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Adaptation of a Commonly Used, Chemically Defined Medium for Human Embryonic Stem Cells to Stable Isotope Labeling with Amino Acids in Cell Culture

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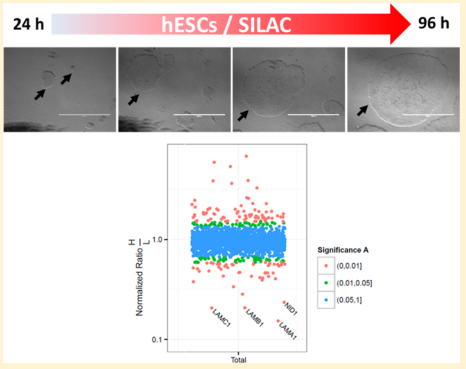
Supporting Information

ABSTRACT: Metabolic labeling with stable isotopes is a prominent technique for comparative quantitative proteomics, and stable isotope labeling with amino acids in cell culture (SILAC) is the most commonly used approach. SILAC is, however, traditionally limited to simple tissue culture regimens and only rarely employed in the context of complex culturing conditions as those required for human embryonic stem cells (hESCs). Classic hESC culture is based on the use of mouse embryonic fibroblasts (MEFs) as a feeder layer, and as a result, possible xenogeneic contamination, contribution of unlabeled amino acids by the feeders, interlaboratory variability of MEF preparation, and the overall complexity of the culture system are all of concern in conjunction with SILAC. We demonstrate a feeder-free SILAC culture system based on a customized version of a commonly used, chemically defined hESC medium developed by Ludwig et al. and commercially available as mTeSR1 [mTeSR1 is a trade mark of WiCell (Madison, WI) licensed to STEMCELL Technologies (Vancouver, Canada)]. This medium, together with adjustments to the culturing protocol, facilitates reproducible labeling that is easily scalable to the protein amounts required by proteomic work flows. It greatly enhances the usability of quantitative proteomics as a tool for the study of mechanisms underlying hESCs differentiation and self-renewal. Associated data have been deposited to the ProteomeXchange with the identifier PXD000151.



KEYWORDS: *human embryonic stem cells, metabolic labeling, SILAC, chemically defined medium*

1. INTRODUCTION

Human embryonic stem cells (hESCs) are derived from the inner cell mass of blastocyst state embryos and are considered pluripotent, i.e., possessing the potential to differentiate into any cell type present in the adult organism.[1] The study of hESCs is thus relevant for regenerative medicine, which focuses on restoring or replacing damaged tissue through transplantation of functional hESCs or tissues derived from them[1−3] as well as for fundamental research into developmental processes.[4] Additionally, hESCs-equivalent, human induced pluripotent stem cells,[5] offer the potential to study the cellular and molecular hallmarks of disease in tightly controllable in vitro systems.[6]

As much of the cellular machinery relies on proteinaceous effectors, extensive efforts have been devoted to characterize and comparatively quantify the protein complement of biological systems, much of it by using mass spectrometry based proteomics and SILAC.[7−11] SILAC refers to the metabolic labeling of the entire proteome of a given cell population with "heavy", nonradioactive, isotopic variants of amino acids, thus rendering its proteome distinguishable from the unlabeled state by mass spectrometric (MS) analysis.[12] Two or more differentially labeled populations of proteins from different cellular states can be mixed and analyzed in a single MS experiment that allows accurate comparative quantitation of the proteins.[13] Samples may be mixed even prior to cell lysis, thus avoiding potential quantitation artifacts deriving from parallel sample processing, as can be observed in alternative techniques such as chemical isotopic labeling.[14] This flexible strategy has been used for the characterization of a plethora of complex biological phenomena and notably extends easily to

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the analysis of post-translational modifications.[15−23] In research centering on hESCs, however, this powerful platform for quantitative proteomics has yet to be utilized to its full potential, which is largely due to the complex nature of hESCs culture.[24] Maintenance of hESCs in culture traditionally requires growing the cells on a feeder layer of mouse embryonic fibroblasts (MEFs) to preserve the undifferentiated pluripotent state.[25] Feeder cells represent a source of unlabeled amino acids that ultimately decreases the SILAC labeling efficiency of the ES cells, thus considerably compromising the accuracy of quantitation.[10] In addition, culture in the presence of MEFs implies potential sample contamination with molecules derived from mouse cells. One exemplary effect of this is the faulty quantitative assessment of peptides common to both species.[26]

Efforts to make hESCs amenable to metabolic labeling in general and SILAC specifically have included elimination of feeders[8] by culturing the cells using dialyzed mouse embryonic fibroblast-conditioned medium (MEF-CM).[7,9,10,27,28] MEF-CM, however, contains a variety of undefined xenogenic factors provided by MEFs,[29,30] which may complicate data interpretation and hinder quantitation of subtle molecular characteristics of differentiation and self-renewal of hESCs. Additionally, the preparation of MEF-CM for SILAC applications involving dialysis represents a demanding, multistep protocol.[7,31] To circumvent the use of feeder cells all together, Wang et al.[8] formulated an entirely chemically defined medium for SILAC labeling of hESCs. The protocol employs dissociation into single cell suspension and supplementation with synthetic Rho-associated kinase (ROCK) inhibitor to increase yield of labeled cells. These culturing conditions may interfere with signaling pathways in hESCs[32] and represent a parting from commonly used routine procedures in hESC culture.[33−36]

In answer to the general need for feeder-independent and defined culturing systems for hESCs, Ludwig et al.[37] developed a culturing system based on a medium commercially available as mTeSR1. The medium is a serum-free, complete, and defined formulation[37] for feeder-independent maintenance and expansion of human embryonic stem cells (hESCs) in an undifferentiated state. It contains recombinant human transforming growth factor β and human basic fibroblast growth factor and is designed to be used with a Matrigel-coated cell culture surface.

The utility of mTeSR1-based hESC culture has been confirmed in studies covering a wide spectrum of applications, including derivation[37,38] and maintenance of both hESCs and induced pluripotent stem cells (iPSCs),[39] prevention of apoptosis in stem cells,[40] iPSC-based tissue engineering,[41,42] study of signaling pathways[43] as well as cell surface interaction,[44] controlled differentiation,[45] cell programing,[46] and reprograming.[47] The popularity of the system and its chemically defined nature render mTeSR1 an attractive candidate for adaptation to metabolic labeling in general and more specifically to SILAC. We report a strategy for straightforward SILAC labeling of hESCs, aiming for an efficient protocol yielding in-depth characterized hESCs, that may serve for direct comparative proteomic analyses as well as a complex biogenic spike-in standard[48] in future proteomic studies. A comparable strategy has recently been reported for the competing reagent DMEM/F12 from Invitrogen.[49]

2. MATERIALS AND METHODS

2.1. hESCs Culture

ES4 cells[50] were purchased from WiCell Research Institute (Madison, WI). Permission to use the cell line was obtained from the Cornell/Rockefeller/Sloan Kettering tri-institutional ESC research oversight committee. Funding was secured from nonfederal funding sources. ES4 cells were cultured on growth factor reduced Matrigel (BD Biosciences, Franklin Lake, NJ) in chemically defined mTeSR1 medium (STEMCELL Technologies, Vancouver, BC, Canada) as previously described[38] and supplemented with 100 units/mL penicillin/streptomycin (PAA, Pasching, Austria). Cultures were passaged in 4-day intervals. Following 15−20 min of Dispase exposure [1 mg/mL, 166 μL per well of a six-well plate (STEMCELL Technologies, BC, Vancouver, Canada)], 2 mL of medium was added to each well, and cells were dispersed by repeated aspiration. Colonies were pelleted at 100 rcf for 3 min at room temperature, the supernatant was discarded, and 1.66×10^5 cells were further dispersed by gentle pipetting in fresh media prior to plating into Matrigel-coated wells of a six-well plate. Cells were fed once a day with fresh, prewarmed medium.

