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Monitoring Differentiation of Human Embryonic Stem Cells Using Real-Time PCR

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ABSTRACT

There is a general lack of rapid, sensitive, and quantitative methods for the detection of differentiating human embryonic stem cells (hESCs). Using light microscopy and immunohistochemistry, we observed that morphological changes of differentiating hESCs precede any major alterations in the expression of several commonly used hESC markers (SSEA-3, SSEA-4, TRA-1-60, TRA-1-81, Oct-4, and Nanog). In an attempt to quantify the changes during stochastic differentiation of hESCs, we developed a robust and sensitive multimarker quantitative real-time polymerase chain reaction (QPCR) method. To maximize the sensitivity of the method, we measured the expression of up- and downregulated genes before and after differentiation of the hESCs. Out of the 12 genes assayed, we found it clearly sufficient to determine the relative differentiation state of the cells by calculating a collective expression index based on the mRNA levels of Oct-4, Nanog, Cripto, and α-fetoprotein. We evaluated the method using different hESC lines maintained in either feeder-dependent or feeder-free culture conditions. The QPCR method is very flexible, and by appropriately selecting reporter genes, the method can be designed for various applications. The combination of QPCR with hESC-based technologies opens novel avenues for high-throughput analysis of hESCs in, for example, pharmacological and cytotoxicity screening. STEM CELLS *2005;23:1460–1467*

INTRODUCTION

Populations of pluripotent human embryonic stem cells (hESCs) can be derived from the inner cell mass of blastocysts and have the capacity for indefinite, undifferentiated proliferation in vitro [1–5]. Differentiation of hESCs may occur spontaneously in vitro, especially during suboptimal culture conditions. In addition, hESCs can be coaxed to differentiate in a directed fashion along specific pathways forming a variety of specialized cell types. However, relatively little is currently known about how to control and manipulate hESC differentiation to produce exclusive populations of specific cell types. Besides their importance in basic research, promising future applications of hESCs and their derivatives include cell-replacement therapies [6]. In addition, the hESC technology platform holds tremendous potential in novel approaches for drug discovery and in vitro toxicology [7, 8].

To maintain hESCs in an undifferentiated state in vitro, the cells are usually cultured on top of a feeder layer obtained either from animal [1–3, 5] or human sources [9–13]. In addition, feeder-free conditions for hESC culture have also been reported [14, 15]. Furthermore, efficient propagation of undifferentiated hESCs is

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also critically dependent on timely passaging of the cells. Normally, this time interval is between 4–7 days, depending on the culture conditions [3, 5, 14]. Despite controlled and standardized culture conditions, hESCs may undergo spontaneous differentiation during in vitro propagation. Differentiating hESCs can be identified based on changes in the morphology of the cells, their downregulation of expression of stem cell–specific markers, and concomitant upregulation of markers for differentiated cell types [16, 17]. Consequently, visual inspection of hESC colonies in concert with immunohistochemical evaluation of the cells is instrumental for quality control of hESC cultures. Furthermore, various labor-intensive and time-consuming tests can be performed in vitro and in vivo to demonstrate the pluripotency of hESCs [1, 2, 18].

Gene-expression analysis of hESCs is a valuable complement to the approaches indicated above. Global gene-expression profiling has been performed by several independent investigators using a variety of hESC lines in attempts to define a set of universal "stemness" genes [19–26]. Although some progress in defining genes associated with the pluripotent state has been made, based on the results from these studies it is obvious that there are indeed substantial differences in the gene-expression profiles between individual hESC lines. This is, however, not surprising since all hESC lines are derived from different embryos, each representing a unique genetic background. In addition, the differences in culture conditions used by the various laboratories obscure the interpretation of the data. Thus, the list of genes that can be considered as common molecular markers for undifferentiated hESC is currently relatively short, and among them are the transcription factors Oct-4 and Nanog [27–29]. On the other hand, derivatives of hESCs can be identified by a number of genes that are expressed exclusively by differentiated cells.

The future use of hESCs in drug development and for in vitro toxicity testing will require sensitive and quantitative methods for determination of the differentiation state of the cells. Importantly, these assays should be possible to implement in high-throughput analysis. Quantitative real-time polymerase chain reaction (QPCR) fits these requirements and has emerged as a very attractive large-scale screening technique [30].

