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Proteomic Analysis of Mouse ES Cells

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1. Introduction

Embryonic stem (ES) cells have generated enormous interest because of their capacity to self-renew and differentiate into various cell types *in vitro*. Although numerous problems are encountered in the use of ES cells for regenerative medicine, such as ethical issues associated with the use of stem cells established from terminated human embryos and immunorejection due to transplantation of allogenic ES cell-derived cells into patients, recent technologies to generate induced pluripotent stem (iPS) cells from adult somatic cells have provided alternative ways to access pluripotent stem cells (Takahashi et al., 2007). However, the practical application of these pluripotent stem cells has yet to emerge, and regulatory mechanisms are not well known. Moreover, precise differentiation methodologies of ES and iPS cells have not been developed. These problems cause difficulties in the manipulation of pluripotent stem cells and derivation of functionally differentiated cells. Detailed analysis of the transcriptome has allowed elucidation of transcription networks that regulate the pluripotency of these stem cells. However, the specific nuclear infrastructures that maintain the pluripotent stem cell-specific transcription network have not yet been elucidated. We used proteomics to analyze the nuclear protein machinery in stem cells and identified some crucial components for the maintenance of pluripotent stem cells. In addition, various growth factors and extracellular matrix components regulate the pluripotency and differentiation of stem cells. Therefore, the cell surface receptors that bind these regulatory factors are important for the precise regulation of stem cells. We have also explored stem cell-specific cell-surface markers by proteomic analysis of mouse ES cells. These cell-surface membrane proteins can be useful to manipulate pluripotent stem cells. In this chapter, we describe some examples of new findings elucidated by proteomic analysis of ES cells.

2. Quantitative analysis of proteins by proteomics

To identify proteins from complex samples, there are three major approaches (Fig. 1). One is a 2-dimensional polyacrylamide gel electrophoresis (2D-PAGE)-based method, and the other two are shotgun-based methods using 2D-liquid chromatography (2D-LC). The first approach, which involves 2D-gel electrophoresis followed by identification of isolated

proteins by mass spectrometry (MS), has been used for a long time. To obtain comparative data of two different samples, 2-dimensional difference gel electrophoresis (2D-DIGE) has been developed (Unlu, et al., 1997). In this method, protein samples that are covalently conjugated with different fluorescent dyes are combined, resolved by 2D-PAGE, and differentially expressed protein spots are quantified according to the intensity of each fluorescent color by a laser scanner. Differentially expressed protein spots are then excised from the gel, trypsinized, and subjected to MS analysis. To identify the proteins, molecular weight information of the digested peptides are compared with databases such as the National Center for Biotechnology Information (NCBI) and Swiss-Prot. In contrast, in the case of the shotgun-based methods, protein samples are first digested with a specific protease, and the resulting huge number of peptides are fractionated by 2D-capillary chromatography followed by automated analysis by MS (Nagele, et al., 2004, Wu, et al., 2006). For quantitative analysis by the shotgun method, isobaric tag for relative and absolute quantitation (iTRAQ) have recently been developed as a labeling reagent for the digested peptide fragments (Ross, et al., 2004). During tandem mass spectrometric (MS/MS) analysis, the isobaric tags are readily cleaved from the peptides, and the generated reporter fragments from different samples give different molecular weight peaks. By comparing the intensity of these reporter fragments, the relative protein quantity of each protein sample can be calculated. In contrast, peptide fragments without reporters are used to identify peptide sequence by MS/MS analysis. Alternatively, SILAC (stable isotope labeling with amino acids in culture) can be used when comparing proteins in cells cultured under different conditions, such as with or without growth factors, chemicals, or at different time points. In this case, cells are cultured in normal medium or medium replaced with selected amino acids synthesized with ^{13}C and ^{15}N (Chen, et al., 2000, Ong, et al., 2002). Under these conditions, the labeled "heavy" amino acid will be incorporated into most of the proteins in the cell. After harvesting, these labeled and non-labeled cells are combined and processed as the normal shotgun method without labeling. Quantification of proteins can be done by comparing the intensity of light and heavy MS peaks of each peptide. In the present study, we applied quantitative proteomics to identify critical proteins in regulating the pluripotency-specific transcription network.

3. Proteomic analysis of pluripotency-specific nuclear proteins expressed in mouse embryonic stem cells

To identify specific proteins involved in the regulation of pluripotent stem cells, we used mouse ES cells. Although mouse ES cells are normally cultured on mouse embryonic fibroblast feeder cells, some mouse ES cell lines, such as D3 cells, can be maintained without feeder cells. We used D3 cell line for our analysis to avoid contamination of feeder cells. Leukemia inhibitory factor (LIF) is a crucial factor for maintenance of undifferentiated mouse ES cells. Culturing ES cells without LIF on a gelatin-coated dish for 7 days induces spontaneous differentiation. These pluripotent ES cells and the differentiated cells cultured without LIF were disrupted in a hypotonic buffer, centrifuged, and nuclear and cytoplasmic fractions were prepared. Proteins in these fractions were analyzed by 2D-DIGE. Proteins prepared from pluripotent cells or differentiated cells were labeled with different fluorescent dyes, separated by 2D-PAGE, and expression of the resolved protein spots were quantitatively analyzed by scanning the fluorescent intensity of the labeled protein spots. Differentially expressed protein spots were extracted and identified by MS (Fig. 2). More

