Stem Cell Research (2008) 1, 219–227

available at www.sciencedirect.com

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Identification of a membrane proteomic signature for human embryonic stem cells independent of culture conditions

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Received 10 February 2008; received in revised form 25 June 2008; accepted 25 June 2008

Abstract Proteomic profiling of human embryonic stem cells (hESC) can identify cell fate determination and self-renewal biomarkers. Employing Fourier transform LC-ESI-MS/MS and MS^3 mass spectrometry, we obtained a membrane proteomic signature overlapping between hESC cultured on mouse embryonic fibroblast (MEF) feeders and those grown under MEF-free culture conditions. We identified 444 transmembrane or membrane-associated proteins, of which 157 were common between both culture conditions. Functional annotation revealed CD antigens (10%), adhesion proteins (4%), proliferation-associated proteins (4%), receptors (41%), transport proteins (21%), structural proteins (5%), and proteins with miscellaneous functions (15%). In addition, 15 CD antigens and a number of surface marker molecules not previously observed in hESC at a proteome level, e.g., Nodal modulator 1, CD222, transgelin-2, and CD81, were identified. In conclusion, we describe the first membrane proteome profile of hESC that is independent of culture conditions. These data can be used to define the phenotype of hESC.

Introduction

One of the major issues facing the understanding of the biology of human embryonic stem cells (hESC) and their use in therapy is the absence of well-defined hESC-specific markers that can be employed to ensure quality control of hESC, to compare cell lines obtained from different laboratories, and to identify differences between hESC lines propagated under different culture conditions. Previous studies have tried to identify a "molecular signature" of hESC based on gene expression profiling (Abeyta et al., 2004; International Stem Cell Initiative (ISCI), 2007; Laslett et al., 2007; Sato et al., 2003). The first global transcriptional profile of a single hESC line, the Wisconsin H1 cell line, grown

Abbreviations: hESC, human embryonic stem cell; LC-ESI-MS/MS, liquid chromatography electrospray ionization mass spectrometry[2]; LC-FTICR, liquid chromatography Fourier transform ion cyclotron resonance; MEF, mouse embryonic fibroblast; MG, Matrigel; TMHMM, transmembrane helix prediction based on a Markov model.

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doi:10.1016/j.scr.2008.06.001

on mouse feeder cells identified the presence of 918 genes enriched in undifferentiated cells compared with differentiated cells (Sato et al., 2003). Also, a comparison of H1 cells to undifferentiated mouse embryonic stem cells showed several genes expressed in common, suggesting the presence of a core pluripotency program (Sato et al., 2003). In another study, transcriptome analysis using Affymetrix oligonucleotide microarrays of various, independently derived hESC grown on mouse embryonic fibroblast feeders demonstrated that the majority of genes expressed in hESC lines were similar, but a small number showed a unique expression profile, which could be explained only by either culture conditions or intrinsic genetic differences (Abeyta et al., 2004). A recent microarray study focusing on gene expression profiles of early lineage committed hESC has reported that in addition to genes associated with pluripotency, lineage-specific transcription factors were coexpressed during early differentiation stages (Laslett et al., 2007). Recently, the International Stem Cell Initiative (ISCI, 2007) employed low-density arrays and identified a common expression profile for 59 hESC lines. While these studies are useful, the identified markers cannot provide a complete picture of the functional proteins being expressed.

Mass spectrometry-based proteome analysis is a powerful tool for identification of proteins by sequencing of peptides from complex mixtures and has been employed to describe the biological nature of a variety of cell types, including adult stem cells (Adachi et al., 2007; Cox and Mann, 2007; Foster et al., 2005). However, application of this technology to hESC has been limited. Two studies have reported proteome analyses of human embryonic stem cells using whole-cell lysates. The first, a study by Baharvand et al. (2006) employed 2D gel electrophoresis followed by MALDI TOF/TOF mass spectrometry. The second study, by van Hoof et al. (2006), employed 1D gel separation, in-gel digestion, and LC-FTICR-MS/MS analysis. While these studies have provided interesting data regarding the pluripotent proteome profile of the cells, they were not directed toward identification of new surface markers, since no specific procedure was performed to enrich for membrane proteins.

Applying proteome analysis for the identification of novel cellular surface markers to a reasonable proteome coverage has, traditionally, been hampered by the poor probability of obtaining peptides from low-abundance plasma membrane (PM) proteins in a complex mixture. A previously published PM isolation procedure from our group (Foster et al., 2005) demonstrated its suitability for identifying novel low-abundance surface markers of human mesenchymal stem cells (hMSC). The aim of the current study was therefore to apply the same methodology with modifications to obtain low-abundance membrane proteins of hESC. Since hESC are currently cultured under different *in vitro* conditions, we wanted to identify a common proteome profile of hESC independent of culture conditions. We employed hESC populations obtained from two different but standard and commonly employed culture conditions, feeder-containing and feeder-free cultures, and we carried out membrane proteome analysis using a method based on LC-ESI-FTICR-MS^2 and MS^3. Our data present a "membrane proteome profile" of hESC, which provides insights into self-renewal programs characteristic of hESC and identifies novel surface markers characteristic of hESC.

