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Qualification of Embryonal Carcinoma 2102Ep As a Reference for Human Embryonic Stem Cell Research

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Key Words: Embryonic stem cells • Embryonal carcinoma • Standard • Pluripotent • Human

ABSTRACT

As the number of human embryonic stem cell (hESC) lines increases, so does the need for systematic evaluation of each line’s characteristics and potential. Comparisons between lines are complicated by variations in culture conditions, feeders, spontaneous differentiation, and the absence of standardized assays. These difficulties, combined with the inability of most labs to maintain more than a few lines simultaneously, compel the development of reference standards to which hESC lines can be compared. The use of a stable cell line as a reference standard offers many advantages. A line with a relatively unchanging hESC-like gene and protein expression pattern could be a positive control for developing assays. It can be used as a reference for genomics or proteomics studies, especially for normalizing results obtained in separate laboratories. Such a cell line should be widely available without intellectual property restraints, easily cultured without feeders, and resistant to spontaneous changes in phenotype. We propose that the embryonal carcinoma (EC) line 2102Ep meets these requirements. We compared the protein, gene, and microRNA expression of this cell line with those of hESC lines and alternative reference lines such as the EC line NTERA-2 and the karyotypically abnormal hESC line BG01V. The overall expression profiles of all these lines were similar, with exceptions reflecting the germ cell origins of EC. On the basis of global gene and microRNA expression, 2102Ep is somewhat less similar to hESC than the alternatives; however, 2102Ep expresses more hESC-associated microRNAs than NTERA-2 does, and fewer markers of differentiated fates.

INTRODUCTION

The field of human embryonic stem cell (hESC) research holds much promise, ranging from furthering our understanding of basic mechanisms of human development to the development of novel therapeutics. However, advances in this young field have been slowed by scientific, as well as political, roadblocks. Developing inexpensive, reliable technology that can be used in routine screens is a challenge. An equally great challenge is to coordinate results from multiple investigators, ensure a consistent supply of cells, reach consensus about markers used for testing, and develop mechanisms to freely share data. Historically in the U.S., the Federal Government has played a leading role in establishing standards, hosting databases and data sets, and developing and enforcing guidelines. However, in the case of hESC research, the U.S. government’s role has been limited as it has adopted a dual-track policy, funding hESC research only on cell lines established before a particular date (August 9, 2001). This policy, together with the current patent situation in the U.S., has led to a fragmentation of the field and has limited the Federal Government to a facilitative rather than a leadership role.

Several formal and informal efforts have been initiated to begin to standardize protocols and provide a common data set for hESC research. Perhaps the most ambitious to date is the work of the International Stem Cell Initiative, under the auspices of the International Stem Cell Forum (http://www.stemcellforum.org), which has begun the work of characterization of 75 different hESC lines [1]. We have undertaken a parallel effort to comprehensively characterize limited sets of pre- and post-August 9, 2001 hESC lines, using molecular tests such as single nucleotide polymorphism analysis, gene expression profiling, methylation profiling, and microRNA (miRNA) expression [2–4]. In this effort, we have used the human embryonal carcinoma (EC) line NTERA-2 cl.D1 [5] and the karyotypically abnormal hESC line BG01V as reference standards. EC lines have several advantages as standards for comparison between laboratories: they grow without feeder cells, are relatively simple to passage, resist spontaneous differentiation, and are a rich source of the proteins and mRNAs used to characterize hESCs. Long before ESCs had been derived from early embryos, researchers discovered pluripotent EC cells within mouse and human germ cell-derived teratocarcinomas (reviewed in [6]). Some EC lines derived from these tumors were able to colonize a host blastocyst and contribute widely to tissues of a chimera mouse [7–9]. The markers that are today used to demonstrate the undifferentiated state of hESC were originally defined as antigens of human EC cells [10]. Among these are stage-specific embryonic antigen-3 (SSEA-3) [11],...
SSEA-4 [12], and tumor rejection antigens (TRA)-1-60 and TRA-1-81 [13]. We have made use of these properties of EC cells to have a ready supply of positive controls and comparison material for our own research. We have further proposed the use of NTERA-2 and BG01V as reference standards for interlaboratory data comparisons, reasoning that both lines are freely available to the research community from The American Type Culture Collection (ATCC) [Manassas, VA, http://www.atcc.org] and both have been characterized in detail in side-by-side comparisons [14, 15]. The global gene expression of NTERA-2 is highly similar to undifferentiated hESC but is outside the range of variation among hESC lines [15]. In contrast to EC lines, the hESC line BG01V shares all of the pluripotent differentiation potential of normal hESC lines along with virtually undistinguishable gene expression [14]. Although BG01V recovers well from thawing and is thus easier to grow than most hESC lines, it is still very sensitive to culture conditions that can vary between laboratories.

Another cell line that is already being used as a standard in numerous laboratories is the EC 2102Ep, which was derived from a primary human testicular teratocarcinoma and later subcloned [16]. This relatively nullipotent line is easily cultured without feeders or mitogens other than serum. It shows little spontaneous differentiation in vitro unless seeded at low density, does not differentiate in response to retinoic acid, and when xenografted into nude mice forms tumors entirely composed of EC cells [17–19]. Several monoclonal antibodies that recognize surface markers of undifferentiated hESC were generated using 2102Ep as the immunogen [12, 13, 20]. The surface antigens of this and other EC lines have been extensively characterized [21].

