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REVIEW

Proteomics applications in next generation induced pluripotent stem cell models

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ABSTRACT

Introduction: Induced pluripotent stem (iPS) cell technology has transformed biomedical research. New opportunities now exist to create new organoids, microtissues, and body-on-a-chip systems for basic biology investigations and clinical translations.

Areas Covered: We discuss the utility of proteomics for attaining an unbiased view into protein expression changes during iPS cell differentiation, cell maturation, and tissue generation. The ability to discover cell-type specific protein markers during the differentiation and maturation of iPS-derived cells has led to new strategies to improve cell production yield and fidelity. In parallel, proteomic characterization of iPS-derived organoids is helping to realize the goal of bridging in vitro and in vivo systems.

Expert Opinions: We discuss some current challenges of proteomics in iPS cell research and future directions, including the integration of proteomic and transcriptomic data for systems-level analysis.

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1. Introduction

The discovery of iPS cells in mice and in humans has had a significant impact on biomedical research [1,2]. The ability to reprogram mature somatic cells from adult donors back to pluripotency, then in turn direct their differentiation into different adult-like terminally differentiated cells in vitro, has enabled a virtually unlimited source of otherwise inaccessible cells such as human cardiomyocytes and neurons in the laboratory. Compared to embryonic stem (ES) cells, iPS cells circumvent critical ethical issues associated with cell procurement, and moreover, capture the genetic background of the donors to allow personalized study of disease mechanisms and drug responses in a dish. Many protocols have been established to promote the reprogramming, fidelity, and maintenance of pluripotency in iPS cell lines [3–5], whereas advances continue to be made that allow the differentiation from iPS cells of an ever-increasing variety of terminally differentiated cell types that compose the adult body. As a result, iPS cells are now widely used in biomedical research and to understand basic cell and developmental biology.

Enabling next-generation applications in disease modeling, drug testing, and regenerative medicine will require another leap, one that goes from producing individual cell types in a dish toward producing complex microphysiological systems. Rapid progress is under way to construct multi-lineage iPS-derived cells forming into organoids, engineered tissues, and body-on-a-chip systems [6,7]. The success of these endeavors will require a fuller knowledge of how to orchestrate microtissues from multiple cell types that can function in synchrony and capture the complexity of regulation in higher organisms. Despite progress, several limitations continue to pose significant challenges to the use of iPS cells. iPS-derived cells often lack the developmental and functional maturity of adult cells and resemble fetal cells rather than their primary cell counterparts, which impedes the clinical translation and cell therapy goals of mimicking adult cell types. The purity and yield of many differentiation protocols remain to be fully characterized, and the applicability of protocols to diverse iPS cell lines from different genetic background remains to be established. Effective tissue engineering in turn requires further understanding the modality of communication and crosstalk between cells.

Methods that can probe the total protein composition of cells and tissues would be fundamental to overcoming these challenges. Proteins are the primary effectors of most biological processes and confer structure and function to cells and tissues. Although protein abundance is moderately correlated to the transcriptome of a sample [8–10], many post-transcriptional and post-translational regulations exist that uncouple mRNA and protein level, including the differential translation rates of transcripts and degradation rates of proteins. This non-correlation has important implications for accurately assessing the across-condition functional changes from transcript data (e.g. upon perturbation or across individuals in a population). Proteomics techniques, spanning both mass spectrometry (MS) and non-MS approaches, provide a direct readout of protein abundance (Box 1). Moreover, coupled to protein enrichment and isolation strategies, proteomics can reveal the spatial distribution (e.g. cell surface localized) of proteins and paint a more comprehensive picture of cell-type transitions that may be missed in gene and/or transcript analysis. Proteomics studies that directly measure the abundance of proteins therefore provide unique information into the identity and physiology of iPS cells and their derivatives.

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Article highlights

- Opportunities now exist to create organoids and microphysiological systems from human induced pluripotent stem (iPS) cells as the next generation of biomedical reesarch models.
- Application of proteomics techniques can help advance iPS models by providing readouts of cell differentiation yield, purity, and maturity.
- Improvements to both mass spectrometry and non-mass spectrometry methods have drastically improved the proteomic characterization of iPS cell models.
- Continued development of secretome and surfaceome profiling techniques will avail characterization of cell identity and crosstalk at the protein level.

Box 1. Non-MS based proteomics methods

MS remains the most versatile method for the unbiased identification and quantification of proteins. Non-MS approaches are also in use, including affinity-based platforms like reverse phase protein array (RPPA) [101], SomaScan [102], and Olink [103], which can be used separately or in conjunction [104] to quantify hundreds to thousands of pre-determined protein targets in upward of thousands of samples. These techniques achieve particularly impressive throughput and sensitivity in clinical samples of liquid biopsies. However, a drawback is that they typically target only commonly studied proteins in model organisms and questions linger about the target specificity of some reagents [105].

This review aims to highlight the potential and utility of proteomics toward the continued advances of iPS technologies. Section 2 gives a much-abbreviated overview of some common proteomics approaches and considerations. Section 3 reviews some 'classic' applications of understanding iPS cell reprogramming and pluripotency, whereas Section 4 and 5 focus on the use of proteomics toward the ongoing goals of improving the production of mature cells and organoids to enable advanced applications (Figure 1). To limit scope, we largely omit studies that compare protein abundance profiles to study diseases, which have been reviewed comprehensively elsewhere [11,12].