Here we describe an approach, based on QPCR, for the quantitative evaluation of differentiating hESCs. By measuring the relative mRNA levels of Oct-4, Nanog, Cripto, and α-fetoprotein (AFP) in the same hESC sample and combining these values into an expression index, it is possible to discriminate between undifferentiated hESCs and their early derivatives. We evaluated the method using several independent hESC lines maintained in feeder-dependent and feeder-free conditions and demonstrated that the method is very robust and generally applicable for all cell lines tested. The combination of QPCR and hESC technologies provides novel opportunities for high-throughput analysis of hESCs.

Materials and Methods

hESC Culture and Differentiation

hESCs Maintained on Feeder Layers

The hESC lines SA001, SA002, AS034, AS034.1, SA121, SA181, and SA202 were established as described [5] and maintained at Cellartis AB (Göteborg, Sweden, http://www.cellartis.com) using mitomycin-C inactivated mouse embryonic fibroblast (MEF) feeder layers and VitroHES™ medium (Vitrolife AB, Kungsbacka, Sweden, http://www.vitrolife.com) supplemented with 4 ng/ml human recombinant basic fibroblast growth factor (hrbFGF). Undifferentiated hESCs were passaged every 4–5 days with fresh medium and MEF by mechanical dissociation using a "Stem Cell Tool" (Swemed Lab International AB, Billdal, Sweden, http://www.swemed.com).

Spontaneously differentiating hESC cultures were obtained by maintaining the hESCs on the feeder layers for up to 24 days without passaging of the cells. Medium change was performed every 2–3 days. For subsequent analyses, the hESCs were either fixed for immunohistochemical evaluation or rapidly harvested by mechanical dissociation and frozen at –80°C for RNA extraction.

Feeder-Free Culture of hESCs

The hESC lines BG01, BG02, and BG03 were cultured in laboratories at the Cellular Neurobiology Branch (National Institutes of Health [NIH]) as described elsewhere [4, 31] with minor modifications. For feeder-free culture, the cells were maintained in MEF-conditioned hESC-medium (Dulbecco's modified Eagle's medium/F12, 1:1 supplemented with 20% knockout serum replacement, 2 mM nonessential amino acids, 2 mM L-glutamine, 50 μg/ml Penn-Strep, 0.1 mM β-mercaptoethanol, and 4 ng/ml of bFGF). The hESCs were cultured on fibronectin-coated plates and passaged using EDTA-free trypsin.

Spontaneously differentiated hESC cultures were obtained by dissociating the feeder free–maintained hESCs into small clumps with EDTA-free trypsin and transferring the cells to cultures using medium similar to hESC-medium except that 20% fetal bovine serum and 2 ng/ml bFGF were used. Medium change was performed every second day. The cells were harvested for subsequent RNA extraction after 5, 7, 9, 11, and 15 days of differentiation.

Immunohistochemistry

Undifferentiated and spontaneously differentiated hESCs maintained on MEF were fixed in 4% paraformaldehyde. After permeabilization and blocking, the cells were incubated for 20 hours at 4°C with primary antibodies directed against the following antigens (final antibody concentration indicated within

parentheses): SSEA-1 (1 μg/ml), SSEA-3 (1 μg/ml), SSEA-4 (1 μg/ml), TRA-1-60 (1 μg/ml), TRA-1-81 (1 μg/ml), Oct-4 (1 μg/ml), Nanog (0.4 μg/ml), and AFP (1 μg/ml). Anti-Nanog was from R&D Systems (Minneapolis, http://www.rndsystems.com), anti-AFP was from Sigma-Aldrich (St. Louis, http://www.sigmaaldrich.com), and all other antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, http://www.scbt.com). Negative controls were included in which the primary antibodies were omitted. Fluorescein isothiocyanate – or Cy-3–conjugated secondary antibodies and 4,6-diamidino-2-phenylindole were used for detection of the primary antibodies and for nuclei staining, respectively. The stained hESC cultures were mounted and visually inspected in an inverted fluorescence microscope. For semiquantitative evaluation, an hESC colony was scored as positive if greater than 50% of the cells within the colony were stained by the antibody used.

