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Assessing self-renewal and differentiation in hESC lines

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Abstract

Like other cell populations, undifferentiated human embryonic stem cells (hESCs) express a characteristic set of proteins and mRNA that is unique to the cells regardless of culture conditions, number of passages and methods of propagation. We have sought to identify a small set of markers that would serve as a reliable indicator of the balance of undifferentiated and differentiated cells in hESC populations. Markers of undifferentiated cells should be rapidly down-regulated as the cells differentiate to form embryoid bodies (EBs), while markers that are absent or low during the undifferentiated state but are induced as hESCs differentiate could be used to assess the presence of differentiated cells in the cultures. In this manuscript we describe a list of markers that reliably distinguish undifferentiated and differentiated cells. An initial list of approximately 150 genes was generated by scanning published MPSS, EST scan and microarray datasets. From this list, a subset of 109 genes was selected that included 55 candidate markers of undifferentiated cells, 46 markers of hESC derivatives, 4 germ cell markers and 4 trophoblast markers. Expression of these candidate marker genes was analyzed in undifferentiated hESCs and differentiating EB populations in four different lines by immunocytochemistry, RT-PCR, microarray analysis and quantitative RT-PCR (qPCR). We show that qPCR with as few as 12 selected genes can reliably distinguish differentiated cells from undifferentiated hESC populations.

Keywords

Human embryonic stem cell; Embryoid body; Differentiation

Introduction

Currently, more than 100 distinct human embryonic stem cell (hESC) lines have been derived and efforts at new derivations are ongoing. Approximately 20 lines from the 78 derivations undertaken before August 9, 2001 are available in sufficient quantities for general research use (NIH stem cell registry, http://stemcells.nih.gov/research/registry). Of these, only a small subset of lines is available for detailed characterization [1-8]. As expected, various hESC lines have a number of similarities. For example, undifferentiated hESCs are similar in expressing surface antigens and markers characteristic of the undifferentiated ESC state including Oct4 (POU5F1), Nanog, UTF1, DPPA5, TERT, gap junction proteins, SSEA and TRA antigens [1-8]. hESCs are also similar in their ability to proliferate and differentiate into cell types of

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the three germ layers in vitro and in vivo [9-16]. Properties of hESCs have also been compared using microarray, EST scan, SAGE and MPSS [4;17-27]. These studies suggest that it is likely that markers shared by hESC lines but absent in other cell populations exist.

Although these studies have highlighted similarities among hESC lines and markers that distinguish them from mouse ESCs, it is likely that differences also exist. These include potential differences in methylation patterns [28;29], likely HLA differences [25], allelic differences, variability of X-inactivation and adaptation of cells to different culture conditions [17,2;30;31]. Indeed, important differences between hESC lines in growth rates, methods of propagation and karyotype have been reported using a variety of different techniques, suggesting that while shared markers may exist, care will be needed to identify them. Identifying such shared markers, however, remains an easier task than the technically challenging experiments of direct comparing hESCs under identical culture conditions-experiments that are being undertaken at the Stem Cell Center at the NIH (Dr. McKay) and at the International Stem Cell Initiative (Dr. Andrews) to identify fundamental differences among cell lines. Such experiments are beyond the scope of our laboratories. The available data, however, indicate that identifying a common pattern of gene expression that is conserved independent of culture conditions and reflects the fundamental properties of hESC is possible.

Several other experiments suggest that a unique molecular signature can be defined to distinguish undifferentiated hESCs from their differentiated progeny, and that this signature can be used to define the states of hESCs [17-19]. These experiments used MPSS, EST scan, and microarray data to suggest that a large pool of potential markers that could distinguish embryoid bodies (EBs) and other differentiated cells from hESCs exist. We have reasoned, therefore, that at the current level of resolution of techniques, it is possible to identify a core set of genes that are conserved and/or required to maintain hESC identity. These genes should be expressed irrespective of the conditions of culture, numbers of passages and methods of propagation as long as undifferentiated hESCs are present. Moreover, these core markers should be present regardless of the methods of hESC derivation and ethnic phenotypes of the blastocysts used. If present at lower levels, they should be detectable by RT-PCR as well as by SAGE/MPSS, and if robust by SAGE and microarray. Furthermore, if the expression of such genes is examined in EBs, then a subset of markers that are rapidly downregulated or rapidly induced as cells differentiate can be identified [19]. A combination of such markers can serve to reliably assess the states of ESCs and EBs.

To test this hypothesis we have performed a meta-analysis of published reports examining hESCs and EBs, and identified a list of potential markers. We tested a substantial number of these markers by quantitative RT-PCR (qPCR) and immunocytochemistry and identified a combination of markers to distinguish hESCs from EBs.

Materials and Methods

hESC culture and in vitro differentiation via EB

hESC lines BG01, BG02, BG03 and I6 were either maintained on inactivated mouse embryonic fibroblast (MEF) feeder cells in medium comprised of Dulbecco's Modified Eagle's Medium/ Ham's F12 supplemented with 20% knockout serum replacement, 2 mM non-essential amino acids, 2 mM L-glutamine, 50 μg/ml Penn-Strep (all from Invitrogen; Carlsbad, CA; http://www.invitrogen.com), 0.1 mM β-mercaptoethanol (Specialty Media; Phillipsburg, NJ; http://www.specialtymedia.com), and 4 ng/ml of basic fibroblast growth factor (bFGF, Sigma; St. Louis, MO; http://www.sigmaaldrich.com), or on fibronectin (BD Biosciences, Bedford, MA;http://www.bdbiosciences.com) coated dishes in medium conditioned with MEF for 24 hours as previously described [4;25].

Differentiation via EB formation was performed as preciously described [4]. Briefly, hESCs were dissociated into small clumps by collagenase IV (Sigma) and grown as floating spheres in hESC medium without bFGF for up to 2 weeks, with a medium change every second day.

RT-PCR and qPCR analysis

Total RNA was extracted from undifferentiated hESCs or EBs (7-day and 14-day) using RNA STAT-60 (Tel-Test Inc., Friendswood, TX). cDNA was synthesized by using a reverse transcription kit (RETROscript, Ambion, Austin, TX) according to the manufacturer's recommendations. The PCR primers are listed in Supplementary Table 1.

Real-time qPCR was used to quantify the levels of mRNA expression of 12 selected genes (Oct4, Nanog, Sox2, UTF1, DPPA5, Lin41, Sox1, DCN, H19, IGF2, GATA4 and Hand1) in hESCs or EBs at different times of differentiation. PCR reactions were carried out by DNA Engine Opticon Fluorescence Detection System (MJ Research, Waltham, MA) using a DyNAmo SYBR Green qPCR kit according to the manufacturer's instructions. The content of selected genes was normalized to the content of 18S-rRNA and standard curves were generated using 10 to 1000 ng cDNA per 20 μl reaction volume. All PCR products were checked by melting curve analysis to exclude the possibility of multiple products or incorrect product size. PCR analyses were conducted in triplicate for each sample.

Immunocytochemistry

Immunocytochemistry and staining procedures were as described previously [32]. Briefly, hESCs grown either on MEF feeder cells or under feeder-free conditions, 7-day and 14-day EBs either attached or floating were fixed with 2% paraformaldehyde for half an hour. Parts of EBs were embedded in O.C.T. Blocks were cut on a cryostat to obtain 8 μm sections. Fixed cells and sections were blocked in blocking buffer (5% goat serum, 1% BSA, 0.1% Triton X-100) for 1 hour followed by incubation with the primary antibody at 4°C overnight. Appropriately coupled secondary antibodies (Molecular Probes) were used for single and double labeling. All secondary antibodies were tested for cross reactivity and non-specific immunoreactivity.

The following antibodies were used: Nanog (1:1000, R&D Systems AF1997), ITGB1 (CD29/Integrin β1, 1:1000, Chemicon MAB1951), CDH1 (E-Cadherin, 1:2500, R&D Systems MAB1838), PODXL (Podocalyxin, 1:500, R&D Systems MAB1658), Sox2 (1:1000, R&D Systems MAB2018), Oct4 (1:1000, R&D Systems AF1759), Brachyury (1:1000, R&D Systems AF2085), ACTC (Cardiac Actin, 1:50, Research Diagnostics, Inc., PRO61075), GATA4 (1:100, Santa Cruz sc-25310), GATA6 (1:100, Santa Cruz sc-9055), AFP (alpha-fetoprotein, 1:500, Sigma A8452) and TUBB3 (βIII tubulin 1:2000, Sigma T8660). Bis-benzamide (Dapi, 1:1000, Sigma) was used to identify the nuclei. Images were captured on an Olympus fluorescence microscope.

Microarray analysis using BeadArray platform

RNAs from undifferentiated BG01, BG02 and BG03 cells and the matched 14-day EBs were hybridized to prototype Illumina RefSeq-8 BeadChips, which contained more than 24,000 genes [33]. A detailed description of the BeadChip system has been provided elsewhere [33]. Samples were coded and run in duplicates and the results were analyzed using standard bioinformatics tools and the Bead Studio, a tool kit developed by Illumina. A detailed analysis of these and other samples will be reported elsewhere [Jeanne Loring, Burnham Institute, personal communication].

Results

Meta-analysis procedure

A large dataset on gene expression of undifferentiated hESCs and EBs that differentiated from them using large scale array experiments including MPSS, EST enumeration and microarrays has been generated and described by a variety of investigators [17-24]. To generate a list of genes characteristic of undifferentiated hESCs and differentiated EBs, we examined three published reports on gene expression in hESCs and EBs. These were: 1) a list of ninety-two genes identified as “stemness” genes which are expressed at high levels in six hESC lines as assessed by a 16,695 seventy base pair oligonucleotide microarray [18], 2) a comprehensive list of genes common to undifferentiated hESCs obtained by MPSS analysis using pooled hESCs samples [17], and 3) a large list of genes that are highly expressed in differentiated EBs detected by both MPSS and EST scan [19] (A complete list of EST scan data are obtained from http://www.ncbi.nlm.nih.gov/UniGene/library.cgi?all=yes&ORG=Hs&LID=14183 http://www.ncbi.nlm.nih.gov/UniGene/library.cgi?all=yes&ORG=Hs&LID=14184).

The criteria for selection were that the levels of expression should differ between hESCs and EBs by at least three fold by array data or by five fold for EST scanning and MPSS, the levels of expression should be high (>10 tpm for MPSS, >5 by EST scan) and that the genes must be detected by at least one independent method and in more than one hESC line. One exception to these selection criteria is that we included some genes that were not detected by MPSS or EST scanning but are used as ESC marker by convention, and a few genes that were highly expressed in both hESCs and EBs or expressed at higher level in EBs but were reported as ESC specific genes by microarray analysis when compared to human universal RNA [18] (Table 1), or because of being a potential cell surface marker. A list of several hundred genes generated after this selection was pruned to a list of about one hundred by examining published literature with the goal of including known genes with approximately half of them being candidate genes highly expressed in undifferentiated hESCs (but not in EBs or down-regulated in EBs), and the other half being those that are expressed in EBs (but lower or absent in hESCs). We did not include any unknown genes even though many of them were significantly differentially expressed in hESCs and EBs [19].

