RESOURCE

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Characterization of human embryonic stem cell lines by the International Stem Cell Initiative

The International Stem Cell Initiative[1]

The International Stem Cell Initiative characterized 59 human embryonic stem cell lines from 17 laboratories worldwide. Despite diverse genotypes and different techniques used for derivation and maintenance, all lines exhibited similar expression patterns for several markers of human embryonic stem cells. They expressed the glycolipid antigens SSEA3 and SSEA4, the keratan sulfate antigens TRA-1-60, TRA-1-81, GCTM2 and GCT343, and the protein antigens CD9, Thy1 (also known as CD90), tissue-nonspecific alkaline phosphatase and class 1 HLA, as well as the strongly developmentally regulated genes *NANOG*, *POU5F1* (formerly known as *OCT4*), *TDGF1*, *DNMT3B*, *GABRB3* and *GDF3*. Nevertheless, the lines were not identical: differences in expression of several lineage markers were evident, and several imprinted genes showed generally similar allele-specific expression patterns, but some gene-dependent variation was observed. Also, some female lines expressed readily detectable levels of *XIST* whereas others did not. No significant contamination of the lines with mycoplasma, bacteria or cytopathic viruses was detected.

Since 1998, when the first human embryonic stem (hES) cell lines were reported, a plethora of genetically diverse cell lines have been derived from human blastocysts. Techniques for their derivation and subsequent cell culture have varied from laboratory to laboratory[1]. Combined with the intrinsic genetic variation in the human samples, any of these additional sources of variation could lead to the selection of hES cell lines with substantially different properties. The available data also suggest differences between human and mouse ES (mES) cells[2–4]. Despite the opportunity for genetic and environmental influences affecting the phenotype of hES cell lines isolated by different investigators, few lines have been subjected to in-depth analysis, and new isolates are often published without detailed characterization data. As a consequence it is uncertain whether the different isolates of hES cells are indeed very similar, or whether significant differences exist between the various lines. To address this issue, The International Stem Cell Initiative (ISCI)[5] was established by the International Stem Cell Forum (http://www.stemcellforum.org.uk) to carry out a comparative study of a large and diverse set of hES cell lines derived and maintained in different laboratories worldwide. The goals of this study, the results of which we now report, were to assess the similarities and differences in the expression of commonly used markers of hES cells among as many as possible hES cell isolates, and to identify a set of well-validated markers to establish hES cell identity.

Several approaches have been used to characterize hES cells, but the most widespread are analyses of cell surface–antigen phenotype, often by flow cytofluorimetry, and gene expression studies, commonly assessed by RT-PCR and, increasingly, by microarray analyses, for example[6–11]. Many cell-surface antigens used to identify hES cells were first detected using antibodies prepared against preimplantation mouse embryos and/or against mouse or human embryonal carcinoma (hEC) cells[3,12]. Likewise, several gene products that are functionally associated with maintenance of the undifferentiated ES cell state were first recognized in mES cells, most notably *POU5F1* (refs. 13–15), *NANOG* (refs. 16,17) and *SOX2* (ref. 18). Other developmentally regulated genes, such as *TDGF*[19], and many identified in microarray studies have been postulated to play key functions in hES cells, but there is less evidence to support their role in maintenance of the undifferentiated state. Nevertheless, they may represent useful markers for identification of pluripotent stem cells. Several studies have compared patterns of gene expression in various hES cell lines, with some reporting similarities[6–8,20,21] and others emphasizing differences[10,11,22] between different hES cell lines. However, these studies used only small numbers of hES cell lines.

ES cells are derived from early embryos at a time when epigenetic reprogramming is occurring[23], and it is unclear whether their epigenetic status is stable or subject to perturbation. Imprinted genes, whose parent-of-origin–dependent expression is epigenetically regulated, have variable allele-specific expression and methylation patterns in some mES cells[24,25]. In contrast, studies of a limited number of hES cells suggest that genomic imprinting in hES cells is rather more stable[26,27], but the generality of this conclusion remains in question.

To provide a more comprehensive assessment of the hES phenotype, the present ISCI study analyzed 59 independently derived hES cell lines, from 17 laboratories in 11 countries, for expression of 17 cell-surface antigens and 93 genes, chosen as potential markers of the undifferentiated stem cells or their differentiated derivatives. In addition, the allele-specific expression status of ten imprinted genes was examined in those cell lines with distinct alleles. The microbiological status of the lines was also assessed, and the histology of a

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sample of xenograft teratomas was reviewed. Overall, the data show that all the independent hES cell lines studied exhibited a common expression pattern for a specific set of marker antigens and genes, despite their different genetic backgrounds and their derivation in different laboratories using different techniques. Although more subtle differences between the lines, either in phenotype or in behavior upon differentiation, cannot be excluded, this study provides no evidence for markedly distinct subsets of hES cell lines.

RESULTS

Cell-surface antigens

We examined the expression of a panel of cell-surface antigens widely used as markers of hES and hEC cells (**Fig. 1a**, **Supplementary Tables 1** and **2** online). An example of the expression patterns in one line, HUES1, is provided in **Supplementary Figure 1** online. These are: the globoseries glycolipid antigens SSEA3 (ref. 28) and SSEA4 (ref. 29); the keratan sulfate antigens TRA-1-60, TRA-1-81 (ref. 30), GCTM2 and GCTM343 (ref. 31); and a miscellaneous set of protein antigens comprising the two liver alkaline phosphatase antigens TRA-2-54 and TRA-2-49 (ref. 32), Thy1 (ref. 33), CD9 (ref. 34), and HLA class 1 antigens[35,36]. Several antigens commonly expressed following hES differentiation[33], SSEA1, A2B5, CD56 (NCAM), GD2 and GD3, were also analyzed. These all showed substantially lower expression than those commonly used as hES markers (**Fig. 1a**). In addition, expression of the pan-human antigen TRA-1-85 (Oka)[37] was assessed.

The relationship between the different cell lines was examined by cluster analysis (**Fig. 1b**). Overall, a high proportion of cells in all the hES cell lines examined expressed cell-surface antigen markers previously associated with undifferentiated stem cells, whereas few cells expressed markers associated with differentiated derivatives. The former group of antigens includes SSEA3 and SSEA4, which appear to be expressed on a lower proportion of the hES cells than the keratan sulfate–associated antigens, a finding consistent with the notion that that SSEA3 and SSEA4 expression is more rapidly downregulated on differentiation than the keratan sulfate antigens[33,38]. Only subtle quantitative differences were found between the various hES cell lines, most likely reflecting variation in culture conditions; for example, the cell lines CA1 and CA2, when assayed after culturing in two different media, appeared in separate clusters, but these were based upon only small quantitative changes in levels of expression and

