RESEARCH ARTICLE

Assessing the impact of minimizing arginine conversion in fully defined SILAC culture medium in human embryonic stem cells

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We present a fully defined culture system (adapted Essential8™ [E8™] medium in combination with vitronectin) for human embryonic stem cells that can be used for SILAC purposes. Although a complete incorporation of the labels was observed after 4 days in culture, over 90% of precursors showed at least 10% conversion. To reduce this arginine conversion, E8™ medium was modified by adding (1) L-proline, (2) L-ornithine, (3) N^{ω}-hydroxy-nor-L-arginine acetate, or by (4) lowering the arginine concentration. Reduction of arginine conversion was best obtained by adding 5 mM L-ornithine, followed by 3.5 mM L-proline and by lowering the arginine concentration in the medium to 99.5 μM. No major changes in pluripotency and cell amount could be observed for the adapted E8™ media with ornithine and proline. However, our subsequent ion mobility assisted data-independent acquisition (high-definition MS) proteome analysis cautions for ongoing changes in the proteome when aiming at longer term suppression of arginine conversion.

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1 Introduction

SILAC, developed in 2002 by the laboratory of M. Mann, is used to quantify differences in protein abundance between two cell culture conditions by means of incorporating stable isotopically labeled or "heavy" amino acid(s) (AA) in a culture (e.g., $^{13}C_6$ lysine in one culture and $^{12}C_6$ lysine (K) in the other) [1]. $^{13}C_6$ lysine and $^{13}C_6$ arginine are commonly used as heavy AA in order to quantify every peptide during MS/MS since most digests are performed with trypsin [2, 3].

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Abbreviations: **AA**, amino acid; **DDA**, data-directed acquisition; **E8™ medium**, Essential8™ medium; **FC**, flow cytometry; **FM**, fluorescence microscopy; **HDMS^E**, high-definition MS; **hESC**, human embryonic stem cells; **NO**, nitric oxide; **Nor-NOHA**, N^{ω}-hydroxy-nor-L-arginine

*Both the authors contributed equally to this work.
Colour Online: See the article online to view Fig. 1 in colour.

Significance of the study

SILAC is an invaluable label-based quantitative proteomics approach. The fact that biological samples can be merged at the onset of the pipeline allows for indefinite extension of the downstream sample handling without any increase in technical variation. In this perspective, it can be seen as the exact opposite of label-free approaches. However, the heavy amino acids used can be metabolically converted in living cells or organisms and the degree to which this occurs depends on the proteome of the model under investigation. Here, we show that human embryonic stem cells (hESC) are specifically prone to such conversion of arginine, resulting in uninformative and interfering peaks at, for example, 5 Da for peptide sequences containing proline and glutamic acid. We compared both previously reported and a new way to reduce this conversion, and for the first time also investigate the impact of this counter measure on the proteome of these cells. As can be expected, hESC are very sensitive to such interventions. Together, we conclude that SILAC might not be the best method for longer term quantitative proteomics studies of hESC and we propose to include a full proteome screening when trying to intervene with SILAC metabolic conversion in all models.

Importantly, incomplete incorporation of heavy AA can lead to skewed light-over-heavy ratios. For most cell types, five population doublings are therefore performed for near-complete incorporation (~97%) of the heavy AA into the cell proteome [3].

A second problem is the metabolic conversion of AA during SILAC experiments, resulting in incorrect light-over-heavy ratios. Metabolic conversion of L-arginine (R) to L-proline (P) and, to lesser extent, L-glutamate (E) generates (an) extra peak(s) of +5 Da per heavy P or E in the peptide in the MS spectrum [2]. Especially fast metabolizing cell types such as human embryonic stem cells (hESC) suffer greatly from this arginine conversion problem [4]. Arginine titration as well as a proline titration can be used to reduce this arginine conversion [3, 4]. In hESC cultures, however, lowering the arginine concentration can induce differentiation and cell death [4]. For this reason, P titration was used as a solution to overcome this problem, since the addition of P can theoretically reduce its formation out of R [4]. However, these reports used serum in their media, making the culture conditions intrinsically undefined [4–6]. Additionally, the impact of these approaches on pluripotency or the rest of the proteome was not assessed. To date, no fully defined culture medium for SILAC application is available for hESC, which makes it difficult for laboratories to use SILAC directly out of literature for their own applications. Recently, Essential8™ (E8™) medium in combination with vitronectin was developed for culturing hESC in a full xeno-free and defined way [7]. Besides arginine or proline titration to prevent arginine conversion, analysis of the pathway mediating the conversion from R to P and E (Fig. 1), indicates that arginine conversion could also be inhibited by the addition of L-ornithine to the media. Another solution is to inhibit arginase, an enzyme that is responsible for the conversion of arginine to ornithine. Arginase can be inhibited by N^{ω}-hydroxy-nor-L-arginine (Nor-NOHA) [14]. Nor-NOHA works as a competitive inhibitor of arginase at physiological pH and is around 40 times more potent in inhibiting arginase than the endogenous formed N^{ω}-hydroxy-L-arginine in murine macrophages [16, 17]. This inhibition of arginase theoretically blocks the conversion of L-arginine to L-ornithine. L-Ornithine is among others a precursor of polyamines needed for cell proliferation in a growing fetus [17].

