Supporting Information

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SI Materials and Methods

Protein Expression and Purification Platform. Production of secreted protein for screening was carried out on the Protein Expression and Purification Platform (PEPP), a custom robotics platform. PEPP enables mammalian cell culture, transient transfection, protein-expression, harvest, and purifications to be completed in a fully automated and traceable manner. The expression module (Fig. S1) consists of two bulk cell-culturing units each designed to maintain a 10-L culture of mammalian or insect cells at a consistent cell density to provide cells for transfection. After the dispensing of bulk cells into culture flasks, protein expression is performed by transfection of expression plasmids and transfer into one of two incubator units. Cells are maintained in suspension using an optimized shaking pattern to achieve higher cell density and protein production. Protein is purified online by centrifugation of the culture flask, removal of the culture supernatant, and processing over a metal affinity resin. Purified proteins are characterized by a combination of automated and manual methods to evaluate concentration, purity, integrity, and endotoxin content. The bioanalytic module contains stations for liquid handling, chromatography, UV/visible/fluorimetric assays, and mass spectrometry (MS). A nonintegrated capillary-electrophoresis system provides information on protein purity and molecular mass. In a significant number of cases, the protein band was fairly diffuse, likely because of glycosylation. Obtaining interpretable results for analytic-sizing chromatography depended on the concentration and degree of modification. In most cases, mass-spectral data were not taken because of the low likelihood of obtaining signal with glycosylation.

Cloning and Preparation of Genes for Transient Transfection. The polymerase incomplete primer extension (PIPE) cloning method (1) was used, as previously described, to clone target ORF sequences minus their native signal peptides and, in the specific case of type I and type II transmembrane proteins, their non-extracellular domains into three derivatives of pRS. The vectors share the following features: a CMV promoter, human IgK signal peptide (SP), C-terminal FLAG-His tag (FH), bovine growth hormone (BGH) polyA, ampicillin and Zeocin resistance markers, and *ori*P and pUC origins of replication. The vectors differ by either the inclusion or exclusion of a noncleavable mouse IgG1-Fc fusion protein. Vector pRSh (SP-*ccdB*-FH) does not include the Fc fusion. Vector pRSnFch (SP-Fc-*ccdB*-FH) encodes an N- terminal Fc fusion. Vector pRScFch (SP-*ccdB*-Fc-FH) encodes a C-terminal Fc fusion. After cloning, the target sequence of interest replaced the *ccdB* cassette. Clone inserts were verified by sequencing. Transfection-grade DNAs were made from sequence-positive clones using HiSpeed Plasmid Maxi Kits (QIAGEN) per manufacturer's recommendation, and then, they were stored as single-use aliquots using 2D-barcoded tubes (Micronic).

Expression, Purification, and Characterization of Proteins for Screening. Most protein expression and purification experiments were performed using the PEPP robotics platform (GNF Systems). High-density suspension cell culture for PEPP was maintained in two 15-L pilot-scale Techfors-S bioreactors (Infors HT; Appropriate Technical Resources, Inc.). For expression of proteins, Freestyle HEK 293F cells (Invitrogen) were cultivated at densities between 3.0×10^5 and 3.0×10^6 cells per mL in Freestyle 293 media in custom Autoflasks (Greiner); the Autoflasks had a flange suitable for handling by a Stäubli robotic arm, a septum for delivery of culture, a gas-exchange membrane, and a v-pocket to facilitate separation of the cell pellet from supernatant by low-speed centrifugation. The Autoflasks were seeded with culture from the bioreactors using peristaltic pumps and cannulae at a density of 1.5×10^6 cells per mL and a total volume of 35–50 mL; a typical seed of 96 flasks uses 2 hours and 30 minutes of robotic time. Cells were maintained in suspension using an optimized shaking pattern for maximal cell growth in an incubator at 37 °C, 5% CO_2, and 75% relative humidity.

