# Intact Cell Mass Spectrometry as a Quality Control Tool for Revealing Minute Phenotypic Changes of Cultured Human Embryonic Stem Cells

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## Abstract

The stability of in vitro cell cultures is an important issue for any clinical, bio-industrial, or pharmacological use. Embryonic stem cells are pluripotent; consequently, they possess the ability to differentiate into all three germ layers and are inherently prone to respond to differentiation stimuli. However, long-term culture inevitably yields clones that are best adapted to the culture conditions, passaging regimes, or differentiation sensitivity. This cellular plasticity is a major obstacle in the development of bio-industrial or clinical-grade cultures. At present, the quality control of cell cultures is limited by the lack of reliable (epi)genetic or molecular markers or by the focus on a particular type of instability such as karyotype abnormalities or adverse phenotypic traits. Therefore, there is an ongoing need for robust, feasible, and sensitive methods of determining or confirming cell status and for revealing potential divergences from the optimal state. We modeled both intrinsic and extrinsic changes in human embryonic stem cell (hESC) states using different experimental strategies and addressed the changes in cell status by intact cell mass spectrometry fingerprinting. The analysis of spectral fingerprints by methods routinely used in analytical chemistry clearly distinguished the morphologically and biochemically similar populations of hESCs and provided a biomarker-independent tool for the quality control of cell culture. Stem Cells Translational Medicine 2018;7:109–114

## Significance Statement

Safe biomedical applications of cells require predictable and well-defined cell populations. This study describes intact cell mass spectrometry combined with chemometric and artificial intelligence approaches as a simple, robust, and hypothesis-free quality control tool for determining induced and spontaneous alterations in genetically and morphologically uniform embryonic stem cell populations in routine cell cultures.

## Introduction

Cell-based applications are dependent on rigorous conditions including predictable and stable cell populations. Human embryonic stem cells (hESCs) paved the road for the development of state-of-the-art technologies for regenerative medicine and bio-industry applications. Derived from the pluripotent inner cell mass of a pre-implantation blastocyst, hESCs are inherently unstable and prone to rapid, spontaneous differentiation in vitro. Current culture protocols are focused on maintaining the pluripotent character of hESCs by repressing their differentiation and supporting sustained self-renewal. In culture, hESCs cumulatively acquire various alterations on both the genetic and non-genetic levels [1] and despite sophisticated culture techniques, culture-adapted clones are inevitably selected during long-term in vitro cultures [2]. However, these changes could remain unnoticed until they substantially alter the cell karyotype or cell phenotype, even in case of the stable expression of stemness-associated transcription factors including c-Myc, Sox-2, Klf4, Nanog, or Oct3/4, or their differentiation capacity or typical morphology. Further, molecular, genetic, and/or light-microscopy analyses can fail in the case of the genetically or karyotypically silent changes that arise in cultured cells.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and its derivatives have diverse applications in classic analytical and structural chemistry and, thanks to their universality, have been adapted for analysis and determination of complex biological samples, including bacteria, yeasts, or mammalian cells [3, 4]. For monitoring of cell cultures, two possible approaches for analyzing mass

spectra are available. The "top-down" approach is based on revealing and identifying individual unique peaks and their use as biomarkers that are correlated with phenotypes or cell states, such as pluripotency, reprogramming events, or differentiation cues [5]. However, despite the precise identification of specific biomarkers that could be statistically associated with cell parameters, their applicability is often based on a random correlation among myriads of other dynamically changing molecules in a cell- or culture-dependent context. Also, we have recently documented that using one or two dominant peaks for quantitative analysis of a cell type reveals non-linear relationships that can be easily influenced by the technical or biological variability in the measurement or the sample itself [6]. The alternative approach assumes that a full spectral fingerprint is sufficiently robust to eliminate any undesired variability but contains enough information to discriminate species, cell types, or even cell states differing in chemical composition, even without the identification of individual molecules. Thus, the analysis without a preceding subjective reduction to several major peaks and their identification can eliminate the bias of the random correlation of individual molecules to a phenotypic change or a cell status and avoid potential misinterpretation. Avoiding the fractionation of cells also helps to reduce the unwanted variability while improving the overall consistency of the mass spectra and their reproducibility.

