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iPSCs are transcriptionally and post-transcriptionally indistinguishable from fESCs

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1. ABSTRACT

Induced pluripotent stem cells (iPSCs) are generated by reprogramming mouse or human somatic cells to a pluripotent state by introducing key transcription factors and have great therapeutic potential. It has been illustrated that the transcriptional and post-transcriptional profiles of nuclear-transferred embryonic stem cells (ntESCs) is identical to those of embryonic stem cells derived from fertilized blastocysts (fESCs). Although iPSCs seem to be indistinguishable from fESCs, the degree of transcriptomic and proteomic similarity among iPSCs, ntESCs, and fESCs has not yet been elucidated completely. To investigate whether iPSCs and fESCs have similar therapeutic potential, we compared mRNA and protein profiles of mouse iPSC, ntESCs, and matching fESCs lines using microarray technology, iTRAQ method, and bioinformatic analyses. Real-time PCR, two-dimensional LC, and MS/MS analyses were further conducted to study the expression of specific transcripts and identify and quantitate 929 proteins. Our results demonstrate that, like ntESCs, the iPSC and matching fESCs lines have very similar transcriptional and protein expression profiles. This is consistent with their similar developmental potential.

2. INTRODUCTION

Embryonic stem cells (ES cells, ES is derived from fertilized blastocysts, fESCs), which have pluripotency and could be passaged indefinitely, are derived from the inner cell mass of blastocysts (1-3). ES cells could be differentiated into different types of somatic cells belonging to three embryonic germ layers, that is, the endoderm, ectoderm, and mesoderm. The differentiated cells that are committed to each of these germ layers give rise to the tissues of adult body, such as the brain, intestine, or cardiac muscle (4). Therefore, human ES cells might be a good model to study the mechanisms associated with those of diseases. They are useful to screen effective and safe drugs, and treat patients of various diseases and injuries. As immune rejection might occur after transplantation, fESCs were not appropriate for transplantation based therapy. Pluripotent stem cells are generated through three important ways: fESCs are isolated from the inner cell mass of blastocyst; ntESCs are derived from somatic cell nuclear transfer into denucleated egg cells (5,6) ; and iPSCs are pluripotent stem cells derived from somatic cells by inducing "forced" expression of specific genes (7-11) .Genetically matched ES cells can be derived from somatic cells of diseased individuals by

nuclear transfer technology, which can be differentiated into a host of cell types for cell replacement therapy. This therapy has been applied to different animal models, and the clinical application of human ntESCs cells represents a promising approach in the treatment of various medical conditions (12, 13) . However, in animals, aberrated gene expression patterns of donor nuclear cells (e.g. the failure to induce critically early embryonic development genes or to silence specific somatic genes) significantly affects most pre-implantation nuclear-transferred embryos. This was associated with an embryonic and fetal lethality, leading to severe phenotypic and transcriptional abnormalities of surviving clones (14-19). The differentiation state of the donor nucleus can influence the gene expression patterns in newborn clones (14, 20-22). The gene expression abnormalities in the somatic tissues of cloned animals have raised concerns in terms of therapeutic application of ntESCs when compared to fESCs. However, it has been suggested that the process of ES cell derivation, entailing strict selection for *in vitro* proliferation, allows for the survival clones lost the "memory" of donor nucleus. This renders ES cells derived from nuclear-transferred blastocysts equivalent to those derived from fertilized counterparts (23-24) .iPSCs were first generated by introducing Oct3/4, Sox2, c-Myc, and Klf4 in mouse embryonic or adult fibroblasts under ES cell culture condition. This was done by introducing the same 4 transcription factors in adult human dermal fibroblasts (7, 10-11). This approach led to artificially reprogramming a non-pluripotent cell into pluripotent state. This has been cited as an important milestone in stem cell research, as it may allow researchers to obtain pluripotent stem cells without any controversial use of embryos. Unlike embryonic stem cells, iPSCs don't have any issue associated with graft-versus-host disease and immune rejection because they are derived entirely from the patient. It has been shown that iPSCs are able to produce viable chimeras when injected into developing embryos (9). Moreover, Ding's group has reported small-molecule combination facilitating the mouse and human iPSCs generation (25). Thus, iPSCs hold great promise in investigating various diseases by using stem cells derived directly from patients of interest.

Recent studies have systemically compared the transcriptional and post-transcriptional levels of ntESCs and fESCs, indicating that, similar to their pluripotency, the transcriptional and post-transcriptional profiles of these two stem cell lines with same background are highly comparable (24,26) . However, the transcriptional and post-transcriptional status of iPSCs and the similarity of iPSCs with ntESCs and fESCs need to be elucidated. Herein, we investigated the mRNA and protein profiles of iPSCs, ntESCs, and fESCs to address whether iPSCs cells are truly comparable with the other stem cell lines. Very similar mRNA and protein profiles were shown in these three stem cell lines with same background by performing mRNA microarray and proteomics analyses. This result provides strong evidence to support the notion that iPSCs are transcriptionally and post-transcriptionally indistinguishable from ntESCs and fESCs.

