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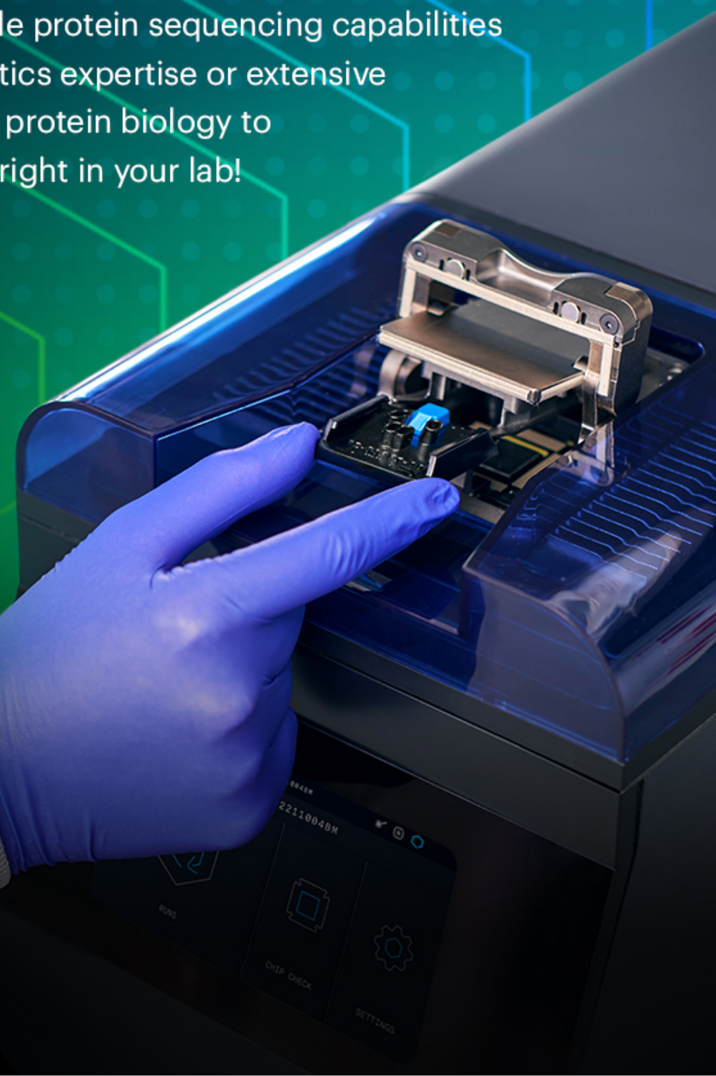

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Review

Proteomics of cell–cell interactions in health and disease

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The mechanisms of cell–cell communications are now under intense study by proteomic approaches. Proteomics has unraveled changes in protein profiling as the result of cell interactions mediated by ligand/receptor, hormones, soluble factors, and the content of extracellular vesicles. Besides being a brief overview of the main and profitable methodologies now available (evaluating theory behind the methods, their usefulness, and pitfalls), this review focuses on—from a proteome perspective—some signaling pathways and post-translational modifications (PTMs), which are essential for understanding ischemic lesions and their recovery in two vital organs in mammals, the heart, and the kidney. Knowledge of misdirection of the proteome during tissue recovery, such as represented by the convergence between fibrosis and cancer, emerges as an important tool in prognosis. Proteomics of cell–cell interaction is also especially useful for understanding how stem cells interact in injured tissues, anticipating clues for rational therapeutic interventions. In the effervescent field of induced pluripotency and cell reprogramming, proteomic studies have shown what proteins from specialized cells contribute to the recovery of infarcted tissues. Overall, we conclude that proteomics is at the forefront in helping us to understand the mechanisms that underpin prevalent pathological processes.

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Abbreviations: **CM**, conditioned medium; **CTAP**, cell type–specific labeling using amino acid precursors; **DDA**, data-dependent acquisition; **DIA**, data-independent acquisition; **EMT**, epithelial to mesenchymal transition; **EVs**, extracellular vesicles; **IGF**, insulin growth factor; **IL**, interleukin; **iPS**, induced pluripotent stem cells; **IRI**, ischemia/reperfusion injury; **miRNAs**, microRNAs; **MSCs**, mesenchymal cells; **Prdx**, peroxiredoxin; **TGF-β1**, transforming growth factor-β1; **VEGF**, vascular endothelial growth factor

1 Introduction—Mechanisms of cell–cell communication: The direct and the secretome worlds

Dynamic communication between cells is also called “education” [1], a phenomenon—and a concept—based on the ability of cells to change their surrounding microenvironment, influencing behavior and activity of neighboring cells. Mechanisms of cell–cell communication—and therefore of this “education”—are crucial for the comprehension of physiological homeostasis and their deviation toward pathological conditions. They can be classified as: (i) contact-dependent

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signaling through gap junctions or ligand–receptor interaction; (ii) endocrine signaling mediated by hormones acting at distant sites; and (iii) paracrine signaling associated with the secretion of soluble molecules acting on adjacent cells [2]. Recent evidence has established a role for cell-derived extracellular vesicles (EVs) released into the microenvironment as another system of cell communication [3]. Since their discovery almost 30 years ago [4], EVs have been purified from almost all cell types, including primary cells of the nervous and immune systems [5,6], stem cells [7–9], including numerous tumor cells [10, 11]. This emerging field in proteomics is discussed in more detail below.

Most of these mechanisms—if not all—involve protein–protein interactions and are, therefore, clearly represented in the field of functional proteomics [12]. For this reason, the proteomics of the influence of external and intracellular factors deserves intense investigation. As some examples, we could mention those related with the still poorly understood conditions involving prions, scrapie [13], and Creutzfeldt–Jacobs diseases [14]. These investigations demonstrated how a broad knowledge of several proteins and those binders help in the understanding of the pathogenesis of incurable diseases.

In the case of the gap junction proteome, connexins are prominently involved, these being proteins that assemble into complexes called connexons, thus allowing permeation of ions and small molecules crucial for communication between cells [15] as well as in the organization of different epithelia, including skin [16]. In this regard, proteomics has revealed some key processes by which adjacent cells respond to injury and treatment to produce the so-called "bystander" effect [17]. Gerashchenko et al. [17] pioneered the concept of multifunctional proteins in vast and differentiated fields of the proteome world. For example, their studies allowed the detection of one enzyme that plays a central role in cell adaption in a number of processes, viz. α-enolase. A general role of this protein in cell responses to a range of stress challenges seems to be confirmed by its upregulation in cultured human renal cells after hypoxia when cocultured with mesenchymal cells (MSCs) [18]. As mentioned above, ligands are important mediators of cell–cell communication, and also for some interacting processes in which extracellular proteins—circulating or not—are involved. In the case of membrane-located receptors, it is crucial to distinguish isoforms, splicing variants, and post-translational modifications (PTMs) essential for protein regulation [19]. Interesting approaches have been developed for the proteomics of ligand–receptor interactions. The combination of biochemical and proteomic protocols, such as that represented by affinity purification coupled with multidimensional LC-MS/MS and bioinformatics, helped identify extracellular ligand interactions [20].

Of particular interest is to analyze the ligand–receptor interactions in living cells. A proteomic approach using a chemoproteomic bifunctional molecule, which simultaneously binds to an amino acid residue in the ligand and another in the glycosylated receptor, leads to their efficient conjugation. A biotin tag of the same molecule (TRICEPS) allows further purification and quantitative MS analysis. This ingenious approach succeeded in identifying several surface proteins and their corresponding ligands, including some with potential therapeutic applications, like vaccines [21].

