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Article *in* Expert Review of Proteomics · October 2018

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To cite this article: Faezeh Shekari, Chia-Li Han, Jaesuk Lee, Mehdi Mirzaei, Vivek Gupta, Paul A. Haynes, Bonghee Lee, Hossein Baharvand, Yu-Ju Chen & Ghasem Hosseini Salekdeh (2018) Surface markers of human embryonic stem cells: a meta analysis of membrane proteomics reports, Expert Review of Proteomics, 15:11, 911-922, DOI: 10.1080/14789450.2018.1539669

To link to this article: https://doi.org/10.1080/14789450.2018.1539669

EXPERT REVIEW OF PROTEOMICS
2018, VOL. 15, NO. 11, 911–922
https://doi.org/10.1080/14789450.2018.1539669

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REVIEW

Surface markers of human embryonic stem cells: a meta analysis of membrane proteomics reports

Faezeh Shekari[a,b], Chia-Li Han[c], Jaesuk Lee[d], Mehdi Mirzaei[e,f,g], Vivek Gupta[g], Paul A. Haynes[e], Bonghee Lee[d], Hossein Baharvand[b,h], Yu-Ju Chen[c] and Ghasem Hosseini Salekdeh[a,e,i]

[a]Department of Molecular Systems Biology at Cell Science Research Center, Royan Institute for Stem Cell Biology and Technology, ACECR, Tehran, Iran; [b]Department of Developmental Biology, University of Science and Culture, ACECR,, Tehran, Iran; [c]Chemical Biology and Molecular Biophysics Program, Institute of Chemistry, Taipei, Taiwan, Republic of China; [d]Center for Genomics and Proteomics, Lee Gil Ya Cancer and Diabetes Institute, Gachon University, Incheon, Republic of Korea; [e]Department of Molecular Sciences, Macquarie University, Sydney, NSW, Australia; [f]Australian Proteome Analysis Facility, Macquarie University, Sydney, NSW, Australia; [g]Department of Clinical Medicine, Macquarie University, Sydney, NSW, Australia; [h]Department of Stem Cells and Developmental Biology at Cell Science Research Center, Royan Institute for Stem Cell Biology and Technology, ACECR, Tehran, Iran; [i]Department of Systems and Synthetic biology, Agricultural Biotechnology Research Institute of Iran (ABRII), Agricultural Research, Education, and Extension Organization, Karaj, Iran

ABSTRACT

Introduction: Human embryonic stem cells (hESCs) have unique biological features and attributes that make them attractive in various areas of biomedical research. With heightened applications, there is an ever increasing need for advancement of proteome analysis. Membrane proteins are one of the most important subset of hESC proteins as they can be used as surface markers.

Areas covered: This review discusses commonly used surface markers of hESCs, and provides in-depth analysis of available hESC membrane proteome reports and the existence of these markers in many other cell types, especially cancer cells. Appreciating, existing ambiguity in the definition of a membrane protein, we have attempted a meta analysis of the published membrane protein reports of hESCs by using a combination of protein databases and prediction tools to find the most confident plasma membrane proteins in hESCs. Furthermore, responsiveness of plasma membrane proteins to differentiation has been discussed based on available transcriptome profiling data bank.

Expert commentary: Combined transcriptome and membrane proteome analysis highlighted additional proteins that may eventually find utility as new cell surface markers.

ARTICLE HISTORY
Received 19 July 2018
Accepted 19 October 2018

KEYWORDS
Human embryonic stem cells; membrane proteomics; cell surface markers; meta-analysis; plasma membrane

1. Introduction

Human embryonic stem cells (hESCs) provide an excellent model system to study cell-differentiation during human embryonic development. As cell therapy remains one of the important goals of hESC applications [1], it is critical to have appropriate molecular analysis and characterization of these cells performed to ensure that we are able to assess their purity [2] and we are able to correlate observed therapeutic effects to the cellular changes . Membrane proteins are amongst the most important subset of hESC proteins since they can be used as cell surface markers for sorting or isolation [3]. Further, these proteins are the ones which can act as receptors or anchors for several membrane and extracellular molecules. Membrane proteins can be viewed as molecular toll-booths on the cell surface, regulating influx and efflux of different intracellular and extracellular signaling molecules. Despite their important roles in cellular signaling and cell-cell interactions, our understanding of membrane proteins is limited due to the inherent difficulties associated with their purification, cloning, and crystallization [4]. A generally accepted notion is that most of the existing membrane markers are not absolutely specific for embryonic stem cells [3]. For example, Thy1 has been considered as a marker for hESCs [5–7], but is also well expressed on the surface of fibroblasts [8,9]. Thus, identification of membrane protein markers that are specific to particular cell type, stage, and lineage remains a research priority.

Recent developments in omics technologies provide us with tools for large-scale molecular profiling of hESCs. Various studies illustrating transcriptomics changes in hESCs have been performed [10–17]; however, there is little protein evidence to correlate the mRNA level changes with protein expression and their cellular localization [18,19]. Gundary et al. examined 34 global proteome profiles of human and mouse pluripotent cells and observed that amongst more than 7000 proteins that were identified, only 169 were common in 10 or more studies [20]. The poor correlation between mRNA and protein expression profiles [21] further which highlights the urgency of the need for in-depth proteomic analysis of underrepresented membrane proteins in hESCs.

CONTACT Ghasem Hosseini Salekdeh hsalekdeh@yahoo.com; salekdeh@royaninstitute.org Department of Molecular Systems Biology at Cell Science Research Center, Royan Institute for Stem Cell Biology and Technology, ACECR, Tehran, Iran; Yu-Ju Chen yujuchen@gate.sinica.edu.tw Chemical Biology and Molecular Biophysics Program, Institute of Chemistry, Taipei, Taiwan, Republic of China; Hossein Baharvand Baharvand@royaninstitute.org Department of Developmental Biology, University of Science and Culture, ACECR, Tehran, Iran

Supplemental data can be accessed here.