The final list of 109 potential markers included 55 stem cell markers in which most of them were highly expressed in undifferentiated hESCs. For potential markers of the EB state, 46 markers were selected including representative genes from each germ layer with 12 ectoderm, 15 mesoderm and 19 endoderm markers that are likely over-expressed in EBs. In addition, 4 trophoblast markers and 4 germ cell markers were included (Tables 1 and 2, Supplementary Table 1).

Markers of the ESC state

Forty-four genes highly expressed in undifferentiated hESCs but down-regulated in EBs were selected as markers of stem cells to represent the undifferentiated ESC state (Table 1). Several of these genes such as Oct4, Nanog and TERT are generally accepted markers of pluripotency. Many genes in the list including UTF1, Sox2, Lin28, Lin 41, PODXL, LeftyB, GJA1, FoxD3 and Rex1 (ZFP42) have recently been reported to be expressed in undifferentiated hESCs by several research groups [4;17-19;34]. Other undifferentiated markers included genes that encoded transcriptional factors, growth factors, signal transducers, cell surface antigens and receptors. In addition, 11 genes were included as ESC markers although they did not meet our selection criteria. Of them, eight genes, NOG, TFCP2L1, CommD3, TERT, NR5A2, DPPA5, NODAL, ITGB1BP3, were selected by convention. Two genes, GJA1 and IMP2, were selected because they expressed at significant higher levels in hESCs when compared to human universal RNA by microarray analysis, although their expression were equally high in EBs.

Finally, despite higher level expression in EBs, ITGB1 was included because it is a cell surface receptor which may bind fibronectin that has been reported to be a substrate capable of supporting hESC growth [35;36].

Markers of the EB state

To represent the complexity of EBs, we included as many types of early markers of differentiation as possible, and selected the following: 1) 12 ectoderm markers including markers for neural precursors such as nestin and Sox1, and for terminal differentiated neural cells like Tuj1 (TUBB3), TH and GFAP, 2) 19 endoderm markers including pancreatic marker insulin, imprinted maternally expressed gene H19, HNF and AFP, 3) 15 mesoderm markers including collagen, Brachyury and ACTC, 4) Four trophoblast markers, KRT1, EOMES, GCM1 and CDX2, and 5) Four germ cell markers SYCP3, DDX4, IFITM1, IFITM2 (Table 2).

Expression of candidate markers by RT-PCR

Our selection criteria indicated that candidate markers will be expressed in the appropriate stage of development and should be detected by RT-PCR. We therefore generated a PCR primer list for all 109 genes (Supplementary Table1) and tested 35 genes (Fig. 1 and data not shown, highlighted in Supplementary Table 1) using three different cell lines. Representative results for a subset of markers for the I6 line are shown. Of the 7 ESC markers shown, all were expressed in undifferentiated hESCs. A subset (Dppa5, UTF1 and ZFP42) were undetectable in 14-day EBs while the others were down-regulated (GAL, Lin28, Lin41 and TDGF1) in EBs (Fig. 1, left panel). Likewise, the 7 EB specific markers (AFP, FoxA2, Hand1, HGF, IGF2, Msx1 and MSI1) were expressed in EBs but absent (AFP, HNF3b, IGF2 and Msx1) in undifferentiated hESCs or only slightly expressed (Hand1, HGF and MSI1) in undifferentiated hESCs (Fig. 1, right panel). These results showed the relatively specific pattern of expression of candidate ESC and EB markers and indicate the suitability of using some or all of these to assess the overall state of cultured cells.

Antibodies to test the expression of candidate markers

Of the 109 genes, we were able to locate commercially available antibodies for about two thirds (76) with reactivity against human antigens and these antibodies and their source are provided in Supplementary Table 1. We tested a subset of these commercial available antibodies by immunocytochemistry and at least 15 of them worked well (highlighted in Supplementary Table 1). Representative staining of ESC or EB specific genes in 7-day and 14-day differentiated EBs and undifferentiated hESCs are shown in Figs. 2 and 3. In general, all the ESC markers (Nanog, Oct4, ITGB1, CDH1 and PODXL) were strongly positive in undifferentiated hESCs but weakly expressed in 7-day EBs, and not expressed in 14-day EBs (Fig.2, panel A-L). The one exception was Sox2, which was expressed in both undifferentiated hESCs and the two stages of EBs (Fig. 2, panel M-O). All the markers of differentiation examined (Brachyury, ACTC, AFP, GATA4, GATA6, TUBB3) were strongly expressed in both 7-day and 14-day EBs but negative or only weakly expressed in undifferentiated hESCs (Fig. 3). In addition to these markers, known pluripotency markers SSEA (SSEA3 and 4) and TRA (TRA-1-60, 1-81) were down-regulated in differentiated EBs and Table 3 summarizes the results of immunocytochemistry. Thus antibodies to most of the candidate markers exist and a significant subset can be used for immunocytochemistry. We notice, however, that ITGB1 was strongly positive stained in undifferentiated hESC but down-regulated in EBs which is in conflict with the MPSS and array data. This suggests that not all genes could be used in all methodologies.

Monitor differentiation by microarray

Although offering sufficient resolution, RT-PCR and immunocytochemistry are difficult to perform for a large number of genes and cannot be easily automated. To test whether differences in gene expression were of a sufficient magnitude that they could be detected by a more global and less quantitative measurement, we assessed the expression of candidate ESC and EB markers by analyzing their expression in three hESC lines derived by BresaGen (three undifferentiated hESC samples of BG01, 02 and 03 and EBs derived from them) using the Illumina BeadArray containing about 48,000 unique features. All samples were examined in duplicate and only data from duplicates samples that showed 99% or greater correlation was used. The present results were focused on expression of the genes that were selected as candidate markers of the ESC and EB state.

Global pairwise comparisons between different hESC lines (Fig. 4, panel A, and Supplementary Table 2) or different EBs (Fig. 4, panel B), showed similar levels of gene expression and around 90% of the genes detected at greater than 99% confidence limit were expressed at approximately similar levels (within the 2.5-fold range) (Fig. 4F). Pairwise comparisons of hESCs with hESCs or EBs with EBs showed a high degree of similarity of samples (correlation coefficient greater than 0.90). Most of the differential expression seen in Fig.4 A and B is the result of biological differences between the cultures; technical replicates have correlation coefficients greater than 0.90 (Loring, et al., in preparation). Comparisons of hESCs to EBs showed a much lower degree of similarity (correlation coefficient <0.8, Fig. 4, panel C). This suggests that different hESCs are similar to each other and that this similarity is greater than that between hESC and EB derived from the same line. The entire comparison is presented in Supplementary Table 2 and a restricted list of genes which were selected as ESC or EB markers that showed a greater than 2.5-fold difference in expression is shown in Fig. 4 (Panel G and H). The large difference between hESCs and EBs detected by this global comparison indicates that arrays can readily distinguish hESCs from EBs derived from them.

To further test whether the similarity in gene expression between hESC lines can be generalized, we analyzed an additional hESC lines H9, obtained from WiCell Institute (Madison, WI, http://www.wicell.org) rather than from BresaGen. As shown in Fig. 4D, gene expression profiles of H9 was remarkably similar to the BG lines, with a correlation coefficient of 0.93 when compared to BG01. This suggests that gene expression profiles in hESC lines derived from different laboratories are similar.

qPCR to monitor differentiation

Our results suggested that a global assessment by a relatively non-quantitative method such as RT-PCR or microarray could be used to detect differentiation. Given the dramatic differences in gene expression, we reasoned that assessing a smaller number of markers using a more quantitative measurement could be sufficient in monitoring the overall state of hESCs. To test this hypothesis, we selected a small number of genes from the 109-list and tested their expression in undifferentiated hESCs and in two differentiating stages of EBs (7-day and 14-day) using BG03. These included 6 undifferentiated ESC markers (Oct4, Nanog, UTF1, DPPA5, Lin 41 and Sox2) and 6 markers of differentiation with at least one gene from each germ layer (Sox1, DCN, H19, IGF2, GATA4 and Hand1). The expression level of these genes were determined as the ratio to the level of 18S RNA, and differential expression of these genes in undifferentiated hESCs and EBs were shown as the ratio of expression in hESC to EB (ESC markers) or EB to hESC (EB markers).

As expected, the expression of ESC markers Oct4, Nanog, UTF1, DPPA5 and Lin41 were higher in undifferentiated hESCs than in EBs, whereas the expression of EB markers Sox1, DCN, H19, IGF2, GATA4 and Hand1 were up-regulated in EBs compared to in

undifferentiated hESCs (Fig. 5). In particular, expression of UTF1 and Nanog was rapidly down-regulated upon differentiation with more than a 10-fold decrease in 7-day EBs and 200-fold lower in 14-day EBs. Down-regulation of Oct4, an important gene for the maintenance of pluripotency in both hESCs and mESCs, was less marked with only 5-fold decrease in 14-day EBs. Sox2 was expressed in both undifferentiated hESCs and differentiated EBs (3-fold higher in 14-day EB and 5-fold higher in 7-day EB) which is expected as Sox2 is known to express in neural stem cells which are present in EBs. Interestingly, expression of Lin41 was rapidly decreased in 7-day EBs but the expression level increased in 14-day EBs (Fig. 5).

All of the differentiation markers, except for Decorin, were rapidly induced as the cells differentiated. The most dramatic changes were seen for an imprinted gene IGF2 whose levels were several thousand fold higher in EBs than in undifferentiated hESCs. Expression of the imprinted gene H19 as well as Hand1 and GATA4 was also rapidly increased as the cells underwent differentiation.

qPCR detects changes that may be missed by immunocytochemistry

To test whether our qPCR assay can detect more subtle changes in hESC cultures that affect the undifferentiated state, we examined hESC cultures maintained with bFGF and cultures where bFGF was withdrawn for a period of 72 hours by qPCR and immunocytochemistry. For qPCR assay, we chose to analyze two markers of the ESC (UTF1 and Nanog) and EB (IGF2 and Hand1) state as expression of these four genes changed most significantly upon differentiation in our qPCR analysis (Fig. 4). No change in expression of either ESC (SSEA4 and Oct4) or EB markers (AFP) could be detected in this time period by immunocytochemistry (Fig. 6). However, qPCR readily detected a significant change in cultures maintained without bFGF for 72 hours: IGF2 and Hand1 were expressed 3.6-fold and 2.3-fold higher in hESC cultures without bFGF, whereas no significant changes were observed for the two most differentially expressed ESC markers (UTF: 1.4-fold and Nanog: 1.5-fold) detected by our qPCR analysis. These changes, despite smaller than those seen in 7-day and 14-day EBs, were similar in profile to changes when cells undergo differentiation.

Discussion

Since the first derivation of hESC lines in 1998, information on gene expression in hESCs and other human stem cells has been accumulated rapidly using a variety of techniques such as microarray, SAGE, EST scan and MPSS [17-21;24]. A large number of genes have been identified that are expressed at high levels in undifferentiated hESCs and “stemness” genes that define a stem cell state have been proposed [18;37]. Expression of many of these genes is down-regulated as hESCs differentiate, and parallel to this, many genes are induced during differentiation. Nevertheless, there are no defined set of markers that can be routinely used for assessing the different states of hESCs, i.e., the undifferentiated ESC stage and different stages of differentiating EBs. In the present study, we compared published reports on gene expression in ESCs and EBs and selected a set of 109 known genes including 55 stem cell, 12 ectoderm, 19 endoderm, 15 mesoderm, 4 trophoblast and 4 germ cell markers as potential ESC or EB specific markers. We show that this set of genes can serve as indicators of the states of hESCs using four independent methods, qPCR, immunocytochemistry, RT-PCR and microarray, using at least three different hESC lines for each method.