2.2. Titration of Arginine and Lysine in hESCs Culture and Characterization of Proliferation Kinetics

To titrate the amount of SILAC amino acids required by hESCs, 1.66×10^5 cells per well of a six-well plate were plated into standard mTeSR1 and customized mTeSR1 devoid of arginine and lysine supplemented with 100% (0.548 mM Arg, 0.391 mM Lys), 50%, 33%, 25% and 0% of the arginine and lysine concentrations found in the standard medium. Cells were cultured for 96 h, and medium was changed daily. On the final day medium was aspirated, and cells were washed once with PBS and trypsin was added (1 and 0.5 mL per well of a six-well plate, respectively). After 10 min of incubation at 37 °C, 0.5 mL of medium was added to quench trypsin activity. Individualized ES4 cells were counted using a TC10 cell counter (Bio-Rad, Philadelphia, PA). For each culturing condition, cells from three individual wells of a six-well plate were counted. Additionally, phase contrast images of three randomly chosen colonies from separate wells were taken per culturing condition with 4× magnification using an inverted microscope (EVOS fl; AMG, Bothell, WA). On the basis of the resulting micrographs, colony area was calculated in 24 h intervals.

Evaluation of the amino acid titration was extended by recording growth curves. Cells (1.66×10^5 per well) were plated on day 0 of each passage into standard mTeSR1 and customized mTeSR1 devoid of arginine and lysine supplemented with 25% of the arginine and lysine concentrations found in the standard medium (customized mTeSR1-25%) in six-well plates in duplicate. Cells were cultured for 96 h, passaged, and cultured for 4 more days, with daily change of medium. At time of harvesting, cells were counted. Throughout the experiment, micrographs of selected cell colonies were taken daily as described above. In total, 182 colonies were tracked (85 and 97 colonies in mTeSR1 and customized mTeSR1-25%, respectively) and 728 images were analyzed. See the Supporting Information for details on the data collection protocol.

2.3. SILAC Labeling of hESCs

For labeling, customized mTeSR1 devoid of arginine and lysine was supplemented with heavy, isotopically labeled forms of arginine and lysine (Arg10, $^{15}N_4{}^{13}C_6$; Lys8, $^{15}N_2{}^{13}C_6$; Cam-

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bridge Isotope Laboratories, Andover, MA) to a concentration of 137 μM for arginine and 97.75 μM for lysine, corresponding to 25% of the concentrations found in the standard medium. Cells were cultured as described above for two passages (at 96 and 192 h) with daily medium changes. Cells harvested at the first passage (after 96 h incubation) were used for SILAC quantitation experiments.

2.4. Cell Lysis and in-Solution Digest

Prior to LC–MS/MS experiments, cell pellets were resuspended in one pellet volume of 6 M urea, 2 M thiourea in 10 mM Hepes (pH 8.0) and sonicated (3 × 5 s, intensity 10%) on ice using a sonifier (w-250 D; Branson, Danbury, CT). Protein concentration in the resulting extract was determined using a Bradford assay (see the Supporting Information). The lysate was cleared by centrifugation. Extracts for SILAC quantitation were mixed at a 1:1 ratio according to the protein concentration measured. Following reduction and alkylation with 1 mM DTT and 5.5 mM iodoacetamide, proteins were digested with Lys-C (1 μg/50 μg of protein,Wako Chemicals, Neuss, Germany) for 3 h at room temperature. The urea/thiourea concentration was reduced to 2 M by dilution with 50 mM ammonium bicarbonate and digestion continued using trypsin (1 μg/50 μg of protein, Promega, Madison, WI) for 12 h at room temperature. Proteolysis was stopped by addition of trifluoroacetic acid to pH <2.5. Peptide samples were desalted and stored at 4 °C on C18 StageTips.[51]

2.5. Evaluation of SILAC Labeling Efficiency and Arginine to Proline Conversion

Cells were harvested after 72, 96, 168, 192, and 216 h of metabolic labeling and exposed for 10–15 min to Dispase. After addition of 2 mL medium into each well, cells were dispersed by repeated aspiration. Cells were collected by centrifugation at 400 rcf for 5 min at room temperature, and the supernatant was discarded. Cells were washed four times in PBS and clumps broken up by gentle pipetting. PBS was discarded after centrifugation. Harvested cell pellets (typically from five wells of a six-well plate per culturing condition) were snap frozen and stored at −80 °C for 10 min. Cells were lysed, extracts digested as above, and samples subsequently subjected to LC–MS/MS.

2.6. LC–MS/MS Analysis

Peptides were eluted twice from StageTips using 20 μL of 60% acetonitrile, 0.5% acetic acid. Solvent was evaporated to 5 μL using a vacuum concentrator (Eppendorf, Hamburg, Germany) and the peptide solution acidified with 5 μL of 2% acetonitrile, 0.1% trifluoroacetic acid. All LC–MS/MS experiments were performed essentially as previously described.[52] Briefly, peptides were separated using an EASY-nLC system (Proxeon Biosystems/ThermoFisher Scientific, Odense, Denmark). A 6 μL sample was loaded with constant flow of 250 nL/min onto a 20 cm fused silica emitter with an inner diameter of 75 μm packed in-house with ReproSil-Pur C18-AQ 3 μm resin (Dr. Maisch GmbH, Ammerbuch-Entringen, Germany). Peptides were eluted with a segmented gradient from 10 to 60% solvent B (80% acetonitrile, 0.5% acetic acid) over 100 min with a constant flow of 250 nL/min. The HPLC system was coupled to a QExactive mass spectrometer (ThermoFisher Scientific, Bremen, Germany) via a nanoscale LC interface (Proxeon Biosystems/ThermoFisher Scientific, Odense, Denmark) equipped with a column oven (PRSO-V1, Sonation, Biberach, Germany) set to 30 °C. The temperature of the heated capillary was set to 250 °C and the spray voltage to 2.1 kV. Survey MS spectra (m/z 300–1650) were acquired with a resolution of 70 000 at m/z 300 and using the automatic gain control (AGC) to set a target value of 3 000 000 charges. The 10 most intense ions from the survey scan were sequenced by higher energy collisional dissociation (HCD) (normalized collision energy 25%) at a resolution of 17 500 with an AGC target value of 100 000. Maximal filling times were set to 20 ms for the full spectra and 120 ms for MS/MS spectra. Precursor ion charge state screening was enabled, and all unassigned charge states as well as singly charged species were rejected. Precursor ions were selected according to isotopic matching and further fragmented as described above. Fragmented precursors were then placed on a dynamic exclusion list for 25 s (repeat count = 1). Data were acquired using the Xcalibur software (version 2.2.44) and are available at ProteomeXchange.org using the identifier PXD000151.