Due to limited information regarding hESC membrane proteins, particularly the expression and posttranslational modification related changes they undergo during cellular differentiation, here we comprehensively review membrane protein profiling of hESCs from literature and discuss their responsiveness to differentiation based on transcriptome profiling [22]. In this report, we have used 'membrane protein' as a general term for proteins associated with any kind of subcellular membrane and cell surface proteins to emphasise plasma membrane localization.

2. The role of membrane proteins in hESCs

An in-depth knowledge of the membrane proteome is not only critical for evaluation of the role of membrane proteins in regulation of self-renewal and pluripotency of hESC, but also in profiling cell surface membrane proteins for biomarker research. Membrane proteins perform some of the most important functions in hESCs, including control of their survival and death, as listed below.

2.1. Attachment

Chief amongst apparent differences between mouse and human ESCs is the observation of dissociation induced apoptosis in the later. Chen et al. [23] and Ohgushi et al. [24] showed that this phenomenon relies on enhanced actomyosin contraction. Furthermore, loss of E-cadherin and consequent dysregulation of attachment resulted in blebbing and apoptosis [24]. The extracellular matrix and integrin regulation of stem cells has been reviewed elsewhere [25–28]; interestingly, a consistent observation is that high levels of integrin α6β1 expression in hESCs supports their self-renewal [29]. Laminin α5 (subunit of laminin-511) is the primary ligand of integrin α6β1 and has been shown to be associated with the functionality of feeder cells in hESC culture [30] and promotion of self-renewal [31]. Silencing laminin α5 resulted in reduced expression of integrin α6 and reduced Oct4 levels [29]. Modulating the cell–cell and cell–matrix interactions with substrate or ligands by selective activation of signaling pathways can be used to differentiate hESC [32].

2.2. Ion channels and transporters

Acquisition of electrophysiological features is a prominent manifestation of the differentiation of cells into neural and muscular lineage. However, pluripotent human stem cells (hESCs and induced pluripotent stem cells) functionally express specialized ion channels [33,34] which play important roles in the regulation of cell proliferation [as reviewed in [35,36]]. The expression analysis of ion channels in hESCs at the transcriptomics level has revealed approximately 100 voltage-gated ion channel genes, ten transcripts of which were significantly expressed [33]. Potassium is known to be a pluripotency- associated inorganic element in human pluripotent cells and changes in intracellular potassium concentration due to altered membrane permeability are known to perturb the core components of pluripotency signaling and cell reprogramming [37]. The proliferation of ESCs is also regulated by the mobilization of calcium concentration via different calcium channels [38].

2.3. Recognition

Antibodies recognizing cell-surface proteins are frequently used to identify and characterize various cell types. The CD markers CD9, CD24, CD133, CD29, CD90, and CD117 are expressed in hESCs, and panels of various CD markers have been used to characterize undifferentiated hESCs and identify their neural derivatives [39]. Recently, comparative quantitative proteomics approaches have been utilized to search for surface markers that specifically label the ISL1+ cardiac progenitor cells derived from hESCs [40] .

2.4. Receptors

Binding of Activin A/Nodal to their plasma membrane receptors is important to maintain hESCs in their undifferentiated state [41,42] and promote self-renewal during elevated PI3K/AKT conditions [43]. FGF2 (also known as bFGF) or IGF-2 can activate the PI3K/AKT pathway [for review see 43] and both FGF and IGF receptors are known to be hESC surface markers (supplementary Table S1, https://figshare.com/s/6a101da5be9949141082).

BMP binding to its receptors can induce cell differentiation into extra-embryonic [44] or mesoderm lineages [45]. As discussed in a previous study from our laboratory, the role of Wnt signaling in hESCs is debatable [46], as it is implicated in both maintenance of stem cell properties [47–50] and cellular differentiation [51,52]. We have also shown that the activation of the noncanonical pathway of Wnt signaling might be mediated through Frizzled and its coreceptors ROR and VANGL proteins [46].

2.5. Enzymess

Alkaline phosphatase, a membrane bound enzyme, is a traditional marker of both mouse and human ESCs which is downregulated during differentiation [53]. However, its importance and function in ESCs remains ill-defined. CD38, another membrane enzyme that helps to convert nicotinamide adenine dinucleotide (NAD) to cyclic adenosine diphosphoribose (cADPR), is required for the neural [54] and cardiomyocyte [55] differentiation in the mouse ESCs.

3. Why human ESC proteomics?

Major breakthroughs in hESC research have been made by the identification of proteins such as cell-surface CD molecules. Ghazizadeh et al. used comparative proteomics to identify a surface marker that enabled the isolation of LIM-homeodomain transcription factor ISL1 ($ISL1^+$) progenitor cells. ISL1 marks multipotent cardiac progenitors that give rise to cardiac muscle, endothelium, and smooth muscle cells. Using a genetic selection strategy, they enriched $ISL1^+$ cells derived from hESCs and performed comparative quantitative proteomic analysis of enriched $ISL1^+$ cells. They identified ALCAM (CD166) as a surface marker that enabled the isolation of $ISL1^+$ progenitor cells. Transplantation of $ALCAM^+$ progenitors to a

rat model of myocardial infarction enhanced tissue recovery, restored cardiac function, and improved angiogenesis [40].

Recently, Fathi and colleagues used a shotgun proteomic approach to find reliable membrane protein markers for isolation of midbrain dopaminergic neurons. They generated a LIM homeobox transcription factor 1 alpha (LMX1A) knock-in GFP reporter human embryonic stem cell (hESC) line that marks the early dopaminergic progenitors during neural differentiation. Further quantitative proteomics analysis of purified GFP positive (LMX1A+) cells identified several membrane-associated proteins including contactin 2 (CNTN2), enabling prospective isolation of LMX1A+ progenitor cells. Transplantation of the purified CNTN2 + progenitors has been shown to enhance dopamine release from transplanted cells in the host brain and alleviated Parkinson's disease-related phenotypes in animal models [56].