In addition to MS-based quantitative cell line authentication that we reported recently [6], here we demonstrate that the whole mass spectrum acquired from intact hESCs contains sufficient information to provide an unambiguous fingerprint also of a cell state in an otherwise uniform cell population and offers a simple tool for the monitoring and quality control of pluripotent stem cells in culture.

## MATERIALS AND METHODS

### Cell Culture

Undifferentiated CCTL-14 hESCs (hPSCreg: MUNIe007-A, RRID: CVCL_C860), passage numbers 29, 72, and 269) were maintained in 6-cm Petri dishes (TPP, Trasadingen, Switzerland) on mitotically inactivated mouse embryonic fibroblasts (MEFs) in Dulbecco's modified Eagle's medium/F12 supplemented with 15% knockout serum replacement (both from Invitrogen, Life Technologies, Carlsbad, CA), L-glutamine, minimum essential medium, nonessential amino acids, 0.5% penicillin-streptomycin (both from PAA Laboratories, Pasching, Austria), 2-mercaptoethanol (Sigma-Aldrich, Prague, Czech Republic), and 10 ng/ml fibroblast growth factor-2 (PeproTech, Rocky Hill, NJ). The hESCs were maintained in an incubator at 37°C in a humidified atmosphere containing 5% $CO_2$ and the media was exchanged daily. Retinoic acid was purchased from Sigma-Aldrich (Prague, Czech Republic) and added to the fresh culture medium to the final concentration of $10^{-8}$ M.

### Isolation of RNA, Reverse Transcription, and Quantitative PCR

Total RNA from hESCs was extracted using an RNeasy Mini Kit (Qiagen) and quantified using a NanoDrop (Thermo Scientific). cDNA was synthesized from 1 μg DNase I-treated total RNA using a First Strand Reverse Transcription Kit (Roche, Prague, Czech republic). Relative expression was quantified using exon-spanning primers and UPL probes specific for *SOX2, NANOG, OCT4, PAX6, SOX1, GATA4*, and *BRACHYURY*. Glyceraldehyde 3-phosphate dehydrogenase was used as the housekeeping gene control (Roche, Prague, Czech Republic). Results were expressed as relative ratio units. All PCR reactions were performed in triplicate from three independent experiments, and reverse transcriptase-negative and template-negative controls were included.

### Immunofluorescent Microscopy

The hESC colonies were manually harvested under visual control, washed in 1× phosphate-buffered saline (PBS), fixed in 4% formaldehyde for 15 minutes, washed three times in 1× PBS, permeabilized in 0.1% Triton-X100 in PBS for 10 minutes/RT, blocked in 3% BSA in PBS for 1 hour. Then, cells were incubated with anti-Sox2 (AB5603, Merck Millipore, Prague, Czech Republic, dilution 1:500) or anti-Oct3/4 (sc-5279, Santa Cruz Biotechnology, Heidelberg, Germany, dilution 1:100) primary antibodies overnight at 4°C. The cells were then washed three times in 1× PBS and incubated for 60 minutes room temperature with secondary antibody conjugated with AlexaFluor 568 (Sox2) or 488 (Oct3/4) and diluted 1:1,000 (Life Technologies/ThermoFisher Scientific, Prague, Czech Republic, A11004 or A11008, respectively), followed by additional wash in 1× PBS. Hoechst 33342 stain was added to the final concentration 5 μg/ml. Images were acquired using an automated microscope with ×10 objective (Image Xpress MicroXL, Molecular Devices).