Regarding circulating protein containing receptors in their structure, as with monoclonal antibodies, identification of sites with potential therapeutic activity can be achieved by a method that combines LC-MS/MS with a "universal surrogate peptide" that has now been identified in human antibodies [22], and that gives a clear example of how the proteome of interactions can be useful in the development of preclinical work involving Igs. In the same field, the combination of micro- and microplate-based ligand-binding techniques fosters investigation at the proteome level of potential sites for new drugs [23]. This approach gives theoretical and technical support for the development of the "polypharmacology" concept, now becoming of considerable importance in the discovery of new drugs [24].

A specific case of ligand–receptor interactions is represented by hormones and their targets. Hormones are specialized secreted cellular products, of which one of the two main classes is represented by peptides of different structure and size incorporated in the secretome (the total molecules—organic or otherwise—secreted by different cells in a living organism). Thus, the proteomics of these polypeptides helps anticipate the type of interaction they may have with specific targets, and the processes and pathways they activate, thereby reflecting the secretory profile of the target cells. Several proteomic approaches have been used to examine this type of interaction in key metabolic processes, including those related with the storage and release of energy in physiological and highly prevalent diseases. The pioneering proteomics work of Zhou et al. [25] elucidated the mechanism by which insulin regulates secretion of proteins by adipocytes, some being related with the development of obesity and insulin resistance. LC-MS/MS and 2D LC-MS/MS in combination with the cleavable ICAT provided information regarding long-range effects of the secreted proteins on other tissues. In the field of the secretome, there are also examples of hormone-mediated communications that modify the proteome of cancer cells, leading to an understanding of the mechanisms of tumor progression. The thyroid hormone, T_3, modulates a number of metabolic processes after binding to specific receptors, and the profile of secreted proteins differs between normal and cancer cells. In cultured hepatocellular carcinoma cells overexpressing TRα1, SILAC-based quantitative proteomics showed that $>$1700 proteins are differentially expressed by a specific T_3/TRα1 interaction [26].

This introductory section ends with a brief consideration of cell–cell communication mediated by soluble factors, focusing on interactions of injured cells with stem or MSCs, a rapidly growing field in proteomics. MSCs secrete a wide range of soluble factors that have autocrine and paracrine actions capable of stimulating differentiation, survival, and

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proliferation in tissues that have suffered a range of injuries of different etiology [27]. Even though the secretome profile has not been determined, it has been clearly demonstrated that factors secreted by MSC partially restore urinary bladder function in a model of incontinence [28]. One of the most promising proteins secreted by MSC—although its mechanisms of induction and action remain unknown—is the so-called “hypoxia and Akt induced stem cell factor” that promotes cardiomyocyte proliferation and arrests cell death [29]. This protein seems to coordinate a synchronized network of specialized proteins that include εPKC, caspases, and those involved in transient permeability pore in mitochondria, possibly a unique example of interacting cell–cell communication involving signaling pathways from different cells and subcellular organelles. Cell–cell communication mediated by EVs and microRNAs (miRNAs), as well as the proteome of their interactions, will deserve a special coverage below.

2 Thinking on experiments—How researchers have constructed the more challenging models and circumvented the pitfalls

Classical proteomic studies are based on characterization of protein content in cells from different tissues before and after exposure to different stimuli, or in pathological and physiological conditions. This approach has led in the last couple of decades to considerable knowledge regarding the proteome of many cell types. Nearly complete coverage has been obtained in some cases, as demonstrated by some investigations that were capable of identifying >10 000 proteins from common cancer cell lines, a number close to the estimated total in a human cell [30]. This work has recently led to drafts of the complete human proteome [31]. Moreover, in the past few years, important advances have been achieved in the field of proteomics, with the development of new types of high-throughput technologies and novel protocols for existing ones, expanding the possibilities beyond the large-scale analysis of total protein expression. This now includes analysis of protein location, turnover, structure, and activity, and enables the dynamics of several protein systems in different physiological or physiopathological processes to be followed. Therefore, our understanding regarding cell–cell interactions emerges as a potential application for proteomics techniques through the identification of secreted proteins, changes in protein localization and activity, and their activation status of different signaling pathways, notable when target cells are directly or indirectly exposed to others cells.

Secreted proteins represent approximately 10% of the human genome [32] and are key mediators of cell–cell communication. It is, therefore, evident that there is a great interest in their characterization to improve our understanding of biological processes that involve indirect cell communication, as has been demonstrated for MSC-mediated tissue repair [33, 34]. Due to technical difficulties of in vivo experiments, most human proteomic analysis of cell communication was carried out with cell lineages grown in complete medium [35]. Cells were then, after extensive washing, usually incubated in serum-free medium to create a conditioned medium (CM) without interference from serum proteins that can be present in much larger amounts than secreted proteins. This CM is examined for protein identification by various techniques [27], as shown in Fig. 1A.

It is often of interest to characterize shifts in protein secretion when cells are exposed to other cells, and also in the intracellular mechanisms triggered by such interactions. This is generally achieved by coculturing of two cell types and analyzing the resulting CM. One interesting strategy is the use of the porous transwell membranes to separate two cell populations, allowing free diffusion of soluble factors, and permitting recovery of the conditioned media without actual cell–cell contact [18, 33] (Fig. 1B).

Another way to evaluate the differential expression of proteins is through SILAC or cell type-specific labeling using amino acid precursors (CTAP). SILAC is based on the incorporation of amino acids labeled with nonradioactive isotopes into newly synthesized proteins [36], while CTAP relies on the expression of enzymes to convert isotopically labeled amino acid precursors into essential amino acids [37]. Conditioned culture media, tagged with different labels, are then mixed and concentrated, proteins are digested with specific proteases such as trypsin, and the sample is analyzed by LC-MS/MS (Fig. 1C). These methods are of great advantage in discriminating unlabeled serum proteins from those secreted by cells, and have been used to examine the secretome of different cell types [38–40]. SILAC allows distinguishing cell-specific networks within mixed and interacting cell populations in complex tissues. This possibility was exploited by Jørgensen et al. [41] for the study of bidirectional (forward and reverse) signaling processes involving receptor ligands and tyrosine kinase-mediated phosphorylations. Identification of specific, interacting, and differentially activated networks and targeting opened the possibility for understanding how an intracellular signal originating in one type of cell (in this case ephrin-B1$^+$ cells expressing the membrane-bound ligand) impacts a different, neighboring cell type (in this case EphB2$^+$ cells) [41]. The authors demonstrated that kinase-mediated phosphorylations of tyrosine residues are different depending on whether the cells are stimulated by soluble factors or by direct cell-to-cell contact. This study is an example of how SILAC contributed to the comprehension of the widely distributed and complex signaling networks involving tyrosine kinases during cell-to-cell communications.