2.7. Processing of Mass Spectrometric Data

Mass spectrometric data was analyzed using the MaxQuant suite of algorithms (version 1.3.0.5).[53] The data was searched against the *Homo sapiens* UniProtKB protein sequence database (downloaded on 10/24/2012; 68 108 entries including canonical and isoform sequence data). Enzyme specificity was set to trypsin, allowing for cleavage N-terminal to proline and between aspartic acid and proline.[52] Carbamidomethylcysteine was set as a fixed modification, and oxidized methionine and N-acetylation were set as variable modifications. Labeled Arg10 and Lys8 were used as peptide modifications for quantitation. MaxQuant's requantitation option was not used where incorporation efficiency and arginine to proline conversion were analyzed. The maximum mass deviation allowed[54] was set at 20 ppm for the initial search and 6 ppm for the main search for monoisotopic precursor ions; the mass error tolerance was set at 20 ppm for MS/MS peaks. A maximum of two missed cleavages and three labeled amino acids (arginine and lysine) was allowed. The required false discovery rates were set to 1% at the peptide and protein level (the protein level filter was disabled for incorporation efficiency and arginine to proline conversion testing) and the minimum required peptide length was set to six amino acids. Files documenting the MaxQant parameters used in analyses studying incorporation, proline conversion, and protein quantitation are deposited at ProteomeXchange.org along with the raw data (PXD000151).

2.8. Matrigel and hESCs Protein Separation by One-Dimensional SDS–Gel Electrophoresis

Samples containing 3 μL of Matrigel and 147 μL of SDS solution [4% SDS (w/w) in 0.1 M Tris/HCl, pH 7.6] were heated for 3 min at 95 °C and sonicated (w-250 D; Branson, Danbury, CT; 3 × 5 s, intensity 10%) on ice. In parallel, pellets of hESCs (ES4 cells, SILAC labeled for 96 h) were resuspended in 1 pellet volume of SDS solution and treated as described above. The lysates were cleared by centrifugation, and protein concentration was determined using a BCA protein assay kit (Thermo Scientific, Rockford, IL) (see the Supporting Information). Prior to loading on the gel, extracts (amounts described below) were mixed with 7 μL of LDS sample buffer (NuPAGE, Life Technologies, New York, NY) and 3 μL of DTT (10 mM in 50 mM ammonium bicarbonate), and the volume was made up to 30 μL with deionized H_2O, of which 25 μL was loaded.

Proteins extracted from cell pellets (70 and 100 μg) and Matrigel (7.5, 6.0, 4.5, 3.0, 1.5 μg) were separated by SDS–

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PAGE using the XCell SureLock Mini-Cell system and NuPAGENovex Bis−Tris 4−12% precast gels in NuPAGE MOPS−SDS running buffer containing antioxidant as per the manufacturer's instructions (Life Technologies, New York, NY). Gels were stained with a Colloidal Stain Kit (Life Technologies, New York, NY) and visualized using a Geliance 600 imaging system (Perkin-Elmer, Waltham, MA). The experiment was replicated three times.

2.9. Evaluation of Stemness

2.9.1. Antibody Array. Expression of stemness related proteins was verified in SILAC-labeled hESCs and in unlabeled hESCs as a control. The Human Pluripotent Stem Cell Array Kit (R&D Systems, Minneapolis, MN)[55] was used for the analysis according to the manufacturer's instructions. Briefly, all cells were cultivated as previously described, collected, frozen at −80 °C for 15 min, and lysed in the lysis buffer provided. Proteins were quantified using the Bradford assay (see the Supporting Information); 400 μg of protein extract were loaded per array. Arrays were developed using horseradish peroxidase (HRP) and chemiluminescent peroxidase substrate (Sigma-Aldrich, St. Louis, MO). Data were collected using a Geliance CCD camera (Perkin-Elmer, Waltham, MA) and analyzed using ImageJ software, version 1.45s, (Wayne Rasband, National Institute of Health).[56] Array pictures were inverted, and background was subtracted. Signal was captured as the median pixel density value in an area of 110 μm diameter (black = 10 000 units, white = 1 unit) for each spot. HeLa cells (passage 6) were used as a negative control, following the same protocol.

2.9.2. Immunostaining. hESCs were washed three times after 96 h of culture with PBS, fixed with paraformaldehyde [4% PFA in PBS (w/v), 1 mL/well of six-well plate] for 10 min, washed three times with PBS, and immunostained using a StemLight Pluripotency Antibody Kit (#9656, Cell Signaling, Boston, MA) according to the manufacturer's instructions. Briefly, after paraformaldehyde fixation, cells were permeabilized by incubation in ice-cold methanol for 10 min at −20 °C and rinsed with PBS for 5 min. The specimen was blocked using BSA solution [5% BSA in PBS (w/v) containing 0.3% Triton X-100 (w/v), 1 mL per well of six-well plate] for 60 min. The primary antibody was prepared by adding 5 μL of antibody to 995 μL of antibody dilution buffer [1% BSA (w/v) and 0.3% Triton X-100 (w/v) in PBS]. The blocking solution was aspirated and the diluted primary antibody was applied (1 mL per well of a six-well plate). Cells were incubated for 12 h at 4 °C, rinsed three times in PBS for 5 min, and then incubated for 2 h in diluted fluorochrome-conjugated secondary antibody (5 μL of antibody in 995 μL of antibody dilution buffer per well) at room temperature in the dark. Cells were rinsed three times in high-salinity PBS (0.39 mM NaCl in PBS) for 5 min. Specimens were microscopically examined under 10× magnification (EVOS fl; AMG, Bothell, WA), and GFP (470 nm excitation, 525 nm emission), RFP (531 nm excitation, 593 nm emission), and transmitted light channels were recorded. Prior to imaging, samples were stored at 4 °C protected from light. The following antibodies were used: Oct-4A (clone C30A3) rabbit mAb, SOX2 (clone D6D9) XP rabbit mAb, NANOG antibody rabbit IgG, SSEA4 (clone MC813) mouse mAb, TRA-1−60(S) [clone TRA-1−60(S)] mouse mAb, and TRA-1−81 (clone TRA-1−81) mouse mAb (Life Technologies, New York, NY). All primary antibodies were used at a dilution of 1:200. For surface antigens SSEA4, TRA-1−60, and TRA-1−81, permeabilization with methanol was omitted. Secondary antibodies were used as follows: Alexa Fluor 594 donkey anti-mouse IgG for SSEA4; Alexa Fluor 488 goat anti-mouse IgG for TRA-1−60 and TRA-1−81; and Alexa Fluor 488 goat anti-rabbit IgG for Oct-4, SOX2, and NANOG (Life Technologies, New York, NY).

2.9.3. Alkaline Phosphatase Staining. hESCs were washed three times with PBS after 96 h of culture, fixed with paraformaldehyde (4% w/v in PBS, 1 mL per well of a six-well plate) for 2 min, and washed three times with PBS. Alkaline phosphatase[57] activity was assayed using a Leukocyte AP Kit (Sigma-Aldrich, St. Louis, MO) as instructed by the manufacturer. Briefly, the fixed cells were incubated in the provided working solution (1 mL per well of a six-well plate) for 15 min at room temperature in the dark. Cells were rinsed three times in PBS for 5 min. Images were acquired using an Aficio MP C4000 (Ricoh, Malvern, PA) scanner. Wells were additionally screened for differentiated colonies using inverted microscopy (EVOS fl; AMG, Bothell, WA).