Secreted proteins were expressed on PEPP by transient transfection using 25-kDa linear polyethyleneimine (PEI; PolySci) reagent with plasmid DNA prepared as above. One microgram of DNA per 1.5×10^6 cells was combined with PEI at a ratio of 1:4 (wt/wt), respectively, and then, it was incubated with Freestyle media for 5 minutes at room temperature. The DNA-PEI precipitate was added to the flasks, mixed, and placed in an incubator for a 96-hour period. A typical transfection efficiency of >70% was consistently achieved with this method. At the time of harvest, flasks were retrieved from the incubator in clusters of four and pelleted by centrifugation at $1{,}000 \times g$ for 5 minutes. Before purification, a buffer concentrate was added to the supernatants to give a final concentration equivalent to 10 mM Tris-Cl (pH 7.4), 150 mM NaCl, and 5 mM imidazole (in addition to the Freestyle media components). The supernatants (35–50 mL) were loaded onto 0.2 mL nickel affinity-chromatography resin (Ni-NTA Superflow; QIAGEN) in gravity-flow mode. The resin was washed with 50 column volumes of 20 mM Tris-Cl (pH 7.4), 137 mM NaCl, Tris-buffered saline (TBS), and 5 mM imidazole. Target proteins were then eluted with five column volumes of TBS with 250 mM imidazole, desalted using Nap-10 columns (GE LifeSciences) equilibrated with TBS, and maintained at 4 °C until final dispensing and storage at −80 °C.

Characterization of Secretomics Protein Samples. All purified proteins were taken through a standard set of characterization assays. The sample aliquots designated for analysis were freeze-thawed the same number of times as the samples going into the cell assays. The assays were enabled by a robotic analytical platform consisting of a TECAN Evo200 interfaced to a Safire UV/Vis/Fluor plate reader, two automated analytical HPLCs, an automated HPLC interfaced to an LCT-Premier TOF mass spectrometer (Waters), and a stand-alone LC90 capillary electrophoresis system (Caliper). Protein concentration was determined using BCA assay (Pierce) at two sample volumes to cover for variances in protein yield. Endotoxin level was determined using the Kinetic-QCL kit (Lonza). Protein purity, molecular weight, and in some cases, concentration were assessed by LC90 electrophoresis. Protein in-solution molecular weight and aggregation state were assessed by analytic size-exclusion chromatography as previously described (1) for proteins that were reexpressed as part of the hit confirmation process.

Immunocytochemistry. Cultures were fixed with 4% paraformaldehyde and blocked in 1× PBS containing 0.2% Triton X-100 and 2% BSA. The cells were incubated with the primary antibody in 0.1% Triton X-100 in PBS at 4 °C overnight. Secondary antibody labeled with Alexa 488 or 594 (Invitrogen) was then added and incubated at RT for 45 min. After staining with DAPI, cells were visualized with a fluorescence microscope. Primary antibodies to POU51/OCT4, SSEA-4, Tra-1–81, and pigment epithelium-derived factor receptor (PEDFR) were obtained from Santa Cruz Biotechnology and NANOG was from R&D Systems. For the quantification of OCT4, Nanog, SSEA-4, Alkaline Phosphatase, and TRA-1–81-expressing cells, at least ten images for each treatment were taken using an Opera high-content confocal image system. Quantification for each

treatment was done for three independent experiments. Data present mean values ± SD.

Immunoblotting. Lysates from 1.5×10^6 cells were electrophoresed on 4–20% gradient polyacrylamide gels along with biotinylated standards and transferred to nitrocellulose (Bio-Rad Laboratories). Filters were blocked for 1 hour at RT in 1× blocking buffer (Sigma-Aldrich) in PBS. Anti-human PEDFR primary antibodies (R&D Systems) were diluted 1:500 or 1:100 in 1× blocking buffer and probed for 1 hour at room temperature. Filters were washed 2 times for 5 minutes each in Tris-buffered saline/Tween 20 [TBST; 10 mM Tris-HCl (pH 7.9), 150 mM NaCl, and 0.05% Tween-20]. Dilutions (1:2,000) of appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies(Santa Cruz Biotechnology, Inc.) along with 1:5,000 dilutions of avidin-HRP (Bio-Rad Laboratories) in 1× blocking buffer were added and probed for 1 hours at room temperature. Filters were washed 2 times for 5 minutes in TBST, and signals were detected by chemiluminescence with enhanced chemiluminescence reagents (GE Healthcare)