Separation of SSEA-4–Positive and –Negative hESCs

hESCs maintained on MEF for 7 days without passaging were dissociated into a single-cell suspension in 0.5 mM EDTA for 30 minutes and resuspended in 0.1% bovine serum albumin in phosphate-buffered saline to a final concentration of 100,000 cells per ml. Magnetic Dynabeads (CELLection Pan Mouse IgG; Dynal Biotech, Oslo, Norway, http://www.dynal.no) were coated with anti-SSEA-4 antibodies (Santa Cruz Biotechnology) following the instructions from the manufacturer and subsequently incubated for 1 hour at 4°C with the cell suspension at a cell-to-bead ratio of 1:25. The SSEA-4–positive cells that bound to the beads were separated from the nonbound cell population by placing the tube in a magnetic holder. The SSEA-4–positive and –negative cell fractions were collected and frozen at –80°C until used for RNA extraction.

RNA Extraction and Reverse Transcription

Extraction of total RNA from undifferentiated and differentiating hESCs maintained on MEF was performed using RNeasy Mini Kit (Qiagen, Hilden, Germany, http://www1.qiagen.com) according to the manufacturer's instructions. DNase treatment was performed on-column using RNase-free DNase Kit (Qiagen).

Extraction of total RNA from undifferentiated and differentiating feeder free–maintained hESCs was performed using RNA STAT-60 (Tel-Test Inc., Friendswood, TX, http://www.isotexdiagnostics.com) according to the manufacturer's recommendations.

Reverse transcription was performed using 1 μg of total RNA in a final volume of 20 μl, using iScript First Strand Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, http://www.bio-rad.com) and a Rotorgene 3000 (Corbett Research, Sydney, Australia, http://www.corbettresearch.com). Each RNA sample was reverse-transcribed in duplicate, and appropriate negative controls were included in each run.

QPCR

Gene-specific primer pairs were designed and evaluated for an annealing temperature of 60°C using freely available Web-based software (Primer3, Netprimer, Beacon Designer 2.1, mFold, and Oligonucleotide Properties Calculator). Primers were designed for the following genes: *POU5F1* (Oct-4), *NANOG*, *Cripto* (TDGF1), *DNMT3B*, *SOX-2*, *GDF-3*, *Lin28*, *OC90*, *AFP*, *Nestin*, *Desmin*, and β-*III-tubulin*. The optimized assays, including reference material, are available from TATAA Biocenter (Göteborg, Sweden, http://www.tataa.com).

PCR conditions were optimized and standard curves were generated as described elsewhere [32]. All QPCRs were performed with SYBR Green I chemistry in a Rotorgene 3000. The authenticity of the PCR products was verified by melt-curve analysis and agarose-gel electrophoresis.

For QPCRs, 1x Jump Start Buffer x10 (Sigma-Aldrich), 3 mM $MgCl_2$ (Sigma-Aldrich), 0.3 mM dNTP mix (Sigma-Aldrich), 0.4x SYBR Green (Molecular Probes, Inc., Eugene, OR, http://probes.invitrogen.com), 0.4 μM forward primer (MWG Biotech, Ebersberg, Germany, http://www.mwg-biotech.com), 0.4 μM reverse primer (MWG Biotech), 0.04 U/μl Jump Start *taq* polymerase (Sigma-Aldrich), and 2 μl cDNA template were used in a final volume of 20 μl. After an initial denaturation/activation step of 3 minutes at 95°C followed 45 cycles of 20 seconds at 95°C, 20 seconds at 60°C, and 20 seconds at 72°C. The detection of fluorescent signal was performed at 72°C in each cycle. Ct (threshold cycle) values were calculated using the Rotorgene software.

Quantification of Gene Expression Using QPCR

Quantification of gene expression was based on the Ct value for each sample. The Ct values were calculated as the average of duplicate measurements. A mathematical model, previously described in detail [32], was used to determine the expression ratio of two or more genes. The general mathematical formula to calculate an expression index, based on the geometric average of several reporter genes, is given by (Equation 1):

$$Index = K_{RS} \frac{\sqrt[n]{\left(1+E_{gene(1)}\right)^{CT_{gene1}} \cdot \left(1+E_{gene(2)}\right)^{CT_{gene2}} \cdot \ldots\ldots \cdot \left(1+E_{genen}\right)^{CT_{genen}}}}{\sqrt[m-n]{\left(1+E_{gene(n+1)}\right)^{CT_{gene(n+1)}} \cdot \left(1+E_{gene(n+2)}\right)^{CT_{gene(n+2)}} \cdot \ldots\ldots \cdot \left(1+E_{genem}\right)^{CT_{genem}}}}$$

E is the PCR efficiency, Ct is the threshold cycle, and n and (m-n) are the numbers of genes that are up- and downregulated, respectively, upon differentiation of hESCs. The PCR efficiencies were evaluated from dilution series of purified PCR products [32]. K_{RS} is the relative sensitivity constant that, among other things, accounts for the differences in the fragment lengths of templates. It did not affect relative comparisons of samples and was not determined.