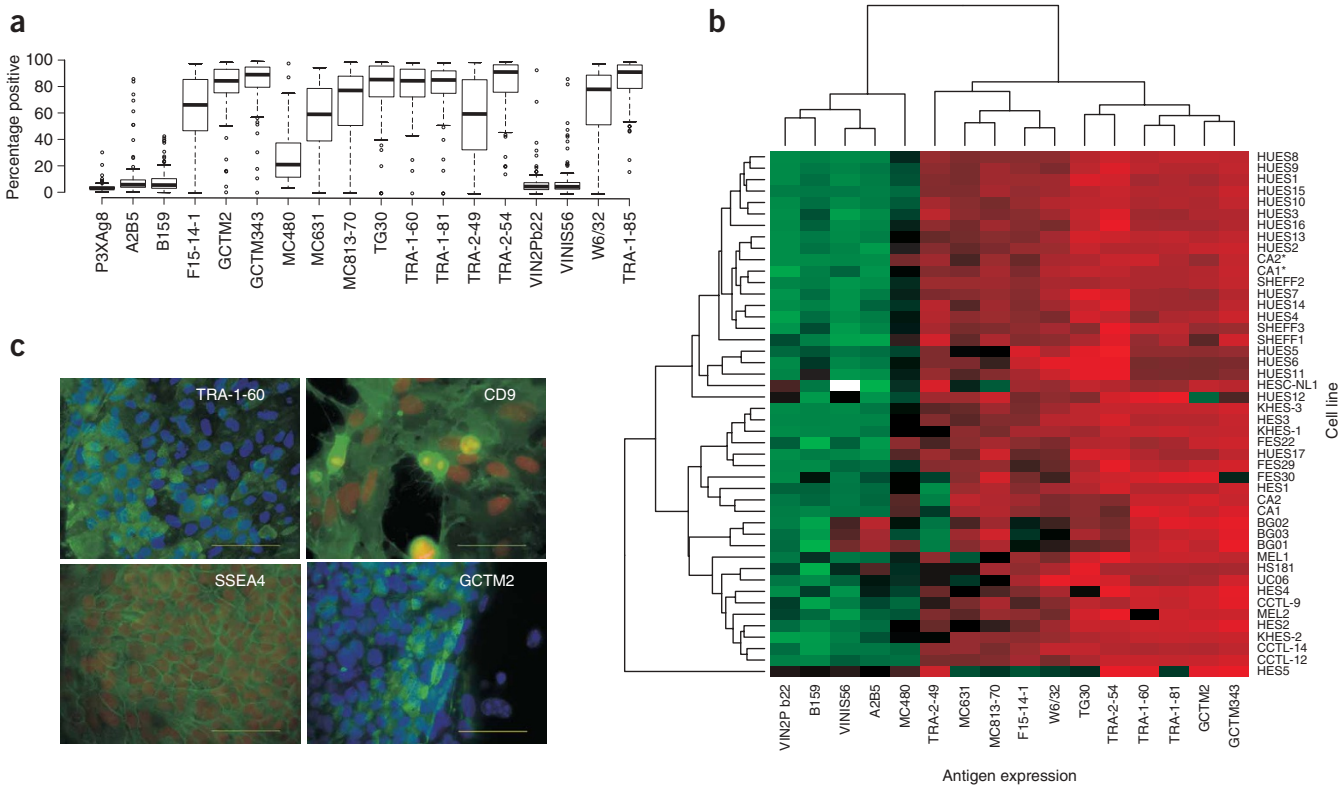

Figure 1 Surface-antigen expression patterns of undifferentiated hES cells. (**a**) Box and whisker plots illustrating the range of percent antigen positive cells in cultures of undifferentiated human ES cells. This analysis was carried out on data from those cell lines (44 cell lines from 12 laboratories), which met FACS quality control criteria defined using the reference standard hEC cell line, 2102Ep (**Supplementary Table 2**). The open circles indicate outlying data points. The antigens detected by each antibody are shown in parentheses: P3XAg8 (negative control), A2B5 (ganglioside GT3), B159 (NCAM), F15-14-1 (Thy-1), GCTM2 (GCTM2), GCTM343 (GCTM343), MC480 (SSEA1), MC631 (SSEA3), MC813-70 (SSEA4), TG30 (CD9), TRA-1-60 (TRA-1-60), TRA-1-81 (TRA-1-81), TRA-2-49 (liver alkaline phosphatase), TRA-2-54 (liver alkaline phosphatase), VIN2Pb22 (ganglioside GD2), VINIS56 (ganglioside GD3), W6/32 (HLA-A, B, C), TRA-1-85 (Oka). (**b**) Clustering of cell-surface antigen expression versus cell line. The heat map shows a two-way cluster analysis, using Euclidean distance measures, carried out on the data from those cell lines (44 cell lines from 12 laboratories), which met FACS quality-control criteria defined using the reference standard hEC cell line, 2102Ep, and for which two time points were available (**Supplementary Table 2**). Two cell lines (CA1 and CA2) were grown in both standard and nonstandard (alternate) conditions (indicated by an asterisk). For each antibody, the average percent cells scored positive (logit transformed) in two independent assays for each cell line is colored from green (low % positive) to red (high % positive); white indicates missing data. (**c**) Immunofluorescence detection of surface antigens in hES cells. Fixed colonies of hES cells were stained with antibodies TRA-1-60 (HES-2), TG30 detecting CD9 (HES-2), SSEA4 (H1), and GCTM-2 (HES-2). Nuclei were counterstained with DAPI (TRA-1-60 and GCTM2, blue; TG30 and SSEA4, red). Scale bar, 85 μm.

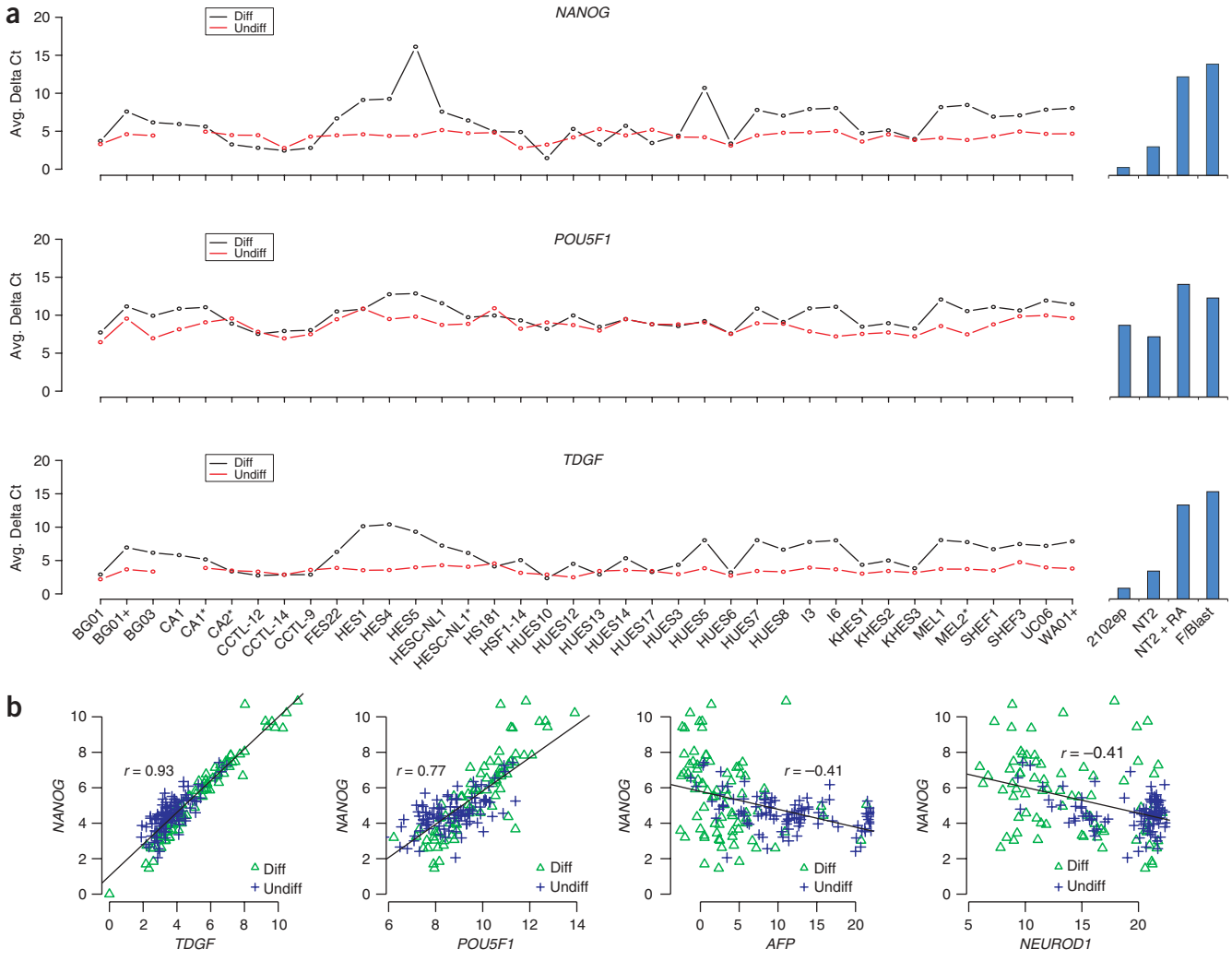

Figure 2 Expression profiles of the archetypal hES genes, *NANOG*, *POU5F1*(*OCT4*) and *TDGF*. (**a**) The expression of *NANOG*, *POU5F1* and *TDGF* was estimated by quantitative RT-PCR and is shown as DeltaCt values (average of the two time points for each cell line), relative to β-actin, for both undifferentiated hES cells (red line) and differentiated embryoid bodies derived from them (black line). Cell lines for which only single samples were available were excluded from the analysis, though they, too, showed comparable levels of gene expression (see **Supplementary Tables 3** and **4**). The results for several lines grown under both 'standard' and 'alternate' (*) conditions are included. For comparison, the corresponding bar charts indicate gene expression in the undifferentiated hEC cell lines 2102Ep and NTERA2, as well as in differentiated NTERA2 cells induced by exposure to retinoic acid (NTERA2.RA) for 7 d, and in the human foreskin fibroblast cell line, HS27. Note that an increase in DeltaCt value of 1.0 denotes a twofold decrease in mRNA level. (**b**) Correlation of gene expression (DeltaCt values) for *NANOG* expression with that of *POU5F1* and *TDGF*, genes expected to mark undifferentiated hES cells, and with that of *AFP* and *NEUROD1*, genes that mark differentiation into endodermal and neural cell types, respectively. Because the undifferentiated hES cultures and the embryoid bodies derived from them both appeared to contain variable proportions of undifferentiated cells and their differentiated derivatives, data from both sets were combined in each scatter plot to estimate the coefficient of correlation for the expression of each gene with NANOG. In this analysis, the data from the two time points were not averaged; undifferentiated and differentiated samples are indicated by blues crosses and green triangles, respectively. Note the strong positive correlation of *NANOG* with *POU5F1* and *TDGF*, consistent with the downregulation of all three genes upon differentiation, and the negative correlation of *AFP* and *NEUROD1* expression with that of *NANOG*, consistent with their up-regulation upon differentiation.

not on any significant qualitative differences. We could discern no significant clustering linked to passaging technique. One cell line, HES5, clustered separately from all the others, most likely the result of spontaneous differentiation in this culture; that is, a higher proportion of HES5 cells expressed the differentiation-marker antigens and a lower proportion, the stem cell antigens. Thus, the panel of antigens used in this study allows robust identification of undifferentiated hES cells.