Teratoma Formation. Approximately 10^4 human embryonic stem cells (hESCs) were injected beneath the kidney capsule of adult male SCID mice. After 21–90 days, mice were killed, and teratomas were dissected, fixed in Bouin's fixative overnight, processed for paraffin sections, and stained with H&E. Sections were examined for evidence of tissue differentiation using bright-field light microscopy and photography as appropriate. Ethical approval for all works on animals was obtained from the Animal Research Committee of The Burnham Institute for Medical Research.

PEDFR Short Hairpin RNA Knockdown. The Lenti-Lox3.7(pLL3.7) was used to express a short hairpin RNA (shRNA) under the U6 promoter (2).The target sequences for PEDFR are 5′- AGAAGACGTGGAACATCTT -3′ (PEDFR shRNA A) and 5′- TTTCCTGCTGAAGGTCCTT -3′(PEDFR shRNA B). pLL3.7 vector expressing a shRNA for luciferase 5′-CTTACGCTGAGTACTTCGA-3 was used as a control. The lentiviruses were prepared as described previously (2). Briefly, we cotransfected pLL3.7 and packaging vectors (pΔR8.9 and pVSV-G) into 293 T cells (Invitrogen) and collected the resulting supernatant after 48 hours. We recovered virus after ultracentrifugation for 1.5 hours at 85,00 × *g* in a Beckman SW28 rotor and resuspension in PBS (15–200 μl) to produced titers of 1×10^8 infectious units per milliliter. Titers were determined on HT1080 cells. For examination of PEDFR knockdown efficiency, hESCs maintained in unconditioned medium (UM) plus PEDF (100 ng/mL) on Matrigel were transduced by a single round of infection overnight with a 25× multiplicity of infection plus 6 μg/mL polybrene (hexadimethrine bromide; Sigma). Seventy-two hours later, infected cells were selected by FACS for GFP-positive cells and pooled onto Matrigel-coated plates containing UM plus PEDF (100 ng/mL). Seven days after plating, Western blot and immunofluorescence assays were performed.

1. Klock HE, Koesema EJ, Knuth MW, Lesley SA (2008) Combining the polymerase incomplete primer extension method for cloning and mutagenesis with microscreening to accelerate structural genomics efforts. *Proteins* 71:982–994.

2. Rubinson DA, et al. (2003) A lentivirus-based system to functionally silence genes in primary mammalian cells, stem cells and transgenic mice by RNA interference. *Nat Genet* 33:401–406.

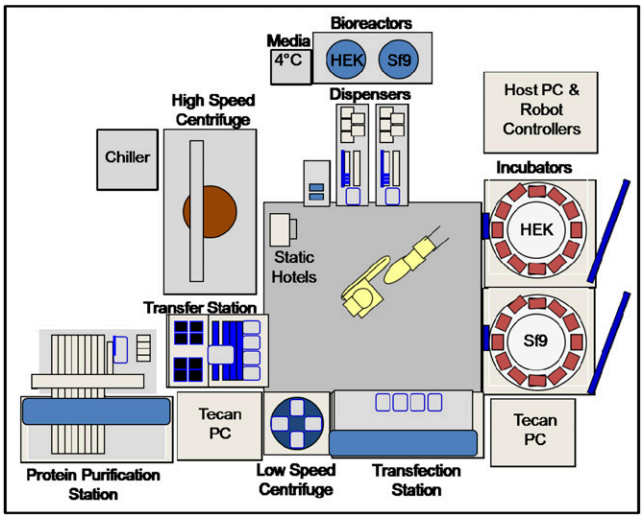

Fig. S1. PEPP robotics platform for protein expression and purification.

