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Proteomics and Glycoproteomics of Pluripotent Stem-Cell Surface Proteins

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ABSTRACT

Pluripotent stem cells are a unique cell type with promising potentials in regenerative and personalized medicine. Yet the difficulty to understand and coax their seemingly stochastic differentiation and spontaneous self-renewal has largely limited their clinical applications. A call has been made by numerous researchers for a better characterization of surface proteins on these cells, in search of biomarkers that can dictate the developmental stages and lineage specifications, and can help formulate mechanistic insight of stem-cell fate choices. In the past two decades, proteomics has gained significant recognition on profiling surface proteins at high throughput. This review will summarize the impact of these studies on stem-cell biology, and discuss proteomic techniques used in these studies. A systematic comparison of all the techniques and their results is also attempted here to help reveal pros, cons, and the complementarity of the existing methods. This awareness should assist a better selection of suitable strategy for stem-cell related research, and shed light on technical improvements that can be explored in the future.

INTRODUCTION OF PLURIPOTENT STEM CELLS

Pluripotent stem cells (PSCs) can give rise to all three germ layers and have the potential to form chimeric multicellular organisms in vivo[1]. Ex vivo, these cells can infinitely renew themselves and differentiate into any cell types in the body. Closely related to PSCs are somatic or adult stem cells that are classified according to their origin and multipotency [2]. A few well studied somatic stem-cell types include hematopoietic stem cells (HSCs), mesenchymal stem cells (MSCs), and neural stem cells (NSCs). Comparing to PSCs, somatic stem cells have limited potential to differentiate. This review will focus primarily on the PSCs and mention briefly the somatic stem cells when their information is necessary to support the PSC studies.

PSCs can refer to a broad range of cells with varied tissue origins [3-4]. The earliest studied PSCs are embryonal carcinoma cells (ECCs) isolated from tetratocarcinomas [4-5]. To date, the most studied PSCs with highest pluripotency and germline transmission efficiency are embryonic stem cells (ESCs) derived from the inner cell mass of blastocyst [6-7]. Others such as epiblast stem cells (EpiSCs) can be obtained from the post-implantation embryo [8-9], and it is now widely believed that human ESCs (hESCs) are similar to mouse EpiSCs in many functions and behaviors [8-9]. Pluripotency can also be established from spontaneous reprogramming in the long-term culture of bone marrow cells [10]. Parthenogenesis of unfertilized eggs [11-12] as well as in vitro culture of neonate and adult spermatogonial cells [13-14] were also reported to form PSCs. Terminal differentiated cells can also be reprogrammed back to pluripotent stages, and the 2012 Nobel Prize of physiology or medicine was awarded to Drs. Gurdon and Yamanaka for their contribution to the reprogramming technology. Three major methods are possible to install pluripotency in somatic cells, i.e. the somatic-cell nuclear transfer (SCNT) [15], the fusion of somatic cells with ESCs, and the derivation of induced pluripotent stem cells (iPSCs) by activating cellular specific transcription pathways [3]. The iPSC technique was first succeeded by

Takahashi et al. in 2006 [16] using transgenes to enforce the expression of four transcription factors (Oct 4, Sox 2, c-Myc, and Klf4). Later on, the induction has been extended to the use of DNA-free systems such as proteins and RNA molecules [17].

The success of iPSC technology has impacted the entire PSC field [1, 18-19]. It breaks the ethical barrier that had limited the studies of hPSCs for decades. The possibility of using patient-match stem cells to investigate disease mechanisms and to develop specific therapies excelled "personalized medicine". The process of de-differentiating various somatic cells into iPSCs also offers a window to peek developmental biology with ramifications in cancer and aging. The unlimited cell source enabled by the self-renewal and differentiation potential of patient-specific iPSCs will benefit the pharmaceutical industry in their study of drug efficacy, toxicity and mechanisms in action [20].

Driven by the promises of ESCs and iPSCs, the development and studies of these cells as tools in biology and regenerative medicine have been accelerated in the recent years. The initial success in establishing ESCs was achieved in mouse in 1981 [6-7] and about 17 years later in human in 1998 [21]. Whereas, the success of forming mouse iPSCs [16]and human iPSCs [22-23]was only one year apart, in 2006 and 2007 respectively. From ESCs to iPSCs is a big leap in stem-cell field, yet currently the iPSCs do not possess the same level of homogeneity and potency as ESCs do, such as the germ-line transmission and differentiation capacity [1, 24]. Genetic and epigenetic differences in autogenic iPSCs further raise concerns on their complete replacement of the ESCs [1]. In general, the applications of PSCs in cell replacement therapy, tissue engineering, drug toxicity screening, and developmental biology have been limited[25-27], due in part to our incomplete understanding of their biology and the mechanisms regulating their fate decisions.

Cell-fate decisions are not only an intrinsic transcriptional process as demonstrated in the reprogramming, but also an outcome influenced by extrinsic cues initiated from the microenvironment outside of stem cells known as the "niche" [28]. The inextricable across talk between intrinsic and extrinsic signals in regulating stem-cell fate manifested in many processes in development and regeneration, such as the migration of ESC and MSC in vivo [29-30], the homing and differentiation of HSC [31], and the paracrine signaling mediated by MSCs in immune/inflammatory suppression and the recruitment of macrophages[32]. The balance developed from this interplay will be a key for us to ultimately control the lineage specification of stem cells. Various culture conditions have emerged with demonstrated effects in maintaining pluripotency or coaxing the directed differentiation [33-34]. Yet, because of the lack of in-depth understanding how these environmental factors impact stem cells, regardless the current progress no expansion methods are available to provide large-quantity and high-quality homogeneous PSCs or fate-committed cells derived from PSCs that can meet the clinical needs [33-34].