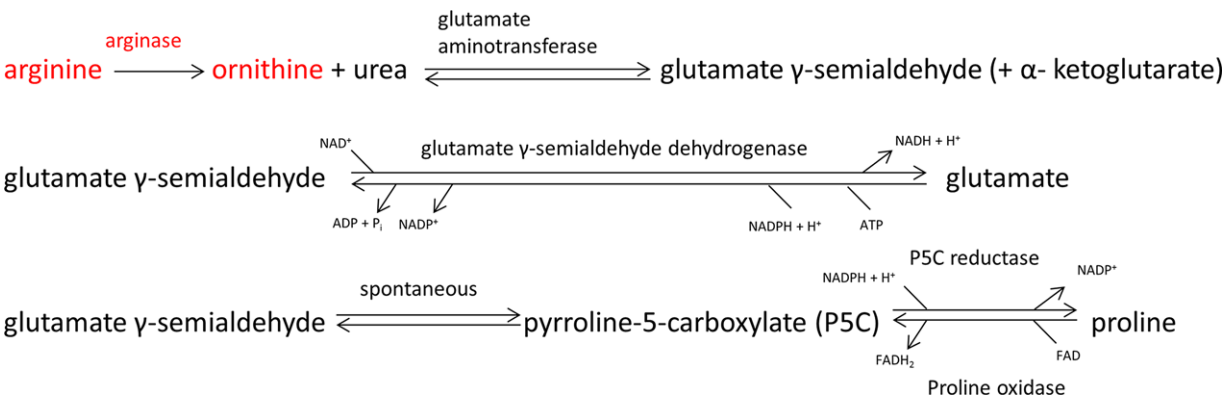

Figure 1. Arginine conversion (based on [15]). L-arginine is converted to L-proline and L-glutamate by several intermediate steps. The steps in which we intervene for inhibiting the arginine conversion are indicated in the figure as bold red.

Here, we present the comparison between these different arginine conversion reducing strategies on hESC in a fully controlled experimental pipeline. As stem cells are extremely sensitive to changes in their surroundings, we monitor pluripotency and general proteome changes following the different treatments intended to minimize conversion of heavy arginine. For this, an OCT4–eGFP Knock-In hESC line (WiCell Research Institute, Madison, WI, USA) was used, and pluripotency was monitored by means of flow cytometry (FC) and fluorescence microscopy (FM) [8]. To reduce the conversion, a completely defined culture for SILAC was supplemented by, respectively, adding different concentrations of L-proline, L-ornithine, or Nor-NOHA acetate to the E8 culture medium, or by lowering the L-arginine concentration in this medium. Within the time span of the experiment (4 days, complete heavy label incorporation), only the addition of L-ornithine and L-proline had no impact on OCT4 expression and cell number. The highest reduction in arginine conversion was found in culture media with 5 mM ornithine, 3.5 mM proline, or 99.5 μM arginine. Finally, the effect on total proteome of the two best culture conditions that had no effect on cell number and pluripotency and had the highest reduction in arginine conversion (5 mM ornithine, 3.5 mM proline) was analyzed using a label-free HDMS^E analysis. Even at 4 days of culturing, however, the culture conditions already had a detectable effect on the proteome. As we anticipate that these minor differences might well exacerbate over longer time periods, we here caution for premature adaptation of protocols that reduce arginine conversion in such sensitive cells as hESC.

2 Materials and methods

2.1 Experimental design

Incorporation was assessed by triplicate harvest at days 3–4–5 after growth in heavy medium. Four different strategies for reducing metabolic conversion of arginine were tested in triplicate on OCT4–eGFP Knock cells that allow the use of FC as well as FM to monitor cell (and culture) pluripotency. Full proteome screening was done on triplicate harvest of cells grown in the presence of the compounds best attenuating the arginine conversion. All products were purchased from Life Technologies (Carlsbad, CA, USA) unless stated otherwise.

2.2 Feeder-free culture of hESC

WA01 OCT4–eGFP Knock-In hESC (WiCell Research Institute) were plated on a precoated xeno-free vitronectin (VN XF, Primorigen Biosciences, Madison, WI, USA) 6-well plate (coating concentration = 0.5 μg/cm^2) and cultured in E8^{TM} medium (37°C, 5% CO_2, and 5% O_2). E8^{TM} medium was made by diluting E8^{TM} 50× supplement 1:50 with "arginine and lysine-free" DMEM/F12 (Thermo Scientific, Rockford, IL, USA) supplemented with 398 μM L-arginine HCl and 499 μM L-lysine HCl (both from Sigma-Aldrich, St. Louis, MO, USA). Splitting was performed every 4–5 days with 0.5 mM ethylenediaminetetraacetic acid in Dulbecco's PBS according to the manufacturer's protocol of culturing hESC in E8^{TM} medium.

2.3 SILAC labeling of hESC and culture conditions

2.3.1 Incorporation of heavy labels into the proteome

For labeling, E8^{TM} medium was supplemented with $^{13}C_6$ lysine and $^{13}C_6$ arginine (both from Thermo Scientific) in the same concentrations as the light variant. hESC were harvested after 4, 5, or 6 days in culture (three, four, or five population doublings in which a population doubling is defined as a doubling of the amount of cells between two consecutive days) to examine the time needed for a full incorporation of heavy labels. No splitting was performed during the time of the experiment. Media were changed daily. The experiment was performed in triplicate.

2.3.2 Inhibition of the arginine conversion to P and E

The composition of the heavy labeled E8^{TM} medium was changed to examine the effect on inhibition of arginine conversion. Four different culture conditions were examined: (1) addition of L-proline (3.5–6.9–10.4–13.9 mM; Sigma-Aldrich), (2) L-ornithine HCl (0.05–0.5–1–2–5 mM; Sigma-Aldrich), (3) Nor-NOHA acetate (50–100 μM; Enzo Life Sciences, Farmingdale, NY, USA), and (4) L-arginine concentration was decreased from 398 μM (concentration in no adapted E8 medium) to, respectively, 199 and 99.5 μM. hESC grown in heavy isotopically labeled E8^{TM} medium were used as a control for all SILAC-related experiments. The experiment was done in triplicate for FC analysis and a fourth well was used for FM analysis to assess colony morphology (Section 2.4).