3. MATERIALS AND METHODS

3.1. iPSCs, ntESCs, fESCs and MEFs preparation

After nuclear transplantation, testicular sertoli cells were collected from B6129F1 (C57BL/6×129/sv) mice to perform derivation of ntES cell lines(NC6) as donor cells. NC6 was performed as described (27,28). The same strains of mice were used to collect fertilized embryos to derive the matching fES cell lines(FC3). NC6 and FC3 cells used were passaged about 20 generation. Mouse Primary iPS Cells-WP5 (genetic background is C57BL/6×129/sv) was purchased from Stemgent (Catalog Number 08-0007). The culture of WP5 was according to manufactory's instruction.The ES cell lines were cultured on 6-well plates preplated with a layer of mitomycin C-treated mouse embryonic fibroblast cells(MEFs). The mESC medium consisted of 80% DMEM supplemented with L-glucosamine (Invitrogen), 15% FBS (Hyclone), 0.1 mM nonessential amino acids (Invitrogen), 0.1 mM 2-mercaptoethanol (Sigma-Aldrich), 1000U/mL leukemia inhibitory factor (Chemicon).

For primary mouse embryonic fibroblasts (MEFs) isolation, uteri isolated from 13.5-day-pregnant mice (genetic background is C57BL/6×129/sv) was washed with phosphate-buffered saline (PBS). The head and visceral tissues were removed from isolated embryos. The remaining bodies were minced with the help of a pair of scissors, trypsinized for 20 min at 37°C, and then were dissociated with the help of pipette. 1×10^6 cells were collected through centrifugation and resuspended into fresh medium. In this study, we used MEFs within three passages to avoid replicative senescence.Three stem cell lines and MEFs were cultured for three times and then sent for RNA microarray and protein analysis independently. These stem cells were pelleted after feeder cell depletion by pre-plating, and were collected in serum-free media after being washed in ice-cold PBS for three times. Then, the cells were frozen in liquid nitrogen and stored at -80 °C until RNA and protein extraction.

3.2. RNA Isolation, Microarray Experiment and Data analysis

Total RNA was isolated from the samples using Trizol reagent (Invitrogen, Carlsbad, CA), and cleaned up using RNeasy Micro Kit (Qiagen, Valencia, CA) techniques. The samples were hybridized to the Affymetrix® Mouse 430 2.0 Genechip. The computer data files to be used in data analysis (*.dat, *.cel, *.chp) were generated using the Affymetrix GeneChip Operating Software (GCOS) (Affymetrix®). The differentially expressed genes were selected by ANOVA test and further clustered with the help of hierarchical methods.

3.3. Quantitative and semiquantitative RT-PCR analysis

Total RNA was isolated from cell pellets using RNeasy Mini Kit (Qiagen). First strand cDNA was synthesized using Sensiscript T Kit (Qiagen). mRNA expression of *Pou5f1*, *Sox2*, *Klf4*, *Foxd3*, *Otx2*, *Lin28*, *H19*, *Grb10*, *Mdm2*, *Tsc1*, *Apc*, *Trp53* and *Cdknla* was

determined by real-time PCR using SYBR Green (Applied Biosystems).

3.4. Cell lysates, in-solution digestion, and iTRAQ labeling

For whole cell proteomic analysis, iPSCs, ntESCs, fESCs, and MEFs were lysed in 0.5% SDS and subsequently sonicated for 3 minutes on ice. (Duty cycle 30%, output control at 3, on Sonifier 250, Branson).The cells were sheared by Dounce homogenizing 150 strokes in buffer containing 5mM HEPES, pH 7.4, 0.5 mM EDTA, 250 mM sucrose, and freshly prepared 1mM PMSF. According to manufacturer's instructions, peptides from iPSCs, ntESCs, fESCs, and MEFs were differentially labeled using iTRAQ reagent (Applied Biosystems).

3.5. 2D-LC MS/MS

Chromatographic separation of the pooled samples was performed on an ACQUITY Ultra Performance LC system (Waters, USA). Tryptic digested and labeled peptides were first fractionated by strong cation exchange liquid chromatograph (SCX) using a 0.5 x 23 mm, 5 µm, 300Å Column (Waters, USA). Sample was loaded onto the column and stepwise elution was conducted by injecting salt plugs of 10 different molar concentrations, that is, 25, 50, 75, 100, 150, 200, 300, 400, 500, 1000 mM of NH_4AC. Ten fractions were collected from the SCX column. Each of these fractions was then loaded onto a reverse phase (RP) column, ZORBAX 300SB-C18 column (5 µm, 300Å, 4.6 x 50 mm, Agilent, USA). Buffer A was 5% acetonitrile, 95% water, and 0.1% formic acid, while Buffer B was 95% acetonitrile, 5% water, and 0.1% formic acid. Elution was performed using a gradient ranging from 5% to 45% Buffer B over 90 min.

The LC eluent was subjected to positive ion nano-flow electrospray analysis using a Qstar XL MS/MS system (Applied Biosystems, USA) in an information-dependent acquisition mode (IDA). In IDA mode, a TOFMS survey scan was acquired (m/z 400-1800) with up to 6 most intense multiply-charged ions in the survey scan that were sequentially subjected to product ion analysis. Product ion spectra were accumulated for 2 s in the mass range m/z 100-2000 with a modified and enhanced all mode Q2 transition, setting the favoring of low mass ions, so that the reporting iTRAQ ion (114,115,116, and 117 m/z) intensities were enhanced for quantification.