FUNCTIONAL IMPORTANCE OF CELL SURFACE PROTEINS

Proteins on the plasma membrane situate at the interface of this intrinsic-extrinsic interplay, and can sense and relay molecular information cross lipid bilayers[35]. The changes undergone in either side of the plasma membrane can be reflected by surface proteins through their dynamic expression, localization, structure remodeling, and interactions with other molecules. Based on function, Almen et al. catalogued surface proteins into four types [36]: transporters (such as channels, solute carriers, and active transporters), receptors (such as G-protein coupled receptors, receptor type tyrosine kinases, receptors of the immunoglobulin superfamily or related, and scavenger receptors and related), enzymes (oxidoreductases,

transferases, hydrolases, lyases, isomerases, and ligases), and miscellaneous (adhesion molecules, ligands, and others). These proteins' surface expression is constantly regulated by intracellular events such as endocytosis [37] and exocytosis, as well as extracellular activities such as enzymatic remodeling. As a result, cell surface proteins resemble both environmental conditions and intracellular states, function importantly in signaling transduction as well as cell-cell communication. The consequence can be observed in all major cellular processes from cell division to growth, from proliferation to differentiation, from senescence to apoptosis, from adhesion to migration, from immune response to attack, and so on [38].

These highly coordinated activities are orchestrated by tightly regulated surface-protein networks. Some critical regulations are achieved through the immense post-translational modifications occurred on plasma-membrane proteins, especially the bulky and complex glycosylation that is sensitive to environmental conditions and can be drastically remodeled during development, aging, and pathogenesis [39-40]. Glycosylation is one of the most abundant post-translational modifications. Except for O-GlcNAcylation, other types of glycosylation including N-glycosylation and O-GalNAcylation take place in the secretory pathway and modify most cell surface and secreted proteins. Remarkably, these abundant and diverse attachments only add to the ectodomain of membrane proteins. Several excellent reviews [41-46] have entailed the biological functions of glycosylation. Yet their unique ectodomain orientation and ubiquity on surface proteins also provide an interesting handle for specially designed methods to grip and to enrich membrane proteins, which are generally termed as "glycocapture" here, and will be further discussed in the methodology section below.

The first step towards a holistic understanding of the coordinated events occurring at cell surface is to molecularly profile stem-cell surface proteins. Shotgun proteomics based on liquid

chromatography and mass spectrometry (LC-MS) analyses is capable of characterizing complex protein mixtures in high throughput with exclusive accuracy and specificity that antibody-based assays cannot achieve[47-49]. The advancement in quantitative and sensitive proteomics has made the technology capable of analyzing stem cells in small numbers [50]. The discovery phase of proteomics has helped to establish the molecular map of many stem cells, including human and mouse ESCs and iPSCs, adult stem cells, their differentiated progenitor and precursor cells [51-54]. Further studies on protein interaction networks, and on protein modifications such as phosphorylation and epigenomic modifications are also actively pursued by several groups with fruitful outcomes that are summarized in several recent reviews [55-56]. Efforts have also been focused on molecular comparison of different stem cells, such as ESCs and ECCs[57-58]. Yet membrane proteins have under-represented in the global profiling of the whole proteome of stem cells [59-60].

Increasing attention has been paid to enrich and characterize membrane and plasma-membrane proteins of PSCs in search of surface markers that can define stem cells, and their developmental stages [61-64]. Previous success on identifying the "cluster of differentiation" (CD) markers in leukocytes coming out of the Human Leukocyte Differentiation Antigens Workshop (HLDA) [61, 65] encouraged the surface proteomics of PSCs. Many techniques have been explored to characterize membrane proteomes of PSCs as summarized in a few reviews in 2008[58, 61, 63-64]. Ever since, the studies in this field have grown and allowed us to gain substantial knowledge towards stem-cell surface proteins. The following sections of this review will summarize these recent efforts, and highlight their biological impacts. Most importantly, this article will provide the pros and cons of the used techniques, and pinpoint the challenges faced by researchers.

IMPACT OF MEMBRANE PROTEOMICS TO STEM CELL BIOLOGY

A summary of recent and past membrane proteomic efforts on PSCs is listed in Table 1. After sporadic publications prior to 2008, more than a handful studies have been conducted on membrane proteome of PSCs. The main biological outcomes from these studies can be generalized into at least four aspects: i.e. 1) the biomarker discovery, 2) cell-type relationships, 3) culture conditions, and 4) mechanistic insights. First is the discovery of a plethora of surface markers for identification of different stem cells and their subpopulations. The heterogeneous nature of pluripotent and adult stem cells demands the use of surface markers to isolate a relatively pure population[66]. Yet, the paucity of available markers encourages more thorough characterization of surface proteins to populate the surface-marker pool. Therefore, membrane proteomics has been largely focused on this aspect. Nagano et al. had summarized the surface markers known to stem cells [64] in the past, and Prokhorova et al. further expanded the list by analyzing the membrane proteome of hESC and their differentiated cells using SILAC (Stable Isotope Labeling by Amino acids in Cell culture) [67]. In this study, the authors identified several additional markers, such as glypican-4, neuroligin-4, ErbB2, and PTPRZ that are specific to undifferentiated hESCs and 17 other proteins that are unique to differentiated cells[67].