### Flow Cytometry

The hESC colonies were manually harvested under visual control, disaggregated using TryPLE Express (1×) for 2 minutes at 37°C, cells counted and resuspended in FACS buffer (5 mM EDTA, 2% FBS in 1× PBS). Prior staining of intracellular Sox2, cells were fixed using 3.7% formaldehyde in 1× PBS and permeabilized in BD Phosflow Perm Buffer III (BD Biosciences, Prague, Czech Republic). For blocking, 10% mouse serum in 1× PBS was used. Sox2 was stained using anti-Sox2 antibody (Abcam, Cambridge, UK, Ab97959), diluted 1:200 and incubated at 4°C/overnight and anti-rabbit IgG conjugated with Alexa Fluor 488 (Life Technologies/ ThermoFisher Scientific, Prague, Czech Republic, A11008).

### Sample Preparation for Mass Spectrometry

The hESC colonies were manually harvested under visual control, disaggregated using TryPLE Express (1×) for 2 minutes at 37°C, washed in 1× PBS and cells counted. To eliminate traces of PBS, the cells were centrifuged (200*g*, 2 minutes) and washed three times in 1 ml of 150 mM ammonium bicarbonate buffer. The cell numbers were determined using a Cedex XS cell counter operated with Cedex Control Center software v. 1.0.3. (Innovatis AG, Roche Life Sciences, Prague, Czech Republic). Finally, the cells were resuspended in 150 mM ammonium bicarbonate buffer to the final concentration of $100 \times 10^6$ cells per ml. The cell suspension was then mixed with an acidified matrix containing sinapinic acid (30 mg ml$^{-1}$) and 2,2,2-trifluoroacetic acid (7.5% vol/vol) in a 2:1 sample to matrix ratio and applied to a target plate that was purified in an ultrasonic bath before spotting the samples.

### Mass Spectrometry

The mass spectra were recorded in linear positive ion mode over the 2,000–12,000 m/z range using an AXIMA-CFR mass spectrometer from Shimadzu Biotech (Kratos, U.K.) equipped with a nitrogen laser (337 nm). The time pulse length of the laser was 3 ns. The maximum laser fluency was 60 mJ per pulse. The full laser power was indicated on the instrument as 180 arbitrary units

(a.u.; 6 mW). The irradiated spot size was approximately 150 μm in diameter. An external calibration was performed using standard mixtures of peptides and proteins (TofMix, PepMix, LaserBio, France) and standard bacterial extracts (BTS standard) from Bruker BioSpin AG (Fällanden, Switzerland).

### Processing of Mass Spectra

The spectral data (Supporting Information Figs. S1, S4) were exported using Launchpad software and imported into MATLAB 2014 Student edition from The MathWorks Inc. (Natick, Massachusetts, USA). The mass spectra in the complete data matrix were reduced to a uniform distribution of the 10,000 data points over the 2,000–12,000 m/z range and aligned using the peak alignment by the fast Fourier transform method. The baseline was subtracted, negative values were set to zero, and an average spectrum was calculated. Afterward, the normalization to the area under the curve or to the total ion count was performed.

### Statistical Analysis

Pearson's correlation and principal component analysis (PCA) were performed in STATISTICA 12 (StatSoft Inc., Tulsa). For the artificial neural network (ANN) analysis, Trajan Neural Network Simulator, Release 3.0 D 1996–1998 from Trajan Software Ltd. (Durham, U.K.) was used. We constructed an ANN containing four neurons in one hidden layer. The intensities of the processed mass spectra served as the input, whereas the sample categories represented the output. The "*learning*" of the ANN was performed using the back-propagation training algorithm as previously described [7, 8]. The back-propagation was achieved by iteratively adjusting the values of the connection weights in order to minimize the difference between the ANN calculated output value and the experimental value. The optimal ANN architecture was confirmed by plotting the root mean square of the sum of residuals value against the number of neurons in the hidden layer(s) and the number of training cycles (epochs). The ANN input variables (peaks) were selected upon PCA analysis as the peaks covering more than 95% of the variability in the dataset.