The purpose of this article is to propose 2102Ep as a useful tool in the many types of quality control assays performed on hESC lines. To that end, we provide a comprehensive characterization and comparison of the 2102Ep line and the other potential standard lines NTERA-2 and BG01V. Although any of these lines may be appropriate as positive controls for particular assays, we find that 2102Ep is a stable and rich source of the genes and proteins commonly used as markers of undifferentiated hESC. It also highly expresses miRNAs specific to hESC and EC lines. The ease of growing 2102Ep in the undifferentiated embryonic-like state makes it a convenient reference material for normalization of hESC data and potentially a benchmark for relating quantitative marker expression to the functional potency of hESC.

**Characterization of 2102Ep**

BG01V hESCs (P10) were obtained from BresaGen, Inc. (San Diego, http://www.novocell.com) as a frozen stock. Approximately $1 \times 10^6$ BG01V cells were plated into each of two $9.5\text{cm}^2$ wells of a six-well culture dish (Corning Life Sciences) containing a feeder layer of mitomycin C-treated CF-1 mouse embryonic fibroblasts (MEF) (ATCC). Cells were cultured at 37°C, 5% CO$_2$. The growth medium used was DMEM/F-12 (ATCC) supplemented with ES-Qualified FBS (15%) (ATCC), knockout serum replacement (5%) (Invitrogen Corporation, Carlsbad, CA, http://www.invitrogen.com), l-α-aminolevulinic acid (2.0 mM) (ATCC), minimal essential medium nonessential amino acids (1×) (ATCC), β-mercaptoethanol (0.1 mM) (Sigma-Aldrich, St. Louis, http://www.sigmaaldrich.com), penicillin (100 IU/ml)/streptomycin (100 μg/ml) (ATCC), and fetal bovine serum (FBS) (ATCC) (Manassas, VA, http://www.atcc.org). Daily medium changes began after the first 48 hours in culture. Colony formation was visible within 2–3 days. Cells were passaged every 4–5 days using collagenase IV (200 units per milliliter) (Invitrogen Corporation) for 15 passages (P25). BG01 and BG03 cells were maintained under feeder-free conditions on fibronectin-coated plates in hESC medium (as above) that had been conditioned by MEF for 24 hours. HUES lines were obtained from Harvard University and cultured as instructed (http://www.mcb.harvard.edu/melton/hues/).

**Cytogenetics**

The karyotype analysis was performed using a standard G-banding technique. Cells cultured in a T75 culture flask were treated with 0.05 μg/ml colcemid (Invitrogen Corporation) for 1 hour, followed by dissociation using 0.25% trypsin/0.53 mM EDTA in Hanks’ balanced salt solution (HBSS) without calcium or magnesium (ATCC 30-2101). The cells were then collected by centrifugation (5 minutes at 240g) and gently resuspended in a 0.06 M KCl hypotonic solution and placed in an incubator at 37°C for 25 minutes. The hypotonic effect was halted by the addition of 3:1 Carnoy’s fixative (methanol/glacial acetic acid). The cells were collected by centrifugation, resuspended by gentle mixing, and run through a series of fixes prior to slide preparation. Metaphase spreads were prepared on glass microscope slides exposed briefly to a 2% Enzar-T trypsin (40×) (Mediatech, Herndon, VA, http://www.tepnel.com) and allowed to dry. The FTA card lyses the Minneapohs, (http://www.rndsystems.com). Daily medium changes began after the first 48 hours in culture. Colony formation was visible within 2–3 days. Cells were passaged every 4–5 days using collagenase IV (200 units per milliliter) (Invitrogen Corporation) for 15 passages (P25). BG01 and BG03 cells were maintained under feeder-free conditions on fibronectin-coated plates in hESC medium (as above) that had been conditioned by MEF for 24 hours. HUES lines were obtained from Harvard University and cultured as instructed (http://www.mcb.harvard.edu/melton/hues/).

**Short Tandem Repeat Analysis**

Short tandem repeat (STR) analysis was performed using either isolated genomic DNA or frozen cells. In the case of frozen cells, the cells were first resuspended in phosphate-buffered saline (PBS) (ATCC SCRR-2201), and then a 20-μl aliquot was spotted on a labeled FTA card (Whatman plc, Brentford, Middlesex, U.K., http://www.whatman.com) and allowed to dry. The FTA card was placed on the cells on contact and binds the DNA to the paper surface. Prior to polymerase chain reaction (PCR), a portion of the dried spot was removed with a Harris punch, washed three times with purified reagent (Whatman plc), washed once with TE buffer (Tris-EDTA, pH 8.0), and allowed to dry. STR analysis was conducted using a multiplex-PCR-based PowerPlex 1.2 kit (Promega Corporation, Madison, WI, http://www.promega.com). Loci analyzed include 13 STRs on the chromosomes (D5S818, D13S317, D7S820, D16S539, von Willebrand factor A (vWA), TH01, Amelogenin, TP0X, and CSF1P0. Electropherogram data were collected on an ABI 310 Genetic Analyzer. Data were analyzed using Genescan 3.1 and Genotyper 2.0 (Applied Biosystems, Foster City, CA, http://www.appliedbiosystems.com). The resulting profiles were imported into an in-house database and screened against all other baseline profiles of all samples tested by ATCC.