It is clear that no single marker is sufficient to define the state of hESCs. Several surface antigens including SSEA and TRA are useful markers for undifferentiated hESCs as their level of expression is down-regulated as hESCs differentiate. The genes encoding them however, have yet to be identified. Other pluripotency genes including two well-characterized transcription factors, Oct4 and Nanog, are good markers to assess the presence of undifferentiated hESCs. Oct4 and Nanog are essential for the maintenance of pluripotency in

both hESCs and mESCs and knock-out or knock-down of either gene causes differentiation [38;39]. Oct4 and Nanog are, however, not uniquely expressed in undifferentiated hESCs: Oct4 is expressed in germ cells and Nanog has recently been reported to be expressed in mature tissues [40;41]. Moreover, the expression of Oct4 declines slowly as cells differentiate and the change in levels is small. For example, we have detected Oct4 expression in hESCs that underwent differentiation for a week or underwent neuronal differentiation on PA6 cells for 2 weeks (unpublished results). Our qPCR results likewise showed only a moderate decrease in Oct4 expression in 7-day EBs and only a 5-fold decrease in 14-day EBs. Taken together, we believe that while SSEA, TRA, Oct4 and Nanog are useful for distinguishing undifferentiated hESCs from their differentiated progeny, expression of these markers alone or in combination is not enough to define the undifferentiated hESC populations. Likewise, expression of a single marker is not a definitive indicator of the differentiated EB state. Indeed, some of the early differentiation genes including keratin, actin and tubulin were expressed at low levels in undifferentiated hESCs; however, their expression was strongly up-regulated as hESCs formed EBs [19]. Since the differentiated progeny of hESCs include a number of cell types, it is important to assess EBs using a combination of endoderm, mesoderm and ectoderm markers.

Our assessment of RT-PCR as a method of examining hESC cultures showed that even though it is not quantitative, it is quite robust provided appropriate genes are selected for assessment. Primers were designed to all 109 genes and the expression of 35 candidate ESC and EB genes was confirmed by RT-PCR. Our data showed that undifferentiated hESCs could be readily distinguished from differentiating EBs by assessing 10-20 markers using semi-quantitative RT-PCR. The relatively specific expression of ESC and EB markers in undifferentiated hESCs and in EBs provides a simple method to assess the quality of RNA samples for different purposes and to estimate the level of differentiation in hESC culture.

In addition to the confirmation of differential expression in hESCs and EBs by RT-PCR, we examined the expression of many of these genes by immunocytochemistry. These included markers that have not previously been analyzed by antibody staining in hESCs such as PODXL, ITGB1 and Nanog. Nanog, PODXL, ITGB1 and CDH1 were down-regulated in 7-day EBs, and further decreased in 14-day EBs. Similarly, expression of differentiation markers such as AFP, GATA4 and GATA6 were strongly up-regulated in 7-day EBs. These markers, together with the SSEA and TRA surface markers can reliably detect the differentiation of hESCs and can be used for routine examination of differentiation in hESC cultures. Although immunostaining is relatively more time consuming, it offers unprecedented resolution allowing one to rapidly assess the degree of contamination or the extent of differentiation.

RT-PCR and immunocytochemistry, however, are not suitable for scaling up or processing of a large number of markers. We therefore examined if the genes identified as candidate markers can be used to assess differentiation using a microarray platform. Our results showed that many though not all genes show detectable changes in gene expression even in a relatively poor quantitative method such as microarray. For example, Oct4, Lin28, TDGF1 and GDF3 were present at significantly higher levels in hESCs than in EBs; while Col2A1, Col1A1 and SerpinA1 were present higher in EBs using the Illumina BeadArray. The difference in gene expression were present in five hESC lines tested in this study and in six other cell lines evaluated (Jeanne Loring, Burnham Institute, personal communication), indicating that these genes may serve as a standard measure of changes irrespective of the cell line being used. Other genes that were expected to serve as useful markers and showed utility in immunocytochemistry and RT-PCR were not as useful in this microarray format. Such genes included Nanog, Sox2 and Sox1 (see Supplementary Table 2 for a complete list), indicating that candidate genes will have to be assessed in each individual platform to determine if they are adequate within the limitations of that particular technology.

Among the 109 genes, six of each undifferentiated and differentiated markers (at least one marker of each germ layer) were further examined by qPCR in undifferentiated hESCs and two stages of EBs (7-day and 14-day). We reasoned that careful quantitation may allow one to use only a small subset of markers. Indeed our results showed that as few as six markers may be sufficient provided both positive and negative markers were used. Whereas down-regulation of Oct4 was gradual during differentiation, expression of Nanog and UTF1 declined more than 200-fold in 14-day EBs, suggesting that these markers are good indicators of the undifferentiated ESC state. Dramatic up-regulation of expression in EBs was also found for an imprinted gene IGF2, and for Hand1. Expression of IGF2 and Hand1 were quickly up-regulated in 7-day EBs by several thousand folds and by day 14, the expression levels were 3 million-fold higher for IGF2. It therefore seems that undifferentiated hESCs and their derivatives can be discriminated by examining a few genes using a quantitative method, if the genes are appropriately selected. UTF1 and Nanog are excellent candidates for markers of the undifferentiated state, whereas IGF2 and Hand1 are good markers for differentiated EBs. The dramatic changes in expression level of these four genes upon differentiation can be reliably used for assessing the undifferentiated ESC and differentiated states. Moreover, negative markers (differentiated EB markers) are more sensitive than positive markers (undifferentiated ESC markers) in detecting differentiation in hESCs. These conclusions are supported by experiments designed to detect smaller changes of differentiation in hESC cultures where bFGF was removed for a period of three days. Whereas immunocytochemistry could not detect any difference in bFGF treated and bFGF withdrawn cultures using ESC or EB markers, bFGF withdrawn hESC cultures could be readily distinguished from their sister culture maintained with bFGF by qPCR using negative markers (IGF2 and Hand1).

As more data on hESCs are collected, additional markers for undifferentiated and differentiated cells will undoubtedly be identified. For example, a large number of novel genes or genes of unknown function that show a similar robust alteration in expression levels as hESCs differentiate [18] may be included in future arrays or qPCR sets to provide an additional level of sensitivity to allow a finer dissection of the state of differentiation. The present lists, however, provides useful information for evaluating the states of hESC populations, extent of differentiation or for quality control of hESC cultures. Our results suggest that any of the four methods we describe here can be used to monitor the transition of undifferentiated hESCs to differentiated EBs when a combination of ESC and EB markers from our list were tested.

Conclusion

Our strategy of including a combination of genes that are down-regulated and up-regulated during differentiation which includes genes that represent different cell types (undifferentiated cells and cell types of the three germ layers, as well as trophoblast and germ cells) allows one to identify a set of markers that can be readily assessed by routine molecular or cellular biology methods. We believe that any of the methods we tested is sufficient to monitor the state of hESC but each method has its advantages and disadvantages. If qPCR is used, a small number of genes are sufficient provided both positive and negative markers are used [see present results and 42]. However, the most cost-effective method for the wealth of information obtained may be a focused array that includes many markers such as the genes we have described. Efforts to generate such an array are in progress [43, Ian Lyons, Invitrogen, personal communication]. Alternatively, microfluidic plates allow to custom design markers and have the advantages of being able to be adapted to very small number of cells.

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References

1. Thomson JA, Itskovitz-Eldor J, Shapiro SS, et al. Embryonic stem cell lines derived from human blastocysts. Science 1998;282:1145–1147. [PubMed: 9804556]
2. Carpenter MK, Rosler E, Rao MS. Characterization and differentiation of human embryonic stem cells. Cloning Stem Cells 2003;5:79–88. [PubMed: 12713704]
3. Mitalipova M, Calhoun J, Shin S, et al. Human embryonic stem cell lines derived from discarded embryos. Stem Cells 2003;21:521–526. [PubMed: 12968106]
4. Zeng X, Miura T, Luo Y, et al. Properties of pluripotent human embryonic stem cells BG01 and BG02. Stem Cells 2004;22:292–312. [PubMed: 15153607]
5. Amit M, Itskovitz-Eldor J. Derivation and spontaneous differentiation of human embryonic stem cells. J Anat 2002;200:225–232. [PubMed: 12033726]
6. Heins N, Englund MC, Sjoblom C, et al. Derivation, characterization, and differentiation of human embryonic stem cells. Stem Cells 2004;22:367–376. [PubMed: 15153613]
7. Park JH, Kim SJ, Oh EJ, et al. Establishment and maintenance of human embryonic stem cells on STO, a permanently growing cell line. Biol Reprod 2003;69:2007–2014. [PubMed: 12930726]
8. Ginis I, Luo Y, Miura T, et al. Differences between human and mouse embryonic stem cells. Dev Biol 2004;269:360–380. [PubMed: 15110706]
9. Carpenter MK, Inokuma MS, Denham J, Mujtaba T, Chiu CP, Rao MS. Enrichment of neurons and neural precursors from human embryonic stem cells. Exp Neurol 2001;172:383–397. [PubMed: 11716562]
10. Kaufman DS, Hanson ET, Lewis RL, Auerbach R, Thomson JA. Hematopoietic colony-forming cells derived from human embryonic stem cells. Proc Natl Acad Sci U S A 2001;98:10716–10721. [PubMed: 11535826]
11. Kehat I, Kenyagin-Karsenti D, Snir M, et al. Human embryonic stem cells can differentiate into myocytes with structural and functional properties of cardiomyocytes. J Clin Invest 2001;108:407–414. [PubMed: 11489934]
12. Odorico JS, Kaufman DS, Thomson JA. Multilineage differentiation from human embryonic stem cell lines. Stem Cells 2001;19:193–204. [PubMed: 11359944]
13. Rambhatla L, Chiu CP, Kundu P, Peng Y, Carpenter MK. Generation of hepatocyte-like cells from human embryonic stem cells. Cell Transplant 2003;12:1–11. [PubMed: 12693659]
14. Reubinoff BE, Itsykson P, Turetsky T, et al. Neural progenitors from human embryonic stem cells. Nat Biotechnol 2001;19:1134–1140. [PubMed: 11731782]
15. Xu C, Police S, Rao N, Carpenter MK. Characterization and enrichment of cardiomyocytes derived from human embryonic stem cells. Circ Res 2002;91:501–508. [PubMed: 12242268]
16. Zhang SC, Wernig M, Duncan ID, Brustle O, Thomson JA. In vitro differentiation of transplantable neural precursors from human embryonic stem cells. Nat Biotechnol 2001;19:1129–1133. [PubMed: 11731781]
17. Brandenberger R, Khrebtukova I, Thies RS, et al. MPSS profiling of human embryonic stem cells. BMC Dev Biol 2004;4:10. [PubMed: 15304200]
18. Bhattacharya B, Miura T, Brandenberger R, et al. Gene expression in human embryonic stem cell lines: unique molecular signature. Blood 2004;103:2956–2964. [PubMed: 15070671]
19. Miura T, Luo Y, Khrebtukova I, et al. Monitoring early differentiation events in human embryonic stem cells by massively parallel signature sequencing and expressed sequence tag scan. Stem Cells Dev 2004;13:694–715. [PubMed: 15684837]
20. Sperger JM, Chen X, Draper JS, et al. Gene expression patterns in human embryonic stem cells and human pluripotent germ cell tumors. Proc Natl Acad Sci U S A 2003;100:13350–13355. [PubMed: 14595015]
21. Abeyta MJ, Clark AT, Rodriguez RT, Bodnar MS, Pera RA, Firpo MT. Unique gene expression signatures of independently-derived human embryonic stem cell lines. Hum Mol Genet 2004;13:601–608. [PubMed: 14749348]
22. Robson P. The maturing of the human embryonic stem cell transcriptome profile. Trends Biotechnol 2004;22:609–612. [PubMed: 15542146]