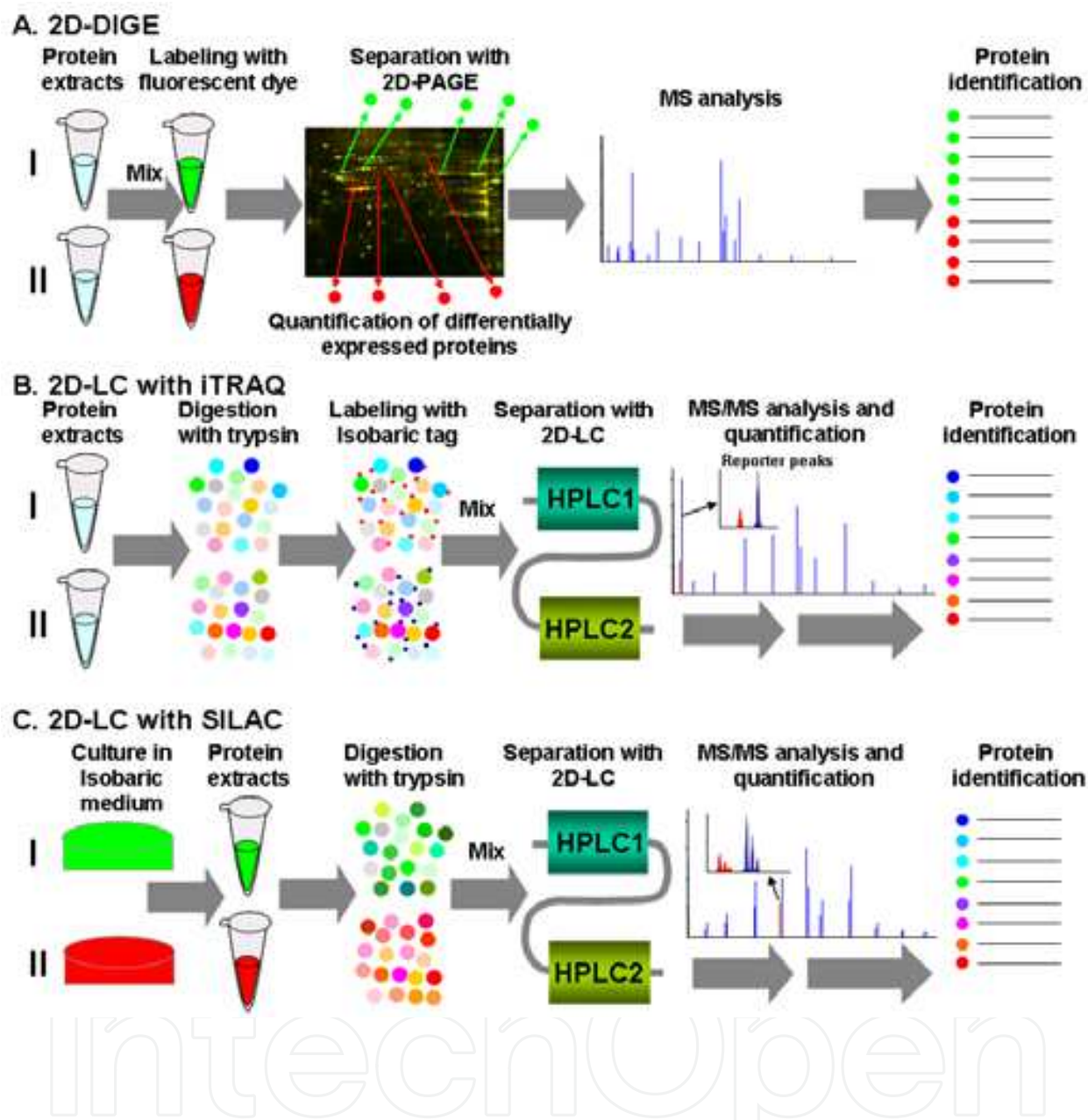


Fig. 1. Three major approaches for quantitative proteomic analysis.

(A) 2D-DIGE is a 2D-PAGE-based method using fluorescently labeled protein samples. Quantification of protein expression is performed on a 2D-PAGE gel and differentially expressed proteins are further analyzed by MS. (B) 2D-LC method with iTRAQ reagents. Protein samples are first digested with a protease and then labeled with isobaric tag reagents. Labeled peptide samples are mixed, fractionated by 2D-LC, and analyzed by MS/MS. Quantification is based on the relative intensity of the reporter fragments of iTRAQ reagents. (C) 2D-LC method with metabolically labeled samples. Cells are cultured with isobaric amino acids, and harvested cells are combined and processed as in (B) without the labeling step. Quantification of proteins is based on the intensity of each MS peak.

