Detailed protocol for the maintenance of hESCs in DC-HAIF medium

- **Collagenase passaging of human ES cells in DC-HAIF**
  - Listed volumes are for cells grown in 60 mm dishes
  - Warm appropriate amount of **Collagenase IV 10 mg/ml (~2000 U/ml)**, **DC-HAIF** and **BSA Wash medium** to 37°C in water bath.
  - Set up hESCs plate on a dissecting microscope in a biosafety cabinet or laminar flow so that it is comfortable to see the colonies.
  - If necessary, cut out and remove any overtly differentiated colonies with a 21 1/2g needle.
  - Aspirate the medium and gently add 1-2 ml of collagenase.
  - Leave for 3 mins to remove cells from in between colonies and round up colony edges.
  - Gently tap the sides of the flask/dish to dislodge cells.
  - Remove collagenase, rinse colonies with PBS then add 2 ml BSA Wash Medium.
  - Gently scrape colonies off the dish using a 1000 µl pipette tip.
  - Gently transfer clumps into a 15 ml tube using a 5 ml pipette.
  - Wash plate with 3 ml of BSA wash medium and add to the cells in the 15 ml tube.
  - Spin cells at 1000 rpm (200 g) for 4 mins at room temperature.
  - Gently aspirate medium and flick tube to loosen cells from the bottom.
  - Gently resuspend the cells in 1 ml medium using a 1 ml or 5 ml seriological pipette. Do not triturate excessively.
  - A confluent plate is typically split 1:3. Transfer cells to new **1:200 matrigel coated** plates. Make up the final volume to 5 ml DC-HAIF per plate.
  - Place the plates into an incubator set at 37°C with 5% CO₂, and mix plates gently to evenly spread out the clumps.
  - Feed cells gently the next day to remove excess cells and every day thereafter.
  - Observe cells every day and passage by the above protocol whenever required (5-7 days).

- **Medium Recipes**
  - Defined Medium: DC-HAIF
    - DMEM:F12
    - 2% BSA
    - 1× Pen/Strep
    - 1× NEAA
    - 1× Trace Elements A, B & C
    - 50 µg/ml Ascorbic Acid
    - 10 µg/ml Transferrin
    - 0.1 mM β–ME
  - Add immediately prior to use:
    - 10 ng/ml Heregulin (1 µl/ml of 10 µg/ml stock)
    - 10 ng/ml Activin A (0.4 µl/ml of 25 µg/ml stock)
    - 200 ng/ml LR³-IGF (0.2 µl/ml of 1 mg/ml stock)
    - 8 ng/ml FGF2 (0.32 µl/ml of 25 µg/ml stock)
- **Collagenase**

- **1:200 Matrigel Plates**
  - Thaw matrigel vial slowly at 4°C. Dilute 1:1 with DMEM:F12 and aliquot into 1 ml aliquots. Store at −20°C until needed.
  - Slowly thaw Matrigel aliquots at 4°C for at least 2 hr to avoid the formation of a gel.
  - Dilute the Matrigel aliquots 1:100 in cold DMEM:F12 (for a final dilution of 1:200).
  - Add appropriate amount of Matrigel solution to coat each plate. (2.5 ml/60 mm or T25, 1.5 ml per well of a 6 well plate/35 mm plate).
  - Incubate the plates 1-2 hr at RT before use.
  - The plates with Matrigel solution can be stored sealed at 4°C for 1-2 weeks. Do not use if dried out. Incubate 1-2 hr at RT after removing from 4°C.
  - Remove Matrigel solution immediately before use. Do not rinse. Do not re-use Matrigel.

- **BSA Wash Medium**
  - Prepare 0.1% BSA in DMEM:F12. Add Pen/Strep to preserve.
<table>
<thead>
<tr>
<th>Component Information</th>
<th>Distributor</th>
<th>Cat. Numbers</th>
<th>Stock Solution</th>
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<tr>
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<td>Invitrogen</td>
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<td>*BSA. Fraction V, fatty acid free</td>
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<td>R&amp;D Sytems</td>
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<tr>
<td>Growth Factor reduced Matrigel</td>
<td>BD Biosciences</td>
<td>35 6231</td>
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*Fatty acid free, Fraction V BSA. Do not substitute for lower quality BSA.
Working with DC-HAIF

While hESCs grow well in DC-HAIF and undifferentiated cultures can be maintained easily, there are a number of critical factors that have a strong influence on how successfully cells can be maintained while the approach is being learned.