2.9.4. Undirected Differentiation of SILAC-Labeled hESCs. Three germ layer differentiations were performed using hESCs that were SILAC-labeled for 96 h in a six-well plate as described above. After 96 h of culturing, the SILAC media was aspirated and replaced with either 10% FBS DMEM containing media or with HuESM without basic FGF [knockout DMEM supplemented with 20% knockout serum replacement, nonessential amino acids, β-mercaptoethanol, L-glutamine, and penicillin/streptomycin; all reagents were purchased from Invitrogen (Grand Island, NY)]. After 4 weeks of culture, cells were harvested, fixed with paraformaldehyde [4% PFA in PBS (w/v), 1 mL/well of a six-well plate] for 15 min at rt. Nondirected differentiation of cells was assayed for markers of the three embryonic germ layers (endoderm, mesoderm, and ectoderm) by immunohistochemistry for alpha-1-fetoprotein (AFP), smooth muscle (αSMA), and beta III tubulin (TUJ1). Cells were incubated with a blocking solution of 10% donkey serum (Jackson ImmunoResearch, West Grove, PA) in PBST [PBS with 0.1% Triton-100 (Sigma Aldrich, St. Louis, MO)] for 1 h at rt, followed by an overnight incubation at 4 °C with the following primary antibodies: AFP (cat. # MAB1368-rabbit, working dilution 1:100), purchased from R&D systems (Minneapolis, MN), αSMA (cat. # AB5694-rabbit, working dilution 1:100) purchased from Abcam (Cambridge, MA), and TUJ1 (cat. # 09-0020-mouse, working dilution 1:500) purchased from Stemgent (San Diego, CA). Cells were then incubated for 1 h at rt with the corresponding secondary antibodies purchased from Stemgent (San Diego, CA) and used with a dilution of 1:1000: Rb Dylight 488 (cat. # 09-0034), Ms Dylight 488 (cat. # 09-0033), and Rb Cy3 (cat. # 09-0037). Cells were washed and counterstained with DAPI (Invitrogen, Grand Island, NY) (1:1000 in PBS) for 15 min at rt.

2.10. Differential Expression Analysis

Peptides corresponding to 2214 protein groups and corresponding SILAC ratios were identified from three biological replicates using MaxQuant as described above. To characterize differential protein expression, a significance score, termed "significance A",[53] was calculated for data sets of each replicate using an R implementation of the original algorithm (available in the Supporting Information). Protein groups with a p-value of less than 0.05 were considered to be significantly differentially expressed (SDE).

The depth of the proteomic analysis performed in combination with the default minimum ratio bin size employed by the commonly used outlier determination algorithm "significance B"[53] (default bin size 500) motivated the usage of the nonintensity binned equivalent significance A to score ratio changes for significance.

DAVID[58,59] and BioVenn[60] were used to determine whether gene products involved in arginine (KEGG Id 00330) or lysine metabolism (KEGG Id 00310) are reported as differentially expressed. We also performed gene enrichment analysis using DAVID and pathway analysis using Ingenuity Pathway Analysis (IPA) software version 8.6 (Ingenuity Systems, Inc., Redwood City, CA) on the list of genes intersecting in three replicates. Significant enrichment and impacted pathways were calculated using the default parameters of DAVID and IPA. We report here the functional classification table from DAVID and the canonical pathways from IPA.[59]

2.11. Statistical Analysis

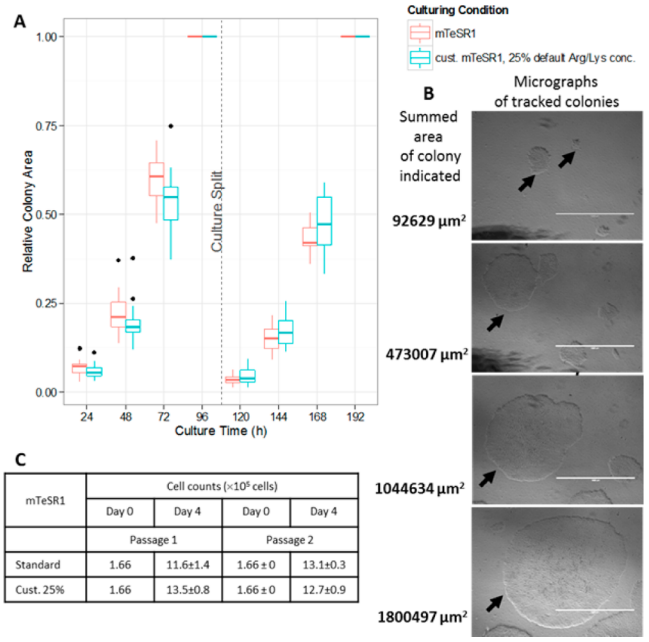
Statistical analysis and plotting were performed using the R Statistical Programming Environment.[61] The analyses performed are documented in a package provided with the raw data at proteomeCommons.org (PXD000151) and based on a publicly available collection of tools (RCFPD, URL: http://sourceforge.net/projects/rcfpd/). Numerical results are given as means ± SEM (n = sample size). Statistical significance was assessed using a Student's t test.

3. RESULTS AND DISCUSSION

3.1. Culturing the Cells Using Reduced Concentrations of Arginine and Lysine

For economic reasons and to avoid arginine to proline conversion, we first sought to culture hESCs under conditions of reduced L-arginine and L-lysine concentrations as compared to the standard medium. Titration experiments were performed with unlabeled amino acids using 100%, 50%, 33%, and 25% of the nominal amount of arginine and lysine. We included a control experiment using culture medium devoid of arginine and lysine to reveal hidden sources of arginine and lysine in the culture regimen (see Figure S1, Supporting Information).

To assess cellular growth rate under varying experimental conditions, two independent methods were used: the count of individualized cells in suspension and the evaluation of colony size evolution. Given the fact that only 40% of hESCs clusters adhere to the Matrigel-coated surface,[62] the precise counting of seeded cells is problematic. In contrast, tracing colony growth through micrographs assures that only viable and dividing cells are considered for growth rate evaluation (see Figure 1B). Both methods of growth rate evaluation led to the conclusion that under all conditions tested, with exception of the absence of arginine and lysine, cells grew at rates comparable to that of standard mTeSR1 culture. At an interval of 96 h, cell number increased more than 6-fold according to cell count, while colony size increased 18-fold (see Figure S1, Supporting Information). We thus proceeded with 25% of the amino acid concentrations in subsequent experiments, while default L-arginine and L-lysine concentrations were used as a control [see Figures 1, S1, and S2 (Supporting Information) for results of all experimental replicates]. To test if the concentration of labeling amino acids may be reduced beyond 25%, we cultured ES4 cells in custom mTeSR1 devoid of arginine and lysine supplemented with 10% and 5% of the arginine and lysine concentrations found in the standard medium. In both cases, abnormal colony



mTeSR1	Cell counts (×10⁵ cells)			
	Day 0	Day 4	Day 0	Day 4
	Passage 1		Passage 2	
Standard	1.66	11.6±1.4	1.66 ± 0	13.1±0.3
Cust. 25%	1.66	13.5±0.8	1.66 ± 0	12.7±0.9