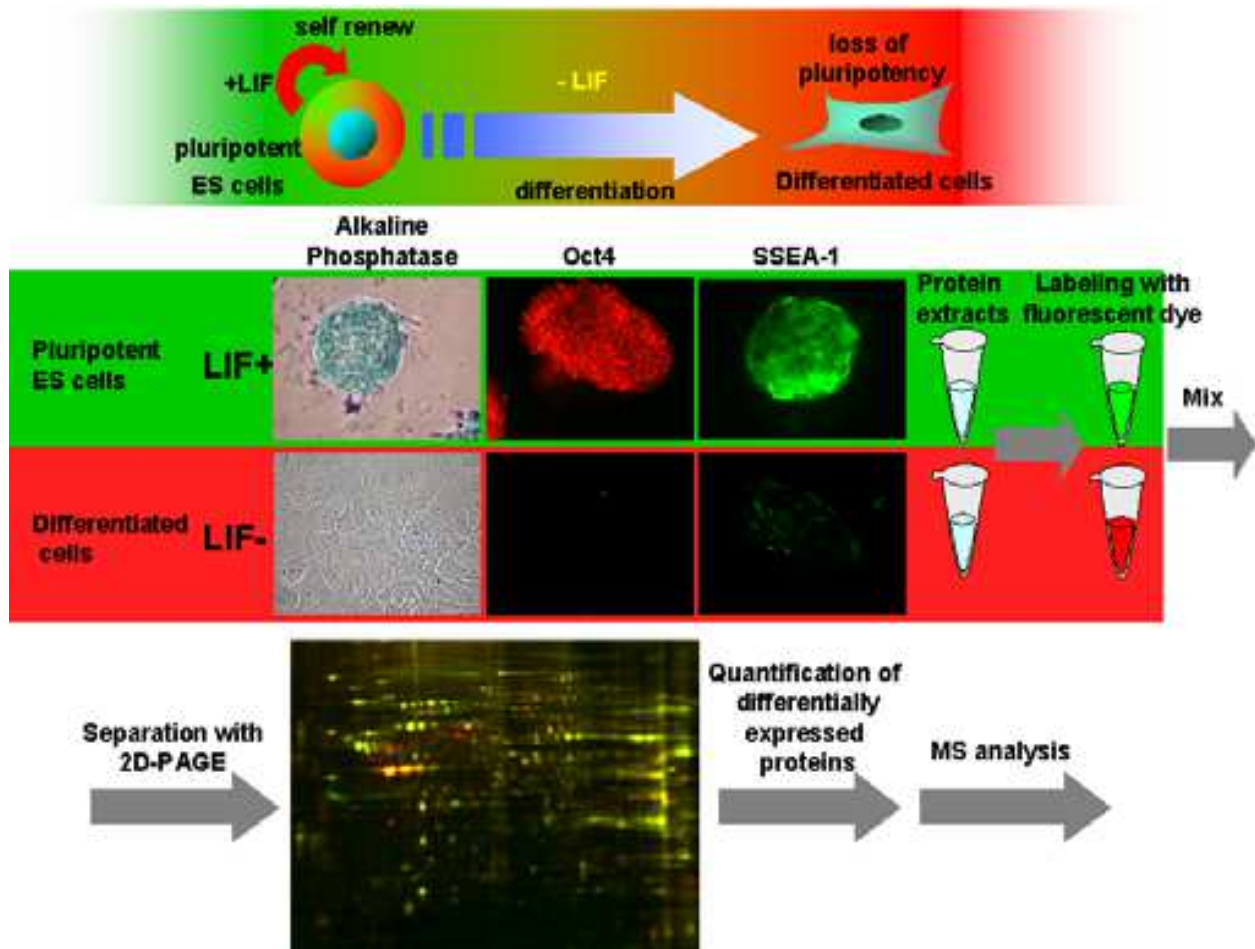


Fig. 2. Proteomic analysis of pluripotency-specific proteins expressed in mouse ES cells. Mouse ES cells cultured with or without LIF for one week were used as the source of pluripotent ES cells and differentiated cells, respectively. Pluripotent ES cells showed alkaline phosphatase activity and were positive for Oct4 and SSEA-1. In contrast, the differentiated cells were negative for these markers. The extracted proteins were labeled with green or red fluorescent dyes, mixed, and analyzed by 2D-DIGE.

than 100 proteins specifically expressed in pluripotent ES cells were identified from nuclear and cytoplasmic extracts. This study was the first detailed proteomic analysis of pluripotency-specific nuclear proteins in mouse ES cells (Kurisaki, et al., 2005). Interestingly, many of the pluripotent stem cell-specific nuclear proteins were related to chromatin functions. For example, we identified the 60-kDa subunit of the SWI/SNF complex (BAF60a/Smarca1), a component of a chromatin-remodeling complex, which slides nucleosomes along the DNA helix in an ATP-dependent manner and functions to expose genomic DNA to transcription factors or chromatin modifiers (Roberts, et al., 2004). We also identified one of the high-mobility group proteins (HMG-B2), which loosens the DNA helix thereby enhancing the accessibility to chromatin-remodeling complexes and possibly to transcription factors (Travers, 2003). Amine oxidase flavin-containing domain protein 2 (AOF2), also known as lysine-specific demethylase 1 (LSD1), is a demethylase for histone H3K4. Methylation of histone H3K4 is linked to active transcription. A recent report indicated that LSD1 regulates the expression and appropriate timing of key developmental regulators during early embryonic development (Foster, et al., 2010). Transcriptional intermediary factor

1 β (TIF1 β /KAP1/Trim28) has been reported to be a universal corepressor that forms a complex with histone methyltransferase SETDB1, which methylates histone H3K9 within euchromatin (Ivanov, et al., 2007, Sripathy, et al., 2006). RbAp48 is a histone-binding protein, which is often found in various histone modifying enzyme complexes (Wolffe, et al., 2000). MSH2 is a DNA mismatch repair protein, and recent reports have suggested that this protein could function as a coactivator of transcription (Wada-Hiraike, et al., 2005).

In the same year, a large proteomic dataset of proteins expressed in the E14 mouse ES cell line was analyzed by 2D-LC-based proteomics (Nagano, et al., 2005). Although the expression of the identified proteins was not systematically compared with other non-ES cells or differentiated cells, 1790 proteins in total were identified, including 365 potential nuclear proteins, such as pluripotency-specific transcription factors Oct4 and Sox2, as well as chromatin-related proteins TIF1 β and Smarcd1. Very recently, another group has performed extensive proteomic analysis using ES-like embryonic carcinoma cells (F9) and differentiated cells (NIH3T3), and identified a number of chromatin-remodeling factors highly expressed in F9 cells (Singhal, et al., 2010).