The same antigens were also assayed by indirect immunofluorescence of fixed hES cell cultures (**Fig. 1c**). In general the results qualitatively matched the observations from flow cytometry, with most cells in a given culture expressing the antigens associated with the undifferentiated state, but with isolated cells or clusters of cells expressing antigens associated with differentiated derivatives. For example, antigens showing high levels of mean fluorescence intensity by flow cytofluorimetry (e.g., SSEA4, TRA-1-60, GCTM2 and CD9) were readily detected by *in situ* immunostaining, whereas detection of SSEA3 reactivity was more variable. Some antigens, such as SSEA4 and CD9, showed staining patterns typical of integral membrane components, whereas others, such as the keratan sulfate antigens recognized

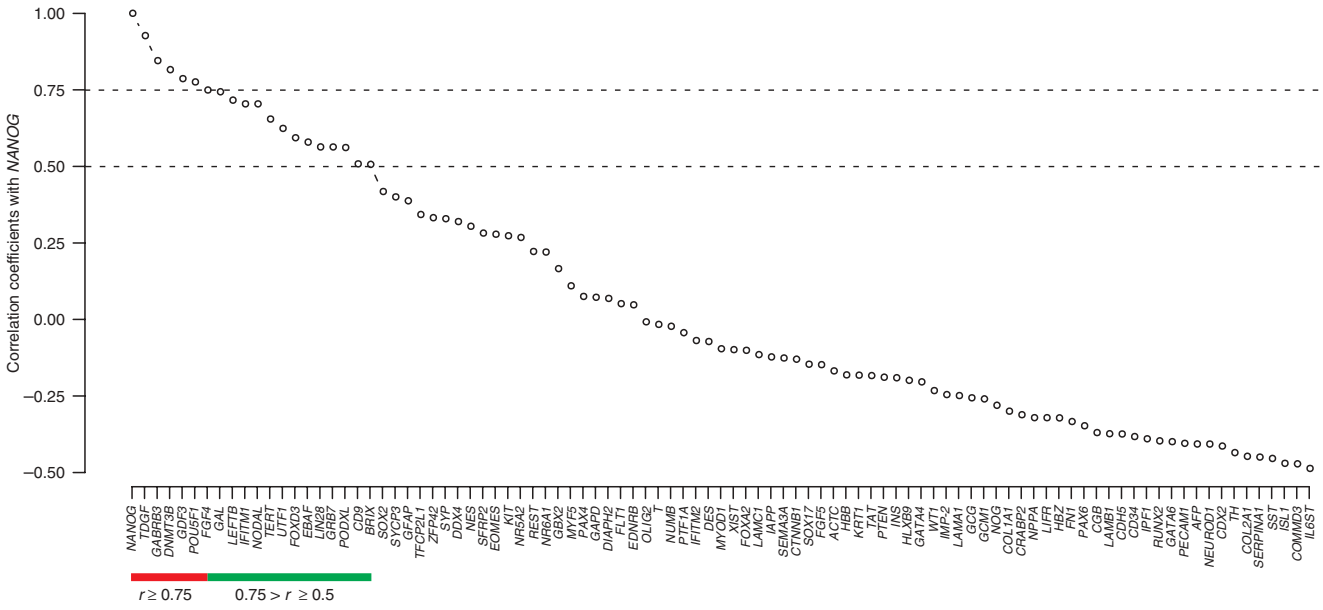

Figure 3 Pairwise correlation of expression of *NANOG* with that of all tested genes. The pairwise correlation coefficient of *NANOG* expression with the expression of each gene analyzed was calculated from the combined data for the undifferentiated hES samples and differentiated embryoid body samples, and plotted in descending order. In this analysis, the data from the two time points were not averaged. Expression of five genes, *TDGF*, *POU5F*, *GABRB3*, *GDF3* and *DNMT3B*, showed very strong correlations with *NANOG* expression ($r \geq 0.75$), whereas 14 additional genes, *FGF4*, *GAL*, *LEFTB*, *IFITM1*, *NODAL*, *TERT*, *UTF1*, *FOXD3*, *EBAF*, *LIN28*, *GRB7*, *PODXL*, *CD9* and *BRIX* showed strong but lower correlation ($0.75 > r \geq 0.5$).

by GCTM2 and TRA-1-60, showed a granular pattern of reactivity around the cell and on the culture surface characteristic of proteins deposited in the pericellular matrix (**Fig. 1c**).

Gene expression

Quantitative RT-PCR was used to assay the expression of a series of genes in stock cultures of undifferentiated hES cells and in differentiated embryoid bodies derived from them (**Supplementary Table 3** online). These genes had been previously suggested as markers of undifferentiated hES cells or of their differentiated derivatives. Among them, *NANOG*[16,17], *POU5F1* (ref. 13) and *TDGF*[39] are well known to be closely associated with the pluripotent state in both mES and hES cells, and to be strongly downregulated upon their differentiation. They can be regarded as archetypal pluripotent stem cell markers. The level of expression of these three genes was remarkably consistent across all the undifferentiated hES samples tested (**Fig. 2a**, red lines, and **Supplementary Table 4** online). Slightly higher levels (that is, lower DeltaCt values), particularly of *NANOG* and *TDGF*, were evident in the two undifferentiated hEC cell lines, 2102Ep and NTERA2, included for comparison, probably a reflection of the greater tendency for spontaneous differentiation in the hES cultures (**Fig. 2a**, bar charts on right hand side). Rather more variation in the levels of these three genes was seen in the hES embryoid body samples (**Fig. 2a**, black lines), although their continued expression suggested the persistence of undifferentiated stem cells in these preparations. For reference, the differentiated NTERA2 cells induced by retinoic acid, and a human fibroblast line, expressed substantially lower levels of all three genes compared with the undifferentiated hEC cells. In undifferentiated hES and differentiated embryoid body samples analyzed separately (data not shown), and in combined datasets (**Fig. 2b**), the levels of expression of the three archetypal stem cell genes, *NANOG*, *POU5F1* and *TDGF*, were strongly positively correlated whereas their expression was negatively correlated with the levels of expression of *AFP* and *NEUROD1*, markers commonly associated with differentiation to extraembryonic endoderm and neurectoderm, respectively. This is consistent with variable levels of expression of the pluripotency genes among different cell lines being a result of variable levels of spontaneous differentiation.

We next tested pair-wise correlations in expression with other genes in the panel, using *NANOG* as a reference, and the combined data from 'undifferentiated' and 'differentiated' (embryoid body) samples (**Fig. 3**). This combination provides a dataset containing a wider range of stem-to-differentiated cell proportions than either dataset alone, and so a more powerful test to discriminate between genes that mark the undifferentiated and differentiated cells. In this analysis expression of five genes showed a high correlation with that of *NANOG* ($r \geq 0.75$): *TDGF*, *POU5F1*, *GABRB3*, *GDF3* and *DNMT3B*. All of these genes have been previously identified as expressed in undifferentiated hEC and hES cells[8], and as showing developmental regulation upon hES differentiation[40]. We suggest that these six genes constitute a core set of markers that could be used to define undifferentiated hES cells. Among the 59 independent hES cell lines from the primary laboratories and the four duplicates from the secondary laboratories studied here, none failed to express these genes.

