Proteomics

Investigating the Secretome
Lessons About the Cells That Comprise the Heart

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The cell-environment interface is composed of the proteins of the plasma membrane that face the extracellular space and the proteins secreted directly by the cell of origin or by neighboring cells. The secreted proteins can act as extracellular matrix proteins or autocrine/paracrine proteins. This report discusses the technical aspects involved in the identification and characterization of the secreted proteins of specific cell types that comprise the heart. These aspects include the culturing of the cells, cell coculturing and quantitative labeling, conditioned media collection, dealing with highly abundant serum proteins, posttranslational modification enrichment, the use of protein separation methods and mass spectrometry, protein identification and validation, and the incorporation of pathway analysis to better understand novel discoveries on the background of already known experimental biological systems. Proteomics methods have a firm place in cardiovascular research, and identification of proteins secreted by cardiac cells has been used in various applications, such as determination of specificity between secretomes of different cell types (eg, cardiac stem cells and cardiac myocytes); for global secretome screening of, eg, cells as human arterial smooth muscle cells; for mapping of the beneficial effect of the conditioned medium of 1 cell type on another cell type, eg, the effect of the conditioned medium of human mesenchymal stem cells on cardiac myocytes; and to search the candidate paracrine factors and potential biomarkers.

Interaction among different types of cells is a common response of the heart to injury. Interplay between various cell types constituting the heart or the vasculature occurs either through direct cell contact (eg, gap junctions or adhesion molecules) or via paracrine and autocrine action. Cardiac myocytes and fibroblasts, the primary cells that comprise the heart, as well as resident cardiac stem cells (CSCs), respond to changes in their local environment, whereas it is the cell surface that translates the extracellular signal into a cellular response through direct or indirect interactions. The main site of this response is the cell-environment interface, which includes both cell surface proteins and proteins that are secreted by cells as a reaction to these changes. Various methods have been developed to identify these subproteomes and clarify protein alterations in vivo. The ability to target and define the proteins responsible for the repair process could lead to improved therapy and to the development of potential biomarkers for disease diagnosis and prognosis. In this article, we will discuss the technical issues related to identification of 1 of these subproteomes, ie, the proteins secreted by cardiac cells as a response to the alteration in their environment caused by cardiovascular diseases.

Secretome Proteomics

The proteins secreted by a particular type of cell, secretomes, play important roles in the regulation of many physiological processes via paracrine/autocrine mechanisms, and they are of increasing interest as potential biomarkers and therapeutic targets in diseases. The proteins released by cells into conditioned media in vitro have been studied to better understand pathological conditions and mechanisms in vivo. For damaged myocardium, current therapeutic approaches are limited, because postnatal cardiac myocytes have minimum or no regenerative capacity. Thus, the ability to regenerate the myocardium by use of cell therapy has potential, and several experimental and clinical studies have demonstrated an improvement in cardiac function after myocardial infarction and heart failure. In addition, soluble proteins secreted by cardiac myocytes, fibroblasts, and/or (cardiac) stem cells into the damaged myocardium microenvironment have a functional benefit, seen in studies of cell therapy, and identification of secretomes that have salutary effects on injured cardiac myocytes and CSCs introduces the possibility of administering “off-the-shelf” protein therapy. The proteins secreted by various cardiac cells have been identified and characterized over the years and used, for example, for determination of the specificity between secretomes of different cell types such as CSCs and cardiomyocytes or peripheral blood–derived smooth muscle progenitors and aortic smooth muscle cells; for the global secretome screening of arterial smooth muscle cells, endothelial cells, atheroma plaque, and early proangiogenic cells; for map-
ping of the beneficial effect of the conditioned medium of 1 cell type on another cell type, such as the conditioned medium of mesenchymal stem cells on cardiac myocytes13; and to investigate potential paracrine/autocrine factors.12,14,23,24 With technology development, proteomics has become a powerful identification tool, and mass spectrometry (MS) is the commonly applied technique for detection of proteins in cell secretomes. Even though each cell type has its own challenges, many of the issues and considerations described herein for secretome proteomics apply regardless of differences in the cell type under investigation.