Besides distinguishing pluripotent and differentiated cells, membrane proteomics is also effective in delineating cell subpopulations as illustrated in a few recent efforts. For example, intensive surface proteomics on CXCR4-sorted endoderm progenitor cells that are differentiated from ESCs helped to identify another 51 surface proteins that can indicate the CXCR+ and CXCR- populations in differentiated ESCs[68]. Among them, CD61 was verified to further indicate a subpopulation in CXCR+ cells carrying smooth muscle genes and extracellular matrix (ECM) genes from gene ontology (GO)[69] and RT-qPCR (Reverse Transcription-Quantitative real time

Polymerase Chain Reaction) analyses, and with elevated functions of vasculogenesis/angiogenesis. A separate analysis of hMSCs [70] identified a set of membrane proteins, among which 25 showed distinct patterns upon differentiation to adipocyte and osteoblast in two weeks. In a different study, Graneli et al. [71] examined lineage specification during hMSC differentiation. Through a series of careful follow-up validations, they pinpointed three novel markers (CD10, CD92, and CRYaB) on hMSCs, in which CRYaB+ hMSCs had a specificity to osteogenesis but not to adipogenic and osteogenic differentiation, whereas CD10+ and CD92+ cells showed both osteogenisis and adipogensis. In addition, their results were opposed to the known marker, CD166, but supported another known protein, CD49e, in osteo-transition of hMSCs. Interestingly, Yan et al. through their membrane proteomics discovered that CD166 was better than CD44 in indicating the stem-cell like cells in head and neck squamous cell carcinoma [72]. The authors verified their results through both in vitro microsphear formation and in vivo tumorigenesity, as well as through clinical stratification of patient outcome and relapse rate [72]. In a different study, a characterization of surface proteins of cord and periphery blood monocytes identified MOSC-1[73] that can distinguish a subpopulation in CD34+/CD45- cells with monocyte/granulocyte differentiation potency. Surface markers that can guide the isolation of iPSCs from mouse embryonic fibroblast during reprogramming were also unveiled [74].

To note, an encouraging piece of work coming out of a comparative membrane proteomics examined four closely related stem cell lines derived from mouse embryos, i.e. embryonic, epibalst, trophoblast, and extraembryonic endoderm stem cells [59]. In this study, Rugg-Gunn et al. successfully identified a large set of surface markers for each lineage. The in vivo assay on E4.5 and E5.5 mouse embryos using these markers demonstrated a clear lineage

restriction occurred during early embryo development. The identified markers also helped isolate viable cells from developing embryos for further functional and molecular characterizations.

In all the surface proteomic studies addressed here, one trend is the thorough verification of the selected proteins. This necessity is because of the less stringent membrane selection of most methods, which will be further discussed in the result-comparison section below. Western blotting and flow cytometry have been the standard verification approches. In many cases, in vitro and in vivo functional assays were also deliberately carried out to explore the biological roles played by these proteins. Furthermore, the supporting and opposing information to the prior knowledge coming out of different studies is constantly emerging when increased attention has focused on similar stem-cell types and biological processes. New insight that can explain these differences will be a matter of time. The pool of new stem-cell markers and subpopulation identifiers will also excel the follow-up biological studies and clinical applications as demonstrated by Yan et al. in their clinical application of CD166 as a stratification factor in head and neck carcinoma patients[72].

The second contribution of membrane proteomics to stem-cell biology is the understanding of relationships between different cell types. Traditionally, this connection has been built based on the observed phenotypes such as the proliferation potential between cancer cells and stem cells. Now, with the high-throughput membrane proteomics, such understanding can be built at membrane-proteome level as being demonstrated in the study of the “malignant” cancer stem cells (CSCs) and the “benign” stem cells [57]. Additional examples also include the quest of the similarity between human ESCs and sperms [75], the neural lineage bias towards rat bone-marrow mesenchymal stromal cells [76], and the expression of tissue-specific proteins in ESCs [77]. Besides similarities, differences can be revealed. For instance, van Hoof et al.

discovered marked difference between in vitro ESC-derived cardiomyocyte-like cells and in vivo developed fetal cardiomyocytes using a well designed SILAC membrane proteomic strategy[78]. Along a different thread, the dedifferentiation of breast epithelial carcinoma to mesenchymal like cells were also hypothesized in GPI (glycophosphatidylinositol)-anchored membrane proteomic study of breast tissues and cell lines [79].

The third contribution of membrane proteomics to stem-cell biology is the facilitation of the optimization of culture conditions. Stem cells are poised for self-renewal and differentiation, and their fate commitment can be regulated by external environment as being introduced above. Membrane proteomics has been used to investigate surface proteins of stem cells growing in different conditions, such as with fibroblast growth factor in hMSC culture [80], and the characterization of extracellular matrix proteins to hESCs [81-82]. Further analyses of hESCs surface proteins that are independent of culture conditions have also been conducted [83].