3.6. MS Data Analysis

All LC-MS/MS data were acquired in Analyst QS 1.1 (Applied Biosystems, USA). MS/MS data were analyzed using Protein Pilot v 3.0 (Applied Biosystems) which uses the Paragon algorithm to perform database searching. The search results were further processed by the Pro Group Algorithm to remove redundant hits and comparative quantitation so that the minimal set of justifiably identified proteins could be found. The protein database used for all searches was IPI v 3.55 mouse. Loading error was normalized by bias correction that was calculated using Protein Pilot. All the reported data were based on 95% confidence for protein identification, as determined by Protein Pilot (Prot Score >=1.3). The relative protein quantitation was calculated in terms of an average ratio. The confidence level of the altered expression of proteins was calculated by Protein Pilot as p-value, which allows the results to be evaluated based on the confidence level of expression change, and not just the magnitude of the change.

3.7. Data processing and statistical analysis

Stanford University developed Cluster 3.0 soft was used to make cluster analysis on gene expression, and the results were visualized with TreeView software.The pathway analysis was carried out using KEGG database (29). Two-side Fisher's exact test and X2 test were used to classify pathway analysis, and the false discovery rate (FDR) was calculated to correct the P-value. P-value < 0.05 and FDR < 0.05 were used as a threshold to select significant KEGG pathways.

4. RESULT

4.1. Development potency of stem cell lines

To determine the developmental potential of nuclear-transferred ES cell line (NC6), fertilization-derived ES cell lines (FC3), we used tetraploid (4n) blastocyst complementation: the most stringent assay for ES cell pluripotency. The results suggested that viable clonal mice can be produced by tetraploid blastocyst and grow up to adult. The commercial WP5-induced pluripotent stem cells were available and have been proofed for its pluripotency (30) . Thus, the ES cell lines in this study could be used as a model to study the pluripotency. Moreover, these stem cell lines were of the same genetic background: C57BL/6×129/sv.

4.2. Transcriptional profiles of iPSCs, ntESCs and fESCs are highly similar

iPSCs are generally assumed to be functionally equivalent to embryonic stem cells (ESCs) derived from fertilized embryos (fESCs) or ESCs generated through somatic cell nuclear transfer (ntESCs). This was manifested in terms of their appearance, expression of pluripotency markers, ability to form teratomas, and generate chimaeras or mice derived completely from iPSCs through tetraploid complementation (7-11, 30). However, the compare of the transcriptom between the iPSCs and ESCs (ntESCs or fESCs) has not been done. . We compared the expression levels of over 39,000 transcripts in ES cell lines that were derived from cells including iPSCs, ntESCs, and fESCs using microarray technology.In this regard, the mean probe signal levels of iPSCs were compared with the correspondingly mean signal values of ntESCs and fESCs, as well as fESCs and ntESCs. As shown in figure 1, a high degree of transcriptional similarity between iPSCs and ntESCs or iPSCs and fESCs as well as fESCs and ntESCs was reported (Pearson's coefficient of correlation: iPSCs vs. fESCs, r = 0.9971; ntESCs vs. fESCs, r = 0.9927; iPSCs vs. ntESCs, r = 0.9912).Thus, the data indicated that there is no significant change at the transcription level of iPSCs in contrast to ntESCs or fESCs.

Unsupervised hierarchical data set clustering was applied to assess differences and similarities in expression

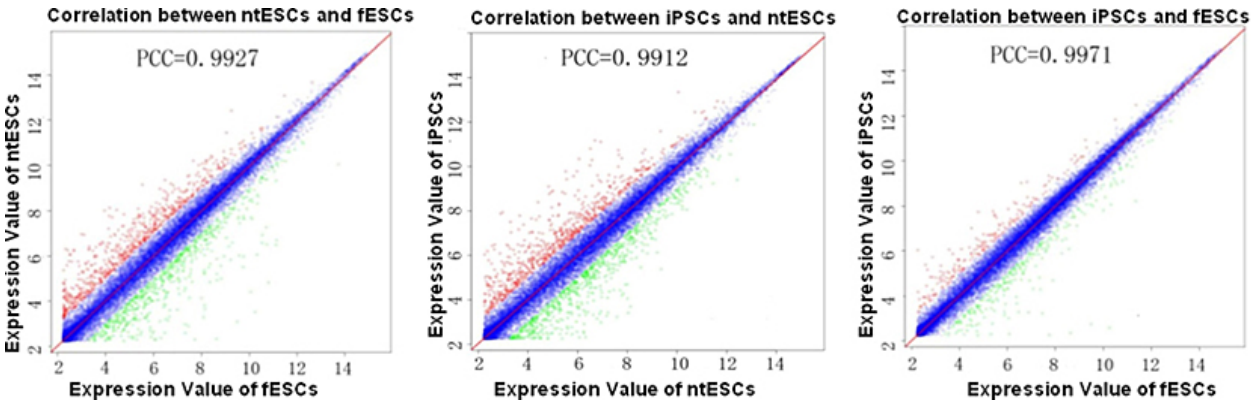

Figure 1. Analysis of expression profiles from iPSCs, ntESCs and fESCs. Mean signal intensities (MSI) of iPSCs line (WP5) with three biological repeats were plotted against the corresponding MSI of ntESCs (NC6) with three biological repeats and that of fESCs (FC3) with three biological repeats. Pearson's coefficient of correlation was shown in the figures.