2.4 OCT4 expression and cell count

The influence of the different conditions on hESC differentiation was examined by analyzing OCT4 expression by means of FM and FC. Daily monitoring of OCT4 expression was assessed by noninvasive monitoring by means of FM. To assess influence of stress, a plate without daily monitoring (FM) but with FC at the end of the experiment was investigated to obtain information about OCT4 expression/cell and cell number as described earlier [8].

2.5 Cell lysis and digest

After culture medium removal, cells were detached with 0.25% trypsin-ethylenediaminetetraacetic acid (4 min, 37°C) and trypsin was subsequently inactivated with trypsin inhibitor (1:1 w/w; Sigma-Aldrich). In total, 10% of the cells were transferred to another Eppendorf for FC analysis. After centrifugation (200 × *g*, 5 min), cells were resuspended in PBS with 1% w/v BSA and kept on ice until FC analysis. After centrifugation (200 × g, 5 min) and a wash step with 1× PBS, remaining cells (90%) were lysed in a protein LoBind Eppendorf containing 250 μL of 500 mM triethylammonium bicarbonate (Sigma-Aldrich) supplemented with 1× Halt protease and phosphatase inhibitors (Thermo Scientific), 125 units benzonase nuclease (Sigma-Aldrich), and 1% w/v sodium deoxycholate (Sigma-Aldrich) as a detergent. The Eppendorf was vortexed (30 s) and subsequently sonicated (10 min, on ice, Transsonic 460, Elma).

Cell lysates were centrifuged for 10 min at 17 968 × *g* and supernatant was further analyzed. Protein content of the supernatant was determined by means of a Coomassie Bradford Assay (standard curve obtained using BSA [0–2000 μg/mL in ten times diluted cell lysate buffer], Thermo Scientific). The cell lysate was digested overnight at 37°C in 500 mM triethylammonium bicarbonate, 1% sodium deoxycholate (w/v), 1 mM $CaCl_2$, 5% ACN v/v, and trypsin/lysC (25:1 protein:enzyme ratio w/w; Promega, Madison, WI, USA) after reduction with 10 mM dithiotreitol for 60 min at 60°C and blocking with 10 mM methyl methanosulfonate for 10 min at room temperature. Sodium deoxycholate was subsequently removed by means of acid precipitation. Detailed information about this method is described in [9].

2.6 LC-MS/MS

After vacuum drying in a Centrivap®, peptides were dissolved in H_2O with 0.1% v/v formic acid. A trapped HPLC system, Dionex Ultimate 3000 (Thermo Scientific), was used to separate the peptides (1 μg loaded) on an Acclaim PepMap 100® C18 column (75 μm × 25 cm; Thermo Scientific) at a flow rate of 300 nL/min. The LC gradient used for elution was obtained by a combination of mobile phase A (H_2O + 0.1% v/v formic acid) and mobile phase B (80% v/v ACN + 0.1% v/v formic acid): 4–100% B in 66 min. Data-directed acquisition (DDA) on a Triple TOF™ 5600 mass spectrometer (Sciex) with a NanoSpray source operating in positive ESI mode was used to assess MS and MS/MS data in dynamic accumulation mode. In short, the scan range for MS ranged from m/z 400 to m/z 1250 with a 250 ms accumulation time. In MS/MS, a scan range from m/z 65 to m/z 2000 with a minimum of 25 ms accumulation time was used. Rolling collision energy was used in MS/MS. DDA was triggered for m/z with a charge state from 2+ to 4+ which exceeds 50 cps. Former target ions were excluded for 30 s.

2.7 Data analysis of incorporation and conversion

RAW DDA data (wiff files) were loaded into MASCOT Distiller (Matrix Science) and processed. A MASCOT search was subsequently performed with the following parameters: enzyme specificity was set to trypsin with maximum two missed cleavages. Methylthio (on cysteine) was used as fixed modification and deamidation (on asparagine and/or glutamine) and oxidation (on methionine) as variable modifications. The precursor tolerance was set to 20 ppm and the MS/MS tolerance to 0.1 Da. The human database from Swiss-Prot was used (downloaded in November 2015, 20 210 sequences). Identification was considered positive with a p-value < 0.05. After identification, incorporation rate was determined by analysis of the L/H ratio. This ratio was determined by defining the light component as a peptide having a $^{12}C_6$ arginine or/and $^{12}C_6$ lysine and the heavy component as a peptide having a $^{13}C_6$ arginine or/and $^{13}C_6$ lysine. In addition, the conversion of heavy arginine to heavy proline is taken under consideration by defining the heavy proline as a satellite modification group. The ratio was accepted by Distiller by applying thresholds to two measurements: correlation (threshold = 0.9) and fraction (threshold = 0.5).

2.8 LC-HDMS^E as label-free method to analyze the effect of the conditions on the proteome

To study the effect of these different treatments on the proteome, a label-free HDMS^E was used on nonlabeled cells. To validate the impact of treatment on the proteomes, culture media were supplemented with either no additive or 5 mM ornithine, 3.5 mM proline, or 99.5 μM arginine. Cell lysis and digestion was performed as described above. After digestion, dried peptides were dissolved in H_2O with 0.1% v/v formic acid. Peptides (100 ng loaded) were separated on a NanoACQUITY system (Waters Corp., Manchester, UK) with direct injection on a NanoACQUITY column (UPLC® 1.7 μm BEH130 100 μm × 100 mm C18) at a flow rate of 300 nL/min. The column temperature was maintained at 35°C. The LC-gradient (1–40% B in 60 min followed by 7 min on 85% B) was obtained by a combination of mobile phase A (H_2O + 0.1% v/v formic acid + 3% v/v dimethyl sulfoxide) and mobile phase B (ACN + 0.1% formic acid). All samples were analyzed by high-definition MS (HDMS^E) with an in-house optimized collision energy look up table (ultradefinition MS) on a Synapt G2Si instrument (Waters Corporation) [10]. The sample list was randomized and interspersed with QC samples to assess system performance.