Figure 1. Titration of SILAC amino acids (arginine and lysine) in hESCs culture suggests that a 4-fold reduction of concentration does not affect hESC expansion as compared to default concentrations of L-arginine and L-lysine. (A) Area-based quantitation (ImageJ) of tracked hESCs colonies indicates comparable growth rates for arginine and lysine concentrations tested. Cells were cultured in standard mTeSR1 and customized mTeSR1 devoid of arginine and lysine supplemented with 25% of the arginine and lysine concentrations found in the standard medium (not isotopically labeled). For each tracked colony, the area was normalized to the area of that colony recorded at 96 or 192 h. (B) Example of tracked colonies (scale bar: 1 mm), the number corresponds to the area occupied. (C) Cell number expansion using reduced arginine and lysine concentrations. Numbers of dissociated cells are reported (×10^5 cells) from triplicate wells at passages 1 and 2.

morphology was observed (see Figure S3, Supporting Information), reaffirming the choice of mTeSR1-25% for further experiments.

To examine if titration of arginine and lysine affects average colony size, we cultured ES4 cells in custom mTeSR1 devoid of arginine and lysine supplemented with 100, 50, 25 and 0% of the arginine and lysine concentrations found in the standard medium. After 96 h of culture, cells were fixed with paraformaldehyde [4% PFA in PBS (w/v), 1 mL/six-well plate well], culture plates scanned, and colony sizes determined by ImageJ particle analysis. The amino acid concentrations used do not display a prominent effect on colony size and no trend toward smaller colonies is observable with diminishing concentration (see Figure S4 of the Supporting Information for results and details of the experiment).

3.2. Efficiency of SILAC Labeling

Having established that the growth rate of hESCs was unaffected by a 4-fold reduction of L-arginine and L-lysine, we next examined the incorporation efficiency of the labeled amino acids.

As shown in Figure 2A, hESCs were labeled both with respect to arginine and lysine after 96 h of culturing (approximately five doublings) in SILAC medium containing 30.2 mg/L heavy arginine (Arg10, $^{15}N_4{}^{13}C_6$) and 22 mg/L heavy lysine (Lys8, $^{15}N_2{}^{13}C_6$). Labeling efficiency was estimated to be on average 96.02 ± 0.34% for arginine and 94.69 ± 0.15%

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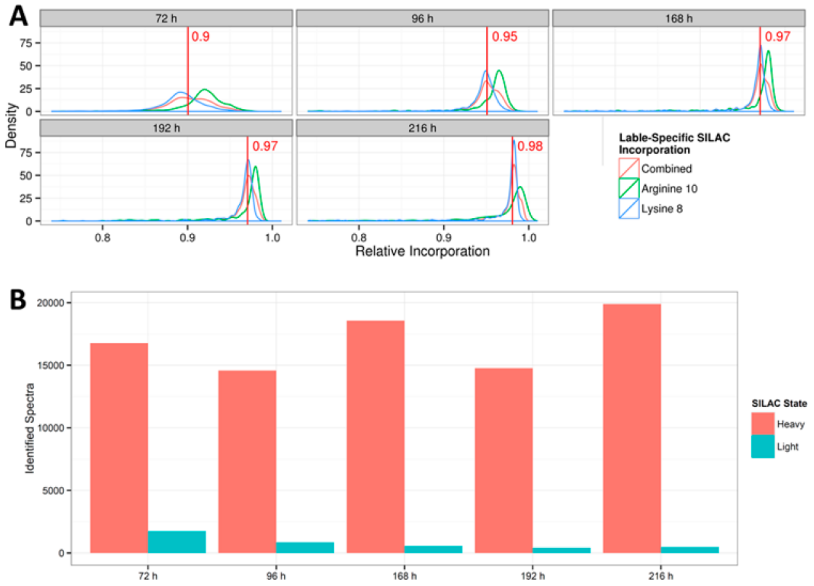

Figure 2. Full SILAC labeling is achieved for hESC after 96 h of culture at arginine and lysine concentrations of 30.23 and 22.20 mg/L, respectively (25% of the normal concentration). (A) Overall incorporation efficiencies evaluated by the assessment of SILAC ratios between unlabeled and labeled versions of arginine and/or lysine-containing peptides. The red vertical line marks median incorporation for combined peptides. (B) Number of identified spectra corresponding to peptides containing heavy and light forms of lysine and arginine.

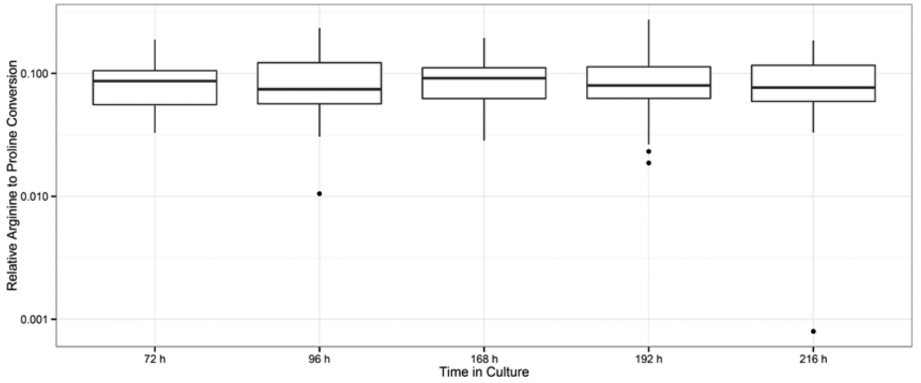

Figure 3. Metabolic arginine to proline conversion in SILAC-labeled hESCs. Heavy arginine and lysine were supplied at 30.23 and 22.20 mg/L, respectively (25% of the normal concentration). The extent of conversion was estimated by manual assessment. For the 50 most intense proline-containing peptides per time point, the intensity of heavy proline satellite peaks was estimated relative to the most intense isotope peak of the parental isotope distribution. Neighboring full MS scans were checked to ensure usage of spectra with maximal peptide intensity.

for lysine [see Figures 2 and S5 (Supporting Information) for results of all experiment replicates], close to the maximal labeling efficiency achievable, given the supplier-provided purity of the labeled amino acids used (99% for both ^{15}N and ^{13}C).

3.3. Arginine to Proline Conversion

In many cell types, including hESCs, arginine is metabolically converted to proline. [$^{13}C_6$,$^{15}N_4$]arginine becomes [$^{13}C_5$,$^{15}N_1$]-proline, which leads to the generation of a satellite peak for proline-containing heavy peptides and finally to underestimation of the intensity of the [$^{13}C_6$,$^{15}N_4$]arginine-containing peak,[13,31,63,64] which in turn compromises ratio determination. Several strategies have been proposed to deal with this problem,[63,65−68] including correction by computational approaches or prevention of conversion, for instance, by increasing the concentration of available proline in SILAC labeling media.[31,49] The simplest approach to this problem is a reduction of labeled arginine in the system, balancing a potential for starvation[64] with decreasing the kinetics of arginine to proline conversion. According to the titration experiments above (Figure 1), a 4-fold reduction of L-arginine did not affect cell proliferation compared to the default concentration. We thus explored arginine to proline conversion at this concentration by manual inspection of heavy proline satellite peak intensities in survey spectra of the 50 most intense proline-containing peptides per evaluated culture time point (see Figure 3).