Technology
To identify cell-specific secreted proteins, investigators often begin their experiments in cell culture. Yet the goal is the more complex in vivo setting, which consists of multiple cell types that exist in a 3-dimensional structure and includes local environment changes. For example, within the in vivo setting, cocultures can mimic the coexistence of multiple cell types, and cells grown in culture on a scaffold can mimic the existence of a 3-dimensional structure. In the subsequent sections, we discuss the issues and challenges that lie in the path of the ultimate goal of identification and characterization of secreted proteins in cell conditioned media.

Cell Culture
To ensure the optimal growth and viability of the cells, cells cultured in vitro are generally grown in media that contain bovine serum. Although high-abundance serum proteins may mask the presence of low-abundance secreted proteins, making their detection and identification by MS difficult, there are a number of strategies by which to handle this issue. The first is to reduce the amount of serum in the medium by moving toward serum-free conditions. However, there is a need to minimize cell death, and this requirement often results in the reduction but not the complete elimination of the serum. Thus, it is important to have strategies for depletion of the serum, including affinity techniques to deplete or remove at least the high-abundance serum proteins, or protein fractionation methods such as SDS-PAGE, 2-dimensional gel electrophoresis (2-DE), or liquid chromatography (LC), in which serum albumin is physically separated from the remaining proteins. Most common is the replacement of serum-rich medium with conditioned medium with lower serum content or with serum-free medium.25,26 Although many cells tolerate starvation conditions for a short period of time (usually 12–48 hours), it is essential to monitor and optimize the starvation condition for each particular cell type. The important step between changing the serum-rich medium to conditioned medium with lower or no serum concentration is a rinsing step to ensure that any contamination from high serum content is eliminated. On the other hand, extensive rinsing or the choice of an improper washing buffer can also cause cell death. Generally, cells are washed several times with PBS buffer or with reduced or serum-free culture medium. Recently, a comparison of the efficiency of BSA removal from medium of rat vascular endothelial cells by 3 rinsing techniques was published.27 The study showed that the most effective technique to change from medium with 20% fetal bovine serum (FBS) to serum-free medium appeared to be the combination of 2 rinses with Dulbecco’s PBS containing calcium and magnesium and 1 rinse with serum-free medium. Even when strict precautions are followed and the protocol is optimized, handling the cells in vitro results in cell necrosis, and the percentage of dead cells and cell viability in reduced-serum medium should be measured and evaluated, because the intracellular proteins released by necrotic and apoptotic cells distort the results obtained for secreted proteins. In addition, the medium itself should be analyzed the same way as conditioned medium and used as a control to eliminate the proteins delivered by medium. Recently, a technique has been published by which secreted proteins can be distinguished from proteins derived from media residual fetal calf serum and proteins released by dead cells.28 The method is based on the combination of in vitro metabolic labeling of cells ([35S]-labeled methionine and cysteine) and subsequent detection of proteins by fluorescence analysis and autoradiography. Whereas fluorescence analysis detects all proteins present in medium, autoradiography detects only proteins synthesized by living cells during the metabolic labeling period (secreted proteins).

Coculture and Quantitative Labeling
Numerous studies have been published on the identification of proteins in cardiac myocyte, nonmyocyte, and stem cell secretomes and on the contribution of secreted proteins to functional improvement in various cardiac pathologies. Secretomes obtained from either single cultures29,30 or cocultures of various cells31 have been studied in vitro, as well as changes induced in 1 cell type conditioned by secretomes of other cell types.33,23,32–35 Although the latter method is technically easier and most often used, the cell coculture more closely reflects the situation in vivo and enables the evaluation of the possible cross talk between both cell types. In coculture studies, especially when secreted proteins are to be assigned to the cells from which they originated, the experimental design is more challenging. First, labeling methods are used in which 1 of the cell types is labeled to distinguish between the same proteins delivered from 2 different cell types mixed together. Second, the labeling should not alter the cells and secretome. Third, the heterogeneity of both cell systems complicates the coculturing conditions in terms of such things as the selection of the type of medium, serum concentration, and culture dish coating.