- **Starter culture**
  - This **must** be a high quality culture. There must be a high density of cells, and they must be primarily undifferentiated. The starter culture can be cells maintained on matrigel in MEF-CM, or on MEFs. Attempt to minimize initial carry-over of MEFs to DC-HAIF conditions.

- **Passaging**
  - Passaging the cells is the most likely point of difficulty. It is absolutely critical to achieve high plating/survival of colony pieces. The colony pieces must be a bit smaller than typical collagenase passaging of cells on matrigel/MEF-CM.
  - Some cell death at passaging is normal, but wide-scale cell death is not (i.e. `<20% survival`), and typically indicates a technically poor split.

- **Timing of passaging**
  - This is critical. Do not passage the cells too early, they will plate poorly and differentiate. The cultures need to grow to near-confluence, i.e. a day or two longer than when the colonies are just touching. At this level of confluency ~5-8 × 10^6 cells should be present per 60 mm dish.

- **hESCs in DC-HAIF** are sensitive to over exposure to collagenase. This will cause poor plating and induce differentiation. Do not expose longer than 3 mins. Do not use lower concentrations of collagenase and treat for longer periods.

- **Density**
  - The cultures should be maintained at a high density. This means 200+ colonies in a 60 mm dish. If the density of colonies drops, the culture may deteriorate: elevated differentiation may occur, and the culture will take longer between splits. If this happens, leave the culture longer to proliferate to near-confluence before splitting.

- **BSA**
  - The quality of the BSA is critical. Do not substitute lower-grade BSA for the recommended fatty acid-free Cohns fraction V BSA. We recommend using batches with certified low endotoxin levels (0.1 or less EU/ml), and individual batches should be tested.

- **Transfer of hESCs from MEFs or MEF-CM to DC-HAIF**
  - hESCs may take 2-3 passages to adjust fully to initial culture in DC-HAIF, after which the culture stabilizes and passaging is more routine. This is a critical period and it is more difficult to rescue cultures that start to deteriorate during this period.
o We routinely use 1:2 split ratios for the first 2-3 passages and 1:3 or 1:4 ratios thereafter.

- Feeding
  o DC-HAIF cultures should be fed every day. Do not exhaust the medium by not feeding. Badly differentiated areas should be scraped out with a pipette tip. Mistreated cultures will differentiate.
**Human RTK arrays**

Proteome Profiler™ human phospho-RTK antibody arrays (R&D Systems, Cat#ARY001) were used according to the manufacturer’s instructions. Protein lysates were prepared in 1% NP-40, 20 mM Tris-HCl (pH 8.0), 137 mM NaCl, 10% glycerol, 2.0 mM EDTA, 1.0 mM sodium orthovanadate, 10 µg/ml Aprotinin, and 10 µg/ml Leupeptin. 500 µg fresh protein lysates were incubated overnight with nitrocellulose membranes dotted with duplicate spots for 42 anti-RTK antibodies and 5 negative control antibodies, as well as 8 anti-phosphotyrosine positive control spots (Supplemental Figure 1). The arrayed antibodies capture the extracellular domains of both phosphorylated and unphosphorylated RTKs, and bound phospho-RTKs are detected with a pan anti-phospho-tyrosine antibody conjugated to horseradish peroxidase (HRP) using chemiluminescence. X-ray film was scanned and spots were quantified using NIH-image (http://rsb.info.nih.gov/nih-image/). Blots were normalized using the 8 pan-antiphosphotyrosine antibody control spots per filter, and normalized values were averaged.