Results

Culture and Differentiation of hESCs

The hESC lines maintained on MEF and used in this study have been extensively characterized previously, and they express cell-surface antigens and transcriptional markers expected for undifferentiated hESCs as well as exhibiting in vivo and in vitro pluripotency [5] (Cellartis AB, unpublished results). As illustrated in Figures 1A and 1D, 5-day-old hESC colonies displayed the morphology characteristic for undifferentiated hESCs (i.e., large, compact, multicellular colonies of cells with a high nucleus-to-cytoplasm ratio). At this time point, the hESC cultures are normally passaged by mechanical dissociation. However, upon extended in vitro culture, without passaging, the hESCs differentiate spontaneously and generate heterogeneous populations of cells with a variety of morphologies. Figure 1 (1B, 1C, 1E, and 1F) shows differentiating cells at days 14 and 21 after passage. The expression of markers indicative of endo-, ecto-, and mesodermal derivatives has previously been demonstrated in these cells [5]. In addition, we observed that undifferentiated hESC colonies efficiently formed simple and cystic embryoid bodies when placed in suspension cultures [18], whereas this ability was substantially lower in differentiating cells (data not shown). Taken together, these data indicate that the hESCs remain pluripotent at least up to 5 days after passage, whereas the cultures at days 14 and 21 consist of heterogeneous populations of undifferentiated and differentiating cells.

Immunohistochemical Analysis of Differentiating hESCs

The temporal expression of several frequently used hESC markers was evaluated using immunohistochemistry. The hESCs maintained on MEF were fixed at different time points after passage. Subsequently, the cells were stained using antibodies directed against SSEA-3, SSEA-4, TRA-1-60, TRA-1-81, Oct-4, Nanog, SSEA-1, and AFP. Representative staining patterns obtained for SSEA-3 and TRA-1-60, SSEA-1, and AFP are shown in Figure 2. Semiquantitative evaluation of the staining intensities was performed as described in Materials and Methods, and the results are presented in Figure 3. The results showed that SSEA-3, SSEA-4, TRA-1-60, TRA-1-81, Oct-4, and Nanog were expressed by the vast majority of the cells in the undifferentiated hESC colonies at 4–5 days after passage. During differentiation, the expression of these antigens decreased as expected, although the kinetics of disappearance appeared to be different between the antigens. Whether these results reflect the actual expression levels in the differentiating hESCs or a difference in turnover of the antigens remains to be investigated. Interestingly, none of these antigens disappeared completely in the hESC colonies within the time span for differentiation used in this study. Importantly, markers for differentiated hESCs, such as SSEA-1 and AFP, were not detected in undifferentiated hESCs (5-day-old colonies), but at 9 days after passage these antigens were observed in some regions of differentiating colonies (Figs. 2, 3). Extended in vitro culture resulted in increased expression of SSEA-1 and AFP, and at day 22, the majority of the cells in approximately 50% of the hESC colonies expressed these antigens. Interestingly, based on morphological evaluation and immunohistochemical analysis, undifferentiated hESCs were also identified in certain regions of differentiating colonies even after 24 days of in vitro culture, suggesting that hESCs can undergo several cell divisions in "differentiation-promoting" culture conditions while maintaining their pluripotent phenotype [17].

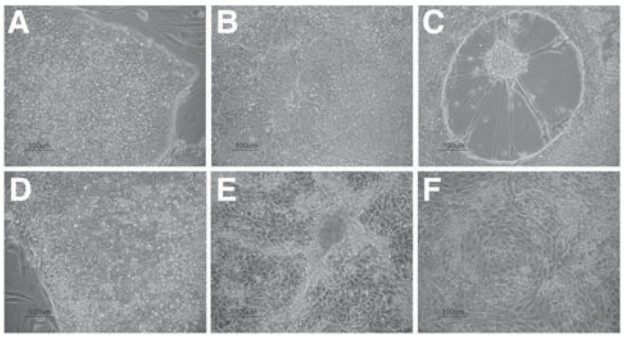

Figure 1. Representative illustrations of the morphology of undifferentiated and differentiating human embryonic stem cells (hESCs): **(A)** SA002 at day 5, **(B)** SA002 at day 14, **(C)** SA002 at day 21, **(D)** AS034 at day 5, **(E)** AS034 at day 14, and **(F)** AS034 at day 21. The cells were cultured on mouse embryonic fibroblasts using VitroHES medium supplemented with basic fibroblast growth factor and passaged mechanically every 4–5 days. Extended culture of the hESCs without passaging initiated differentiation, and a mixture of early hESC-derivatives was obtained. Magnification ×100.