There are a number of available labeling strategies by which these challenges can be addressed. In addition to chemical derivatization, which introduces a mass tag into proteins/peptides (eg, tandem mass tag [TMT] and isobaric tag for relative and absolute quantification [iTRAQ]),36,37 the other stable isotope–labeling methods are applied based on metabolic labeling of living cells.58 In 2002, the SILAC (stable isotope labeling by amino acids in cell culture) method was introduced59 for MS-based quantitative proteomics. The SILAC method is based on a metabolic labeling approach and can be used in any cell culture system for incorporation of a specific amino acid tag into all mammalian proteins. With this method, the cells are grown in media
lacking a standard essential amino acid but supplemented with a nonradioactive, isotopically labeled form of that amino acid ("heavy" amino acid, eg, $^{13}$C- and $^{15}$N-Arg). Complete incorporation of "heavy" amino acid occurs after several doublings in cell lines and does not affect the growth and morphology of cells compared with cells in normal medium. Because free amino acids present in serum-containing medium can interfere with results, dialyzed serum should be used. When "heavy" and "light" amino acid (eg, arginine or lysine) cell populations are mixed, they are distinguishable by MS (eg, 6 Da for $^{13}$C$_6$-Arg), and protein abundances are determined from the relative MS signal intensities.$^{40}$ Compared with other metabolic or chemical labeling methods ($^{18}$O, $^{15}$N, iTRAQ, and tandem mass tag), SILAC has several advantages, such as no differences in labeling efficiency between samples (100% tag incorporation), no chemical labeling or affinity purification steps and thus no sample loss during handling, and, because proteins are uniformly labeled, several peptides from the same protein can be compared to ensure that the magnitude of change is the same. Despite these advantages, there are some issues with SILAC labeling; for example, the method does not work for every cell type and can alter the proliferation of some cell types, and the labeling requires sufficient cell doubling to incorporate the tag and obtain 100% labeling efficiency, which can be problematic, especially for ventricular cardiac myocytes, for which no dividing cell culture system exists. The method has been shown to be compatible with gel electrophoresis$^{59}$ and LC methods$^{41}$ followed by MS, and it has been used as a tool for many applications.$^{42}$–$^{44}$ Because it was designed for comparative proteomic experiments, the benefit of SILAC application in coculture secretome identification studies is apparent, and the method is being tested in several laboratories for this purpose.

The use of an insert-well coculture system is another possible approach by which to evaluate cross talk between different cell types.$^{45}$–$^{46}$ Although the purity of each tested cell is preserved in this system, the cells can be separated easily, and the components of this system can be easily changed to achieve the desired experimental conditions, there are limitations; for instance, the effect of direct cell-to-cell contact between different types of cells cannot be evaluated, and it is difficult to identify which cell type is responsible for the secretion of a particular protein. Thus, the coculturing setting depends on the goal of each particular study. When the origin of the proteins is not an issue or direct cell-to-cell contact is desirable, various cell types can be cocultured without protein labeling either together or in various insert arrangements. However, to be able to distinguish among proteins originated from different cell types, protein labeling is required (SILAC) in the coculture. Two approaches are used primarily: Either 1 cell type is labeled in the cell coculture, or the secretome of 1 cell type is labeled and used as a conditioned medium for the other cell type (the labeling can be vice versa).

**Conditioned Medium Collection**

After conditioning, cell supernatants are collected and centrifuged or filtered to remove nonadherent cells. Conditioned media are then stored at \(-80^\circ\text{C}\) before analysis. Before fractionation by separation methods, conditioned medium can be processed further, eg, high-abundance serum proteins can be depleted, and medium can be concentrated. Because secreted protein concentration in the conditioned medium is usually low and the proteins are in a soluble form, it can be desirable to retain solubility during concentration and use methods that do not involve precipitation, eg, centrifuge filtration, dialysis, or evaporation. Centrifuge filtration with microconcentrators can result in protein binding to the membranes (even when low-protein-binding membrane devices are used), and the proteins of interest can be lost. In addition, the membrane cutoff must be chosen to be well below the size of the proteins of interest. During dialysis, the protein can precipitate because of low salt concentration in the dialysis buffer. In our group, we primarily use SpeedVac concentrator to concentrate conditioned medium approximately 6- to 10-fold before analysis by reverse-phase liquid chromatography (RPLC), and we do not experience any protein precipitation.