4. TIF1 β regulates the pluripotency of embryonic stem cells

To isolate crucial regulatory components for the maintenance of ES cell pluripotency, the proteins identified by MS analysis were stably expressed in ES cells and further functional screening was performed according to their prolonged alkaline phosphatase activity in the absence of LIF. Among these chromatin-related proteins, we found TIF1 β as a functional regulator of pluripotency, which prolonged the pluripotency of ES cells after withdrawal of LIF. Recently, other groups have also identified TIF1 β as an essential gene for mouse ES cells by RNAi-based screening (Fazio, et al., 2008, Hu, et al., 2009). However, the mechanism by which TIF1 β regulates ES cell pluripotency has not been well elucidated.

When TIF1 β was knocked down in mouse ES cells, the cells lost their tight, compact morphology and adopted a stretched-out shape even in the presence of LIF. The growth of TIF1 β -knockdown ES cells was significantly decreased, and the expression of pluripotency markers SSEA1 and Nanog was diminished. In contrast, expression of the primitive ectoderm marker gene, *Fgf5*, and relatively weak expression of the extraembryonic ectoderm marker gene, *Eomes*, was induced. These results indicate that TIF1 β is an indispensable factor for the maintenance of pluripotency in ES cells, which preferentially inhibits the differentiation of ES cells into primitive ectoderm cells.

When we carefully analyzed the expression of TIF1 β in ES cells, we found that TIF1 β is specifically phosphorylated at serine 824 (S824), which induces active relaxation of chromatin (Ziv, et al., 2006). Phosphorylation at S824 was dramatically decreased when the cells were differentiated. As shown in Fig. 3, TIF1 β was highly phosphorylated in the inner cell mass of embryos during early development, from which ES cells can be established (Seki et al., 2010). Ataxia telangiectasia mutated (ATM), a serine/threonine protein kinase, has been reported to be a specific kinase for S824 of TIF1 β upon DNA double-strand breakage (Ziv, et al., 2006). We confirmed that ATM could phosphorylate the C-terminus of TIF1 β as transfection of ATM shRNA significantly decreased the phosphorylation of TIF1 β . Concomitantly, protein levels of Oct4 and Nanog were also decreased by knockdown of ATM in mouse ES cells, further supporting the importance of TIF1 β phosphorylation.

Unexpectedly, TIF1 β had distinct effects on ES cells in a phosphorylation-dependent manner. First, phosphorylated TIF1 β promotes the pluripotency of mouse ES cells. Stable

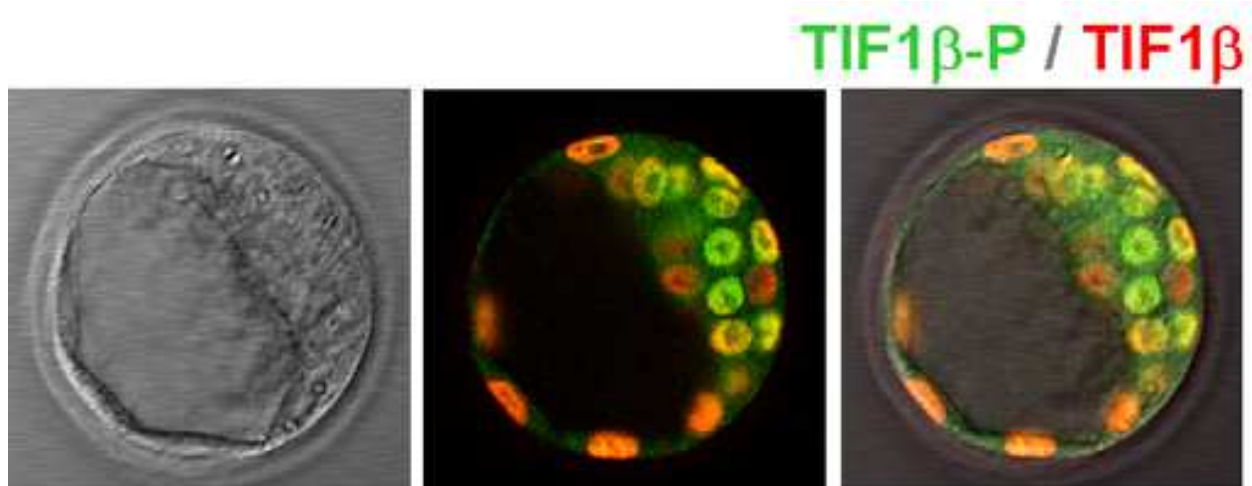


Fig. 3. Specific localization of phosphorylated TIF1 β in mouse embryos. Mouse embryos (E3.5) were immunostained with TIF1 β (red) and phosphorylated TIF1 β (green) antibodies. Phosphorylated TIF1 β is highly concentrated in the inner cell mass of blastocysts (quoted from Seki et al., 2010).