Last but not the least, the mechanistic studies of membrane-protein functions in, for instance, the signaling transduction during stem cell differentiation, cancer-stem-cell chemoresistance, and karyotype instability, have been pursued in conjunction with biomarker discovery in membrane proteomics [84-86]. Particularly, Cao et al. characterized the change of membrane proteome during astroglial potentiation of NSCs, and revealed activated metabolic pathways in cell growth and development besides the identification of transferring receptor protein 1 as a marker for NSCs [84]. Membrane proteomic efforts in characterizing CSC-like MDA-MB453 cells also discovered CD 147/Emmprin that can function in chemo-resistance pathway in cancer stem-like cells[86]. Further studies of karyotypical normal and abnormal cells using membrane proteomics have also been conducted [85]to elucidate the molecular difference for insight on the genetic instability that hinders stem-cell applications and therapies.

DIVERSE PROTEOMIC METHODOLOGIES:

The rich knowledge addressed above is built upon a large collection of membrane proteomic tools. Because of the low abundance of membrane proteins, previous reviews have been focused on the enrichment techniques of the plasma membrane[61, 63]. Yet, the challenges of studying membrane proteome do not stop at enrichment step alone, but also propagate in several downstream steps such as the dissolution and digestion of membrane proteins, the removal of the associated proteins, and the separation of membrane proteins or their derived peptides for more effective MS characterization. To better reflect the numerous choices in membrane proteomics, this review follows the sequence of proteomic sample preparation and dissects the entire MS-based proteomic strategy into three sections: enrichment, dissolution, and separation.

Figure 1 enlists the methods of choice under each section. For separation and/or enrichment of membrane and plasma-membrane proteins, physical methods based on density, such as gradient or differential centrifugation, have been popular. To improve the selectivity of plasma-membrane proteins, affinity and chemical enrichments are also devised. The affinity enrichment has included the use of antibody affinity to surface antigens, the avidin affinity to biotinylated surface proteins [59, 75, 77, 87], the lectin affinity to glycosylated proteins [88], and the use of toxin affinity to the GPI-anchored plasma-membrane proteins [79]. Covalent-bond based chemical enrichment, on the other hand, is more selective than affinity interactions because of the strength from the chemical bonding [38]. Hydrazide chemistry has been frequently used to study stem-cell membrane proteins, because most surface proteins are glycosylated as aforementioned, and for details please find them from a recent review[38]. Several studies using glycocapture including those from us as well as others have been carried out on stem cells [60, 74,

[89]. For all the existing enrichment methods, recent trend is to combine the coarse separation achieved by centrifugation together with affinity or chemical enrichment for better removal of abundant cytosolic proteins and for high selectivity to membrane proteins [60, 74, 87, 89].

Yet the enrichment of surface proteins is not limited to what has been listed in Figure 1, there are numerous other methods to collect membrane proteins [38, 61, 63], such as the use of colloidal particles and enzymetic shedding. For glycoenrichment, besides hydrazide chemistry and lectin-affinity, a wide range of other methods also exist, including the use of boronate or click chemistry, and the use of bioorthogonal probes or chromatography separation[38]. However, these approaches have not been widely applied in stem-cell membrane proteomics, and the reasons are complicated. Many of these techniques lack the comprehensiveness to label or to enrich all the surface proteins or glycoproteins. In addition, the labeling procedures such as the metabolic labeling can perturb sensitive stem cells. As a large number of stem cells, in most cases, are difficult to obtain than that of other cells, methods with less sensitivity have not been favored in stem-cell proteomics[61].

Following the enrichment, a series of means have been deployed to dissolve and/or digest membrane proteins, and in these steps the choices of detergent, the wash and digestion conditions have been detailed in reviews [58, 90-91]. The compatibility of the detergent to downstream MS detection will affect the design of the separation and sample cleaning-up procedures. In most dissolution steps, a combination of a few choatropics is common. In shot-gun proteomics, digestions are always carried out to obtain peptides. Methods such as enzymetic and non-enzymetic digestions are available [90], and trypsin proteolysis has been the most frequently used digestion method in stem-cell membrane proteomics for its high specificity, robustness, and well characterized nature.

The last most important step in proteomics prior to MS analysis is the separation of membrane proteins or peptides. Many methods are available and these methods have contributed the most to the diversity of membrane proteomics especially in recent years. Originally, 2D-PAGE was widely employed for the separation of intact proteins in proteomic analysis. With the computer controlled alignment and automatic spot picking, 2D-PAGE was popular in early quantitative proteomics[92]. Later, this technique was evolved to use fluorescence, and termed 2D-DIGE (Differential In Gel Electrophoresis) with marked improvement in quantitation accuracy. Recently LC based separation has quickly replaced gel-based separation for its high automation and exquisite separation power. Common LC separation schemes include strong cation exchange, size exclusion, HILIC (hydrophilic interaction chromatography), and reverse phase partition chromatography. Intoh et al. [93] also reported the use of zwitterionic chromatography for membrane proteomics with improved separation efficiency on the membrane proteome of mESCs. Yet 2D-DIGE has one unique advantage over LC is that the MS detection can be carried out only to the differentially expressed proteins in the immobilized protein gel, which greatly saves the instrument time.

