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Embryonic stem cell proteomics

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Human embryonic stem cells potentially represent an unlimited source of cells and tissues for regenerative medicine. Understanding signaling events that drive proliferation and specialization of these cells into various differentiated derivatives is of utmost importance for controlling their behavior *in vitro*. Major progress has been made in unraveling these signaling events with large-scale studies at the transcriptional level, but analysis of protein expression, interaction and modification has been more limited, since it requires different strategies. Recent advances in mass spectrometry-based proteomics indicate that proteome characterization can contribute significantly to our understanding of embryonic stem cell biology. In this article, we review mass spectrometry-based studies of human and mouse embryonic stem cells and their differentiated progeny, as well as studies of conditioned media that have been reported to support self-renewal of the undifferentiated cells in the absence of the more commonly used feeder cells. In addition, we make concise comparisons with related transcriptome profiling reports.

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Derivation & culture of embryonic stem cells

Embryonic stem cells (ESCs) possess the combined abilities to divide and self renew indefinitely *in vitro* and to differentiate into all somatic cells [1], as well as germ cells [2], of the adult individual. ESCs are derived from the inner cell mass of blastocyst-stage embryos (FIGURE 1A), and are generally cocultured on a monolayer of mitotically inactivated feeder cells to inhibit spontaneous differentiation and promote self renewal (FIGURE 1B). The first ESC lines were generated 25 years ago from mouse embryos [3,4] and have since been the subject of research that has varied from serving as a model system to study early differentiation in the mammalian embryo to a vehicle for modifying gene expression in the germ line in mice. Despite apparent generic similarities between human and mouse, it took 17 years before the procedure of mouse ESCs (mESCs) derivation was properly adjusted and successfully applied to human embryos [5–7], although the development of legislation and guidelines for the use of human ESC (hESC) research contributed to the delay. The difficulties encountered during attempts to produce hESCs are indicative of significant dissimilarities between the two species. For instance, in contrast to hESCs [8], mESCs retain their undifferentiated phenotype in the absence of feeder cells when cultured in serum-containing medium supplemented with leukemia inhibitory factor (LIF) [9–12]; hESCs do not respond to LIF at all. Likewise, signaling pathways mediating differentiation to specific cell lineages have been identified in mESCs [13], but their activity is only partially conserved in hESCs [14,15]. Moreover, multiple differences exist between individual ESC lines derived from the same species; these are most likely caused by different isolation procedures and culture conditions. Signaling pathways that support self-renewal of mESCs grown on feeder cells may differ from those active in mESCs cultured without feeder cells in serum-containing medium supplemented with LIF [16]. Similar differences probably exist between hESCs cultured on either mouse or human feeder cells. In addition, differences may also arise between hESCs passaged mechanically (i.e., by cut-and-paste method) [6] versus those passaged by enzymatic dissociation (i.e., using trypsin) [7].

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Insight into hESC behavior is of immense interest, as they may represent an unlimited source of cells for tissue replacement in regenerative medicine. Thus, extensive characterization to define similarities and differences between multiple hESC lines is an essential prelude to their clinical application. It may even become possible in the future to predict which hESC line is most suited to which purpose and develop prospective markers for their differentiation potential. Recently, an international consortium (International Stem Cell Initiative) was established to set standards and benchmarks for the uniform characterization and categorization of the many hESC lines generated to date [17]. The results of this initiative are likely to shed light on the variability between cell lines that arise between the same line cultured in different laboratories.

The analytical methodologies used at present are predominantly based on transcriptome characterization using microarray analysis and conventional protein analyses, such as western blotting, immunofluorescence microscopy and fluorescence-activated cell sorting. However, the assumption of a direct correlation between the amount of mRNA and protein does not always hold; moreover, the fate of ESCs is not solely determined by the abundance of specific proteins. Although protein expression is regulated at the mRNA level (i.e., transcription and splicing), the production and activity of proteins depends on translation, post-translational modifications (e.g., phosphorylation) and degradation. Thus, to gain insight into pathways activated during proliferation and maintenance, as well as differentiation of ESCs, extensive analysis of the proteome is pivotal. Current state-of-the-art mass spectrometric (MS) techniques have convincingly demonstrated that MS-based proteome analysis has matured sufficiently for identification of protein benchmarks and post-translational modifications on a large scale [18–22]. Complementation of microarray studies with protein analyses at such levels may, therefore, provide the missing link between gene transcription and cell behavior.

Proteome profiling of mouse embryonic stem cells

Despite their relatively long history, mESCs (FIGURES 2A & 2B) have only recently become the subject of multiple large-scale screens to identify ESC-specific markers. Microarray [16,23–28], serial analysis of gene expression [29] and massive parallel signature sequencing [30] techniques have been used to generate extensive transcriptome profiles of mESCs, distinguishing genes important for ESC maintenance. The first tentative MS-based analysis of mESCs was applied to identify proteins involved in differentiation of mESCs (line phenylketonuria [PKU]) to neural cells upon 4 days of suspension culture followed by 4 days of incubation with retinoic acid [31]. Proteins differentially expressed in these mESCs and differentiated cells were selected from 2D electrophoresis gels and analyzed by matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) protein mass fingerprinting (PMF). Although a comparative experimental setup allows proteins specifically expressed in either ESCs or differentiated cells to be distinguished, the number of proteins described in this study was rather small (i.e., 24 proteins, 15 of which had no known function or name).

