Editorials

Proteomics: Its Potential in the Post-Genome Era

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As the enormous complexity of the human genome is unraveled, science is another step closer to unlocking some of the most complex processes involved in cellular function, including cell signaling, apoptosis, differentiation, as well as determining the underlying genetic and molecular bases of many diseases. In our aim to fully comprehend how the cell carries out both simple and complex functions, identification and characterization of genes involved in given processes will only provide part of the "overall picture". For most mammalian cells, the genome is a static body which contains the information used in all cells at all times; the genome does not evolve with the progression of disease, nor does it change in response to perturbations in the cell's environment or activity. Scientists are now seeking "post-genome era" tools that may allow us to gain a deeper understanding of cellular function and the mechanisms or pathways leading to various diseases.

In contrast to the genome, cellular protein composition and activity always correlates with changes in overall cellular function. This follows from the fact that proteins catalyze the overwhelming majority of cellular reactions and are almost completely responsible for cellular function; variations in protein-protein interactions, protein expression levels, and protein post-translational modifications result in global functional changes. Thus, full elucidation of cellular function and disease requires an understanding of the greater context and conditions in which genes are expressed, their function, and the other protein players that they influence. This dictum is at the core of the developing field of proteomics.

The cell's "proteome" is defined as the full complement of expressed proteins and their respective post-translational states at one time point[1]. At the heart of "proteomics" is the effort to understand the dynamics of protein function; by studying the proteome of a cell at various time points or under different conditions, one can gain information about proteins that are relevant to certain processes, alterations in their post-translational modification and expression level, and their interactions with other proteins that lead to the observed cellular phenotype. The tools of proteomics include a mix of "old" and "new" technologies, such as two-dimensional electrophoresis, protein microarrays, mass spectrometry, immunoprecipitation, yeast two-hybrid screens and chromatography. New applications of these techniques allow proteomics researchers to gain a massive amount of integrative information; together, these methods have the potential to generate large volumes of data quickly, and to facilitate the development of high-

throughput systems useful in the analysis of complex biological systems.

The field of proteomics is considered one of the most important post-genome era approaches applied to understanding cellular function and dysfunction[2], and has shown great promise in the areas of cancer, neurologic, and cardiovascular research. Proteomics offers researchers a means of gaining information that cannot be gained by studying only the genome. Gene array "chips" are becoming popular tools in studying cellular transcriptional changes in response to stimuli; these chips measure changes in the amount of a set of mRNA species in the cell at a given time-point. However, increased or decreased transcription of a specific gene does not necessarily lead to a proportional change in protein level[3]; sometimes, changes in mRNA levels for a particular gene are accompanied by no change in protein level, and in some cases, transcription and translation rates are inversely related[4]. Given that changes in transcription upon experimental manipulation are typically used to infer changes in protein function, data from gene arrays must be carefully applied and validated by other methods. Compared to "transcriptome" analysis using gene array methods, proteomic analysis is an indisputably more direct way to measure expression levels.

Another advantage of proteomics lies in its ability to dissect out the time course of protein behavior, as well as characterize whether post-translational modifications are present and relevant to a given process. While the explosive field of bioinformatics allows researchers to roughly predict protein structure, function and likely protein binding partners from the deduced amino acid structure, the actual timing of expression and activity cannot be predicted. In addition, DNA sequence information can yield information for possible post-translational modification sites on proteins, but cannot predict whether those sites are physiologically relevant, nor what role relevant sites play in the cell. Post-translational modifications are sometimes the only cellular change that occurs in response to some types of stimuli, especially with regard to transient signaling events; for processes that do not typically elicit gene transcription, such as synaptic transmission or muscle contraction, genomics cannot offer any information. Thus, proteomics offers several major advantages to researchers by measuring exactly the circumstances and behavior of a protein of interest.