Expression of an additional 14 genes (*FGF4*, *GAL*, *LEFTB*, *IFITM1*, *NODAL*, *TERT*, *UTF1*, *FOXD3*, *EBAF*, *LIN28*, *GRB7*, *PODXL*, *CD9*, *BRIX*) showed a marked, though weaker correlation ($r < 0.75$; $r \geq 0.5$) with that of *NANOG*. Each hES line studied here expressed each of these genes. Most of these have also been previously associated with undifferentiated hES cells (**Supplementary Table 3**), but the lower correlations with expression of *NANOG* most likely reflect their more widespread expression in derivatives differentiating from hES cells. A notable instance is *SOX2*, which is not included in this set of genes as its expression showed an even slightly lower correlation with that of *NANOG* ($r = 0.47$); *SOX2* is thought to play a role in undifferentiated hES cells, and is closely linked in a regulatory loop

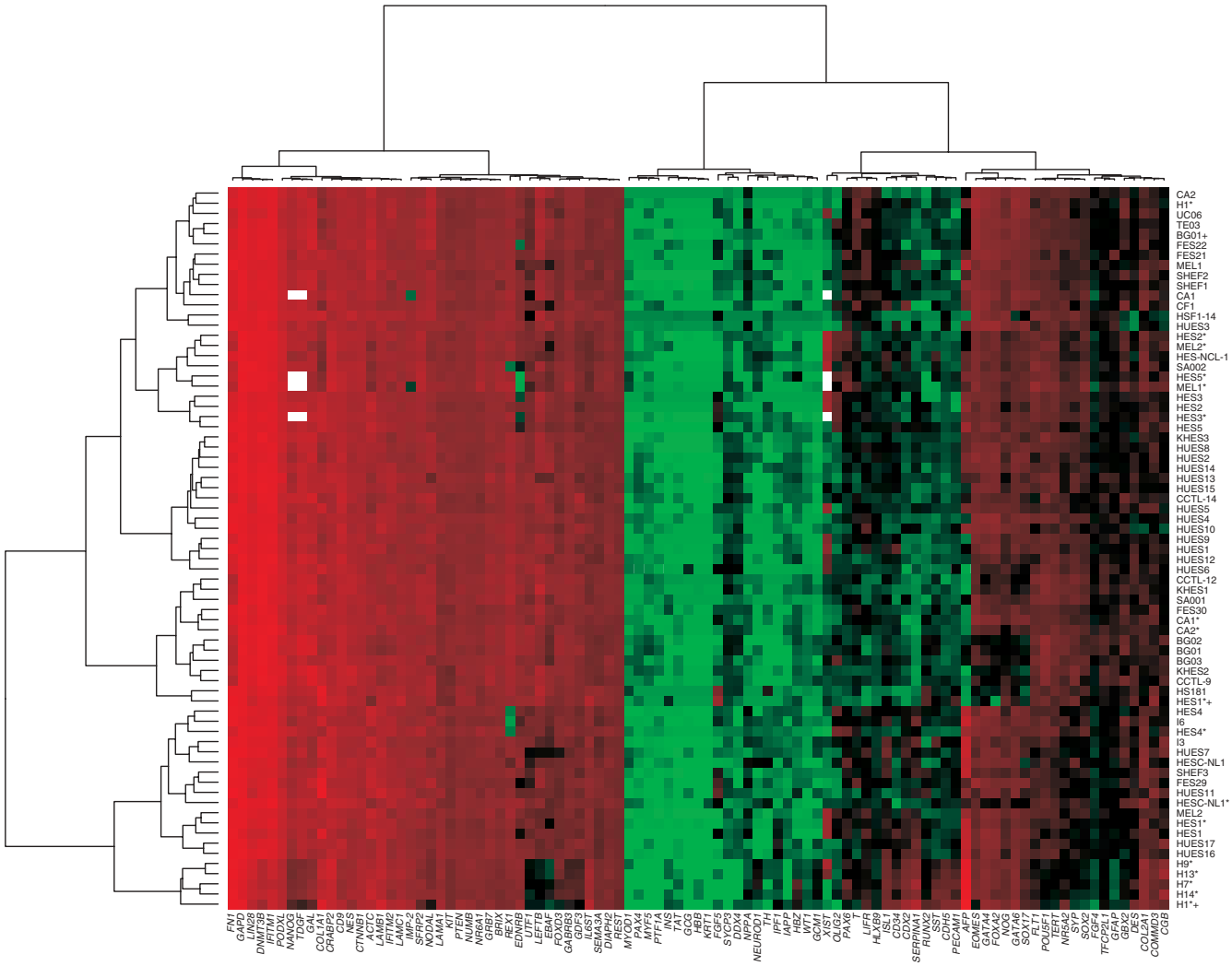

Figure 4 Two-way cluster analysis of hES cell lines with respect to gene expression. Hierarchical clusters were generated for the cell lines and gene expression levels from the undifferentiated hES cell samples; when data for two time points for a given cell line were available the results were averaged (**Supplementary Table 4**). The levels of gene expression relative to β-actin are indicated by the color change from red (high expression levels; low DeltaCt) to green (low expression levels, high DeltaCt); white indicates missing data. The results for several lines grown under both 'standard' and 'alternate' (*) conditions, or grown in separate primary and secondary laboratories (+) are included. Note cell line CF-1 is now renamed KCL-003-CF-1.

with *NANOG* and *POU5F1* (refs. 41,42), but it is also expressed in the neurectoderm lineage[43].

Apart from the expression of a number of genes showing weak positive correlations with that of *NANOG*, the expression of several exhibited strong negative correlations, consistent with the view that they are not expressed by the undifferentiated cells and are good markers of differentiation. Indeed these genes had been selected, a priori, as candidates to identify the appearance of particular differentiated lineages. For example, genes whose expression showed a negative correlation with that of *NANOG* ($r < -0.25$) included *CDX2* and *CGB* (encoding the beta chain of human chorionic gonadotropin, *βHCG*), associated with trophectoderm differentiation[44]; *GATA6* (ref. 45) and *AFP*[46], associated with extraembryonic endoderm; and *PAX6* (ref. 47) and *NEUROD1* (ref. 48), associated with the neural lineage.

We next examined whether a two-way cluster analysis of gene expression in the undifferentiated samples would reveal any significant groupings of genes or significant differences between groups of cell lines (**Fig. 4** and **Supplementary Table 4** online). Indeed, this analysis revealed a substantial cluster of genes expressed by essentially all the undifferentiated hES cell samples, and included those genes that were downregulated upon differentiation, identified in the above correlation analysis. It also included a set of genes that are commonly associated with differentiated cell lineages, such as *FN1*, *ACTC*, *LAMA1*, *AFP*, *EOMES*, *GATA4*, *FOXA2*, *GATA6*, *SOX17*, *FLT1*, *SYP*, *COL2A1* and *COL1A1*. Whether these are expressed in the undifferentiated stem cells themselves or in a set of differentiated derivatives that are always present in association with the undifferentiated cells as a result of spontaneous differentiation cannot be ascertained from the present data. It is notable that many of these genes are thought to be expressed by extraembryonic endoderm, which might be an inevitable early derivative of ES cells to appear on differentiation. In this regard, *CGB (HCG-β)*, *GCM1* and *CDX2*, which are markers of trophectodermal cells, the other extraembryonic lineage produced by hES cells, were not inevitably present.

However, this cluster analysis provided no evidence of any discrete subsets of the hES cell lines in which the undifferentiated cells differed qualitatively with respect to the expression of the core stem cell genes identified above. Some clustering of lines was apparent, but inspection suggested that this was largely due to subtle quantitative differences in levels of gene expression and could be most easily explained by varying amounts of spontaneous differentiation occurring in the cultures from which RNA was isolated. This was most marked in a cluster comprising H14, H7, H13 and H9. These showed somewhat higher expression of genes associated with differentiation, most strikingly *AFP*, and lower levels of various stem cell associated genes, suggesting that the particular cultures analyzed contained a