The fast advancing MS techniques with increased scan speed of tens of milliseconds, such as those of TOF (time-of-flight) and Orbitrap based instruments, have drastically dropped the analysis time. Yet, the in-gel digestion time used to extract proteins out of the SDS-PAGE based separation systems cannot be easily reduced. As a result, LC approaches are gradually outpaced 2D-PAGE, and the difference between the two techniques have been demonstrated by research groups using the same samples [80, 93-95]. In addition, hybrid multi-dimensional separations are often observed in membrane proteomics, with the first dimension using gel-based

separation such as SDS-PAGE [75, 77] and IPG-IEF (Immobilized pH Gradient Isoelectric Focusing) [96], and the rest of separations using LC.

Because of the low cell number that is common to stem-cell studies, sensitive analyses are always highly desired. The sensitivity of current membrane proteomics can analyze as few as 0.5 million cells, which has been demonstrated by Dormeyer et al. [57] using differential centrifugation, stringent wash, miniaturized microseparation, and a sensitive MS instrument, i.e. LTQ-orbitrap.

Given the large number of choices in each proteomic step, it is often challenging to decide which method, or what combination of methods, will form the best strategy and help address a specific biological question. With the overview of these methods in this section, and comparison of the resolved data from the following section, I hope this challenge can be ameliorated to certain extent.

COMPARISON OF RESULTS OBTAINED BY DIFFERENT TECHNIQUES

Because of the existence of many methods, it is important to understand the advantages and disadvantages of each method by examining their final results. However, not enough efforts have been focused on accurately evaluating the differences and commonality among these results. A fair comparison can be extremely challenging as the published analyses were usually carried out on different biological samples and the research focuses varied drastically from comprehensiveness centric, sensitivity centric, to selectivity centric. Technically, besides the sample-preparation difference, other variables also include the sampling strategy, the MS instrumentation, the detection method, and the downstream bioinformatics. In the past, attention has been raised to standardize LC-MS analysis to ensure the quality of proteomics, and MIAPE

(Minimum Information About a Proteomics Experiment) [97] has been developed as part of the HUPO (Human Proteome Organization) standard initiative, in which minimum information on reporting LC[98], gel electrophoresis[99], MS[100], and bioinformatics[101] in proteomics have been proposed. From these exercises, it is clear that each step in proteomics is a multivariable procedure that it is non-trivial to standardize. To evaluate the entire proteomic strategy that includes the sum of all above is almost impossible.

In the past, attempts in stem-cell membrane proteomics have been pursued to compare the total number of the identified proteins based on Gene Ontology (GO)[85, 96]. This type of efforts provides general information on the overall performance and the sensitivity of the method; however, this comparison is insufficient to offer more in-depth insight to the class of proteins being enriched, and is lack of information on the method selectivity including the potential bias or omission to certain classes of membrane proteins. These hidden factors can be critical to certain biological questions.

For example, in an extremely sensitive study led by Gu et al., the authors identified more than 1500 membrane proteins in mESCs, from which they discovered many low-abundance tissue-specific membrane proteins[77]. A result sheds light on the complexity of ESC membrane proteome, and the potency of ESCs to differentiate. Yet, the selectivity of the method was about 50%, since the total identified proteins from this study were more than 3000. Therefore, this method would not be a top choice for studies concerning high selectivity of membrane proteins, such as those related to surface biomarker discovery without thorough validation of protein localization.

A few studies such as that of Nunomura et al. have deliberately analyzed the non-membrane proteins [87] and pointed to the potential sources of these non-membrane

contaminations. Many studies [59, 89] have published the total protein list as well as the filtered protein list to assist other researchers to better evaluate and understand the performance of the used methods.

We have developed in the past an enrichment analysis to compare results from different studies [60] to a fixed set of factors. The enrichment analysis considers not only the total number of protein identified, but also the category of proteins being identified, which is effective to expose the pros and cons of different techniques. If the analyzing samples are drastically different, such as ESCs versus red blood cells, the comparison can also disclose sample differences[60]. To ensure the fairness of the comparison, it is critical to select the same set of factors (such as GO terms) to all the comparing datasets. Based on the pattern obtained from all the datasets and all the comparison factors (i.e. GO terms), both method- and sample-specific information can be inferred, which is inaccessible or not obvious when examining the total protein number alone.

To demonstrate the capacity of this method, I compared 14 published ESC membrane proteomes with mouse or human origin in their enrichment of the GO terms related to cellular components. Figure 2 is the heatmap summary of the top-10 enriched GO terms in each dataset. The enrichment was performed using DAVID (Database for Annotation, Visualization and Integrated Discovery) [102-104]. In Figure 2, three clusters of GO terms can be observed (marked in red, yellow, and blue bars), in which the red cluster mainly includes the cell surface and extracellular proteins as well as proteins in the secretory and endocytic pathways that are closely related to the plasma membrane, with representative GO terms such as “cell surface”, “plasma membrane”, “intrinsic to membrane” and “integral to membrane”, “lysosome”, and “vacuole”. The blue cluster, however, mainly includes cytosilic components, such as “cytosol”, “ribosome”,

and “mitochondria”, which are generally considered as contaminants in plasma membrane protein analysis.

In both the red and blue clusters, glycocapture-based methods clearly distinguish themselves from biotinylation- and centrifugation-based approaches, suggesting a high selectivity to membrane proteins. This observation has been consistent with original publications where these results were obtained [60, 87, 89]. As explained above, inside a cell glycosylation has been used as a signal for cellular proteins to traffic to the cell surface or to be secreted, therefore it is without surprising to observe the high selectivity to surface and secretory proteins in glycocapture results than in centrifugation-based results. In the blue cluster, biotinylation methods show improved selectivity than those of centrifugation but not as good as those of glycocapture. The less optimal selection of surface proteins using labeling strategy such as biotinylation has been addressed in the past [87], which is partially due to the natural cell death [89] and the engulfing of the labeling agent through the endocytosis of intact cells [105]. Either process can expose cytosolic proteins to the labeling reagent, a result which can interfere with the final membrane selectivity.