Results and discussion

Characterization of hESC-OD3

hESC-OD3 cells were grown successfully both on inactivated mouse feeder cells and under feeder-free culture conditions, forming colonies that expressed the pluripotency markers Oct-4, Nanog, Sox2, Tra1-81, and alkaline phosphatase (ALP), visualized by immunocytochemistry. Also, hESC-OD3 cells expressed Tra1-60, Tra1-81, and SSEA4 as demonstrated by FACS analysis (Supplementary Fig. 1). In addition, the cells expressed Oct4, Sox2, Nodal, ALP, and hTERT mRNA as evidenced by RT-PCR. Similar expression levels were observed in cells grown without feeders (data not shown) and in hESC-HUES9 grown on inactivated feeders (Supplementary Fig. 2). Analysis of Giemsa-banded hESC-OD3 chromosomes revealed a karyotype of 46XX inv(9)(q20) in 20 metaphase spreads. In addition, hESC-OD3 were able to form embryoid bodies (EB) that contained cells from all three germ layers as evidenced by immunocytochemical positive staining for albumin and CK18 (endoderm), TUJ-1 (ectoderm), and CD31 and CD34 (mesoderm). hESC-OD3 obtained from MEF feeder-cell cultures were able to form teratomas when implanted under the kidney capsule and subcutaneously in NOD-SCID mice (Prokhorova et al., 2008). Data showing the characterization from colony staining, flow cytometry, karyotyping, and EB can be found in Supplementary Fig. 1. Side-by-side comparison of hESC-OD3 with a commonly employed hESC line, HUES9 hESC, revealed similar phenotypic characteristics (Supplementary Fig. 2).

Proteome analysis and functional classification of the membrane fraction

The Mascot search engine initially matched sequenced peptides to 3133 protein identifications before data validation. Following validation by the MSQuant program (for criteria see Materials and Methods), we obtained a total of 1075 proteins (539 nonredundant proteins detected in the hESC–MEF and 536 in the hESC–MG populations). Filtration of the 1075 proteins for membrane and cell surface proteins resulted in 240 proteins selective for the hESC–MEF fraction and 204 proteins selective for the hESC–MG fraction, of which 157 were found under both culture conditions (Fig. 1). Manual clustering for cellular compartmentalization using the SwissProt/UniProt and Ensembl databases of the unfiltered dataset provided information about the efficiency of the membrane-enrichment procedure.

Seventy-seven percent of the proteins shared between the hESC–MEF and the hESC–MG growth conditions were integral membrane proteins, proteins with known or predicted membrane anchors, or membrane-associated proteins, whereas the percentage of nuclear or nuclear membrane-bound proteins was lower than 7% (Fig. 2a).

Common proteins detected under the hESC–MEF and hESC–Matrigel conditions

We considered the group of 157 proteins shared between hESC–MEF and hESC–MG as a potential profile of membrane proteins independent of culture conditions. Functional

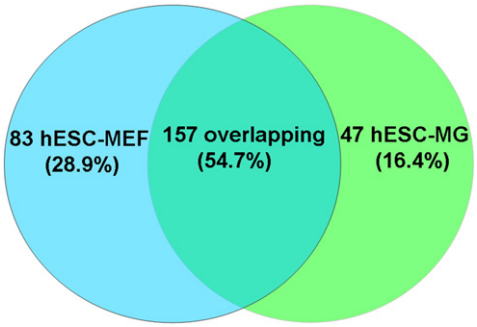

Figure 1 Common and distinct proteins found in the proteomic profile. A schematic diagram illustrating the overlap of proteins found under the two culture conditions: human embryonic stem cells cultured on a feeder layer of mouse embryonic fibroblasts (hESC–MEF) or hESC cultured under feeder-free conditions on Matrigel (hESC–MG). After validation in MSQuant and filtration using ProteinCenter, 157 (54.7%) proteins were found to be common to both profiles. Two groups of proteins, which were not found common to both conditions, were also identified, with 83 (28.9%) proteins found in the hESC–MEF set and 47 (16.4%) in the hESC–MG set.

clustering using SwissProt, UniProt, and Ensembl revealed several categories: 10% CD antigens, 4% adhesion proteins, 4% proliferation-associated proteins, 41% receptors, 21% transport proteins, 5% structural proteins, and 15% proteins with miscellaneous functions (Fig. 2b). Semiquantitation by XIC (extracted ion current) scoring of the 157 overlapping identifications revealed that the protein ratios compared between the two culture conditions did not vary more than 15%. For this analysis we calculated the protein ratios for proteins recurring in the double determinations of hESC–MEF files and hESC–MG files, respectively, and generated the XIC scores. Peptide ratios were calculated and then averaged to a protein ratio and a standard deviation (SD) was determined based on the double determinations. The average SD for the protein ratios of the hESC–MEF files was 28.25% and for the hESC–MG files was 33.25% (see Supplementary Fig. 3). Average retention time standard deviation was also calculated for the hESC–MEF files (3.16%) and for hMSC–MG (4.45%), indicating good machine performance (Fig. 3). Values plotted show the changes in abundance between corresponding peptides (Da×s) dividing integrated values of hESC–MG over hESC–MEF for peptide pairs. Eighty-five percent of proteins were found to decrease or increase by 100% with an estimated 30% variation (see Materials and Methods), or twofold increase/decrease, in a semiquantitative setting. Seven proteins (4.5%) were above the upper limit and 15 (9.6%) were below, giving levels of inaccuracy between the differences of the two states.