**Receptor Inhibition Studies**

For experiments using the IGF1R blocking antibody, H1 hESCs grown on Matrigel were incubated with 10 µg/ml A12 (ImClone Inc.) or control human IgG (Sigma) in CM. IGF1R expression was evaluated by flow cytometry after 4 hours of incubation with A12 or control antibody. For proliferation assay, H1 hESCs were starved and pre-incubated with A12 or control, the cultures were fed after 4 hours, and the experiment conducted in the same conditions with daily medium changes. Triplicate cultures were split 1:2 on day 3 and 6, and colonies were counted and graded morphologically on days 2, 5, and 9. Colonies exhibiting more than 20% differentiated cells were categorized as differentiated (Supplemental Figure 2). Morphological assessment of differentiation was confirmed by SSEA-3 staining using flow cytometry at day 3. Apoptosis was also evaluated by TUNEL staining, 3 days after addition of A12. Inhibition of ERBB2 with AG825 (Calbiochem) used hESCs growing in CM on Matrigel diluted 1:30. For cell counting studies, 5×10^5 hESCs were plated per well in 6-well trays, after being split to single cells using Accutase (Innovative Cell Technologies) (Armstrong et al, Hum Mol Genet. 2006 15(11):1894-913). Triplicate wells were counted after overnight culture in CM (day 1, baseline count), then 50 µM AG825 or DMSO carrier was added, and the culture fed daily with fresh medium/treatment. Triplicate experimental wells were counted on day 4. For cell cycle and apoptosis analysis, hESCs were plated using dispase passaging. After 4 days of culture with AG825 or DMSO control in CM, with daily fresh medium/treatment change, hESCs cells in triplicate wells were collected for PI staining and TUNEL assay.

**Flow Cytometry and Immunofluorescence**

Cytometric analyses or sorting were performed using a FACScan, FACSanto Cytometer or FACS Aria cell sorter (BD Biosciences). The FlowTACS kit (R&D system) was used to detect apoptotic cells, according to the manufacturer’s instructions. Primary antibodies used to detect cell surface antigens were: anti-SSEA-3 (Chemicon or R&D Systems), anti-OCT4 and anti-SSEA4-APC (R&D Systems), anti-IR-PE and anti-IGF1R-PE antibodies (BD Biosciences), anti-ERBB2 (clone 9G6.10) (Lab Vision Corporation), and anti-ERBB3 (R&D Systems). Appropriate FITC- or PE-conjugated secondary antibodies (SouthernBiotech or Jackson ImmunoResearch) were used to detect unlabeled primary antibodies, and isotype-matched primary antibodies and secondary-only antibodies were used as negative controls (BD Bioscience). Cell cycle and DNA content analysis was performed using propidium iodide
staining and analysis with the Modfit 3.0 software (Verity House Software). Immunofluorescence analysis of hESC cultures for OCT4, SSEA-4, TRA-1-60 and TRA-1-81 was performed as described previously (Schulz et al, 2004, Stem Cells 22:1218-1238).

**RT-PCR Analysis**
Total cellular RNA was extracted using the RNeasy kit (Qiagen) and reverse transcription carried out using the Superscript III (Invitrogen) or iScript (BioRad) cDNA synthesis kits according to the manufacturer’s instructions. PCR was performed using the SYBR green Q-PCR mastermix (Applied Biosystems) or Taqman universal master mix (Applied Biosystems) and an ABI 7300 Real Time PCR system (Applied Biosystems), or with REDTaq ReadyMix PCR Reaction Mix (Sigma). PCR products were separated and visualized by gel electrophoresis. Amplification of GAPDH or omission of RT served as positive and negative controls. The primers used were:

**Human IGF1**
Forward 5’-GCTGGTGGATGCTCTTCAGTTC-3’
Reverse 5’-AGCTGACTTGGCAGGCTTGAG-3’

**Human IGF2**
Forward 5’-TGCCCTCCTGGAGACATACTGTGC-3’
Reverse 5’-TTGGAAGAAGAGACTTGAGAAGGATTC-3’

**Human IGF1R**
Forward 5’-TCGACATCCGCAACGACTATC-3’
Reverse 5’-AGGGCGTAGTTGTAGAAGAGTTT-3’

**Human IR**
Forward 5’-CTTGGCGATGGTGGGAATGTG-3’
Reverse 5’-CGGAGATGACCAGCGACTC-3’

**Human GAPDH**
Forward 5’-ACGACCACCTTTGTCAAAGCTCATTTC-3’
Reverse 5’-GCAGTGAGGGTCTCTCTCTCTTCTTCT-3’

**Mouse IGF1**
Forward 5’-ATGAGTGTTGGCTTCCGGAGCT-3’
Reverse 5’-AGCGGGCTGCTTTTGTAGG-3’

**Mouse IGF2**
Forward 5’-GCATGCTTGGCAGAAGGCT-3’
Reverse 5’-CGAAGAGGCTCCCCCGT-3’

**Mouse β-actin**
Forward 5’-ATCCTCACCACTGAAGTACC-3’
Reverse 5’-ATTCCCGCTCGCGCTGGT-3’
Taqman gene expression assay primers (Applied Biosystems) used were:

IGF1R: Hs00181385_m1  
OCT4: Hs01895061_u1  
Human HPRT1 (4333768F) was used as endogenous control.