A similar MS analysis approach was applied 3 years later in an attempt to generate a more comprehensive map of the mESC (line R1) proteome [32]. Using three different 2D electrophoresis gels with diverse pH gradients (pH 3–10, 4–7 and 6–11) and varying acrylamide concentrations, 600–1000 protein spots derived from these mESCs were resolved. A total of 218 unique proteins were identified by MALDI-TOF PMF and electrospray tandem MS (MS/MS) from 600 manually selected spots. Although no known ESC-specific proteins were detected, the data set contained several uncharacterized proteins (i.e., with no known function), some of which might be specific for mESCs.

A more extensive data set, comprising 1790 proteins expressed in mESCs (line E14–1), was generated using fully automated microscale multidimensional liquid chromatography (LC) and high-resolution hybrid MS [33]. Comparison of this protein data set to transcriptome analyses distinguished 60 protein products among 485 transcripts previously defined as ESC-specific [24]. The resulting subset included known ESC-specific proteins, such as alkaline phosphatase, and low-abundance transcription factors, such as octamer-binding transcription factor (Oct4) [34] and undifferentiated embryonic cell transcription factor (UTF1) [35], which indicate the potential of this experimental strategy to identify novel ESC-specific markers.

In a subsequent study, cell-surface proteins of mESCs (line D3) were labeled with biotin and purified using sucrose density gradients [36]. The purified proteins were digested with trypsin, after which the biotin-labeled peptides were isolated by avidin affinity chromatography and subsequently analyzed on an automated 2D LC-MS/MS system. Repeating the procedure yielded a composite data set of 324 proteins, 200 of which contained one or more transmembrane domains, as predicted with the SOSUI program [37]. To validate the confidence by which these putative plasma membrane proteins were identified, four candidates (RIKEN (Japan) cDNA B430119L13; a trophoblast plasma membrane glycoprotein; glycoprotein A33; and the hypothetical protein D7Ertd458e) with a single predicted transmembrane segment were selected for examination at the cellular level. The mESCs were transiently transfected with cDNA constructs of these proteins, which were tagged with FLAG. Immunofluorescence microscopic analysis showed that all four proteins colocalized with CD9, indicating that they are associated with the plasma membrane.

Although the numbers of proteins comprising current data sets seem quite impressive, it remains to be investigated whether the proteins, including those with no known function, are somehow involved in maintaining the undifferentiated state of ESCs. Generating a similar protein profile of differentiated cells would allow exclusion of proteins expressed in both ESCs and differentiated cells, thus creating a data set of proteins uniquely identified in ESCs [38]. Alternatively, uncharacterized proteins identified in ESCs could be selected for further analysis at the expression level using quantitative PCR or *in situ* hybridization and at the functional level using RNA interference (RNAi). The current absence of these