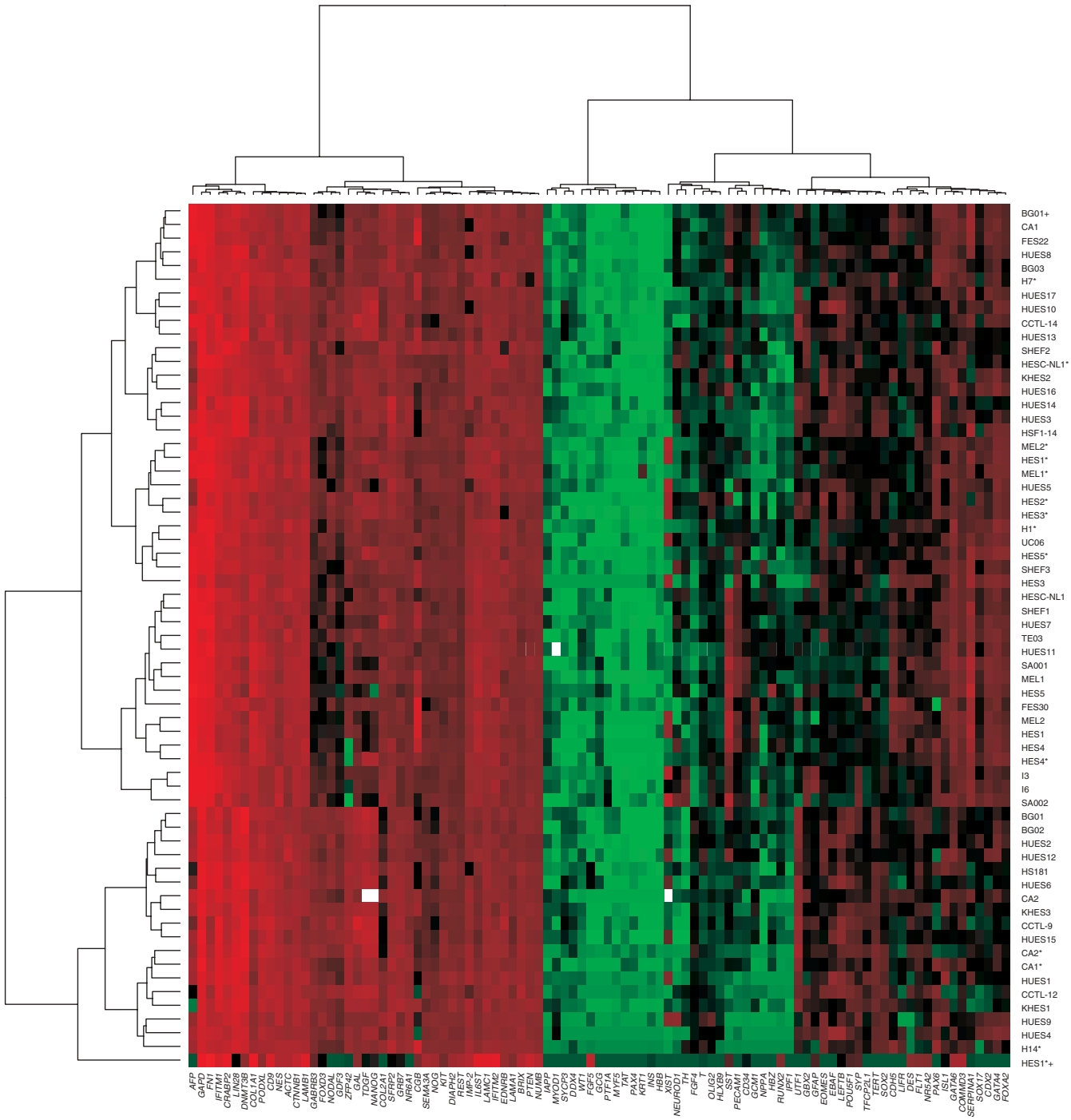

Figure 5 Two-way cluster analysis of differentiated hES cell lines with respect to gene expression. Hierarchical clusters were generated for the cell lines and gene expression levels from the differentiated hES cell samples; when data for two time points for a given cell line were available, the results were averaged (**Supplementary Table 4**). The levels of gene expression relative to beta-actin are indicated by the color change from red (high expression levels; low DeltaCt) to green (low expression levels, high DeltaCt); white indicates missing data. The results for several lines grown under both 'standard' and 'alternate' (*) conditions, or grown in separate primary and secondary laboratories (+) are included.

Table 1 Summary of allele-specific imprinted gene expression analysis[a]

	Imprinting status of samples analyzed			
	Monoallelic (0–14% minor allele)	Partial-allelic (15–29% minor allele)	Biallelic (30–50% minor allele)	Number of different hES cell lines analyzed
Paternally expressed genes				
SNRPN	66	0	0	24
IPW	30	0	0	14
KCNQ1OT1	54	0	0	23
PEG3	8	0	0	3
IGF2[b]	37	5	10	20
MEST[c]	9	9	20	16
Maternally expressed genes				
H19	53	0	1	20
NESP55	11	0	1	9
MEG3[d]	9	0	3	7
SLC22A18[e]	1	6	16	11
Totals (349 samples)	278 (79.7%)	20 (5.7%)	51 (14.6%)	46 different lines

[a]The contribution of 'minor' allele expression to total expression of each gene was determined by calculating the area under the peak from cDNA sequencing chromatograms. Results were grouped into monoallelic (0–14% minor allele contribution to total gene expression), partial-allelic (15–29% minor allele contribution) and biallelic (30–50% minor allele contribution). The number of individual samples analyzed does not equal the number of hES cell lines studied; this is because each of the hES lines was represented by samples at more than one time point or differentiation status, and conversely, not all of the samples produced analyzable DNA and RNA (see **Supplementary Table 5** for a detailed summary of the time points, status and yield of samples for each hES line). [b]Twenty cell lines (out of 20 analyzed) provided one or more samples that were monoallelic for *IGF2*, with 12 lines monoallelic in every sample. Six cell lines provided one or more samples that were biallelic for *IGF2*, but none of the lines examined were biallelic in every sample. [c]Five cell lines (out of 16 analyzed) provided one or more samples that were monoallelic for *MEST*, with three lines monoallelic in every sample. Eleven cell lines provided one or more samples that were biallelic for *MEST*, with six lines biallelic in every sample. [d]All samples of the hES line HES-4 were biallelic for *MEG3* ($n = 3$). [e]Eight cell lines (out of 11 analyzed) provided one or more samples that were biallelic for *SLC22A18*, with six lines biallelic in every sample.

higher proportion of differentiated cells than the average across all the samples. When we examined cases where the same lines were grown in standard and alternate conditions (e.g., the two lines CA1 and CA2 grown with and without plasminate, and the HES1-5 lines grown in KSR or FCS), there were some quantitative differences in gene expression levels, but no consistent clustering that could be ascribed to variations in the media. More likely, these differences also reflected random fluctuations in the levels of spontaneous differentiation.

We finally examined a two-way cluster analysis of gene expression in the differentiated samples (**Fig. 5**). All expressed those genes identified above as characteristic of undifferentiated hES cells, indicating the continued presence of undifferentiated stem cells in these embryoid body preparations. In addition, there were significant variations in expression of a number of genes associated with distinct pathways of differentiation. For example, *PAX6*, a neural lineage marker, was expressed strongly in the embryoid bodies of some lines but not others. However, given the stochastic nature of differentiation in embryoid bodies and the difficulties of standardizing this method, these results cannot be taken to indicate intrinsic differences between the hES cell lines in their capacity for differentiation. More detailed studies would be required to test such a hypothesis.

Imprinting and X inactivation

To address the epigenetic status of hES cell lines, we assessed the allele-specific expression of ten genes subject to genomic imprinting (**Table 1**). Of the 59 independent hES cell lines submitted to the ISCI, 46 provided informative heterozygous polymorphisms within one or more of these imprinted genes. Occasionally, allele-specific imprinted gene expression patterns were different between samples of the same hES cell line, perhaps reflecting epigenetic changes between different time points. However, because the structure of the study did not permit detailed follow-up, it was not possible to exclude technical problems in individual cases. Accordingly, conclusions have been drawn from the data as a whole on the basis of samples analyzed rather than from individual cell lines (**Table 1** and **Supplementary Table 5** online).

Overall, monoallelic expression of imprinted genes was the most common result observed. Thus, of the six paternally expressed genes studied, four (*IPW*, *SNRPN*, *KCNQ1OT1* and *PEG3*) showed monoallelic expression in all samples of all the cells analyzed. Of the four maternally expressed genes studied, two (*H19* and *NESP55*) were predominantly monoallelically expressed, although in each case one sample from a single cell line was scored biallelic (**Table 1**). Of the remaining genes, the paternally expressed gene *IGF2* and the maternally expressed gene *MEG3* were mostly monoallelically expressed, although a substantial proportion of samples ($\sim$20–30%) were scored as partial- or bi-allelically expressed. On the other hand, the paternally expressed gene *MEST* and the maternally expressed gene *SLC22A18* were predominantly bi-allelically expressed, albeit with a substantial proportion ($>$20%) of mono- and partial-allelic samples.

As a further assessment of epigenetic status, we looked for evidence of X chromosome inactivation in female cell lines. In both hES and mES cells, the absence of X inactivation in the undifferentiated cells, with subsequent inactivation upon differentiation, has been reported[49,50]. However, this is not invariably the case, and instances of inactive X chromosomes in undifferentiated cells have also been reported[40,50,51]. We therefore tested the expression of *XIST* in all the hES samples submitted to the ISCI (**Fig. 6**). With the exception of the undifferentiated samples of one line, H13, all the karyotypically male hES lines expressed low or undetectable levels of *XIST* (DeltaCt $>$ 13) (**Fig. 6a**). Within the context of the current screen, the individual significance of the one outlier cannot be assessed. By contrast, the female hES lines fell into two broad groups (**Fig. 6b**), one group in which *XIST* levels were low or undetectable (DeltaCt $>$ 13), matching the male lines, and a second group in which *XIST* was readily detectable (DeltaCt $<$ 10). These results indicate marked heterogeneity