### RESULTS

First, we investigated if intact cell MALDI-TOF MS, without any previous sample fractionation or identification of dominant peaks, could discriminate between morphologically uniform, but inherently different hESCs. As a well characterized model, we employed hESC line CCTL-14 cultured on CF-1 MEFs [9]. During long-term culture, CCTL-14 line developed chromosomal abnormalities and also showed increased expression of CD44 and CD30 surface markers (Supporting Information Fig. S2), previously associated with culture adaptation and genomic instability, respectively [10, 11]. In parallel, expression of Sox2, marker associated with undifferentiated phenotype, remained constant (data not shown). To test our hypothesis, low (P29), medium (P72), and high (P269) passage number CCTL-14 hESCs were cultured under identical conditions, harvested 48 hours after seeding, and processed immediately for MS. The final normalized spectral dataset contained 255 m/z values with assigned peak intensities, which were subjected to statistical analyses. Pearson's correlation of the average mass spectra obtained from the low, medium, and high passage hESCs indicated disparities in the spectral fingerprints (Fig. 1A). Using PCA, the correctly clustered P29-hESC and P269-hESC populations were clearly identified, with the p72-hESCs

represented a transition population (Fig. 1B). To exclude potential contribution of MEFs to overall spectral heterogeneity, otherwise identical hESCs were seeded on different MEF batches and had their mass spectra recorded. No significant alterations in their mass spectra were detected due to this manipulation, documenting no distortion due to MEFs (data not shown). Thus, the complex mass spectral fingerprint based on a large number of non-dominant peaks provided sufficient information to discriminate the hidden phenotypes associated with long-term culture adaptation.

Next, we were curious if we could determine shifts in the hESC phenotypes shortly after induction of differentiation. We designed a hESC model using retinoic acid (RA) as a potent inducer of differentiation. It has been well documented in vitro that RA induces the differentiation of hESCs to the ectodermal phenotype, including neural lineages and keratinocytes or extra-embryonic tissues [12–14]. Thus, we picked the RA-driven differentiation model to evaluate the subtle changes that occur in hESC populations during early differentiation. We stimulated the CCTL-14 hESCs with $10^{-8}$ M RA for a period of 12, 24, or 48 hours (Supporting Information Fig. S3A).

We found that the mass spectra from the RA-treated and control cells were highly correlated and lacked obvious specific peaks that would allow a straightforward search for biomarkers (Fig. 2A). Interestingly, the Pearson's correlation matrix obtained from the average mass spectra revealed a decreased correlation within RA-treated P269-hESCs when compared to RA-treated P29-hESCs and P72-hESCs (Fig. 2B). We then performed the PCA of the control and RA-treated P29-hESCs and compared them with the P269-hESCs. The results revealed that the hESCs without any apparent morphological or molecular alterations were clearly clustered based only on the mass spectral fingerprint (Fig. 2C). Although the cluster analysis only allows for the analysis of an enclosed dataset, we wanted to test whether MS data could be used to predict and correctly categorize an unknown spectral sample. Thus, we constructed and trained a back-propagation ANN [7] based on a reduced dataset containing 23 peak intensities revealed by variable selection approaches (Fig. 2D). Critically, within these time intervals, the hESCs that were induced by RA to differentiate still possessed a typical uniform morphology, maintained high levels of Sox-2, Oct-4, and Nanog, and were completely indistinguishable from the untreated controls. Still, increased level of neuroectodermal determinant Pax6 and downregulation of mesodermal marker Brachyury, as determined by qRT-PCR, clearly spoke for RA-driven entry into differentiation pathway (Fig. 2E, Supporting Information Fig. S3B). The ANN correctly assigned the unknown spectrum to the appropriate sample. On the other hand, using the intensities of only 23 input peaks, the PCA failed to correctly cluster the samples (data not shown). This suggests that non-linear, self-learning approaches are suitable for handling complex data, allowing simple application for the monitoring of routine cultures or for quality control in current good manufacturing practice (CGMP) facilities.