In the yellow cluster, the nonglyco-methods show better enrichment than the counterpart in the endomembrane-related GO terms, such as the ER and Golgi membrane, which appears counterintuitive. As glycosylation takes place in the ER and Golgi and many enzymes in these endomembrane systems are glycosylated, glycocapture methods should enrich these proteins. A careful examination of all the results showed that the enrichment was dominant in human samples (highlighted in purple dots in Figure 2) but not in mouse samples. As enrichment p-value was calculated using species-match gene background, the observed difference may be caused by different backgrounds. To verify this, I converted our mESC membrane

glycoproteome [60] (labeled as "Sun" in Figure 2) to its human orthologues, and analyzed the converted data using Homo sapiens background. The result is included in Figure 2 and labeled as "Sun-HU homo". As expected, after the background conversion, the glycoproteome shows better enrichment in the yellow cluster (Figure 2), which suggests that the used background can affect the final result. This difference indicates the sensitivity and the breadth that can be achieved by this analysis.

Many datasets used in Figure 2 are the total protein list. The filtering of membrane proteins will improve the quality of the results substantially, such as the data from Gu et al. for both their human and mouse ESC results[75, 77] and the data from Harkness et al. [83] The complete lists were not provided in these studies, so their results in Figure 2 are relatively clean. However, these authors did provide the total number of identified proteins. Based on their numbers, the non-membrane proteins are around or beyond 50% of the total identified proteins [75, 77, 83], and are similar to other studies included in Figure 2 using the same method.

To sum up the methodology discussion of membrane proteomics on stem cells, the choices are many and each has its unique characteristics, in which glycocapture provides the best membrane selectivity, whereas the biotinylation and centrifugation provide better coverage based on the total number of identified membrane proteins. What is not focused is the antibody-based affinity approach to profile stem-cell surface markers, such as those using the known CD protein antibody library [68, 106-107]. The challenges and limitation of antibodies have been known for their varied specificity and affinity that can likely introduce bias to the characterization. The protein information coming out of the discovery-based membrane proteomics will without doubt supply antibody library with new candidates. Nevertheless, at the current time for any method, there is a substantial space for further improvement on both the sensitivity and the selectivity.

REMARKS

It is evident from this review that membrane proteomics has been quickly spread in the PSC field since 2008 after a few leading-field reviews. Six-year effort has greatly promoted the molecular understanding of stem-cell membrane, with potential markers for different stages of stem-cell development and lineage specification. Our knowledge of the heterogeneity in stem-cell population is also constantly growing, especially with the help of surface markers identified through membrane proteomics. Because of the holistic molecular view offered by membrane proteomics, it is anticipated that this discovery-based technology will advance our mechanistic insights of stem-cell fate commitment, and help define appropriate culture conditions for maintenance, expansion, and differentiation of these cells in vitro for research and clinical applications. These discovery-based researches will also provide ample protein candidates to improve the existing targeted analyses. Immunoafffinity based assays as well as other targeted functional assays can quickly access the heterogeneity of cell population or cellular functions; however their potentials have been hampered by the sparse targets that are available for studying. A thorough and accurate profiling of stem-cell surface proteome will not only provide biomarkers to isolate specific subpopulation for further functional and behavioral analyses, but can also stimulate the development of various functional assays to further characterize the population heterogeneity.

Several challenges have emerged from current membrane proteomics. First is the less-than-ideal selectivity to membrane proteins in many studies. The poor selectivity requires thorough validation of membrane location of identified proteins. This validation needs orthogonal approaches than discovery-based LC-MS. Immuoassays, including

immunofluorescence aided flow cytometry, imaging, and Western blot, have been widely used. The availability and quality of antibodies have confined the testable proteins to be those that are well known in biological studies. The high cost and low successful rate for raising new antibodies against novel proteins have hindered the large-scale validation of results coming from the high-throughput proteomic analyses. Methods with high membrane-selectivity such as glycocapture approaches will be more advantageous than those with poor membrane-selectivity for lowering the cost and shortening the time used in developing new targeted bioassays especially antibody assays.

Secondly, the obtained stem-cell proteomic results are lack of consistence. Not limited to the obtained membrane proteomes, the results of global proteomes of PSCs have shown small overlaps [66]. The reasons are several folds. Besides technical differences as addressed here, biological difference among different PSCs used in these studies is another dominant factor. Stem cells are prone to genetic changes and susceptible to environmental perturbations as introduced above. Currently, a range of stem-cell strains exist, and each has its own optimized culture conditions. The biological samples used for obtaining proteomic results not only have different genetic backgrounds, but also have different culture history including the passage numbers. For example, as the culture condition alone, there are feeder and feeder-free culture, serum and serum-free media, as well as chemically defined media to avoid potential xenogeneic contamination and the instability of biologically defined media [33-34]. In addition, to mimic natural stem-cell niche, various natural as well as synthetic extracellular matrices have also emerged to help expand and differentiate pluripotent and adult stem cells [33-34]. Due to the poor understanding of stem cells themselves, no standards have been created in stem-cell culture, which has

contributed in part to the large variation and little overlap observed in current stem-cell proteomes.

