sites on 60 different mitochondrial proteins using a biotin switch assay to enrich SNO modified peptides from mitochondria-enriched rat heart lysates (36). This study is the first comprehensive analysis of SNO-modifiable proteins in cardiac mitochondria and the results add to the expanding regulatory role for this modification in cardiovascular biology (11). Wang *et al.* utilized bottom-up MS in combination with an antibody-based enrichment strategy to characterized the 20S proteasome acetylome (55). In addition to the identification of 16 sites of acetylation, the authors confirmed that the acetylation at five of the sites could be induced by treatment with histone deacetylase (HDAC) inhibitors and that proteasomal activity was increased as a consequence (55). Therefore, treatment with HDAC inhibitors may represent a novel therapeutic strategy to rescue compromised proteolytic activity in failing hearts (55). While these studies highlight the usefulness of affinity-based enrichment strategies for the identification of PTMs via bottom-up proteomics, the limited sequence coverage and loss of connectivity between PTMs remain significant problems facing bottom-up MS-based PTM identification (Table 1) (60).

In contrast, top-down MS is an ideal method for the comprehensive characterization of PTMs owing to the fact that intact proteins, rather than peptides, are analyzed and, thus, no information is lost regarding the modification status of the protein (Table 1). Our group has

utilized top-down MS with ECD for the complete mapping of all of the PTMs of cardiac troponin I (cTnI) isolated from mouse, rat, pig, and human hearts (5, 14, 44, 57, 59, 61). In the case of mouse cTnI, high-resolution top-down analysis of cTnI immunoaffinity purified from mouse hearts revealed the existence of un-, mono-, and bis-phosphorylated cTnI proteoforms and subsequent MS/MS analysis localized the sites of phosphorylation to Ser22/Ser23 (the numbering here and below corresponds to human cTnI sequence with the removal of N-terminal Met), two well-known PKA phosphorylation sites (5). Phosphorylation of cTnI was not detected by either top-down MS or Pro-Q diamond gel analysis for cTnI from a transgenic mouse in which cTnI Ser22/Ser23 were replaced with non-phosphorylatable alanine residues, suggesting that Ser22 and Ser23 are the only sites that are basally phosphorylated in wild-type mouse cTnI (5). Similarly, top-down MS studies of rat, pig, non-human primate, and human cTnI purified from normal myocardium also unambiguously identified Ser22/Ser23 as the only sites of basal phosphorylation (44, 57, 61, 62). While these findings seem to suggest that cTnI phosphorylation sites other than Ser22/Ser23 are merely “fancy” rather than “fact” (49), we recently identified Ser41/Ser43 phosphorylation (two well-established PKC phosphorylation sites) in cTnI purified from the myocardium of spontaneously hypertensive rats, suggesting that these sites may play a role in cardiac dysfunction (Figure 2) (14). In addition to the general identification of PTMs, top-down can also be used to decipher the order of sequential PTM addition and to identifying positional isomers, which are often problematic for bottom-up proteomics (60). Our top-down MS analysis of cTnI purified from human heart tissue revealed Ser23 phosphorylation only in the presence of Ser22 phosphorylation, suggesting a sequential phosphorylation mechanism (59). Similarly, we have localized the sites of phosphorylation of a recombinant mouse cMyBP-C to Ser283, Ser292, and Ser312 using a combination of top-down and middle-down MS (which relies on limited proteolysis to generate larger protein fragments than are traditionally analyzed using bottom-up MS) (18). Interestingly, although bis- and tris-phosphorylated forms of the protein were observed, the only mono-phosphorylated species identified in our study was phosphorylated at Ser292, which suggests that phosphorylation of Ser292 is required for the phosphorylation of Ser283 and Ser312 (18).

Furthermore, top-down MS can be used for the identification of modifications in the amino acid sequence of proteins (e.g. polymorphisms) as well as the characterization of protein isoforms. Our lab has identified sequence variations in myofilament proteins including cTnI and tropomyosin (Tm) isolated from normal swine myocardium using top-down MS (39, 61). We also recently identified multiple Tm isoforms in the myocardium of rejected donor hearts including α -Tm, β -Tm and κ -Tm, with α -Tm being the predominant isoform and minor isoforms of β -Tm and κ -Tm (40). This study represents the first comprehensive analysis of Tm isoforms in the human hearts. These examples showcase the capabilities of top-down MS for the comprehensive characterization of protein modifications including PTMs and sequence variations.

Protein quantification

A systems level understanding of biological processes and the signaling pathways that regulate them, necessitates the ability to assess how the elements of the system change over a range of perturbations (15). Thus, the quantification of protein expression and PTM

occupancy changes is of the utmost importance for understanding complex syndromes such as HF at the systems level.