23. Sato N, Sanjuan IM, Heke M, Uchida M, Naef F, Brivanlou AH. Molecular signature of human embryonic stem cells and its comparison with the mouse. Dev Biol 2003;260:404–413. [PubMed: 12921741]
24. Richards M, Tan SP, Tan JH, Chan WK, Bongso A. The transcriptome profile of human embryonic stem cells as defined by SAGE. Stem Cells 2004;22:51–64. [PubMed: 14688391]
25. Brimble SN, Zeng X, Weiler DA, et al. Karyotypic stability, genotyping, differentiation, feeder-free maintenance, and gene expression sampling in three human embryonic stem cell lines derived prior to August 9, 2001. Stem Cells Dev 2004;13:585–597. [PubMed: 15684826]
26. Wei CL, Miura T, Robson P, et al. Transcriptome profiling of human and murine ESCs identifies divergent paths required to maintain the stem cell state. Stem Cells 2005;23:166–185. [PubMed: 15671141]
27. Bhattacharya B, Luo Y, Miura T, et al. Comparison of the gene expression profile of undifferentiated human embryonic stem cell lines and embryoid bodies differentiated from them. BMC Dev Biol. 2005accepted.
28. Humpherys D, Eggan K, Akutsu H, et al. Epigenetic instability in ES cells and cloned mice. Science 2001;293:95–97. [PubMed: 11441181]
29. Dean W, Bowden L, Aitchison A, et al. Altered imprinted gene methylation and expression in completely ES cell-derived mouse fetuses: association with aberrant phenotypes. Development 1998;125:2273–2282. [PubMed: 9584126]
30. Xu C, Inokuma MS, Denham J, et al. Feeder-free growth of undifferentiated human embryonic stem cells. Nat Biotechnol 2001;19:971–974. [PubMed: 11581665]
31. Rosler ES, Fisk GJ, Ares X, et al. Long-term culture of human embryonic stem cells in feeder-free conditions. Dev Dyn 2004;229:259–274. [PubMed: 14745951]
32. Zeng X, Chen J, Sanchez JF, et al. Stable expression of hrGFP by mouse embryonic stem cells: promoter activity in the undifferentiated state and during dopaminergic neural differentiation. Stem Cells 2003;21:647–653. [PubMed: 14595124]
33. Kuhn K, Baker SC, Chudin E, et al. A novel, high-performance random array platform for quantitative gene expression profiling. Genome Res 2004;14:2347–2356. [PubMed: 15520296]
34. Wong RC, Pebay A, Nguyen LT, Koh KL, Pera MF. Presence of functional gap junctions in human embryonic stem cells. Stem Cells 2004;22:883–889. [PubMed: 15536180]
35. Amit M, Shariki C, Margulets V, Itskovitz-Eldor J. Feeder layer- and serum-free culture of human embryonic stem cells. Biol Reprod 2004;70:837–845. [PubMed: 14627547]
36. Draper JS, Moore HD, Ruban LN, Gokhale PJ, Andrews PW. Culture and characterization of human embryonic stem cells. Stem Cells Dev 2004;13:325–336. [PubMed: 15345125]
37. Ramalho-Santos M, Yoon S, Matsuzaki Y, Mulligan RC, Melton DA. “Stemness”: transcriptional profiling of embryonic and adult stem cells. Science 2002;298:597–600. [PubMed: 12228720]
38. Zaehres H, Lensch MW, Daheron L, Stewart SA, Itskovitz-Eldor J, Daley GQ. High-efficiency RNA interference in human embryonic stem cells. Stem Cells 2005;23:299–305. [PubMed: 15749924]
39. Mitsui K, Tokuzawa Y, Itoh H, et al. The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. Cell 2003;113:631–642. [PubMed: 12787504]
40. Niwa H, Miyazaki J, Smith AG. Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. Nat Genet 2000;24:372–376. [PubMed: 10742100]
41. Hart AH, Hartley L, Ibrahim M, Robb L. Identification, cloning and expression analysis of the pluripotency promoting Nanog genes in mouse and human. Dev Dyn 2004;230:187–198. [PubMed: 15108323]
42. Noaksson K, Zoric N, Zeng X, et al. Monitoring differentiation of human embryonic stem cells using real-time PCR. Stem Cells. 2005In Press.
43. Yang AX, Mejido J, Luo Y, et al. Development of a focused microarray to assess human embryonic stem cell differentiation. Stem Cells Dev. 2005In Press.
44. Rosner MH, Vigano MA, Ozato K, et al. A POU-domain transcription factor in early stem cells and germ cells of the mammalian embryo. Nature 1990;345:686–692. [PubMed: 1972777]

45. Yuan H, Corbi N, Basilico C, Dailey L. Developmental-specific activity of the FGF-4 enhancer requires the synergistic action of Sox2 and Oct-3. Genes Dev 1995;9:2635–2645. [PubMed: 7590241]
46. Cui L, Johkura K, Yue F, et al. Spatial distribution and initial changes of SSEA-1 and other cell adhesion-related molecules on mouse embryonic stem cells before and during differentiation. J Histochem Cytochem 2004;52:1447–1457. [PubMed: 15505339]
47. Anisimov SV, Tarasov KV, Tweedie D, Stern MD, Wobus AM, Boheler KR. SAGE identification of gene transcripts with profiles unique to pluripotent mouse R1 embryonic stem cells. Genomics 2002;79:169–176. [PubMed: 11829487]
48. Lee YS, Kim HK, Chung S, Kim KS, Dutta A. Depletion of human micro-RNA miR-125b reveals that it is critical for the proliferation of differentiated cells but not for the down-regulation of putative targets during differentiation. J Biol Chem 2005;280:16635–16641. [PubMed: 15722555]
49. Okuda A, Fukushima A, Nishimoto M, et al. UTF1, a novel transcriptional coactivator expressed in pluripotent embryonic stem cells and extra-embryonic cells. Embo J 1998;17:2019–2032. [PubMed: 9524124]
50. Lei W, Hirose T, Zhang LX, et al. Cloning of the human orphan receptor germ cell nuclear factor/retinoid receptor-related testis-associated receptor and its differential regulation during embryonal carcinoma cell differentiation. J Mol Endocrinol 1997;18:167–176. [PubMed: 9134503]
51. Hanna LA, Foreman RK, Tarasenko IA, Kessler DS, Labosky PA. Requirement for Foxd3 in maintaining pluripotent cells of the early mouse embryo. Genes Dev 2002;16:2650–2661. [PubMed: 12381664]
52. Chambers I, Colby D, Robertson M, et al. Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. Cell 2003;113:643–655. [PubMed: 12787505]
53. Rogers MB, Hosler BA, Gudas LJ. Specific expression of a retinoic acid-regulated, zinc finger gene, Rex-1, in preimplantation embryos, trophoblast and spermatocytes. Development 1991;113:815–824. [PubMed: 1821852]
54. Feldman B, Poueymirou W, Papaioannou VE, DeChiara TM, Goldfarb M. Requirement of FGF-4 for postimplantation mouse development. Science 1995;267:246–249. [PubMed: 7809630]
55. Kim SK, Suh MR, Yoon HS, et al. Identification of developmental pluripotency associated 5 expression in human pluripotent stem cells. Stem Cells 2005;23:458–462. [PubMed: 15790765]
56. Schmid E, Tapscott S, Bennett GS, et al. Differential location of different types of intermediate-sized filaments in various tissues of the chicken embryo. Differentiation 1979;15:27–40. [PubMed: 93557]
57. Sharma MK, Saxena V, Liu RZ, et al. Differential expression of the duplicated cellular retinoic acid-binding protein 2 genes (crabp2a and crabp2b) during zebrafish embryonic development. Gene Expr Patterns 2005;5:371–379. [PubMed: 15661643]
58. Kolodkin AL, Matthes DJ, Goodman CS. The semaphorin genes encode a family of transmembrane and secreted growth cone guidance molecules. Cell 1993;75:1389–1399. [PubMed: 8269517]
59. Good P, Yoda A, Sakakibara S, et al. The human Musashi homolog 1 (MSI1) gene encoding the homologue of Musashi/Nrp-1, a neural RNA-binding protein putatively expressed in CNS stem cells and neural progenitor cells. Genomics 1998;52:382–384. [PubMed: 9790759]
60. Garner CC, Tucker RP, Matus A. Selective localization of messenger RNA for cytoskeletal protein MAP2 in dendrites. Nature 1988;336:674–677. [PubMed: 3200318]
61. Reeves SA, Helman LJ, Allison A, Israel MA. Molecular cloning and primary structure of human glial fibrillary acidic protein. Proc Natl Acad Sci U S A 1989;86:5178–5182. [PubMed: 2740350]
62. Zhou Q, Wang S, Anderson DJ. Identification of a novel family of oligodendrocyte lineage-specific basic helix-loop-helix transcription factors. Neuron 2000;25:331–343. [PubMed: 10719889]
63. Malas S, Duthie SM, Mohri F, Lovell-Badge R, Episkopou V. Cloning and mapping of the human SOX1: a highly conserved gene expressed in the developing brain. Mamm Genome 1997;8:866–868. [PubMed: 9337405]
64. Hockfield S, McKay RD. Identification of major cell classes in the developing mammalian nervous system. J Neurosci 1985;5:3310–3328. [PubMed: 4078630]
65. Lee JE, Hollenberg SM, Snider L, Turner DL, Lipnick N, Weintraub H. Conversion of Xenopus ectoderm into neurons by NeuroD, a basic helix-loop-helix protein. Science 1995;268:836–844. [PubMed: 7754368]

66. Masserano JM, Weiner N. Tyrosine hydroxylase regulation in the central nervous system. Mol Cell Biochem 1983;53:54–129.