At labeling time points ranging from 72 to 216 h, arginine to proline conversion was found to produce a satellite peak of approximately 10% of the intensity of the most intense peak in the corresponding peptide isotopic cluster of the peptide (see Figure S6 of the Supporting Information for results of three experimental replicates).

3.4. Matrigel as a Potential Contaminant

Given the dynamic range limitations of mass spectrometry based proteomics workflows, the culture of hESCs on Matrigel, a proteinaceous matrix, has the potential to severely interfere with sample analysis. To explore this possibility, Matrigel alone and whole cell lysates of cells grown on Matrigel were analyzed

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by SDS−PAGE. Figure 4 shows that no Matrigel signal is observable in separated whole cell lysate from harvested hESCs,

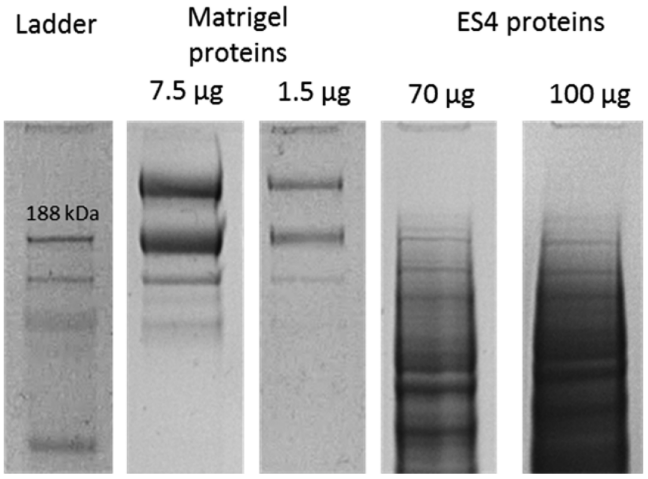

Figure 4. Cell lysates of hESCs grown on Matrigel are free of Matrigel contamination. Matrigel and harvested hESCs after 96 h culture on Matrigel were dissolved, lysed in SDS, and subjected to PAGE analysis followed by colloidal Coomassie staining (see Materials and Methods for details).

indicating that Matrigel-derived proteins are a minor fraction of the total pool of proteins obtained from cell culture and are unlikely to interfere with LC−MS analysis. It is also noteworthy that the SILAC labeling efficiency described above excludes significant contribution of amino acids to the cellular pool by Matrigel (see Figure S7 of the Supporting Information for results of all experiment replicates).

3.5. Stemness is Preserved in SILAC-Labeled hESCs

As the SILAC labeling protocol presented here introduces slight modifications to media and culture conditions, it is of utmost importance to characterize hESCs cultured using it with respect to proliferative capacity and maintained stemness. Comparing cell counts, as well as colony growth of standard and reduced arginine and lysine (25% of standard) conditions, as shown above, indicates that neither was negatively affected. Maintenance of stemness was evaluated as follows. After culture of hESCs in conventional mTeSR1 or customized medium (mTeSR1-SILAC) for 96 h, the cells exhibited typical undifferentiated colony morphology [Figures 5 and S7 (Supporting Information)]. Fluorescence immunocytochemistry analysis of the stemness markers NANOG, Oct4, SSEA4, SOX2, TRA-161, and TRA-1−81[57,69,70] indicated equivalent marker expression between standard and SILAC culturing conditions [Figures 5 and S8 (Supporting Information)]. Expression remained comparable at least up to 40 days in culture (data not shown). Expression profiles of stem cell markers were also analyzed using a commercial human pluripotent stem cell antibody array. In whole cell lysates prepared from labeled and unlabeled hESCs, the relative expression levels of stem cells markers were found to be comparable between both conditions [see Figures 5B,C and S9 (Supporting Information) for results of all experiment replicates]. The list of cell markers tested includes NANOG,

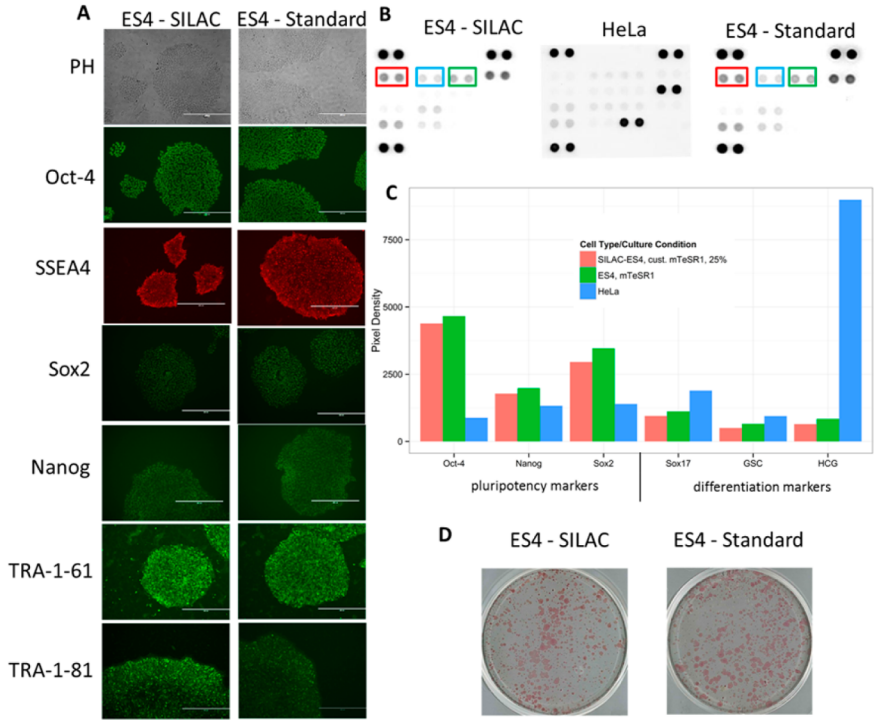

Figure 5. SILAC-compatible mTeSR1 culture preserves stemness of hESCs. (A) hESCs in mTeSR1-SILAC exhibit typical undifferentiated colony morphology (first row, phase contrast images) and express pluripotency markers (second row and rows below, immunofluorescence images) (scale bars: 400 μm). (B) Human pluripotent stem cell array demonstrates the detection of multiple stem cell markers in ES4 cell extracts; HeLa cell extract was used as a negative control. Red, blue, and green boxes represent Oct4, NANOG, and SOX2 protein markers, respectively [see Figure S9 (Supporting Information) for complete annotation of protein markers]. (C) Protein marker quantitation from panel B by ImageJ (mean gray value; calibration 10 000 units for blackness, 1 unit for whiteness). The average of the two signals per array is plotted. (D) Alkaline phosphatase activity staining for hESCs (ES4) on Matrigel-coated dishes in the presence of standard mTeSR1 as well as mTeSR1-SILAC.