Although a large number of methodological approaches can be used for characterization of cell secretome, including EVs (comprehensively reviewed in [42, 43; see also Section 4.1]), MS is undoubtedly the most common strategy. MS and the development of numerous bioinformatics tools for spectral analysis have increased their applicability and effectiveness in protein detection, as discussed in the next section. Further information is obtained with tools for

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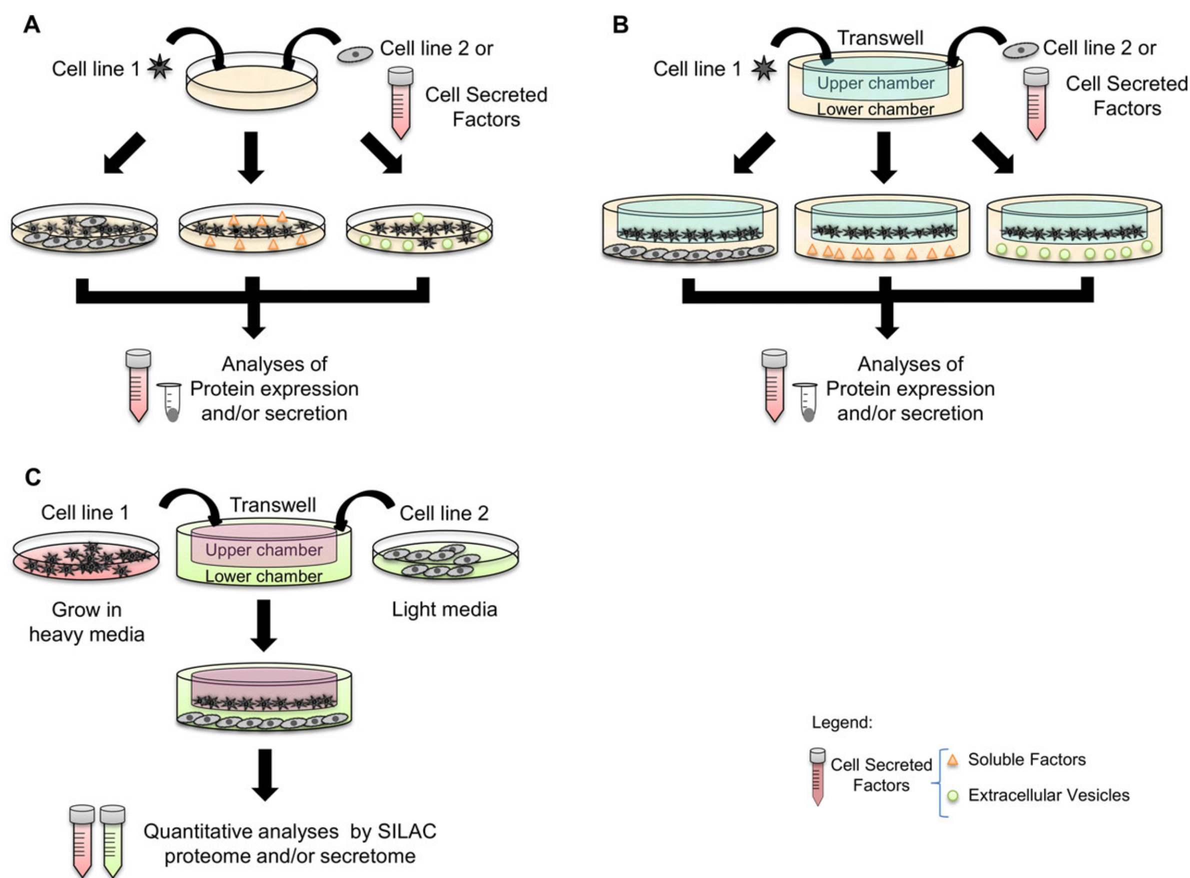

Figure 1. Overview of different methodologies to study cell–cell communication. (A) Direct cell line cocultured with another cell line (symbols shown), or exposed to soluble factors (yellow triangles) or extracellular vesicles (green circles). (B) Indirect coculture using cell lines (symbols shown) separated by an inserted transwell into upper and lower chambers, allowing no physical contact between the two cell types. Soluble factors or EVs (symbols as in A) can permeate the transwell and interact with the cells in the upper chamber. (C) Direct coculture using SILAC-labeled cell lines to examine the proteins involved in cellular communication.

biological network analysis, evaluating possible pathways associated with a given protein set [44]. This is of particular interest in interpreting a secretome, since signaling pathways triggered by these soluble factors, as well as by EVs, can be inferred, as well as their final outcome. The signaling pathways are important in understanding how communication mechanisms between cells are carried out both in the same environment and at a distance.

Finally, what are the pitfalls that these proteome studies face today? Certainly there are at least two. One is the *sample preparation* and the other *bioinformatics*. Fortunately, a range of new technologies are becoming available, as already mentioned, those increasingly used for the challenging proteomic studies of receptor/ligand interactions [20], and the use of biotin-tag molecule that simultaneously bind to the receptor and the associated ligand, allowing the identification of ligand partners and their purification [21]. With respect to bioinformatics, we believe that the creation of a mass spectra data bank is one the most fascinating challenges for comparing and identifying differentially expressed proteins, now mainly based on peptide sequences. Interactome analyses—possibly the weaker element in proteomics—also requires refinements that need biological validation.

3 Proteomic tools used in cell–cell communications and other studies

In the last few years, the MS-based proteomic approach has been a useful and powerful tool for investigating communication pathways. This approach provides (i) an overview of proteins present in cell, tissue, or media; (ii) measurement of protein level changes related to a wide variety of processes including, for instance, signaling and secretion; (iii) information regarding PTMs.

The mass spectrometers most usually applied in proteomics are MALDI-TOF-TOF and nanoLC coupled to MS/MS (nanoLC-MS/MS). In MALDI-TOF-TOF, peptides are embedded in a matrix and loaded onto a target plate, which is subjected to laser pulses (also called laser shots). The energy from the laser promotes peptide ionization and desorption. Ions are accelerated by an electromagnetic field

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toward the TOF tube, which separates them according to their *m/z* ratio [45]. NanoLC-MS/MS, however, is most widely used for quantitative analyses in MS-based proteomics. Its configuration is a chromatographer coupled to a mass spectrometer (ESI-(oa)Quadrupole/TOF, ESI-LTQ/Orbitrap, or others (where LTQ is linear trap quadrupole)) and assists in the separation of peptides on a chromatography column followed by their ionization by electrospray into the mass spectrometer [46].

There are several proteomic methods that can be employed, among the most widely used being gel-based proteomics (2DE) and gel-free proteomics, which allow the separation of proteins/peptides from a complex sample [47–50]. These separation techniques are coupled to different types of MS analysis. The choice of techniques should take the most suitable approach to address the question of the project to be executed and how the data should be analyzed. The main advantages and disadvantages are related to reproducibility of the method, the identification of differentially expressed or secreted proteins, sensitivity, and quantification [51].

The traditional approach for protein separation in proteomic analyses is gel based. 2DE gel analysis separates proteins according to their p*I* (first dimension) and molecular weight (second dimension). The proteins spots that can be resolved and quantified are very broad. The advantages of this approach include the visualization of protein isoforms, differential PTM, and changes in protein expression/secretion level [47, 48]. However, there are notable limitations of this technique, such as the inability to detect low levels of proteins and proteins of extremely high or low molecular weight or/and p*I*s. Furthermore, hydrophobic proteins as well as membrane proteins are difficult to analyze because of poor solubility [50].

To improve the sensitivity and accuracy 2D-DIGE has been employed in proteomics [47]. This method labels proteins from two distinct samples using different fluorescent dyes, such as Cy3 and Cy5, the pool of both samples being labeled with another fluorophore, such as Cy2. Samples are run on the same gel and the analyses for each sample relates to the specificity of fluorescence emission. Thus, the problem of reproducibility, even as the time spent decreases, makes the analyses much more sensitive. But limitations remain associated with gel-based techniques, as described above [47, 48]. In 2DE gel approaches, the protein spots are hydrolyzed in the gel after their separation and then analyzed by MS.