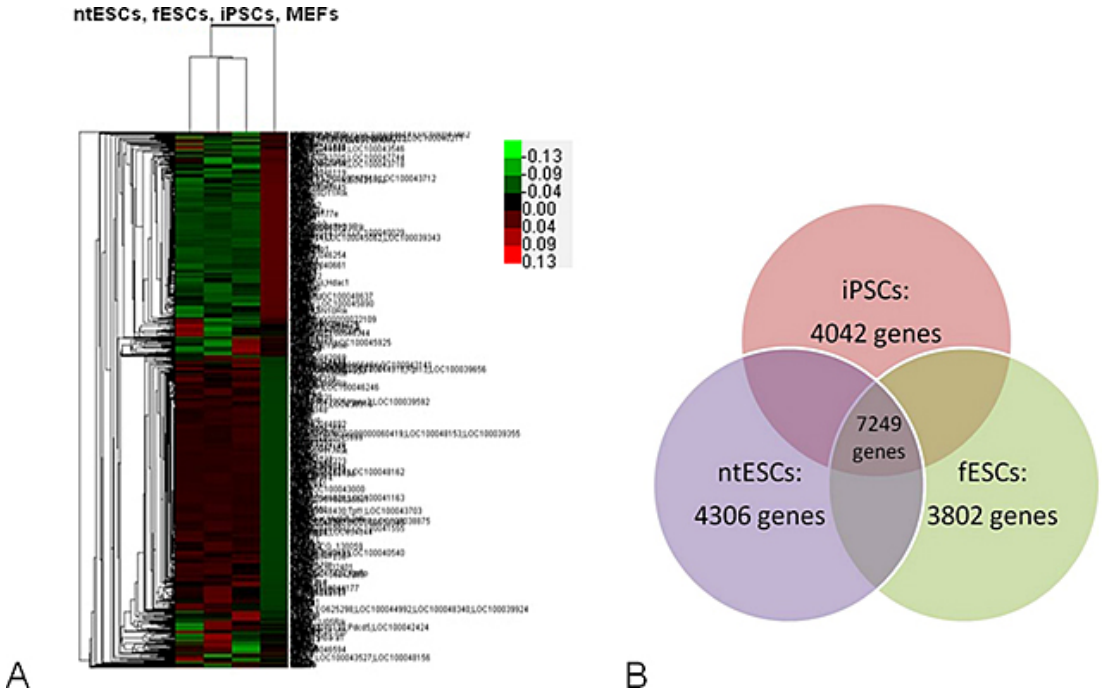

Figure 2. Expression profiles from iPSCs, ntESCs and fESCs. (A) Hierarchical clustering of from iPSCs, ntESCs and fESCs (with three repeats respectively) expression profiles. Heat map of clustering results (green, no or very low expression; black,, low expression; red, high expression). (B) iPSCs, ntESCs and fESCs specific genes were got by the compare between the gene expression of these cells with MEF cells that is a control differentiated cells. The concordance of these pluripotent stem cells specific gene expression profile were shown.

profiles of stem cell lines derived by three different protocols (iPSCs, ntESCs and fESCs) in an unbiased way. This grouping of transcriptional profiles according to their overall similarities is less sensitive to outliers than the average signal comparison approach and can identify transcriptionally with similar subsets of cell lines. As a result, no marked difference was found in expression profiles of all three stem cell lines. While the differentiated mouse embryonic fibroblasts(MEFs), indicating obviously distinct transcription profile (Figure 2A). Furthermore, the unsupervised hierarchical clustering analysis indicated that the transcriptional profile of fESCs is more similar with iPSCs compared with that of ntESCs (Figure 2A).

To further investigate the gene expression of these stem cell lines, we firstly found the differential genes of each stem cell line by comparing them with MEFs (with P-Value<0.01, FDR<0.01), and then analyzed the characteristic of genes differentially expressed by iPSCs, ntESCs, and fESCs. As a result, there were 4042, 4306, and