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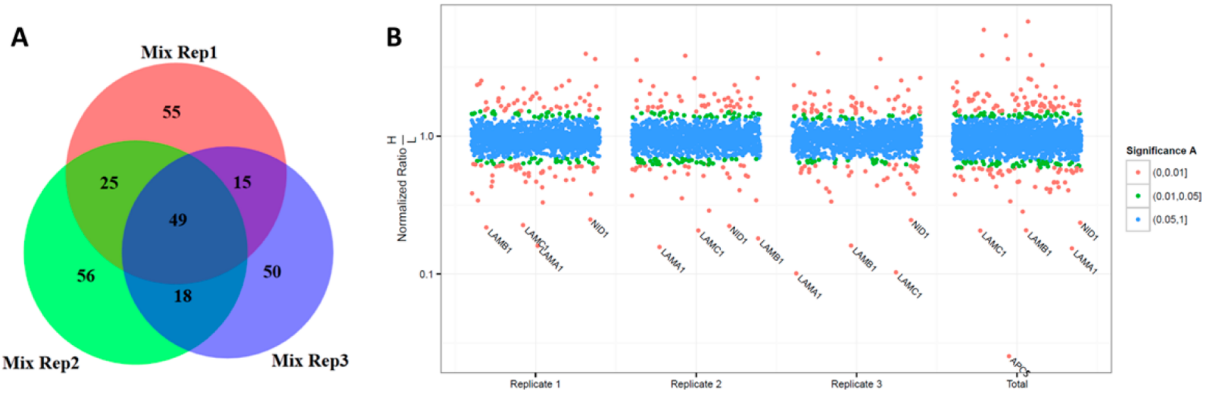

Figure 6. Proteome of SILAC-labeled hESCs. (A) Intersection of proteins identified in three replicate analyses. (B) Distribution of protein ratios reflecting protein expression changes between SILAC-labeled and unlabeled cells. Lysates were mixed after protein quantitation and processed together prior to high-resolution LC–MS/MS. Ratios associated with potential Matrigel contamination (LAMA1, LAMB1, LAMC1, APCS; derived from ref 83) have been annotated. Note that serum amyloid P-component (APCS) passes the quantitation quality requirements applied only in the combined data set and with a single peptide.

Oct4, and SOX2 as pluripotency markers, as well as SOX17, HCG, and GSC as differentiation markers. SOX17 belongs to the SOX family of DNA-binding transcription factors.[71] It is known to be a key factor in various developmental processes involving the formation of definitive endoderm[72] and mesoderm.[73] It is thus expected that SOX17 is overexpressed in HeLa as compared to pluripotent cell systems. GSC is an endoderm differentiation marker[74] and HCG is a trophectoderm differentiation marker.[75] Both GSC and HCG are expected to have low expression in hESCs,[76] which is consistent with our findings. High levels of HCG expression in HeLa have been reported earlier.[77]

NANOG is a 305 amino acid protein belonging to the homeobox gene family. It has been described as a transcription factor essential for maintaining pluripotency in embryonic stem cells.[78] It is presumed that NANOG, in cooperation with Oct4 and SOX2, maintains equilibrium between monomeric and active dimeric forms that help maintain ES cell self-renewal.[78−80] Being a pluripotency marker, the expression of NANOG is expected to be significantly higher in ES cells than in HeLa. A recent study by Ambady et al.[81] demonstrated that, for HeLa cells, assay sensitivity to NANOG strongly varies depending on the type of antibody used. In their study, Western blot analysis in HeLa and hESC cells using two commercially available antibodies raised against NANOG revealed dramatically different relative ratios in expression of NANOG.

To independently confirm higher levels of NANOG expression in labeled and unlabeled hESCs versus differentiated cells in the current study, we carried out qPCR analysis using differentiated ESCs as a control (see the Supporting Information). qPCR confirmed that NANOG is 40-fold more expressed (based on the RQ values) in differentiated than in nondifferentiated cells (Figure S9, Supporting Information).

Additionally, alkaline phosphatase (AP) activity, thought to indicate the absence of spontaneous differentiation[57] was observed to be equivalent between hESCs grown in mTeSR1-SILAC and standard mTeSR1 [see Figures 5D and S10 (Supporting Information) for results of all experiment replicates]. It is noteworthy that no colony in a state of differentiation was observed under either culture condition, as indicated by colony morphology, marker expression and alkaline phosphatase activity. To reinforce AP results quantitatively, colonies were AP stained and scanned as described before. The mean gray value was evaluated using ImageJ (see Figure S11, Supporting Information).To overcome assay limitations such as variation in background intensity, scanning quality, and matrigel thickness; we grew the ES4 cells in standard and SILAC conditions in the same six-well plate for 96 h.

To further characterize SILAC-labeled hES cells, we assessed their capacity to undirectedly differentiate into monolayer embryonic bodies (EB) as a measure of retained in vitro differentiation potential. Following differentiation for 4 weeks under standard differentiation conditions, cells were fixed and assayed for markers of ectoderm, endoderm, and mesoderm by immunocytochemistry for TUJ1, AFP, and αSMA, respectively (Figure S12, Supporting Information). Formation of all three germ layers was verified, demonstrating maintenance of differentiation potential through SILAC-compatible hESC culture.

3.6. Comparative Quantitative Proteomics of SILAC-Labeled hESCs

We next conducted global MS-based proteomics analyses to qualitatively and quantitatively compare the proteomes of SILAC-labeled hESCs and the unlabeled cells grown in standard mTeSR1. The goal was to survey whether substantial changes were introduced into the proteome by the SILAC labeling procedure of human ES cells.

In a first experiment, equal protein amounts from urea/thiourea generated whole cell lysates of unlabeled cells grown in conventional mTeSR1 and corresponding Lys8/Arg10-labeled hESCs were mixed. Following in-solution digestion, samples were subjected to high-resolution LC–MS/MS analyses using a Q Exactive mass spectrometer, and the raw data were analyzed using MaxQuant. The analysis yielded more than 2000 distinct hESCs protein groups, at a false discovery rate (FDR) of 1% as estimated by reverse database searching,[82] of which more than 85% were quantified. Protein ratios measured were clustered tightly around the expected 1:1 value, and the ratio distribution indicated no major changes in the SILAC-labeled hESCs proteome compared to the mTeSR1-hESCs proteome with respect to protein expression levels (Figure 6).

Out of 2114 identified protein groups, 269 were significantly differentially expressed in at least one biological replicate (see Supplementary Table 1, Supporting Information). However,

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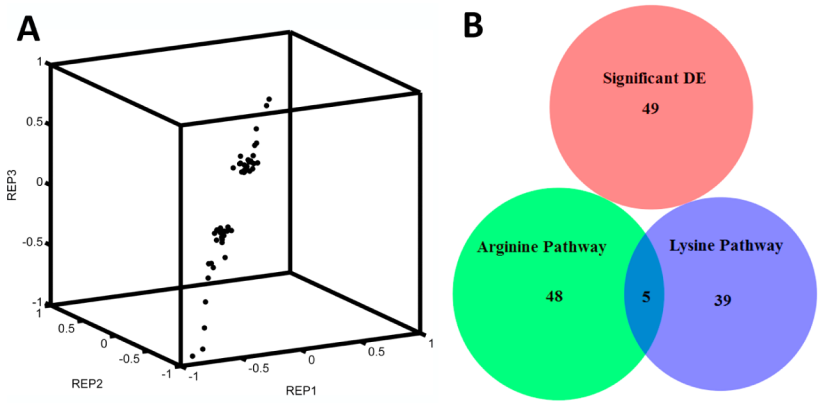

Figure 7. Analysis of differential expression. Out of 2214 protein groups identified by mass spectrometry, 49 were significantly differentially expressed (SDE) at a p value <0.05, found in all three biological replicates. (A) 3D scatter plot showing good quantitative agreement among the set of 49 proteins repeatedly SDE within three biological replicates (REP1−3). The ratios are presented in log-scale. (B) The set of 49 SDE protein groups did not overlap with genes involved in either lysine or arginine metabolism.

only 49 out of those 269 protein groups were significantly differentially expressed (SDE) in all three replicates. We focus subsequent analysis on the set of 49 reproducible SDE protein groups (Figure 6), for which we found excellent quantitative agreement (the first principal component accounts for 98.9% of the total variance) among the replicates (Figure 7A). The comparatively small fraction of reproducibly SDE proteins (49 out of 269) suggests that biological noise may be a dominant source of differential expression.