2. Brief overview of proteomics methods and analysis

Comprehensive tutorials of MS-based proteomics methods have been written elsewhere [13,14]. We give here only a brief commentary on some recent improvements that may be broadly beneficial for cell analysis. Firstly, sample preparation for proteomics has continued to advance, and a renewed attention is driven by the goal of analyzing minute samples such as from single organoids or single cells. Clearly, sample preparation is crucial to acquiring accurate data and supporting meaningful interpretation. The diversity of sample types and their procurement (e.g. single cells, tissues, homogenate, conditioned media) makes it necessary to adopt protocols specific to a sample type that produces downstream-compatible (e.g. detergent and salt free) analytes while having sufficient power to extract and solubilize proteins. Workhorse methods like filter aided sample preparation (FASP) [15] have been joined by approaches such as single-pot, solid-phase-

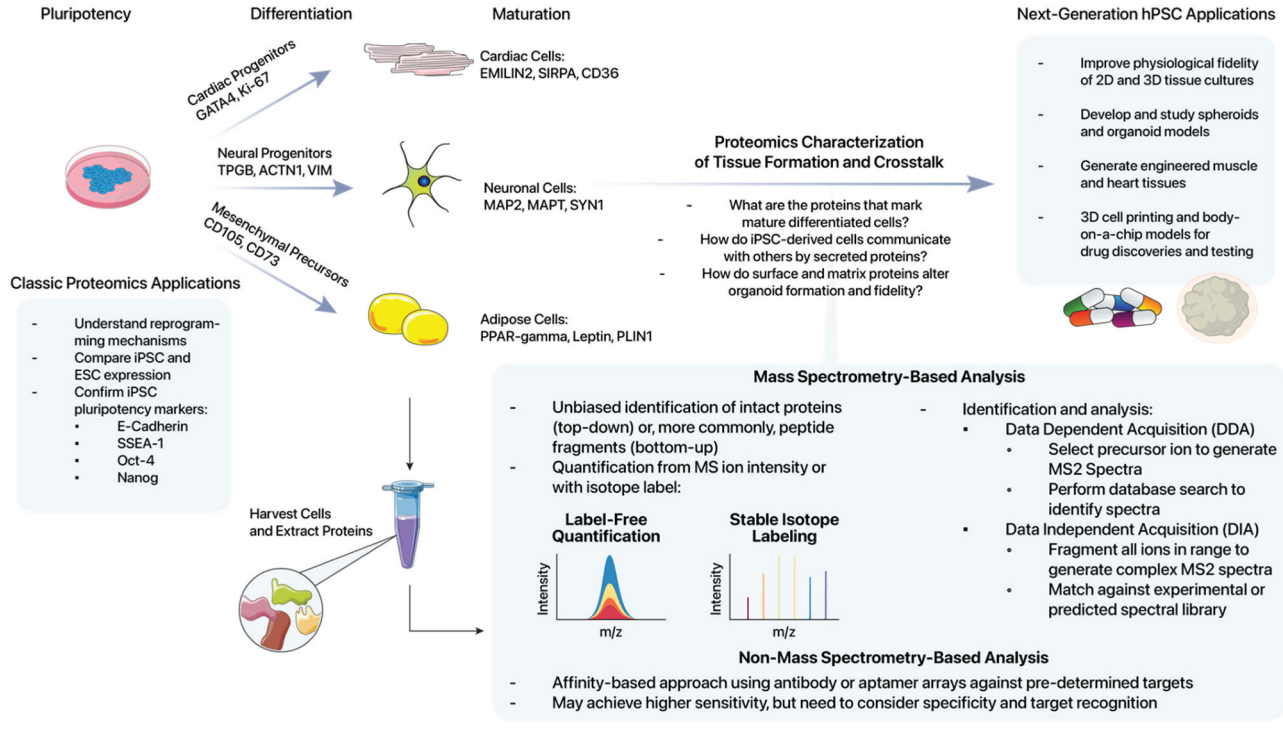

Figure 1. Proteomics applications to enable next-generation iPS cell applications.

enhanced sample preparation (SP3) [16] which uses hydrophilic interaction to purify and recover peptide samples. To enable single-cell analysis, one-step, one-pot protocols are being developed that aim to reduce loss of minute quantities of proteins [17,18]. The automation or semi-automation of protein chemistry steps using liquid handling robots is becoming standard, with the aim of reducing variability and increasing throughput. Robot-assisted workflows have been applied to protein digestion of bulk or single-cell samples [19–21], new protein synthesis capture [22,23], surface protein capture [24], and other protocols.