While the potential for discovery is great, investigators probing the field of cellular proteomics face three major challenges that genomics researchers do not. First, there is no such thing as *one* cellular proteome, in stark contrast to the genome. Cellular proteomes are highly dynamic, ever-evolving bodies; changes in the proteome occur simultaneously with changes in cellular function. The state of the cellular proteome reflects the dynamics of moment-to-moment cellular function. For example, while "housekeeping gene" expression demonstrates little variation under different conditions, other proteins, such as cell signaling pathway proteins, exert short-lived but powerful influences upon cell function and effect abrupt changes in the proteome. The ability of proteomics to detect the moment-to-moment cellular protein changes and modifications is both a great asset and a great liability, because these dynamic fluctuations demand that great care be taken in standardizing results and interpreting data. The second challenge is that in contrast to DNA and RNA, which are thoroughly homogenous chemicals whose physical properties vary only with their sequences and lengths, proteins vary significantly in molecular weight, partition coefficients (hydrophilicity/ hydrophobicity), electrostatic charges, ion chelation properties, and post-translational modification (glycosylation, lipid conjugation, and phosphorylation). In other words, while all RNA and DNA is made of guanine, cytidine, adenine, thymidine, and a few derivatives of those bases, proteins can possess nearly every imaginable type of chemical modification. Therefore, proteomics researchers face an arguably infinite number of specific, individual protein chemistries, necessitating the arduous task of developing conditions optimal for each set of experiments or set of proteins. As an example, one recent study reported that the heat-shock protein Hsp27 can be post-translationally modified in 59 distinct ways[5]. Although this is exceptional, every cellular protein is theoretically able to be modified in several ways, illustrating the challenges in proteomics research. The third challenge is that there is not yet a single standard, efficient and reliable method available to study the entire cellular proteome at once. The methods used in proteomics are themselves technically difficult and time-consuming, and as a consequence, proper proteomic analysis is a major undertaking. Inherent technical difficulties have slowed the implementation of strict standards in proteomic analysis, though techno-

logical improvements in the last decade have improved reliability and efficiency of the main proteomic methods.

With the promise of a new informatic approach to cell function, proteomics stands as the next major step forward in studying cellular function on a global level. As the human genome is unlocked, we will move from reading the cell's instruction manual to uncovering all of the machinery for which it codes.

Specific methods

Proteomic analysis can be divided into two broad categories: a) expression proteomics; and b) functional proteomics. Both have great potential to elucidate complex molecular processes in regard to both normal cellular function and disease pathogenesis.

a) Expression Proteomics

The crux of expression proteomics is to separate, visualize, identify and quantify protein changes in the whole proteome under various conditions or time points[6]. In the ideal case, one would be able to observe every protein expressed in the cell at a given time point, along with its post-translational modifications (PTMs), and then be able to identify the changes that occur upon specific experimental manipulations. Once changes in the proteome are observed, such as differences in expression level or phosphorylation of a protein, one can correlate these changes with alterations in cellular function and confirm their functional significance using additional methods.

The power of expression proteomics lies in its ability to elucidate cellular function and disease at its most fundamental level, in a manner that does not exclude any component of the cell. All too often, the choice of the set of signaling pathway proteins to examine is biased by current signaling models and the availability of reagents such as antibodies or vectors[7]. Expression proteomics sidesteps these problems by "zooming out" sufficiently to become all-inclusive. This inclusiveness helps greatly in understanding the pathogenesis of diseases, which are multifactorial in nature, variable in phenotype, or possess only a partial genetic basis. These types of diseases often require the activation of multiple signaling pathways, many of which intersect or feed into the same terminal effector[8]; expression proteomics allows these diseases to be broken down into phases, based on the cell's protein expression profile. One can simply observe the course of the disease and track the concomitant evolution of the proteome, and even correlate the severity or onset of disease with its true molecular markers.

Two-dimensional electrophoresis (2DE) has become the primary tool used to study the proteome. In 2DE, proteins are separated on the basis of their isoelectric point and their molecular weight and then visualized; this method provides a high resolution "snapshot" of the cellular proteome. Methodologically, proteins in the sample to be analyzed are first absorbed to a narrow gel strip which possesses a fixed pH gradient, stretching from one end to the other. Once absorbed, an electric field is applied to this gel, which causes the proteins to migrate to their respective isoelectric points. Following isoelectric focusing (IEF), the pH gradient strip is placed directly on top of a conventional polyacrylamide gel and is overlaid with polyacrylamide or agarose. The proteins enter the gel and are electrophoretically separated by molecular weight. Finally, the proteins are visualized by silver or Coomassie Brilliant Blue staining. The final gel appears as a constellation of spots.

In a typical experiment, 2D gels are processed for control and experimental samples in parallel. Subsequently, computer programs are used to quantify protein spot size/intensity as well as to localize the spot to a standard set of coordinates. Control and experimental gels are then compared to each other. Using the computer-generated average protein spot locations and intensities, minimal changes in protein levels, protein spot location, and the appearance or disappearance of protein spots upon experimental manipulation can be detected (Figure 1).