67. Burgoyne RD, Cambray-Deakin MA, Lewis SA, Sarkar S, Cowan NJ. Differential distribution of beta-tubulin isotypes in cerebellum. Embo J 1988;7:2311–2319. [PubMed: 2461292]

68. Kornblihtt AR, Vibe-Pedersen K, Baralle FE. Isolation and characterization of cDNA clones for human and bovine fibronectins. Proc Natl Acad Sci U S A 1983;80:3218–3222. [PubMed: 6304699]

69. Fisher LW, Hawkins GR, Tuross N, Termine JD. Purification and partial characterization of small proteoglycans I and II, bone sialoproteins I and II, and osteonectin from the mineral compartment of developing human bone. J Biol Chem 1987;262:9702–9708. [PubMed: 3597437]

70. Ruoslahti E, Pihko H, Seppala M. Alpha-fetoprotein: immunochemical purification and chemical properties. Expression in normal state and in malignant and non-malignant liver disease. Transplant Rev 1974;20:38–60. [PubMed: 4135842]

71. Lohi J, Leivo I, Franssila K, Virtanen I. Changes in the distribution of integrins and their basement membrane ligands during development of human thyroid follicular epithelium. Histochem J 1997;29:337–345. [PubMed: 9184849]

72. Smyth N, Vatansever HS, Murray P, et al. Absence of basement membranes after targeting the LAMC1 gene results in embryonic lethality due to failure of endoderm differentiation. J Cell Biol 1999;144:151–160. [PubMed: 9885251]

73. Wozney JM, Rosen V, Celeste AJ, et al. Novel regulators of bone formation: molecular clones and activities. Science 1988;242:1528–1534. [PubMed: 3201241]

74. Kurachi K, Chandra T, Degen SJ, et al. Cloning and sequence of cDNA coding for alpha 1-antitrypsin. Proc Natl Acad Sci U S A 1981;78:6826–6830. [PubMed: 7031661]

75. Kendall RL, Thomas KA. Inhibition of vascular endothelial cell growth factor activity by an endogenously encoded soluble receptor. Proc Natl Acad Sci U S A 1993;90:10705–10709. [PubMed: 8248162]

76. ten Dijke P, Ichijo H, Franzen P, et al. Activin receptor-like kinases: a novel subclass of cell-surface receptors with predicted serine/threonine kinase activity. Oncogene 1993;8:2879–2887. [PubMed: 8397373]

77. Arceci RJ, King AA, Simon MC, Orkin SH, Wilson DB. Mouse GATA-4: a retinoic acid-inducible GATA-binding transcription factor expressed in endodermally derived tissues and heart. Mol Cell Biol 1993;13:2235–2246. [PubMed: 8455608]

78. Novak U, Wilks A, Buell G, McEwen S. Identical mRNA for preproglucagon in pancreas and gut. Eur J Biochem 1987;164:553–558. [PubMed: 3569278]

79. Andik I, Donhoffer S. The effect of insulin on food intake and selection of mice. Z Vitam Horm Fermentforsch 1949;3:208–212. [PubMed: 14782629]

80. Newman PJ, Berndt MC, Gorski J, et al. PECAM-1 (CD31) cloning and relation to adhesion molecules of the immunoglobulin gene superfamily. Science 1990;247:1219–1222. [PubMed: 1690453]

81. Lowe JB, Boguski MS, Sweetser DA, Elshourbagy NA, Taylor JM, Gordon JI. Human liver fatty acid binding protein. Isolation of a full length cDNA and comparative sequence analyses of orthologous and paralogous proteins. J Biol Chem 1985;260:3413–3417. [PubMed: 3838313]

82. Chartier FL, Bossu JP, Laudet V, Fruchart JC, Laine B. Cloning and sequencing of cDNAs encoding the human hepatocyte nuclear factor 4 indicate the presence of two isoforms in human liver. Gene 1994;147:269–272. [PubMed: 7926813]

83. Crossley PH, Martin GR. The mouse Fgf8 gene encodes a family of polypeptides and is expressed in regions that direct outgrowth and patterning in the developing embryo. Development 1995;121:439–451. [PubMed: 7768185]

84. Zarnegar R, Michalopoulos G. Purification and biological characterization of human hepatopoietin A, a polypeptide growth factor for hepatocytes. Cancer Res 1989;49:3314–3320. [PubMed: 2524251]

85. Yamada S, Zhu Q, Aihara Y, et al. Cloning of cDNA and the gene encoding human hepatocyte nuclear factor (HNF)-3 beta and mutation screening in Japanese subjects with maturity-onset diabetes of the young. Diabetologia 2000;43:121–124. [PubMed: 10672453]

86. Termine JD, Kleinman HK, Whitson SW, Conn KM, McGarvey ML, Martin GR. Osteonectin, a bone-specific protein linking mineral to collagen. Cell 1981;26:99–105. [PubMed: 7034958]

87. Russell MW, Baker P, Izumo S. Cloning, chromosomal mapping, and expression of the human eHAND gene. Mamm Genome 1997;8:863–865. [PubMed: 9337404]

88. Robert B, Sassoon D, Jacq B, Gehring W, Buckingham M. Hox-7, a mouse homeobox gene with a novel pattern of expression during embryogenesis. Embo J 1989;8:91–100. [PubMed: 2565810]

89. Gunning P, Ponte P, Blau H, Kedes L. alpha-skeletal and alpha-cardiac actin genes are coexpressed in adult human skeletal muscle and heart. Mol Cell Biol 1983;3:1985–1995. [PubMed: 6689196]

90. Suzuki E, Evans T, Lowry J, et al. The human GATA-6 gene: structure, chromosomal location, and regulation of expression by tissue-specific and mitogen-responsive signals. Genomics 1996;38:283–290. [PubMed: 8975704]

91. Glant TT, Hadhazy C, Mikecz K, Sipos A. Appearance and persistence of fibronectin in cartilage. Specific interaction of fibronectin with collagen type II. Histochemistry 1985;82:149–158. [PubMed: 3997552]

92. Lau ET, Kwok YK, Chui DH, Wong HS, Luo HY, Tang MH. Embryonic and fetal globins are expressed in adult erythroid progenitor cells and in erythroid cell cultures. Prenat Diagn 2001;21:529–539. [PubMed: 11494285]

93. Herrmann BG. Action of the Brachyury gene in mouse embryogenesis. Ciba Found Symp 1992;165:78–86. [PubMed: 1516477]discussion 86-91.

94. Call KM, Glaser T, Ito CY, et al. Isolation and characterization of a zinc finger polypeptide gene at the human chromosome 11 Wilms′ tumor locus. Cell 1990;60:509–520. [PubMed: 2154335]

95. Braun T, Buschhausen-Denker G, Bober E, Tannich E, Arnold HH. A novel human muscle factor related to but distinct from MyoD1 induces myogenic conversion in 10T1/2 fibroblasts. Embo J 1989;8:701–709. [PubMed: 2721498]

96. Li ZL, Lilienbaum A, Butler-Browne G, Paulin D. Human desmin-coding gene: complete nucleotide sequence, characterization and regulation of expression during myogenesis and development. Gene 1989;78:243–254. [PubMed: 2673923]

97. Kangawa K, Matsuo H. Purification and complete amino acid sequence of alpha-human atrial natriuretic polypeptide (alpha-hANP). Biochem Biophys Res Commun 1984;118:131–139. [PubMed: 6230082]

98. Marotta CA, Forget BG, Cohen-Solal M, Weissman SM. Nucleotide sequence analysis of coding and noncoding regions of human beta-globin mRNA. Prog Nucleic Acid Res Mol Biol 1976;19:165–175. [PubMed: 1019344]

99. Ogawa E, Maruyama M, Kagoshima H, et al. PEBP2/PEA2 represents a family of transcription factors homologous to the products of the Drosophila runt gene and the human AML1 gene. Proc Natl Acad Sci U S A 1993;90:6859–6863. [PubMed: 8341710]

100. Bell GI, Merryweather JP, Sanchez-Pescador R, et al. Sequence of a cDNA clone encoding human preproinsulin-like growth factor II. Nature 1984;310:775–777. [PubMed: 6382021]

101. Ryan K, Garrett N, Mitchell A, Gurdon JB. Eomesodermin, a key early gene in Xenopus mesoderm differentiation. Cell 1996;87:989–1000. [PubMed: 8978604]

102. Suh E, Chen L, Taylor J, Traber PG. A homeodomain protein related to caudal regulates intestine-specific gene transcription. Mol Cell Biol 1994;14:7340–7351. [PubMed: 7935448]

103. Yamada K, Ogawa H, Honda S, Harada N, Okazaki T. A GCM motif protein is involved in placenta-specific expression of human aromatase gene. J Biol Chem 1999;274:32279–32286. [PubMed: 10542267]

104. Cserhalmi-Friedman PB, Squeo R, Gordon D, et al. Epidermolytic hyperkeratosis in a Hispanic family resulting from a mutation in the keratin 1 gene. Clin Exp Dermatol 2000;25:241–243. [PubMed: 10844506]

105. Yuan L, Liu JG, Zhao J, Brundell E, Daneholt B, Hoog C. The murine SCP3 gene is required for synaptonemal complex assembly, chromosome synapsis, and male fertility. Mol Cell 2000;5:73–83. [PubMed: 10678170]

106. Castrillon DH, Quade BJ, Wang TY, Quigley C, Crum CP. The human VASA gene is specifically expressed in the germ cell lineage. Proc Natl Acad Sci U S A 2000;97:9585–9590. [PubMed: 10920202]

107. Deblandre GA, Marinx OP, Evans SS, et al. Expression cloning of an interferon-inducible 17-kDa membrane protein implicated in the control of cell growth. J Biol Chem 1995;270:23860–23866. [PubMed: 7559564]

108. Tanaka SS, Matsui Y. Developmentally regulated expression of mil-1 and mil-2, mouse interferon-induced transmembrane protein like genes, during formation and differentiation of primordial germ cells. Gene Expr Patterns 2002;2:297–303. [PubMed: 12617817]

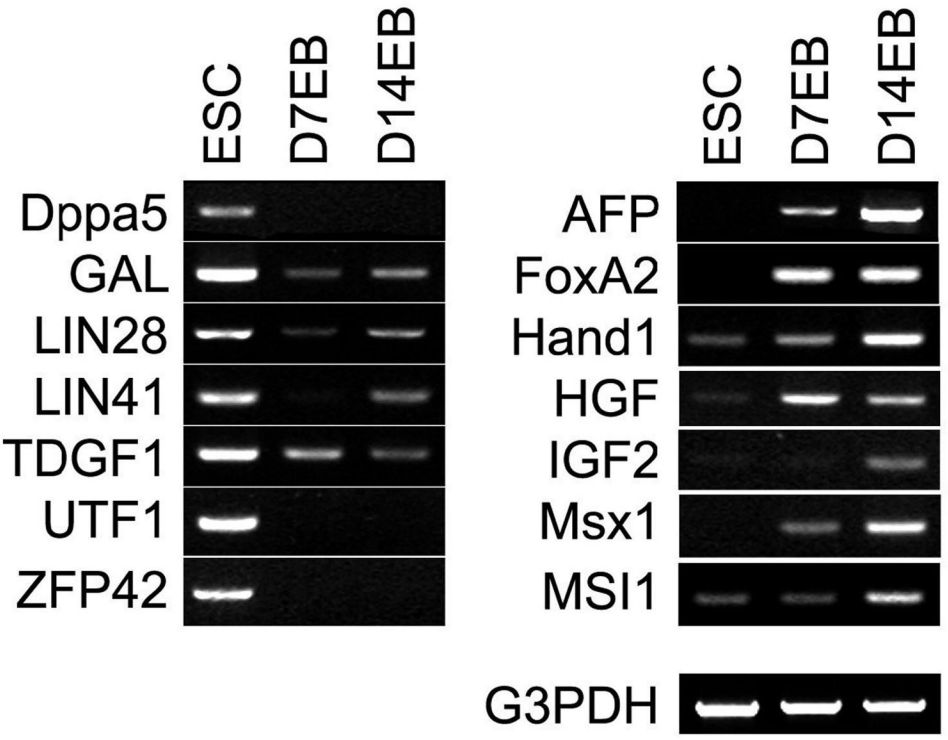


Fig 1.