Qualified primer sets purchased from SuperArray were:

- Human ERBB1 (Cat# PPH00138A, 171 bp)
- Human ERBB2 (Cat# PPH00209A, 185 bp)
- Human ERBB3 (Cat# PPH00463A, 167 bp)
- Human ERBB4 (Cat# PPH00460A, 189 bp)
- Mouse ADAM19 (Cat# PPM05659A, 117 bp)
- Mouse NRG1 (Cat# PPM57587A, 157 bp)

Primers for Human ABCG2, DPPA5, ZFP42, NANOG, OCT4, FOXD3, TERT and CRIPITO have been described previously (Brimble et al 2004, Stem Cells, 13:585-596; Zeng et al 2004, Stem Cells, 22:292-312).

**shRNA Vector Design and Lentiviral Infection**

A stem-loop structure oligonucleotide containing an IGF1R-targeting sequence 5′-CCGAAGATTTCACTGCTCAA-3′ [target site is 3476-3494 (NM_000875.2)], was designed using the RNAi codex program (http://codex.cshl.org/scripts/newmain.pl) and cloned under the control of the human U6 promoter in lentiviral vectors, which also contained a GFP reporter. The control shRNA sequence was 5′-GCTGGAAACTCTTCTACAA-3′, which is complimentary to a different site within IGF1R [target site is 368-386 (NM_000875.2)], but has no effect of IGF1R expression. To demonstrate the effectiveness of shRNA inhibition, lentiviral plasmids were transfected into H1 hESCs using the GeneJuice reagent (Novagen). 48 hours after transfection, GFP+ and GFP- cells from the IGF1R-targeted transfection, and GFP+ cells from the control transfection, were sorted by FACS Aria and IGF1R mRNA expression was examined by qPCR. Lentiviruses were produced in 293D cells as described (Rubinson et al 2003, Nat Genet 33:401-406) and viral supernatants were concentrated by ultracentrifugation to produce stocks with titers of 1 × 10^8 infectious units per milliliter. Titers were determined on HT1080 cells. For examination of shRNA-mediated IGF1R blockade on longer term culture, H1 hESCs maintained in CM on Matrigel were transduced by single round of infection overnight with a 25× multiplicity of infection. Transduced cells were cultured for 22 days and evaluated periodically for GFP and IGF1R expression by flow cytometry.

**Cell and colony counting assays in DC-HAIF medium**

For cell counting studies, 10^5 hESCs were plated per well in 12-well trays in DC-HAIF, after being split to single cells using Accutase (Innovative Cell Technologies) (Armstrong et al 2006, Hum Mol Genet. 15(11):1894-913). Triplicate wells were counted after overnight culture (day 1, baseline count), and the experimental wells were carefully washed and fed with fresh media with different combinations of 10 ng/ml HRG-1β, 10 ng/ml ActA, 200 ng/ml LR3-IGF1, and 8 ng/ml FGF2. Triplicate wells were counted on day 7. A duplicate experiment was fixed and immunostained with OCT4 and DAPI to quantify the proportions of remaining undifferentiated cells. Three random 10× magnification fields were imaged and counted for each condition.
(average counts per field per condition: 346 (AF), 828 (HAF), 1015 (AIF), 1552 (HAIF) cells). For comparison of these growth factor combinations during serial passaging, parental starter cultures were grown on MEFs and the experiment was initiated by passaging in a ratio equivalent to 1:2, to parallel 35 mm dishes with collagenase IV as described above. HESCs were plated directly in the different growth factor combinations, on dishes coated with Matrigel diluted 1:200. The proportion of undifferentiated and differentiated colonies was determined within a dish based on morphological appearance 3-4 days after plating, and all the cultures were passaged 1:2 on day 5 into the same conditions. The same grading and passaging cycle was repeated twice. For comparison of CM and DC-HAIF conditions during serial passaging, parental starter cultures were grown on MEFs and split to parallel 60 mm dishes in CM or DC-HAIF conditions (p0 cultures). Counting experiments were initiated by passaging to parallel 6-well trays, in a ratio equivalent to 1:3 (p1 cultures). After 4 days, three wells from each condition were disaggregated to single cells with Accutase and counted. The remaining wells were passaged with collagenase IV to new 6-well trays with split ratios appropriate for each condition (p2 cultures). Cell counting was performed at passage 1-3 and total cell numbers were plotted after correction for split ratios.