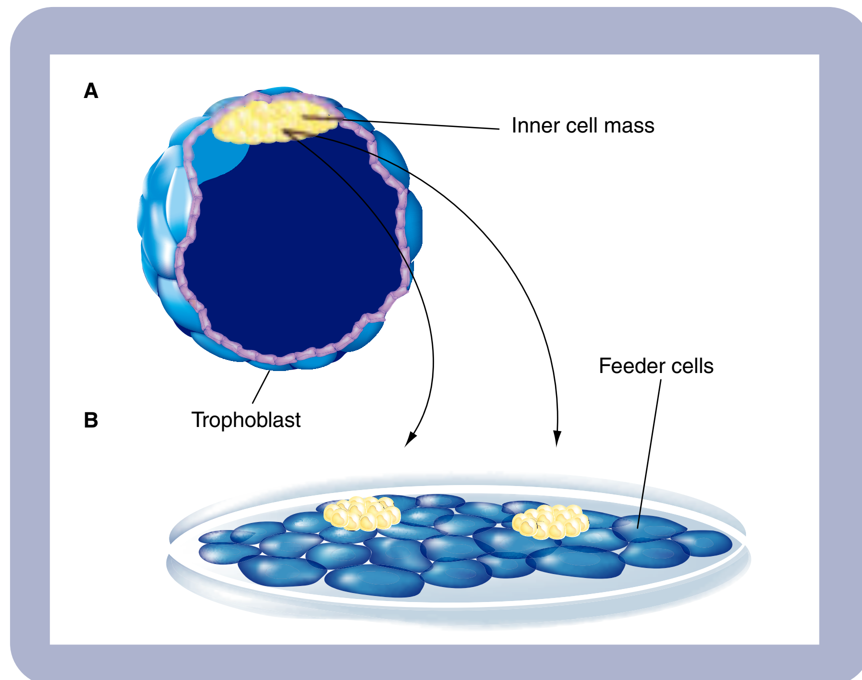

Figure 1. Derivation of human and mouse embryonic stem cells. The inner cell mass is isolated from blastocyst-stage embryos **(A)** and cultured on a monolayer of mitotically inactive feeder cells **(B)** that support growth and inhibit differentiation of the inner cell mass cells. After several passages, some cells develop the ability to proliferate and self-renew as well as differentiate into derivatives of the three primary germ layers (i.e., ectoderm, endoderm and mesoderm). These are considered pluripotent embryonic stem cells.

additional studies limits the interpretation of results based solely on a single data set to speculation. In order to unravel complex processes such as signaling pathways that are active during ESC maintenance, it is essential to conduct comparative analyses of cells grown under ESC-sustaining and differentiation-inducing conditions.

In contrast to hESCs [8], mESCs retain their undifferentiated state in the absence of feeder cells when cultured in serum-containing medium supplemented with LIF [9,10,12] via the signal transducer and activator of transcription (STAT)-3 pathway. In addition, a combination of bone morphogenetic proteins and LIF can replace the requirement for feeder cells and serum entirely for mESCs [39], but not for hESCs. These intriguing discrepancies suggest that distinct signaling pathways are active in ESCs derived from different species. To define the LIF-induced pathway in mESCs, downstream targets of STAT-3 were investigated by microarray-based kinetic studies comparing LIF-stimulated mESCs (line Gs2) with mESCs induced to differentiate by shutting down STAT-3 [16]. STAT-3 activation in these mESCs was inhibited either by removing LIF from the culture medium or expressing a dominant-negative mutant form of STAT-3. Independently, a similar strategy was simultaneously applied to analyze mESCs (line D3) at the protein level [40]. The latter investigation focused on the differential expression of cytosolic and nuclear proteins in the presence or absence of LIF using 2D difference in-gel electrophoresis (DiGE) [41]. Proteins extracted from undifferentiated D3 cells were labeled with a green fluorophore and were mixed with those extracted from D3 cells grown for 1 week without LIF and labeled with a red fluorophore. Over 100 spots showing a fluorescent intensity differing for the green and red dye were excised from the gels, digested with trypsin and analyzed by MALDI-TOF. The nuclear samples of mESCs were predominantly enriched in chromatin-related proteins that were downregulated during differentiation; expression dynamics of some of these proteins (high mobility group box (HMG-B)-2, amine oxidase flavin-containing (AOF)-2, mutS homologue (MSH)-2, Nanog and the 60 kDa subunit of the switching/sucrose nonfermenting [SWI/SNF] complex) were confirmed by real-time reverse-transcriptase PCR [40]. Notably, approximately half of the proteins identified by MS [40] matched with corresponding transcripts from the microarray data of mESCs studied under analogous conditions [16].

Removal of LIF generally initiates spontaneous differentiation of mESCs into a variety of somatic cells *in vitro*. However, *in vivo* differentiation events are tightly controlled by extracellular factors that direct the fate of undifferentiated cells to a specific cell type. As previously described, the development of neural cells can be mimicked *in vitro* by suspension growth of mESCs in the presence of retinoic acid [31]. Coculture of mESCs on PA6 stromal cells in the presence of retinoic acid has similar effects, giving rise to motor neuron-like cells. Conversely, when cocultured on these PA6 cells in the absence of retinoic acid, mESCs preferentially differentiate into dopaminergic neurons. To analyze global proteome changes during the latter differentiation process, mESCs (line E14) differentiated for 10 days on PA6 cells were lysed and subjected to 2D gel electrophoresis [42]. Approximately 1200 spots were resolved and analyzed with LC–MS/MS. Among the proteins identified, 23 were found to differ more than twofold in expression level between mESCs and *in vitro*-differentiated dopaminergic neurons, as determined from 2D electrophoresis gels. Western blotting was used to verify these differences for some of the proteins (tumour protein translationally controlled [TCTP], class III β-tubulin [Tuj1] and α-tubulin). These same proteins were also found to be differentially expressed in motor neuron-like cells (i.e., E14 cells grown for 10 days on PA6 cells in the presence of retinoic acid).