Identified proteins, along with their International Protein Index (IPI) accession numbers, UniProt numbers, localization, and function and, for the common protein set, XIC values and relevant references have been provided in Supplementary Table 1.

Cluster of differentiation antigens

CD antigens are cell surface molecules with a number of functions, including ligands, receptors, transporters, and cell adhesion molecules. We identified 15 different CD markers in the common profile: CD 8b, 9, 29, 36L2, 44, 66, 71, 81, 98, 107a, 147, 222, 298, 321, and 326. The known biological functions of several of the identified CD markers suggest an important role in early embryonic development. CD107a or LAMP1 is thought to be involved in blastocyst implantation (Weitlauf and Knisley, 1992). CD147, an IgG family glycoprotein, is the regulatory subunit of the γ-secretase complex, which may play a role in blastocyst implantation (Ding et al., 2002). CD326 has functions related to signaling, cell migration, proliferation, and differentiation as well as adhesion. The interplay of CD326 with E-

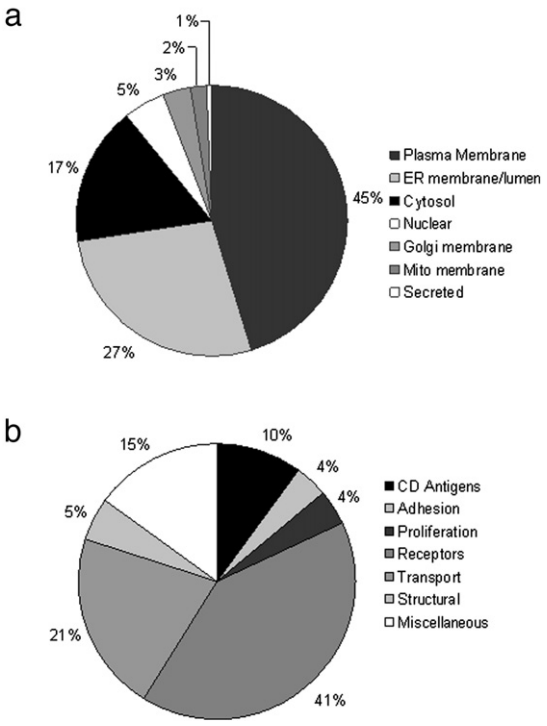

Figure 2 Subcellular protein distribution and functional clustering of membrane-enriched fractions from the combined hESC cluster. (a) The combined percentages of overlapping proteins analyzed from the membrane-enriched fraction of hESC cultured on a feeder layer of MEF or hESC cultured under feeder-free conditions on Matrigel. Analysis using ProteinCenter provided the subcellular distribution of proteins overlapping both culture conditions. Annotation of cellular compartmentalization was performed in ProteinCenter, applying the filter definition "membrane and cell surface." The pie chart shows that 45% of proteins were identified with the plasma membrane and 27% with the ER membrane or lumen, 3% were associated with the Golgi membrane and 2% with the mitochondrial membrane. 17% of proteins analyzed were cytosolic and 5% were of nuclear origin. (b) A pie chart illustrating the functional clustering of proteins found in the overlapping set. Analysis using SwissProt, UniProt, and Ensembl for each individual protein found in the combined group of overlapping proteins provided functional clustering. 41% of proteins were clustered as receptors, 21% as transport proteins, 10% as differentiation antigens, 5% as structural proteins, 4% as adhesion proteins, and 4% as proteins associated with proliferation, and 15% had miscellaneous functions.

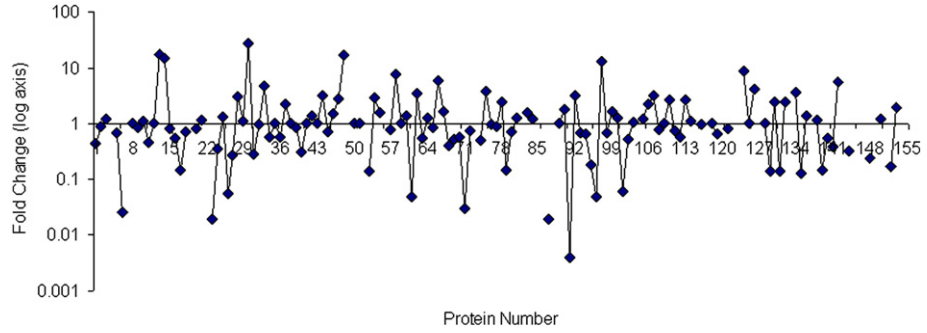

Figure 3 XIC scoring from the combined group of common proteins. A graph illustrating XIC values of the 127 quantified, overlapping proteins analyzed from the membrane-enriched fraction from hESC cultured on feeder layer of MEF or hESC cultured under feeder-free conditions on Matrigel presented on a log-scale *y* axis. XIC scores were calculated directly by the MSQuant program (http://msquant.sourceforge.net/).

cadherin and CD9, as well as the influence of CD326 on NF-κB, c-myc, and E-FABP, provides a putative link for its involvement in cell migration and cell signaling controlling the hESC pluripotent state (Trzpis et al., 2007).