expression of the TIF1 β -S824D mutant, which mimics phosphorylated TIF1 β and induces constitutive chromatin relaxation, maintained Oct4 and Nanog protein expression after 8 days in culture without LIF. In contrast, expression of TIF1 β -S824A, which cannot be phosphorylated, in ES cells did not show these effects. Second, TIF1 β inhibits the differentiation of mouse ES cells in a C-terminal phosphorylation-dependent manner. Embryoid bodies prepared from dissociated ES cells were cultured in suspension in serum-free medium and then differentiated by adhesion culture on a poly-L-lysine, laminin, and fibronectin coated dish. Although the cells transfected with the control vector and TIF1 β -S824A-expressing cells showed significant outgrowth of neurofilament-200- and TuJ1-positive neurites projected from embryoid bodies, TIF1 β -S824D-expressing cells did not show such outgrowth. Third, phosphorylation of TIF1 β is important for induction of iPS cells from somatic cells. Retroviral gene transfer of TIF1 β -S824A dramatically decreased the generation of ES-like colonies from mouse embryonic fibroblasts infected with four transcription factors, Oct4, Sox2, Klf4, and c-Myc. Moreover, the colonies were very difficult to establish as iPS cell lines, and ES cell-specific markers were not expressed in these cells. In contrast, the induction of iPS cells was increased when TIF1 β -S824D was introduced in addition to the above four transcription factors. Interestingly, the induced iPS cells generated with TIF1 β -S824D showed more complete expression of ES cell-specific marker genes. The phosphorylation of TIF1 β , which induces relaxation of chromatin, seems to affect the efficiency of iPS induction and the quality of established clones of iPS cells.

TIF1 β has been reported to be involved in transcriptional repression with heterochromatin protein 1 (HP1) and localized to heterochromatin foci in differentiated cells (Sripathy, et al., 2006). However, in pluripotent ES cells, TIF1 β is diffusely localized in the nucleoplasm and is not localized with heterochromatin foci. On the other hand, phosphorylated TIF1 β (S824) showed punctate staining in the nucleus. This characteristic localization of phosphorylated TIF1 β was partially colocalized with transcriptionally activated euchromatin markers such as histone H3K4me³ and H3K9Ac, but not with the heterochromatin markers histone H3K9me³ and HP1 α . These data suggest that TIF1 β could play a distinct role in pluripotent ES cells as compared to other differentiated cells.

What is the function of phosphorylated TIF1 β in the specific nuclear spots? We hypothesized that phosphorylated TIF1 β might be involved in transcriptional activation of pluripotency-specific genes in the activated chromatin foci of pluripotent stem cells. In fact, ectopic expression of TIF1 β -S824D, but not TIF1 β -S824A, in ES cells markedly induced various pluripotency-specific genes such as *Nanog*, *Sox2*, and *Dax1*. Moreover, knockdown of TIF1 β resulted in an increased number of H3K9me³ and HP1 α foci, suggesting that endogenous TIF1 β inhibits H3K9me³ and HP1 α foci formation in pluripotent mouse ES cells. These results suggest that TIF1 β can selectively activate the expression of various pluripotency-specific genes in a phosphorylation-dependent manner.

Biochemical analysis suggests that TIF1 β functions as a transcriptional activator of pluripotency specific genes by forming a complex with pluripotency-specific transcriptional factors at promoter regions. Indeed, co-immunoprecipitation assays revealed that TIF1 β specifically forms a complex with Oct4 in a C-terminal phosphorylation-dependent manner. In contrast, another pluripotency-specific transcription factor, *Sox2*, was not detected in this complex. In addition to these transcription factors, *Smarca1*/BAF60a was found to interact with TIF1 β , although this interaction was not dependent on the phosphorylation of TIF1 β . As mentioned above, our quantitative proteomic analysis has previously identified *Smarca1* as a highly expressed protein in undifferentiated ES cells (Kurisaki, et al., 2005). *Smarca1* functions as an ATP-dependent SWI/SNF chromatin remodeling factor that modulates chromatin structure. Recently, *Smarca1* has been shown to be a component of esBAF, an ES-specific BAF (Brg/Brahma-associated factors) ATP-dependent chromatin remodeling complex, which is essential for ES cell self-renewal and pluripotency (Ho, et al., 2009). Endogenous TIF1 β also formed a complex with other esBAF components, such as Brg-1 and BAF155. TIF1 β -S824D specifically induced transcriptional activation of a proximal *Nanog* promoter reporter construct with Oct4. A ChIP assay confirmed that TIF1 β forms a complex on the endogenous *Nanog* promoter in a phosphorylation-dependent manner. TIF1 β -S824D was shown to recruit endogenous Oct4 to form an active complex on the *Nanog* promoter. Microarray analysis revealed that one third of Oct4 target genes are specifically regulated by phosphorylated TIF1 β . Several chromatin remodeling factors such as *Suz12*, *Chd9*, *Pcaf*, and *Smarca1* were also induced by TIF1 β -S824D. Our data suggest that phosphorylated TIF1 β forms a unique complex with Oct4 on pluripotency-specific genes and promotes expression of pluripotency-specific transcriptional factors such as *Nanog*, *Sox2*, and Oct4. Recent studies by ChIP-ChIP analysis revealed that TIF1 β interacts with half of the promoters occupied by Oct4 and *Sox2* (Jin, et al., 2007), suggesting that TIF1 β might be an important regulator of Oct4-dependent transcription in ES cells.