### DISCUSSION

The quality of stem cell cultures is an essential prerequisite for applications in which the product variability, lot-to-lot consistency, composition, contamination, or phenotype stability are significant. Here, we present a quality control tool based on the combination of intact cell MS (MALDI-TOF MS) and sophisticated statistical analysis that revealed the hidden variability in hESCs. Current approaches for routine cell identification or characterization use either DNA-based techniques, such as short tandem repeat (STR) profiling or the analysis of the expression of a defined set of
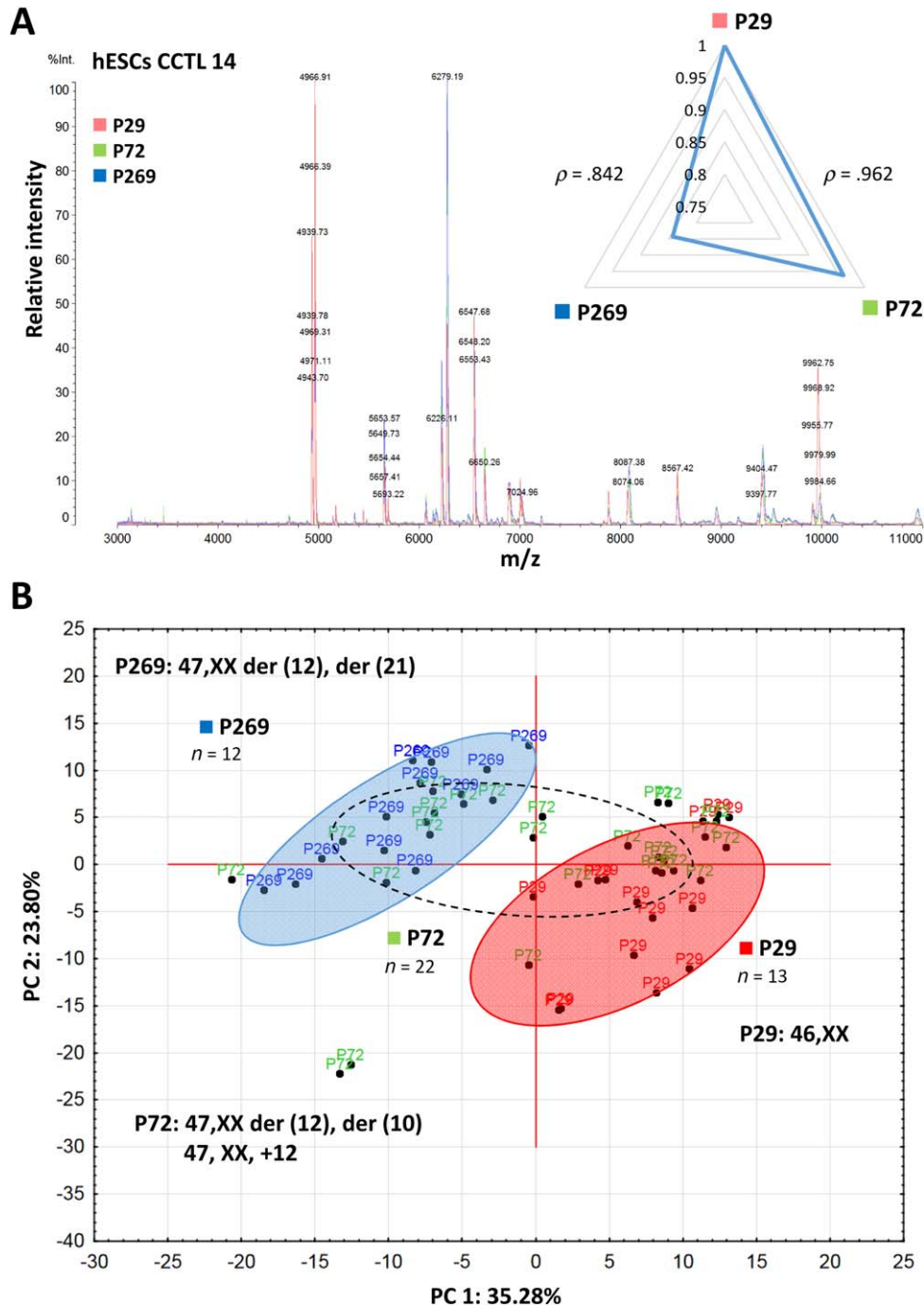
**Figure 1.** **(A):** Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry mass spectra obtained from intact hESCs of different passage numbers (P29, P72, and P269). The inset shows the Pearson's correlation of the peak intensities in the average spectra between the P29, P72, and P269 experimental groups. **(B):** PCA of spectral datasets. Each point in the PCA plot represents a unique biological sample. Abbreviations: hESCs, human embryonic stem cell; PCA, principal component analysis.