Second, there has been increasing attention placed not just on the identification of a large number of proteins but also on quantifying protein abundance with high throughput and accuracy, using both label-free and isotope-labeled approaches. Labeled analysis such as using isobaric tags (e.g. tandem mass tags) has become commonplace for quantifying changes in protein abundance, and allows multiplexing of multiple samples (up to 18-plex from tandem mass tags, and up to 54 or more from 'hyperplexing' strategies) within a single MS experiment. This has the effect of improving the quantitative accuracy and throughput of MS analysis for profiling of complex cellular proteomes while reducing some of the data missingness that can come from the stochastic nature of data-dependent acquisition (DDA) analysis [25]. This benefits, however, only extends to a certain number of samples. When samples number in the hundred and require multiple blocks of isobaric tags to be bridged from one another, the problem of missing data (label quantity acquired in one block but not another) returns. Hence, a parallel trend has been the increasing adoption of data independent acquisition (DIA) mode of bottom-up MS [26]. DIA differs from DDA in how MS2 spectra containing peptide sequence information are generated. In DDA, the mass spectrometer selects a specific precursor ion in the MS1 scan to generate MS2 spectra and gather sequence information. This selection process is susceptible to run-to-run differences, especially when high abundance peptides mask low abundance peptides from being selected. By contrast, DIA MS uses predefined mass windows to isolate multiple precursors and fragment all precursors within the mass window at the same time. This expands the number of product ions that can be monitored and reduces the missing data issues of DDA, allowing higher sensitivity and reproducibility. After acquisition, peptides can then be identified from the acquired spectra using search engines, such as DIA-NN [27] and FragPipe [28] that can consider multiple sequences in one spectrum with the aid of spectral libraries and quantified from precursor ions in MS1. Statistical analysis of peptide quantity can then be performed to determine differential protein abundances across sample using bioinformatics tools and packages, including MSStats [29], which is designed to account for MS runs that involve multiple biological or technical replicates as well as unbalanced designs that might have an unequal number of replicates in the sample mixture.

3. Applications of proteomics in iPS cell characterization

3.1. Comparison of iPS and ES cells

Early applications compared iPS and ES cells to assess how faithfully the former recapitulate the properties of natural pluripotent cells. Although iPS cells are postulated to carry largely the same differentiation potential as ES cells, at least in mice, their actual differentiation efficiency is often reported to be lower than ES cells, suggesting some minor differences between the two cell types. Proteomics experiments that compare iPS and ES cells have found about 50 to 300 proteins significantly different [30,31]. MS comparison that focused on membrane proteomics also corroborated minor differences, with proteins including amino acid transporters and vesicular trafficking proteins to be differentially expressed [32], whereas proteins related to focal adhesion processes may be incompletely repressed in iPS cells from the original cell type [33]. These differences may be attributable to the persistence of epigenetic memories, as iPS cells derived from different types of adult cells retain some signatures of their originating cell types [34]. However, other factors including genetic differences and laboratory culture conditions cannot be ruled out, and overall these differences may be relatively minor with ~98% proteins not significantly different between iPS and ES cells [30].

3.2. Identification of proteins involved in self-renewal and pluripotency

After it was known that OSKM can be used to reprogram somatic cells into iPS cells, many mechanistic details of the reprogramming steps remained incompletely understood [2]. Understanding the cellular pathways involved and their synergistic interaction became an important goal for optimizing reprogramming [35,36]. The induction of pluripotency expectedly led to huge changes in proteome in iPS vs. precursor cells [30]. Like many other techniques, proteomics has been employed to analyze the regulatory factors that control pluripotency and differentiation [37]. In one study [38], a simultaneous measurement of protein expression and thermal stability changes is used to identify key differences between iPS cells and their progeny embryoid bodies. From this combined analysis using tandem mass tag multiplexing and high-pH reversed phase peptide fractionation, it was found that ribosomal protein expression may play a triggering role in iPS differentiation, where an increase in ribosomal protein thermal stability is reflective of a change in ribosomal structure. Notably, iPS cells show lower translation than other cell types, suggesting an emphasis of energy usage on maintenance of pluripotency over protein synthesis. Moreover, a ribosomal associated protein SBDS is found to be deficient in iPS cells to hamper protein synthesis and may help maintain pluripotency, suggesting a potential avenue for controlling pluripotency regulation and exit. In another study, a label-free proteomics screen using DDA MS analysis found

that repressive H3K9 marks impair reprogramming, and that active chromatin markers occur late in the pluripotency trajectory from pre-iPS to iPS cells [39]. Quantification of post-translational modifications of histones revealed a varied active chromatin character between iPS cells and differentiated cell types such as fibroblasts as well as pre-iPS cells, with decreased heterochromatin protein-1γ (Cbx3) and H3K9 methylation in pluripotent cells.

Another study by Hansson et al. [37] leveraged in-depth quantitative proteomics to track the changes in the cellular proteome during somatic cell reprogramming. To do so, the authors extracted proteins at different time points during the process of reprogramming secondary mouse embryonic fibroblasts, followed by digesting and labeling the peptides with stable isotopes. Because of the complexity of the samples, to achieve greater profiling depth, the authors performed deep fractionation and identified 7,918 unique protein groups, among which 5,601 proteins were quantified across all time-points. Many transcriptional regulators and chromatin modifiers were identified that have been implicated in maintaining pluripotency. Additionally, the observation of global proteome changes across several time points enabled the identification of a proteome shift or resetting between the initial and later phases of reprogramming. Identification of such proteome dynamics enabled a better understanding of directional protein expression changes to achieve successful reprogramming.

3.3. Characterization of iPS cell quality and variability

Proteomics has been applied toward the quality assurance of iPS lines. One study discovered that X chromosome inactivation erosion leads to changes in the iPS cell proteome during culture and contributes to RNA-protein non-correlation [40]. Another study used proteomics to develop of a marker panel comprising 22 proteins that can be used to ascertain cell quality and pluripotency [41]. Comparing proteins in initial vs. intentionally prolonged culturing led to the detection of proteins that are indicated during initial growth, differentiation, as well as at later formation of embryoid bodies [41]. The establishment of these markers provides a method for monitoring the physiological state of iPS cells, which have implications in quality assurance during the development of cell-based therapies.