In addition to secreted proteins, conditioned medium may also contain shed membrane proteins and secretory vesicles (eg, microvesicles, exosomes, and membrane particles) after its collection. Because the size of the secretory vesicles is typically in the range of tens of nanometers to 100 nm (except for microvesicles, which are 100–1000 nm), the filters used for removal of nonadherent cells and debris (typically 40 \(\mu\text{m}\) in size) will not remove the secretory vesicles from the medium. In addition, the centrifugation of the medium after collection (typically 450–600 \(g\)) will leave the secretory vesicles in the medium, because they constitute (together with Golgi membranes) the slowest-sedimenting membranes. They are retained in the supernatant fraction after centrifugation at 17 000 to 25 000 \(g\) for 15 minutes and collected in the pellet fraction after sedimentation at 100 000 \(g\) for 60 minutes. Thus, secretory vesicle proteins can be released into conditioned medium during processing and constitute a fraction of the secretome.$^{48}$–$^{51}$

It is also possible to consider analysis of the in vivo secretome. Certainly, there are instances in which proteomics has successfully identified proteins in fluid obtained from proximal tissue or cells of interest (such as with cancer in which the tumor drainage can be obtained),$^{52}$,$^{53}$ and the pericardial fluid could be a source for enrichment of cardiospecific secreted proteins. Furthermore, one can envision that with injury, there would be an increase in secreted proteins. However, the proteins secreted could originate from 1 or more of the cell types that comprise the heart and coronary vasculature. The blood sample will also contain proteins that originate from other organs in the body, because they can respond in a systematic way. The other challenges that were outlined for cell culture in vitro apply as well: Ensuring that the proteins being detected in vivo do not arise from the death of the cell, the need to maximize proteome coverage, and quantification of the proteins.

**Dealing With High-Abundance Serum Proteins**

Because media that contain serum (eg, FBS) in concentrations ranging from 10% to 20% are generally used for in vitro cell cultures, the identification of low-abundance secreted...
proteins in the presence of serum proteins (albumin and immunoglobulins) presents a challenge. A relatively easy solution is to decrease the serum concentration in conditioned media or use serum-free media during cell conditioning, as mentioned above. Another option is to keep the serum in conditioned media at the same concentration level as was used for the cells in the culture, with albumin and/or other high-abundance serum protein removal before 2-DE, LC, and subsequent MS. Numerous methods have been developed and are commercially available for high-abundance protein removal from serum that can be applied for FBS in secretomes as well. Most often, antibody affinity depletion methods for the removal of various numbers of top serum proteins are applied, as well as fractionation of the samples by chromatography methods without the necessity of albumin or IgG depletion. Recently, new ligand-based (not antibody-based) affinity columns were developed to remove albumin from serum (www.proteabio.com). These columns are highly efficient (99% human albumin and 80% bovine albumin depletion) and are not based on an antibody but rather on a unique motif found in other species. Figure 1 shows the results of albumin depletion in smooth muscle cell medium containing 1% FBS. A, Image of SDS-PAGE gel after silver staining. Lane 1, bovine serum albumin (6 μg loaded); lane 2, medium containing 2% FBS before albumin depletion (5 μL loaded is equal to 1.25 μg of total protein); and lane 3, medium containing 2% FBS after albumin depletion (5 μL loaded). B, Chromatograms from RPLC of medium containing 10% FBS before albumin depletion (solid arrow; 100 μL loaded is equal to 56 μg of total protein) and after albumin depletion (dashed arrow; 100 μL loaded). Inset, Example of spectrum of 1 peptide, DAFLGSFLYEYSR, identifying bovine albumin (accession #P02769; ALBU_BOVIN) present in FBS culture medium. ProteaPrep albumin depletion sample prep kit was from Protea Biosciences (Morgantown, WV; catalog #SP-200-12), albumin was from Sigma (St Louis, MO; catalog #A7517), and medium was Clonetics smooth muscle cell basal medium (Lonza, Walkersville, MD; catalog #CC-3181).
miment, as well as on the type of medium and the medium components, because they can interfere with the depletion assay. In addition, most of the depletion methods will result in sample losses and in removal of the proteins bound to albumin. This can be overcome in part with agents that will break the bonds between albumin and its bound proteins before depletion, provided that this will not interfere with the purpose of the protein study. Because it is a very important step in secretome analysis, we recommend optimization of the depletion step for each particular case. This is also true if other samples such as coronary sinus serum or plasma are analyzed instead of cell conditioned media.