marker genes [15], or molecular and functional characterization of phenotype alterations [16]. However, both approaches focus on a limited set of variables (e.g., gene expression or STR profiling), or in theory do not cover unknown or uncharacterized parameters. The identification of individual biomarkers in large, high-dimensional datasets can also be biased by the formulation of an initial hypothesis, the biological background, or the selection of candidate biomarkers, or can be limited to a particular experimental scenario. For instance, a recent comparison of mouse induced

pluripotent cells (miPSCs) and mouse ESCs using high-throughput lectin arrays coupled with surface plasmon resonance imaging revealed specific metabolomic profiles unique to miPSCs and mESCs, indicating differences in the global metabolism of cultured stem cells [17].

Recently, multiple reaction monitoring-mass spectrometry (MRM-MS) was introduced by Baud et al. for the rapid determination and quantitation of pluripotency markers in iPSCs [18]. MRM-MS precisely determined proteotypic peptides generated by
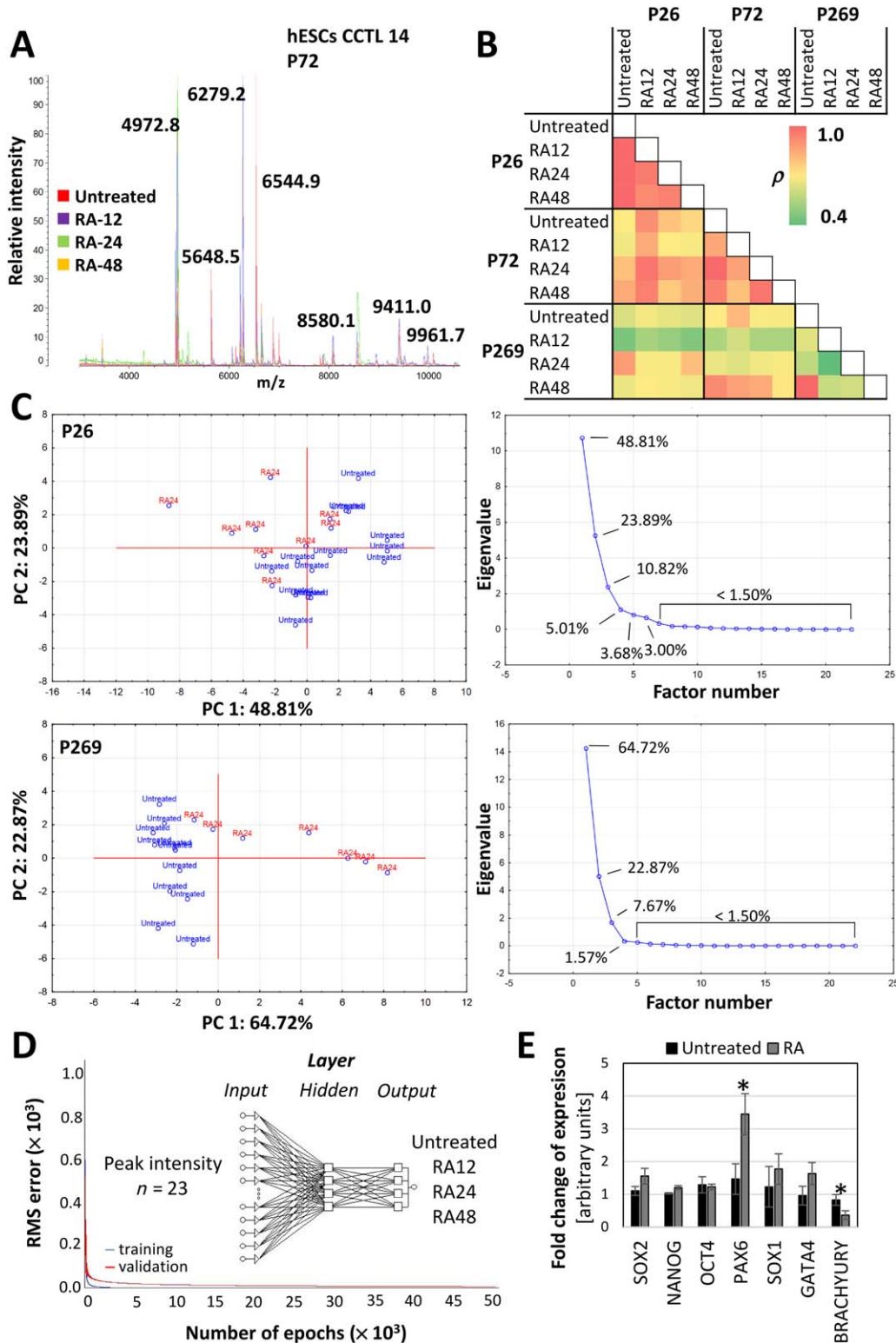
**Figure 2.** **(A):** Representative matrix-assisted laser desorption/ionization time-of-flight mass spectrometry mass spectra recorded from intact hESCs (P72) treated with RA for 12, 24, or 48 hours. **(B):** Heat map depicting the Pearson's correlation matrix of the peak intensities obtained from the average mass spectra. **(C):** PCA plots of the control and RA-treated P29 or P269 hESCs. Scree plots documenting the contribution of individual factors to the overall variability. **(D):** Training and leave-one-out verification plots of the RMS versus the number of training cycles (epochs). The first 50,000 iterations are shown. The inset shows the architecture of the artificial neural network used for the prediction of the sample category. **(E):** Relative normalized expression of stemness (Sox-2, Nanog, Oct-4), neuroectoderm (Pax-6, Sox-1), endoderm (GATA-4), and mesoderm markers (Brachyury) as determined by qRT-PCR. hESCs were cultured with $10^{-8}$ M RA for 48 hours, or left untreated. The asterisk indicates statistically significant changes in gene expression ($p < .05$). Abbreviations: hESCs, human embryonic stem cell; PC, principal component; PCA, principal component analysis; RA, retinoic acid; RMS, root mean square.