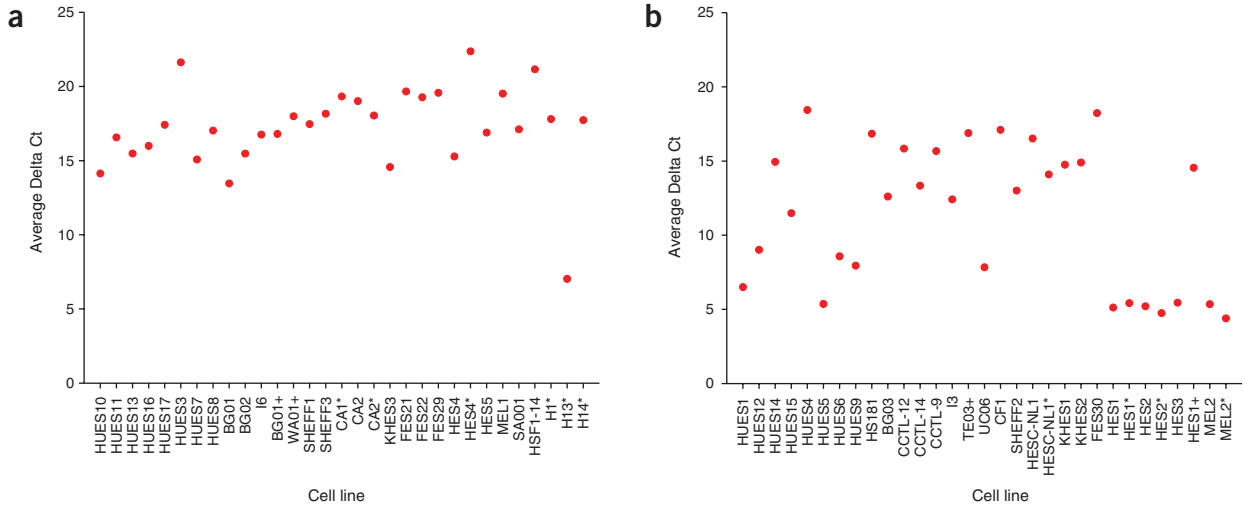

Figure 6 *XIST* expression in male and female hES cell lines. (**a**,**b**) The level of *XIST* expression (estimated as DeltaCt values relative to β-actin) is shown separately for those karyotypically male (**a**) and female (**b**) undifferentiated hES cell lines. When results from two time points were available, these have been averaged. Note that an increase in DeltaCt value of 1.0 denotes a twofold decrease in mRNA level. Note cell line CF-1 is now renamed KCL-003-CF-1. The results for several lines grown under both 'standard' and 'alternate' (*) conditions, or grown in separate primary and secondary laboratories (+) are included.

between the female hES cell lines with respect to X-chromosome inactivation, a conclusion reflected by the contrasting results previously reported[40,50,51]. The reasons for this variability might relate to the X-chromosome inactivation state of the particular embryonic cells from which individual hES lines were derived, but it may be related to either cellular differentiation within the hES cell culture[50] or adaptation to culture conditions[40].

Microbiology of feeder cells and hES cell lines

Sixteen human and mouse feeder cell samples were submitted from different laboratories, representing the feeder cells on which the hES cells were maintained. All tested negative for bacterial, fungal and mycoplasma contamination. Also, in cell line inoculation tests, with subsequent testing for hemadsorption of chick and guinea pig red blood cells, no evidence of viral infection was revealed, and these same cells showed no evidence of viral particles, inclusion bodies or aligned or unaligned viral nucleoprotein by transmission electron microscopy. Of the 59 independent hES cell lines studied, samples from 58 were submitted from the primary laboratories for microbiology, together with an additional four from the secondary laboratories. One sample was positive for fungal contamination; none were positive for bacteria or mycoplasma. In a minority of cases (eight cell lines; **Supplementary Table 6**) antibiotics had been used in growth media sampled for sterility tests. Consequently, in these few cases, we cannot exclude the presence of low-level contamination, which could have been masked by the presence of antibiotics. None of the hES lines showed any evidence of viral infection when assayed, as described above for the feeder cells.

Xenografted tumors

A systematic study of the conditions that affect the development of xenografted human ES cell–derived tumors was not planned within the present study. Nevertheless, we were able to obtain a review by a well-qualified clinical pathologist, with long experience of teratoma biology, of 37 histological slides from xenograft tumors produced from 15 independent hES lines in immunocompromised mice by the participating laboratories. In almost all cases the tumors were teratomas, benign tumors composed of somatic tissues derived from each of the three embryonic germ layers—ectoderm, mesoderm and endoderm (**Supplementary Fig. 2**). Ectodermal and mesodermal tissues predominated, most with the appearance of mature somatic tissues. However, neural tissue was often present in the form of immature embryonic neural rosettes. Mesodermal tissues were found in all grafts, and included fibroblasts and capillaries, smooth muscle, striated muscle, cartilage, bone and fat cells. Endodermal tissues included gland-like structures lined with columnar or cuboidal epithelium, which occasionally contained mucus-secreting goblet cells or ciliated cells, and even cells resembling intestinal Paneth cells. Three grafts contained foci of undifferentiated cells, which had the morphologic features of hEC cells. The nature of these EC-like cells could not be determined, but they might represent either residual, undifferentiated hES cells or hES cells that had undergone malignant transformation into true embryonal carcinomas. In one case, the investigator had explanted the tumor and observed outgrowth of karyotypically abnormal cells with a phenotype consistent with both hES and hEC cells[52].

DISCUSSION

The current study is an initial attempt to provide a systematic and comparative survey of the many different human ES cell lines established and maintained by various groups around the world. Previously, some smaller-scale comparisons of lines maintained in single laboratories have been undertaken[22]. It is difficult to know exactly how many lines have so far been derived, although estimates range from 200 to >300 (refs. 53,54). The current panel of 59 independent lines provides a representative sample of those readily available worldwide. Overall, the microbiological assays pointed to the high standards of cell culture in the different laboratories and the absence of significant contamination from adventitious agents.

In such a survey, detailed studies of individual lines are not possible and, inevitably, obtaining complete sets of data for each submitted line is subject to the vagaries of experimental variation. Thus, fluorescence-activated cell sorting (FACS) or gene expression data were not obtained for all time points for every cell line. In particular, difficulties

in standardizing FACS assays of surface-antigen expression in different laboratories became apparent as we analyzed the data collected, despite the provision of common batches of antibodies and protocols. This points to a need to develop standards for these tests, which will be addressed in the second ISCI that is currently being planned.

Certainly, there were differences between the hES cell lines included in the current ISCI study, for example, variable *XIST* expression in the female lines, and differences in the imprinting status of some genes. Nevertheless, a global analysis of all the data collected indicates a similar pattern of expression of a series of characteristic surface antigens and genes by hES cells that have been derived by different laboratories, using various protocols, from different gene pools of donated embryos. The similarities were evident despite the small variations in culture media and conditions used by some laboratories. All the lines examined expressed a comparable spectrum of genes and surface marker antigens characteristic of hES cells, suggesting that there is a common set of markers that can be used in general to monitor the presence of pluripotent stem cells. Contrary to a recent suggestion that SSEA3 and SSEA4 may not be acceptable markers for hES cells because they are not essential for human ES cell pluripotency[55], the expression of these antigens by all tested hES cells makes them valuable operational markers of this cell type. Of course, rare variants may emerge as more lines are studied. For example, the known polymorphism for SSEA4 expression on red blood cells[56] suggests that about 1% of hES cells derived from populations of European ancestry may prove to be SSEA4(–), although for most purposes SSEA4 is a good indicator for undifferentiated hES cells. Also, the results do not exclude more subtle variations among the cell lines, either with respect to quantitative levels of expression of specific antigens or genes, or in differentiation. One observation that might impinge on such potential variability is the evident potential for culture adaptation and genetic instability of hES cell lines during prolonged passage[57,58].

The results of the current study demonstrate the existence of both similarities and differences between hES and mES cells, as well as the closely related molecular properties of hES and hEC cells. Thus, the SSEA1(–)/SSEA3(+)/SSEA4(+) phenotype of hES cells versus the SSEA1(+)/SSEA3(–)/SSEA4(–) phenotype of mES cells is confirmed[3,12,59]. Also the presence of markers of trophectodermal differentiation in hES cell cultures agrees with the apparent increased ability of hES cells to adopt this lineage of differentiation in contrast to their murine counterparts[60], although the failure to detect trophectodermal differentiation in the hES cell xenograft tumors that were examined is notable. On the other hand, of the group of genes whose expression correlates closely with that of *NANOG* and appears to be characteristic of the hES cells (*NANOG*, *POU5F1*, *TDGF*, *GDF3* and *GABRB3*), all are also expressed by mES cells, with the possible exception of *GABRB3* (refs. 13,16,17,61,62).

It was notable that several of the genes that showed a positive correlation with *NANOG* expression (*EBAF*, *PODXL*, *NODAL*, *ZFP42*, *LIN28*, *EOMES* and *SFRP2*) were identified in ChIP on CHIP experiments as potentially direct targets of *NANOG*[42]. Also, several other genes identified in that study as potential *NANOG* targets (*ISL1*, *PAX6*, *CDX2*) showed a negative correlation in the present study. Thus, combining the correlation data from gene expression experiments with target-binding assays for pluripotency-specific targets may provide insight into positive and negative control in the regulatory networks that affect pluripotency in hES cells.