With scaled-up cell production, very large panels of multiple iPS lines can be compared with each other to understand the variability of intra-clonal, inter-clonal, and inter-donor effects on iPS cell proteomes. Such heterogeneity can lead to differences in iPS-derived cells that would be important to understand [42], whereas inter-donor variability can also be used as a natural model to understand genetic architectures. Using HILIC chromatography and spectral library generation through DDA/DIA MS workflow proteomics, a study by Brenes et al. in the HipSci consortium [43] evaluated the differential protein expression of 217 lines of iPS cells from 163 donors across various molecular pathways and determined that iPS lines expressing lower BMP4 signaling had higher pluripotent capacity and lesser vulnerability to apoptotic signaling. A report from the same consortium [44] measured protein pluripotency markers in 307 lines and observed the sources of genotypic and phenotypic variation in iPS cells and developed a map of regulatory variants and pluripotency markers that can be used to optimally generate pluripotent cell lines. Finally, combining the proteome profiles of 202 iPS cell lines and integrated with genome sequence data, Mirauta et al. used the iPS cell panel to discover protein quantitative trait loci (pQTL) that cannot be detected at the mRNA level [45].

The National Institute of Health (NIH) also developed the NeuroLINCS initiative as a part of its Library of Integrated Network-based Cellular Signatures (LINCS) program to study human iPS cells and differentiated neuronal cultures from healthy donors, as well as spinal muscular atrophy and amyotrophic lateral sclerosis patients [46]. DIA MS was used to quantify around 3,000 proteins from multiple iPS lines to identify pluripotency markers. The proteomics data generated was further validated through the analysis of cell line and culture protocol assessments. Coefficient of variation analyses was used to identify the outliers for both technical and biological replicates, which were excluded from biological analyses to reduce experimental variability. This in-depth proteomic profiling enables a method to determine pluripotency and evaluate the stability of differentiation protocols across lines.

4. Applications of proteomics toward iPS-derived cell differentiation and maturation

A critical utility for iPS cell models is the ability to direct the differentiation of pluripotent cells toward the cell types of interest. The studies of the surfaceome (i.e. plasma membrane) [47–49] have found important applications. Cell surface proteins often play a significant role as markers of iPS cell-generated cellular sub types that allow the selective recognition and isolation of target cells, and the characterization of cell fate transition events. The divergence of transcript and protein information is especially pertinent here because expressed transcripts, even if translated into proteins, may not be translocated or discoverable on the cell surface [50]. Proteomics technologies that can capture cell surface proteins provide a direct method to discover cell surface proteins. In the past decade, emerging proteomics methods are allowing this challenging 'sub-proteome' to be profiled with greater depth and finesse (see Box 2)

4.1. Use of proteomics to improve cell differentiation yield and fidelity

The development and optimization of directed differentiation protocols benefit from cell-specific markers that can be reliably detected in the target derived or transition cell types. Despite advances, directed differentiation protocols typically produce a mixture of cell types with significant cell to cell heterogeneity [51,52]. For instance, even though protocols to derive cardiomyocytes can yield 95% or greater proportion of cardiac troponin T expressing (cTnT+) cardiomyocytes, this canonical marker masks the underlying heterogeneity where protocols may yield a mixture of atrial, ventricular, pacemaker, and conductive cardiomyocytes, as well as fibroblast-like cells. This illustrates the

Box 2. Proteomics technologies to map the cell surface markers of iPS models

Box 2.1 Analysis by cell surface capture MS

Traditional approaches to screen for cell surface proteins use immunoreagent panels to recognize the clusters of differentiation (CD) molecules. However, other cell surface proteins exist that are not CD proteins. Most cell surface proteins are heavily glycosylated. Wollscheid and colleagues [106] demonstrated the cell surface capture (CSC) method, where hydrazide chemistry is used to specifically label cell surface glycans with biotin. The glycosylated proteins can then be captured by streptavidin beads, deglycosylated, and analyzed by mass spectrometry. This approach provides an unbiased approach to identify cell surface markers. A large-scale application of CSC proteomics to 41 human cell types including iPS cell lines led to an atlas of 1,492 cell surface proteins providing a resource for understanding cell surface specificity [107]. CSC analysis of iPS cell surfaces found over 500 surface proteins, with up to 80% of non-CD molecules, highlighting the importance of unbiased discovery [49,50].

Box 2.2 Analysis by mass cytometry

Mass cytometry, or CyTOF, leverages the principles of both MS and flow cytometry to characterize both the cell surface and intracellular proteins of single cells through metal-conjugated antibodies [108]. Cells tagged with these metal isotopes are then vaporized in the mass spectrometer to give a readout of up to several dozens of parameters (i.e. proteins) on each cell simultaneously, overcoming the laser channel limit of fluorophore-based flow cytometry. CyTOF has been utilized to monitor proteome changes during reprogramming [109] to identify various pluripotency and cell cycle markers involved. Markers identified through CyTOF are further analyzed using computational tools such as PhenoGraph, diffusion mapping, and statistical correlation and dimensional reduction by SPADE. Another study by Zunder et al. [110] evaluated several markers related to pluripotency, differentiation, cell-cycle status, and cellular signaling through CyTOF and a time-resolved progression analysis. Cell samples collected across a time course were prepared into a single-cell suspension that was multiplexed through mass-tag cell barcoding prior to mass cytometry and further downstream analysis was carried out by SPADE and FLOW-MAP algorithms. Common reprogramming markers, such as Oct4, Klf4, CD54, were identified by the algorithms, reflecting the utility of this method in facilitating a deeper understanding of the molecular processes.