Since bottom-up MS is not inherently quantitative, a number of quantification strategies have been developed to allow for both the relative and absolute quantification of proteins. Here, we will only briefly outline these strategies but, for more extensive reviews on this subject, see (6, 38). Three general strategies are employed for bottom-up protein quantification: chemical tagging, metabolic labeling, or label-free quantification. In chemical tagging strategies, which include the prototypical chemical tagging strategy isotope-coded affinity tags (iCAT), thiol- or amine-reactive tags containing “heavy” or “light” isotopes are used to label proteins from cell or tissue lysates representing different experimental conditions and relative quantification is achieved by direct comparison of the signal intensities for the labeled peptides in MS mode (6, 38).

A second type of chemical tagging strategy in which quantitative data is obtained in MS/MS mode also exists. So called isobaric mass tagging strategies, which include isobaric tags for relative and absolute quantification (iTRAQ) and tandem mass tags (TMT), rely on a direct comparison of the signal intensities of reporter ions that are released following fragmentation of the labeled peptides for the relative quantification of protein species (6, 38). Warren *et al.* utilized bottom-up MS with iTRAQ to identify potential tissue biomarkers of MI in a sub-proteomic fraction enriched in cardiac sarcomeric proteins (56). Using this approach, they identified 22 unique sarcomeric proteins that exhibited at least a 20% change in expression in myocardium from a rat model of MI; thus, uncovering a slew of potential biomarkers for acute cardiac injury (56). In addition to the quantification of protein expression, chemical tagging strategies have also been employed for the determination of PTM occupancy. Using bottom-up MS with a cysteine-reactive TMT labeling procedure, Kohr *et al.* discovered a 2- to 3-fold increase in SNO occupancy in mouse hearts following ischemic preconditioning, thereby shedding light on a potential mechanism underlying ischemic preconditioning-associated cardioprotection (25).

Metabolic labeling strategies, as exemplified by stable isotope labeling by amino acids in cell culture (SILAC), are similar to chemical tagging strategies with a few exceptions. First, amino acids (typically lysine or arginine) labeled with heavy and light stable isotopes are utilized rather than chemical tags and, second, the labeling of cellular proteins is achieved by culturing cells in media supplemented with the labeled amino acids (6). Although metabolically labeled samples can be combined at the level of intact cells, which makes this strategy less prone to errors arising during sample preparation than chemical tagging strategies (6), metabolic labeling is primarily restricted to cell culture, which severely limits the usefulness of this strategy in HF research because there are almost no commercially available cardiac cell lines.

Label-free quantification can be grouped into two separate categories: those strategies that use 2DGE for protein quantification prior to MS analysis and those that utilize either the number of fragmentation spectra obtained for a given protein (spectral counting) or the intensity of precursor ions from extracted ion chromatograms (XICs) for protein quantification (6, 26, 38). Using a combination of 2DGE and bottom-up MS, Agnetti *et al.*

identified 17 mitochondrial proteins that exhibited differential expression upon cardiac resynchronization in a canine model of dyssynchronous HF (3). The increase in expression noted for anaplerotic enzymes in this study, implicates improved mitochondrial bioenergetics as a salutary mechanism underlying cardiac resynchronization therapy (3). Utilizing a similar approach, Mayr *et al.* identified 17 proteins that differed in their expression in the myocardium of patients with AF in comparison to myocardium from patients with sinus rhythm (31). Thus, in this study, the use of bottom-up proteomics in combination with quantitative 2DGE shed light on metabolic adaptations occurring in the myocardium of patients with persistent AF (31).

In addition to 2DGE, other label-free quantitative approaches have also been employed in HF research. For example, Addona *et al.* identified over 100 potential biomarkers of cardiac injury in the serum of patients undergoing planned MI as a treatment for hypertrophic cardiomyopathy using an XIC-based label-free approach (1). Although the biomarker discovery pipeline developed in this study was used to identify markers of cardiac injury, the basic method can be employed for the study of a number of different diseases (1). Similarly, Duan *et al.* used an XIC-based label-free method to quantify expression level changes in the mitochondrial proteomes of healthy swine versus those with hibernating myocardium (16). Interestingly, only 5% of the proteins quantified in this study exhibited a statistically significant change in expression in hibernating myocardium suggesting that many biological activities remain intact despite reduced contractility (16). Using XIC-based label-free quantitative bottom-up MS, Monte *et al.* identified 176 proteins from a sub-proteome of chromatin that exhibited altered expression in a mouse model of cardiac hypertrophy and failure (34). The identification of factors involved in the regulation of chromatin in this study provides insights into the molecular mechanisms regulating the re-expression of fetal genes in HF (34). The fact that label-free quantitative strategies are low-cost, and require no additional sample manipulation beyond protein extraction and MS analysis, has made them the method of choice for quantitative proteomics studies in HF research; however, this strategy is not without shortcomings, which include an increase in instrument time as well as very stable instrumentation (both LC and MS).