Uniform optimal processing parameters (low energy, high energy, intensity) for HDMS^E analysis were first determined with Protein Lynx Global Server Treshold Inspector and the data were subsequently analyzed with Progenesis 2.0 software (Nonlinear Dynamics, Waters).

Peak picking was performed and data were filtered by charge state (only 2–4+ MS precursors were used for

analysis). Next, normalization was performed to all proteins. After processing, the data were searched against a human databank (Swiss-Prot, downloaded in November 2015, 20 210 sequences) with methylthio (on cysteine) as fixed modification and deamidation (on asparagine and/or glutamine) and oxidation (on methionine) as variable modifications. The enzyme specificity was set to trypsin, with maximum one missed cleavage. False discovery rate was set to 1% at the protein level, corresponding to a $HDMS^E$ score threshold in our search environment of ±5.4. For identification of $HDMS^E$ data, Progenesis 2.0 relies on the Protein Lynx Global Server ion accounting algorithm, which is intrinsically dependent on decoy hits during the search for the above-mentioned FDR estimation.

After removal of any outliers in the replicas by means of Multivariate Statistics (Principal Component Analysis, Progenesis software), proteins with a minimum of two peptides from which at least one was unique were kept for analysis. A p-value ≤ 0.05 was used to identify proteins that were significantly different between control and condition (L-proline/L-arginine or L-ornithine). These proteins were exported and were further analyzed with Reactome wherein only those pathways were considered in which all proteins (with a minimum of 3) were consistently up- or downregulated [11, 12]. The MS proteomics data have been deposited to the ProteomeXchange Consortium [13] via the PRIDE partner repository with the dataset identifier PXD002859.

3 Results and discussion

3.1 Incorporation and conversion of heavy labels into the proteome

Before any SILAC analysis can be performed, it is not only recommended to check the incorporation of the heavy labels into the proteome, but equally to verify the occurrence of any conversion of the heavy label to another AA (e.g., L-arginine conversion to L-proline/L-glutamate) [3]. While some corrective data analysis tools are available, full incorporation without conversion remains the preferred point of departure to obtain correct quantitative information. WA01 OCT4–eGFP Knock-In hESC grown in $E8^{TM}$ medium were therefore analyzed after 4, 5, or 6 days in culture (three, four, or five population doublings). Importantly, no measurable effect on OCT4 expression was observed when using heavy arginine in comparison to light arginine. Cell number analysis showed a population doubling rate of 24 h between two consecutive days (*data not shown*).

Full incorporation was attained, as seen by the MASCOT Distiller Quantitation Toolbox on the roughly 3000 identified peptides wherein the light peptide was consistently $\leq 5\%$ of the total peak area (heavy + light). Of note, some of the completely light peptides (2% in total) were identified as keratins, which are most probably contaminants from sample preparation.

With complete incorporation being achieved, arginine conversion was investigated as second possible bottleneck when using SILAC. Arginine conversion can be quantified and over 90% of all annotated precursors suffered an R to P or E conversion of at least 10% of the total MS intensity of that peptide, confirming that arginine conversion in the hESC line is a major issue (Fig. 3), while these peak pairs were practically absent in samples not containing heavy AAs. Importantly, around 50% of all tryptic peptides (between 700 and 6000 Da) contain at least one proline according to the human database of the international protein index [4]. For this reason, it is important to inhibit this arginine conversion.

3.2 Effect of the different conditions on OCT4 expression and cell number

Because hESC are known to start differentiating spontaneously upon changes to their culture environment, the different conditions (L-proline/L-ornithine/Nor-NOHA acetate/L-arginine) were first analyzed for their effect on the differentiation status of the hESC by means of an optimized screening method using FM and FC (Fig. 2) [8]. A low eGFP expression (fluorescence signal $<10^1$), correlating to a low OCT4 expression, indicates differentiation. When using FM, both colony shape and fluorescence signal/surface unit can be measured in a noninvasive manner. This can give important additional information on the impact of an experiment on hESC. In such experiments, the overall fluorescence of the colonies increases with time when no differentiation is induced, as we described earlier (Fig. 2A) [8]. Normal $E8^{TM}$ medium was used as a control. No decrease in OCT4 expression or change in colony shape was observed with FM in all tested conditions within the analyzed time period of 4 days needed for complete AA incorporation. For FC, only a small but insignificant loss in eGFP signal could be seen (OCT4 expression) at L-proline concentrations above 10.4 mM (Fig. 2B).

