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Supporting Information for Proteomics

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A practical guide for the identification of membrane and plasma membrane proteins in human embryonic stem cells and human embryonal carcinoma cells

1. Materials and experimental procedures

Cell culture

hESCs, line HUES-7 (a kind gift from D. Melton, Harvard University), were cultured under feeder-free conditions on Matrix Growth Factor Reduced Matrigel (BD Biosciences)-coated flasks in MEF (strain CD1, 13.5 dpc)-conditioned medium and passaged enzymatically using trypsin. For the preparation of MEF-conditioned medium, MEFs were cultured in D-MEM High Glucose (Invitrogen) supplemented with 10% Fetal Bovine Serum (FCS; BioWhittaker), 2 mM glutamine (Invitrogen) and 1% non-essential amino acids (Invitrogen). MEFs were mitotically inactivated for 2.5 h with 10 µg/ml mitomycin C (Sigma) at passage #5, and seeded at 6×10^4 cells/cm^2. Twenty-four hours after treating the MEFs with mitomycin C, their medium was replaced by D-MEM:F12 Glutamax (Invitrogen) supplemented with 15% KNOCKOUT Serum Replacement (Invitrogen), 100 µM β-mercaptoethanol (Invitrogen), 4ng/ml basic fibroblast growth factor (basic FGF; Peprotech), 1% non-essential amino acids. This MEF-conditioned hESC medium was collected every day for 7 consecutive days, and re-supplemented with 4 ng/ml basic FGF after each collection. hECCs, line NT2/D1, were cultured in D-MEM:F-12 Glutamax with 10% FCS (Invitrogen) and passaged enzymatically using trypsin-EDTA (Invitrogen) every 2-3 days.

Preparation of the membrane-enriched fraction of hESCs and hECCs

hESCs (passage 22-26) and hECCs (passage 39-41) were were washed twice with PBS and harvested by scraping. Harvested cells were pelleted at 1100 g for 5 min at room temperature, resuspended in lysis buffer (50 mM Tris pH 7.8, 250 mM sucrose, 2 mM EDTA) with protease inhibitor cocktail (Roche Diagnostics, Switzerland), and incubated on ice for 10 min. Cells were lysed by 30 passes through a 30½ gauge needle at 4°C. Cell debris, unbroken nuclei, ER membranes and mitochondrial membranes were removed by centrifugation at 1000 g for 10 min at 4°C. The postnuclear supernatant was layered onto a 60% sucrose cushion and centrifuged at 160000 g for 1 h at 4°C in a MLS50 rotor (Optima,

Beckman-Coulter, Netherlands). The membrane fraction on top of the sucrose cushion was collected, diluted 1:2 with cold 50 mM Tris pH 7.8 and pelleted at 100000 g for 1 h at 4°C in a TLA55 rotor (Optima, Beckman-Coulter, Netherlands). The supernant was discarded and the membrane pellet was washed for 1 h at 4°C with 100 mM Na_2CO_3 pH 11.5, rinsed twice with cold H_2O and pelleted at 20000 g for 30 min at 4°C.

Sample preparation steps

Washes were performed with 100 mM Na_2CO_3 pH 11.5 for 1 h at 4°C. Trypsin pre-digests were performed overnight at 37°C with 0.1 µg Trypsin per 1×10^5 cells in 25 mM NH_4HCO_3 pH 8.0. After washes or predigests the membranes were rinsed twice with cold H_2O and pelleted at 20000 g for 30 min at 4°C. For deglycosylation membrane pellets were incubated for 10 min at 95°C in denaturation solution (20 mM NH_4HCO_3 pH 8.0, 0.2% SDS, 135 mM β-mercaptoethanol) and cooled down on ice. 0.6 U PNGase F was added and deglycosylation was performed for 2 h at 37°C. After deglycosylation, the membranes were washed with cold H_2O and pelleted at 20000 g for 30 min at 4°C. For delipidation acetone/methanol 8:1 was added to the membrane pellets and membrane proteins were precipitated for 2 h at -20°C. After delipidation the membrane proteins were washed with cold H_2O and pelleted at 20000 g for 30 min at 4°C. For tube gel digests, the membrane pellets were solubulized in 2% SDS, 25 mM NH_4HCO_3 pH 8.0 for 1 h at 4°C. The solution was casted directly into a 10% tube gel. The gel was cut into pieces and thoroughly washed with 50% ACN and H_2O, and dehydrated with ACN. After reduction and alkylation of reactive cysteines, in-gel digestion with Trypsin was performed overnight at 37°C.