Figure 2 exemplifies how 2D gels can be used to detect changes in the proteome upon experimental manipulation. In the panel on the left, cardiac homogenates of wild-type mice were run on a 2D gel and compared to an identical preparation of cardiac homogenates from mice lacking a specific gene thought to be important in regulating contractility. The figure shows a difference in the two spot patterns; it could be that the knockout mouse expresses far less "protein X" than wild type and far more "protein Y", or that a post-translational modification caused a rightward shift in the isoelectric point of spot X.

Spots that change compared to controls, such as the change occurring above, may be excised, de-

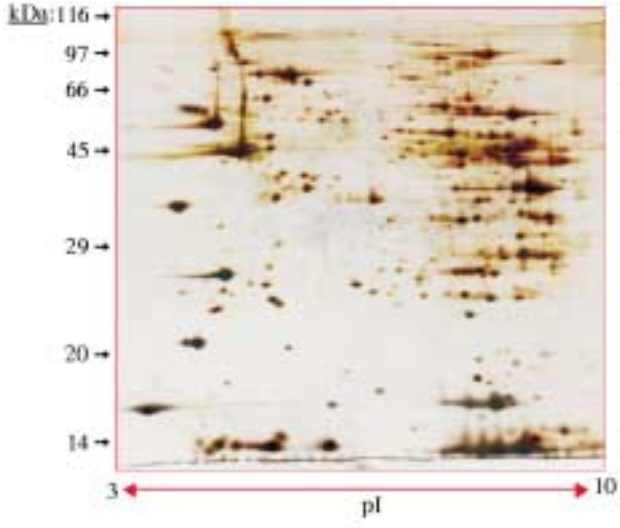

Figure 1. A silver-stained 2D gel. Each spot represents one or more proteins that have been separated by molecular weight and pI.

stained, and digested by trypsin in order to be identified by mass spectrometry. Once the spot or spots are identified, the protein's physiologic role may be assessed using various methods. While MS-identification is the most straightforward way to determine protein identity, an alternative approach championed by many researchers is to compile a database of 2D gels[9,10]. This "inventory" would list every protein's pI and molecular weight, as well as standard migration coordinates, and it has the potential to make identification of proteins rather simple and efficient.

As with any research method, limitations exist for two-dimensional electrophoresis. Since two-dimensional electrophoresis analysis is utterly dependent on the detection of every protein, gel-running conditions must be optimized for the subset of proteins of specific interest. Difficulties with 2DE arise primarily during isoelectric focusing (IEF) of

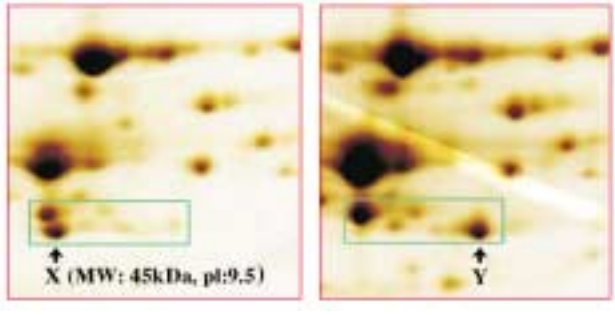

Figure 2. 2D gel spot comparison in wild-type mice compared to a genetic model.

the protein sample. Highly hydrophobic proteins have classically been difficult to visualize, because the non-denaturing conditions required for IEF disallow these proteins to migrate to their proper isoelectric point. In addition, ion-binding proteins may not focus correctly because their extra charges interfere with IEF. As a secondary difficulty, low-abundance proteins may not be observed in gels. The difference in expression level among proteins in a specific sample may be as high as seven or eight orders of magnitude[11], indicating that low-abundance proteins are below the limit of visualization. Finally, on one 2-dimensional gel, one may reasonably distinguish ~1000 spots; the human genome project now estimates that our DNA codes for about 30000 genes. It is therefore impossible to resolve every expressed protein on one gel. Due to these factors, it is not possible to obtain a "complete" picture of the proteome using only one set of gel-running conditions.