When assessing the effect of different treatments on cell number (Fig. 3C), addition of proline or ornithine to the media displayed no significant effect on cell growth. Second, when 50 or 100 μM Nor-NOHA acetate was added, a positive effect on cell growth could be observed (p-value ≤ 0.05). The latter can be explained by the fact that inhibition of arginase would theoretically lead to an increase in arginine. Deamidation of arginine by nitric oxide (NO) synthase will form NO. Dependent on the available concentration of NO, NO promotes cell proliferation (picomolar and nanomolar) or induces cell arrest (micromolar) [18]. The cell proliferation as observed in our experiment can therefore be explained if NO is indeed present in low concentrations. Of note, a higher cell amount with Nor-NOHA acetate was also reported in neural stem cells [19]. When decreasing the arginine concentration, complete loss of cells could occasionally be observed, making this an unreliable method. These observations are in line with previous reports [4]. In Fig. 2C, only the results from

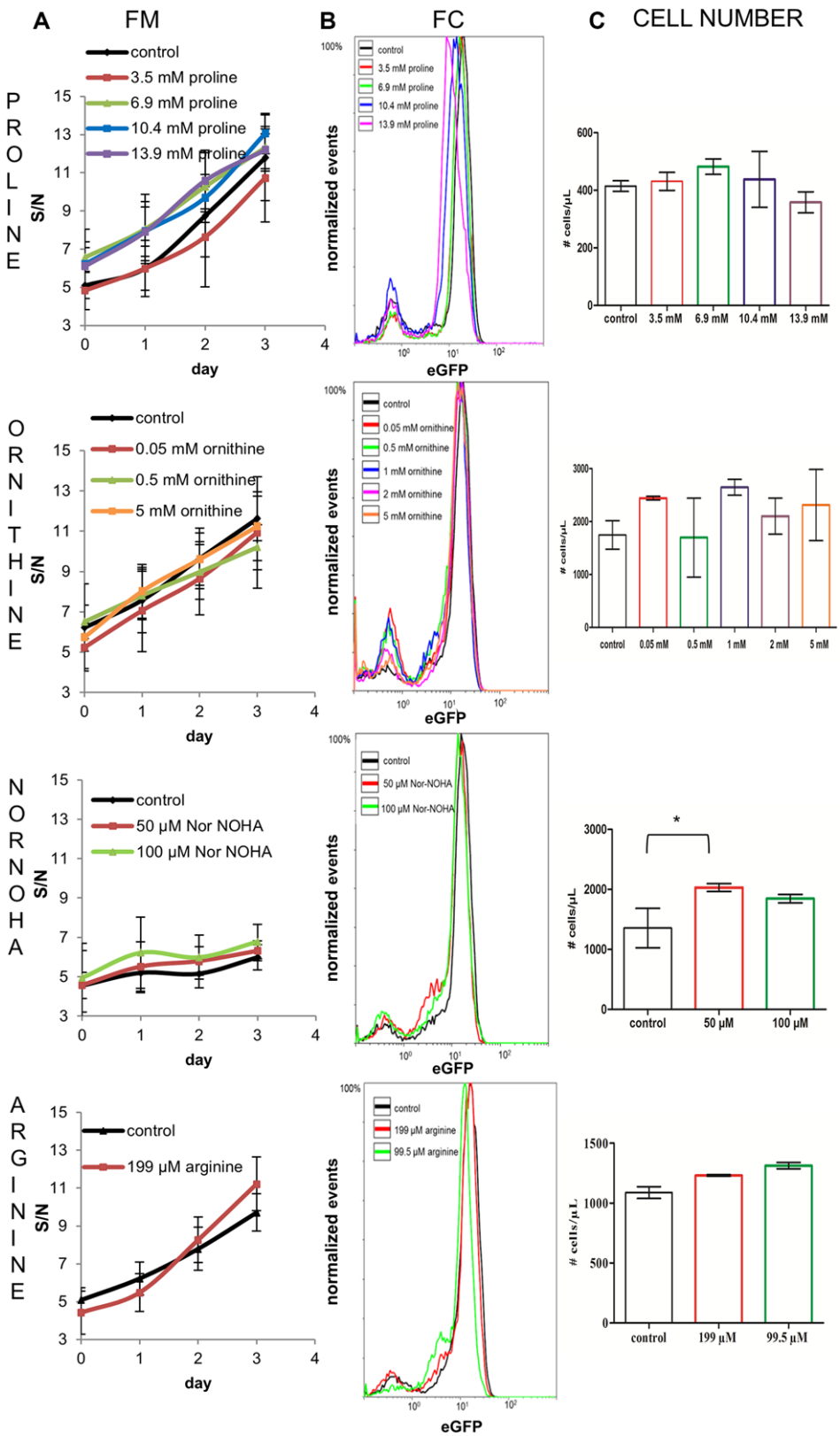

Figure 2. Analysis of the different conditions (L-arginine/L-proline/L-ornithine/Nor-NOHA) on OCT4 expression (FM [A] and FC [B]) and cell number (C). (A) FM results of the different conditions. The *y*-axis represents the mean of the fluorescence (eGFP signal) of five hESC colonies/surface unit (= background; S/Ns); the *x*-axis represents the day of the experiment. A loss in eGFP expression and thus of S/N indicates loss of pluripotency. Because of the 3D growth of hESC colonies, an increase in S/N is observed during the time of the experiment in undifferentiated colonies as described in Scheerlinck et al. [8]. In none of the conditions, significant loss in eGFP was observed. (B) FC results. At the last day of the experiment (day 4), a part of the cell was analyzed with FC in addition to FM analysis to confirm FM results and to obtain information concerning cell amount. The expression of eGFP signal in all tested conditions was measured (a minimum of 10 000 events was analyzed). No significant loss of eGFP in any of the conditions was observed in comparison with the control, confirming FM results. (C) Cell number results (FC, day 4). The amount of cells per microliter (*y*-axis) was determined by adding a known number of fluorescent beads as spike-in to the FC samples. Only the addition of Nor-NOHA induced a significant increase in cell number. Inconsistent results were observed when arginine concentration was decreased. The asterisk depicts a *p*-value ≤ 0.05 (by unpaired *t*-test).