Proteins, which are key players in the cell, have diverse features that are not predictable based on gene sequences or transcript levels. For example, post-translational modifications, protein-protein interactions, and subcellular localization affect the function and activity of proteins and hESC commitment; however, these features are difficult to predict using genomics or transcriptomics technology. Additionally, numerous questions remain in the field of hESC research, which can be most efficiently answered by employing proteomics analysis. For example, cell surface proteins, signaling cascades of hESCs and their differentiated progenies are largely unknown, as are the pluripotency maintenance or differentiation-specific proteins that can be used as biomarker for the intermediate or terminal steps of cell differentiation [57]. Thus, proteomics and hESC research need to be performed concurrently to maximize the potential for generation of important new knowledge.

4. Current common hESC membrane protein markers

Success in hESC derivation, isolation or sorting depends on stage- and lineage-specific markers, preferably located on the cell surface. Such markers are also required for efficient ESC maintenance or differentiation protocols. Numerous review articles have summarized various hESCs markers [3,5,58–61]; however, most of our knowledge about hESCs membrane markers is based on immunocytochemistry or flow cytometry followed by PCR confirmations at the mRNA level. Supplementary Table S1 lists the commonly used hESCs membrane markers.

https://figshare.com/s/6a101da5be9949141082

From a structural perspective, we initially sought to determine whether these reported markers were integral membrane proteins containing transmembrane (TM) domains. We predicted the TM helices using TMHMM version 2.0 [62] followed by SignalP (version 4.1) confirmation for single TM containing proteins [63]. Our computation results showed that most of these markers lack TM helices (Supplementary Table S1,

https://figshare.com/s/6a101da5be9949141082).

Although these commonly available markers are valid enough to accurately isolate hESCs from other kind of cells or their derivatives, many results have not been reproduced in other hESC lines or differentiated progenies. One critical factor is the specificity of these reported hESC markers.

Some hESCs surface markers (Supplementary Table S1, https://figshare.com/s/6a101da5be9949141082) have been reported in other cells [64], which might imply that they are expressed developmentally. A typical example is CD90 (Thy1), which is a stem cell marker [5–7] and also a well known fibroblast marker [9,65–68]. A number of hESC surface markers have been used for other cell types. For example, BMPR1A is considered to be a promising ganglion cell marker [69]. Connexin-43 is widely expressed in the adult astrocytes [70,71] and is a major connexin in breast tissue [72]. CDH1 is a specific marker for undifferentiated type A spermatogonia [73] and is expressed in numerous adult cell types [74]. CD9 is a marker of plasma cells [75] while non-tissue specific isozyme of alkaline phosphatase is particularly abundant in hepatic, skeletal, and renal tissues.

Most characterized membrane markers are shared between embryonic stem cells and cancer cells [3], which may reflect the similarities between these two types of cells. Since 1984, HER2 (ERBB2) has been known to have an important role in cancer biology [76] and is amplified in about 20% of breast cancers [77]. ERBB3 somatic mutations were reported in approximately 11% of colon and gastric cancers, in addition to their role in breast cancers [78]. For more than 30 years, IGF1R has been known as a marker for various cancer lines (for review, see [79–82]). Fibroblast growth factor receptors (FGFRs) are found in several tissue types and are reported to be over-expressed in cancer tissues (for review, see [83]). Integrin alpha 6 is present in endothelial cells and is up regulated during angiogenesis [84]. Integrin a6 is not only an enrichment marker, but also a promising therapeutic target in certain cancer cell types such as glioblastoma [85]. Moreover, a6 integrin has been shown to be required for the growth and survival of breast cancer stem cells [86], and is also used as a prostate cancer stem cell marker in prostate carcinoma cells [87,88]. Ep-CAM is expressed in numerous epithelial tissues and up-regulated in tumor cells, specifically hepatocellular carcinoma [89], and is a marker for cancer-initiating cells [90,91]. CD133 (PROM1) is found in epithelial cells [92–94] and is also expressed in both cancer stem cells and differentiated tumor cells [95], but is not limited to adult stem or progenitor cells [96]. Podocalyxin is a marker of colorectal cancer [97,98] where its function is dependent upon the tumor location [99]. TDGF1 is a well-known target for cancer immunotherapy [100,101].

The existence and role of commonly used hESC membrane markers in many other cell types, especially cancer cells, will need to be elucidated for the improved clinical application of ESCs, that needs well characterized and purified cells [3].

5. 5. hESC membrane proteomics studies

The rapid progress in stem cell technology, accompanied by the necessity for discovery of valid new markers, has brought high throughput omics techniques to the forefront of hESC research and applications. Previously, Cell Surface Capture (CSC) technology has been used to generate a mass-spectrometry derived Cell Surface Protein Atlas of 41 human and 31 mouse cell types [102] . Recently an excellent review of cell surface proteomic enrichment has been

published which discussed cell-surface proteomics for the identification of novel therapeutic targets in cancer [103]. Unbiased in-depth analyses of transcriptomes and proteomes are expected to continue to exert dramatic changes in stem cell biology, that are difficult to be achieved through traditional trial and error-based methods. Various proteomic technologies have been used to decode the membrane proteome of hESCs (Supplementary Table S2, https://figshare.com/s/6a101da5be9949141082), and their potential role in the key biochemical and cellular signaling networks.

Since the exact localization of membrane proteins is experimentally difficult to define, we performed a meta analysis of published annotations for membrane proteins using four bioinformatics parameters obtained from protein databases and computation tools: subcellular localization as 'cell membrane' from UniProt, subcellular compartment as 'plasma membrane' from Gene Ontology (GO: 0005886) [104], prediction of TM helix by TMHMM [62] and signal peptide prediction by SignalP [63] as reported previously [46]. According to these parameters, four protein categories have been described: (1) plasma membrane proteins (PM proteins); (2) probable peripheral membrane proteins (PPM proteins) (3) probable membrane proteins (PMem proteins); and (4) non-membrane proteins (NonMem proteins), which comprised all other proteins. The first three categories can be considered membrane associated proteins (MAPs).