Separation Methods

Because proteomes of biological samples consist of a large number of different proteins in broad dynamic ranges, separation methods are used to fractionate and reduce the complexity of the samples, enhance the proteome coverage, and improve protein identification by subsequent MS. In addition, they can be used to physically separate the high-abundance serum proteins (eg, albumin) from the remaining proteins. The proteomic methods used most frequently for fractionation of complex protein samples are 2-DE19,21 and LC methods,30,59 which can also be used for fractionation of proteins in secretome samples.17,27 Despite disadvantages such as poor separation of membrane and hydrophobic proteins and the inability to analyze high- or low-molecular-mass proteins, 2-DE is the method of choice in many laboratories because of the relatively inexpensive equipment, its versatile applicability to a wide range of samples, and the fact that it is one of the few methods that enables the determination of posttranslational modification and specific protein isoforms. The method is based on the separation of proteins by isoelectric points (pIs) in the first dimension and by molecular masses in the second dimension.60 Protein identification (or confirmation) can be accomplished by use of Western blot or MS analysis. Before MS, the protein spots are excised from gel, destained if the gel was Coomassie blue– or silver-stained, reduced, alkylated, and subjected to enzymatic digestion.

The modification of 2-DE called 2-dimensional difference gel electrophoresis (2-D DIGE) was designed for reproducible detection and quantification of differences in protein levels or expression between 2 proteomes61 and should also be considered as a method for evaluation of protein changes between conditioned media of different types of cells. In this method, 2 protein samples are tagged fluorescently with 2 different cyanine dyes; samples are mixed and run on the same 2-DE gel; the gel is scanned with the excitation wavelength of each dye; and gel images are then superimposed to compare the differences in protein composition. Because the samples are run on the same gel in both dimensions and thus subjected to the same procedure and environment, 2-D DIGE is highly reproducible, without the interference caused by gel-to-gel variation. The combination of 2-D DIGE and shotgun proteomics has been used recently for characterization and mapping of the proteome and secretome of human early proangiogenic cells.22

The other methods frequently used in proteomics are LC methods, which can be used either as a single method or which can be combined and used sequentially in various multidimensional arrangements either offline or online. Most often, RPLC is the final separation method in a multidimensional arrangement, because it is compatible with downstream MS. In RPLC, proteins or peptides are separated in a chromatographic column on the basis of differences in retention magnitude of the protein or peptide to the column stationary phase by use of a relatively nonpolar stationary phase and polar solvent. The proteins or peptides are eluted from the column in order of increasing degree of hydrophobicity, and elution times depend on the percentage of organic solvent and the type of stationary phase. The use of gradient elution (linear or discontinuous, primarily with increasing concentrations of organic solvent) is preferred for complex samples to speed the elution of strongly retained proteins or peptides and to obtain sufficient resolution. Recently, we used RPLC for fractionation of intact proteins in conditioned media of rat CSCs and neonatal cardiac myocytes (which contained 1% of FBS) before MS/MS protein identification.17 Because a low amount of secreted protein is usually present, the cell conditioned media are concentrated after collection and mixed with a solvent compatible with RPLC (eg, 20% acetonitrile [ACN], 1% trifluoroacetic acid [TFA] in final concentration, pH 2.3), centrifuged at 18 000g for 30 minutes at 4°C, and injected into an RPLC column. For secretome protein separation with reduced FBS (1%) in media, we used a linear AB gradient from 0% to 100% B, where solvent A was water/0.1% TFA (vol/vol) and B was ACN/0.08% TFA (vol/vol) with a C18 column. The eluted fractions were collected (the albumin-rich fraction was excluded), concentrated, neutralized by ammonium bicarbonate, and digested (250 ng of enzyme per fraction) before MS analysis.