Among CD antigens not previously reported in hESC proteomic profiles, we identified CD9 (cell adhesion) and CD81 (16-kDa Leu 13 protein receptor), which are members of the transmembrane 4 tetraspanins superfamily (Levy and Shoham, 2005). Similar to other members of this superfamily, these proteins are involved in diverse biological processes including cell activation, cell proliferation, cell adhesion, and motility. Specifically, CD9 and CD81 have been shown to be essential in sperm–egg fusion to mediate fertilization (Rubinstein et al., 2006). High levels of CD9 expression have previously been detected at a transcriptional level in hESC (Carpenter et al., 2004) and it has been reported as a marker of stemness (Cai et al., 2006; ISCI, 2007). Interestingly, CD9 associates with the integrin β1 precursor (Rubinstein et al., 1997), also known as CD29, which has previously been described as a marker of undifferentiated hESC (Cai et al., 2006), and has been described as essential for embryogenesis (Rohwedel et al., 1998). Another molecule thought to be of importance for hESC self-renewal is CD44, a hyaluronic acid (HA) receptor. CD44 is a mediator of HA-induced proliferation and it has been linked to implantation due to its expression in early embryos (Gerecht et al., 2007). Other CD antigens found with potential roles in implantation are CD66 and CD298. CD66 has been linked to cellular growth, proliferation, and cell survival (Gray-Owen and Blumberg, 2006) and has been postulated to be involved in blastocyst implantation (Bamberger et al., 2006), while CD298 has been found to be transcriptionally present in bovine embryos, with increasing levels recorded between the two-cell and the blastocyst stages (Goossens et al., 2007). We also identified IGF2R (IMP2 or CD222), which has previously been reported as a marker of the transcriptional regulation of the stemness state in hESC lines (Cai et al., 2006; ISCI, 2007). Recently, IGF-2 and FGF have been reported to establish cooperatively the regulatory stem cell niche of pluripotent hESC (Bendall et al., 2007).

Adhesion-related proteins

We identified seven adhesion proteins in the dataset, representing 4.4% of the proteins classified. Among these seven proteins, five were transmembrane and two had multiple cellular localizations. Adhesion proteins are known to mediate cell–cell or cell–matrix interactions as well as playing a role in early development patterning. They may be important in mediating the pluripotent state of hESC as evidenced by the observation that single-cell suspension of hESC usually leads to induction of hESC differentiation (Karp et al., 2006; Sathananthan and Trounson, 2005) and that plating efficiency of hESC without a matrix or feeders is low (Ludwig et al., 2006). One of the adhesion proteins identified was podocalyxin, a surface membrane glycoprotein with a known transcriptional correlation to "stemness markers" (Cai et al., 2006; ISCI, 2007). It has recently been demonstrated that the purified 200-kDa podocalyxin binds to the Tra1-60 and Tra1-81 antibodies commonly used to define a human embryonic stem cell (Schopperle and DeWolf, 2007). The Na^+/H^+ exchanger regulatory factor-1 (NHERF-1) has been shown to be a selective ligand of podocalyxin, regulating the ability to block or enhance cell adhesion (Tan et al., 2006). We identified some membrane-associated cell adhesion molecules previously reported by van Hoof et al. (2006), including DSG2 (desmoglein-2), RAC1 (Ras-related C3 botulinum toxin substrate), and ATP2A2 (putatively an isoform of sarcoplasmic/endoplasmic reticulum calcium ATPase 2). DSG2 is thought to be necessary for normal ES cell proliferation and early embryonic survival (Eshkind et al., 2002).

Proliferation-related proteins

Seven (4.4%) of the hESC–MEF and hESC–MG common proteins were associated with cell proliferation (one nuclear, four membrane, one ER, and one nontransmembrane (predicted in TMHMM http://www.cbs.dtu.dk/services/TMHMM/). Two of the membrane proteins have previously been reported: glypican-4 and TXNDC1 (Van Hoof et al., 2006). The membrane-associated proteins identified were glypican-4 (K-glypican), CAP-1, TRAP-α, and TXNDC1 (thioredoxin-related transmembrane protein). Glypican-4 is a membrane-bound GPI-anchored glycoprotein that binds heparan sulfate proteoglycan that plays a role in developmental morphogenesis and has been described as one of the 25 most significantly expressed genes in hESC (Sperger et al., 2003). Most work on localization and function of glypican-4 has been carried out after day E7.5 (mouse) of development and glypican-4 has been identified during the development of the extraembryonic membranes and gastrulation. The activity scope of growth factors and morphogens, such as FGFs, BMPs, Wnts, hedgehogs, and IGFs,

all of which are putatively important in the regulation of hESC pluripotency, seems to be activated or determined by glypicans (De Cat and David, 2001).