The SWI/SNF2-like chromatin complex (BAF complex) is a huge complex with ATP-dependent chromatin remodeling activity, which is about 2 M Da in size. Components in this complex, such as Brg-1, BAF155, and BAF47, are essential in early development. Knock-out mice of these genes lead to peri-implantation lethality and failure to generate both the inner cell mass and trophoblast. Brg has been purified from *Xenopus* egg extracts and promoted the reprogramming of somatic cells into ES-like stem cells (Hansis, et al., 2004). Ho et al. reported that the ES-specific BAF complex (esBAF), which is required for self-renewal and pluripotency of mouse ES cells, was different from that in mouse embryonic fibroblasts (MEFs) or newborn mouse brain. The esBAF contains Brg-1, BAF155, and BAF60a, but not *Brm*, BAF170, or BAF60c. Pluripotency-specific transcription factors Oct4 and *Sox2* also associated with the esBAF complex (Ho, et al., 2009). Recently, other groups have reported that a couple of proteins, which are components of the chromatin remodeling

complex, promote the establishment of iPS cells from MEFs (Singhal, et al., 2010). They performed differential proteomics of the nuclear proteins extracted from NIH3T3 cells and F9 embryonic carcinoma cells by SILAC method. After computer-assisted Gene Ontology analysis of more than 5,000 identified proteins, they found specific expression of chromatin remodelling factors in pluripotent ES cell. Among them, Brg-1 and BAF155, which have been suggested to be crucial factors for maintenance of pluripotency of ES cells, promoted demethylation of pluripotency-specific transcription factor promoters such as the Oct4 and Nanog locus during the induction of iPS cells. Their results also supported the importance of the chromatin remodeling protein complex for the establishment of iPS cells from somatic cells.

5. Interactome of transcription factors identified by proteomics

In addition to describing a total set of proteins expressed in a population, proteomics are quite powerful for the analysis of protein components in a certain complex. Identification of functional protein complexes of transcription factors is important to elucidate transcription networks. Wang et al. constructed a protein interaction network surrounding the pluripotency factor Nanog (Wang, et al., 2006) by taking advantage of unique ES cells that express *Escherichia coli* biotin ligase, BirA. In this ES cell line, Nanog protein was N-terminally tagged with Flag and a biotin acceptor sequence was biotinylated *in vivo*. Thus, the Nanog protein complex was readily isolated by tandem purification with Flag antibody beads and streptavidin beads. In total, 266 proteins were identified. The constructed network was enriched for nuclear factors important for maintenance of the ES cell state and co-regulation of differentiation. Recently, an extended protein network that interacts with Oct4 was also reported using a different epitope-tagging affinity purification strategy (Pardo, et al., van den Berg, et al., 2010). A combination of the 3× FLAG epitope and a calmodulin binding peptide (CBP) separated by a TEV cleavage site was inserted into the C-terminus of the Oct4 coding region of a BAC clone containing full-length Oct4. This BAC construct was then integrated into the Hprt locus of mouse ES cells. In this system, expression levels of the Oct4-fusion protein were less than that of endogenous Oct4. In another study, van den Berg et al. used a single N-terminally 3× FLAG epitope-tagged Oct4 as the probe. Both studies succeeded in describing a detailed Oct4-centered interactome network in mouse ES cells. Although Oct4, Sox2, and Nanog form a positive feedback loop to maintain the ES cell-specific transcription network, such protein interactomes constructed by proteomics helps to locate Oct4 networks in known signaling pathways. Interestingly, both studies identified chromatin-related proteins, such as SWI/SNF chromatin remodelling factors, the NuRD complex, and the LSD1 complex, rather than pluripotency-enriched transcription factors. These results suggest the importance of epigenetic modifying complex associated with transcription factors in ES cells.

6. Application of pluripotent stem cells for regenerative medicine

Recently developed iPS cell technology enabled to obtain patient-derived pluripotent stem cells by reprogramming adult somatic cells with four transcription factors that can reorganize the ES-like transcription network (Takahashi, et al., 2007). Although iPS technology can overcome two critical problems, such as immunorejection due to mismatch of HLA types and ethical issues associated with using ES cells established from destroyed

human embryos, there are still many problems that need to be addressed. First, these stem cells are not standardized; not all pluripotent stem cells can efficiently differentiate into specific cells. Moreover, insufficient differentiation methodologies for stem cells pose an increasing problem. Differentiation of pluripotent stem cells has been mainly studied with mouse and human ES cells. However, the specificity and efficiency of differentiation of these stem cells by current methods is still insufficient. Moreover, most of the differentiated cells are embryonic-type cells, not adult-type, and may not effectively function in patients. Therefore, in addition to establishing quantitative and specific differentiation methods, maturation protocols for differentiated cells may be required to obtain the targeting cells *in vitro*.

One of the most serious concerns for their application to regenerative medicine is the tumorigenic property of iPS cells. After transplantation of differentiated cells derived from iPS cells, occasionally teratomas can form from transplanted cells. Two major reasons have been suggested for this phenomenon. One is due to reactivation of the integrated tumorigenic transgene c-Myc in iPS cells. Human iPS cells have been established by the stable introduction of crucial transcription factors, Oct4, Sox2, Klf4, and c-Myc into the genome using retroviral or lentiviral gene transfer. Induction of iPS cells from fibroblasts would be much less efficient if c-Myc was excluded. However, genomic incorporation of transgenes by these viruses itself destroys multiple loci of the endogenous genome, which increases the potential risk of tumor formation by disruption of tumor suppression genes. Recently developed alternative integration-free induction methods, such as protein (Kim, et al., 2009, Zhou, et al., 2009), RNA (Fusaki, et al., 2009, Nishimura, et al., 2011, Warren, et al., 2010), and chemical compound-based induction methods (Li, et al., 2009) are expected to overcome this problem. In addition to reactivation of the Myc transgene during differentiation, residual contamination of undifferentiated iPS cells in the differentiated cell population may also lead to tumor formation (Nakagawa, et al., 2008). Transplantation of pluripotent ES cells into immunodeficient mice by definition generates teratomas *in vivo* that contain tridermally differentiated tissues. For the differentiation of both human and mouse ES/iPS cells, an embryoid body-based method has been widely adopted. Differentiation by embryoid body formation is an effective and convenient method for ES/iPS cell differentiation. However, there could be some residual differentiation-resistant pluripotent stem cells inside the embryoid body, and these stem cells may continue to grow after transplantation into the patient. Therefore, contamination of a small number of pluripotent stem cells causes high risk of tumor formation after transplantation (Miura, et al., 2009).