Differential expression of ESC or EB specific genes by RT-PCR. Expression of selected markers of ESC (Dppa5, GAL, LIN28, LIN41, TDGF1, UTF1, and ZFP42) and EB (AFP, FoxA2, HAND1, HGF, IGF2, Msx1, MSI1) was examined by RT-PCR in undifferentiated hESCs, 7-day and 14-day EBs. Consistent with other independent analyses, all the ESC markers are highly expressed in hESCs but quickly down regulated in the two EB populations, whereas all the EB markers are detected in EB samples, but not or slightly expressed in undifferentiated hESCs.

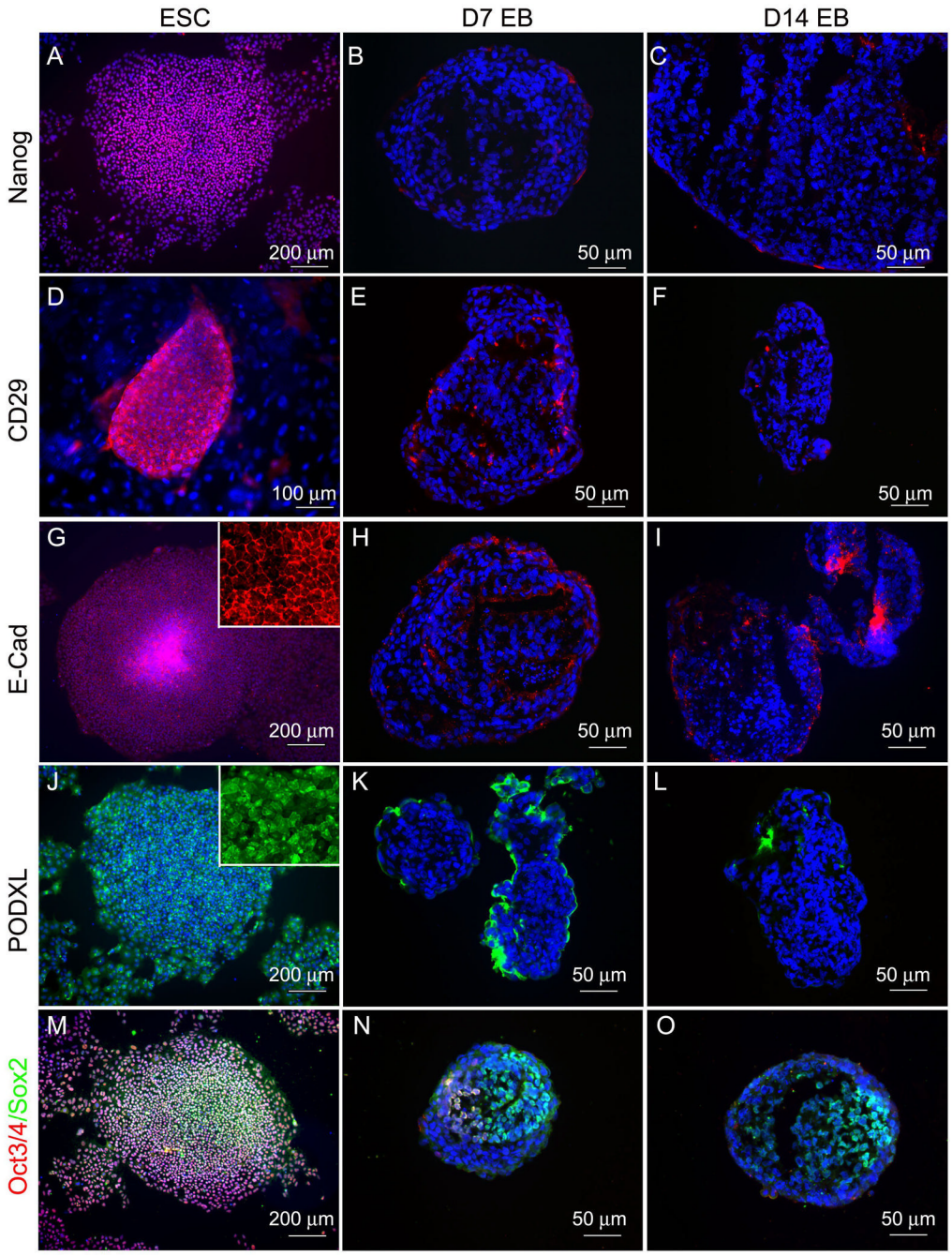


Fig 2.

Staining of ESC markers on undifferentiated hESCs, 7-day and 14-day EBs. ESC markers Nanog, ITGB1, CDH1, PODXL, Oct4, and Sox2 are expressed by most of undifferentiated hESCs (A, D, G, J, M), while their expression were downregulated in both 7-day and 14-day EBs (B-C, E-F, H-I, K-L, red in N-O) except Sox2 (green in N-O), which is also a neural stem cell marker.

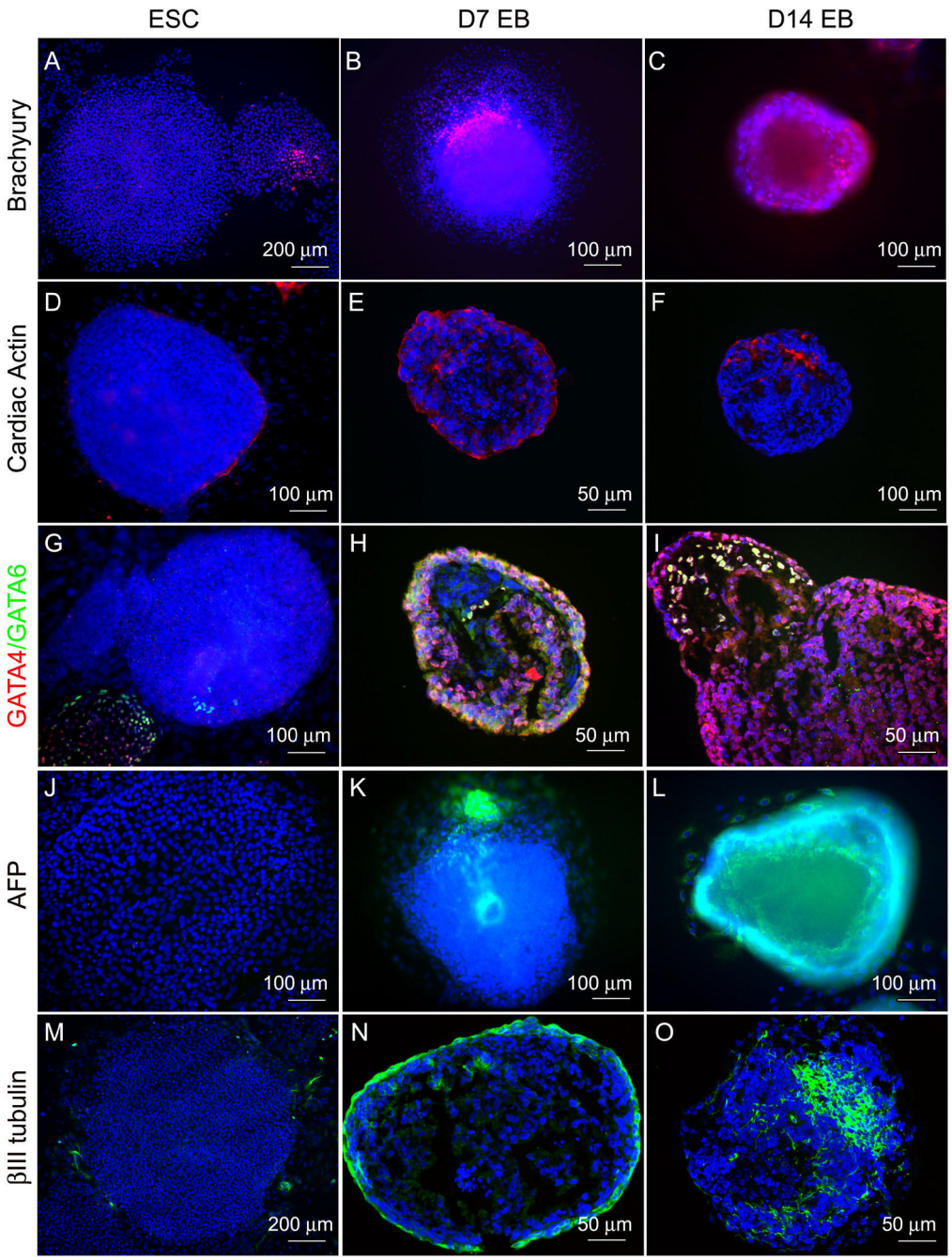
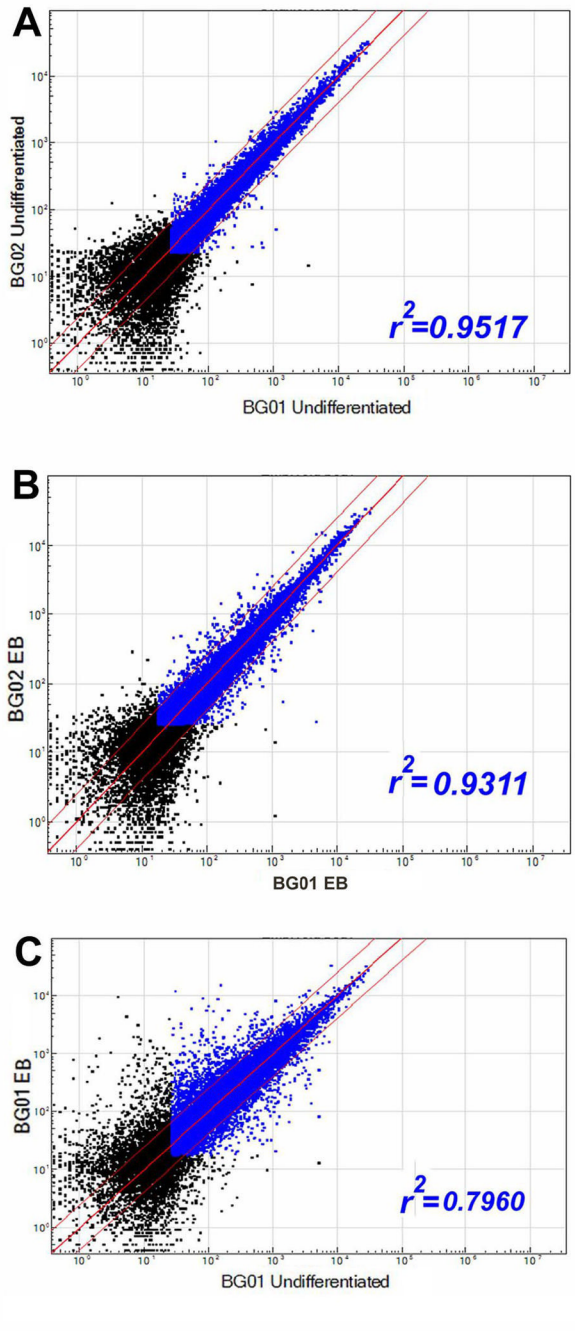


Fig 3.