QPCR Analysis of Undifferentiated and Spontaneously Differentiated hESCs

QPCR systems were designed and optimized for the group of genes indicated in Materials and Methods. Subsequently, we analyzed the mRNA levels of these genes in undifferentiated and differentiating hESCs maintained on MEF. The cells were harvested at days 5, 14, and 21 after passage and total RNA was extracted. After reverse transcription and QPCR analysis, Ct values were obtained for all individual samples (data not shown). Among the genes tested, *Cripto*, *Oct-4*, and *Nanog* were all significantly downregulated upon differentiation of the hESCs. On the other hand, the mRNA level of *AFP* was substantially increased during differentiation of the hESCs. Due to their consistent and reproducible expression patterns in all five hESC lines tested, we included these four genes as reporter genes in the final QPCR assay. The remaining genes analyzed displayed either inconsistent expression profiles when comparing the different hESC lines or there were little or no changes in their relative mRNA levels during the timeframe used here.

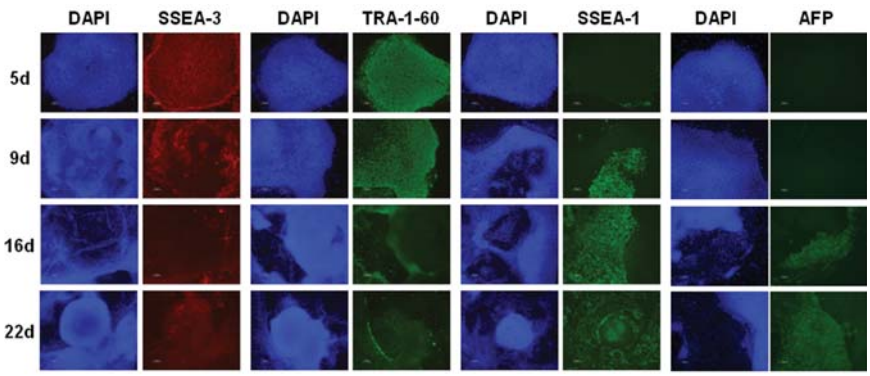

Figure 2. Undifferentiated and spontaneously differentiated human embryonic stem cell colonies maintained on mouse embryonic fibroblasts were analyzed using immunohistochemistry as described in Materials and Methods. The panels show representative 4,6-diamidino-2-phenylindole (DAPI) stainings and the corresponding specific antibody staining for SSEA-3 (SA181), TRA-1-60 (SA181), SSEA-1 (SA181), and AFP (SA121) as indicated. The cells were fixed and analyzed at the time points indicated on the left. Magnification ×100.

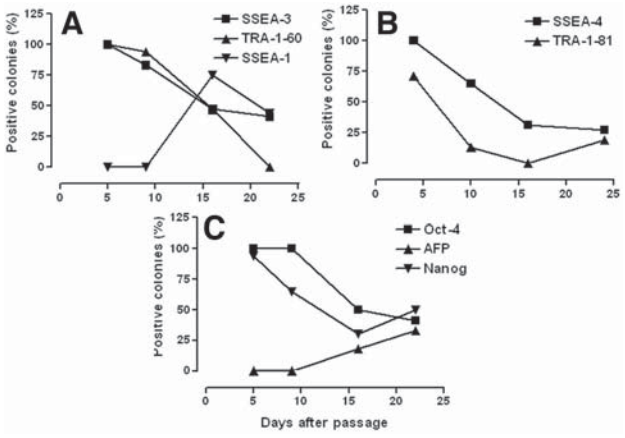

Figure 3. Semiquantitative evaluation of the immunohistochemical staining of undifferentiated and differentiating human embryonic stem cells (hESCs) was performed as described in Materials and Methods: **(A)** SA181, **(B)** SA202, and **(C)** SA121. For each data point, 12–32 individual hESC colonies were evaluated. Abbreviation: AFP, α-fetoprotein.