In attempting to overcome the limitations of protein separation by gel-based approaches, gel-free methodologies have been developed [50]. In-solution digestion is the most commonly used gel-free approach, which consists of enzymatic digestion (usually trypsin) of all proteins in the sample before any separation. With this method it is possible to identify a large number of proteins from each sample. This strategy is normally combined with peptide separation by LC coupled to a mass spectrometer [18]. One of the main advantages of this approach is reduction in preparation time and sample handling, minimizing the losses in these steps. On the other hand, the disadvantage of this method is due to protein solubilization and denaturation because some of the efficient detergents are incompatible with mass spectrometric analysis. To resolve this problem, the filter-aided sample preparation method was developed, which helps sample preparation by a filter-aided principle, at least for proteins that are soluble in SDS. After this step, SDS buffer is exchanged for urea by centrifugation. In addition, proteins are hydrolyzed in the same apparatus and the resulting peptides analyzed by MS [52]. The difficulty in this method is the yield of peptide recovery after many centrifugation steps.

To separate peptides, LC is generally used; this is most frequently applied in quantitative proteomics, being coupled to mass spectrometer. There are different types of LC analysis, such as RPLC and SCX chromatography. These approaches can be combined to improve the separation of peptides (e.g., SCX-RPLC or RPLC-RPLC) [53]. RPLC is usually the last step on peptide separation, because the peptides are desalted when eluted, which allows direct online analysis in the mass spectrometer. This approach detects lower levels of proteins, such as cytokines, unlike other techniques [27].

After separation and protein digestion, the peptides are submitted to MS for identification and/or quantification. MS determines the *m/z* ratio and MS/MS helps determine the amino acid sequence of the peptide and recognize some PTMs [44, 45, 47]. In recent years, the nanoLC-MS/MS mass spectrometer has mostly been employed in proteomics. In this way of identifying and quantifying peptides, several modifications of MS were made that increase the resolution and accuracy without losing the speed of analysis, its robustness and sensitivity [54].

Depending on the nanoLC-MS/MS mass spectrometer, the data acquisition method can be classified into "data-dependent acquisition", DDA, or "data-independent acquisition", DIA [55]. In DDA, the most abundant ions are selected for fragmentation and the selected precursor peptide ions (MS1) of a survey scan are subsequently fragmented (MS2). Some parameters need to be adjusted to generate reliable and reproducible quantitative results, for example number of acquired fragment ion spectra, MS acquisition time and fragmentation method. One disadvantage concerns the suppression of low levels peptides, which decreases the dynamic range of detection. By contrast in DIA, whole peptide precursors that have been set in the window ranges of the mass spectrometer are fragmented. That is, no selected peptide ions are done that are based on a precursor ion scan. The DIA technique improves confidence in data quality, quantification, and protein coverage [55].

Quantitative analyses can use label-free or target proteomic strategies with labeling tags. Label-free quantification methods can be done either by DDA or DIA. Quantification is possible by spectral counting or peptide ion intensity measurement, which provide peptide clusters of proteins from the samples that were treated by these approaches that allow analysis of differentially expressed proteins [56]. Other quantitative methodologies, especially the use of isobaric tags for

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absolute and relative quantification (i.e., iTRAQ, ICAT, and others) have also gained popularity latterly, allowing simultaneous analysis of several samples and improving detection of low frequency transcripts.

Since MS data are usually a long list of identified proteins, much effort is necessary for its validation and interpretation to extract any biological significance. One approach is ontology-based analysis [57], which is based on categorization of the proteins in a dataset in hierarchical groups organized by biological process, molecular function, and cell compartment, and can use several bioinformatics tools, including AmiGo [58], DAVID [59], Panther [60], Scaffold [61], Ingenuity Pathway Analysis software (IPA®), GeneGO MetaCore software, BiNGO [62], and many others. This classification is supported by annotated reports based on experimental or computational evidence that associate a protein with a particular term. The results are statistically validated by enrichment analysis tools, given critical *p*-value providing information about which biological processes are most likely to be related to the particular disease or condition under examination. Clearly, standardization of annotation vocabulary and constant updating of the databases greatly influence the results obtained from these tools. Further information is obtained using biological network analysis tools, evaluating possible pathways associated with a given protein set. This is of particular interest in interpreting a secretome, since signaling pathways triggered by these soluble factors and their final outcome can be inferred.

Of particular interest in the field of direct cell-to-cell contact is the approach called cross-species proteomics, which brings to the fore the reality of the complexity of tissues and organisms. Approaches that combine MS, bioinformatics, and—initially—reduced database information were especially useful in early studies of proteomics when there were a great number of unsequenced genomes [63]. Cross-species proteomic studies have been useful in a variety of proteome fields and in biology as a whole, especially in cases where direct cell-to-cell contacts are particularly relevant. One example is that of cancer biology, since tumors are composed of heterogeneous populations of cells that dynamically interact with the surrounding stroma [64]. It is noteworthy that stroma has emerged as a target in antineoplastic therapies during the past few years [65]. The cross-species approach has illuminated possible mechanisms underlying the differential behavior of anticancer compounds against potential intracellular targets (kinase-mediated signaling pathways, e.g., [64] in vitro and in vivo, which is relevant for the development of effective anticancer drugs. In the field of developmental biology, cross-species proteomics has helped to understand how present-day species evolved, differentiated, reproduced, and survived [63, 66, 67].

Combinations of one or more of the above techniques have been frequently used to gain insight into interaction between cells and cell–cell communication (Fig. 2). We will now describe how proteomics approaches have contributed to the knowledge of a many different processes in models used by researchers.

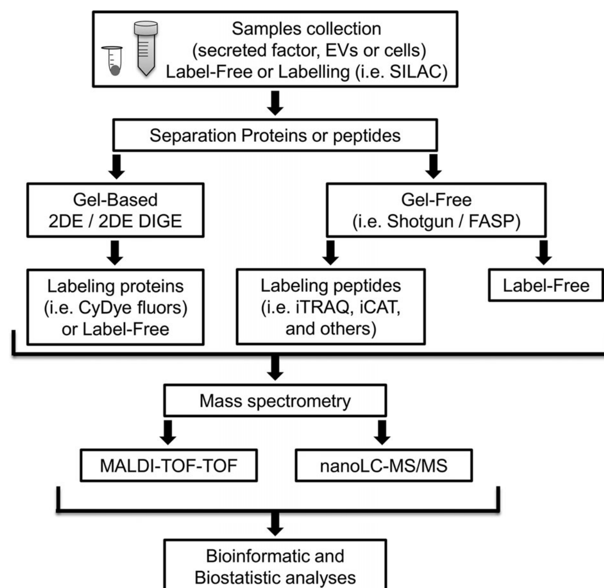

Figure 2. Proteomic workflows to investigate cell–cell communication. In this scheme we depict different proteomic approaches (for details and abbreviations, see text).

4 What does proteomics tell us about cell–cell communications?

4.1 Secretome-mediated interactions: Role of EVs and noncoding RNAs

EVs have been classified as a new mechanism of cell-to-cell communication [2]. They have been purified from almost all cell types, and correlated with physiological and pathological conditions in different organs. To define EVs composition, activity and purification methods have become an important research area, aimed at using EVs as therapeutic agents or promising biomarkers for repair processes in many diseases. EVs are considered to act mainly as carriers for a range of biologically active molecules, including lipids, proteins, and RNAs that can be transferred into target cells and mediate EV activity [2].