Unlike the bottom-up approach, top-down MS is believed to be inherently semi-quantitative in regard to the relative quantification of modified versus unmodified proteoforms (60). This stems from the fact that the addition of modifying groups to intact proteins has little to no influence on their overall physiochemical properties because of the large mass difference between the protein and the modification; thus, the relative abundances of modified and unmodified proteoforms of interest can be determined by comparing the relative ratios of the signal intensities for these species (60). Using a direct comparison of the top-down MS signal intensities for un-, mono-, and bis-phosphorylated cTnI, we have quantified changes in the phosphorylation status of cTnI in hearts with varying stages of chronic HF (62). Quantitative top-down MS analysis revealed a significant decrease in the phosphorylation of cTnI isolated from failing hearts in comparison to cTnI from healthy hearts, suggesting cTnI phosphorylation as a candidate biomarker for chronic HF (62). In addition, we have also used top-down MS to quantitatively assess the levels of cTnI phosphorylation a spontaneously hypertensive rat (SHR) model of hypertension and HF (14). Top-down MS

analysis revealed a significant increase in cTnI phosphorylation in SHR hearts in comparison to cTnI from control hearts, which was in good agreement with Western blot results (Figure 2) (14). Surprisingly, in addition to increased phosphorylation at Ser22/Ser23, phosphorylation of Ser42/Ser44, two PKC sites, was also observed in cTnI immunoaffinity purified from SHR hearts, which represents the first observation of Ser41/Ser43 phosphorylation *in vivo* (Figure 2) (14).

The future of proteomics in HF research

Both bottom-up and top-down MS have already provided key insights into the cellular and molecular mechanisms underlying HF and HF-associated diseases; however, significant challenges associated with systems level proteomics still preclude a comprehensive understanding of this devastating disease. Unlike the genome, which is considered to be largely static, the proteome is highly dynamic owing to a large dynamic range of protein expression as well as considerable complexity that can be attributed to a myriad proteoforms arising from mutations/polymorphisms, truncations, alternative splicing events, and the addition of PTMs; thus, the proteome must be fractionated into a series of less complex sub-proteomes prior to MS analysis (3). While extensive fractionation can, at least in part, solve the problem of proteomic complexity, the analysis of multiple sub-proteomes is incredibly time consuming; and, consequently, the majority of proteomics studies in HF research have focused on the analysis of a single or small number of sub-proteomes—leaving out important information pertaining to the status of a number of signaling pathways and cellular processes in the disease state. Thus, there is a need for technological advancements that will increase the robustness of MS-based proteomics analyses, thereby increasing the proteomic depth that can be achieved with less fractionation.

Although this review has focused on the advantages and disadvantages associated with bottom-up and top-down proteomics, in order to tackle the complexity of the proteome and elucidate complicated disease mechanisms, it will be necessary to combine these complementary strategies. While top-down MS offers more reliable protein identification, particularly for the identification of proteins with a high degree of sequence homology such as alternatively spliced isoforms, bottom-up proteomics remains the most robust strategy for protein identification—boasting well-established and reliable strategies for proteomic separation as well as mature bioinformatics tools, which remain underdeveloped in top-down proteomics (Table 1). Similarly, the numerous quantification strategies currently available for bottom-up MS allow for both the relative and absolute quantification of hundreds of proteins, whereas quantitative top-down proteomics studies have largely been restricted to the relative quantification of differentially modified proteoforms (Table 1). However, in regards to the identification and analysis of PTMs, bottom-up suffers from several severe drawbacks including incomplete sequence coverage and a loss of connectivity between distant modifications that make bottom-up ill-suited for the analysis of PTMs. Top-down proteomics, on the other hand, is an ideal strategy for the comprehensive analysis of PTMs as intact protein analysis provides a “bird’s eye” view of all modifications and subsequent MS/MS of proteoforms of interest can be used to localize PTMs, determine the order of sequential modifications, and identify positional isomers (Table 1).

Lastly, proteomics although powerful on its own, will be insufficient to comprehensively understand all facets of disease pathophysiology and, thus, in order to truly gain novel insights into disease mechanism it will be essential to correlate/integrate proteomics findings with datasets from transcriptomics and metabolomics studies in addition to functional and biochemical findings using physiological and biochemical methods.