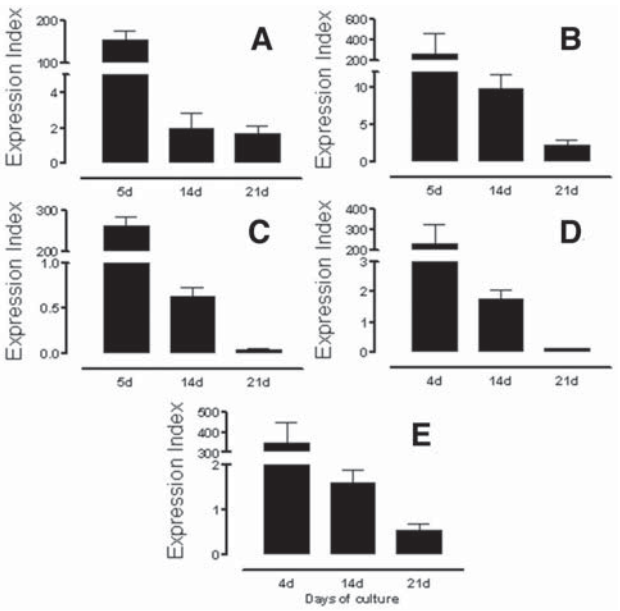

Figure 4. Quantitative real-time polymerase chain reaction (QPCR) analysis of undifferentiated and differentiating human embryonic stem cells (hESCs) maintained on mouse embryonic fibroblasts: **(A)** SA001, **(B)** SA002, **(C)** AS034, **(D)** AS034.1, and **(E)** SA121. The cells were cultured as described in Materials and Methods and harvested at the time points indicated on the x-axis. After RNA extraction, the relative mRNA levels of Oct-4, Nanog, Cripto, and α-fetoprotein were determined using QPCR. The expression index was subsequently calculated using the equation indicated in Materials and Methods (Equation 1). The data are presented as the mean plus SD ($n = 4$).

Quantifying the Differentiation State of hESCs

To obtain a quantitative measure of the relative level of differentiation of the hESCs, the mathematical model (Equation 1) described in Materials and Methods was applied. The input in the equation is the Ct value for each individual reporter gene and the corresponding PCR efficiency, and the output is an index based on the geometric averages between the mRNA levels of down- and upregulated genes. By including several genes, the assay becomes very robust, while maintaining a high precision, and less sensitive to minor fluctuations in the expression levels of the individual genes. Figure 4 shows the calculated expression indexes from the QPCR analyses of hESCs maintained on MEF. For the five cell lines tested, the expression indexes ranged between 150 and 350 for undifferentiated hESCs (day 4–5) and between 0.6 and 10 for differentiating hESCs at day 14. In differentiated progenies from hESCs at day 21, the expression indexes were further decreased and ranged between 0.05 and 2. The quantitatively most striking changes in the expression indexes occurred during the first week of differentiation. The fold changes observed in the expression indexes were between 26 and 415 when comparing cells at day 5 and 14, and the fold changes ranged between 1.2 and 15 when comparing cells at days 14 and 21.

By using monoclonal antibodies immobilized on magnetic beads, we separated SSEA-4–positive and SSEA-4–negative fractions of hESCs from a heterogeneous cell population harvested at day 7 after passage. These cells represent a mixture of undifferentiated hESCs and early progenies thereof. Approximately 60% of the cells were captured by the SSEA-4 antibody–coated beads. These results correlate well with the semiquantitative evaluation of the SSEA-4 immunostainings (Fig. 2B). The fractionated cells were subsequently analyzed using QPCR as indicated above. The

results are shown in Figure 5 and demonstrate that the expression index of SSEA-4–positive cells is about 15-fold higher than the expression index of SSEA-4–negative cells.

Finally, we sought to investigate if the QPCR assay was generally applicable also for hESC lines other than the ones maintained on MEF at Cellartis AB. For this purpose, we determined the expression indexes of three independent hESC lines (BG01, BG02, and BG03) that were maintained and differentiated in feeder-free conditions. Importantly, these cell lines were established and cultured in laboratories separate from Cellartis AB, and the samples were analyzed blindly. Despite the substantial differences in the general procedures of culturing and passaging of the hESCs, it was possible to accurately discriminate between the undifferentiated and differentiating cells using the QPCR method (Fig. 6). The expression indexes for the undifferentiated hESCs ranged between 72 and 100 and were significantly higher compared with the corresponding expression indexes for the differentiating hESCs. In addition, based on the expression indexes, it was possible to correctly rank the group of samples from differentiating hESCs (day 5–15), although a few samples deviated slightly from the expected trend (BG01 at day 7, BG02 at day 9, and BG03 at day 7). Interestingly, these apparent outliers were present within a rather small and specific time interval (7–9 days of differentiation). Further studies are necessary to elucidate the possible biological significance of this observation.