"Education" (Section 1) has been applied to the EV field in different physiological and pathological settings. Aliotta et al. [68] demonstrated that pulmonary hypertension can be induced in healthy mice by injecting EVs obtained from pulmonary hypertensive mice; disease transfer is mainly mediated by EVs. Yamamoto et al. [69] recently found that inflammation-induced EVs could ''educate'' target cells to modulate growth factor production by the transfer of proinflammatory EVs into pericytes. Quesenberry et al. [70] described the occurrence of two notable effects of EVs on target cells. The first was defined as short-term modification by the transfer of originator cell mRNAs into recipient cells using EVs. The second—persistent and associated with possible long-term phenotype changes due

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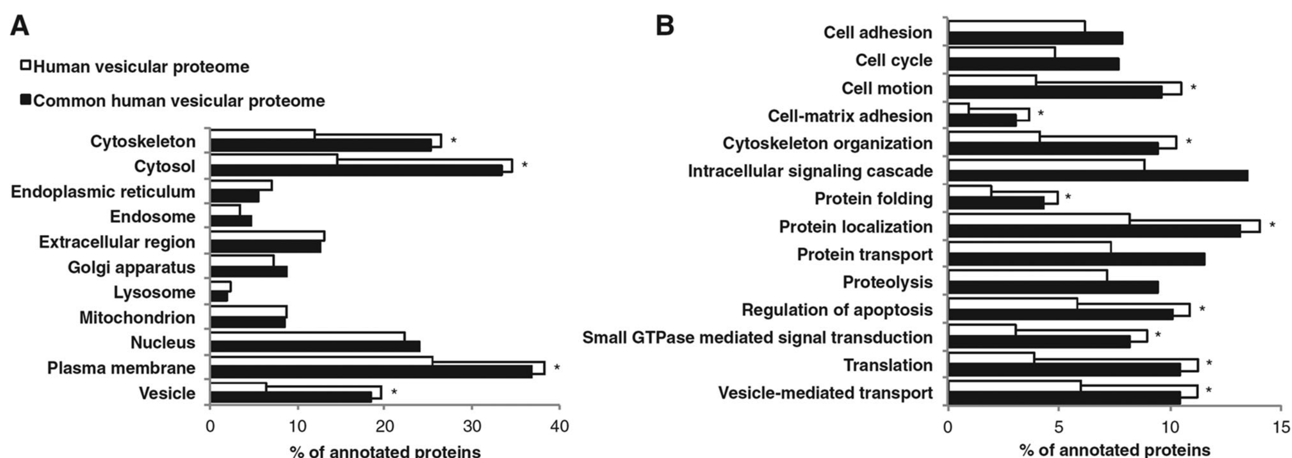

Figure 3. Proteomics of EVs revealing association of cellular components (A) and biological processes (B), the proteins being classified by GO. Reproduced from [71] with permission.

to epigenetic modifications—occurs into EV-treated target cells.

The contents of EVs are involved in modifying target cell phenotype and transcriptome profile. To define the EV contents, high-throughput proteomic and transcriptomic profiles of EVs from different origin recognized more than a thousand vesicular proteins and RNA species [71]. The proteins in EVs are characterized by the presence of specific subsets, shared among vesicles of different origins, such as tetraspanins, membrane transport, RNA-binding, cytoskeletal and ribosomal proteins, and enzymes [71]. GO analyses succeeded in correlating subcellular localization with biological processes in which >7800 human vesicular proteins are involved (Fig. 3). EVs can also transport specific cell-derived proteins, such as the oncogenic form of the epidermal growth factor receptor (EGFRvIII)—found in oncosomes—or the MHC class (MHC I/II) molecules, detected in EVs released by antigen-presenting cells [72, 73]. Different mechanisms for protein release into EVs have been proposed, some of them supporting the importance of interactions between cytoplasmic and vesicular cargo proteins for their cosorting inside EVs [71]. The iTRAQ labeling technique followed by LC-MS/MS has been used to define the protein composition of exosomes released by highly metastatic bladder cancer cells. Jeppesen et al. [72] identified several proteins related to epithelial–mesenchymal transition that can sustain the metastatic cascade, including vimentin, hepatoma-derived growth factor, casein kinase II, and annexin A2 found inside exosomes from metastatic tumor cells. Yang et al. [74], using the complementary techniques of 2DE and LC-MS/MS, analyzed the changing profile of exosomal proteins in the serum of NF-κB$^{-/-}$ mice following ischemia/reperfusion injury (IRI) to the skeletal muscle, demonstrating that NF-κB pathway may be responsible for changes in serum exosomal protein expression following IRI. High-throughput proteomic analyses successfully discriminated urinary exosome protein composition from renal cell carcinoma patients and control healthy subjects [75]. These authors used the same approaches to investigate alteration in the proteome of urinary exosomes isolated from Zucker diabetic fatty rats, a model of type 2 diabetes mellitus, supporting the possibility that urine exosome proteins might act as specific indicators of the pathology [76].

Together with proteins, extracellular RNAs may be another class of vesicle-related molecules involved in EVs activity. Besides uncertain evidence of the relevant abundance of RNA fragments inside EVs, together with a small percentage of intact coding RNAs, some work has addressed the problem of whether EV-associated RNAs remain functionally active when transferred to target cells. The first evidence of RNA transfer to cells using EVs was described by Valadi et al. [77], who demonstrated the capacity of mouse mast cell exosomes to transfer their RNAs to human mast cells. Numerous reports describe the ability of recipient cells to synthetize new proteins of different species after EV internalization, indicating the translation of exosomal mRNA into functional proteins for a short period of time [77, 78]. Others have reported an enrichment of small RNA species in EVs [79]. miRNAs seem to be carried inside EVs [80], and they have been shown on several occasions to have regulatory activity in target cells [81], which may explain EV activity. Until now, numerous mechanisms for RNA incorporation in EVs have been proposed, showing the importance of different RNA-binding protein complexes for RNA retention inside the cells or release into EVs [82]. Together with miRNAs, many other small RNAs seem to be enriched inside immune cell-derived EVs, such as transfer RNAs and small Vault RNAs [83]. Several of these small noncoding RNAs were also detected in EVs isolated from body fluids, supporting their potential use as biomarkers [84]. A functional role of miRNA-containing EVs was explored by Xin et al. [85], who found that MSCs might communicate with brain parenchymal cells by transferring miR-133b to neural cells via EVs, inducing recovery from injury. Ismail et al. [86] showed that miR-223 transfer

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through macrophage-derived EVs could induce differentiation of target cells. Endothelial EVs can transfer miR-503 into tumor cells, providing a system that reduces tumor growth, by regulating CCND2 and CCND3 proteins in the tumor [87]. Momen-Heravi et al. [88] showed that vesicles derived from ethanol-treated hepatocytes horizontally transfer a mature form of liver-specific miR-122 into monocytes, inhibiting HO-1 pathway, sensitized cells to lipopolysaccharide (LPS) stimulation, and increased proinflammatory cytokines. Chen et al. [89] reported that miR-214 transfer via hepatic stellate cell exosomes was significant in blocking fibrosis in steatotic livers through the suppression of CCN2 and its downstream targets, including alpha smooth muscle actin or collagen.

The possibility of using proteomic approaches has been recently proposed as a captivating strategy for experimental identification of miRNA targets [90]. In fact, the posttranscriptional effect of miRNAs on protein expression does not correlate perfectly with mRNA regulation [71]. Other phenomena, such as different half-lives of proteins and mRNAs, and the presence of regulatory mechanisms that affect the efficacy of miRNA-mediated gene silencing independently from the mRNA/miRNA levels, illustrate the importance of protein analysis in miRNA biology [91]. Among the most used quantitative proteomic analyses, iTRAQ and SILAC should be mentioned, because they help not only to define the components of miRNA machinery, but facilitate the discovery of new miRNA targets [92–94]. In fact, individual targets and entire signaling networks affected by miRNAs have been identified by quantitative proteomic analysis. Yang et al. [93], using a SILAC-based strategy, identified miR-21 potential targets after silencing it in breast cancer cells. Kaller et al. [94], using a variation of the classical SILAC technology called pulsed SILAC, analyzed the effects of miR-34a, a p53 signaling regulator, on mRNA and proteome expression. Computational prediction together with iTRAQ-based quantitative strategy has been also used to identify novel targets of miR17–92 clusters in lung cancer cells [95]. A global protein profile using the SILAC method helped define proteins modulated by different families of miRNAs in numerous cancers [96, 97].