Because surface receptors, that are responsible to chemical and physical growth conditions such as various growth factors and hormones, are low in abundance, and also because stem cells are difficult to obtain a large amount, current techniques need to be further improved on their sensitivity to closing the gap between the detectable membrane proteome and the expressed membrane proteome. Several avenues can be pursued to improve analysis sensitivity, such as the improvement of separation and detection techniques. Currently, both capillary electrophoresis-MS and the newly developed high-sensitivity MS instrumentation have demonstrated the improved sensitivity to low-abundance proteins[108-109]. Further increasing the selectivity of sample-preparation methods to membrane proteins will also help a sensitive detection by eliminating the shielding effect from the high-abundance cytosolic proteins to membrane proteins as well. In addition, a prevention of sample loss should improve the sensitivity of analysis, especially when limited quantity of sample is available.

Ultimately, stem cells should be understood at single-cell level where the population heterogeneity is completely eliminated. A goal has been actively pursued by several research labs in characterizing the whole proteome of stem cells [110-112], and it can be a reachable goal for membrane-proteomic researchers as well in the near future.

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LEGENDS:

Figure 1. Summary of membrane proteomics strategies that were applied to study pluripotent stem cells.

Figure 2. GO enrichment of results obtained by different membrane proteomics methods on mouse and human embryonic stem cells and induced pluripotent stem cells, in which results from hESCs are highlighted by purple dots.