Discussion

We have developed a QPCR-based approach to determine the differentiation state of hESCs cultured in vitro. To the best of our knowledge, this is the first report on such a device designed specifically for hESCs. By measuring the relative mRNA levels of Oct-4, Cripto, Nanog, and AFP and combining these values into an expression index, we clearly distinguish between undifferentiated hESCs and their early derivatives. In contrast to most other methods based on measuring relative mRNA levels, here we avoid dependence on the expression of housekeeping genes, which recently has been questioned [33–35]. The method is robust in detecting changes in different hESC lines grown under quite different culture conditions. Interestingly, the expression indexes for undifferentiated hESCs maintained in feeder-free conditions were slightly lower than the expression indexes for hESCs maintained on MEF (Figs. 4, 6). The explanation for this observation remains speculative at this point. Notably, the cell lines used in this study display the common markers of hESCs and thus, the cells are in that regard indistinguishable from each other [5, 31]. However, the differences in culture conditions appear to affect the state of differentiation of the cells that can be detected using the QPCR method. Whether the observed differences in the expression indexes between the undifferentiated hESCs cultured with or without MEF have any biological significance remains to be determined. In additional experiments, we separated hESCs into SSEA-4–positive and SSEA-4–negative cells from a heterogeneous cell population comprised of undifferentiated hESCs and early derivatives thereof, and demonstrated that these can be clearly discriminated using QPCR (Fig. 5).

The genes included in the assay were selected from a number of genes previously reported to be down- or upregulated in differentiating hESCs [21, 24, 27–29, 36, 37]. It is clear that other genes

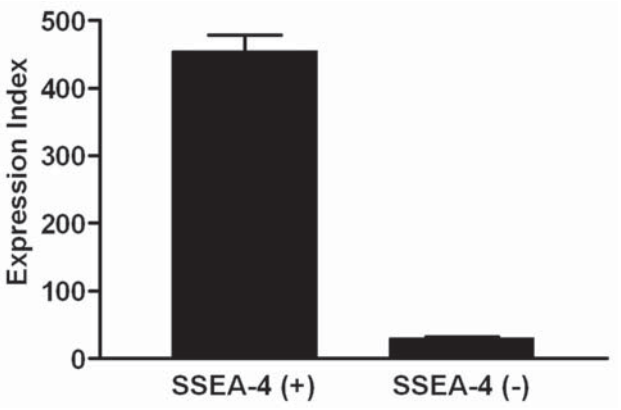

Figure 5. Quantitative real-time polymerase chain reaction (QPCR) analysis of SSEA-4–positive and SSEA-4–negative human embryonic stem cells. The cells (SA121) were cultured as described in Materials and Methods, harvested at day 7, and fractionated using SSEA-4-antibody–coated magnetic beads. After RNA extraction, the relative mRNA levels of Oct-4, Nanog, Cripto, and α-fetoprotein were determined using QPCR. The expression index was subsequently calculated using Equation 1. The data are presented as the mean plus SD ($n = 2$).

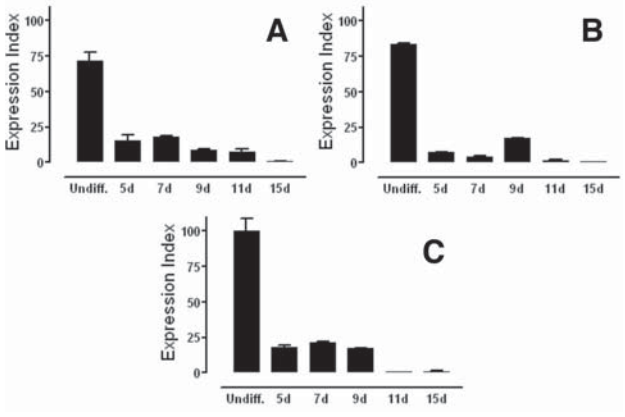

Figure 6. Quantitative real-time polymerase chain reaction (QPCR) analysis of undifferentiated (Undiff.) and differentiating human embryonic stem cells maintained in feeder-free conditions: **(A)** BG01, **(B)** BG02, and **(C)** BG03. The cells were cultured as described in Materials and Methods and harvested at the different time points indicated on the x-axis. After RNA extraction, the relative mRNA levels of Oct-4, Nanog, Cripto, and α-fetoprotein were determined using QPCR. The expression index was subsequently calculated using Equation 1. The data are presented as the mean plus SD ($n = 2$).