The same approaches were used by Panagopoulos et al. [98] to analyze the phenotypic changes of protein levels in normal prostate cells treated with EVs from prostate cancer cells. The authors showed the occurrence of a bidirectional exchange of information between the normal and cancer cells through EVs, accounting on one side for reversion of the malignant phenotype and on the other side, for promotion of disease progression [98]. For this reason, quantitative proteomic strategies seems to be an important tool needed for characterizing direct targets of miRNAs delivered through EVs. The possibility of using proteins/RNA-containing EVs for the treatment of many pathologies is now a promising application in regenerative medicine. Identification of EV subpopulations is becoming relevant in defining the pharmacological composition responsible for EV functioning into target cells. Tauro et al. [99] described the isolation of two exosome populations, as well as microvesicles, from the same human colon carcinoma cell line. Characterization of their protein [93] and RNA typing [94] showed significant differences between the expression profiles of the three EV subtypes. Moreover, the same group also showed differences in the effectiveness of EV populations in promoting invasiveness of fibroblast cells [100]. This finding supports the view that the heterogeneous molecular composition of EVs is accountable in determining EV distinctive subpopulations and correlated activity.

4.2 Proteome of altered signal transduction in vitro models of myocardial infarction and ischemic renal injury

Analysis of signaling events gives insight about cellular function, and for many decades, researchers have devoted their attention on unraveling such networks in health and disease. However, the classical approaches, to date, such as the use of specific antibodies, usually allow the investigation of only one pathway/molecule, although it is well known how complex and intricate these cellular pathways can be. Therefore, the need arose for a comprehensive view on signaling events taking place at the cellular level in order to gain more insight into responses to different stimuli and environments during the processes of "education." In this regard, proteomics represents a unique approach to studying signal transduction, since one can then evaluate all three levels of protein changes occurring during signal propagation, that is, (i) PTMs, (ii) protein–protein interactions, and (iii) protein expression changes, with the advantage of not requiring a previous hypothesis [46].

In this section, we discuss some studies that have applied proteomics to investigate signaling networks and their alterations in ischemia, a phenomenon that underpins a whole variety of pathological processes in most, if not all, tissues and organs (Fig. 4). Far from a comprehensive review of the subject, our aim is to exemplify how proteomic approaches can improve our understanding of signal transduction alterations, using models of myocardial and renal ischemic injuries as examples.

IRI, as defined above, is a complex phenomenon that is divided into two steps. Ischemic conditions lead to a decrease in mitochondrial ATP, loss of selective permeability of cell membranes, and necrotic cell death. During reperfusion, accumulation of metabolic intermediates results in an increase of ROS that leads to oxidation of vital cell components. Intracellular activation of the apoptotic cascade is triggered at this stage and also an inflammatory response [101]. Ischemia arises from an impairment of the blood supply and reduced oxygenation of tissues and cells. Hypoxia has a major impact especially on tissues such as heart and kidney that have a high energy demand, which can culminate in cell death by apoptosis or necrosis [102–104]. In recent years, proteomic

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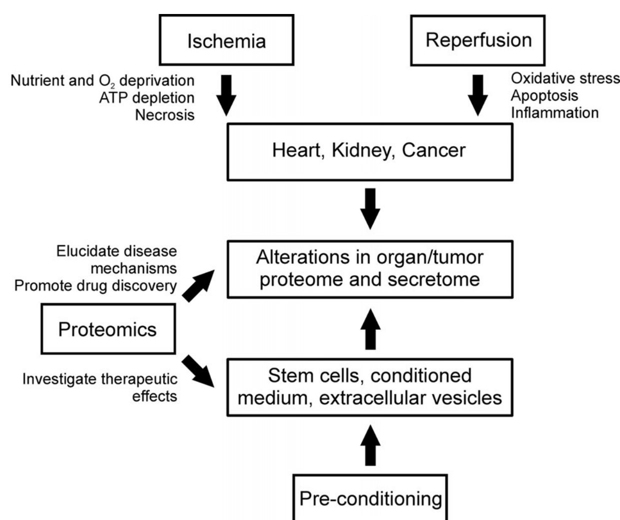

Figure 4. Organogram showing the importance of proteomic studies in understanding the mechanisms involved in tissue injury and those related to beneficial actions of stem cells administration and/or their secretions (soluble factors and EVs).

approaches have helped investigate cell death by hypoxia, and shown that PTM, such as phosphorylation, plays a significant role in the underlying mechanisms of injury (reviewed in [105, 106]). Schechter et al. [107] compared the phosphoproteomic profiles of myocardial tissue from patients diagnosed with ischemic and nonischemic heart failure. They used a TiO_2 column to enrich proteins samples for phosphopeptides and then applied nanoLC-MS/MS to identify and quantify proteins. There was a >twofold alteration in phosphorylation of proteins from ischemic compared to nonischemic tissue. These were proteins involved in important cellular functions that are affected during ischemia, including RNA processing, cytoskeleton structure, apoptosis, and energy metabolism [107].

Proteomic tools have also been used to investigate the central role of εPKC in protecting cardiac tissue from ischemic injury in an ex vivo rat heart model of cardiac ischemia [108], one of the most relevant discoveries in the field of IRI. An εPKC-specific activator peptide together with phosphoproteomics was used to identify proteins whose phosphorylation was altered by this selective activation. A different proteomic approach was applied in which proteins were separated by 2DE; after selection of phosphoproteins by specific staining, they were identified by MS/MS. εPKC protected cells during ischemia through the phosphorylation of mitochondrial proteins involved in glucose, lipid metabolism, and oxidative phosphorylation. Another protein kinase, Src kinase, has been implicated in ischemia-induced oxidative stress of H9C2 cardiomyocytes [109]. Antiphosphotyrosine affinity purification and LC-MS/MS were used to identify protein targets of stress-induced phosphorylation. Src kinase inhibitor appeared to prevent the modification of cell adhesion structures and the cytoskeleton, deadhesion, and apoptosis.

Glycosylation is another PTM of interest regarding ischemic models. iTRAQ and dimethyl labeling was used to examine the glycosylation profile of proteins from a myocardium subjected to 40 min of ischemia followed by 20 min of reperfusion [104, 110]. A total of 108 glycopeptides were differently expressed in the ischemic myocardium compared to normal tissue, most of which were components of the extracellular matrix and basement membrane (laminins and integrins) [110].

Investigation of protein–protein interactions during IRI can also involve proteomics [111]. Ahn et al. [112] looked at the role of peroxiredoxin 5 (Prdx 5) as a regulator of kidney response to hypoxia by direct interaction with others proteins in kidneys of hypoxic mice, using immunoprecipitation and LC-MS/MS. Dihydrolipoamide branched chain transacylase E2, a protein involved in mitochondrial metabolism, interacted more strongly with Prdx 5 under this stress.