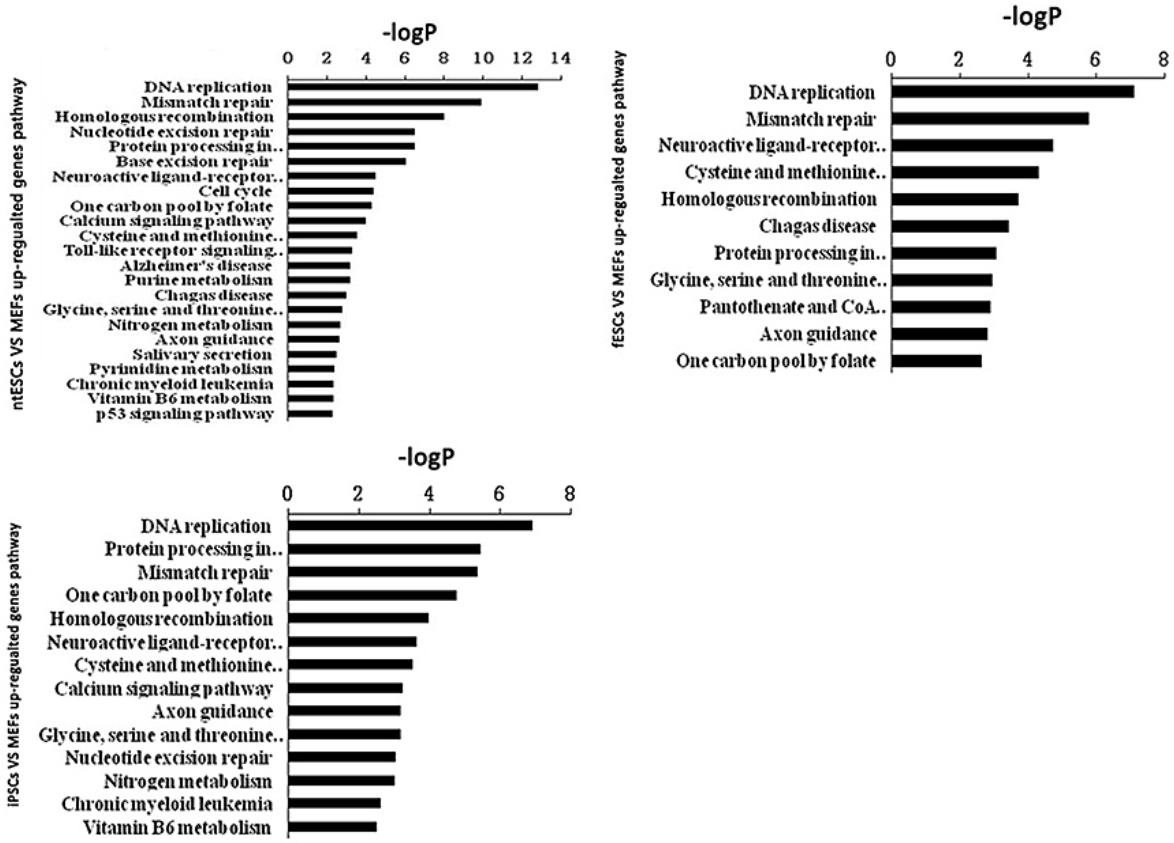

Figure 3. Significantly up-regulated genes were analyzed for their pathway enrichment based on KEGG database. Functional classification of all the iPSCs, ntESCs and fESCs specific genes which were compared by MEFs.

3802 differentially expressed transcripts indentified in iPSCs, ntESCs, and fESCs, respectively. Apart from this, there were 7249 genes (3273 genes up-regulated and 3976 genes down-regulated) that significantly changed in all three sublets (Figure 2B).

To further study the involved pathway by the differentially expressed genes in each stem cell lineage, we analyzed these genes based on Kegg database (Fisher analysis P-Value <0.01, FDR < 0.01). Although there is some difference in the different stem cell lines, the overall associated pathways among iPSCs, ntESCs, and fESCs is similar (Figure 3, 4). Taken together, the transcriptional profiles of iPSCs, ntESCs, and FESCs are highly similar, and is associated with similar pluripotency of these stem cell lines.

4.3. Similar levels of transcriptional variability in iPSCs, ntESCs and fESCs

To investigate the variability in the mRNA expression in iPSCs, ntESCs, and fESCs, the transcription levels of a subset of genes was measured by real-time PCR. *Pou5f1, Sox2, Klf4,* and *Foxd3* encode transcription factors play an important role in embryonic development and maintaining the pluripotency of stem cells (31-34). *Otx2* encodes transcription factor that is required for proper development of forebrain (35); *Lin28* encodes a highly conserved RNA binding-protein which participates into the mouse embryo development (36); *H19 and Grb10* are important imprinted genes during embryonic development (37-39) . *Mdm2, Tsc1, Apc, Trp53, and Cdknla* are oncogenes: tumor-suppressor genes that are critical in cell cycle and differentiation of embryonic stem cells (40-44) . Here, there was no marked difference in the mean expression levels or variability of gene expression across iPSCs, ntESCs, and fESCs for gene testing (Figure 5A).

To further examine whether a global increase in gene expression variability could be detected in iPSCs in contrast to ntESCs and fESCs, the standard deviations of all probe signal levels obtained in these stem cell lines were sorted and plotted. This was done by calculating the standard deviation values for different percentiles of these sorted data sets. As shown in figure 5B, the standard deviation levels between the data sets of iPSCs, ntESCs, and fECSs are quite similar, but not the standard deviation levels between the data sets of iPSCs, ntESCs, and fECSs with MEFs. The values obtained for the 95th to 100th percentile indicate a slightly higher variability in gene expression in iPSCs. Thus, the data suggests that the iPSCs examined here do not display an increased overall variability in gene expression levels, as compared to other pluripotent stem cells, i.e. ntESCs and fESCs.