However, these difficulties can be kept to a minimum using a few selective strategies. The key to overcoming solubility and protein abundance issues is to pre-fractionate the sample to be analyzed. In doing so, gel image complexity is reduced and a more complete picture of the proteome can be obtained. To generate a complete picture, the whole cellular proteome can be fractionated into subsets of proteomes. As a first pass, a battery of protein solubilization conditions consisting of various detergent and salt concentrations may be used to select for proteins with different chemical properties[12]. Varying the ionic strength of detergents, the salt concentration, and the presence of other solubilizing agents such as urea and thiourea can greatly enhance resolution of the cellular proteome by differentially solubilizing . To delve more deeply into subproteomic changes, individual organelles can be purified and analyzed[13], or methods used to exploit proteins' individual chemical characteristics can be selected, such as separation on the basis of hydrophobicity or charge[14,15]. In this way, the information that the gels provide is more amenable to analysis, and "inventories" can be created of every protein present in the particular extraction. In contrast, using a battery of different solubilization conditions in an effort to cater to subsets of proteins of special chemical properties is also an effective method to visualize the proteome. Finally, low-abundance proteins can be visualized and analyzed by loading high protein concentrations on the gel with increased amounts of

sample and then manipulating the computer software analysis to search for new spots while ignoring high abundance spots.

Though two-dimensional electrophoresis is an overall cumbersome and difficult procedure, a wealth of data can be obtained by using this technique. As technology advances, two-dimensional electrophoresis has the potential to boost proteomics to the forefront of science.

Functional proteomics

Functional proteomics is concentrates on elucidating the interaction between proteins assembled in complexes during the execution of their respective functions. Proteins with enzymatic activity rarely function in isolation; they are usually associated with other proteins which influence their function or perform structural or anchoring roles. The quantity and composition of a given complex may dramatically affect the physiologic state of the cell. Thus, understanding which proteins form functional complexes and how these complexes change to affect cell physiology is of great interest.

Functional proteomics is narrower in scope than expression proteomics, and intentionally biased. Methods focus on the isolation of one protein complex at a time, in order to assess changes in its composition or the post-translational modification state of its members. By observing those changes, one can begin to understand its functional role and relevance to particular cellular processes. Once again, this information must be coupled with established physiological and biochemical assays to definitively assess complex function. However, functional proteomics stands as an excellent means to dissect molecular pathways.

Two techniques used in functional proteomics are immunoprecipitation and protein microarray assays. Immunoprecipitation involves fixing the F_c region of antibodies specific for a protein of interest to sepharose beads, incubating an appropriate sample with the beads to allow the antibody to bind to the protein of interest, and then washing away all unbound components. The sepharose beads will extract the protein of interest as well as every protein that it binds; subsequently, a one- or two-dimensional gel may be run, and the bands or spots subjected to mass spectrometry to identify the proteins present.

Protein microarrays work in a similar way[16]. The protein "chip" can be manufactured to possess a surface coated with ions, molecules, or antibodies/ proteins of interest; experimental sample is incubated on the chip surface, and after washing, complexes which have an affinity for the selected surface remain (Figure 3).

Figure 3. A Protein Microarray Chip. The surface of the chip may be coated with any type of molecular or metal, incubated with sample, and analyzed to assess the composition of large protein complexes.

The proteins are then directly identified by mass spectrometry, a major advantage over immunoprecipitation. Protein chip experiments are generally faster and technically easier than immunoprecipitation. Thus, they have the potential to provide a great amount of data for protein complexes. Just as gene array chips are a relatively new and imperfect technique, protein chips also require validation before their use is widely accepted[17].

These two methods have few drawbacks. Naturally, the possibility of false positives turning up among "true" data does exist, but this is most often compensated for by the use of negative controls, and by increasing the rigor of the binding conditions. Though studies yield less overall information than expression proteomics, functional proteomics offer a powerful method to identify and quantify complex protein-protein interactions, lending new insight into enzymatic function.

Cardiovascular relevance

Proteomic analysis is amenable to all fields of study, and has already been put to great use in the cardiovascular research field[18]. Three examples of the use and power of proteomics in cardiovascular biology are illustrated below.

Functional alterations that occur in genetic models of disease are prime candidates for proteomic analysis. The animals' full phenotypes are not usually

accounted for by the absence or overabundance of a single gene product, since compensation often occurs as a result of the genetic manipulation. Kernec et al[19] investigated changes in the mitochondrial sub-proteome of mice deficient in the three isoforms of creatine kinase compared to wild-types. Normally, creatine kinase regenerates ATP from phosphocreatine and ADP, when other ATP-generating pathways cannot keep up with cellular demand for energy. Mice lacking each of the three isoforms of creatine kinase show no cardiac phenotype, though cardiac metabolic alterations occur; thus, the authors sought to uncover what compensation occurs in the proteome that preserves heart function. In this study, mitochondria were isolated from cardiac homogenates using differential centrifugation in a sucrose gradient. The samples were subjected to 2DE and the resultant gel images were standardized and analyzed for differences.