Proteome dynamic analysis in terms of protein expression could also provide insight of cell signal transduction patterns, which is the most common approach used so far to identify key proteins involved in the pathogenesis and recovery from ischemic insults. The proteome and secretome of the cardiac cell line, HL-1, under hypoxia were investigated [113]. Cells nutrient starved for 8 h, corresponding to ischemia, and recovered with fresh medium for 16 h, corresponding to reperfusion. Using 2DE and MS, ischemia altered proteins related to apoptosis, oxidative stress, structure, and energy metabolism, which were partially reversed after reperfusion [113]. For secretome analysis, H9c2 cells were made hypoxic for 16 h followed by 24 h of reperfusion; secreted proteins were quantified by iTRAQ and MS [114]. A total of 2165 proteins were detected in the culture supernatants of these cells under three different conditions (normoxia, hypoxia, and reperfusion). When compared, 130 proteins were significantly altered in response to hypoxia, while 106 proteins were modulated after reperfusion. In hypoxia, proteins involved in extracellular matrix organization and cell adhesion were upregulated, whereas those related to cell metabolism and intracellular organelle organization were downregulated. However, after reperfusion, proteins related to cell metabolism were upregulated, whereas those involved with cell adhesion and apoptosis were downregulated [114].

MSC are capable of shifting cellular signaling from an injury to a recovery pattern in an in vitro model of ischemic renal injury [18, 33]. In one of these complementary studies [18], label-free high-definition 2D-NanoESI-MS^E was used to compare differentially expressed proteins in renal cells made ischemic insult and subsequently exposed to human MSCs. Addition of MSCs after ischemia leads to overexpression of proteins involved in antiapoptotic response, normal ROS handling, energy production, cytoskeleton organization, protein synthesis, and cell proliferation, compared with cell that were not exposed to MSCs.

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4.3 The proteome of stem cell mediated regeneration after ischemia

Adult stem cells have been widely investigated over the last two decades as an alternative therapy for several diseases. At first, the proposed mechanism of action for these cells was through regeneration promoted by transdifferentiation into unrelated cell types [115, 116]. However, this capacity was soon challenged [117, 118], and a paracrine effect subsequently became the accepted mechanism [119], which postulates that stem cells sense their environment and respond by secreting molecules having a therapeutic effect. Proteomic analysis of MSC secretion showed it contained a number of cytokines and growth factors that induce proliferation, protect cells from apoptosis, and stimulate angiogenesis, acting as an important mechanism for tissue regeneration [102, 104].

CM (Fig. 4) obtained from MSC also promotes beneficial effects on heart tissue after myocardial infarction. MSC CM reduced collagen density, apoptosis, and oxidative stress, and stimulated angiogenesis and vascularization, resulting in the preservation of cardiac function in pig model [120]. Different approaches to proteomic analysis of MSC CM identified several molecules that may be involved, such as insulin growth factor (IGF), epidermal growth factor, interleukin-6 (IL-6), IL-8 (IL-8), and transforming growth factor-β1 (TGF-β1) [27]. Assessment of the influence of CM treatment on TGF-β signaling in vivo leads to reduced expression of phosphorylated SMAD2, indicating that this suppression was via ALK-5, thereby suggesting TGF-β signaling may be involved in CM-mediated cardioprotection [114].

The effects of MSCs have also been widely studied in kidney IRI models [121]. Identification of the MSC secretome profile [27] pointed to important factors—including several proteins—potentially involved in renal regeneration. MSCs can protect the kidney not only by acting directly on renal cells, but through its anti-inflammatory and immunomodulatory properties following paracrine secretion. MSCs suppress B lymphocyte proliferation, antibody production, and inhibit activation of dendritic cells [122]. They also secrete TGF-β1 and indoleamine 2,3-dioxygenase that suppress T-cell proliferation. Special mention should be made of growth factors secreted by MSCs, namely IGF-1 and hepatocyte growth factor, which are epithelial mitogens and morphogens that promote renal blood flow and protect against ischemic injury [123, 124]. Interaction between MSCs and renal cells can also be mediated by paracrine secretion; in vitro experiments showed that lack of contact interactions between these cells resulted in a stimulus to proliferation and reduction apoptosis following ATP depletion [18, 33]. Vascular endothelial growth factor (VEGF), also present in MSC secretion, acts directly on renal epithelial cells by binding to the VEGFR-2 receptor, thereby stimulating proliferation and promoting survival [125]. Therefore, in this context, MSCs support tissue regeneration by interacting with renal surviving cells that proliferate, which helps replace cells lost during injury.

Another important aspect of MSC cell-based therapy is the use of preconditioning strategies, the rationale being strongly supported by proteomic discoveries. Hypoxic preconditioning modifies the MSC secretome, causing the release of angiogenic factors, and improving cell survival after transplantation [126]. Modification of culture conditions from 2D to 3D structures also increases the release of angiogenic cytokines [127].

Finally, we must mention that quantitative proteomics can reveal differential regulation of protein expression in recipient myocardium after transplantation of three lineages of cells derived from human-induced pluripotent stem cells (iPS) [128], which opens up an avenue that can be exploited in other tissues and organs. After the pioneering work of Yamanaka et al. in cell reprogramming [129, 130], iPS became a potential source of suitable lineages of specialized cells for regenerative purposes in injured tissues and organs. In an interesting study by Chang et al. [128], trilineage transplantation in a recipient myocardium was found to be accompanied by a proteome change toward ameliorating overall cardiac function and downregulating proteins associated with cardiac failure (Fig. 5).

4.4 The proteome of cell fate after injury: Regeneration after ischemia or malignant evolution?

Tissue regeneration processes are associated with the replacement of lost cells during injury for fully functional newly differentiated cells. An important step is epithelial to mesenchymal transition (EMT), where surviving epithelial cells lose their epithelial characteristics (loss of polarity, adherents junctions, tight junctions, cytokeratin intermediate filaments) and acquire the properties of MSCs. This phenotypical change allows surviving tissue cells to dedifferentiate, proliferate, and redifferentiate into a mature functional phenotype [131]. However, these changes can be altered by the presence of bioactive molecules in the injured microenvironment, resulting in differentiation toward fibroblasts. EMT can promote progression toward fibrosis by generating MSCs that may expand the population of interstitial fibroblast and reduce the epithelial cell population. EMT is triggered by a combination of cytokines secreted by the immune system cells associated with molecules coming from proteolytic digestion of basement membranes [132]. Phosphoproteomic analysis in renal tubular epithelial cells using the iTRAQ technique showed that TGF-β upregulates 19 phosphoproteins, most being associated with cytoskeletal reorganization. TGF-β also led to downregulation of phosphoproteins related to adhesion molecules. Both modulations are consistent with the action of EMT on proximal epithelial cells [133]. In addition, Fsp1, a fibroblast-specific protein associated with MSC morphology and motility, supports the idea that EMT is the main source of fibroblasts. In this regard, Okada et al. [134] showed that cultured renal epithelial cells in the presence