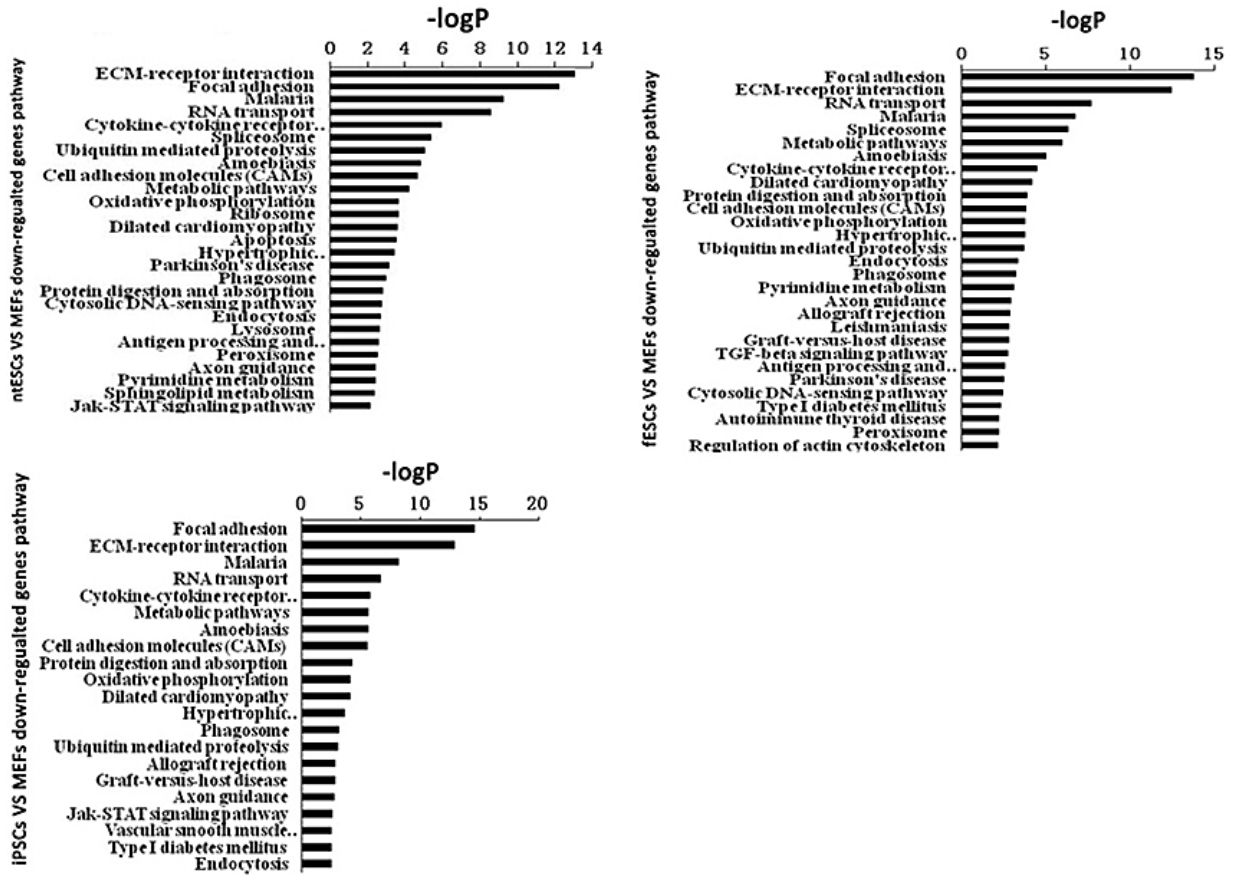

Figure 4. Significantly down-regulated genes were analyzed for their pathway enrichment based on KEGG database. Functional classification of all the iPSCs, ntESCs and fESCs specific genes which were compared by MEFs.

4.4. The protein profiles of iPSCs, ntESCs and fESCs are highly similar

Here, we further evaluated whether iPSCs are similar to their embryonic counterparts ntESCs and fESCs at protein levels by applying iTRAQ coupled with LC-MS/MS analysis. Peptides from iPSCs, fESCs, ntESCs, and MEFs were labeled with reagents containing 114, 115, 116, and 117 iTRAQ reporters. LC-MS/MS analysis of 20 SCX fractions from whole cell lysates preparations generated a total of >100000 M/MS spectra. A total of 929 proteins were identified from 26967 distinct peptides by using confidence cutoff score ProtScore value 41.3 (95% confidence). Here, we set MEFs as a control and compared differentially expressed protein characteristic of iPSCs, fESCs, ntESCs with MEFs. We further analyzed the characteristic protein expression among these three stem cell lines, and a high degree of similarity was observed in their protein profiles (Pearson's coefficient of correlation: iPSCs vs. fESCs, r = 0.9971; ntESCs vs. fESCs, r = 0.9927; iPSCs vs. ntESCs, r = 0.9912) (Figure 6). More importantly, only 41 proteins were differentially expressed in these three stem cell lines. The pathway analysis showed that there was no significant pathway to enrich from the 41 proteins (data not shown). These results suggest that the protein profiles of iPSCs, fESCs, ntESCs are highly similar.

5. DISCUSSION

In this study, we have compared the gene and protein expression patterns of iPSCs with that of genetically matched ES cells derived from either nuclear-transferred or fertilized blastocysts by mRNA microarray, iTRAQ coupled LC-MS/MS and related bioinformatics analysis. Our results revealed no marked differences in expression profiles of gene and protein between these stem cell lines. We observed no elevated levels of transcriptional variability in the tested iPSCs as compared to ntESCs and fESCs. These data support the notion that the iPSCs are transcriptionally and post-transcriptionally indistinguishable from the ntESCs and fESCs. Our study provides molecular evidence to support the biological observations that iPSCs have similar developmental potential with fES and ntES cells as evidenced by their ability to generate mice through tetraploid complementation asssay.