tryptic digestion of 15 marker proteins associated with self-renewal of reprogrammed cells constituting a pluripotency fingerprint. The intact cell MS approach we propose, employs rather a global mass spectrum as the classification input instead of searching for individual biomarkers, and might represent an independent technique that reflects the rational scale of the intrinsic heterogeneity of the cells and is likewise sensitive enough to reveal small changes that are below the detection limits of other techniques. Interestingly, using this technique we visualized the potential phenotypic drifts that occurred during long-term culture that might contribute to the phenomena of culture adaptation [19]. The intact cell MS, followed by the straightforward multivariate analysis, could therefore discriminate unapparent but critical alterations in stem/progenitor cells that are not detected by other techniques, which has clear application for quality control in routine cell cultures or CGMP.

## CONCLUSION

In summary, we introduced a simple, robust, and hypothesis-free quality control tool for determining induced and spontaneous alterations in genetically and morphologically uniform embryonic stem cell populations in routine cell cultures.

## AUTHOR CONTRIBUTIONS

P.V.: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing; L.K. and L.J.: collection and/or assembly of data; L.P. and E.M.P.-M.: data analysis and interpretation; J.H. and A.H.: conception and design, data analysis and interpretation, financial support, final approval of manuscript.

## DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicated no potential conflicts of interest.

## NOTE ADDED IN PROOF

This article was published online on 16 December 2017. Minor edits have been made that do not affect data. This notice is included in the online and print versions to indicate that both have been corrected 28 December 2017.

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