Besides neural cells, mESCs can also be coaxed to differentiate into other cell types, such as endothelial and smooth muscle cells, both of which participate in vessel formation by

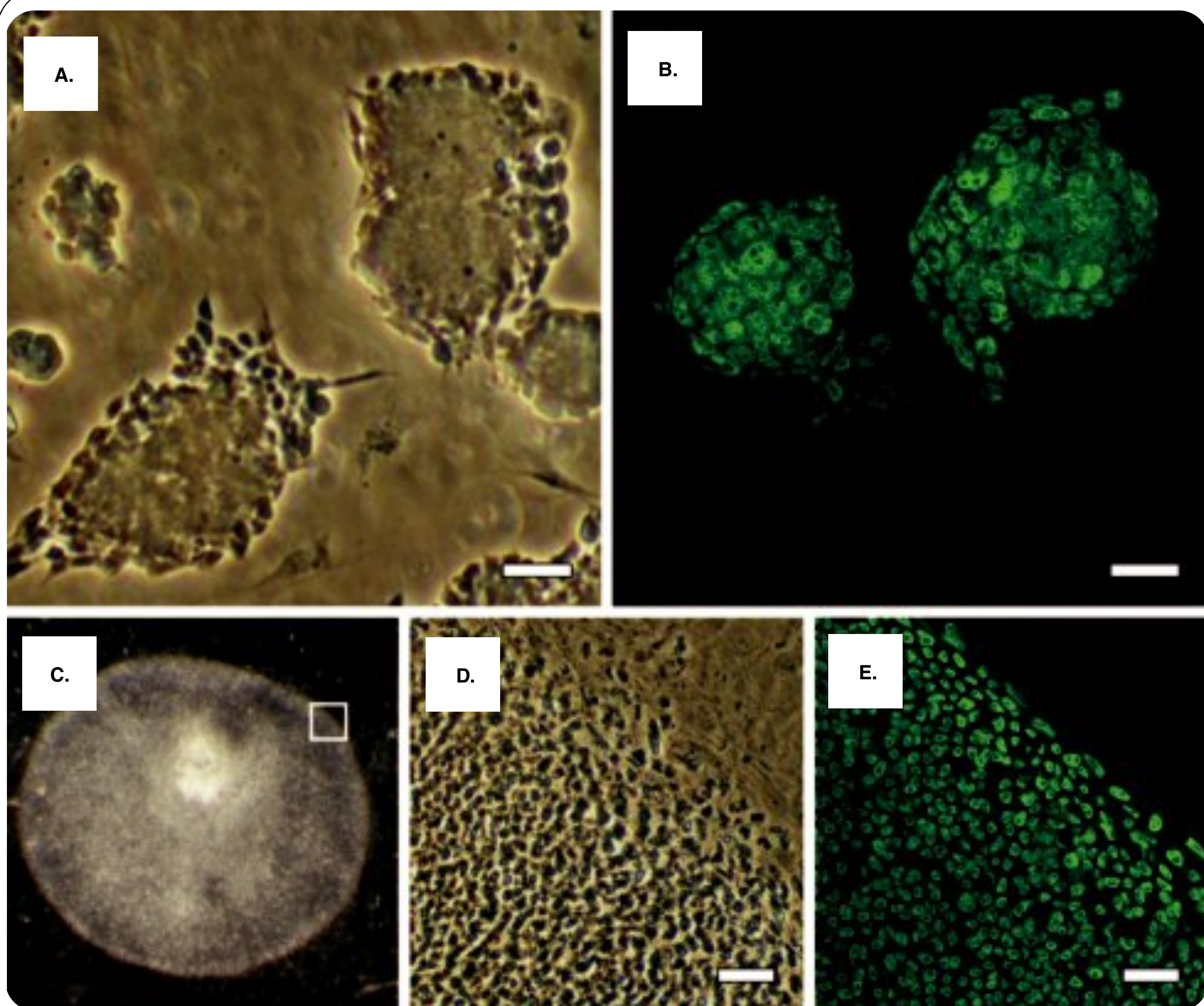

Figure 2. Colonies of embryonic stem cells. (A) Bright field microscopic image of mouse embryonic stem cells that grow as dense clusters of several hundred cells per colony in serum-containing culture medium supplemented with leukemia inhibitory factor in the absence of feeder cells. **(B)** Confocal laser microscopic image of two colonies of mouse embryonic stem cells that were labeled with an antibody against the embryonic stem cell-specific transcription factor Oct4. Due to the compactness of the colony, it is difficult to distinguish the nuclei of the individual cells in which Oct4 is located. **(C)** Macroscopic image of a disc-like human embryonic stem cell colony of ~50000 cells grown on feeder cells. **(D)** Bright field microscopic image of the edge of a human embryonic stem cell colony as shown in C (square). **(E)** Confocal laser microscopic image of human embryonic stem cells labeled with an antibody against the embryonic stem cell-specific transcription factor Oct4 expressed in the nucleus of undifferentiated embryonic stem cells. The feeder cells surrounding the colony do not express Oct4. The scale bars represent 20 μm.

vasculogenesis and angiogenesis *in vivo*. As stem cell antigen (Sca)-1-positive cells isolated from adventitial tissues can develop into smooth muscle cells [43], Sca-1-positive cells obtained from mESCs may serve as a potential source for vascular progenitor cells. mESCs (line D3) were induced to differentiate for 3–4 days, after which Sca-1-positive cells were isolated by magnetic-activated cell sorting using anti-Sca-1 microbeads [44]. Protein extracts of these cells were subjected to 2D gel electrophoresis, after which 300 spots were selected for MALDI-TOF-MS analysis, resulting in the identification of 172 unique proteins. As expected, the percentage of proteins differentially expressed determined from spot intensity on 2D electrophoresis gels was greater between Sca-1-positive cells and mESCs than Sca-1-positive cells and adult arterial smooth muscle cells. Despite the relatively small number of identified proteins, it was additionally concluded that signaling proteins, such Rho GDP-dissociation inhibitor 1, are more abundant in Sca-1-positive cells than mature smooth muscle cells.