The first large-scale analysis of the hESC plasma membrane proteome was published by Harkness et al. In this study, homogenization of hESC-OD3 cells independent of culture conditions followed by tandem high-speed and ultracentrifugation of homogenate was performed to remove the mitochondria and nuclei and to isolate membrane fractions, respectively [105]. The membrane pellet was washed with carbonate and ammonium bicarbonate buffers followed by Fourier transform LC-ESI-MS/MS analysis [105]. Based on the results, 77% of the integral membrane proteins identified were shared between two different growth conditions (Matrigel and feeder) [105]. This report provided an initial description of hESC membrane proteomes. Several of the known hESC membrane-associated markers were not identified in their proteome profiles (Supplementary Table S2, https://figshare.com/s/6a101da5be9949141082).

Dormeyer et al. [106] performed membrane isolation through ultracentrifugation of a sucrose cushion followed by carbonate washing. They examined the efficiency of different hESC sample preparations and digestion procedures, along with their impact on the quality of, and compatibility with, subsequent mass spectrometric analysis. By comparing the membrane proteome of hESCs and human carcinoma cells, the receptors FZD2, FZD6, FZD7, and LRP6, and the modulator SEMA7A, were validated as candidates for studying the differential regulation of Wnt signaling in embryonic and carcinoma stem cells [106]. As shown in Figure 1(a), approximately two-third of proteins identified in that study were MAPs.

Following high-speed ultra-centrifugation, McQuade et al. [107] applied immobilized pH gradients (peptide IPG-IEF) followed by (LTQ) linear ion trap mass spectrometry to increase membrane proteome coverage from a small amount of the hESC line SIVF001 [107]. Approximately 40% of identified proteins by this approach were categorized as MAPs (Figure 1(b)).

Prokhorova et. al. and Sarkar et. al. (Figure 1(c,f)) used a SILAC labeling approach in hESC membrane proteomics [108,109] . Prokhorova and colleagues showed that six membrane proteins (CD133/Prominin-1, Glypican-4, Neuroligin-4, ErbB2, PTPRZ, and Glycoprotein M6B) have greater than three-fold higher expression in the undifferentiated state, which was confirmed by real time PCR analysis and fluorescence-activated cell sorting [109]. Sarkar et al. performed subcellular fractionation of a SILAC labeled H9 hESC line by utilizing a discontinuous sucrose gradient [108], followed by LTQ-Orbitrap XL high-resolution mass spectrometry analysis. Based on the combination of relative protein expression and subcellular localization, the components of a number of signaling pathways such as BMP receptor, FGF and TGF-beta receptor signaling were identified in undifferentiated hESCs [108]. Compared to Prokhrova et al. [109], subcellular fractionation [108] was relatively more successful in resolving MAP proteins (33% compared to 20%, Figure 1(c,f)).

Gu et al. purified membrane proteins from the HUES3 line by biotinylation followed by protein fractionation with SDS-PAGE and identification by LTQ Linear ion trap MS [110]. Among the 400 randomly selected membrane proteins, the expression levels of 328 membrane proteins on hESCs were confirmed by PCR [110] – the highest percentage of PM proteins (25%) was identified by this method (Figure 1(d)).

Gerwe et al. used high speed centrifugation for isolation of a membrane fraction from the hESC lines BG01 and WA09, followed by ion trap FT-ICR mass spectrometric analysis [111]. Based on their criteria, they validated dysferlin as a distinguishing candidate marker showing expression differences among hESC lines, and ciliary neurotrophic factor receptor as a candidate marker specific to WA09-derived human neural progenitor cells [111]. Approximately 80% of proteins identified belonged to the MAP category (Figure 1(e)).

Melo-Braga et al. performed peptide dimethyl labeling of membrane protein extracts of hESCs and NSCs followed by LTQ Orbitrap Velos mass spectrometric analysis and introduced potential NSC markers including Crumbs 2 and several novel proteins [112]. However, Less than 40% of identified proteins in this study were MAPs (Figure 1(g)).

Our group reported subcellular proteomics of hESCs for six fractions; nucleus, mitochondria, cytoplasmic, crude membrane, and light and heavy microsomes [46]. The localization of three novel hESC membrane proteins (ERBB4, GGT1 and ZDHHC13) was confirmed by immunocytochemistry and their functions assessed in terms of pluripotency. Compared to Sarkar et al. [108], we have been more successful in resolving MAPs through these approaches (Figure 1(h)).

Finally Weldemariam et al. achieved identification of 11,970 unique proteins in hESCs from the three subcellular fractions (membrane, nucleus, and cytoplasm) of which they have annotated 6,138 as membrane proteins and mined a total of 296