**Human Leukocyte Antigen Typing**

Genomic DNA was isolated from the cells using the GenElute Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich). Human leukocyte antigen (HLA) DNA typing was performed by using hybridization of PCR-amplified DNA with sequence-specific oligonucleotide probes (Tepnel LifeSciences Corporation, Stamford, CT, http://www.tepnel.com). The target DNA is amplified by PCR and

**Materials and Methods**

**In Vitro Cell Culture**

2102Ep cl.2A6 cells were grown at high density in tissue-culture plastic flasks (Corning Life Sciences, Acton, MA, http://www.corning.com/lifesciences) at 37°C in a humidified chamber containing 5% CO$_2$ in air. The growth medium used was Dulbecco’s modified Eagle’s medium (DMEM) (4 M/l-glutamine, 4.500 mg of glucose/l) (ATCC) supplemented with 10% fetal bovine serum (FBS) (ATCC) and 1% Pen/Strep (ATCC) added. The growth medium was changed every 24–36 hours. The cells were harvested (FBS) (ATCC) and 1% Pen/Strep (ATCC) added. The growth medium used was DMEM/F-12 (ATCC) supplemented as described above for 2102Ep. Cells were cultured at 37°C, 5% CO$_2$, and the medium was changed every 24 hours. Cells were passed every 4 days by scraping.
then allowed to denature and rehybridize to complementary DNA probes conjugated to fluorescently coded microspheres. A flow analyzer identifies the fluorescent intensity on each microsphere, and the determined HLA type is based on the reaction pattern compared with patterns associated with public HLA gene sequences. Assays were performed to determine the HLA-A, HLA-B, HLA-C, HLA-DRB1, and HLA-DQB1 loci.

**Immunophenotyping of Undifferentiated Cells**

Cells were fixed in 4% paraformaldehyde for at least 20 minutes at room temperature, washed in PBS, and then incubated in 3% normal goat serum (NGS) to inhibit nonspecific binding. Saponin detergent (0.5%) was used to permeabilize cell membranes on samples stained for intracellular markers. Cells were then assayed with monoclonal antibodies specific for Oct-3/4 (1:250) (BD Transduction Laboratories; BD Biosciences, San Jose, CA, http://www.bd.biosciences.com), SSEA-4 [12] (1:50) (R&D Systems, Inc.), SSEA-1 [22] (1:100) (Chemicon International, Temecula, CA, http://www.chemicon.com), and TRA-1-60 [13] (1:100) (Chemicon International), washed using PBS/1% NGS to remove any unbound protein, and then incubated with an Alexa Fluor 488-conjugated goat anti-mouse IgG (H+L) antibody (1:750) (Invitrogen Corporation). Positively stained cells were visualized using an epifluorescence microscope.

**Immunophenotyping by Flow Cytometry**

Undifferentiated, adherent cells were enzymatically dissociated using 0.25% Trypsin/0.53 mM EDTA (ATCC 30-2101) and pipetted to single-cell suspension. The cells were pelleted by centrifugation for 5 minutes at 270 rpm, room temperature, washed in 1× PBS, and then fixed in 2% paraformaldehyde/1× PBS at room temperature for 20 minutes. The cells were washed in 1× PBS/1% NGS and transferred to bovine serum albumin-coated microcentrifuge tubes. Samples stained for intracellular markers were treated with saponin (0.5%) to permeabilize cell membranes. Cells were incubated with monoclonal antibodies specific for Oct-3/4 (1:250) (BD Transduction Laboratories; BD Biosciences), SSEA-4 (1:50) (R&D Systems, Inc.) TRA-1-60 (1:100) (Chemicon International), TRA-1-81 (1:100) (Chemicon International), TRA-1-85 [23] (1:100) (Developmental Studies Hybridoma Bank, Iowa City, IA, http://www.uiowa.edu/~dshbwww), and CD30 clone Ber-H2 (1:100) (Dako, Carpinteria, CA, http://www.dakouusa.com) in PBS/3% NGS for 2 hours at room temperature followed by wash using PBS/1% NGS to remove any unbound protein. Cells were then incubated with an Alexa Fluor 488-conjugated goat anti-mouse IgG (H+L) antibody (1:750) (Invitrogen Corporation) for 1 hour at room temperature in the dark. Cell samples were washed using 1× PBS/1% NGS and then resuspended in 1× PBS. Stained cells were analyzed by a FACSAria flow cytometer (BD Biosciences). Data analysis was performed using CellQuest software installed on a Macintosh computer (Apple Computer, Inc., Cupertino, CA, http://www.apple.com).

**Alkaline Phosphatase Activity Assay**

Endogenous alkaline phosphatase activity in 2102Ep and BGO1V cells was detected using the ELF 97 Endogenous Alkaline Phosphatase Detection Kit (Invitrogen Corporation) according to the manufacturer’s instructions. Cells cultured on 12-mm round glass coverslips in 24-well plates (Corning Life Sciences) were treated with 2% paraformaldehyde for 20 minutes at room temperature. The cells were washed with PBS, treated with 0.2% Tween 20 for 20 minutes at room temperature, and rinsed with PBS. Fixed cells were then incubated with a filtered 1:20 dilution of the phosphatase substrate in situ. The immunostained colonies and the reaction were monitored using an epifluorescence microscope. The reaction was terminated using a stop solution consisting of PBS, 25 mM EDTA, and 5 mM levamisole, pH 8.0. Cells were rinsed with PBS before mounting on glass microscope slides.