The results provided additional insight into the epigenetic status of hES cells, for which relatively few lines have previously been studied[26,27,63]. Overall, the results suggest that genomic imprinting appears to be relatively invariant in hES cells, at least at the loci examined here. Nevertheless, imprinting stability, as indicated by the presence or absence of monoallelic expression, appears to be gene dependent, with some imprinted genes showing a consistent pattern in all hES cells and others being variable. In the case of one such variable gene, *IGF2*, contrasting results were obtained between samples of the same line, I3 (TE03), submitted by two separate laboratories (that is, it was consistently monoallelically expressed in samples from one laboratory and biallelically expressed in samples from the other). This result suggests that changes in imprinting patterns of at least the *IGF2* gene and the nearby *H19* gene[26] can occur upon prolonged culture. One possibility is that overexpression of *IGF2*, a likely growth factor for hES cells, might provide variant cells with a selective advantage. However, any conclusions must be tempered by the recognition that detailed information about the allele-specific expression of imprinted genes in the early human embryo is mostly lacking, and extrapolation from mouse embryo data might be misleading. Of note in this connection is the variable genomic imprinting of *SLC22A18* in humans[64,65]. Derivation of hES cells could perpetuate any such variability in imprinting if it were present in the inner cell mass cells of the blastocyst, from which the hES lines were derived.

The studies of *XIST* expression by the hES cells examined provided a mixed picture. Variation in X inactivation in the female lines, inferred from *XIST* expression, matched variation evident from contrasting reports of specific lines. One factor pertinent to this variability is the apparent selective advantage offered by the presence of multiple active X chromosomes in hES cells, suggested both by the loss of X-chromosome inactivation[40] and the appearance of multiple X-chromosomes in late-passage, culture-adapted hES lines[58]. Alternatively, it may be that in hES cells, like in mES cells, X inactivation occurs only after differentiation, and the differences between the various samples result from partial differentiation within the cultures.

The conclusions of the histology studies are based on a limited number of donated samples and must be considered tentative, but nevertheless represent independent and direct comparative analysis of tumors generated in different laboratories. Most xenografts produced from hES cells in immunocompromised mice were teratomas composed of fully differentiated somatic tissues. Strikingly, however, no extraembyonic tissue, such as trophoblast, was identified, although yolk sac–like tissue was tentatively identified around foci of hEC-like cells. Some of the somatic tissues, most notably the neural tubes, were immature, fetal-like, and proliferating, suggestive of a malignant potential. This and also the apparent persistence of undifferentiated hEC-like cells in a small number of xenografts raises the issue of the malignant potential of hES cells and highlights the need for detailed retransplantation experiments coupled with analysis of genomic stability to assess its potential significance.

Studies of the biology of hES cells have developed rapidly over the past eight years since the first reports of their derivation. They clearly offer enormous potential, not only for regenerative medicine, but also for drug discovery and toxicology, human developmental biology and cancer research. Advances in all of these fields require internationally co-ordinated work by numerous researchers to establish common standards and procedures that will allow the ready cross-verification of results between laboratories. The results of the present ISCI study provide a step in this direction, and indicate the commonality of many features of the hES cell lines currently in use. However, they do not exclude the possibility of more subtle differences between lines, for example, with respect to their differentiation potential. The present study also does not address the extent to which the phenotype of hES cells may be influenced by progressive genetic and epigenetic changes

that can occur upon prolonged culture under suboptimal conditions. A new initiative (ISCI2) is now planned to assess the nature of the genetic changes that occur in hES cells on prolonged passage and to compare the performance of various new media that have been proposed for the long-term maintenance of hES cells under more defined conditions. ISCI2 will also continue to maintain and expand the International Stem Cell Forum's Registry of validated hES lines established in ISCI1 (http://www.stemcellforum.org.uk), by providing arrangements for validation and inclusion of additional lines into the future.

METHODS

Study design. Members of the International Stem Cell Forum were asked to nominate laboratories in their countries that had derived hES cell lines. These 'primary' laboratories were invited to participate and to submit their hES cell lines for comparison in the study. Initially 75 lines were registered with the ISCI but, for practical reasons, material from only 63 lines was eventually analyzed (**Supplementary Table 6**). Of these, four were duplicated, with material being submitted both from a primary laboratory, and separately from cultures of the same cells maintained in one of two 'secondary' laboratories. Thus the study comprised 59 independent primary lines, together with the four duplicates included to provide some insights into the stability of lines between laboratories.

Owners of the different cell lines were asked to sign a participant's agreement, indicating that they agreed to inclusion of their hES cell lines in the study, and confirming that the cell lines had been derived in accordance with ethical standards for informed consent to embryo donation for research comparable with those required for hES cell derivation in the UK. These lines are generally available to the scientific community and contact details are provided in **Supplementary Table 7** online. The intention in the ISCI was that analyses should be conducted in as consistent and reproducible a manner as possible. Inevitably in a multilab study of this type, for quality control and other reasons, not every line was represented in every assay. For logistical and other reasons it was not practicable to transfer live cell cultures between laboratories. Accordingly, flow cytofluorimetric analyses of antigen expression were conducted in the individual laboratories of the ISCI participants. All other analyses, however, were carried out by single reference laboratories using samples prepared by the participating laboratories from their own cell cultures. Participants were asked, where possible, to culture their cells using a standard protocol and medium ('Standard' conditions), and to note any significant deviations (see **Supplementary Table 6**). In some cases, they also provided additional data and materials from cells grown under the conditions routinely employed in their laboratories ('Alternate' conditions). Each laboratory analyzed and provided material from two cultures of each of their hES cell lines, separated by at least one month, denoted as Time Point 1 (TP1) and Time Point 2 (TP2). They also carried out a simple differentiation protocol by culturing clumps of ES cells (embryoid bodies[66]) in suspension for 10 d, then providing material from these for gene expression studies (see below), again on two occasions. Because the embryoid bodies were only allowed to develop for 10 d, they were expected to retain a significant population of persisting hES cells, as well as their newly differentiated derivatives. Conversely, experience suggests that hES cell cultures themselves also contain some differentiated cells.

Data collection and analysis. An archive stock of hybridomas, comprising 17 monoclonal antibodies commonly used to study hES cells, was established at the UK Stem Cell Bank (National Institute for Biological Standards and Control, South Mimms, UK), with agreement from the different organizations that own them (**Supplementary Table 1**). Single batches of antibody were prepared, titrated and distributed to the participating laboratories for use in flow cytofluorimetric analyses of antigen expression according to a standard protocol. A well-characterized hEC cell line 2102Ep[67] was provided and cultured by each laboratory as a reference standard; for detailed cluster analysis, data from assays that did not meet specified criteria with respect to antibody reactivity with 2102Ep cells were excluded. Cells were also grown on glass slides, fixed and dispatched to a single laboratory (M. Pera, Monash University, Australia) for *in situ* immunofluorescence. DNA and RNA were prepared centrally from cell lysates submitted by participating laboratories (The UK Stem Cell Bank). Samples were then provided to single reference laboratories for gene expression and DNA fingerprinting (Geneservice) and for imprinting studies (P. Rugg-Gunn and R. Pedersen, University of Cambridge). Flow cytofluorimetric and gene expression data were subjected to statistical analysis (Computational Sciences Unit, The Jackson Laboratory). Culture samples were tested for mycoplasma, bacteria, fungi and viruses (The UK Stem Cell Bank, at National Institute of Biological Standards and Control), and preexisting slides from xenografts were analyzed by a single, experienced pathologist (I. Damjanov, University of Kansas).

Cells and cell culture. Individual participating laboratories chose which cell lines they would contribute to the initiative. Sufficient Knockout Serum replacement (KSR), bFGF and 'Knock-out' DMEM was donated and distributed to each laboratory by Invitrogen from a single lot of each reagent. The lots of KSR and bFGF were selected following a prescreen of two lots of each tested for their ability to support culture of H7 hES cells. Each laboratory was asked, where possible, to culture their cells in a 'Standard' medium, as previously described[68]. Full details of this medium are provide in **Supplementary Table 6**, which also records any significant deviations from this formulation adopted by particular laboratories. Cells were maintained on inactivated feeder cells as used by each group; in most cases these were mouse embryo fibroblasts but a few laboratories used human fibroblast feeders.

Generally, cultures were passaged using collagenase and scraping[68], but exceptions are noted in **Supplementary Table 6**. Several laboratories also provided samples of cells cultured in conditions normally used in those laboratories, denoted 'Alternate' conditions. Generally, the most significant difference between Standard and Alternate conditions was the use of FBS rather than KSR to supplement the medium; details are provided in **Supplementary Table 6**.

Cells were assayed and cell lysates prepared from two such undifferentiated cultures of each hES cell line, passaged for approximately a month between each sampling, denoted Time Point 1 and Time Point 2 (UT1 and UT2, respectively). Two separate batches of embryoid bodies, (denoted DT1 and DT2), were also prepared a month apart from each line by harvesting clumps of hES cells from stock cultures using collagenase and scraping, followed by culture in suspension for ~10 d in the same medium as used to culture the stock, undifferentiated hES cells.