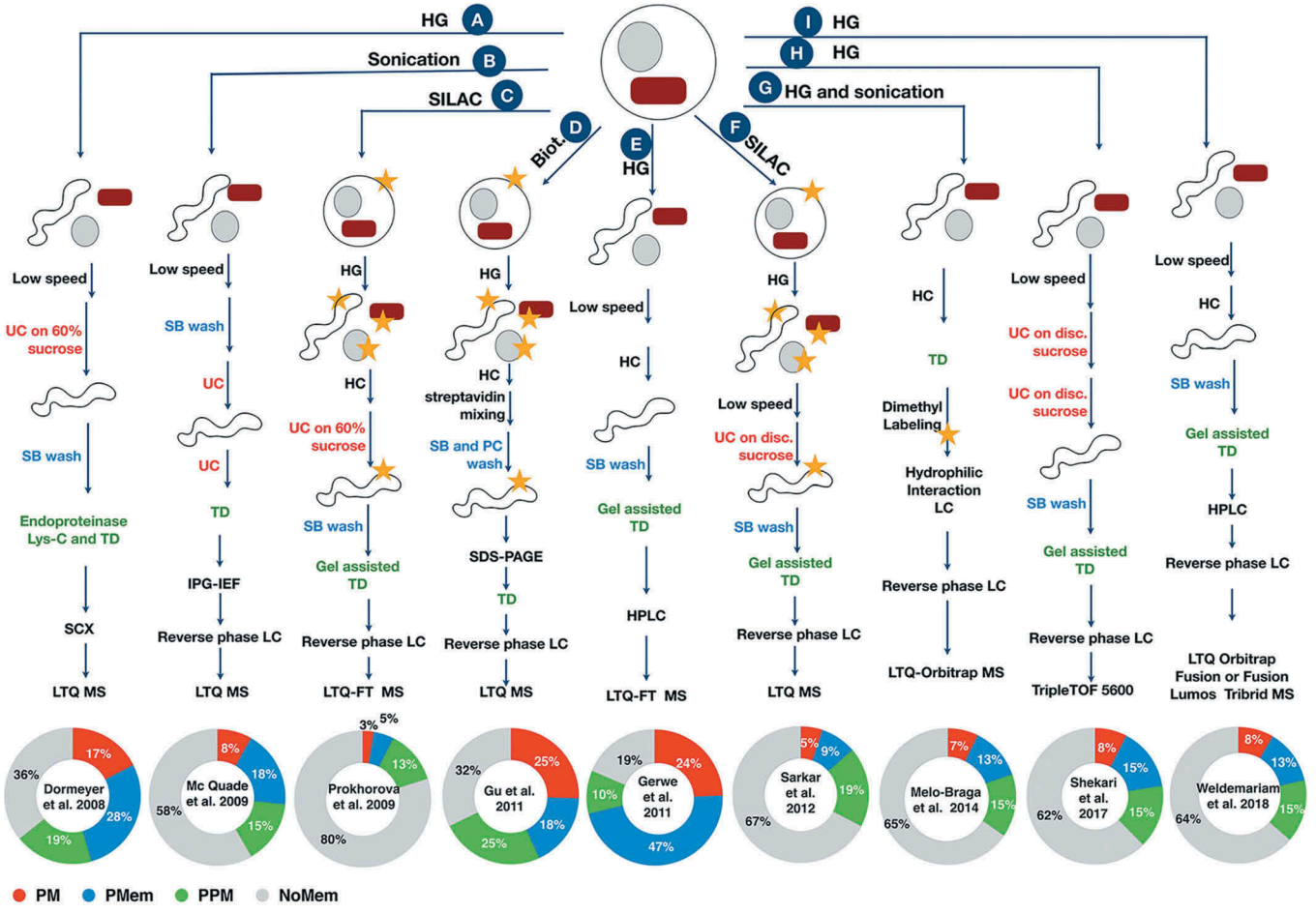

Figure 1. **Flow chart of membrane proteomic analysis approaches and categorization of identified proteins derived from nine membrane proteomics reports presented in Supplementary Table S1)**. As Harkness et al. did not presented identified proteins, we excluded it from this analysis. All identified proteins of nine membrane proteomics reports have been grouped into four categories: PM: plasma membrane, PMem: probable membrane, PPM: probable peripheral membrane, and nonmembrane (NonMem). Gold star and red rectangle indicates labeling and mitochondria, respectively.

Abbreviation: SCX, Strong Cation Exchange; SILAC, Stable Isotope Labeling by Amino Acids in Cell Culture; SB, Sodium bicarbonate; PC, Potassium chloride; HG, Homogenization; UC, Ultracentrifugation; HC, High speed centrifugation (10,000–30,000 Xg centrifugation); Biot., Biotinylation; Disc. sucrose, Discontinuous sucrose gradient; TD, tryptic digestion.

candidate detections of missing proteins [113]. This large-scale proteomic data was one of the only successful report in finding commonly used hESCs membrane markers (Supplementary Table S2, https://figshare.com/s/6a101da5be9949141082).

Compared to the relatively mature transcriptomic technologies, however, proteomic knowledge of hESCs is far from comprehensive. As discussed previously [114], some of the major problems of membrane proteomics are contamination of intracellular components, protein insolubility, low abundance, and loss of hydrophobic peptides, which hinder or interrupt protein identification [114]. In addition, the high cost of growing hESCs, and variation observed in different culture systems makes hESC membrane proteomics analysis more complicated compared to other cell types. This might also reflect to some extent the fact that different reports showed poor overlap of the identified proteins and markers. Each membrane proteomics report shown in Supplementary Table S2 has used a different approach for membrane protein isolation or identification. However, not all commonly used membrane markers of hESCs are reported in most of these membrane proteomics studies. For example, TDGF1 is highly expressed in both mouse and human ESCs [115] and plays an important role in regulating stem cell proliferation and differentiation [116]. However, it has been identified only in large scale proteomic data provided by Weldemariam et al. In contrast, a summary of these studies has shown that ITGA6, PROM1, GJA1, CDH1, EPCAM, CD9, and ALPL are ubiquitous proteins found in most proteome reports (Supplementary Table S2, https://figshare.com/s/6a101da5be9949141082).

Ultracentrifugation has been shown to be a favorite method for membrane isolation, however, Gerwe et al. [111], Gu et al. [110], and Weldemariam et al. [113] presented successful membrane protein isolation without utilizing ultracentrifugation (Figure 1(e, i)). The cell-surface biotinylation approach has also been shown to be a relatively powerful method for enrichment of PM proteins. High resolution MS has been used in most recent reports and an

LTQ orbitrap fusion mass spectrometry-based approach achieved the highest number of MAPs (3212 proteins) [113].

6. When transcriptomics meets membrane proteomics

Analysis of membrane proteins is experimentally challenging due to their high hydrophobicity indices, wide dynamic range and dramatically lower abundance than that of cytoplasmic proteins. All of these factors complicate their solubilization, sample handling, preparation, separation, and analysis. None of the eleven published membrane proteome reports (Supplementary Table S2) provided us with information about differentiation responsive membrane proteins.

Numerous transcriptomic studies have reported gene expression profiling of hESCs [10–17]. Mallon et al performed global transcriptome analysis of 21 hESC lines in two different states, stem cell and differentiated to ectodermal and mesendodermal lineages [22]. In order to obtain statistically confident data, transcripts with an average expression value in at least three lines of less than 7.5 were discarded from NIH transcriptomic report. By applying this criterion, 23,428 probes were selected from a total of 41,000 reported probes. Approximately 1700 transcripts out of 13,441 were pseudogenes without UniProt records.