The earliest signaling events during differentiation of ESCs involve activation and deactivation, mediated by post-translational modifications (mainly phosphorylation) of proteins that are already present in the cells. These initial steps initiate cascades of intracellular processes, resulting in changes in

expression at the transcriptional and translational level. Microarray analytical techniques are excellently suited to track eventual alterations of the transcriptome, detailing preceding processes, whereas identification of post-translational modifications requires analysis at the protein level. To determine which proteins were correlated with cell proliferation and differentiation, phosphorylation states of 31 intracellular signaling network components were obtained under 16 differentiation conditions at three time points (days 0, 3 and 5) [45]. Quantitative western blot analysis and partial-least-squares modeling distinguished proteins activated or deactivated upon proliferation of mESCs (line CCE) and differentiated cells, as well as differentiation of these mESCs upon removal of LIF. In a study that was not restricted to a defined set of proteins as described above [45], phosphoprotein-affinity purification was applied to isolate phosphorylated proteins from extracts of mESCs (line J1) and embryoid bodies formed by suspension culture of these cells for 24 h [46]. Both nano-LC-MS/MS and MALDI-MS/MS were used to analyze 362 spots, most of which exhibited visible changes in intensity or mobility when samples of mESCs and embryoid bodies were compared on silver-stained 2D electrophoresis gels. Among the 108 uniquely identified proteins, 15 protein species were found exclusively or preferentially in mESCs, 20 in embryoid bodies, and 11 showed distinct electrophoretic mobilities when those derived from mESCs and embryoid bodies were compared. Importantly, microarray analysis indicates that the expression levels of these proteins remain relatively unchanged [47]. This suggests that differences in staining intensity of these proteins observed on gel after phosphoprotein affinity purification result from alterations in phosphorylation and not from differences in protein abundance.

Comparison of human & mouse embryonic stem cell proteome profiles

Since hESCs (FIGURES 2C–E) hold great promise for regenerative medicine, many of the hESC lines established to date have been subjected to several transcriptome analysis techniques to define common as well unique characteristics for each line [26,27,30,48–51]. Whereas similarities are indicative of generic processes regulating self-renewal, differences between the numerous hESC lines might explain why some preferentially differentiate into specific cell types. Despite major advancements in proteome profiling techniques and the widespread availability of different lines since the first derivation of hESCs [5], large-scale proteomic analyses of hESCs had, until recently, been missing [38]. Thus far, MS-based proteome analyses of human cells with differentiation capabilities had been limited to cluster of differentiation (CD)-34-positive stem cells [52,53], bone marrow mesenchymal stem cells [54–56], neural stem cells [57], umbilical cord blood-derived stem cells [58,59], adipose tissue-derived stem cells [60], pluripotent embryonal carcinoma cells [61] and hematopoietic stem cells [62].

To our knowledge, we published the first comprehensive profiles of proteins differentially expressed in hESCs and their differentiated derivatives [38]. As described above, the major advantage of our comparative approach is that common proteins (i.e., those identified in both hESCs and differentiated cells) could be eliminated from the hESC protein data set. The resulting proteins are likely to include hESC-specific proteins potentially involved in maintaining the undifferentiated state and promoting self-renewal. Moreover, we used the same strategy to identify mESC-specific proteins, which additionally allowed the comparison of proteins uniquely identified in both human and mouse ESCs. Similar studies comparing human and mouse ESC transcriptomes [26,27,30] revealed conserved and divergent paths that regulate self-renewal in hESCs and mESCs [11]. We started to complement these microarray studies at the translational level.

In summary, we cultured hESCs (line HES-2) [6] on feeder cells and mESCs (line D3) [63] under feeder-free conditions in serum-containing medium supplemented with LIF, as previously described [64]. Differentiation was induced by growing hESCs in the absence of feeder cells and mESCs in medium without LIF for 12 days. Protein extracts were separated by 1D gel electrophoresis followed by nanoflow LC and analyzed by Fourier transform ion cyclotron MS/MS. This resulted in the identification of 1775 nonredundant proteins in hESCs, 1532 in differentiated hESCs, 1871 in mESCs and 1552 in differentiated mESCs [38], with a false-positive rate of less than 0.2%, as determined from a parallel analysis using international protein indexes (IPI) databases with all protein sequences reversed. Comparing the data sets distinguished 191 proteins exclusively identified in both hESCs and mESCs, including well-known ESC-specific protein benchmarks (FIGURE 3A). Interestingly, this subset contained many uncharacterized proteins, some of which may be novel ESC-specific markers or functional proteins.