ongoing need to find additional unbiased markers for cell types and subtypes, which can help improve differentiation quality, decrease variability of batches, and compare differentiation protocols. Proteomics allows an unbiased discovery of cell surface markers that reside selectively on target cell population, upon which antibody-based methods (e.g. FACS) can be developed to isolate these cells. Conversely, the identification of markers in unwanted cell types (e.g. immature cells, fibroblasts) can be used for negative selection. As such, multiple studies have aimed at understanding proteomic changes during differentiation of iPS and ES cells into cardiomyocytes [53], fibroblasts, neurons [54,55], and insulin-producing cells [56].

4.1.1. Neuronal cells

In iPS-neurons, one study used automated sample preparation and deep DIA MS to profile of over 8000 proteins in a single experiment [55]. The results showed a longitudinal rise in mature neuron (e.g. MAPT, MAP2) and synaptic (e.g. SNAP25, SYN1) markers from 0 to 28 days of neuronal differentiation. In another study, Urasawa et al. used DIA MS to examine the temporal proteomic profiles of an iPS-derived neural stem cell protocol [57], identifying a role of trophoblast glycoprotein (TPBG) in promoting neuronal differentiation over proliferative cell fate. Another study used label-free DDA MS to characterize novel biomarkers in the differentiation of iPS-derived neural progenitor cells (NPC), comparing specific cytoplasmic, membrane-specific, and synaptic proteins between in vitro and in vivo models [58]. One study on neuronal differentiation explored the cell surface N-glycoproteome of the neural precursor cells through cell surface capture technology. Confirming and mapping the glycosylation sites of surface proteins helped detect and confirm cell populations during neuronal differentiation [59].

4.1.2. Cardiac cells

In a landmark study, Van Hoof et al. used quantitative MS to identify the elastin microfibril interfacer 2 (EMILIN2) as a specific marker in human ES-derived cardiomyocytes [60]. Another pioneer work used an antibody panel against 370 clusters of differentiation (CD) protein targeting antibodies to identify SIRPA (CD172a) as an iPS-derived cardiomyocyte cell surface marker compared to undifferentiated iPS cells [61]. From this information, the authors developed a cell sorting protocol for SIRPA expressing cells that yielded 98%+ troponin T+ cells from heterogeneous populations. This method has since been widely adopted for the production of iPS-derived cardiomyocytes where purity is of particular concern, such as in scaling up cell production for therapeutics development [62,63]. Markers of cell subtypes (e.g. ventricular specific myocytes [64,65]) have also been explored using unbiased cell surface protein screens.

The applicability of some of the cell surface markers has been debated because they might not be universal across protocols and species. It is important to note that a useful marker will need to account not only for selectivity between undifferentiated iPS cells and the targeted cells but should also be verified in adult primary cells. Moreover, the marked population should be depleted in intermediary cell types as well as possible impure cell populations produced in the directed iPS cell differentiation protocol. In a recent important study, Berg Luecke et al. [53] produced a comprehensive cell surface proteome map of markers across multiple iPS cell-generated cell types with the aid of the computational analysis platform CellSurfer. For instance, the mapping of cardiac cell surface proteins through quantitative cell surface proteome profiling of iPS-derived cardiomyocytes vs. other cell types. Notably, only moderate overlap was found in iPS-derived cardiomyocytes (51%) among the cell surface proteins found in primary cardiomyocytes, highlighting that current differentiation protocols still have significant room for improvement. The authors further compared cardiomyocytes with other cardiac cells, and found LSMEM2 to be a new cell surface cardiomyocyte marker that is not found in primary cardiac fibroblasts, endothelial cells, and coronary artery smooth muscle cells. This provides a new avenue to assess the specificity and fidelity of iPS-derived cardiomyocyte protocols.

4.2. Improvement of differentiated cell maturity

Besides the specificity of cell differentiation, the maturity of the yielded derivative cells is of great importance. Many iPS-

differentiated cell types lack the full structural and functional maturity of primary adult cells and instead resemble their fetal counterparts. This is in part because in vitro differentiation protocols lack the important signaling cues and cellular niches that occur in vivo and are furthermore attempting to recapitulate lengthy in vivo development processes in gestation within a relatively short time in the laboratory. This lack of maturity has important ramification for disease modeling and drug testing because diseases may manifest differently in fetal-like and adult cell types. It has been shown that iPS-derived cells have the potential to gain full maturity when implanted into animals [66], suggesting they contain the potential to become fully mature if given the right cues. Hence, there has been interest in deriving methods that can boost the maturation of iPS-derived cell types in vitro, including culture media or matrix optimization, prolonged culturing, and mechanical or electrical stimulation. These efforts require accurate readouts of what constitutes a mature cell, which would involve protein expression as well as cell surface proteins. Proteomics has been used to compare iPS-derived cells with perceived mature cells, which may come from iPS-derived cells under prolonged culture, iPS-derived cells that have been implanted into an in vivo niche, or bona fide adult primary cell types. Such approaches have been applied to find markers of maturation in iPS-derived hepatocytes [67–69], cardiomyocytes [53,70–72], and pancreatic beta cells [56,73].