**Gene Expression of Undifferentiated Cells Using Quantitative Reverse Transcription-PCR**

Total RNA was isolated by lysing cells in Trizol LS (Invitrogen Corporation) according to instructions. Two micrograms of total RNA was treated with DNaseI (Promega Corporation) 25°C/15 minutes, 65°C/10 minutes, and then reverse-transcribed using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Inc., Hercules, CA, http://www.bio-rad.com). One eightieth of the cDNA synthesis reaction was used as template for each real-time PCR using iQ SYBR Green Supermix (Bio-Rad Laboratories, Inc.). Primer sets used are listed in [14]. For each primer set, T<sub>0</sub> is the annealing temperature determined empirically using template cDNA from NTERA-2 cells. T<sub>i</sub> is the temperature at which the SYBR Green fluorescence is read, chosen by examining the melt curves of the PCR products. PCR was run in an iCycler iQ Real-Time Detection System (Bio-Rad Laboratories, Inc.) for 50 cycles of 95°C/15 seconds, T<sub>i</sub>/30 seconds, 72°C/45 seconds, T<sub>i</sub>/15 seconds. The relative amounts of PCR product were quantified using the relative threshold cycle (ΔΔCt) method corrected for efficiency of each amplification [24]. The gene quantities for each sample were normalized against the geometric mean of expression of the housekeeping genes GAPDH (glyceraldehyde-3-phosphate dehydrogenase), β-actin, and tata-binding protein. Statistically significant (p < .05) differences in gene expression were determined by the Student’s t test.

**Gene Expression of Undifferentiated Cells Using Microarray**

Sample amplification for Illumina BeadArray was performed using 100 ng of total RNA as input material by the method of Van Gelder et al. [25] using the Illumina RNA Amplification kit (Ambion, Inc., Austin, TX, http://www.ambion.com); labeling was by incorporation of biotin-16-UTP (PerkinElmer Life and Analytical Sciences, Boston, http://www.perkinelmer.com) present at a ratio of 1:1 with unlabeled UTP. Labeled, amplified material (700 ng per array) was hybridized to a pilot version of the Illumina Ref-8 BeadChip according to the manufacturer’s instructions (Illumina, Inc., San Diego, http://www.illumina.com). Arrays were washed and then stained with Amersham fluorolink streptavidin-cyanine 3 (GE Healthcare, Little Chalfont, Buckinghamshire, U.K., http://www.gehealthcare.com) following the BeadChip manual. Arrays were scanned with an Illumina BeadArray Reader confocal scanner. Array data processing and analysis were performed using Illumina BeadStudio software.

**Enrichment of miRNA**

miRNA was isolated from either frozen cell pellet or Trizol extracted total RNA by using a Purelink miRNA Isolation Kit (Invitrogen Corporation) according to the recommended protocol. The purity of the isolated miRNA was confirmed by electrophoresis on a 15% NuPAGE urea-Tris/Borate/EDTA gel (Invitrogen Corporation), and the amount was quantified spectrophotometrically.

**NCode miRNA Array and Quantitative PCR**

Five hundred nanograms of the enriched miRNA was labeled with the NCode direct labeling system (dye-swap labeling) and hybridized to replicate NCode multispecies miRNA arrays. Data were background-corrected and normalized using the Latin squares or the dye-swap model by Kerr et al. [26]. The normalized values were then used to rank the miRNA based on their abundance and also to measure the number of differentially expressed markers. The number of differentially expressed markers with p values less than .005 obtained with four independent samples of hESC was pooled to get the average number and standard deviation of differentially expressed markers in the pooled hESC samples in comparison with 2102Ep or Ntera2 cells. Differentially expressed targets were further validated by NCode quantitative reverse transcription-PCR (qRT-PCR) (Invitrogen), and average Ct values were determined from the replicates. ΔCt values were calculated by Ct (hESC) – Ct (2102Ep). Fold change in expression relative to 2102Ep cells was calculated as 2ΔCt, and values less than 1.0 were expressed as its reciprocal with a negative value.
**RESULTS**

Cytogenetics and Cell Culture

The 2102Ep line is easily maintained in vitro. The cells grew as a uniform monolayer of typical human EC cells, with a high nucleus-to-cytoplasm ratio and prominent nucleoli, as previously described [16]. Untreated tissue culture plastic is sufficient for attachment of the cells. 2102Ep cells were maintained at high density and were passaged every 3–4 days using enzymatic dissociation. The NTERA-2 EC line grew very similarly except that mechanical dissociation is preferable, whereas BG01V must be cultured on a MEF feeder layer in colonies characteristic of hESC lines. The doubling time of the 2102Ep cells was 21.8 hours, compared with approximately 36 hours for NTERA-2 (data not shown). The cells froze well and recovered well from freeze (≥85% viability upon thaw). After thawing and plating, BG01V also recovered rapidly and colony formation was visible after 2 days; enzymatic passaging was performed every 4–5 days, enabling efficient expansion of the cell line.

The karyotype of 2102Ep is hyperdiploid. For this cell line, a total of 51 metaphase figures were analyzed at passage 83; the chromosome number ranged from 48 to 58 with a modal number of 56. The modal number agrees with the first description of the line in 1980 at passage 56, and the range observed then was similar (48–61) [27]. In comparison, the NTERA-2 cell line is hypotriploid and contains an average of 12 marker chromosomes with a modal chromosome number of 63, as previously reported [5]. The BG01V hESC line, which evolved from rou- somes with a modal chromosome number of 63, as previously hypo- triploid and contains an average of 12 marker chromosomes with a modal chromosome number of 63, as previously reported [5]. The BG01V hESC line, which evolved from routine enzymatic passaging by BresaGen Inc., exhibits a karyotype of 49 chromosomes (XXX, +12, +17). We have previously reported that this karyotype is stably maintained through at least 25 passages [14].

Identification Assays

Identification assays are of ultimate importance to verify the identity of the cell lines under investigation and to perform meaningful comparisons with previously available data. Therefore, we have performed STR analysis and HLA typing of these lines. Amplification of tandemly repeated elements in the genome is commonly used for genetic mapping, linkage analysis, and human identity testing. ATCC is currently building a database of STR identity information for human cell lines [28]. The test used here allows for discrimination of at least one in 10^8 individuals [29].