Staining of differentiated markers on 7-day and 14-day EBs and undifferentiated hESCs. Mesoderm markers Brachyury and ACTC, endoderm markers AFP, GATA4, and GATA6, and ectoderm marker TUBB3 were detectable in both 7-day and 14-day EBs (A-B, D-E, G-H, J-K, M-N), while their expression were not detected in undifferentiated hESCs (C, F, I, L, O). Spontaneously differentiated hESCs also expressed Brachyury (C), GATA4 and GATA6 (I).



D

No. of genes on the array	~24, 000					
	Undifferentiated hESCs			Differentiated hEBs		
	BG01	BG02	BG03	BG01	BG02	BG03
No. of genes detected at >0.99 confidence	12,757	12,538	14,035	14,569	12,689	10,504
	Higher in hESCs			Higher in hEBs		
	BG01	BG02	BG03	BG01	BG02	BG03
No. of genes present at 2.5-fold difference	551	426	349	819	861	768
No. of genes present at 5-fold difference	110	54	53	261	254	230
No. of genes present at 10-fold difference	33	12	12	91	109	79

E Genes expressed at higher levels by hESCs as compared to hEBs

Gene Symbol	Undifferentiated hESCs			Differentiated hEBs			ES/EB
	BG01	BG02	BG03	BG01	BG02	BG03	
TDGF1	5507.5	6749.8	3750.8	101.9	614	33.8	21.4
LCK	1171	971.7	717.6	25.7	105.3	12.2	20
GABRB3	997.9	693.4	663.6	39.9	89.9	20.5	15.7
EBAF	2007.7	1417.1	1629.8	205.2	88.6	62.6	14.2
GDF3	562.9	507	351.6	43.4	61.4	12.5	12.1
ZNF206	1890.7	2030	1474.8	81.6	330.8	48.7	11.7
CKMT1	729.4	813.9	526.9	58.6	123.8	31.4	9.7
DNMT3B	4133.6	4668.1	3611.5	374.2	830.6	193.9	8.9
GYLTL1B	1846.4	1288.1	1147.3	162.3	299.8	80.4	7.9
PMAIP1	1226.1	1037.1	661.3	77.8	248.2	80	7.2
NTS	1346.3	966.4	631.5	101.7	327.1	62.5	6
POU5F1	534.8	328.3	231.9	42	109.5	39.7	5.7
IFITM1	8092	7238	5010.1	2023.2	2427	865.9	3.8
DIAPH2	1551.5	1110.9	979.3	364.6	451	395.4	3
ZFP42	244.2	227.3	212.2	78.4	141.5	26.7	2.8
LIN28	5071.3	5363.5	4644.3	1798	2890	897.5	2.7
MIBP	165.4	117.5	137.2	47.3	78.4	38.7	2.6
UTF1	367.3	382.1	210.2	135.8	164.3	86.3	2.5

F Genes expressed at higher levels by hEB when compared to hESCs

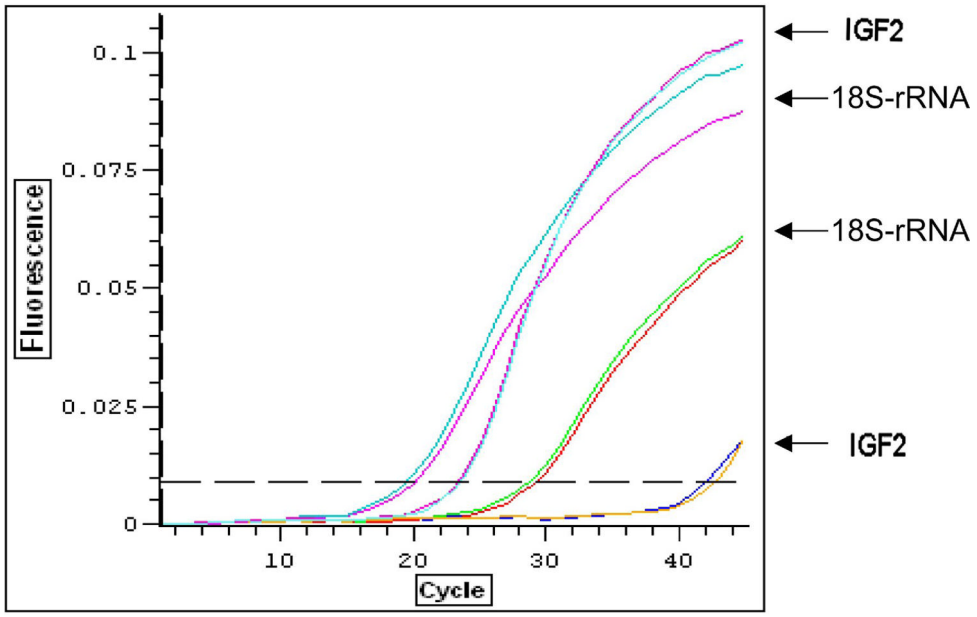
Gene Symbol	Differentiated hEBs			Undifferentiated hESCs			EB/ES
	BG01	BG02	BG03	BG01	BG02	BG03	
HAND1	14703	16475	10019	32.3	51.3	78.3	254.5
AFP	8202	15751	4134.5	107.5	61.7	92.4	107.4
SERPINA1	1063.8	3218.7	212.5	19.6	3	25.7	93.1
IGFBP3	10883.2	11967.7	4693.1	149.5	66	92.9	89.3
MGP	7064.9	2408.7	915.8	99	35.5	23.7	65.7
MSX1	3523.2	3322	2346	39.9	34.6	83.1	58.3
ACTA2	6743.5	2564.4	5094.2	126.7	41.6	95.2	54.7
PAX6	4113.7	1955.7	916.6	40.7	37	102	38.9
CXCL14	3615.6	3121.9	1006.9	141.1	90.8	89.2	24.1
FRZB	4893.7	4261.3	2538.7	174.9	138.4	278.5	19.8
ADAMTS9	1178.3	1083.1	537	65.4	41	41.4	18.9
COL2A1	3304.9	1399	1523.8	81.6	77	205.4	17.1
TGFBI	4764.9	4082.9	2219.5	495.1	152.1	159.4	13.7
OLR1	646	1495.7	216	36.3	61.1	96	12.2
PDGFRB	1197.4	751.7	589.4	95.4	56.1	58.6	12.1
WASPIP	741.5	721.4	496.7	67.2	64.2	75	9.5
FN1	5530.2	6753.9	4773.5	1042.9	409.7	570.5	8.4
CD47	710.1	942.8	305.6	63.9	86.4	107.7	7.6
SERPINF1	733.1	448.3	243.4	72	78.5	62.6	6.7
PTN	6139.3	4693.8	2207.2	558.9	493.5	1018.4	6.3
WNT1	481.4	115.2	162.7	39.4	30.7	59	5.9
GATA6	1464.9	1197.1	535.8	130.9	183.6	247.9	5.7
Hes4	1632.8	900.1	877.2	219.2	159.9	274.2	5.2
VIM	12111	10696	7933.3	3615.2	2481.4	2942.2	3.4
LAMB1	419.3	472.3	217.5	84.7	133.9	123.3	3.2

Fig 4.

Assessment of potential hESC and EB markers using microarrays. Three different ESC lines (BG01, 02 and 03) and 14-day EBs that differentiated from them were compared using an Illumina Bead array. (A-B) Comparisons of gene expression among three hESC lines or three EBs show similar levels with a correlation coefficient (r^2) greater than 0.93. (C) Pairwise comparisons of undifferentiated hESCs with their matched EBs reveal that about 50% of the genes are expressed with at least a 2.5-fold difference. (D) Summary of the numbers of genes detected in this array. (E-F) Selected genes that are differentially expressed in three hESC lines (BG01, 02 and 03) and in their matched EBs. Note that only the genes that are detected at >0.99 confidence (blue dots) are considered valid for further analysis. Dots that fall between the thin

red lines represent genes that are commonly expressed in hESCs and EBs, while dots outside red lines correspond to differentially expressed genes at >2.5 fold.

A



B

Gene	ESC/7-day EB	ESC/14-day EB
ESC marker		
UTF1	65	426
NANOG	12	241
Dppa5	8.7	12.3
Oct3/4	1.2	5.6
SOX2	0.2	0.3
Lin41	98	43
EB marker		
	7-day EB/ESC	**14-dayEB/ESC**
IGF2	5200	3.5E6
Hand1	2393	3347
GATA4	476	762
H19	20	348
SOX1	21	197
DCN	1.9	6.3

Fig 5.

qPCR analysis of 12 genes during hESC differentiation. Expression of 6 markers of each ESC and EB were quantified by qPCR. (A) Amplification curves corresponding to IGF2 and 18S RNA (standard curve) are shown from left to right: IGF2 of 14-day EB (blue and red); 18S RNA of 14-day EB (red and blue); 18S RNA of undifferentiated hESCs (green and red); and IGF2 of undifferentiated hESCs (blue and yellow). (B) The expression level of these genes were determined as the ratio to the level of 18S RNA, and differential expression of these genes in undifferentiated hESCs and EBs were shown as the ratio of expression in hESC to EB (ESC markers) or EB to hESC (EB markers).