Kolle et al. utilized a distinct approach known as membrane-polysome translation state array analysis (TSAA), which is based on the transcript analysis of actively translated, membrane-bound polysomes. Among the 1017 identified proteins, TMHMM predicted 678 TM-containing proteins (up to 35 helices). Among the 88 gene-encoded membrane proteins that marked the pluripotent subpopulation, cell surface immunoreactivity was confirmed for TACSTD1/EPCAM and CDH3/P-cadherin. They also demonstrated that antibodies against EPCAM could be used to enrich the hESCs from heterogeneous cell populations [18].

A total of 10,198 unique proteins have been identified in ten membrane proteome reports (Supplementary Table S3, https://figshare.com/s/6a101da5be9949141082). As illustrated in Figure 2, the transcript of approximately 2400 proteins were not identified in the transcriptome or membrane transcriptome reports. More than 90% of proteins commonly identified

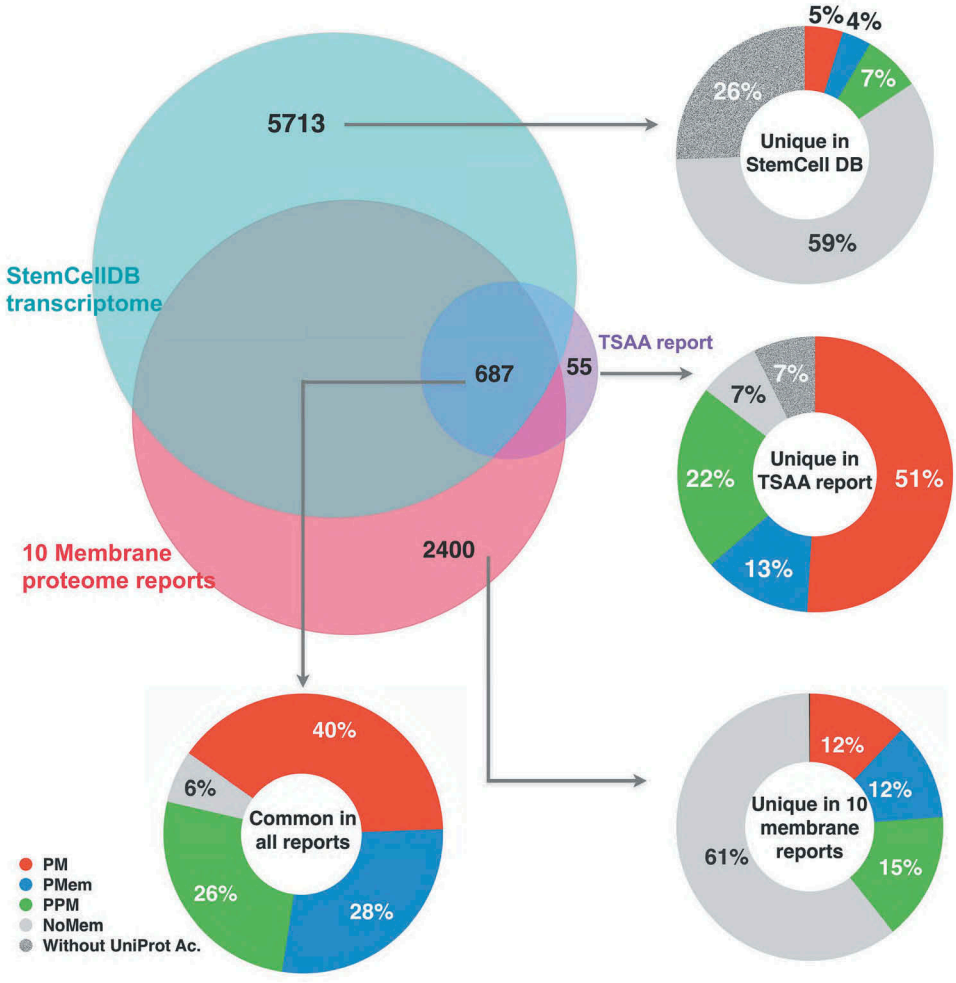

Figure 2. A Venn diagram summarizing the overlap between membrane proteome, transcriptome and membrane transcriptome (TSAA) reports of hESCs. All the uniquely and commonly identified proteins or transcripts have been categorized into four categories: plasma membrane (PM), probable membrane (PMem), probable peripheral membrane (PPM), and nonmembrane (NonMem) and visualized by pie charts.

in membrane proteomics, TSAA, and transcriptomics reports of hESC were also MAPs (Figure 2). Transcriptome analysis of mRNAs bound to actively translated, membrane-bound polysomes (Translation state array analysis or TSAA) seems very successful in resolving membrane associated transcripts since 85% of uniquely identified transcripts belonged to MAPs. However, many of the identified transcripts are pseudogenes without confirmed protein counterpart. The analysis above shows that protein and transcript based experiments are indeed complementary, and are more informative when considered together rather than in isolation.

When we considered up- and downregulated transcripts which were differentially expressed in more than 75% of lines, and categorized these transcripts into the four formerly discussed categories, we found 956 and 971 down- and upregulated transcripts involved in differentiation (Supplementary Table S4,

https://figshare.com/s/6a101da5be9949141082). A comparison between overlap of proteome data and differentially expressed transcripts during differentiation to ectodermal or mesendodermal lineages showed that 76% (475 out of 626) of all differentiation responsive membrane associated transcripts were covered by membrane proteome reports. Approximately 30 PM proteins found in more than four membrane proteomics reports were responsive to differentiation (Supplementary Table S5, https://figshare.com/s/6a101da5be9949141082). Among them, four PM proteins, namely plasma membrane calcium-transporting ATPase 4 (ATP2B4), monocarboxylate transporter 4 (SLC16A3), FXYD domain-containing ion transport regulator 6 (FXYD6) and bone marrow stromal antigen 2 (BST2), were upregulated in both mesendodermal and ectodermal differentiation. It has been shown that BST2 is involved in the osteogenic differentiation via the regulation of the *BMP2* signaling pathway [117]. Seven PM proteins were down regulated in both differentiations according to StemCell DB, and have been identified in more than four membrane proteome reports. These include Feline leukemia virus subgroup C receptor-related protein 1 (FLVCR1), Gastrin-releasing peptide receptor (GRPR), Zinc transporter 1 (SLC30A1), Ectonucleotide pyrophosphatase/phosphodiesterase family member 1 (ENPP1), Gamma-aminobutyric acid receptor subunit beta-3 (GABRB3), Pannexin-1 (PANX1) and Caveolin-1 (CAV1). It has been shown recently that undifferentiated human stem cells express pannexins, with Pannexin-1 showing the highest expression [118]. Therefore, meta analysis of membrane proteome and transcriptomics data revealed some membrane proteins that can be readily identified in proteomics reports and their transcripts are responsive to differentiation. The importance and function of these potentially differentiation responsive membrane proteins remained to be understood.