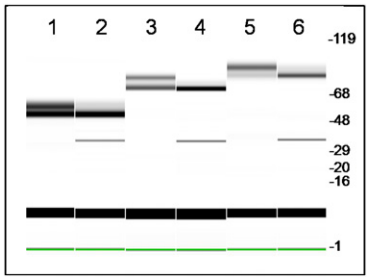

Fig. S2. Capillary electrophoresis of PEDF expression constructs, native and after deglycosylation with N-glycosidase-F. Lanes 1 and 2 are C-Flag-His. Lanes 3 and 4 are N-Fc fusion. Lanes 5 and 6 are C-Fc fusion. The proteins in lanes 2, 4, and 6 are deglycosylated by treatment with PNGase-F for 18 hours at 37 °C.

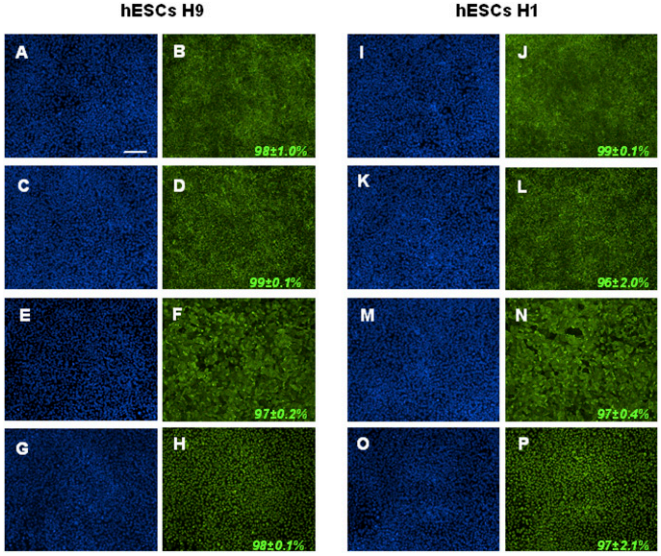

Fig. S3. Analysis of hESC stem-cell marker expression after long-term culture (15 passages) with PEDF. HESC-H9 and H1 cell lines grown with UM plus PEDF (100 ng/mL) for 15 passages were fixed and immunostained with alkaline phosphatase (A/B and I/J), SSEA-4 (C/D and K/L), Tra-1–81 (E/F and M/N), and OCT4 (G/H and O/P) antibodies. Values are the mean ± SD for three measurements. (Scale bar, 100 μM.)

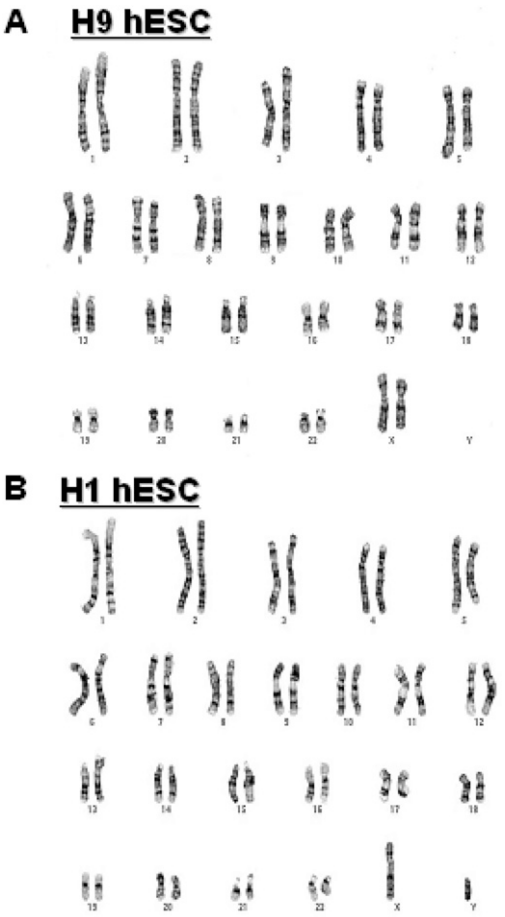

Fig. S4. Karyotypes of hESC-H9 and H1 cells. Cells were grown with UM plus PEDF (100 ng/mL) for 17 passages.

Other Supporting Information Files

Table S1 (DOC)
Table S2 (DOC)
Table S3 (DOCX)