For the final comparison of 5×10^5 hESCs and hECCs, the membrane-enriched fractions were incubated for 10 min at 95°C in denaturation solution (20 mM NH_4HCO_3 pH 8.0, 0.2% SDS, 135 mM β-mercaptoethanol). After cooling down, 0.5 U PNGase F (Sigma-Aldrich, Germany) was added and deglycosylation was performed for 2 h at 37°C. The deglycosylated protein pellet was washed with ice-cold H_2O, pelleted at 20000 g for 30 min at 4°C and dissolved in 8 M urea in 50 mM NH_4HCO_3 pH 8.0. Reduction and subsequent alkylation were performed

with 45 mM DTT for 30 min at 56°C and 100 mM iodoacetamide for 30 min at room temperature in the dark, respectively. 0.5 µg endoproteinase Lys-C (Roche-Diagnostics, Switzerland) was added and digestion was performed overnight at 37°C. After dilution to 2 M urea with 50 mM 100 mM NH_4HCO_3 0.5 µg Trypsin (Roche-Diagnostics, Switzerland) was added and digestion was performed for 8 h. The remaining membranes were sedimented at 20000 g for 30 min at 4°C and the supernatant was stored at -80°C. The pellet was re-dissolved in 80% ACN and re-digested with 0.5 µg Trypsin overnight at 37°C. This second digest was pooled with the first followed by desalting and concentration using Aqua-C18 material (Phenomenex, CA, USA) packed into a ZipTip® (Millipore, MA, USA). The eluate was dried in a vacuum centrifuge and reconstituted in 20% ACN, 0.05% formic acid.

SCX fractionation

SCX fractionation was performed on a system consisting of two Zorbax BioSCX-Series II columns (i.d., 0.8 mm; l, 50 mm; particle size, 3.5 µm), a Famos autosampler (LC packings, the Netherlands), a Shimadzu LC-9A binary pump, and a SPD-6A UV-detector (Shimadzu, Japan). In the first 10 min after injection, unbound material was washed from the column with 100% solvent A (0.05% formic acid in 8/2 (v/v) water/ACN, pH 3.0). The subsequent linear gradient increased with 1.3%/min solvent B (500 mM NaCl in 0.05% formic acid in 25% ACN, pH 3.0) with a flow rate of 50 µl/min. Fractions of 50 µl volume were manually collected, dried in a vacuum centrifuge, and reconstituted in 0.1% acetic acid.

NanoLC-MS/MS

The digests of the four sets of experiments performed for the optimization of the sample preparation and digestion of plasma membrane proteins for MS were analyzed by nanoLC-LTQ-MS using a 45 min LC gradient (Thermo, CA, USA). The SCX fractions of the digests performed for the final comparison of hESCs and hECCs were analysed by nanoLC-LTQ-Orbitrap-MS using a 120 min LC gradient (Thermo, CA, USA). Both systems were run with the following set-up. An Agilent 1100 series LC system was equipped with a 10 mm Aqua

C18 (Phenomenex, Torrance, CA) trapping column (packed in-house, i.d., 100 µm; resin, 5 µm) and a 254 mm ReproSil-Pur C18-AQ (Dr. Maisch GmbH, Ammerbuch, Germany) analytical column (packed in-house, i.d., 50 µm; resin, 3 µm). Trapping was performed at 5 µL/min for 10 min and elution was achieved with a gradient of 0-45% B in 45 or 120 min, respectively, 45-100% B in 1 min, 100% B for 4 min. The flow rate was passively split from 0.4 ml/min to 100 nl/min. Nanospray was achieved using a distally-coated fused silica emitter (New Objective, Cambridge, MA) (o.d., 360 µm; i.d., 20 µm, tip i.d. 10 µm) biased to 1.8 kV. The mass spectrometer was operated in the data dependent mode to automatically switch between MS and MS/MS. Survey full scan MS spectra were acquired from m/z 350 to m/z 1500 on the orbitrap with a resolution of R = 60000 at m/z 400 after accumulation to a target value of 500000 with lock-mass. The two most intense ions were fragmented using collisionally induced dissociation at a target value of 10000.

Protein identification

Spectra were processed with Bioworks 3.2 (Thermo, Bremen, Germany) and the subsequent data analysis was carried out using the Mascot (version 2.1.0) software platform (Matrix Science, London, UK). Protein identification was performed by searching the IPI human database with a precursor ion mass tolerance of 0.9 Da or 5 ppm for data generated by the LTQ linear ion trap or the LTQ Orbitrap, respectively. The fragment mass tolerance was set to 0.9 Da for both instruments. Carbamidomethylation of cysteines and oxidation of methionines were considered as fixed and variable modifications, respectively. For deglycosylated samples, deamidation of asparagines was allowed as variable modification. Fully proteolytic peptides produced by Lys-C, V8, Trypsin and/or Chymotrypsin with 1 maximum miscleavage were accepted. The ion score cut-offs were set to 25 for peptides and to 60 for proteins. For classification, the gene ontology (GO) symbols of the identified proteins were curated using the XRef database, and queried against the gene ontology database using the GoMiner tool (http://discover.nci.nih.gov/gominer/index.jsp). Prediction of

the origin of proteins with unknown cellular localisation was performed using the pTArget algorithm (http://bioinformatics.albany.edu/~ptarget).