4.2.1. Erythroid cells

The proteomes of erythroid cells derived from iPS cells have been compared with their adult erythroid cell counterparts, with ~11% of proteins found to differed between the two cell types by a level of 2-fold change or higher. However, greater than 30 well-defined proteins were also found to be consistent between the two cell types [74].

4.2.2. Hepatic cells

Human iPS-derived hepatocytes have strong utility for pharmaceutical development and drug toxicity research, as liver cells are the primary cell types that metabolize intake compounds. Immature iPS-hepatocytes have limited applications, partly because they have low expression of important liver enzymes such as cytochrome P450s involved in drug metabolism. Cell surface proteomics has identified SLC10A1, CLRN3, and AADAC as highly co-expressed in late hepatocyte differentiation. These findings allowed a proof-of-concept usage of FACS to sort for SLAC10A1 positive cells in hepatocyte differentiation protocols, which show greater similarity to primary cells, including higher expression of liver signature genes such as APOA2 [68]. Another study combined proteome, phosphoproteome, and acetylome profiling to produce a temporal profile of immature and mature hepatocyte-like cells from iPS cells and identified metabolic switch including an increase in ketogenesis, TCA cycle, and glyconeogenesis genes during hepatocyte maturation, whereas most transcription factors decreased on maturation [67]. In a separate study, Hurrell et al. leveraged stable isotope labeled MS-based proteomics to study the proteome of mature hepatocyte-like cells derived from iPS cells during their differentiation over 40 days. A maximum abundance of previously characterized hepatocyte-specific markers was reported between differentiation days 30 and 32, indicating the formation of more mature cell types toward the end of the differentiation process [69].

4.2.3. Cardiac cells

Immature iPS-derived cardiomyocytes do not achieve the elongated morphology, resting membrane potential, and contractile force of primary adult cardiomyocytes [66]. Protein expression level changes longitudinally during the differentiation of iPS- cardiomyocyte, which has been monitored using isotope labeled shotgun proteomics [70]. Analysis of the proteomic data revealed the differential expression of proteins involved in synthesis, ubiquitination, and other metabolic pathways during temporal changes from 32 to 60 days in the maturation process, which may be targets for strategies to make more mature cardiomyocytes.

A different study used MS to compare the proteomic profiles of early, mid, and late (15–90 days post-differentiation) culture of iPS-cardiomyocytes, which continue to maturate in culture following the initial cell-type specification. Poon et al. [72] focused on cell surface markers that are preferentially expressed in more mature cardiomyocytes and identified CD36. This was used to develop a sorting protocol that specifically enriches $CD36^{hi}$ cells from heterogeneous mixture of cells at 45 days post differentiation. Notably, the more mature markers also contain higher mitochondrial DNA content, suggesting they are metabolically more resembling adult cardiomyocytes. Moreover, $CD36^{hi}$ iPS-cardiomyocytes are more sensitive to doxorubicin, a common anti-cancer anthracycline that causes adverse cardiotoxicity in some patients and that is thought to act by targeting mitochondria. Hence, the development of proteomics screen allows the selection of cells that could potentially be more useful for drug screening.

More recently, Berg Luecke et al. [53] further applied their surfaceome approach to find iPS-cardiomyocyte maturation stage markers, including CD36, LPL ASPN, CPBL, and VSIR; by comparing iPS-cardiomyocytes that are in late culture (50+ days post differentiation) with early (10–16 days) and middle (20–31 days) stage cells. In parallel, top-down proteomics capable of resolving full-length proteoforms has also been leveraged to create accurate assays of maturation, including the decreased phosphorylation of alpha-tropomyosin and MLC2a, which can be seen in longer cell culture and also in primary adult heart tissues [71].

4.2.4. Pancreatic cells

Immature iPS-derived pancreatic beta-like cells cannot recapitulate the glucose-stimulated insulin secretion profile of primary human islet cells, which limits their utility for cell therapy. Comparison of the proteomes of iPS-derived beta-like cells and bona-fide human beta cells identified a number of mature beta cell markers that are less expressed in iPS-derived cells, including urocortin 3 [56]. Similarly, a study by Haller et al. [73] leveraged label-free MS to identify markers at each stage of pancreatic endocrine lineage commitment and discovered new features of beta cell maturation following in vivo transplantation that may guide future work to improve glucose-mediated insulin secretion in these cells.

4.2.5. Adipose cells

Like other cell type, the validation of iPS-derived adipocytes against their primary counterpart is an important goal that is not fully met. In one study, Soontararak et al. compared iPS-derived mesenchymal stem cells (iMSCs) to adipocyte derived mesenchymal stem cells (adMSCs) to determine the effect of both in treating inflammatory bowel mouse models [75]. Using flow cytometry, the authors found shared markers between these cells, including Sca-1, CD29 and CD44 which are all common MSC markers and concluded that both derivations of MSCs were sufficient to treat DSS-induced IBD in their mouse model, especially when compared to the requirements by the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy [76]. Though the authors focused on using these stem cells for treatment of IBD, they also found comparable markers between their different sources of MSCs. A review article by Sarantopoulos et al. expands on the cell surface markers of MSCs and adipocyte precursor cells [77], although this article focused on preadipocytes derived from patient adipose tissue.