FIGURES

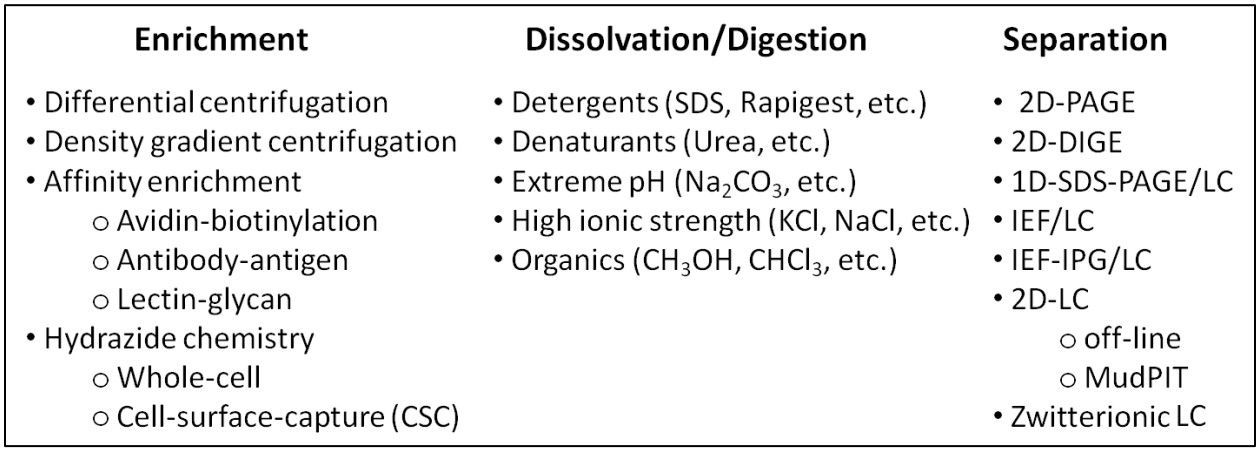

Fig. 1

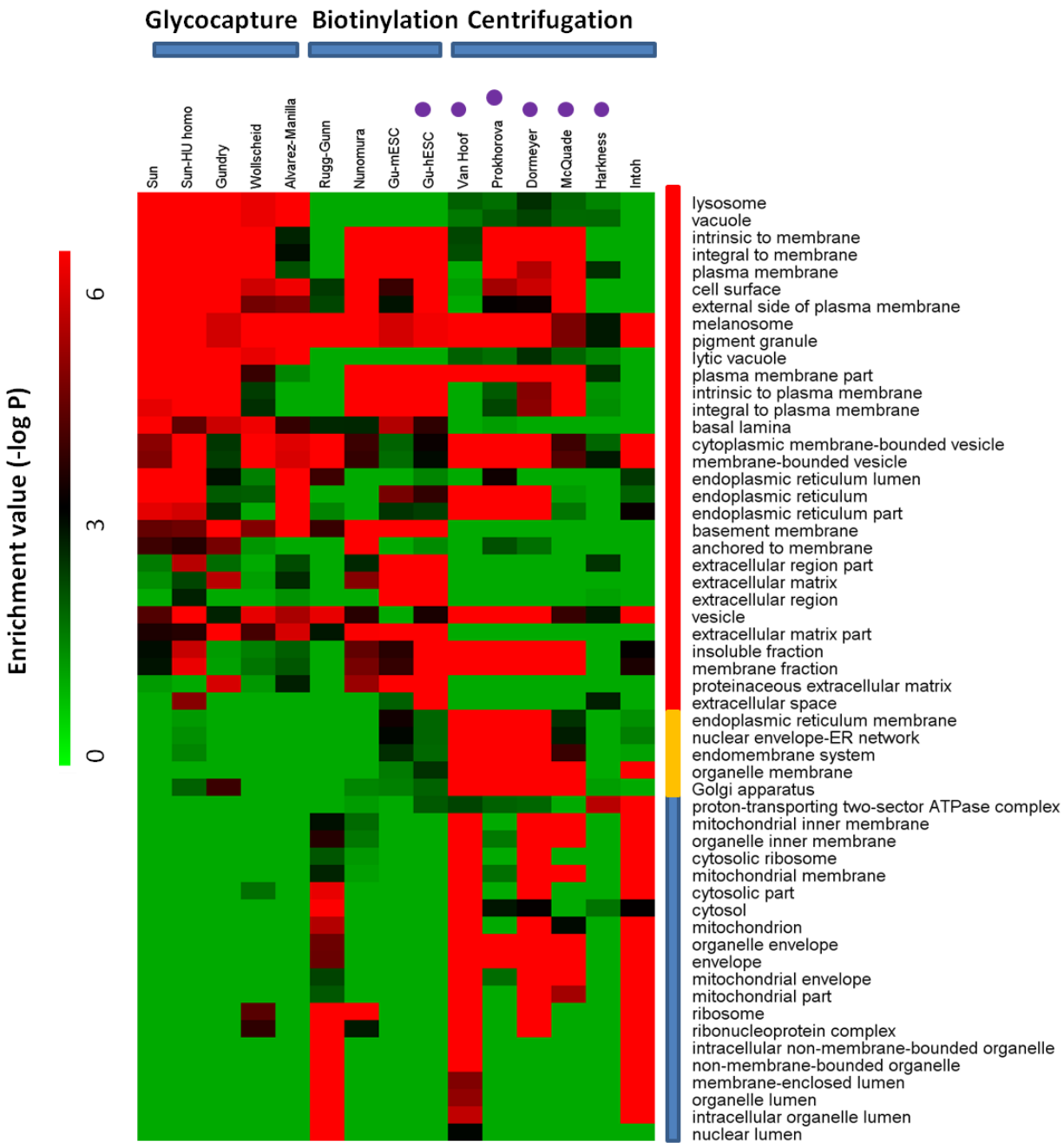
Glycocapture
Biotinylation
Centrifugation
Sun
Sun-HU homo
Gundry
Wollscheid
Alvarez-Manilla
Rugg-Gunn
Nunomura
Gu-mESC
Gu-hESC
Van Hoof
Prokhorova
Dormeyer
McQuade
Harkness
Intoh
Enrichment value (-log P)
0
3
6
lysosome
vacuole
intrinsic to membrane
integral to membrane
plasma membrane
cell surface
external side of plasma membrane
melanosome
pigment granule
lytic vacuole
plasma membrane part
intrinsic to plasma membrane
integral to plasma membrane
basal lamina
cytoplasmic membrane-bounded vesicle
membrane-bounded vesicle
endoplasmic reticulum lumen
endoplasmic reticulum
endoplasmic reticulum part
basement membrane
anchored to membrane
extracellular region part
extracellular matrix
extracellular region
vesicle
extracellular matrix part
insoluble fraction
membrane fraction
proteinaceous extracellular matrix
extracellular space
endoplasmic reticulum membrane
nuclear envelope-ER network
endomembrane system
organelle membrane
Golgi apparatus
proton-transporting two-sector ATPase complex
mitochondrial inner membrane
organelle inner membrane
cytosolic ribosome
mitochondrial membrane
cytosolic part
cytosol
mitochondrion
organelle envelope
envelope
mitochondrial envelope
mitochondrial part
ribosome
ribonucleoprotein complex
intracellular non-membrane-bounded organelle
non-membrane-bounded organelle
membrane-enclosed lumen
organelle lumen
intracellular organelle lumen
nuclear lumen

Fig. 2

Table 1. Summary of membrane proteome studies of pluripotent stem cells.

Author	Year	Cell type	Species	Technique	Reference
Van Hoof et al.	2010	ESC diff.	human	Density gradient	[78]
Gerwe et al.	2011	ESC	human	Density gradient	[85]
Gu et al.	2011	ESC	human	Affinity (Biotinylation)	[75]
Prokhorova et al.	2009	ESC	human	Differential centrifugation	[67]
McQuade et al.	2009	ESC	human	Differential centrifugation	[96]
Sundberg et al.	2009	ESC	human	Affinity (Antibody)	[107]
Harkness et al.	2008	ESC	human	Differential centrifugation	[83]
Rugg-Gunn et al.	2012	ESC	mouse	Affinity (Biotinylation)	[59]
Intoh et al.	2009	ESC	mouse	Affinity (Biotinylation)	[94]
Intoh et al.	2009	ESC	mouse	Differential centrifugation	[93]
Nunomura et al.	2005	ESC	mouse	Affinity (Biotinylation)	[87]
Gu et al.	2010	ESC	mouse	Affinity (Biotinylation)	[77]
Dormeyer et al.	2008	ESC/ECC	human	Differential centrifugation	[57]
Sun et al.	2013	ESC	mouse	hydrazide chemistry (whole-cell)	[60]
Wollscheid et al.	2009	ESC	mouse	hydrazide chemistry (CSC)	[89]
Alvarez-Manilla	2008	ESC	mouse	Affinity (Lectin)	[88]
Gundry et al.	2012	ESC/iPSC	mouse	hydrazide chemistry (CSC)	[74]
Melo-Braga et al.	2014	ESC	human	Centrifugation	[113]

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