Receptors

Of the 64 proteins identified as belonging to the receptor group (41%), 38 were identified as membrane associated. Among the other proteins identified, 14 were found within the cytoplasm, 3 in the nucleus, and 3 in the ER lumen; 1 was secreted and 5 had miscellaneous predicted localizations. Nineteen proteins identified in the membrane-associated group have been reported previously (Van Hoof et al., 2006): PGRMC1 (progesterone receptor membrane component 1), SRPRB (signal recognition particle receptor subunit β), RRBP1 (isoform 3 of ribosome-binding protein 1), CKAP4, RAB11B, RAB5A, ATP1A1, RTN4 (reticulon-4), RAB14, FDFT1, CLIC1, CDC42, CANX, ACSL3, RDH11 (retinol dehydrogenase), FAM62A, RALA, LRRC59, and RPL7A. Some of these proteins have been reported to play an important role in the biological functions of hESC. For example, CDC42 is a plasma-membrane-associated small GTPase known to be involved in epithelial cell polarization and formation of filopodia (SwissProt P60953-1). Also, ESC 3D differentiation of a CDC42-null mouse exhibits defects in cell polarity, cell–cell junctions, cavitation, and survival (Wu et al., 2007). CKAP4 (cytoskeleton-associated protein 4) is a transmembrane receptor that has been reported as a receptor for the frizzled-8 protein (Conrads et al., 2006), suggesting involvement with Wnt signaling known to regulate embryogenesis. Frizzled-8 is involved in cell proliferation and embryonic development and is known to be expressed by hESC lines (Wei et al., 2005). Another of the binding proteins detected was connexin 43, a protein that had not previously been described at the protein level in hESC. However, it has been described at the transcriptional level as a stemness marker (Cai et al., 2006). Cx43 is a major gap junction protein found in various cells and plays an important role in intracellular calcium signaling. It also plays an important role in the formation of the extravillous trophoblast in the developing embryo, contributing to proliferation and the invasive phenotype of the cells (Nishimura et al., 2004).

Transporter proteins

This group includes proteins involved in the movement of ions, small molecules, or macromolecules across the cell membrane. Thirty-four proteins (21.5% of the total combined group) were identified in this group, including 25 membrane-bound, 3 nuclear, 3 cytoplasmic, 1 present in cell tight junctions, 1 in the ER lumen, and 1 within the Golgi apparatus. Nineteen of these proteins have been identified previously (Van Hoof et al., 2006), 13 of which were found to have a membrane association. The majority of proteins found in this group were from the Rab/Ras group. Rab proteins are small GTPases involved in determining organelle identity and participate in membrane fusion reactions. They are well-known effector molecules for the regulation of receptor tyrosine kinase activities. Ras and Rab proteins act as molecular switches related to assorted networks of tethering factors and GTPase activating proteins. Rab proteins have also been interlinked via effector molecules, suggesting coordination of protein transport within the endomembrane system (Markgraf et al., 2007). We identified the following members of this family: RAB1A, RAB1B, RAB5C, RAB13, Ras-related protein RAB10, RAB5B, GTPase RAB14, and Ras-related nuclear protein. Of these, 3 were not located within the membrane (RAB1A, RAB13, and the Ras-related nuclear protein). During gastrulation in zebrafish, regulation of the cohesion of mesendodermal progenitor cells by Wnt11 is mediated by RAB5C (Ulrich et al., 2005). Other groups of proteins identified include 3 solute carriers, solute carrier family 2 (which facilitates glucose transport), solute carrier family 1 (a neutral amino acid transporter), and solute carrier family 16 member 1 (SLC16A1); five proteins associated with vesicle trafficking, VAMP3, VAMP-associated protein B/C (VAPB), vesicle-trafficking protein SEC22b (SEC22B), transmembrane EMP24 domain-containing protein 10 precursor (TMED10), transmembrane EMP24 protein transport domain-containing 9 (TMED9), vesicle-associated membrane protein-associated protein A isoform 2 (VAPA), and ADP ribosylation factor 1 (ARF1). Solute carrier family 2 (also known as GLUT1 or SLC2A1) is thought to have a role as a transporter of glucose in mouse ESC during differentiation and embryonic development (Tonack et al., 2006). SLC16A1 (MCT1) is differentially expressed during development of the embryo and has been demonstrated to have an important role in the regulation of pH and monocarboxylate transport prior to preimplantation. Glucose has an implied role in the control of SCL16A1 expression (Jansen et al., 2006). ARF1 operates in a group of proteins involved in intracellular protein trafficking and has a suggested role in cell adhesion-related processes. Deletion of ARF-related protein 1 leads to embryonic lethality during early gastrulation (Zahn et al., 2006).

Structural proteins

Nine proteins were classified as structural (5.6%) and only three of the nine structural proteins were classified as associated with membranes: myoferlin, a nonspecific alkaline phosphatase inhibitor precursor, and Talin 1. The remaining proteins were classified as cytoplasmic (4), cytosolic (1), or in the ER lumen (1). Talin 1 plays a role in cell proliferation and in the assembly of the extracellular matrix. A study using undifferentiated mouse ESC showed that Talin 1 is fundamental for the assembly of focal adhesions and integrin β1 expression (Priddle et al., 1998).