Induction of iPSCs is a biological process that somatic cells are reprogrammed to a pluripotent state through the ectopic expression of defined transcription factors. Only rare iPSCs were derived from stringent selection e.g. activation of a neomycin-resistance gene inserted into the endogenous *Oct4* (also known as *Pou5f1*)

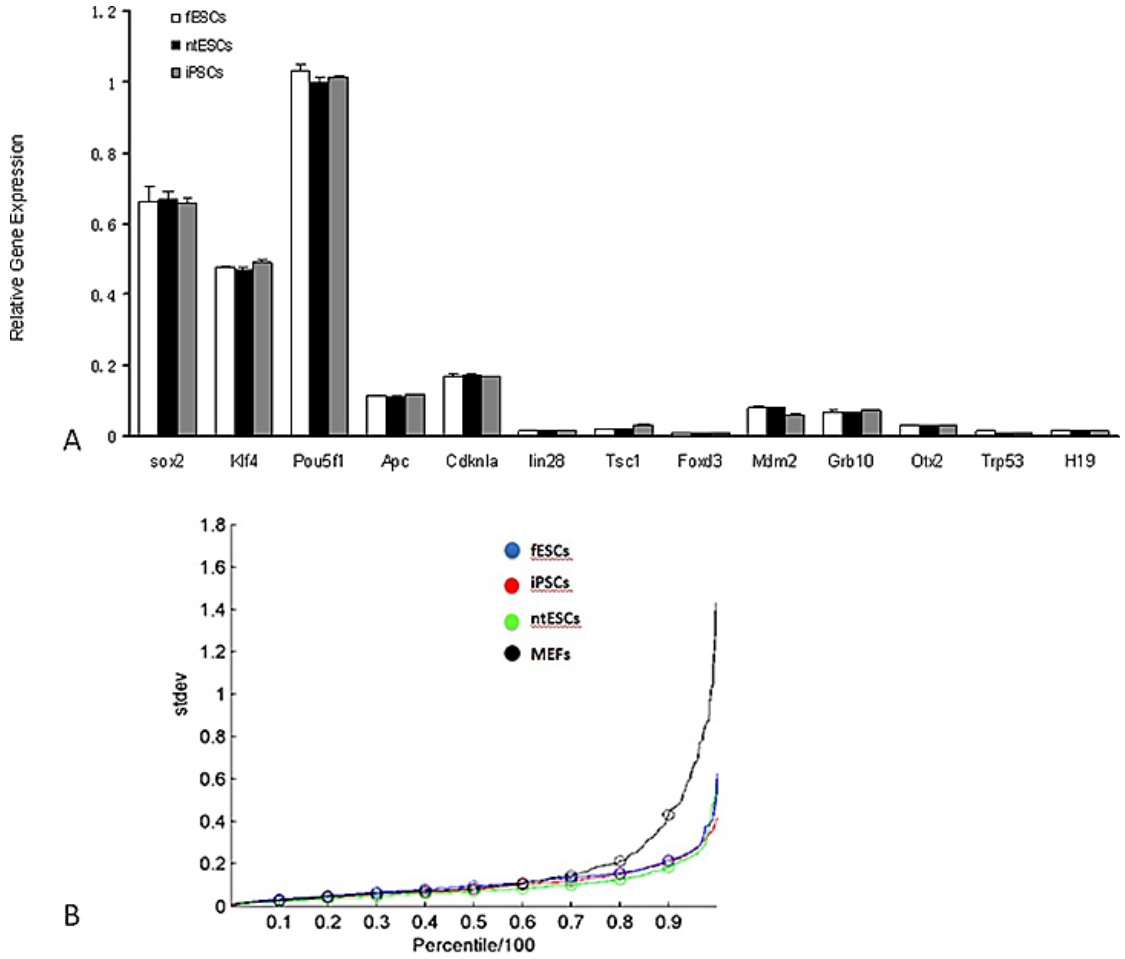

Figure 5. Analysis of variability in gene expression among iPSCs, ntESCs and fESCs. (A) Comparison of gene expression levels in iPSCs, ntESCs and fESCs. Columns display relative gene expression; error bars display standard deviation, (B) Comparison of standard deviation levels across all probes in the data set. As a measure of gene expression variability, standard deviation levels were calculated for the log2 probe signal values for each group. Probes in each group (iPSCs, ntESCs, fESCs and MEFs) were ordered by their standard deviation levels, and then standard deviation levels were compared at different percentiles.

or *Nanog* loci; morphological selection. Majority of transfected cells are partially reprogrammed cells, indicating the reactivation of a subset of stem-cell related genes and incomplete repression of lineage-specifying transcription factors with inefficient DNA de-methylation at pluripotency-related loci (45). Fully reprogrammed cells show gene expression and epigenetic states that are highly similar to embryonic stem cells. In contrast, after explanation *in vitro*, such epigenetic differences are erased during the process of ES cell derivation, rendering both iPSCs and fESCs lines functionally indistinguishable, which is the similar with the process of ntESCs derivation. In fact, it has been known that most inner cell mass (ICM) cells of blastocysts decrease the expression of *Oct4*, a key pluripotency gene, and cease to divide after the blastocysts have been explanted in culture. Only a small fraction of the explanted cells maintain *Oct4* expression giving rise to continuously growing immortal cell lines that are actually embryonic stem cells (46) . It is important that an stem cell line was derived by iPSC induction, nuclear transfer, fertilization, or from the ICM cells undergoing the same stringent selection for *in vitro* survival and proliferation. In this case, our findings have proved that the levels of key genes for pluripotency, that is, *Pou5f1,* Sox2, *Klf4*, *Foxd3 et al* were similar among the pluripotent iPSCs, ntESCs, and fESCs. The overall transcription patterns of three stem cell lines are also highly similar. This is consistent with the notion that ES cell derivation is a highly selective process for rare cells that are able to start and maintain the transcription program for pluripotency under tissue culture conditions. The process of pluripotent iPSCs derivation is associated with the expression of key transcription factors and these factors mediated reprogramming of transcription during the *in vitro* culture selection. Moreover, it is quite promising to know that the small molecules could facilitate the epigenesis and transcription reprogram to archive the pluripotency.