The STR profile of the 2102Ep clearly shows a male line with X and Y chromosome alleles for amelogenin (supplemental online Fig. 1). The karyotypic abnormality of this line is also apparent from the presence of three alleles for the vWA locus on chromosome 12. The profiles for BG01V and NTERA-2 were previously determined and published [14].

Another unique identifier of each line is the HLA profile, commonly used to determine graft donor/recipient compatibility and an important consideration in the diversity of hESC lines. The HLA profile of 2102Ep, which was determined from the genomic DNA, is shown in supplemental online Table 1 with comparison with the previously determined profiles of BG01V and NTERA-2. Although this DNA method shows 2102Ep as heterozygous for most major histocompatibility complex (MHC) loci, it is serologically homozygous for all but DQB1. This is striking because NTERA-2 was found to be homozygous at all MHC loci tested [14].

**Immunophenotyping: Staining and Flow Cytometry**

**Profile and Expression of ESC Markers**

2102Ep cells were strongly positive by immunocytochemistry for the markers of undifferentiated human EC cells and hESCs, Oct3/4, SSEA-4, and TRA-1-60 (Fig. 1). The intensity and high proportion of labeling by these antibodies is comparable with the hESC line BG01V. In addition, 2102Ep cells are positive for alkaline phosphatase activity and negative for the marker of differentiating human EC and hESC, SSEA-1, as previously reported [16].

By flow cytometry, nearly all of the 2102Ep cells were labeled with the markers of undifferentiated human EC cells and hESCs: Oct3/4 (98%), SSEA-4 (98%), TRA-1-60 (93.5%), and TRA-1-81 (91.1%) (Fig. 2). These measurements were virtually unchanged in 2102Ep cells 10 passages later (data not shown). As a comparison, we also ran BG01V in these assays. Because BG01V were grown on a mouse feeder layer, the cells were also stained for the pan-human marker TRA-1-85 to determine the proportion of labeling by these antibodies is comparable with the hESC line BG01V. In addition, 2102Ep cells are positive for alkaline phosphatase activity and negative for the marker of differentiating human EC and hESC, SSEA-1, as previously reported [16].

**Comparison of Gene Expression Patterns by Bead Arrays**

Gene expression profile was examined using an Illumina BeadArray containing 24,131 full-length and splice-variant transcripts from the human RefSeq database. All transcripts are represented on the array by 50-mer probes, which offer higher gene selectivity than lithographic array methods. Each probe is
present on an average of 30 beads within the BeadArray, and this high feature redundancy contributes to reproducibility of results. The total number of genes detected above the 0.99 confidence limit is summarized in Table 1. Intensity results are reported in arbitrary units, and a detailed explanation of the data analysis is provided in Materials and Methods. Using a cutoff of 100 arbitrary units, 2102Ep cells expressed 8,526 transcripts, a number similar to the number seen in other undifferentiated populations (e.g., 9,889 for BG03) (Table 1). Intensity ranged from 10 to 20,000 (a 2,000-fold range), and as with other analyses, the genes with the highest abundance were housekeeping genes, ribosomal genes, and structural genes (see supplemental online File 2 for the entire list). These genes were similar in most samples although relative levels varied.

Expression levels of each individual gene can be compared between two lines (Fig. 3). Such a global pairwise comparison between a single hESC sample (BG03) and RNA from three pooled hESC lines (H1, H7, and H9) gives an estimate of the variability among undifferentiated hESC lines. For the comparison of reference lines, BG03 was chosen as a representative karyotypically normal, undifferentiated line. The correlation coefficient ($r^2$) estimates the relatedness of gene expression in two samples. As can be seen in Figure 3, the EC lines 2102Ep and NTERA-2 can be readily distinguished from hESC lines by their lower correlation of gene expression levels. Of the three reference lines used here, 2102Ep had a lower correlation coefficient for detected genes ($r^2 = .74$) than NTERA-2 ($r^2 = .88$) or BG01V ($r^2 = .90$). However, EC lines model the global gene expression pattern of hESC more closely than any other cell type yet tested and approach the limit imposed by variation

![Figure 2](https://www.StemCells.com)

**Figure 2.** Phenotyping by flow cytometry of 2102Ep cells (A–E) or BG01V grown on mouse embryonic fibroblast feeders (F–J). Cells stained with primary antibodies and detected via indirect immunofluorescence are shown as filled histograms; negative controls (secondary antibody only) are shown as open histograms. The proportion of 2102Ep cells positive for SSEA-4 (A), TRA-1-60 (B), Oct-3/4 (C), TRA-1-85 (D), and TRA-1-81 (E) is shown. For BG01V human embryonic stem cells grown on mouse feeders, the relevant measurement is the percentage of human cells positive for each antigen. Double-staining of SSEA-4 and TRA-1-85 (A) shows that 97.6% (24.4% SSEA-4/25.0% TRA-1-85) of the human cells are SSEA-4-positive. The other monoclonals used here are the same isotype as TRA-1-85, so the percentages can only be normalized to the percentage of TRA-1-85-positive in a separate reaction. Oct-3/4 staining was observed in 21.8% of a culture that was 25.0% TRA-1-85-positive, so the BG01V are approximately 87% Oct-3/4 positive (G, H). A separate culture was 45.8% positive for TRA-1-81 and 43.2% positive for TRA-1-85 in separate reactions, so the BG01V are approximately 100% positive for TRA-1-81 (I, J). Abbreviations: I, intracellular; PE, phycoerythrin; S, surface; SSEA, stage-specific embryonic antigen; TRA, tumor rejection antigen.