7. Tumorigenicity of pluripotent stem cells

To avoid tumor formation caused by contamination of undifferentiated stem cells, some approaches have been proposed. For example, prolonged *in vitro* differentiation of human ES cells into dopaminergic neurons is effective for preventing the formation of teratomas (Brederlau, et al., 2006). Another example is the control of stem cell fate by “stem cell suicide genes”. Stable introduction of a suicide gene such as the herpes simplex virus thymidine kinase gene has been reported (Schuldiner, et al., 2003), which makes ES cells highly sensitive to ganciclovir at low concentrations. The expression of thymidine kinase under the control of the Oct4 promoter is effective for the ablation of undifferentiated ES cells *in vivo* that may produce teratomas (Hara, et al., 2008). However, the long-term stability of

exogenous suicide genes *in vivo* has yet to be evaluated. An alternative approach is separation of residual pluripotent stem cells by cell-surface markers. Several cell-surface markers specific to pluripotent ES/iPS cells, such as SSEA4, TRA-1-60, and TRA-1-81, have been widely used. SSEA-4 is a glycolipid carbohydrate epitope specifically detected in human ES/iPS/EC cells. SSEA-4 is also expressed in some populations of mesenchymal stem cells (Gang, et al., 2007) and spermatogonial cells (Conrad, et al., 2008). Both TRA-1-60 and TRA-1-81 are antigens that are carbohydrate chains of a pericellular matrix proteoglycan, podocalyxin. They were first identified as cell-surface markers expressed in embryonic carcinomas. TRA-1 family proteins are specifically expressed both in human and rodent ES/iPS cells. However, these antigens can be lost by digestion of proteoglycan peptide with trypsin or collagenase treatment, which is required for dissociation of cells after differentiation. Podocalyxin without glycosylation is no longer a pluripotency-specific marker. On the other hand, insufficient dissociation of differentiated cell mixtures in embryoid bodies could increase the risk of contamination of undifferentiated cells that cause uncontrolled growth and teratoma formation after transplantation. Thus, efficient and complete removal of residual ES/iPS cells seems to be important for transplantation of differentiated cells. Mouse and human ES cells are considerably different from each other with respect to growth factor requirements and cell surface markers. Recent reports have suggested that human ES cells are somewhat similar to mouse Epi-stem cells (Epi-SC). Human ES/iPS cells have difficulty surviving after complete dissociation by proteases without a ROCK inhibitor (Watanabe, et al., 2007), and their growth rate is relatively slow. This property of human pluripotent stem cells might be helpful to lower the risk of teratoma formation after transplantation of differentiated cells. However, recently developed culture conditions for human ES cells (Hanna, et al., 2010) may allow easier handling of human ES/iPS cells as mouse ES cells and concomitantly increase the danger of tumor formation.

8. Proteomics approaches to identify cell-surface markers in pluripotent stem cells

Protein analyses by comprehensive proteomics are a potent strategy to elucidate not only identification of marker proteins, but also the molecular dynamics of proteins and their biological implications in cells and tissues. In particular, approximately 20-30% of vertebrate genes encode integral membrane proteins, and especially cell-surface membrane proteins are involved in critical cellular processes and are considered major pharmaceutical drug targets. Despite their biological significance and benefits as cell-surface markers, proteomic analysis of membrane proteins has technical challenges because of their relatively higher molecular weight and hydrophobicity. 2D-PAGE in combination with multicolor fluorescent labeling of proteins for quantitative analysis has limitations in terms of the dynamic range of protein quantity and the molecular mass range of detectable proteins. In addition, difficulties in fully automating 2D-PAGE coupled with MS have hampered its widespread use for large-scale proteomic analysis. Shotgun-based techniques that digest protein samples into small peptides prior to MS analysis have recently been developed for high-throughput proteomic analysis. Protease treatment of protein extracts generates a huge number of peptides. Therefore, a digested peptide mixture should be well fractionated and concentrated for efficient protein identification with low background noise. We have

previously reported an improved method for MALDI-TOF based proteomic analysis of membrane proteins with a combination of zwitterionic hydrophilic interaction liquid chromatography and reverse-phase chromatography, which is a more effective separation method for digested peptide mixtures (Intoh, et al., 2009a). Using this method, we have performed proteomic analysis of membrane proteins specifically expressed in pluripotent mouse ES cells. For quantitative comparison, we used isobaric tags reagents, iTRAQ,