The other potential methods for secretome fractionation are non–gel-based electrophoretic methods, such as free-flow electrophoresis.62 In free-flow electrophoresis based on isoelectric focusing, the proteins are injected continuously into a thin liquid film flowing between 2 parallel glass plates, and isoelectric focusing conditions are established by use of carrier ampholytes by introducing an electric field perpendicular to the flow direction. Proteins are separated on the basis of their different pIs and are collected into fractions for further processing.63 For example, the combination of free-flow electrophoresis to reduce sample complexity and nanoflow LC-MS/MS has been used for the analysis of secretomes of human umbilical vein endothelial cells.20

Posttranslational Modification Protein Enrichment

Because proteins may be secreted by cells in very low concentrations, as mentioned previously, various enrichment methods can be applied. The enrichment method can be based on the posttranslational modification that occurs to secreted proteins, eg, glycosylation. Examples of secreted proteins that are known to be glycosylated and play important roles in cardiovascular diseases are, eg, vascular cell adhesion molecule 1, brain natriuretic peptide (BNP), interleukin 6, ST2 (interleukin-1 receptor-like 1), tumor necrosis factor-α, ma-
trix metalloproteinase 9, and proteins of the intercellular adhesion molecule superfamily. Glycosylated forms of some of these proteins could affect, for example, proBNP-108 processing by furin and thus could have an effect on cardiovascular and cellular function. Recently, a method termed “cell surface capturing” was published that is based on the conjugation of glycopeptides to a solid support by use of hydrazide chemistry and stable isotope labeling of N- and O-linked glycopeptides. The method has been optimized to date for N-linked glycosylation based on the specific release of formerly N-linked glycosylated peptides via peptide-N-glycosidase F (PNGase F). The recovered peptides are then identified and quantified by MS. Although the method was used for the selective isolation of N-linked glycosylation sites from glycoproteins in blood serum, for selective enrichment and detection of the cell surface N-glycoproteomes of T and B cells, and for the identification of the cell surface N-linked glycoprotein subproteome of myoblasts, the use of this method for enrichment of secreted proteins in conditioned medium could be a good approach as well. O-linked glycoprotein and peptide analysis (just like N-linked) can be performed on the basis of lectin affinity and other click chemistry reagents; nevertheless, the methods for O-linked glycoproteins remain challenging.

**MS and Protein Identification**

For protein identification, 2 basic MS approaches are used to analyze the peptides that result from enzymatic digestion of protein samples: Analysis assisted by matrix (matrix-assisted laser desorption ionization–time-of-flight MS [MALDI-TOF-TOF MS] and electrospray ionization MS [ESI-MS/MS]). Today, most scientists use tandem MS instruments that allow determination of the amino acid sequence of the observed peptides. Because MALDI-TOF-TOF MS suppresses the low-abundance proteins in favor of the most abundant proteins and is often successfully used for analysis of simple protein mixtures (unless the peptides are preseparated by LC), the second approach is generally preferred for identification of the proteins in cell secretomes in which RPLC is coupled online directly by an electrospray interface to the MS/MS instrument. With the use of RPLC-ESI-MS/MS or MALDI-TOF-TOF MS, extensive data sets can be generated and various search engines can be used to match the MS/MS fragmentation spectrum with peptides from appropriate databases; thus, the mass accuracy of MS data is of high importance. To gain as high confidence as possible for final protein identification, various criteria and limits are selected for data filtering. In addition, all identified proteins or peptides must be examined for redundancy. In some cases, it is advantageous to use a combination of several different enzymes simultaneously, because this can lead to higher proteome coverage because they cleave the protein at different amino acid sites into a greater variety of peptides.

In addition to MS-based proteomic methods that compare the peptide ratios of 2 proteomes labeled separately with a tag that contains heavy and light isotopes, the other methods with potential utility for secretome analysis are label-free quantification methods, including spectral counting (based on the frequency of identifications) and selected or multiple reaction monitoring (MRM). MRM is a technology for the consistent detection and absolute quantification of specific, predetermined sets of peptides that are representative of their parent proteins in a complex background and in multiple samples. Usually, a triple quadrupole MS instrument is used to monitor parent peptide-fragment ions (transitions) in combination with peptide retention time during MRM assay development. Although a large number of transitions with high reproducibility can be achieved in a single experimental run, development of the assay for each protein is still a time-consuming process, despite the improvements published recently. Nonetheless, the MRM technique has great potential, and as a validation method, it might replace the classic immunoassay approaches, depending on antibody specificity and availability.