### Table 1. Distribution of gene abundances in 2102Ep

<table>
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<tr>
<th>Intensity</th>
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<th>2102Ep</th>
<th>Percentage</th>
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</table>

The total number of genes of the potential 24,000 transcripts present on the array which contained a detectable signal above background was calculated and binned to assess overall distribution of transcripts. Intensity is expressed in arbitrary units. Note that the overall pattern of expression is similar between 2102Ep and a control embryonic stem cell line (BG03) that was processed in parallel.
among hESC lines [30–32] (and data not shown). Expression differences ranged across the entire spectrum of genes, and a list of differences that are 50-fold or higher between 2102Ep and hESC is provided in supplemental online File 3.

An example of how 2102Ep can be used to normalize data sets is shown in Figure 3E. Comparison of array experiments between laboratories can be skewed by systematic errors occurring in one location. We can correct for these by running a universal sample in all laboratories and comparing the data set to data sets run in another setting. To normalize other samples, the expression level of each gene can be divided by the expression level of the same gene in the universal sample and then multiplied by the average expression level for that gene in multiple runs of the universal sample. We have performed this normalization using data from BG03, data from 2102Ep run at the same time, and averaged data from three 2102Ep samples. It is possible to normalize only genes that are confidently detected in both the unique and universal samples. Here, the correlation coefficient (r²) for all genes above the confidence limit is indicated. Dots that fall between the thin red lines represent genes that are similarly expressed in both samples, whereas dots outside the red lines correspond to differentially expressed genes at more than 2.5-fold. Representative data from at least two independent samples of 2102Ep, NTERA-2, and BG01V are shown.

Figure 3. Comparison of global gene expression by BeadArray Expression levels for 24,000 transcripts detected by the Illumina BeadArray compared between BG03 and pooled H1, H7, and H9 (A), between BG03 and 2102Ep (B), between BG03 and NTERA-2 (C), between BG03 and BG01V (D), or between BG03 and the same BG03 data normalized with 2102Ep (E). In the last panel, BG03 data were normalized by first dividing BG03 signal by a simultaneously run 2102Ep sample, then multiplying this ratio by average expression in three 2102Ep samples. Genes detected above the 0.99 confidence limit in both samples are shown as blue dots, those with expression above zero but below the confidence limit as black dots. The correlation coefficient (r²) for all genes above the confidence limit is indicated. Dots that fall between the thin red lines represent genes that are similarly expressed in both samples, whereas dots outside the red lines correspond to differentially expressed genes at more than 2.5-fold. Representative data from at least two independent samples of 2102Ep, NTERA-2, and BG01V are shown.

The expression of known hESC markers was assessed in these samples, and the results are summarized in Figure 4 and supplemental online File 2. Most of these are expressed at similar levels in 2102Ep and BG03, including nucleostemin, TDGF1 (cripto), ABCG2, and DNMT3A. However, 15 of these genes are underexpressed by threefold or more in 2102Ep by comparison with the hESC line, including POU5F1, DNMT3B, FOXD3, LeftyB, and REX-1 (ZFP42). Only three of these genes are overexpressed by threefold or more in 2102Ep: Nanog, GDF3, and UTF1. Nanog and GDF3 are located on the short arm of chromosome 12, in a region frequently amplified in EC lines and germ cell tumors. The nearby gene Stella is not part of the focused set but is also overexpressed in 2102Ep. Overall, the global expression shows that 2102Ep is a useful model of hESC, provided that one remains aware of key differences such as the expression of early germ cell markers and other genes associated with germ cell tumors.

Differences Between 2102Ep, NTERA-2, and BG01V

Two other populations of karyotypically abnormal cells NTERA-2 and BG01V have been suggested as reference standards [34]. We therefore compared gene expression patterns in
of germ cell differentiation, such as PIWIL2 genes and pressed later-appearing germ cell markers such as the summarized in Figure 4B. Overall, it appeared as if NTERA-2 expressed similarly in 2102Ep at passage 95, and BG01V at passage 15. The relative quantity of each gene is calculated by the ΔΔCt method, using a correction for the amplification efficiency of that gene, and normalized to the geometric mean of three housekeeping genes: glyceraldehyde-3-phosphate dehydrogenase, β-actin, and tata-binding protein. Significant differences in 2102Ep between passages 78 and 95 are seen only for TDGF1 (P < .010) and DPPA4 (P < .005). Error bars indicate standard error (n = 3). Significant differences in expression (two-tailed t test) when compared with 2102Ep at passage 78 are indicated (p < .050, ○) (p < .010, ●). hESC state. In the BeadArray experiments, some of these were expressed similarly in 2102Ep and BG03 (TDGF1, DPPA4, and DNMT3B), some were underexpressed in 2102Ep (POU5f1 and REX-1), one slightly overexpressed relative to BG03 (Nanog), and some not detectable by BeadArray (Sox2, hTERT). For five of the seven genes tested, the passage number of 2102Ep did not make a significant difference in expression (P78 vs. P95). The exceptions were significant increases with passage number in expression of TDGF1 and DPPA4. In comparing the two EC lines 2102Ep and NTERA-2, significant differences were observed for the genes Sox2, POU5f1, and hTERT. However, the difference in DNMT3B expression was not confirmed. Also, the ESC line BG01 had significant differences with early-passage 2102Ep in expression of POU5f1, TDGF1, Rex-1, Sox2, and DPPA4. Although qRT-PCR shows the size of most differences between 2102Ep and NTERA-2 or hESC to be smaller than indicated by the BeadArray, this analysis confirms the finding that high expression of the Rex-1 gene differentiates hESC from EC lines.