can also prove useful as makers for hESCs and their derivatives. For example, similar to other reports, we found *DNMT3B* significantly downregulated in differentiating hESCs maintained on MEF (data not shown) [24, 37]. However, we did not observe the corresponding change in the expression of *DNMT3B* in the hESC lines maintained in feeder-free conditions. In these cells, the mRNA levels of *DNMT3B* were not significantly altered during the differentiation process. Thus, *DNMT3B* did not fit our criteria for QPCR reporter genes for hESC differentiation. However, in certain applications and depending on the gene-expression profiles of the specific hESC lines used, genes such as *DNMT3B* can add sensitivity to the QPCR assay. We selected *AFP*, which is commonly used as a marker for early endoderm, as a reporter gene for differentiated hESCs. We found this sufficient for the detection of mixed populations of early hESC progenies. Ultimately, reporter genes for each embryonic germ layer could be included.

In its simplest form, the expression index is a ratio of only two reporter genes, one that is upregulated and one downregulated [32]. However, in the context of hESC biology, there is presently little information regarding genes that are directly associated with pluripotency and self-renewal, and so far no single gene is known that is expressed exclusively by pluripotent cells. Instead, investigators are using a battery of genes to identify undifferentiated hESCs. This is an advantage for the QPCR approach described here, because the robustness of the assay increases by the inclusion of more reporter genes. These genes can represent both up- and downregulated genes.

Spontaneous differentiation of the hESCs was achieved in the present study by extended culturing without passaging of the cells [5]. Visual inspection of hESC colonies using light microscopy revealed alterations in the morphology of the cells which occur during the process of differentiation [2]. The initiation of hESC differentiation is usually associated with an increase in the cell size and the cells appear to flatten and separate. In some cases, the cells start to pile up and the colonies become thick and opaque. Furthermore, areas with organized structures can be observed within individual colonies (Fig. 1). Using immunohistochemistry and specific antibodies directed against known hESC-markers, we strengthened the conclusion that the hESCs underwent differentiation during the extended in vitro culture of the cells. In line with previous reports, we detected downregulation of the expression of SSEA-3, SSEA-4, TRA-1-60, TRA-1-81, Oct-4, and Nanog (Figs. 2, 3) [17, 27–29]. At the same time, the expression of markers reflecting differentiation (SSEA-1 and AFP) increased [17].

Immunohistochemistry is a very powerful technique, with which it is possible to detect and localize a specific antigen on a single cell. However, the possibility for accurate quantification of expression levels, especially in large-scale analysis, is limited and, at best, semiquantitative. In addition, the analysis normally spans 2 days. The data presented in this study indicate that morphological alterations of the hESC colonies preceded any major changes in the antigen expression of the cells detected by immunohistochemistry and thus, more sensitive methods need to be used to detect the presence of early hESC-derivatives in the cultures. With QPCR, requiring only small amounts of RNA, large numbers of samples can be rapidly tested, and accurate results can be obtained within the same day of sample preparation.

The QPCR method presented here is flexible and by careful selection and validation of the reporter genes, it is possible to tune the assay to fit a wide variety of applications. Here we demonstrate that accurately measured levels of the Oct-4, Nanog, Cripto, and AFP transcripts are clearly sufficient to discriminate between pluripotent hESCs and their spontaneously differentiating progenies. This makes the QPCR method attractive for quality control of hESC cultures during expansion of the cells. In this regard, the method can be spiked with control RNA prepared from undifferentiated and differentiating hESCs, providing a simple approach to compare states of hESCs across laboratories. In addition, the method can be used as read-out system when testing and optimizing novel culture conditions for hESCs. Any adverse effects caused by the changes in growth conditions would be discovered at an early stage, leading to significant time and cost savings. Finally, the QPCR method also fits readily into high-throughput applications in which hESCs provide the platform for the screening of potential effects of chemicals and drugs.

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Disclosures

The authors indicate no potential conflicts of interest.

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