As the adipose is a highly active endocrine tissue, studies focused on comparing adipocyte precursors and mature cells derived from either patient or pluripotent stem cells should consider the role of adipocyte secretome including secreted proteins and extracellular vesicle contents [78]. Deshmukh et al. used high sensitivity MS analysis to establish a secretome profile between brown and white adipocytes isolated from different adipose depots in patients. They found 471 secreted proteins between the white and brown adipocytes that originated from many different categories. Among those 471 proteins were 101 proteins specific to brown adipocytes, giving them a unique profile for this cell type [79]. Gupta et al. compared the proteome of extracellular vesicles, which are membrane-bound vesicles with endosomal origins that carry molecular cargoes that are produced by various sources of patient derived and iPS-derived mesenchymal stem cells (MSCs). After characterizing extracellular vesicles from different sources using flow cytometry and confirming common markers through western blots, they analyzed their proteome using isobaric isotope labeling quantitative MS along with cellular localization assessments. Using this analysis, they found 223 differentially expressed proteins in EVs shared between patient and iPS-derived MSCs, with 131 of these being higher in abundance in patient derived MSC-extracellular vesicles [80].

Taken together, proteomics offers a means for researchers to identify and quantify minute changes in protein expression in cells, cell surfaces, and secretomes to enable improvements of iPS-derived cellular models. Knowledge of the similarities and differences between iPS-derived and patient derived primary cells will be broadly useful in further refinement of iPS cell models.

5. Applications of proteomics toward iPS-derived organoid and tissue models

An emerging frontier in stem cell research and regenerative therapeutics is the ability to use iPS-derived cells to produce organoids and engineered microtissues that portray a more physiologically relevant simile of functioning organs. For instance, iPS-derived cells have been used to produce microglia and macrophage cells that promote brain organoid maturation [81], kidney organoids [82], and engineered heart tissues containing myocytes and fibroblasts [83]. Improvement of tissue generation will benefit from understanding the proteomes of the constituent cells, the extracellular matrix in which these cells are embedded, and their paracrine crosstalk.

5.1. Characterization of tissues and organoids

MS has been used to characterize the effect of culture condition on iPS-derived engineered heart tissues by comparing their myofilament protein expression [84]. Another study compared the proteomic similarities between iPS-derived human cerebral organoids and fetal brain tissue through a bottom-up proteomics approach to understand the developmental mechanisms within cerebral organoids [85]. In this study, data generated from MS were processed and analyzed using multiple databases leading subsequently to the identification of major proteins involved in various neuro developmental stages including neurogenesis, synaptogenesis, cortical brain development, and other cell communication and signaling processes. These analyses serve as a template and a molecular map for carrying out additional experiments that enable characterization and treatment of many neurodevelopmental disorders.

Patient-derived iPS cells have been used to construct three-dimensional cerebral organoids to understand complex diseases, and proteomics has been used to better understand cellular heterogeneity, interactions, and architecture of these models. For instance, patient-derived iPS cells were used to produce 3D cerebral organoids with schizophrenic neurodevelopmental pathology [86]. Organoids from 25 donors were subjected to tandem mass tag 16-plex isobaric barcoding and MS to compare schizophrenic and control organoids. The authors found that approximately 2.6% of the total organoid proteome was differentially abundant in schizophrenic donor-derived organoids, with specific neuronal factors such as MAP2 and TUBB3 to be significantly depleted when compared to control organoids. Further, the study also compared the protein expression profile against a notable schizophrenia GWAS loci and detected differential expression of pleiotrophin (PTN) that was previously implicated as a risk factor of the disease.

In another study, iPS cells generated from patients with autism spectrum disorder were differentiated to produce cerebral organoids, and hyperplexed protein analysis was performed to study their proteome phenotype across various timepoints [87]. Differential expression of proteins pertaining to energy metabolism pathways, cell adhesion, cell cycle, and cytoskeleton were identified along with a cluster of proteins that can be used as potential biomarkers for autism spectrum disorder identification. Lastly, label-free MS has been used to map the proteomic changes in cerebral organoid development from trisomy 21 human iPS cells [88]. Altogether, these studies demonstrate the utility of proteomics to validate and characterize organoid models for studying complex neurodevelopmental processes in vitro.

5.2. Secretome and paracrine signals

Proteomics can be used to survey the secretome (Box 3), the complement of molecules secreted by cells into their surroundings that play important roles in mediating cell–cell communications. Measuring the paracrine signaling of cells is of interest for understanding the communication and physiology of different cell types within organoid models. In a landmark study by Pellegrini et al., the authors developed a choroid plexus organoid model from human ES and iPS cells, which contains epithelial and stromal cells and which produces an in vitro secretome that mimics human cerebrospinal fluid (CSF) [89]. This in vitro CSF-like fluid shifts in protein abundance over time that shows sign of organoid maturation, with proteins abundant in early time points (e.g. AFP) resembling embryonic human CSF samples and proteins abundant in later time points (after 60 days) (e.g. SOD1) resembling more like pediatric and adult CSFs. Another study, by Hale et al., involved the generation of 3D human glomeruli from iPS-derived kidney organoids along with organoid-derived podocyte cell population. In comparison to the previously used 2D podocyte cell lines, these 3D models facilitated better podocyte-specific gene expression and polarized protein localization. The organoid-derived glomeruli also retained cell-specific markers longer than their 2D counterparts, indicating the flexibility of the model to be leveraged as a drug testing or toxicity screening platform [90].