Proteins with miscellaneous functions

We identified 24 (15.2%) proteins with diverse functions, which could not be grouped into the above-mentioned main categories. Twenty-three of the proteins have a membrane association. These proteins are involved in a variety of cellular processes including apoptosis, metabolic processes, developmental processes, enzyme activity, and electron transport. They include Erlin2, Erasin, cytochrome P450, a Golgi apparatus protein synaptophysin-like 1, and ribophorin 1 (known to be differentially regulated by FGFs) (Tateossian et al., 2004).

Correlation of the proteomic signature with the transcription signature of hESC

Several of the known membrane-associated pluripotent markers of hESC were not identified in our membrane proteome profile. For example, TDGF1, GABRB3, FGF4 and 5,

IFTIM1 and 2, IL6ST, c-KIT, LIFr, NUMB, and EDNRB are expressed on the hESC cell surface either as membrane-associated or as transmembrane proteins and have previously been demonstrated to be expressed at the mRNA level by hESC and thus related to their stemness (Cai et al., 2006; ISCI, 2007). On the other hand, many of the identified membrane proteins have not been described by a global DNA microarray screen (Abeyta et al., 2004; ISCI, 2007; Laslett et al., 2007; Sato et al., 2003). The difference between our data generated by proteomic analysis and the previously reported gene expression array analysis may be due to differences in protein turnover and regulation of the abundance of cellular mRNA and proteins (Aebersold, 2003). Importantly, the choice of enzyme used for peptide generation dictates the nature, number, and lengths of peptides to be analyzed in the mass spectrometer. In this study we have employed the endoproteinase Lys C enzyme. Lys C cleaves at K residues and thus the K content of a protein determines if fragments suitable for MS analysis are generated. The proteins mentioned above have been cleaved *in silico* (peptide-cutter, SwissProt) and found to generate very few proper-size fragments (data not shown). We have described similar differences between the membrane proteome and the mRNA gene expression data of hMSC (Foster et al., 2005). Thus, at this stage, using proteomic tools and DNA microarrays should be considered complementary, as the outcome of a proteome analysis cannot be predicted from the mRNA gene expression levels.

Strengths of this study

In contrast to previous hESC proteome studies that employed whole-cell lysate, we have chosen a subglobal proteomic approach and PM proteome profiling to define a "proteomic profile" of hESC. Also, in our study we have employed an improved approach that facilitates detection of plasma membrane protein-derived peptides as well as GPI-anchored proteins and other low-abundance membrane-associated proteins. In addition, we performed both MS^2 and MS^3 experiments using the Finnigan linear quadrupole ion trap–Fourier transform (LTQ–FT) ICR mass spectrometer to increase data quality and the certainty of protein identification as estimated by reverse database searches. We employed a reverse database search of our identifications to reduce the number of false positive identifications from the dataset (Schandorff et al., 2007), and we employed the MASCOT search engine using a reversed database (revIPI human) and imported the hits into MSQuant for identification of false positive peptides. Average mass accuracy for our study after calibration in MSQuant (CEBI in-house development at http://msquant.sourceforge.net/) was 2.7 ppm (Zubarev and Mann, 2007), in concert with accuracies given in several high-quality reports of proteome analyses (Adachi et al., 2007). Finally, we have quantified the relative protein abundance for the overlapping identities and shown that they are present at comparable levels.

Comparison of our identified proteins with proteins reported in two previously published studies on hESC (Baharvand et al., 2006; Van Hoof et al., 2006) revealed that the highest degree of consensus between datasets was observed for the proteins identified by the use of a strategy based on LC-MS (Van Hoof et al., 2006; 56.8%) rather than a strategy based on 2D gel and MALDI TOF/TOF (Baharvand et al., 2006; 10%). Our study demonstrates that a plasma-membrane-based proteomic approach is a more useful approach for identification of a larger number of low-abundance proteins.

Limitations of this study

We have performed proteomic identification of membrane proteins from hESC to study similarities in a filtered membrane profile between two common methods of hESC culture. Quantitative proteomic methods are available, e.g., SILAC (stable isotope labeling in cell culture) (Ong and Mann, 2005), and can provide absolute quantitation on differences in proteome profiles among cells cultured under a variety of conditions. Our study provides baseline quantitative guidelines needed to perform these experiments.

It is possible that some mouse peptides derived from the feeder layer of MEF contaminated the proteome profile of hESC–MEF. To obtain an estimate of the contribution of murine peptides in the hESC–MEF and hESC–MG membrane proteins and to evaluate if the sequence coverage of mouse proteins was significantly higher in hESC–MEF compared to hESC–MG, we tested the peptides of the same five highest scoring protein hits that matched peptides (>30 per hit) under both conditions. The five proteins chosen represented both high-sequence-conserved proteins between human and mouse and poor-sequence-conserved proteins. The sequence matching was performed by finding the sequence coverage percentage for the peptides identified, for the five chosen human proteins and for their mouse homologs. This was carried out both for the MEF condition and for the MG condition. In all cases both for MEF and MG, the sequence coverage was better for human sequences than for mouse homologs, suggesting no significant murine contribution to the membrane peptide population.