Comparison of the miRNA Profiles of 2102Ep and NTERA-2 with That of Other ESC Lines and ESC-Derived Populations
Gene expression patterns are only one aspect of the pluripotent stem cell state. Recent publications have revealed that ESCs have a unique chromatin structure and dynamism [36, 37]. Clear differences can be detected in the methylation profiles of hESC compared with other cell types, including EC [38]. It is possible that hESC may have unique methods and patterns of post-transcriptional regulation as well, and this led us to examine the miRNA expression profiles of 2102Ep, NTERA-2, and hESC lines.
post-transcriptional regulation in hESC. We cannot yet confirm whether 2102Ep is a superior model of miRNAs at higher levels than NTERA-2 or BG01V. Given that undifferentiated hESC and that 2102Ep expresses known hESC miRNA species highly expressed in 2102Ep are associated with expression of these miRNA species in multiple hESC lines is together, the miRNA array shows that the majority of the expression of these miRNA species may provide new markers for the pluripotent state of hESC lines compared with either 2102Ep or NTERA-2. The number of differentially expressed microRNAs (P < .005) within each ESC/EC pair is shown.

We first examined the number of differentially expressed miRNAs in hESC lines compared with either 2102Ep or NTERA-2. In this global miRNA comparison (to the limits of miRNA represented on the array), three out of four of the hESC lines we tested showed a slightly closer relationship to NTERA-2 than to 2102Ep cells (Table 2 and supplemental online File 5). Further examination of the most highly expressed miRNAs in each cell type indicated the presence of known pluripotent cell-related miRNAs [39, 40] in all cell types tested (Table 3). Eight of the top 10 miRNAs in 2102Ep cells have previously been found to be specifically expressed in hESC and EC [39]. In contrast, only three of the top 10 expressed miRNAs in NTERA-2 cells identified in this study are known ESC/EC cell type markers. Interestingly, Suh et al. found miR371, miR372, and miR373 in hESCs but not in NTERA-2 [39]. Here, we detect these markers in relatively high amounts in 2102Ep cells and at extremely low but measurable levels in NTERA-2 (supplemental online File 5). Thus, if only miRNA known to be associated with pluripotent cells are considered within the miRNA array data, 2102Ep cells seem to be more closely related to hESC than do NTERA-2.

Quantitation of these hESC-associated miRNAs by qPCR confirms that most are expressed at significantly lower levels in NTERA-2 or BG01V than in 2102Ep (Table 4). However, the expression of these miRNA species in multiple hESC lines is generally similar to or slightly higher than in 2102Ep. Taken together, the miRNA array shows that the majority of the miRNA species highly expressed in 2102Ep are associated with undifferentiated hESC and that 2102Ep expresses known hESC miRNAs at higher levels than NTERA-2 or BG01V. Given that the targets of these miRNAs have not yet been identified, we cannot yet confirm whether 2102Ep is a superior model of post-transcriptional regulation in hESC.

## DISCUSSION

EC cells are the transformed stem cells found within testicular germ cell tumors and have been described as the malignant counterparts of ESCs. The culture methods and surface markers of hESC have been derived from work on EC cells. Therefore, we are seeking to apply EC lines as benchmarks for measuring the pluripotent state of hESC lines. We have applied the same in-depth characterization methods used on hESCs to the EC lines 2102Ep and NTERA-2, in an effort to identify potential reference standards for comparison of hESC results.

We have verified that 2102Ep is easily maintained as a monolayer of cells, nearly 100% of which express the typical cell surface and nuclear markers of undifferentiated hESC. The virtual absence of spontaneous differentiation in culture is shown by the maintenance of alkaline phosphatase activity and the lack of SSEA-1 expression on the cell surface. Such purity of the undifferentiated state is very helpful for analysis of global gene expression patterns by microarray. The global gene expression of 2102Ep is almost as highly correlated with undifferentiated hESC as is that of NTERA-2 cells. In addition, analysis by microarray and qRT-PCR of typical hESC markers shows that many genes associated with the undifferentiated state of hESC are as highly expressed in 2102Ep. The relative null-potency of 2102Ep is mirrored by the observation that this line does not express early markers of germ layer differentiation, whereas NTERA-2 highly expresses the ESC/neural marker Sox-2 as well as the early mesodermal marker brachyury [41].

Similar to the results of the gene expression array, a global screen for miRNA expression indicates that NTERA-2 is more similar to hESC than is 2102Ep. However, several miRNAs specific to undifferentiated hESC are more highly expressed in 2102Ep. The role of endogenous RNA interference in early development is incompletely understood. Processing of miRNAs by Dicer is essential for early development and the maintenance of ESCs [42]. The subsequent differentiation of ESC to multiple lineages is also dependent upon Dicer, likely due to the role of miRNA in post-transcriptional gene silencing [43]. Although several miRNAs are expressed only in hESC and EC cells, the genes they regulate are still unknown [39]. Monitoring of these miRNA species may provide new markers for the undifferentiated quality of hESC and possibly offer measures of their stability and differentiation potential.

The generation of new and more informative markers for hESC and their pluripotent or differentiating states will be greatly facilitated by the use of reference standards. To date, it has been challenging for any laboratory to maintain multiple hESC lines, and comparison between laboratories has been uncertain due to variations in culture conditions and protocols which can subtly affect phenotypic profiles.