In vivo and in vitro Differentiation of hESCs
The generation and analysis of teratomas was performed as described previously (Plaia et al 2006, Stem Cells 24:531-546). 5×10^6 hESCs were injected to the hindlimb of SCID/beige mice, and tumors harvested after 8-12 weeks. Animal research protocols (A2005-10147-c1) were reviewed and approved by the University of Georgia (Athens, GA), and experiments were conducted according to institutional guidelines. Embryoid body experiments were performed as described previously (Brimble et al, 2004, Stem Cells Dev 13:585-596). Briefly, hESCs were harvested using collagenase IV and differentiated in suspension in 15% fetal calf serum/5% KSR medium. HESCs were differentiated to definitive endoderm and foregut endoderm using defined medium formulations, which were based on modifications of existing protocols (D'Amour et al 2006, Nat Biotechnol. 24:1392-1401). Briefly, hESCs growing in DC-HAIF were induced to differentiate to definitive endoderm by exposing cultures to RPMI containing 2% fatty acid-free BSA, 25 ng/ml Wnt3a, 100 ng/ml Activin A and 8 ng/ml FGF2 for 24 hours, followed by RPMI containing 2% BSA, 100 ng/ml Activin A and 8 ng/ml FGF2 for a further 2 days. Definitive endoderm was examined on the third day of differentiation. Further differentiation to foregut endoderm was effected by three days exposure to RPMI containing 2% BSA, 50 ng/ml FGF7 and 0.25 µM KAAD-cyclopamine. Endoderm cultures were examined by qPCR and immunofluorescence as described previously (D’Amour et al 2005, Nat Biotechnol. 23:1534-1541; D'Amour et al 2006, Nat Biotechnol. 24:1392-1401). Expression levels in qPCR analyses were plotted as fold change compared to hESC samples, except for HNF1β and HNF4α, which were below the level of detection in hESCs and were therefore normalized to day 3 samples.

Illumina Bead Array and Transcriptional Analysis
Total cellular RNA was isolated from hESCs using Trizol (Invitrogen) and was treated with DNase I (Invitrogen). Sample amplification was performed with 100 ng of total RNA using the Illumina RNA Amplification kit and labeling was achieved by incorporation of biotin-16-UTP (Perkin Elmer Life and Analytical Sciences) at a ratio of 1:1 with unlabeled UTP. Labeled, amplified material (700 ng per array) was hybridized to Illumina Sentrix Human-6 Expression
Beadchips containing 47,296 transcript probes according to the manufacturer’s instructions (Illumina, Inc.). Arrays were scanned with an Illumina Bead Array Reader confocal scanner and primary data processing, background subtraction, and data analysis were performed using Illumina BeadStudio software according to the manufacturer’s instructions. A minimum detection confidence score of 0.99 (a computed cutoff indicating the target sequence signal was distinguishable from the negative controls) was used to discriminate the presence of or absence of transcript expression. Analysis was performed using parallel approaches described for other hESC samples (Liu et al 2006, BMC Dev Biol 6:20). Hierarchical clustering was performed as described previously (Liu et al 2005, BMC Dev Biol 6:20), and was based on average linkage and Euclidean distances as the similarity metric using differentially expressed genes identified by ANOVA (p<0.05). Detailed descriptions of the sensitivity and quality control tests used in array manufacture and algorithms used in the Bead studio software are available from Illumina Inc. (San Diego, CA).

**Equipment and Settings**
Digital photomicrographs of live, or fixed and immunostained, cells were captured using Nikon TE2000-S and -E inverted microscopes, Q-Imaging Retiga cameras, and the Q-Capture imaging system (www.qimaging.com). Images were processed in Adobe Photoshop to adjust appropriate brightness/contrast, levels, or merge individual monochrome images of an overlapping set. Adjustments were applied evenly across images. Images of histological sections and immunohistochemistry were captured with an Olympus BX41 light microscope, Olympus DP70 digital camera and Image Pro Plus software (Mediacybernetics, Inc).

**Statistical analysis**
The t-test was used for statistical comparisons of cell culture experiments in figures 2 and 3, unless indicated. The alpha level was 0.05 throughout. Figures 2B-D, G-H, J; 3B: n=3, data shown as mean ± s.d. Figure 3H: duplicate spots quantified (n=2), mean and range plotted.