7. Expert commentary: opportunity for mining new potential markers

One of the important goals of high-throughput technologies is hypothesis generation in order to suggest new scope for hypothesis-driven research fields. A membrane protein that has been reported in various membrane proteomics screens can be considered a potentially important target with a special function in hESCs. Accordingly, the differentially expressed profile of proteins can potentially be regarded as a marker of particular cell state.

A Venn diagram of seven membrane proteomics reports (Supplementary Table S1, excluding reports that showed less than ten uniquely identified proteins) showed unique and common identified proteins (Figure 3(b)). The highest number of uniquely identified proteins belonged to Weldemariam et al. [113]. In second place, utilizing biotin ester and peptide dimethyl labeling coupled to mass spectrometry analysis respectively, Gu et al. [110] and Melo-Braga et al. [112] identified the highest number of unique proteins (514 and 551). Since all of these groups reported a high number of total identified proteins as well (Supplementary Table S1, https://figshare.com/s/6a101da5be9949141082), biotin labeling seemed more successful in resolving MAPs (68% compared to 33% and 21% for Melo-Braga and Weldemariam reports, respectively).

This data together with data presented in Figure 1, might indicate the potential of biotin labeling [110] (Figure 1(d)) and HPLC (Figure 1(e and i)) coupled with LTQ mass spectrometry in resolving membrane proteins.

Overall, 18 proteins were commonly identified in all ten membrane proteomics reports (Figure 3(a)). These proteins and their probable localization in cells are illustrated in Figure 3(a), which indicates that all of them are localized in membrane parts of the cell.

The function of these proteins in hESCs has not been reported; however, most are proliferation-related proteins in other cells. A close correlation between solute carrier family 7 member 5 (SLC7A5 or LAT-1) expression and cell proliferation has been shown [119]. For example, after binding to progesterone, progesterone receptor membrane component 1 (PGRMC1) can localize to the cytoplasm to elevate cell responsiveness to the anti-apoptotic action of progesterone [120]. However, PGRMC1 that is not bound to progesterone has the opposite effect in the nucleus by regulating gene expression on behalf of apoptosis [120]. Reticulon 4 (RTN4) regulates apoptosis and tumor development [121]. Cytoskeleton-associated protein 4 (CKAP4 or p63) is involved in the regulation of cancer stem cell metabolism [122]. It has been shown that calnexin enhances the STAT3-mediated transcriptional response to EGF [123]. Basigin (BSG or CD147) is a multifunctional protein that plays key roles in both normal tissue remodeling and cancer microenvironment regulation [124]. A panel of 22 candidate marker proteins in pluripotent cells was developed, including BSG [125].

Interestingly, they were also responsive to differentiation according to StemcellDB (Supplementary Fig. S1). According to the transcriptome results the expression level of ATPase, Na^+/K^+ transporting, beta 3 (ATP1B3), SLC7A5, PGRMC1, solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 5 (SLC25A5), and leucine-rich repeat containing 59 (LRRC59), were all decreased during differentiation in both the ectodermal and mesendodermal lineages. They can be considered as new candidate surface markers for hESCs in the stemness state.