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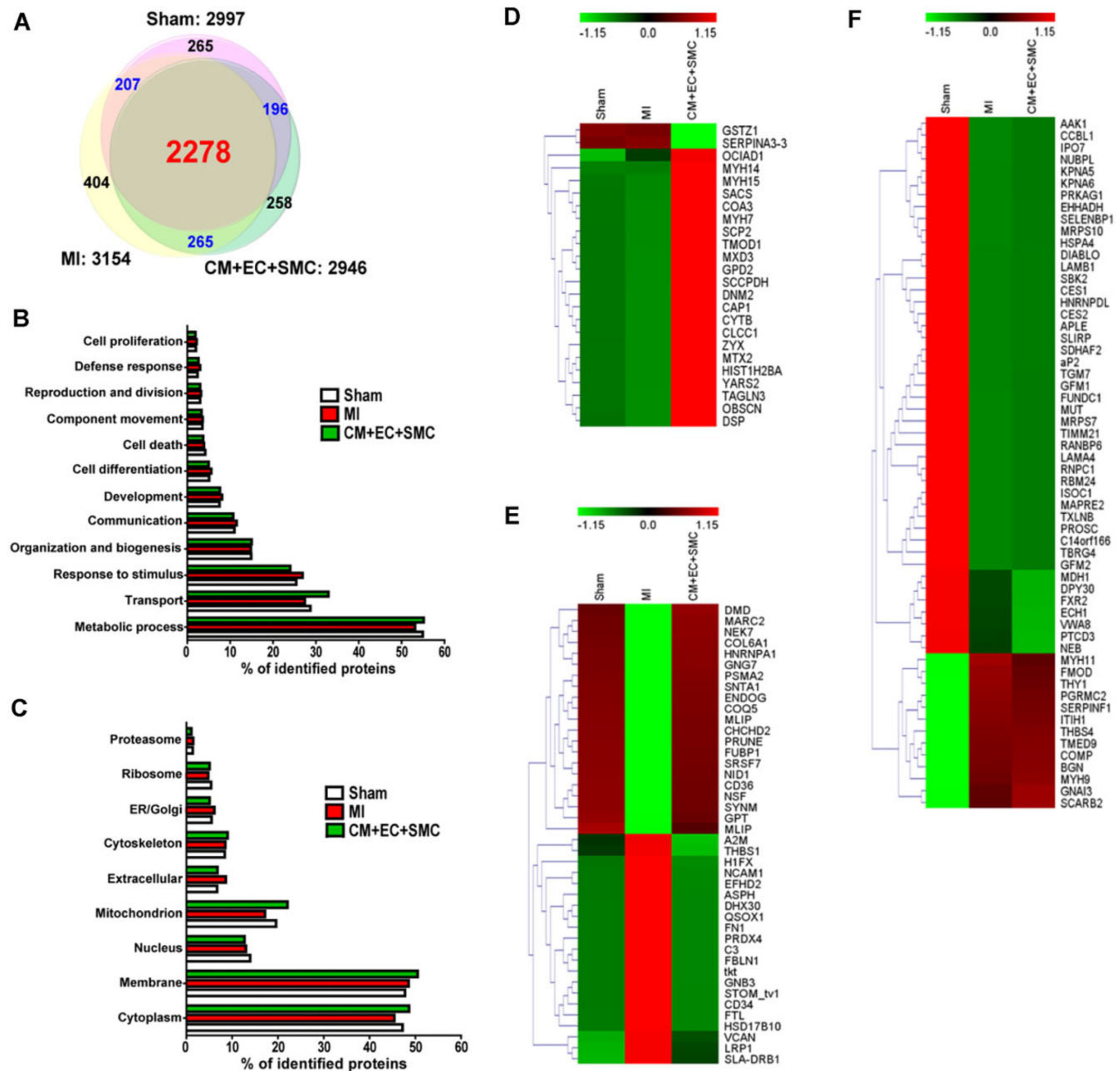

Figure 5. Protein profiling of swine myocardium from Sham, myocardium infarcted (MI), or MI transplanted with iPS-derived cardiomyocytes, endothelial cells, and smooth muscle cells (CM + EC + SMC). (A) Venn diagram showing the identified proteins in the three groups. GO of (B) biological processes and (C) subcellular localization of the identified proteins. (D–F) Heat-maps showing categories of proteins whose expression are altered between the three different groups. (D) No change in MI, but altered after trilineage cell transplantation. (E) Altered in MI, reversed by the transplant. (F) Altered in MI, but without reversion by the transplant. Reproduced from [128] with permission. See Supporting Information from this reference for further details.

of cytokines classically associated to fibrosis (TGF-β and epidermal growth factor) begin to express Fsp1 and participate in the early stages of the pathways toward EMT. These also noted that these fibroblasts can secrete collagen types I and III, sustaining the transformation of more epithelium, leading to tubular atrophy and progressive renal failure.

Another important misdirection of regenerative process also associated with fibrosis is tumor development. The direct relation between regeneration, chronic fibrosis, and cancer progression is well reviewed in [132]. Others have found evidence associating regeneration with cancer-related cellular abnormalities (cancers have been sometimes been referred to as “wounds that never heal” [135]), due to the relationships between carcinogenesis, inflammation, and local tissue repair. Once again, dysregulation of EMT during regeneration seems to be the link to the development of different types of cancers

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(breast, ovarian, colon, and esophageal) and conservation of several aspects of EMT associated with regeneration, for example, cytoskeletal protein changes, downregulation of cytokeratins, and upregulation of vimentin. Inducers of EMT have been associated with tumor development, including TGF-β, Wnt, Snail/Slug, Twist, and Six1; indeed clinical evidence suggests that regulators of EMT in cancer cells correlate with poor patient outcomes and tumor aggressiveness. Proteomic analysis of ovarian cancer indicated an association between EMT-related protein, metastatic status, and the prognosis in cancer patients [136]. There was reduced E-cadherin expression and the presence of nuclear Snail expression in ovarian cancer, suggesting that EMT may promote the dissemination of cells from the tumor mass, and that cells undergoing EMT become invasive and develop resistance to anticancer agents.

An abundance of inflammatory cells is also a common hallmark of cancer and tissue regeneration; inflammation has been described as acting as an initiator on cells acquiring the first mutational hit, sending them toward malignant transformation. The proteome of their secreted components was recently described [137]. An inflammatory microenvironment can increase mutation rate and enhance the proliferation of mutated cells. Activated inflammatory cells serve as sources of ROS and reactive nitrogen intermediates that react forming peroxynitrite, a compound that damages DNA causing genomic instability. p53 mutations, presumably caused by oxidative damage, are found in both cancer cells and in inflamed (but nondysplastic) epithelium, suggesting that chronic inflammation causes genomic changes. Inflammation-induced mutagenesis may also result in inactivation or repression of mismatch repair response genes, and ROS can also cause direct oxidative inactivation of mismatch repair enzymes [138]. In particular, secretome analysis shows that macrophages in noncancerous tissues have predominantly a M1 phenotype, producing high levels of ROS and inflammatory cytokines, resulting in potent antimicrobial and immunostimulatory functions. In tumors, secreted cytokines frequently induce differentiation of macrophages to the M2 phenotype, stimulating angiogenesis and extracellular matrix breakdown through the production of angiogenic growth factors and matrix metalloproteinases, thereby promoting tumorigenesis, cancer-cell motility, and invasion [139]. Therefore, interactions between cells from injured tissue and immune system cells can drive the regenerative process into a tumorigenic one.

5 Concluding remarks and future perspectives

This review shows that proteomics has revealed complex protein networks that are associated with the establishment of a number of prevalent pathologies of importance and, in some cases, insight into their recovery pathways. Sophisticated techniques combining sample preparation, sensitive mass spectra acquisition, and processing analyses using suitable bioinformatics tools, despite some limitations, are essential to our discovery and understanding in greater depth new pathways and targets, and the myriad of cell–cell and protein–protein interactions that can lead to novel strategies for efficient treatments in the future. In a near horizon, these approaches mat enable us to investigate the proteome of a single cell [140, 141], which may answer a naïve question: how does the individual cell function in an ensemble of millions in a tissue? In other words, can we see how the specific proteome of a single-cell resolution integrates and interacts with all its neighboring cells in microenvironments in conditions both of health and disease?

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