For *in situ* antigen staining, each cell line was cultured in chambers (QuadriPerm dishes, Sartorius) on glass slides (ethanol resistant multitest slide with 12 wells, ICN, cat no. 604120E). After growth for several days the slides were fixed in 70% ethanol vol/vol, dried and shipped to a single central laboratory (M. Pera, Monash University) for immunofluorescence analysis.

For control purposes, each participating laboratory was provided with a culture of 2102Ep cl.4D3 hEC cells to be cultured at high density ($>5 \times 10^6$ cells per 75 cm^2 tissue culture flask) as previously described[67]. Limited differentiation of 2102Ep cells was induced by culture at low cell densities (seeding cells at 10^5 cells per 75 cm^2 tissue culture flask). Undifferentiated NTERA2 cl.D1 hEC cells were cultured, and differentiated NTERA2 cells were induced with retinoic acid as previously described[69].

Antibody production and antigen assays. Culture supernatants from each hybridoma were collected and the resulting preparation for each antibody was titrated by flow cytofluorimetry on 2102Ep and NTERA2 EC cells and NTERA2 cells induced to differentiate with retinoic acid, as previously described[70]. Monoclonal antibody MC480 was tested on a mouse EC cell line, P19 (ref. 71).

Aliquots from single batches of each antibody were distributed to each participating laboratory, together with a detailed protocol for flow cytofluorimetry to test for antigen expression (**Supplementary Methods** online). In all cases supernatant from the parental myeloma cell line P3X63Ag8 (ref. 72) was used as a negative control, whereas reactivity with the pan-human antibody TRA-1-85 (anti-Oka)[37] was used as a positive control.

As a quality control for flow cytofluorimetry, each laboratory tested all antibodies on the hEC cell line, 2102Ep[67] and results from assays on hES cells were only included in detailed further analyses if the laboratory scored 2102Ep cells strongly positive (>70%) for reactivity with at least three out of the four antibodies, TRA-1-85 and the anti-keratan sulfate antibodies TRA-1-60,

TRA-1-81 and GCTM2, and negative (<10% positive) for reactivity with the negative control antibody from P3X63Ag8. Undifferentiated hEC cells, like 2102Ep, are typically strongly positive for TRA-1-85, TRA-1-60, TRA-1-81 and GCTM2 reactivity and negative for reactivity with P3X63Ag8 (ref. 73). From the remaining assays, data were excluded if the hES samples were scored <50% positive for reactivity with TRA-1-85. Aliquots of the same antibody preparations were provided to the Central Laboratory (M. Pera), responsible for *in situ* staining of fixed cultures of hES cells.

Gene expression, imprinting and DNA fingerprinting. DNA and RNA were prepared at the UK Stem Cell Bank from the Trizol lysates submitted by participating laboratories. Samples were then provided to single reference laboratories for gene expression and DNA fingerprinting (Geneservice) and for imprinting studies (Rugg-Gunn and Pedersen, University of Cambridge). For gene expression studies, Applied Biosystems (ABI) prepared a batch of custom low density arrays (LDAs) using their proprietary microfluidics Genecard system. All of primers used were designed by ABI to perform with a measured efficiency of 100% ± 10% (http://docs.appliedbiosystems.com/pebiodocs/00113186.pdf) (see **Supplementary Table 3**). These were analyzed using Taqman based reagents[74]. Gene expression is reported as a DeltaCt value. For each individual gene the number of amplification cycles for the fluorescent reporter signal to reach a common threshold value (Ct) was estimated, and then normalized by subtracting the Ct value obtained from the same sample for a positive control transcript (in this case beta-actin), to give the DeltaCt value. Thus an increase in DeltaCt of 1.0 represents a twofold *decrease* in relative gene expression level.

The LDAs allowed for the simultaneous, triplicate analysis of gene expression levels by quantitative PCR, for 96 genes (90 test genes and six internal controls, see **Supplementary Table 3**). In addition quantitative PCR for three additional genes was carried out by individual assays using primers provided by ABI. Genes were selected for inclusion in the study on the following criteria: (i) expression in hEC, primordial germ cells or hES cells, (ii) known role in maintenance of pluripotency in hES cells including *NANOG*, *SOX2*, *POU5F1*, (iii) identification in microarray-based experiments as being correlated with the stem cell state, (iv) expression known to be characteristic of specific differentiation lineages in the post implantation embryo and mature tissues. The primers used were designed by ABI (see **Supplementary Table 3**). For two genes, *NANOG* and *TDGF*, it was not possible to design primers that could work on the LDA card to give unambiguous results. Primers for these genes were therefore designed for use in individual assays. In addition a third individual assay for *XIST* was included in the study due to data published post design and manufacture of the LDA cards indicating potential variability in *XIST* expression even between cell lines of the same sex.

Imprinting. Samples were analyzed for allele-specific expression patterns as previously described[26]: for primers see **Supplementary Table 8** online. Additional material from HES-3 and HES-4 cell lines cultured in BTI, Singapore was also included in the imprinting study. For each gene, the contribution of the 'minor' allele to the total expression of the gene was calculated by measuring the area under each SNP chromatogram peak using NIH ImageJ software. Expression was considered to be monoallelic if the minor allele contributed 0–14% to the total expression of the gene; partial allelic expression was defined as the minor allele expression contributing to 15–29% of the total expression; and expression was defined as biallelic if the minor allele contributed 30–50% of the total expression.

Microbiology. Culture samples were tested for the presence of mycoplasma, bacteria and fungi at the UK Stem Cell Bank using in-house protocols. Samples were also tested for induction of a cytopathic effect when inoculated onto mouse embryo fibroblasts and MRC-5 human fibroblasts[75] and also for hemadsorption of chick and guinea pig red blood cells Selected samples, including any showing apparent cytopathic effects, were also reviewed by EM to assess the presence of any virus-like particles.

Histology of xenograft tumors. Participating laboratories submitted slides from xenografts they had already produced for analysis to a single, experienced pathologist. Some xenografts were produced from cloned lines of hES cells and some were treated and or submitted to special growth conditions that were not known to the pathologist who examined the grafts microscopically. All slides were stained, routinely, with hematoxylin and eosin. Some slides were also stained with the periodic Shiff's reaction, or with antibodies to smooth muscle actin or neurofilament proteins.

Statistical analysis. *General.* Flow cytofluorimetric and gene expression data were subjected to statistical analysis by The Statistics and Analysis Group of the Computational Sciences Unit, The Jackson Laboratory, Bar Harbor, Maine, USA., using R2.3.0 (http://www.r-project.org) software.

FACS data analysis. Boxplots were generated from the average values of data which passed the quality control criteria (see antibody production and antigen assays section). Data were considered to be outlying when falling greater than 1.5 times the interquartile range and are indicated by open circles. The distribution of percentage positive cells in the flow cytofluorimetric assays was skewed to the right, so that a Logit transformation was applied to all the data. The transformed data were used for statistical analysis. Data for both the control cell line (2102Ep) and control antigens (P3X, TRA-1-85) were examined. Data points failing to meet quality control criteria as described above (Antibody Production and Analysis) were excluded. As a result, data were analyzed from 44 cell lines provided by 12 laboratories. Measurements at two time points were averaged and the averaged values were used in clustering analysis. Two-way hierarchical clustering analysis with Euclidean distance as a dissimilarity measure was performed. A heat map plot was generated to show clusters for both antigens and cell lines.

Gene expression analysis. Quantitative PCR expression data for 99 genes from 61 cell lines (57 primary lines and 4 duplicate lines) were recorded in this part of the study. Six out of the 99 genes were control genes. For both differentiated and undifferentiated cell lines, all genes were measured at two time points. At each time point, triplicates were recorded to minimize measurement error and gene expression was normalized to *β-actin* (*ACTB*); the s.d. for each triplicate was calculated. Outliers were excluded from the triplicates with a s.d. >2. Averages of the triplicate gene expression data were used for further statistical analysis. Statistical analysis: Two-way hierarchical clustering with Euclidean distance was performed. Heat map plots were generated to present clusters for both cell lines and genes. Pearson correlations among genes were calculated.

The correlations between *NANOG* and every other gene were calculated taking into account the interaction effects of cell lines, differentiation conditions and time points. Genes that have greater correlations with *NANOG* are expected to have the same expression pattern as *NANOG* across the combination level of cell line, differentiation and time point.

Cell line availability. Supplementary Table 7 online provides contact information and conditions of availability for the cell lines described in this study. Materials transfer agreements indicated in this table are provided in **Supplementary Notes** online.

Note: Supplementary information is available on the Nature Biotechnology website.

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COMPETING INTERESTS STATEMENT

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at http://www.nature.com/naturebiotechnology/.

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