To identify or predict the subcellular location of proteins and to assess which proteins identified by a secretome study are truly secreted, the computational tools have been published to assess proteins that follow both the classic secretory pathways and nonclassic protein secretion. Many proteins are secreted by a classic secretory mechanism, ie, with signal peptide (an N-terminal peptide, typically 15–30 amino acids long), which is cleaved off during translocation of the protein across the membrane, and can be predicted, for example, from the amino acid sequence of the protein as the input by use of bioinformatics tools such as TargetP or SignalP. For secretory proteins that do not have N-terminal signal peptides (eg, fibroblast growth factors, interleukins, and galectins found in the extracellular matrix), alternative pathways called nonclassic secretory pathways exist, and the sequence-based method SecretomeP for the prediction of mammalian secretory proteins targeted to the nonclassic secretory pathway can be used. The method is also capable of predicting signal peptide–containing secretory proteins in which only the mature part of the protein has been annotated or cases in which the signal peptide remains uncleaved.

**Validation and Biological Assays**

After identification by MS, the presence of proteins should be validated qualitatively and/or quantitatively by other methods. One of the methods applied could be the newly developed MRM technique, which is antibody independent and possesses great potential, especially when the antibody for a particular protein is unavailable and because it can be run in the multiplexed format for many proteins simultaneously. Our laboratory recently built an MRM assay for interleukin-1 receptor-like 1 (ST2) protein (Q. Fu, PhD, unpublished data, 2011), which is shown schematically in Figure 2. Unfortunately, the MRM method has not yet been adopted for broad use by the proteomics community, and thus, various immunoassays, such as ELISA and Western blotting, are primarily the methods of choice, at least for proteins for which antibodies are available. For example, the absence of atrial natriuretic peptide (ANP) in conditioned medium of CSCs but its presence in conditioned medium of neonatal rat ventricular myocytes (NRVMs), as revealed by LC-MS/MS, has been confirmed by Western blotting, and in a study by Liao et al, heat shock protein 90-α but not heat shock
protein 90-β was found in conditioned medium of vascular smooth muscle cells by LS-MS/MS, with the results confirmed by Western blotting. Figure 3 shows Western blot validation of ANP as identified by LC-MS/MS. ANP is synthesized as a preprohormone (152 aa; 16556 Da), and within the endoplasmic reticulum, the signal peptide is cleaved to produce the prohormone (128 aa), with ANP hormone consisting of 28 amino acid residues (from the C terminus) with a molecular weight of 3063 Da. Western blots of ANP performed on medium or lysate of NRVMs and medium of CSCs with recombinant protein as control (200 and 500 ng loaded) are shown in Figures 3A and 3B. Three bands corresponding to preproANP, proANP, and ANP were detected in the medium of NRVMs, but none of the ANP bands were found in conditioned medium from CSCs. These findings confirmed the MS results in which 3 peptides were identified exclusively only in medium of NRVMs: 2 peptides that covered part of the ANP prohormone sequence and 1 peptide that matched a partial sequence of the ANP hormone. ANP is considered to be a cardiac lineage marker, because it is expressed early during the differentiation of myocytes and early during heart development. This suggests that because CSCs are like embryonic and other progenitor cells, they do not secrete ANP, but they would during differentiation into cardiac myocytes. Similar to ANP, BNP, another early marker of cardiac differentiation, exists as a prepropeptide that is further processed to a prohormone (proBNP; 108 aa). The proBNP is enzymatically cleaved to form a biologically active C-terminal peptide (BNP-32; 32 aa) and biologically inactive N-terminal peptide (NT-proBNP; 76 aa). It is generally believed that both peptides (BNP-32 and NT-proBNP) are upregulated and released into the circulation in patients with heart failure, and although both peptides are used to monitor the existence and severity of heart failure, very little information is known about the molecular forms of BNP in relation to heart failure. Because BNP is present in the circulation in very low concentrations, both highly sensitive and specific methods are essential for its identification. Recently, studies have been published that have demonstrated the improved separation and detection of BNP and its proteolytic peptides by experimental optimization and by use of MS instruments.