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Abbreviations

HF	heart failure
MS	mass spectrometry
PTMs	post-translational modifications
2DGE	two-dimensional gel electrophoresis
<i>m/z</i>	mass-to-charge ratio
MS/MS	tandem mass spectrometry
MALDI	matrix-assisted laser desorption/ionization
ESI	electrospray ionization
LC	liquid chromatography
CID	collision induced dissociation
AP	affinity purification
ECD	electron capture dissociation
SNO	S-nitrosylation
cTnI	cardiac troponin I
SHR	spontaneously hypertensive rat
WKY	Wistar-Kyoto
iTRAQ	isotope tags for relative and absolute quantification
MI	myocardial infarction
SILAC	stable isotope labeling by amino acids in cell culture
TAC	transverse aortic constriction
MW	molecular weight
MDLC	multi-dimensional liquid chromatography
cMyBP-C	cardiac myosin binding protein-C
TOF	time-of-flight

Q-TOF	quadrupole-time-of-flight
HCD	high-energy collision dissociation
IRMPD	infrared multiphoton dissociation
UVPD	ultraviolet photodissociation
PSD	post-source decay
ETD	electron transfer dissociation
GELFrEE	gel-eluted liquid fraction entrapment electrophoresis
IMAC	immobilized-metal affinity chromatography
TMT	tandem mass tag
FTMS	Fourier transform mass spectrometry
iCAT	isotope-coded affinity tag
HDAC	histone deacetylase
Tm	tropomyosin
XIC	extracted ion chromatogram

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Figure 1. Schematic illustration of the difference between top-down and bottom-up proteomics In top-down proteomics (left), protein are extracted from cell or tissue lysates, separated by either gel or LC, and directly analyzed by MS for a complete view of all proteoforms including those with PTMs and sequence variations. Subsequently, a specific proteoform can be isolated and fragmented by MS/MS to obtain sequence information, which can be used to identify the protein via database searching, and localize PTMs. In bottom-up proteomics (right), proteins extracted from cells or tissue are subjected to proteolytic digestion (often using trypsin)—either in-solution or in-gel—and the resulting peptides are separated using LC and analyzed by MS. Subsequently, the most abundant peptides are fragmented and the peptide sequence information is used to identify the proteins present in the sample as well as map their PTMs although incomplete sequence coverage can preclude PTM analysis with this method. Numerous strategies are also available for both the relative and absolute quantification of proteins/peptides using bottom-up and top-down proteomics.

Figure 2. High-resolution Fourier transform MS (FTMS) analysis of intact rat cTnI purified from Wistar-Kyoto (WKY) and SHR myocardium

(A) Representative FTMS spectrum of cTnI (M^{25+}) purified from age-matched WKY. Dashed arrow indicates the expected position of tris-phosphorylated cTnI (ppp -cTnI) which is not observed in this spectrum. The minor proteolytic fragment, cTnI A[16–205]K, was observed. (B) FTMS spectrum of cTnI purified from SHR-HF. ppp -cTnI was observed in this spectrum. Circles represent theoretical isotopic abundance distribution of the isotopomer peaks. p -cTnI, mono-phosphorylated cTnI; pp -cTnI, bis-phosphorylated cTnI. Calc'd, calculated most abundant mass; Expt'l, experimental most abundant mass. Insets represent the schematic illustration of cTnI with differential phosphorylation in WKY and SHR, respectively. **Quantification of cTnI phosphorylation in WKY and SHR-HF hearts.** The percentages of mono-phosphorylated cTnI components ($\%P_{\text{mono}}$) in (C); bis-phosphorylated cTnI ($\%P_{\text{bis}}$) in (D); and the total amount of cTnI phosphorylation (P_{total} , mol Pi/mol cTnI) in (E). Data points indicate average of triplicates. Average and standard error of the mean (SEM) shown in the graph. * $p < 0.05$; ** $p < 0.001$. Modified based on (14) with permissions.

Table 1

A comparison of the bottom-up and top-down proteomics approaches for protein identification, quantification and the analysis of PTMs.

	Bottom-up MS	Top-down MS
Protein identification	+++ (More robust and high throughput strategy for protein identification with better bioinformatics tools currently available)	+ (More reliable protein identification particularly for proteins with high sequence similarity such as alternatively spliced isoforms, but is of relatively lower throughput).
Protein modification	++ (Amenable for large-scale PTM study, but with limited sequence coverage and loss of connectivity between PTMs resulting from peptide loss during sample preparation and sample digestion)	+++ (Reliable and comprehensive analysis of all types of PTMs simultaneously without <i>a priori</i> knowledge with full sequence coverage but proteins need to be purified or separated prior to detailed MS study)
Protein quantification	+++ (Very well-developed methods available for relative and absolute quantitation of protein expression level but with limitation in quantification of peptides with PTMs)	++ (Accurate relative quantification of multiple proteoforms from the same gene product due to genetic variations, alternatively spliced RNA transcripts and PTMs but the quantification of protein expression level remain underdeveloped.

Ability to identify and quantify proteins as well as characterize PTMs: + poor, ++ moderate, +++ excellent.