iTRAQ coupled Mass spectrum analysis is a powerful approach to punitively compare the expression

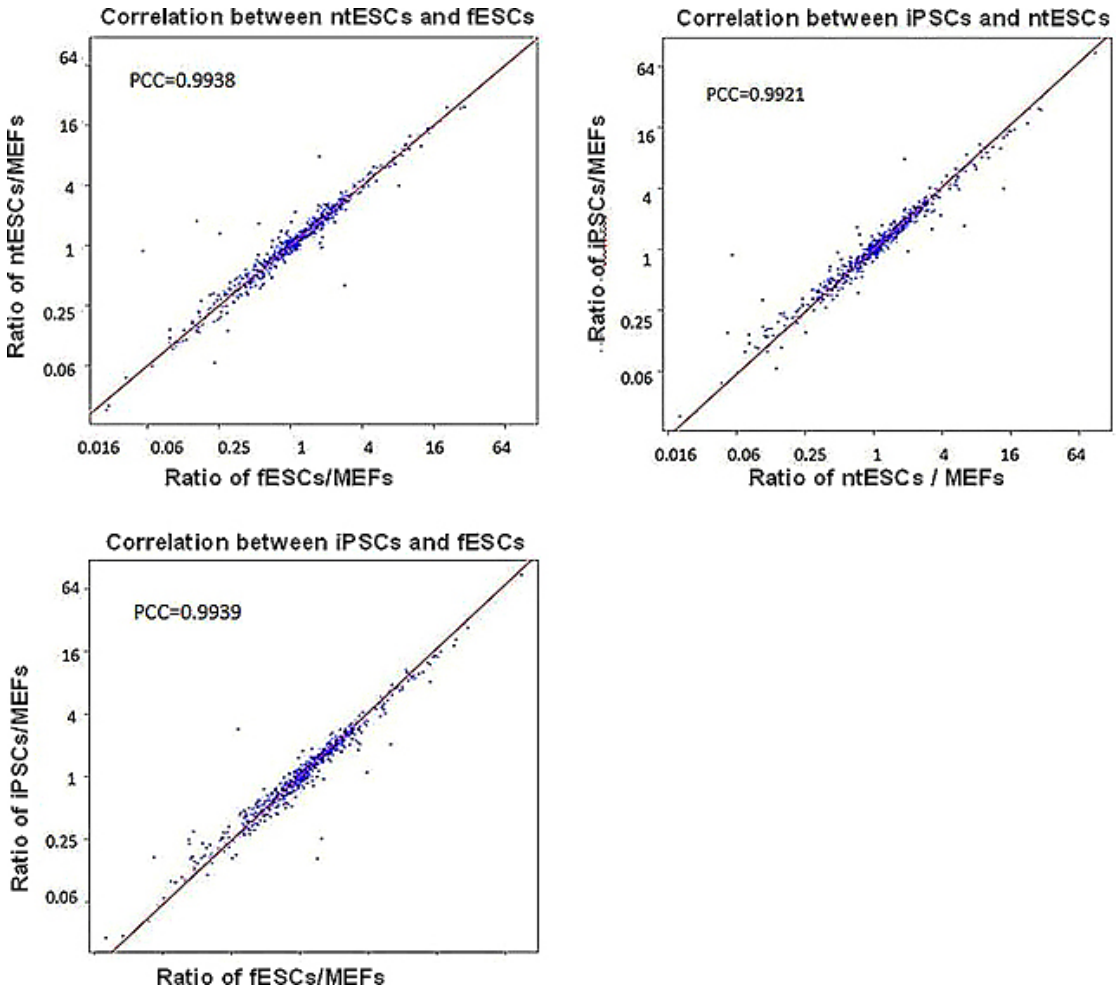

Figure 6. iPSCs, ntESCs and fESCs protein expression compared by that of MEFs. The resulting specific protein expression of iPSCs, ntESCs and fESCs were compared by each other. Pearson's coefficient of correlation was shown in the figures.

level of proteins globally. This approach has been recently used for quantitative comparison of membrane proteomes in human ESCs (47) . As there are lots of post-transcriptional regulation pathways for determining the proteomics of cells, it is important to investigate whether the iPSCs lines are truly comparable with that of ntESCs and fESCs lines in their protein expression profiles. As a result, we only found 41 out of the 929 proteins to be differentially expressed in these stem cell lines. Since the through-put of proteomics is not comparable with transcriptomics by microarray or sequencing analysis, we cannot compare mRNA and its corresponding proteins that are characteristic of iPSCs, ntESCs, and fESCs. However, our study has shown the similarity among the three stem cell lines, which are correlated with their properties.

6. CONCLUSIONS

In conclusion, our data indicates that iPSCs are highly similar to ntESCs and fESCs transcriptionally and translationally. This underline the molecular bases of pluripotency and the application of iPSCs to regenerative medicine is promising.

7. ACKNOWLEDGMENT

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Abbreviations: iPSCs, induced pluripotent stem cells; ntESCs, nuclear-transferred embryonic stem cells; fESCs, embryonic stem cells derived from fertilized blastocysts; MEFs, mouse embryonic fibroblasts; PBS, phosphate-buffered saline. iTRAQ, Isobaric tags for relative and absolute quantitation.

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