As expected, differences were found in the protein profiles of ESCs derived from different species, although there were also differences between different ESC lines from the same species. Comparative microarray studies have repeatedly shown divergent gene expression profiles of multiple ESC lines when established and cultured in different laboratories [26,27,30], which have been explained by:

- Differences in culture and derivation procedures applied in laboratories
- Variations in analysis techniques
- Diverse genetic backgrounds of the multiple ESC lines established to date

Comparison of our mESC protein data set with those previously published [32,33] confirmed these observations. Of the 218 proteins identified in mESCs (line R1) [32], 194 (89%) were also present in our data set of mESCs (line D3) (FIGURE 3B). Notably, the former study was relatively small and was most likely dominated by abundant proteins. Conversely, we identified 842 of the 1790 proteins (47%) detected in mESCs (line

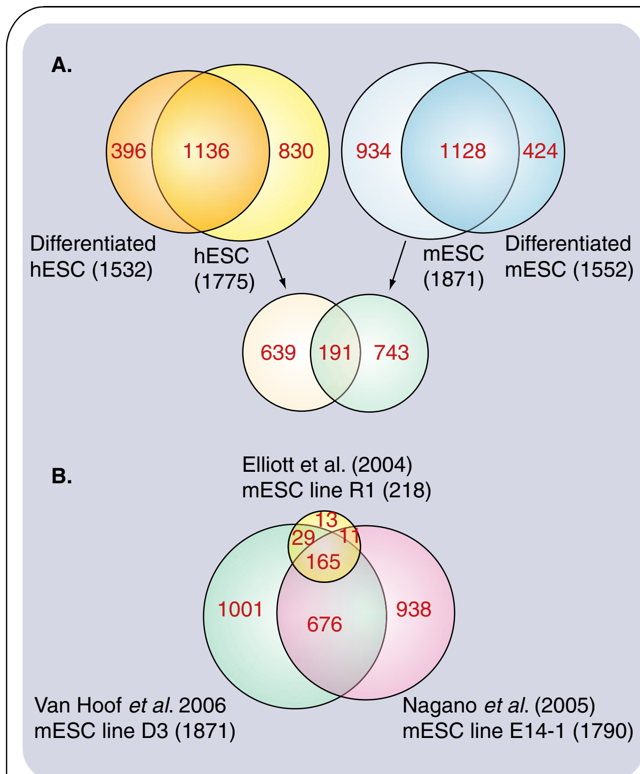

Figure 3. Venn diagrams of proteins profiles of human and mouse embryonic stem cells and their differentiated progeny.
(A) Venn diagrams showing unique and common proteins in human (yellow) and mouse (blue) ESCs (bright) and differentiated ESCs (dark). The overlapping bright yellow and bright blue circles indicate the 191 proteins uniquely identified in both hESCs and mESCs but not in differentiated cells. **(B)** Venn diagram showing proteins commonly identified in different mESC lines by Elliott and colleagues [32] (green circle), Nagano and colleagues [33] (purple circle) and Van Hoof and colleagues [38] (blue circle). The number of proteins in each subset is shown in red (A and B).

E14–1) (FIGURE 3B) [33], 65 of which were among the subset of 191 proteins (34%) uniquely identified in both hESCs and mESCs [38], and thus are likely to be associated with ESC maintenance. Nevertheless, ESC-associated proteins that commonly occur in data sets produced in different laboratories may be highly relevant with respect to generic ESC-sustaining processes, as they appear independent of variation in laboratory-specific culture techniques.

To validate our MS approach, differential expression of a selection of proteins for which antibodies were available was confirmed by Western blotting, immunofluorescence confocal microscopy and fluorescence-activated cell sorting [38]. Furthermore, two other independently isolated and cultured hESC lines, human embryonic stem cell line (HUES)-1 [7] and NL-HESC-01 [65], as well as their differentiated derivatives were monitored for differential expression of selected proteins by Western blotting. Some were found to be exclusively expressed by ESCs of all three human lines and may thus serve as generic hESC markers (e.g., topoisomerase [TOP] 2A). Generally, our approach proved highly effective as a relatively fast search for ESC-specific proteins on a large scale.

Feeder cells sustaining the undifferentiated state of human embryonic stem cells

Medical application of hESC-derived cells requires culture of these cells under complete xeno-free conditions, hence alternatives for mouse feeder cells are being sought. Both mouse and human feeder cells have been shown to support self-renewal and inhibit differentiation of hESCs [66]. Analysis of proteins secreted by these cells may identify extracellular components that activate hESC-sustaining signaling pathways. In a search for these factors, serum-free conditioned medium from STO mouse embryonic fibroblast feeder cells was concentrated and subjected to 2D gel electrophoresis, after which 828 protein spots were examined with MALDI-TOF-MS and electrospray MS/MS [67]. Among the 136 unique identifications were proteins associated with growth and differentiation, but also (residual) bovine serum proteins. Moreover, proteins associated with intracellular structures and processes were detected in the conditioned medium, indicating that a significant proportion of the cells died during extended growth in serum-free medium.