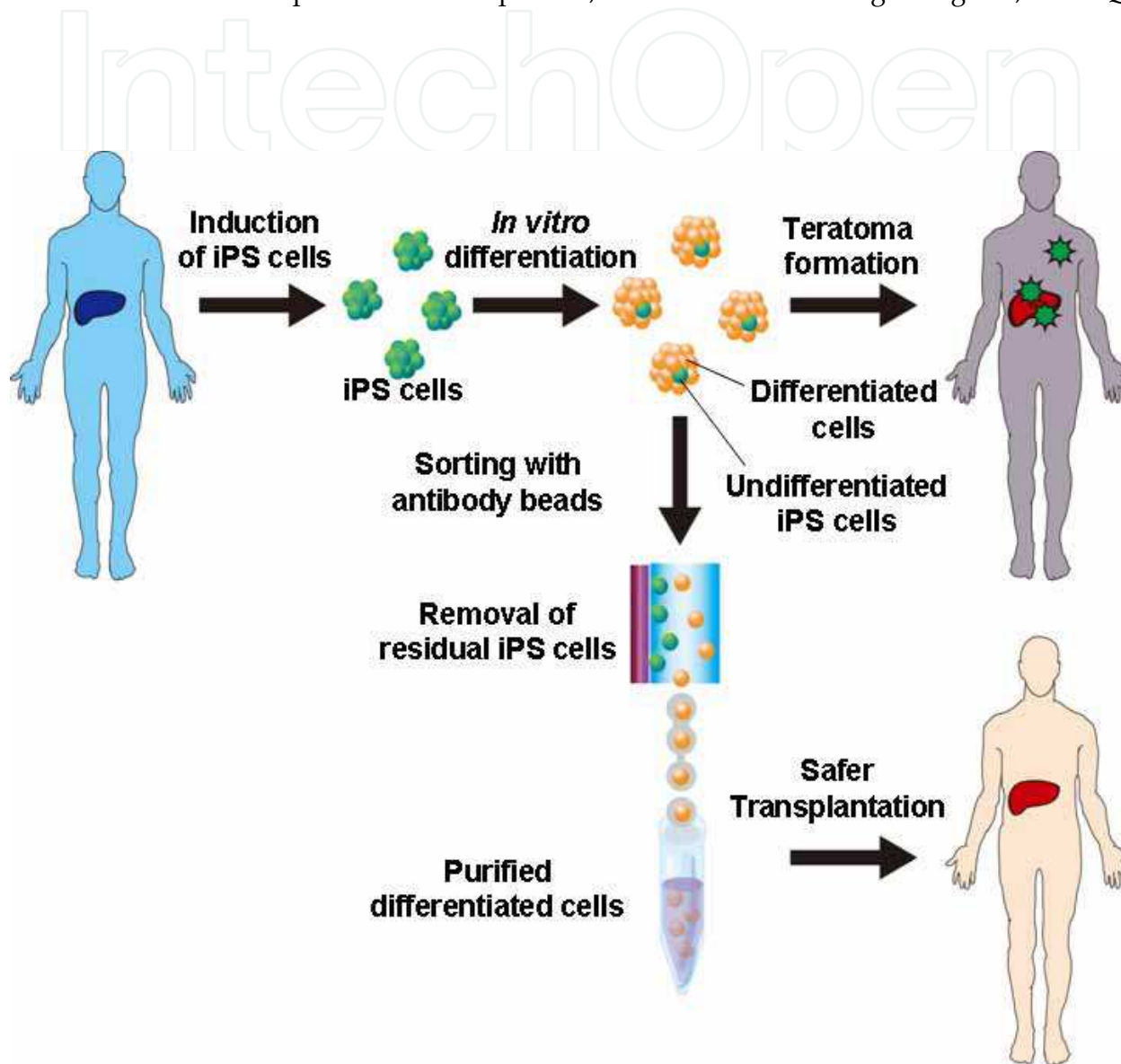


Fig. 4. Safe regenerative medicine using differentiated cells without contamination of tumor-forming pluripotent stem cells. The iPS cells established from patients are processed to differentiate into target cells. However, contamination of a small number of differentiation-resistant pluripotent stem cells in the differentiated cell pool can result in teratoma formation. Pluripotency-specific cell-surface markers may be useful to remove residual pluripotent iPS cells from the differentiated cell pool before transplantation.

to label trypsinized peptides prepared from different cell sources. Simultaneously, we also tested the conventional 2D-PAGE method for identification of membrane proteins for comparison, where extracted proteins were labeled with different fluorescent dyes, combined, and separated by 2D-PAGE. As such, we identified various candidate proteins that are highly expressed on the plasma membrane of pluripotent ES cells and significantly down-regulated during differentiation (Intoh, et al., 2009b). Some of the identified cell-surface proteins were also highly expressed in human ES/iPS cells and down-regulated during differentiation via embryoid body formation. These highly expressed membrane proteins could be useful for separation of residual ES/iPS cells from *in vitro* differentiated cell mixtures. The ultimate goals of safe regenerative medicine using human pluripotent stem cells require purification of targeting progenitors or differentiated cells. They also request complete removal of residual pluripotent stem cells, which could continue to grow and form teratomas *in vivo*. Development of more efficient differentiation methods as well as identification of specific cell-surface markers for both pluripotent and differentiated cells will contribute to safe and effective regenerative medicine in the near future (Fig. 4).

9. Conclusion

In this chapter, we have introduced some examples of proteomic analysis of ES cells. Transcriptome analysis using microarrays or direct sequencing of transcripts reveals the expression of mRNAs. However, the transcriptome does not necessarily correlate with expression levels of the corresponding protein. Moreover, analysis of the protein interactome by proteomics provides significant insight into understanding the mechanisms of how transcription factors function to establish pluripotency-specific functions in ES cells. Thus, proteomics approaches are important for further understanding the regulatory mechanisms of pluripotency and differentiation of ES/iPS cells.

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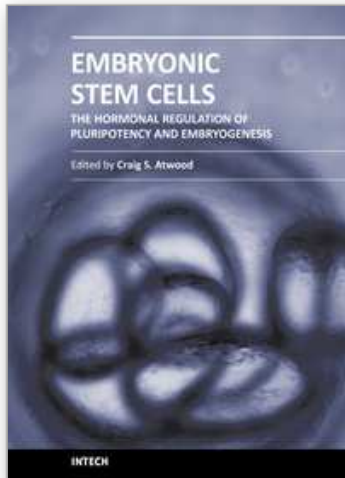
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