A relatively large number of studies have aimed to evaluate and compare the secretomes of various cells under both normal and pathological conditions. Different assays in vitro
have been developed to monitor the influence of various secreted factors and to simulate pathological conditions in vivo, such as the effect of oxidative stress, hypoxia, hyperoxia, cyclic stretch, proliferation, and differentiation. It was demonstrated that cardiotrophin 1 expression is augmented after hypoxic stimulation, that hypoxic conditioned medium presented an enhanced ability to activate the signal transducer and activator of transcription (STAT3) in cardiac myocytes, and that cardiotrophin 1 might play an important role in the pathogenesis of ischemic heart disease. Assays of the conditioned medium of cardiac myocytes subjected to the influence of different concentrations of oxygen have revealed the self-protection of cardiac myocytes against hypoxia and hyperoxia via the production of increasing amounts of angiotensin I and adenosine with oxygen concentrations >6% and <6%, respectively. Placenta growth factor expression was increased 3.88-fold in cultured neonatal rat cardiac myocytes after 12 hours of hypoxia compared with normoxia. Shorter periods of hypoxia, conditioned medium from hypoxic cells and cyclical stretch, did not significantly alter placenta growth factor or its receptor (VEGFR1, vascular endothelial cell growth factor 1) expression. Connective tissue growth factor and ANP proteins identified in the secretome of NRVMs and ST2 protein identified in the secretome of CSCs have been shown to affect CSC proliferation. Whereas NRVM-specific connective tissue growth factor and ANP altered CSC proliferation rates in a paracrine manner (although in an opposite direction), CSC-specific ST2, which inhibits CSC proliferation, acted as an autocrine factor.

In addition, one can consider analysis of the secretome in vivo. As discussed earlier, there are body fluids that are in close proximity to the cells that comprise the heart, and these could capture the changes in their secretomes. These proteins, if quantified, could give rise to circulation biomarkers with diagnostic and prognostic implications.

**Pathway Analysis**

To manage and interpret large-scale proteomic data sets and to model, analyze, and understand complex biological and chemical systems, various pathway analysis software programs have been adopted by the research community. One proof-of-knowledge-based software program that is often used is Ingenuity Pathways Analysis, which helps to understand biology on multiple levels via the integration of data from a variety of experimental platforms and by providing insight into the molecular and chemical interactions, cellular phenotypes, and disease processes of the system being examined. Ingenuity Pathways Analysis contains an extensive library of well-characterized signaling and metabolic pathways that can be used to help find a connection between a novel discovery and known in vivo and in vitro experimental biological systems. It provides information on known protein-protein relationships between members of large data sets through graphical representation. Because numerous papers incorporating pathway analysis have been published in cardiovascular research in recent years, this approach is increasingly being used and appears to be a valuable tool in many research fields, especially in profiling secretomes and potential biomarkers.

**Conclusions**

An increasing number of studies showing the affirmative effect of conditioned media with secreted proteins derived from various stem cells in cardiac regeneration confirm the hypothesis for the use of paracrine/autocrine factors secreted by cells rather than the cells themselves to effect myocardium tissue repair. This has several advantages, such as the potential to generate a therapeutic cocktail that effects in vivo cardiac regeneration and can be administered immediately after injury, thus limiting scar formation and preventing heart failure and arrhythmias, as well as the avoidance of issues such as tumorigenicity and immune compatibility that are associated with ex vivo cell cultures. Thus, identification of these secreted modulators and cardioprotective proteins is of great interest. In addition, secreted proteins can serve as an essential source for biomarker discovery. Despite this, only a few proteomics studies have identified actual protein candidates, presumably because of various technical issues that render analysis of secreted proteins challenging, and some of the challenges have been mentioned in previous sections.

Proteomics is a field that has developed rapidly within the past several years. It represents a broad group of technologies and methods comprising analytic protein biochemistry, analytic separation, MS, and bioinformatics. Many technologies are synergistic and complement each other by providing different data for each proteome. Although there are still many aspects of proteomics that need to be developed further, the sensitive MS instruments, robust methods (eg, enhanced protein and peptide separation and use of multiple enzymes), and expanded databases implemented to date are valuable tools that can be used to better understand the reasons for proteome alteration in the context of health and disease. Proteomics is being incorporated rapidly into cardiovascular research, and as the broader scientific community adopts and applies proteomics methods to common practice, novel
findings are expected to be discovered. This includes further studies on secreted proteins to find new biomarkers and enhance our understanding of the cell-environment interface. The ability to manipulate the cell-environment interface for therapeutic intervention starts with knowing the proteins involved. Thus, the use of proteomics enables better secretome analysis profiling in the context of broader perspectives.

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