In addition to matrigel-based culture systems that replace feeder cells entirely [66], human feeder cells that support hESC propagation may serve as an appropriate substitute [68]. To identify proteins secreted by human feeder cells, serum-free conditioned medium from human neonatal foreskin fibroblast HNF01 cells was collected and concentrated [69]. The TCA precipitate was analyzed by 2D LC-MS/MS and 2D gel electrophoresis followed by MALDI-TOF/TOF, resulting in identification of a total of 102 proteins. Growth factors and proteins associated with differentiation or the extracellular matrix were considered to be important for ESC sustenance. Interestingly, almost half of the proteins identified in conditioned medium of human feeders (43 proteins) [69] matched with orthologous proteins identified in conditioned medium of mouse feeders [67], suggesting that both cells secrete common proteins that might inhibit differentiation of ESCs. However, the majority of this subset consisted of intracellular proteins that are normally not secreted. Furthermore, neither study included coculture of ESCs on the feeder cells, as the conditioned medium could then have been contaminated with factors secreted by the ESCs. Thus, some ESC-sustaining factors that may be secreted only upon interaction between feeder cells and ESCs were not taken into account.

Expert commentary

Since their first derivation in 1998, hESCs have become one of the most promising sources for *in vitro* production of cells for tissue replacement and regenerative medicine. Despite years of extensive research aimed at elucidating and controlling the complex cellular processes that underlie proliferation and differentiation, several hurdles need to be overcome before they can be safely applied in the clinics. Although hESCs have been shown to possess the potential to develop into practically every cell type of the adult individual [1], most of the differentiation protocols currently used in the

laboratory are inefficient at producing homogeneous cell populations. Identifying proteins involved in these processes may significantly increase our understanding of how to manipulate the fate of hESCs in culture and improve differentiation efficiency in a step-wise procedure.

In this review, we have described a variety of proteomic techniques that have been applied to characterize many ESC lines. Although these studies have produced a wealth of data, direct comparison is problematic due to differences in growth conditions of individual cell lines as well as in analytical techniques employed. This problem is generally recognized and also applies to other large-scale analyses, such as transcriptome profiling. Recently, an international initiative has been established, which aims at improving uniformity in culture and characterization of ESC lines [17].

Over the past several years, a wide variety of proteomic techniques have been developed or improved, both in peptide and protein separation (e.g., LC, 2D gel electrophoresis) and MS. Although all approaches have their merits, they differ in both the number and type of proteins they can display or characterize. For instance, 2D gel electrophoresis with high separation power of proteins can be applied with relative ease in most laboratories. However, this separation method tends to show a limited number of proteins due to its restricted dynamic range. Therefore, 2D gels usually display only the most abundant proteins in a sample, which generally do not reflect the proteins that underly differences in differentiation behavior, for example. Although the more sensitive DiGE-based staining procedure shows significant improvements, it is a relatively new technique that still has to prove itself in capturing low abundant proteins (e.g., transcription factors).

Separation of peptides by reversed phase chromatography, especially in a 2D set-up combined with strong cation exchange (SCX) chromatography, provides a broader coverage in protein identification, which may be the preferred method in large-scale analyses. When interested in a particular class of proteins (e.g., membrane or nuclear), enrichment of proteins prior to fractionation is likely to increase the number of significant identifications, as abundant proteins that otherwise may obscure the analysis will be removed.

Considerable differences in reported data sets can also be caused by the type of mass spectrometer used. For comprehensive, large-scale analysis, the trend is to use instruments with high scan speed and high mass accuracy. This is now starting to be applied to ESCs (as well as other stem cells), and those instruments (e.g., fourier transform [FT]-MS and OrbiTrap) are expected to be the most valuable in identifying relevant proteins in ESC biology.

For the identification of biologically relevant proteins, extensive fractionation may not be sufficient. Many biochemical pathways are directed by changes in post-translational modifications (e.g., phosphorylation) rather than by changes in abundance of proteins themselves. For instance, the earliest signaling events, such as activation and inactivation of proteins (e.g., phosphorylation) present in ESCs, occur before regulation of gene transcription. Thus, to influence specific signaling events that direct self-renewal or differentiation, choices of ESCs require the ability to activate and capture the proteins explicitly involved. Identification of phosphorylation targets entails comparative studies to determine relative changes in the phosphorylation state of the proteome before and after differentiation. In our opinion, current MS techniques provide an excellent approach to seeking those candidates [70], but will require specific techniques to specifically isolate phosphopeptides [71]. Once found, culture media can be supplemented or deprived sequentially with extracellular factors that drive these signaling pathways during the whole differentiation process. Whereas studies using model organisms such as mice allow us to evaluate and compare *in vitro* results with the *in vivo* situation, we should be aware that the processes may differ significantly in other species such as humans, as has become apparent over the past few years. Thus, even though ESCs derived from other species may provide generic clues on how proteins interact during signaling events, they must ultimately be validated in their human counterparts.