We next analyzed the set of 49 proteins with manual inspection, functional classification using DAVID,[59] and pathway analysis using Ingenuity Pathway. On the basis of a deep proteomic analysis of Matrigel[83] and with the help of a homology annotation provided by the Ensembl project (http://www.ensembl.org) for the mapping between murine IPI and human UniProt protein identifications, 12 of the 49 SDE proteins with small reproducible ratios ranging from 0.15 to 0.56 (over-representation of the "light" SILAC partner) were classified as Matrigel-derived. Manual inspection of the remaining group led to categorization of one further SDE protein as likely stemming from Matrigel contamination (see Supplementary Table 2, Supporting Information), leaving a group of 36 SDE proteins for further analysis. We next specifically checked if our labeling method, using 25% arginine and lysine, resulted in changes in proteins involved in arginine or lysine metabolism. Proteins involved in arginine or lysine metabolism were obtained from DAVID (see Supplementary Table 3, Supporting Information). We did not find any gene product involved in arginine/proline and lysine metabolisms neither within the 36 likely Matrigel-unrelated candidate SDE protein groups nor the unfiltered superset (Figure 7B). We concluded that the reduction of arginine and lysine under labeling conditions does not result in any significant effects on arginine or lysine metabolism. This is consistent with the comparable growth rates and levels of arginine to proline conversion observed between cells grown in conventional mTeSR1 and SILAC-mTeSR1 culture (Figures 1 and 2). Due to the small number of protein groups involved, global enrichment analysis is unlikely to succeed, and accordingly, neither DAVID's functional classification (see Supplementary Table 5, Supporting Information) nor Ingenuity Pathway Analysis (see Supplementary Table 6, Supporting Information) yielded significantly enriched pathways linked to cellular growth, proliferation, or stemness. Manual inspection, however, reveals that three protein groups upregulated under reduced arginine and lysine concentration are known to be involved in pluripotency and its maintenance: RIF1,[84] SALL4,[85] and UTF1,[86,87] with SILAC ratio averages of 1.69 ± 0.14, 1.66 ± 0.27, and 2.44 ± 0.25. While both RIF1[84] and SALL4[85] have been reported to interact, respectively cooperate with Oct4 and NANOG in transcriptional regulation of many pluripotency-related genes and maintenance of stemness, UTF1 is described as a transcriptional coactivator to Oct4 and SOX2 and has been shown to be required for fine-tuning of stemcell-specific bivalency, keeping key developmental genes poised for expression yet unexpressed.[88,89] The upregulation of this cluster of pluripotency-related gene products under conditions of reduced arginine and lysine invites speculation whether the SILAC-compatible culture conditions may actually strengthen maintenance of stemness. While prior findings that link amino acid homeostasis with maintenance of pluripotency and differentiation[90] may support this hypothesis, following up on it is out of the scope of this study, which uses a wide array of biological readouts to establish the functional equivalency between hESCs mTeSR1-cultured under standard and SILAC-compatible conditions.

4. CONCLUSION

On the basis of advances in culturing technology for pluripotent cells under feeder-free as well as chemically defined conditions, we have established a system for metabolic (SILAC) labeling of human embryonic stem cells in a modified version of the widely published and commonly used mTeSR1 medium. Using 25% of the arginine and lysine concentrations provided by the standard medium, essentially complete labeling was observed after 96 h of culture without any obvious reduction in proliferative capacity as compared to the default medium. Neither immunocytochemistry nor alkaline phosphatase activity assays nor a commercial antibody array probing for markers of maintained pluripotency and differentiation produced any apparent differences between hESCs grown in standard mTeSR1 and under SILAC labeling with reduced concentrations of heavy amino acids. Labeled hESCs also did not display any apparent reduction in undirected differentiation potential. Comparative quantitative proteomics analysis of hESCs grown in default mTeSR1 vs hESCs grown under the established metabolic labeling conditions reaffirmed the

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absence of large proteomic differences as well as of indicators of arginine or lysine starvation. On the basis of the upregulation of RIF1, SALL4, and UTF1 in SILAC-labeled hESCs, culture at reduced arginine and lysine concentrations may even stabilize the pluripotent state.

As reported previously,[63] metabolic arginine to proline conversion was observed to be significant in the SILAC-labeled hESCs, accounting for an estimated 10% of the intensity of the dominant isotopologue of isotope clusters representative of labeled, proline-containing peptides. When using heavy-labeled cells as an actual experimental condition, this conversion rate may significantly impact comparative quantitation of proline-containing peptides and further protocol optimization, such as the addition of unlabeled proline to further kinetically inhibit the conversion,[49] or further, lysine-independent down titration of the arginine concentration used may be necessary. Quantitation interference is, however, negligible when using labeled hESCs as a highly complex biogenic internal standard in cells raised in standard (and light) culture rather than as a bona fide experimental condition.

In conclusion, the present study establishes a well-characterized platform for SILAC labeling of human embryonic stem cells under chemically defined conditions. As the platform is based on the popular mTeSR1 medium, quantitative proteomic studies using cells cultured according to our protocol as a highly complex and biogenic internal standard that covers most, if not all, proteins and modifications thereof are straightforward for many laboratories involved in stem cell research.

Proteomics and in particular SILAC-based quantitative analyses have already contributed significantly to the research in pluripotent cell models.[10,91−96] Adaptation of state of the art feeder-free and chemically defined culture media such as mTeSR1 and others[8,49] to metabolic labeling will make this powerful tool available to the wider research community and enable further insight into the nature of pluripotency, as well as into differentiation pathways and protocols, necessary to work toward future applications in regenerative medicine.[97]

■ ASSOCIATED CONTENT

Supporting Information

Figures S1−S12, Supplementary Tables 1−6, the R package, and Supplementarty Methods, as noted in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

SILAC, stable isotope labeling with amino acids in cell culture; hESCs, human embryonic stem cells; MEFs, mouse embryonic fibroblasts; MEF-CM, mouse embryonic fibroblast-conditioned medium; ROCK, Rho-associated kinase; iPSCs, induced pluripotent stem cells; Arg, arginine; Lys, lysine; AP, alkaline phosphatase; SDE, significantly differentially expressed; IPA, Ingenuity Pathway Analysis; PFA, paraformaldehyde; LDS, lithium dodecyl sulfate; EB, embrionic body; rt, room temperature.

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