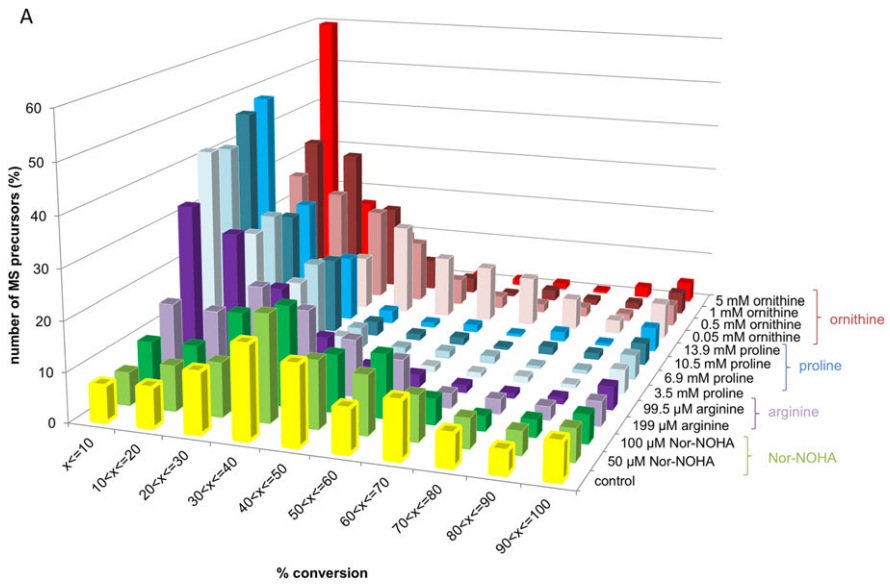

Figure 3. Overview of the impact of the different conditions on arginine conversion. The graph represents the relative number of MS precursors (*y*-axis) with *x*% conversion. The percentage conversion is defined as the intensity ratio of coeluting pairs of precursors (defined as a satellite modification group). Conversion was most inhibited by means of 5 mM ornithine (pink-red bars, 56.85% of all MS precursors with ≤10% arginine conversion), followed by addition of proline (blue bars, 40–45% of all MS precursors) and 99.5 μM L-arginine (purple bars, 33.30% of all MS precursors). In total, 50–100 μM Nor-NOHA acetate (green bars) has no effect on inhibition of the arginine conversion as compared to the control (yellow). (Control = E8 medium in combination with vitronectin.) Supporting Information Fig. 2 shows the conversion rates of peptides containing more than one proline in their sequence.

experiments when no complete cell loss was observed are depicted. In conclusion, the addition of proline and ornithine are the most favorable options to reduce arginine conversion during a SILAC experiment since no significant effect on pluripotency and cell number could be observed in these two conditions.

3.3 Inhibition of the arginine conversion

Next, all conditions were assessed for their ability to inhibit the arginine conversion by defining the heavy proline as a satellite modification group. A frequency plot (Fig. 3) of all MS precursors was made. Arginine conversion was most reduced by 5 mM L-ornithine (56.85% of all MS precursors with ≤10% arginine conversion), followed by L-proline (40–45% of all MS precursors) and 99.5 μM L-arginine (33.30% of all MS precursors). Surprisingly, Nor-NOHA acetate has no effect on inhibition of the arginine conversion. Although spectral counting of identified spectra could be considered less accurate, the reduction in arginine conversion is so intense that the trend is reflected even at the level of the number of identified spectra (Supporting Information Fig. 1).

3.4 After 4 days changes in the proteome are apparent in different treatments

With 5 mM L-ornithine and 3.5 mM L-proline showing no impact on cell proliferation or pluripotency, but with great capacity to reduce the conversion, these conditions were then analyzed for their impact on the total proteome. As conversion of the SILAC label is under study, SILAC itself cannot be used for this purpose. Thus, HDMS^E was used as a label-free quantification technique to compare the unlabeled proteomes of untreated cells with those of cells grown in 5 mM L-ornithine or 3.5 mM L-proline.

Next, significantly different proteins (p-value ≤ 0.05) between control (no change to the medium) and condition (addition of either ornithine or proline concentration) were isolated from Progenesis QI 2.0. Only proteins identified with a minimum of two peptides of which at least one peptide was unique for quantitation were retained (1096 proteins—54.58% of all identified proteins). Finally, significant different proteins (p-value ≤ 0.05) between control and condition were analyzed. Relatively small differences were observed as follows: 82 of 188 proteins (7.48/17.15% of all quantifiable proteins [minimum two peptides of which one unique]) were significantly different between L-ornithine/L-proline and control, respectively. In depth analysis of these potentially different proteins and pathway(s) was performed with Reactome. No obvious gene ontology changes could be defined, but the upregulation of such abundant proteins as histones (H2B and H4) and tubulins and proteins involved in mRNA splicing in comparison with the control do caution for potentially large-scale changes on the longer term. Downregulated pathways were only found in the condition with L-proline: pathways involved in RNA degradation, cellular response to heat stress, and TGF-β receptor complex signaling were possibly affected. All proteins (with their respective normalized abundance after summed intensity normalization) involved in all above mentioned pathways can be found in Table 1. An overview of all proteins (+ proteins with a minimum of two peptides of which one unique) and of all proteins which are significantly different (p-value ≤ 0.05) between control (E8 medium with no change to the medium) and condition (ornithine, proline) can be found in Supporting Information Table 1.