As mentioned previously, comparative studies are vital in a quest for proteins and post-translational modifications that regulate ESC self-renewal and differentiation. As even modest changes in protein expression levels may have profound biological effects, it is important to be able to determine relative expression levels of proteins in various cell types or across a developmental time course. Over the few past years, several MS-based quantitative techniques have been developed that enable the integration of protein identification and quantification. Most of the approaches rely on the incorporation of stable isotopes into proteins of one sample, which is compared with a sample that is untreated [70,72]. Peptides can be labeled chemically at cysteine residues with isotope-coded affinity tags (ICAT) [73], or at peptide N-termini with isobaric tags for relative and absolute quantification (iTRAQ) [74]. A third and increasingly popular method for mass tagging of proteins is by metabolic labeling, also termed stable isotope labeling with amino acids in cell culture (SILAC). To this end, cells are cultured in media supplemented with a particular amino acid labeled with a stable C or N isotope. This is the preferred way of labeling for several reasons, but mostly because any chemical derivatization (including incomplete or side reactions) is avoided. In principle, this method is applicable to ESCs, as they can be cultured in relatively well-defined media. Using such quantitative approaches, relative expression levels of multiple proteins can be studied in time or compared with cells grown under different conditions. This will provide more insight into cellular processes than a qualitative experiment alone.

MS-based quantitative analytical methodologies undergo continuous advancements in technology and accuracy [70,72]. However, these approaches only indicate which candidates might play a role in cellular processes; functional studies are needed to validate their suggested importance. Generating data sets comprising proteins identified in cells under multiple conditions is a prerequisite to unraveling signaling cascades of immense

complexity [75]. However, before specific functions can be annotated to candidate proteins, descriptive and associative studies should be complemented with functional assessments. This can, for instance, be achieved by overexpression of dominant negative mutant forms or RNAi, which is at present only routinely applicable to mESCs.

Besides identification and characterization of paramount proteins that regulate cellular processes *in vitro*, other problems related to current hESC cultures need to be addressed. For instance, hESCs cultured in the presence of nonhuman factors, such as mouse feeder cells and bovine serum, metabolically incorporate substantial amounts of *N*-glycolylneuraminic acid quantification [76]. Normal humans have circulating antibodies specific for this sialic acid, which compromise transplantation of hESC-derived cells grown in the presence of xeno-factors. Whereas human feeder cells in combination with knock-out serum replacement provide an attractive alternative [65], culturing hESCs in clinically applicable numbers may require scaled-up cell-free culture systems that contain serum substitutes and ESC-sustaining factors. We believe that large-scale comparative analyses using high-quality quantitative MS approaches will significantly contribute to our understanding of ESC behavior and development at the cellular level, facilitating optimization of culture requirements and differentiation procedures.

Five-year view

With recent advancements in MS-based quantitative analysis techniques [62,70,77], it is likely that relative protein quantification will soon become the standard evaluation method to identify proteins involved in cellular processes. Comparative quantification strategies as such could readily be used to determine which proteins implicate maintenance and differentiation of ESCs. When combined with the purification of phosphorylated proteins using affinity enrichment [71,75,78,79] at separate time points during development, activation and inactivation of signaling pathways become apparent. Comparison with development of tissues and organs *in vivo* may further elucidate temporal cooperations and interactions between the proteins involved. Once identified, these processes can be mimicked *in vitro* by incubating hESCs in culture medium supplemented with components that either stimulate proliferation and self-renewal or induce differentiation towards specific cells or tissues. In addition, this allows complete omission of non-human components that would otherwise induce host-versus-graft immune responses upon transplantation of hESC-derived cells grown in the presence of xeno-factors. The combined advances in both hESC biology and MS holds great promise for directing *in vitro* studies towards clinical applications within the next few years.

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Key issues

- Large-scale analysis of the embryonic stem cell (ESC) proteome complements characterization at the transcriptional level and may provide the missing link between gene transcription and cell behavior.
- Proteome profiling of mouse ESCs will give insight into proliferation and differentiation of ESCs in general, but cannot serve as a surrogate to study the behavior of human ESCs.
- Comparative proteome analyses of ESCs and their differentiated derivatives are required to characterize differential expression and modification of proteins specific for ESCs and differentiated cells.
- Characterization of signaling cascades that are active during proliferation and self-renewal or differentiation of ESCs will provide clues on how to control these processes *in vitro* culture medium supplements.
- Accurate quantitative methods will be needed to explain differences between ESCs or between differentiation states.

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