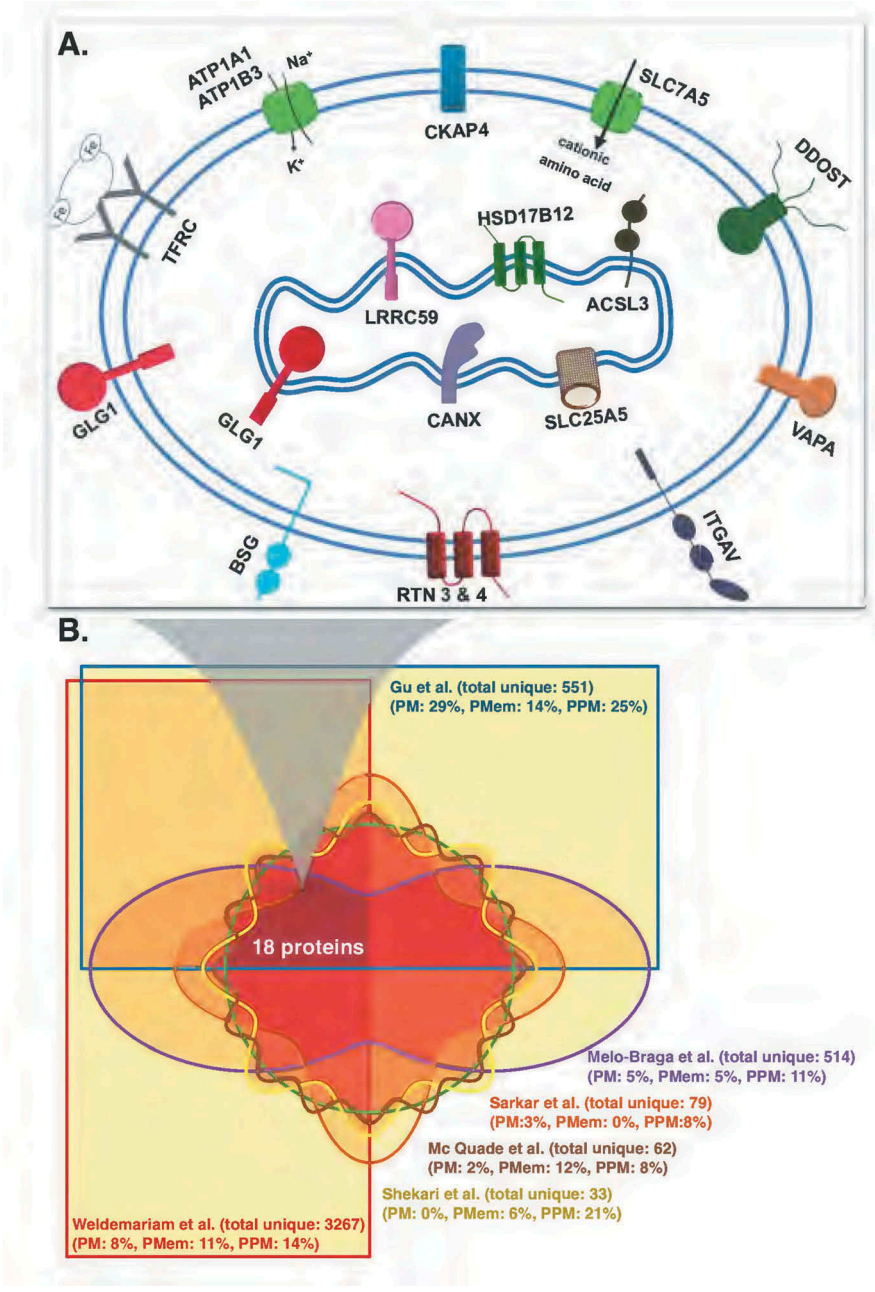

Figure 3. **Common proteins found in all membrane proteomics reports of hESCs**. Localization of 18 common proteins in the plasma or inner cell membranes (a). Venn diagram of eight membrane proteomic reports of hESCs that show 18 proteins found in all reports. The number of proteins uniquely reported in each of reports are shown in parentheses and also membrane protein categorization into our categories: plasma membrane (PM), probable membrane (PMem), probable peripheral membrane (PPM), and nonmembrane (NonMem) (b).

8. Five-year view

ESC technology is a growing field of biotechnology with tremendous potential for advances in medicine and biology. Although numerous hESC lines have been derived, various questions remain regarding their characterization. Considering one of the final goals of hESC biology as cell therapy, specific markers are required to isolate and purify the stem cells, or their derivatives, by sorting. We have shown that although there are various membrane proteome reports, there are few common proteins found in all reports; surprisingly, all of these commonly reported proteins show differential patterns of expression in some hESC lines, which highlights them as potential hESC markers. Among all 18 prevalent membrane proteins, BSG has been recently published as a potential marker for human pluripotent stem cells [125]. Teratoma formation has been a big concern in hESC therapeutic applications [126,127]. Isolating hESC with non-cancer related markers may pave the way to solve the teratoma formation problem in hESC therapy. More than 70% of the commonly used membrane markers of hESCs have been annotated as cancer-related in the Human Protein Atlas [64]. However, among the 18 proteins found in our meta-analysis of all membrane reports of hESCs, just five proteins are marked as cancer-related (Supplementary Fig. S2).

In 2007, the Human Proteome Organization (HUPO) and International Society for Stem Cell Research joint initiative, Proteome Biology of Stem Cells, was established as a collaborative platform to bring together stem cell biologists and researchers in proteomics [128]. During the intervening years, groups associated with this initiative have held workshops aimed at optimization of protocols for hESC sampling as well as MS and bioinformatics analyses. The initiative has successfully chosen hESCs for initial study from the ES cell bank of the International Embryonic Stem Cell Consortium. Another initiative centered on membrane proteins from Asia-Oceania HUPO (AOHUPO) was launched previously and has performed successful collaborative analyses, including sampling for MS analysis of various cell types [129]. There is great promise in the analysis of hESC membrane proteins for standardizing biomarker discovery, which will help to provide standard guidelines for hESC research and application. hESCs can be also used in C-HPP pilot project for functional characterization of identified proteins with no known function [130].

It is also important to study the impact of the growth environment on the hESC cell surface proteome.

Establishing reproducible and versatile large scale hESC production systems may increase the interest in hESC and its derivatives membrane proteome profiling which require a large amount of cells. Furthermore, advances in mass spectrometry will allow identification of more membrane proteins with lesser amount of hESCs.

Key issues

- Membrane proteins perform some of the most important functions in hESCs, including control of their survival and death.
- Success in hESC derivation, isolation, or sorting depends on stage- and lineage-specific markers, preferably located on the cell surface, although most of the existing membrane markers are not absolutely specific for embryonic stem cells and can be found in stem cell derivatives as well.
- Compared to the relatively mature transcriptomic technologies, however, proteomics knowledge of hESCs is far from comprehensive.
- A total of 10,198 unique proteins have been identified in membrane proteome reports.
- In order to identify true plasma membrane proteins, we have categorized all reported proteins into three main membrane associated protein (MAPs) classes: plasma membrane (PM), peripheral to membrane (PPM), and membrane proteins (PMem). More than 90% of proteins commonly identified in membrane proteomics, membrane transcriptomics and transcriptomics reports of hESC were also MAPs.
- Due to limited information regarding hESC membrane proteins, particularly differential expression changes during cellular differentiation, here we comprehensively review, membrane protein profiling of hESCs from literature and discuss their responsiveness to differentiation based on transcriptome profiling.
- Our meta-analysis showed the potential of subcellular fractionation and biotin labeling both coupled with LTQ mass spectrometry in resolving highest number of membrane proteins (MAPs).
- Approximately 30 differentiation responsive PM proteins found in more than four membrane proteomics are reported.
- Overall, 18 proteins were commonly identified in all membrane proteomics reports. These proteins are localized in membrane parts of the cell, are responsive to differentiation and are mostly not cancer related proteins.

Funding

This study was funded by grants provided by the Royan Institute [2016-2731350] and the Korean National Research Foundation (NRF-2017M3A9B4028143).

Declaration of interest

The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

Reviewer disclosures

Peer reviewers on this manuscript have no relevant financial or other relationships to disclose.

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