Together, these early proteome changes caution that reducing the arginine conversion for more accurate quantification using SILAC experiments comes at a cost of potentially

Table 1. Fold-changes of significantly different proteins (p-value ≤ 0.05) in one condition relative to control

Accession number	Name	Normalized abundance (mean ± SD)		
		Proline	Ornithine	Control
Mitose/protein folding/chromatine organization				
P07437	Tubulin beta chain	13.15 ± 0.78	14.08 ± 1.04	**10.50 ± 1.73**
Q13885	Tubulin beta-2A chain	7.53 ± 0.52	7.75 ± 0.35	6.27 ± 0.77
Q9BVA1	Tubulin beta-2B chain	9.36 ± 0.74	9.04 ± 0.79	7.25 ± 0.68
P04350	Tubulin beta-4A chain	0.40 ± 0.16	**0.47 ± 0.28**	**0.24 ± 0.05**
Q9BUF5	Tubulin beta-6 chain	**0.44 ± 0.05**	0.47 ± 0.07	**0.38 ± 0.04**
P62805	Histone H4	37.31 ± 2.45	**33.22 ± 3.44**	**25.56 ± 4.85**
P33778	Histone H2B type 1-B	7.26 ± 0.82	7.79 ± 0.68	5.70 ± 0.74
P42677	40S ribosomal protein S27	3.61 ± 0.23	**3.33 ± 0.37**	**3.06 ± 0.27**
P06493	Cyclin-dependent kinase 1	3.39 ± 0.28	**3.19 ± 0.31**	**2.89 ± 0.20**
P30153	Serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A alpha isoform	1.72 ± 0.16	1.70 ± 0.16	**1.51 ± 0.09**
P20618	Proteasome subunit beta type-1	**1.94 ± 0.44**	**1.93 ± 0.37**	**1.50 ± 0.26**
Q5BJF6	Outer dense fiber protein 2	0.80 ± 0.19	**0.94 ± 0.10**	**1.04 ± 0.12**
P61981	14-3-3 protein gamma	4.05 ± 0.47	**4.17 ± 1.45**	**4.91 ± 0.45**
Q9UNM6	26S proteasome non-ATPase regulatory subunit 13	4.22 ± 0.34	**4.74 ± 0.93**	**5.19 ± 0.52**
P25786	Proteasome subunit alpha type-1	1.74 ± 0.15	**1.97 ± 0.43**	**2.03 ± 0.14**
Membrane trafficking				
Q13885	Tubulin beta-2A chain	7.53 ± 0.52	7.75 ± 0.35	6.27 ± 0.77
Q9BVA1	Tubulin beta-2B chain	9.36 ± 0.74	9.04 ± 0.79	7.25 ± 0.68
P62158	Calmodulin	**0.30 ± 0.07**	**0.27 ± 0.17**	**0.19 ± 0.12**
Q9NP79	Vacuolar protein sorting associated protein VTA1 homolog	**0.70 ± 0.15**	**0.95 ± 0.09**	**0.79 ± 0.14**
Q8NBS9	Thioredoxin domain-containing protein 5	**0.95 ± 0.25**	1.24 ± 0.09	**0.98 ± 0.11**
Q9BUF5	Tubulin beta-6 chain	**0.44 ± 0.05**	0.47 ± 0.07	**0.38 ± 0.04**
Pyrroline-5-carboxylate reductase 1				
P32322	Pyrroline-5-carboxylate reductase 1; mitochondrial	**0.24 ± 0.08**	**0.21 ± 0.05**	**0.16 ± 0.10**
RNA splicing				
P22626	Heterogeneous nuclear ribonucleoprotein A2/B1	29.74 ± 5.82	28.67 ± 4.31	22.09 ± 2.73
P51991	Heterogeneous nuclear ribonucleoprotein A3	6.17 ± 0.35	5.38 ± 1.04	3.81 ± 0.36
Q13242	Serine/arginine-rich splicing factor 9	0.92 ± 0.07	0.93 ± 0.04	**0.71 ± 0.07**
mRNA stability				
Q15717	ELAV-like protein 1	2.95 ± 0.21	**3.23 ± 0.23**	**3.49 ± 0.45**
Q9UNM6	26S proteasome non-ATPase regulatory subunit 13	4.22 ± 0.34	**4.74 ± 0.93**	**5.19 ± 0.52**
P11940	Polyadenylate-binding protein 1	1.00 ± 0.09	**1.05 ± 0.05**	**1.14 ± 0.09**

Table 1. Continued

Accession number	Name	Normalized abundance (mean ± SD)		
		Proline	Ornithine	Control
Q92945	Far upstream element-binding protein 2	4.70 ± 0.21	4.90 ± 0.64	7.50 ± 1.25
P11142	Heat shock cognate 71 kDa protein	37.83 ± 2.98	**42.69 ± 3.37**	**43.63 ± 1.24**
P25786	Proteasome subunit alpha type-1	1.74 ± 0.15	**1.97 ± 0.43**	**2.03 ± 0.14**
Cellular response to heat stress				
P55072	Transitional endoplasmic reticulum ATPase	5.77 ± 0.49	5.92 ± 0.07	**6.46 ± 0.35**
P11142	Heat shock cognate 71 kDa protein	37.83 ± 2.98	**42.69 ± 3.37**	**43.63 ± 1.24**
Q02790	Peptidyl-prolyl cis–trans isomerase FKBP4	2.98 ± 0.10	3.10 ± 0.21	**3.65 ± 0.38**
TGF beta receptor complex signaling				
P62942	Peptidyl-prolyl cis–trans isomerase FKBP1A	6.02 ± 0.74	**7.03 ± 1.69**	**7.73 ± 0.91**
Q16254	Transcription factor E2F4	10.25 ± 0.99	**10.89 ± 2.35**	**13.84 ± 1.95**
O95405	Zinc finger FYVE domain-containing protein 9	0.68 ± 0.04	**0.97 ± 0.17**	**1.15 ± 0.26**