Comparison of the mitochondrial proteome of mice lacking both the muscle and mitochondrial creatine kinase isoforms and wild types revealed that two large protein spots present on wild-type gels were missing from knockouts; these spots were identified by mass spectrometry and proved to be creatine kinase, which gave confirmation of the knockout genotype, and a precursor of aconitase, an enzyme of the Krebs cycle. The change in aconitase levels were unexpected; the authors then measured the activity and total amount of aconitase and found both to be unchanged. Thus, they concluded that the loss of creatine kinase caused a redistribution of aconitase precursor from the mitochondria to the cytosol. The significance of this relocation has not yet been established, but demonstrates the ability of proteomics to uncover unexpected or additional alterations present in genetic models and better understand the functional implications of the genetic perturbations.

Just as genetic models lend themselves to proteomic analysis, understanding environmentally induced changes in cardiocyte physiology is also of great value. Arrell et al[20] investigated the proteome alterations occurring after pre-conditioning rabbit cardiomyocytes. Pre-conditioning causes myocytes to become more resistant to periods of ischemia, reducing cell death and damage. A group of rabbits was chronically administered adenosine to precondition the heart cells, and their hearts were subsequently removed, homogenized and compared to wild-type hearts using two-dimensional electrophoresis.

Two-dimensional electrophoresis revealed five major spot differences between preconditioned animals and control animals. However, low abundance of these proteins and the complexity of the spot pattern precluded immediate protein identification using mass spectrometry. Thus, a second protein extract using tri-fluoroacetic acid was performed to elucidate the spot pattern. Analysis of this extraction revealed a large protein spot at the pI and molecular weight of myosin light chain 1 (MLC1), and an adjacent smaller protein spot suspected to be phosphorylated MLC1. However, there had been no report of any post-translational modification for MLC1 up to this time. Subsequent western blots and mass spectrometry revealed that the minor protein spot was indeed MLC1, and that it was phosphorylated in preconditioned rabbits. This was the very first report of MLC1 phosphorylation, and it constitutes a very important finding, since other reports regarding MLC1 mutations or expression levels have shown that small changes in this protein have a dramatic effect on cardiac function. The implications of this phosphorylation have yet to be characterized, but this study paves the way for a great deal of future work.

Finally, in a study aimed at determining the players involved in the regulation of SERCA2a, the major calcium ATPase of the cardiac sarcoplasmic reticulum, Asahi et al[21] used immunoprecipitation experiments to characterize the interaction of SERCA2a with sarcolipin, a 31-amino acid peptide. Under normal conditions, SERCA2a plays a role in determining the contractile force generated by the heart and decreased SERCA2a function is associated with heart failure. SERCA2a is known to complex with phospholamban, which negatively regulates SERCA2a's ATPase activity; since sarcolipin possesses homology to phospholamban, it was hypothesized that it also negatively regulated SERCA2a activity. SERCA2a, phospholamban, and sarcolipin were each transfected into HEK-293 cells and immunoprecipitated using antibodies against each protein. Sarcolipin was engineered to possess a FLAG epitope for western blot experiments. By first measuring the rate of calcium uptake by SERCA2a, using an established assay, this group showed that sarcolipin weakly inhibits calcium uptake compared to phospholamban, but together, the two proteins caused "superinhibition" of the calcium pump.

In order to understand how this superinhibition took place, the authors examined the manner by which

the proteins bind each other. Immunoprecipitation experiments showed that sarcolipin, phospholamban and SERCA2a each bind one another. Since it is proposed that monomeric phospholamban inhibits SERCA2a more strongly than pentameric phospholamban, the authors concluded that the binding of sarcolipin to phospholamban interferes with phospholamban's pentamer formation, resulting in more monomeric phospholamban and the observed superinhibition of SERCA2a. This study proposed a novel regulatory mechanism for SERCA2a, and sets the stage for further studies of sarcolipin.

What is the future of proteomics?

Proteomics will become a powerful tool in the analysis of cellular function in the near future, owing to its unbiased approach to uncovering cell processes and its wide applicability to nearly any type of study. Incorporation of proteomics within established research framework provides a means of identifying and characterizing complex protein changes, associated with cardiovascular function, dysfunction and other diseases. Proteomics can reveal the dynamic and subtle changes responsible for alterations in global cellular behavior; when coupled with established methods in biochemistry, physiology and pathophysiology, proteomics becomes a powerful tool in unraveling the intricacies of cellular function.

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