Conclusion

A comparison of common cell culture conditions used in hESC growth studies (MEF-feeder and feeder-free) has given a profile of 58.3% of proteins found in common under both conditions. Of these 157 nonredundant proteins, 43% have not previously been reported within a proteomic profile in hESC lines. While the majority of these cell surface proteins have previously been found to be associated with implantation or early fetal development, their roles in the maintenance of pluripotency or in guiding differentiation are yet to be determined.

This report presents the first description and identification of a semiquantitative membrane proteome profile using high-accuracy peptide mass fingerprinting in hESC. Our study provides an initial description of hESC membrane proteome profiles and baseline data needed for future quantitative proteomics studies in hESC.

Methods and materials

Establishment of hESC line OD3

Derivation and characterization of Odense 3 (hESC-OD3), an in-house-derived human embryonic stem cell line, are described in the supplementary methods and materials. The

pluripotent phenotype of hESC-OD3 has been compared side by side with a standard hESC line: HUES9 (kindly provided by D. Melton, Harvard) (Supplementary Fig. 2). hESC-OD3 cells, routinely cultured on inactivated MEF feeders (here termed hESC–MEF) in 15% KOSR/KO-DMEM, were transitioned to Matrigel (made according to the manufacturer's instructions; BD Biosciences, Europe) and MEF conditioned medium (Xu et al., 2001), supplemented, daily, with 8 ng/ml hbFGF (Invitrogen) for five passages (here termed hESC–MG). Whenever wells were subconfluent, the cells were passaged to either fresh Matrigel or feeder-coated wells. hESC from each condition ($20–30\times10^6$) were prepared separately for proteomic studies by growth on MEF and on Matrigel. Cells grown on MEF were detached following incubation with trypsin–EDTA (Invitrogen) for 1 min. Cells grown on Matrigel were mechanically dissociated.

Membrane isolation from hESC

hESC were harvested from six-well culture dishes by washing with 1 ml ice-cold PBS (without Ca^{2+} and Mg^{2+}) containing EDTA-free protease inhibitor cocktail (Roche, Germany). The cells were centrifuged at 500 rcf for 10 min at 4°C. The cell pellet was resuspended using a pipette in 1 ml of ice-cold lysis buffer (255 mM sucrose, 20 mM Hepes, 1 mM EDTA, pH 7.4, EDTA-free protease inhibitor cocktail; Roche). The cell lysate was transferred to a glass homogenizer (Wheaton Douncer) and subjected to 35 strokes with the tight piston. The cellular debris was visually evaluated, microscopically, to confirm that nuclei were intact and that the debris sizes even. The membrane homogenate was transferred to two 2-ml microcentrifuge tubes and centrifuged at 20,800 rcf for 10 min at 4°C to separate mitochondria and nuclei. The supernatants were transferred to two pre-lysis buffer-washed PC4 tubes (Hitachi Koki), placed in a S100AT6-0061 rotor (No.18), and centrifuged in a Sorvall RC M150 GX ultracentrifuge at 245,000 rcf at 4°C for 2 h. After centrifugation the supernatant contained the cytosol fraction and the pellet contained the plasma membranes, endoplasmic reticulum, and Golgi.

The membrane pellet was carbonate-washed by resuspension in 1 ml of ice-cold 100 mM Na_2CO_3 (with EDTA-free protease inhibitors; Roche) and incubated on ice for 30 min with careful, occasional vortexing. A 1PC tube (Hitachi Koki) was prewashed with 100 mM Na_2CO_3, and the membrane sample was then transferred into it. Plasma membranes were harvested by centrifugation at 100,000 rcf, 4°C, for 30 min in a S150AT-0097 (No. 7) rotor (Sorvall). After centrifugation, the membrane pellet was washed once with 25 μl of 500 mM NH_4CO_3, without resuspension, and the supernatant removed again. The procedure was repeated with 25 μl of 50 mM NH_4CO_3 and the pellet was removed. The pellet was resuspended in 6 M urea/2 M thiourea before reduction and alkylation.

In-solution digest

Samples were solubilized in 6 M urea/2 M thiourea (pH 8.0) and centrifuged for 10 min at 9300 rcf (Eppendorf 5415R) to pellet any insoluble material. One microliter of 1 μg/μl dithiothreitol in water was added for every 50 μg of sample and incubated for 30 min at room temperature. One microliter of 5 μg/μl iodoacetamide in 50 mM NH_4HC0_3 was added for every 50 μg of sample and incubated for 20 min (in the dark) at room temperature. Lys C (Wako Pure Chemical Industries) was added at a concentration of 5 μg/ml and incubated overnight at room temperature.

Mass spectrometry

Manufacture of C_{18}-StageTip for peptide isolation

The Empore Disk C18 (No. 2215, 3M, Minnesota, MN, USA) was used. A small disk was transferred into a 250-μl pipette tip (Bioclean).