Box 3. Proteomics technologies to map the secretomes of iPS models

Cells communicate with each other in part through secreted proteins, known as the secretome. The secretomes of iPS cells contain pro-survival molecules that have been associated with therapeutic effects of cell therapies [111], whereas a secretome library screen has also identified molecules including FGF16 that could improve the proliferation of iPS-derived cells [112]. Studying the secretomes of iPS cells and iPS-derived cells and how they compare to primary stem cells or adult cells are therefore an important goal for improving stem cell research. Many proteomics approaches have been applied to enable secretome analysis of cultured cells and tissues [113]. A successful application will need to be able to distinguish bona fide secreted proteins from other background proteins in the conditioned medium (e.g. proteins from culture medium, serum supplement, or cell culture matrix). An example of such methods is secretome protein enrichment with click sugars (SPECS), which involves the click chemistry reaction and pull-down of glycosylated secretory proteins using N-azido-mannosamine (ManNAz), an azido group-bearing sugar [114]. Because ManNAz is metabolically incorporated, proteins in the cell culture matrix or media are excluded. The captured glycoproteins are further quantified and analyzed through DDA or DIA MS. Another innovative technique for secretome analysis involves the stable isotope dynamic labeling of secretomes (SIDLS) [115]. This approach uses the principles of pulsed SILAC to differentiate secretory proteins from intracellular proteins and also reveal information on secretory flux.

6. Expert opinion

Large-scale profiling of proteomes remains an exciting field with rapid progress in addressing the many technical challenges of protein study. Although proteins provide a direct readout of cell state, a full elucidation of the proteome remains highly challenging. Despite progress, sample complexity and heterogeneity still present a challenge to the depth of proteomics analysis such that the overall depths of proteomics experiment still lag those of many RNA sequencing experiments. This challenge is in part due to the lack of means to amplify protein samples unlike in nucleic acids, as well as the much larger copy numbers and dynamic ranges of proteins in the cell. As proteomics moves toward single cell-level analysis [91,92], the challenges outlined above will become more pronounced and will require innovative advances to improve the sensitivity and reproducibility of analysis. The analysis of iPS and iPS-derived cells also face unique challenges. Compared to conventional cell 'monocultures' that largely contain only one homogenous cell type, differentiating iPS cells and tissues contain a heterogeneous population more resembling adult tissues, which introduces additional dynamic range, sample complexity, and batch-to-batch variability concerns. The relatively small size of iPS cells leads to less protein content (requiring higher sensitivity) than other cell types, requiring more cell numbers for bulk analysis and compounding the difficulty of single-cell analysis. For organoid and microtissue analysis, the presence of cell culture substrate matrix proteins can also confound proteomics analysis. While proteomic profiling of organoids is a promising method for establishing *in vivo* and *in vitro* comparisons and associations, the culture conditions of organoids can limit the translatability of analyses. For instance, the presence of common matrix scaffolds for organoid culture has been associated with influencing the proteomic analysis [93]. Developing methods to efficiently dissolve the matrix and recover organoid cells facilitates more accurate proteome profiling that is indispensable for promoting translational organoid models.

Secondly, the full complexity of the proteome is still being investigated. Recent proteomics experiments been able to confidently identify the 'full proteome' as defined by one protein product per expressed gene, but many routine quantification experiments continue to fall short of identifying the full proteome, which makes it difficult to draw inference on low abundance proteins especially some low abundance transcription factors and pluripotency factors. Moreover, the full elucidation of the proteome will require careful consideration of up to millions of 'proteoforms' present in a cell, due to post-translational modifications and glycosylation [94] or alternative splicing [95] that create multiple protein species per gene.

Thirdly, due to the very large scale of data generation in omics experiments, many of the findings remain to be validated by orthogonal methods. It should be noted that contemporary MS analysis is considerably more reliable and rigorous than affinity-based methods using antibodies, which are often poorly validated and may bind nonspecifically or fail to recognize their targets altogether [96–99]. Nevertheless, orthogonal corroboration of findings is one of the cornerstones of the scientific enterprise, and many findings may fail to replicate despite best intentions due to various technical and biological sources of variability. Technologies that can be used in conjunction with proteomics to verify findings in high throughput will likely represent an increasingly important area in the near future.

Looking forward, the integration of multiple omics data types or multi-omics analysis will likewise represent another important frontier for new insights across different layers of biological regulations. Combining proteomics and transcriptomic data

offers a more comprehensive depiction of cellular gene expression regulations and their role in mediating biological functions than either method alone. For instance, the use of RNA-sequencing and MS together can help discover alternative proteins that arise during the differentiation of iPS-derived cells [95]. A study by Connor-Robson et al. involved the generation of a novel integrated proteomics and transcriptomics approach to understand the impact of LRRK2 protein mutations in iPS-derived neurons [100]. Finally, a study by the HipSci consortium using iPS panels showed that pQTL can be found to explain relationships between gene variants and protein levels to identify new genome-proteome associations [45]. More effective integration methods that leverage iPS cells are therefore needed that can combine diverse data types to derive new insights.

In summary, proteomics has proven valuable in revealing the molecular markers and pathways guiding iPS cell generation, reprogramming, differentiation, maturation, and tissue formation.

As iPS cell technologies continue to move toward novel applications in the formation, characterization, and investigation of organoids and microtissues, we expect the technologies of large-scale protein identification quantification to continue to play important roles in understanding the molecular mechanisms that govern cell and tissue behaviors in diverse fields.

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