Identification of reference standards that are readily available and easily used are critical for the rapid advancement of the field. We and others have discussed several possibilities (reviewed in [34]). For gene expression studies, for example, one could develop a publicly accessible database of pooled average expression levels from 10 to 20 hESC lines and differentiated populations derived from them. Efforts are under way to develop such databases [44, 45]. In the U.S., federal money cannot be spent on materials derived from post-August 9, 2001 lines, which hampers comparison of the older and newer lines. In addition, the current patent situation and the control of most lines by companies make establishing such a pooled hESC standard even more difficult [46]. An alternative strategy would be to use a readily available cell line that could be cultured and tested in researchers' laboratories. For this purpose, we have previously proposed the EC line NTERA-2 and the variant hESC line BG01V [14, 47].

EC lines offer many advantages as control lines. The overall similarity between NTERA-2/2102Ep and hESC approaches that between the most distant hESC lines, and many key pathways and markers are conserved. The antibodies that are used to identify undifferentiated hESC can be tested and optimized in EC cells, and small interfering RNA knockdown or blocking reagents for most hESC genes can be tested as well. In the case of NTERA-2, differentiation into neural lineages can be assessed, and it has been shown that the time period required and the steps used to promote differentiation are essentially identical.
to hESCs [15]. RNA from NTERA-2 or 2102Ep can be used as a universal reference for normalization when performing microarray studies, and in studies of undirected differentiation, RNA from EC cells was a much more sensitive control than a human universal RNA pool [33].

Although the case for EC lines as a reference standard is compelling, it is not as clear how to choose one EC line over another. 2102Ep is relatively nullipotent and probably represents the undifferentiated state better than NTERA-2. Although capable of differentiation into all three lineages, NTERA-2 appears to be somewhat biased toward neural differentiation. Even in the undifferentiated state, NTERA-2 expresses early germ layer markers such as SOX2 and brachyury.

However, both EC lines show crucial differences with hESC. These differences include aneuploidy, expression of germ cell markers in both lines, and resistance to spontaneous differentiation in vitro. On the other hand, NTERA-2 and BG01V are much more responsive than 2102Ep to differentiation signals and are more similar to hESC when global gene and miRNA expression patterns are considered. One could speculate that relatively nullipotent lines such as 2102Ep lack the low-level expression of numerous differentiation pathways, which is observed in multipotent stem cells [48]. This promiscuous gene expression, possibly permitted by the recently described “breathing chromatin” of pluripotent cells, could be essential for maintaining developmental plasticity [37].

However the situation arises, 2102Ep shares many of the identified markers of pluripotent hESC while being much less challenging to culture. Unlike hESC lines, 2102Ep is resistant to differentiation when grown without feeders or high concentrations of mitogen. In addition, 2102Ep is widely available, lacking patent protection and burdensome regulations governing its use. These factors, including the considerable descriptive data already available, make 2102Ep a very attractive reference material for the hESC field.

**ACKNOWLEDGMENTS**

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**DISCLOSURES**

The authors indicate no potential conflicts of interest.

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**Table 3. Top expressed microRNAs in NCode analysis**

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<tr>
<th>Name</th>
<th>Ntera2</th>
<th>BG01V</th>
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<th>HuES20</th>
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<td>miR_21</td>
<td>miR_302b</td>
<td>miR_21</td>
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<td>miR_302b</td>
<td>miR_302d</td>
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<td>miR_302b</td>
<td>miR_302a</td>
</tr>
<tr>
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<td>miR_17_5p</td>
<td>miR_302a</td>
<td>miR_302a</td>
<td>miR_302d</td>
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<td>miR_302d</td>
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<tr>
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<td>miR_106a</td>
<td>miR_27</td>
<td>miR_302a*</td>
<td>miR_302*c</td>
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Selected microRNAs detected by array in 2102Ep, NTERA-2 or undifferentiated human embryonic stem cell (hESC) lines. Top 10 microRNA thought to be rare in hESC/EC are in italics. miR_302a* is thought to be derived from the same precursor as miR_302a.

**Table 4. NCode analysis of hESC-associated microRNA expression**

<table>
<thead>
<tr>
<th>Name</th>
<th>Ntera2</th>
<th>BG01V</th>
<th>HuES9</th>
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<tr>
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<td>−25.0</td>
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<td>3.28</td>
<td>−94.40</td>
<td>hESC</td>
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<tr>
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<td>−3.03</td>
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<td>−2.38</td>
<td>−2.66</td>
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<tr>
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<td>−4.7</td>
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<td>10.90</td>
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<tr>
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</tr>
<tr>
<td>miR_367</td>
<td>−6.02</td>
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<tr>
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<td>5.72</td>
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<tr>
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<td>19.77</td>
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<td>8.98</td>
<td>7.40</td>
<td>20.42</td>
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Selected microRNAs detected by array in 2102Ep, NTERA-2, or undifferentiated hESC lines. Ncode polymerase chain reaction validation of known hESC markers and quantification of their amounts relative to 2102Ep cells. Negative numbers represent underexpression relative to 2102Ep; positive numbers represent overexpression. Fold differences greater than 4 were considered to be significant and are presented in bold. Abbreviations: EC, embryonal carcinoma; hESC, human embryonic stem cell; N/D, not determined.
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See www.StemCells.com for supplemental material available online.
Qualification of Embryonal Carcinoma 2102Ep As a Reference for Human Embryonic Stem Cell Research
Stem Cells 2007;25:437-446
DOI: 10.1634/stemcells.2006-0236

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