Use of C_{18}-StageTips for sample desalting and cleaning

The following mass-spectrometry-grade solvents were used during peptide isolation: methanol, trifluoroacetic acid (TFA), buffer A (0.5% acetic acid), and buffer B (80% acetonitrile, 0.5% acetic acid). The digested peptide sample was acidified with TFA. The C_{18}-StageTip was conditioned with 10 μl methanol and the liquid was pressed through at 50 μl/min using a 1-ml plastic fitted syringe. The StageTip was equilibrated using 30 μl buffer A at 50 μl/min. The appropriate volume of sample was loaded at 20 μl/min and the StageTip was washed with 10 μl buffer A at 50 μl/min. The sample was eluted with 10 μl buffer B at a rate of 10 μl/min, dried in vacuum at 45°C to a volume of 1 μl, and diluted 1:8 with buffer A. The sample (6 μl) was loaded onto the analytical column of the LC-MS/MS system.

Nanoflow LC-FTICR-MS^2 and MS^3

Nano-HPLC-MS/MS and MS^3 experiments were performed on an Agilent 1100 nanoflow system and analyzed by electrospray mass spectrometry on a Finnigan LTQ–FT mass spectrometer (Thermo Fisher, Bremen, Germany) fitted with a nanoESspray ion source (Proxeon Biosystems A/S, Odense, Denmark). Peptide mixtures were loaded onto the column with a flow of 0.5 μl/min and eluted with a flow of 0.25 μl/min with a gradient of 10–64% acetonitrile in 0.5% acetic acid over a 140-min gradient with 1 million charges. The mass spectrometer was operated in data-dependent acquisition mode, with multiple charged peptides selected for fragmentation. The three most intense precursor ions of a spectrum were isolated for accurate mass measurement by SIM scans in the ICR call and fragmented in the linear ion trap by collision-induced dissociation. The most intense ions in every MS^2 spectrum (above $m/z=300$) were isolated and further fragmented (MS^3 scan). Previously selected precursor ions were dynamically excluded for 30 s.

Data analysis

Protein identification and validation

Proteins were searched against the IPI human database and for false positive rate estimation against the IPI human reverse database. This was performed using the MASCOT search engine (Matrix Science, London, UK). Peptides were verified and quantified in the MSQuant program (Center for Experimental Bioinformatics, University of Southern Denmark, in-house program; Peter Mortensen, http://msquant.sourceforge.net/). Peptides were searched

with carbamidomethyl as fixed modification and oxidized methionine, N-acetylation, pyroglutamate, and deamidation (arginine and glutamine) as variable modifications. Initial mass tolerance for MS peak identification was set to 5 ppm on MS^2 to 0.6 Da, and three missed cleavages were allowed. Peptides fulfilling the following criteria were accepted: MS^2 score must be over 32 and peptides must have a Lys C cleavage site (K) with a length of at least 7 amino acids. Peptides for which the y-ion or b-ion score was at least 54 were accepted. Protein identification criteria were based on a minimum of two peptides corresponding to an accumulated MS^2 score of at least 64 ($p=0.0001$) and/or an MS^3 spectrum with an MS^3 score of at least 54 ($p>0.0001$); single peptide protein identification required the same as the latter (Olsen and Mann, 2004). Protein identifications were loaded into the ProteinCenter program (Proxeon Biosystems A/S) and filtered by applying the filter combination "membrane and cell surface."

Classification and clustering of the subcellular localization of 1075 proteins were based on annotations in the SwissProt (http://www.expasy.org/sprot/), UniProt (http://www.ebi.uniprot.org/index.shtml), and Ensembl (http://www.ensembl.org/index.html) databases.

Semiquantitative rates estimated by extracted ion currents

LC-FTICR RAW files were loaded into the MGFcombiner program (Center for Experimental Bioinformatics, University of Southern Denmark, in-house program; Peter Mortensen, http://msquant.sourceforge.net/) searched in MASCOT and quantified with no isotope labeling using the MSQuant program. XIC values were retrieved directly for the corresponding peptides m/z signals between the two states. Standard deviations of same-condition intersample XIC rates were calculated from noncombined files. For MEF files the average SD was 28.25%, for MG files 33.25%. For SD distributions of XIC rates for hESC–MEF and hESC–MG, please see Supplementary Fig. 3. SDs are presented as percentages of XIC rates for each protein for which this value was retrievable. SDs were not retrievable for some proteins owing to their either being identified in only one of the technical replicate files or being identified in both technical replicate files but not based on the same peptides. SDs were calculated based on values for each peptide corresponding between the same state, added and averaged to give the SD for each protein.

Acknowledgments

The authors thank members of the IVF Clinic, Odense University Hospital, for donating embryos, Drs. Karin Erb and Per Emil Rasumssen, and Drs. Steen B. Laursen and Ulrik Frandsen for help with the derivation and characterization of the hESC-OD3 line. We thank Ms. Lone Christiansen and Hanne Thayssen for excellent technical assistance, Maria E. O. Nielsen for advice on FT handling, and Morten Bern of Proxeon Biosystems A/S for the use of software analysis tools. The access to the PepMapper program of Dr. Gary Schoenhals, BMB, University of Southern Denmark, is greatly appreciated. This work was supported by grants from the Lundbeck Foundation and the Novo Nordisk Foundation and a local grant supplied by the University Hospital of Odense.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.scr.2008.06.001.

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