Data were obtained as follows: first, HDMS^E data were analyzed with Progenesis 2.0 software (Waters). Only the proteins, identified with a minimum of two peptides of which one peptide was unique, were retained for analysis. Subsequently, only the significantly different proteins (p-value ≤ 0.05) in the conditions (proline, arginine, or ornithine) relative to control (no change to the medium) were further analyzed with Reactome to identify potentially up- or downregulated pathways. In the table, the different proteins (with their normalized abundance value) belonging to the up- or downregulated pathways are displayed (relative to control). Normalized abundances of which the p-value between the condition and control (t-test) was greater than 0.05 (insignificant) are displayed in bold.

influencing other pathways in cellular biology. While previous studies have mainly focused on inhibiting the conversion rate itself, we show that monitoring cell growth, pluripotency, and the overall proteome should also be included to verify the cell-wide impact of this intervention.

4 Concluding remarks

In conclusion, a fully defined medium is presented to perform SILAC experiments on hESC. With the smallest direct effects on the proteome, OCT4 expression, and cell number, we suggest to use 5 mM L-ornithine to reduce arginine conversion in hESC. However, we caution that some cellular changes are being induced during such inhibition and that additional controls should be including when aiming at experiments over longer periods.

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5 References

[1] Ong, S. E., Blagoev, B., Kratchmarova, I., Kristensen, D. B. et al., Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics. *Mol. Cell. Proteomics* 2002, *1*, 376–386.

[2] Ong, S. E., Kratchmarova, I., Mann, M., Properties of C-13-substituted arginine in stable isotope labeling by amino acids in cell culture (SILAC). *J. Proteome Res.* 2003, *2*, 173–181.

[3] Ong, S. E., Mann, M., A practical recipe for stable isotope labeling by amino acids in cell culture (SILAC). *Nat. Protoc.* 2006, *1*, 2650–2660.

[4] Bendall, S. C., Hughes, C., Stewart, M. H., Doble, B. et al., Prevention of amino acid conversion in SILAC experiments

with embryonic stem cells. *Mol. Cell. Proteomics* 2008, *7*, 1587–1597.

[5] Hughes, C., Radan, L., Chang, W. Y., Stanford, W. L. et al., Mass spectrometry-based proteomic analysis of the matrix microenvironment in pluripotent stem cell culture. *Mol. Cell. Proteomics* 2012, *11*, 1924–1936.

[6] Liberski, A. R., Al-Noubi, M. N., Rahman, Z. H., Halabi, N. M. et al., Adaptation of a commonly used, chemically defined medium for human embryonic stem cells to stable isotope labeling with amino acids in cell culture. *J. Proteome Res.* 2013, *12*, 3233–3245.

[7] Chen, G. K., Gulbranson, D. R., Hou, Z. G., Bolin, J. M. et al., Chemically defined conditions for human iPSC derivation and culture. *Nat. Methods* 2011, *8*, 424–429.

[8] Scheerlinck, E., Van Steendam, K., Vandewoestyne, M., Lepez, T. et al., Detailed method description for noninvasive monitoring of differentiation status of human embryonic stem cells. *Anal. Biochem.* 2014, *461*, 60–66.

[9] Scheerlinck, E., Dhaenens, M., Van Soom, A., Peelman, L. et al., Minimizing technical variation during sample preparation prior to label-free quantitative mass spectrometry. *Anal. Biochem.* 2015, *490*, 14–19

[10] Distler, U., Kuharev, J., Navarro, P., Levin, Y. et al., Drift time-specific collision energies enable deep-coverage data-independent acquisition proteomics. *Nat. Methods* 2014, *11*, 167–170.

[11] Fabregat, A., Sidiropoulos, K., Garapati, P., Gillespie, M. et al., The reactome pathway knowledgebase. *Nucleic Acids Res.* 2016, *44*, D481–D487.

[12] Milacic, M., Haw, R., Rothfels, K., Wu, G. et al., Annotating cancer variants and anti-cancer therapeutics in Reactome. *Cancers (Basel)* 2012, *4*, 1180–1211.

[13] Vizcaino, J. A., Deutsch, E. W., Wang, R., Csordas, A., et al., ProteomeXchange provides globally coordinated proteomics data submission and dissemination. *Nat. Biotechnol.* 2014, *32*, 223–226.

[14] Michalski, A., Cox, J., Mann, M., More than 100,000 detectable peptide species elute in single shotgun proteomics runs but the majority is inaccessible to data-dependent LC-MS/MS. *J. Proteome Res.* 2011, *10*, 1785–1793.

[15] Salway, J. G., *Metabolism at a Glance* (2nd ed.). Blackwell Publishing 1999, 111 pp.

[16] Tenu, J. P., Lepoivre, M., Moali, C., Brollo, M. et al., Effects of the new arginase inhibitor N omega-hydroxy-nor-L-arginine on NO synthase activity in murine macrophages. *Nitric Oxide Biol. Chem.* 1999, *3*, 427–438.

[17] Ash, D. E., Structure and function of arginases. *J. Nutr.* 2004, *134*, 2760s–2764s.

[18] Napoli, C., Paolisso, G., Casamassimi, A., Al-Omran, M. et al., Effects of nitric oxide on cell proliferation: novel insights. *J. Am. Coll. Cardiol.* 2013, *62*, 89–95.

[19] Becker-Catania, S. G., Gregory, T. L., Yang, Y., Gau, C. L. et al., Loss of arginase I results in increased proliferation of neural stem cells. *J. Neurosci. Res.* 2006, *84*, 735–746.