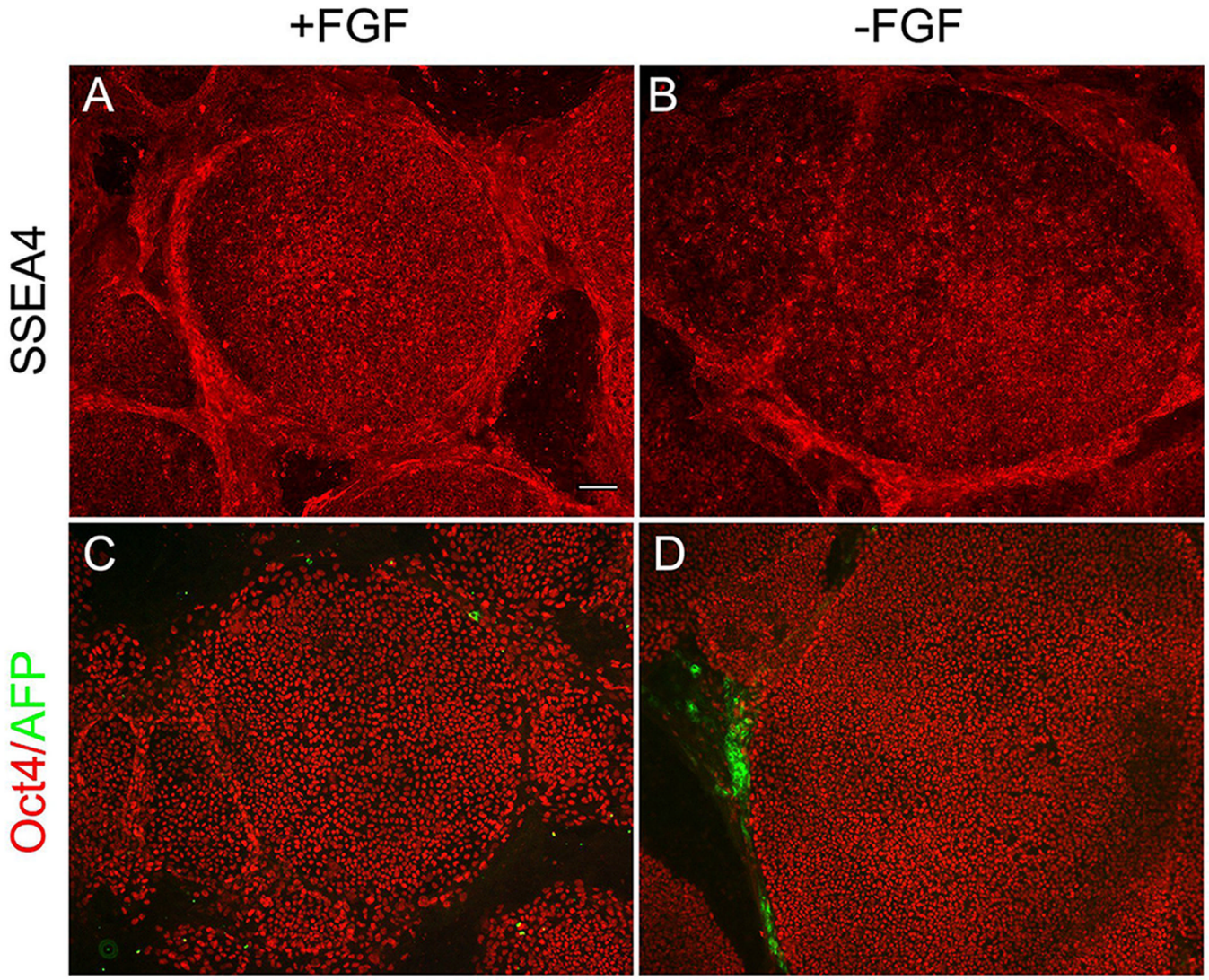


Fig 6.

No difference in ESC and EB marker expression by immunocytochemistry between hESC cultures maintained with bFGF and cultures where bFGF was withdrawn for a period of 72 hours. (A-B) Immunostaining of SSEA4 (red) shows similar expression levels in these two cell populations (with and without supplement bFGF). (C-D) Immunostaining of Oct4 (red) shows most of the cells are positive, while only occasional AFP positive cells (green) are seen outside the colonies in both hESC populations. Scale bar=100 μm.

Table 1

hESC specific markers

Gene symbol	Gene ID (Locus ID)	UniGene	Description	Tpm (hESC)	Tpm (hEB)	Micro-array (hESC)	Micro-array (hEB)	EST scan (hESC)	EST scan (hEB)	Reference
Stem cell marker										
LIN28	79727	Hs.86154	lin-28 homolog (C. elegans)	1962	178	3944.1	1421.6	25	13	24
DNMT3B	1789	Hs.251673	DNA (cytosine-5-)-methyltransferase 3 beta	1274	56	3391	274.5	58	3	24
CLDN6	9074	Hs. 533779	claudin 6	1063	151	2247.2	581.1	14	0	24
IFITM1	8519	Hs.458414	interferon induced transmembrane protein 1 (9-27)	920	221	8844	2188.5	4	1	4
POU5F1	5460	Hs.249184	POU domain, class 5, transcription factor 1	658	20	694.4	28.9	23	1	41
ITGB1	3688	Hs.429052	integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)	545	2246	3726.7	8211.2	16	70	
SFRP2	6423	Hs.481022	secreted frizzled-related protein 2	505	31	1353.9	93.1	7	1	4
GJA1	2697	Hs.74471	gap junction protein, alpha 1, 43kDa (connexin 43)	452	406	2404.4	2926.1	27	24	8
SOX2	6657	Hs.518438	SRY (sex determining region Y)-box 2	344	11	N.D.	N.D.	11	1	42
CD9	928	Hs.114286	CD9 antigen (p24)	221	13	2930.8	1267.2	1	0	43
GAL	51083	Hs.278959	galanin	221	28	1043.3	97.5	N.D.	N.D.	44
LIN41	Non	Hs.517884	LOC131405: similar to abnormal cell LINeage LIN-41, heterochronic gene; Drosophila dappled/ vertebrate TRipartite Motif protein related; B-box zinc finger, Filamin and NHL repeat containing protein (123.8 kD) (lin-41)	165	41	N.D.	N.D.	3	2	45
IMP-2	10644	Hs.35354	IGF-II mRNA-binding protein 2	140	133	1206	1163.2	5	8	4
LECT1	11061	Hs.421391	leukocyte cell derived chemotaxin 1	116	3	N.D.	N.D.	4	0	4
ZNF206	84891	Hs.334515	Zinc finger protein 206	109	0	1504.5	71.3	26	0	
GABRB3	2562	Hs.302352	gamma-aminobutyric acid (GABA) A receptor, beta 3	96	7	482.7	28	5	1	
GYLTL1B	120071	Hs.86543	glycosyltransferase-like 1B	92	0	1066.6	69.3	4	1	
UTF1	8433	Hs.458406	undifferentiated embryonic cell transcription factor 1	90	0	1444.1	81.3	N.D.	N.D.	46
NR6A1	2649	Hs.20131	nuclear receptor subfamily 6, group A, member 1	79	17	N.D.	N.D.	N.D.	N.D.	47
LEFTY1	10637	Hs.278239	left-right determination, factor 1	72	0	6579.6	73.1	4	0	
SCGB3A2	117156	Hs.483765	Secretoglobin, family 3A, member 2	65	0	890.5	12.2	1	0	
KIT	3815	Hs.479754	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene	46	5	188.9	42.2	1	0	
CKMT1	1159	Hs.425633	creatine kinase, mitochondrial 1 (ubiquitous)	42	0	742.4	46.2	1	0	
TDGF1	6997	Hs.385870	teratocarcinoma-derived growth factor 1	38	0	3169	200.2	16	1	18
FOXD3	27022	Hs.546573	forkhead box D3	37	0	N.D.	N.D.	N.D.	N.D.	48
DIAPH2	1730	Hs.226483	diaphanous homolog 2 (Drosophila)	27	4	1971.1	261.4	N.D.	N.D.	
NUMB	8650	Hs.509909	numb homolog (Drosophila)	24	1	618.3	778.9	0	4	
CER1	9350	Hs.248204	cerberus 1 homolog, cysteine knot superfamily (Xenopus laevis)	23	0	151.5	23.4	N.D.	N.D.	
PMAIP1	5366	Hs.96	Phorbol-12-myristate-13-acetate-induced protein 1	23	1	1601.5	336.6	4	0	
CDH1	999	Hs.461086	cadherin 1, type 1, E-cadherin (epithelial)	20	0	1972.9	793.1	11	0	

Gene symbol	Gene ID (Locus ID)	UniGene	Description	Tpm (hESC)	Tpm (hEB)	Micro-array (hESC)	Micro-array (hEB)	EST scan (hESC)	EST scan (hEB)	Reference
Stem cell marker										
			Left-right determination factor 2			944.1	100.1	1	0	
LEFTY2	7044	Hs.520187	(LEFTY2)	16	4					
NANOG	79923	Hs.329296	Nanog homeobox	16	0	56.9	10.7	1	0	36, 49
		Hs.12253, Hs.	SMAD, mothers against DPP homolog			332.8	397.4	1	1	
SMAD2	4087	465061	2 (Drosophila)	13	0					
BRIX	55299	Hs.38114	BRIX	9	0	1833.6	1261.5	3	3	4
REST	5978	Hs.401145	RE1-silencing transcription factor	6	0	18.5	15.4	1	1	
ZFP42 (Rex1)	132625	Hs.335787	zinc finger protein 42	5	0	159.3	34.5	2	0	50
EDNRB	1910	Hs.82002	endothelin receptor type B	0	0	346.8	119.8	0	1	
			phosphatase and tensin homolog			138.4	125.6	1	0	
			(mutated in multiple advanced cancers							
PTEN	5728	Hs.500466	1)	0	0					
NOG	9241	Hs.248201	noggin	0	0	N.D.	N.D.	N.D.	N.D.	
GDF3	9573	Hs.86232	growth differentiation factor 3	N.D.	N.D.	236.5	27.2	N.D.	N.D.	24
GBX2	2637	Hs.184945	gastrulation brain homeo box 2	N.D.	N.D.	44.5	14.7	N.D.	N.D.	
TFCP2L1	29842	Hs.156471	transcription factor CP2-like 1	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	
COMMD3	23412	Hs.534398	COMM domain containing 3	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	
TERT	7015	Hs.492203	telomerase reverse transcriptase	N.D.	N.D.	N.D.	N.D.	1	0	
PODXL	5420	Hs.16426	podocalyxin-like	N.D.	N.D.	498.2	394.2	60	23	4
			fibroblast growth factor 4 (heparin			N.D.	N.D.	1	0	51
			secretory transforming protein 1,							
FGF4	2249	Hs.1755	Kaposi sarcoma oncogene)	N.D.	N.D.					
			nuclear receptor subfamily 5, group A,			N.D.	N.D.			
NR5A2	2494	Hs.33446	member 2	N.D.	N.D.			N.D.	N.D.	
			interferon induced transmembrane			129.2	204	2	0	
IFITM2	10581	Hs.174195	protein 2 (1-8D)	N.D.	N.D.					
GRB7	2886	Hs.86859	growth factor receptor-bound protein 7	N.D.	N.D.	105.4	11.6	2	0	
			developmental pluripotency associated			N.D.	N.D.			52
DPPA5	359942	Hs.125331	5	N.D.	N.D.			N.D.	N.D.	
NODAL	4838	Hs.370414	nodal homolog (mouse)	N.D.	N.D.	N.D.	N.D.	1	0	
			Lymphocyte-specific protein tyrosine			500	10.7			
LCK	3932	Hs.470627	kinase	N.D.	N.D.			N.D.	N.D.	
NTS	4922	Hs.80962	Neurotensin	N.D.	N.D.	1313	137.7	N.D.	N.D.	
ITGB1BP3	9270	Hs.135458	Integrin beta 1 binding protein 3	N.D.	N.D.	N.D.	N.D.	2	0	
			Carbohydrate (N-acetylglucosamine 6-			N.D.	N.D.	2	0	
CHST4	10164	Hs.251383	O) sulfotransferase 4	N.D.	N.D.					

Table 2

EB specific markers

Gene symbol	Gene ID (Locus ID)	UniGene	Description	Tpm (hESCs)	Tpm (hEBs)	Micro-array (hEB)	Micro-array (hEB)	EST scan (hESC)	EST scan (hEB)	Reference
			Ectoderm marker							
VIM	7431	Hs.533317	vimentin	626	9881	6262.9	16146.1	38	114	53
CRABP2	1382	Hs.405662	cellular retinoic acid binding protein 2	26	396	1549.9	3187.6	9	5	54
SEMA3A	10371	Hs.252451	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3A	1	16	214.1	180.2	1	1	55
MSI1	4440	Hs.158311	musashi homolog 1 (Drosophila)	0	11	1968	787.6	5	1	56
MAP2	4133	Hs.368281	microtubule-associated protein 2	0	10	N.D.	N.D.	1	1	57
GFAP	2670	Hs.514227	glial fibrillary acidic protein	0	0	34.7	15	N.D.	N.D.	58
OLIG2	10215	Hs.176977	oligodendrocyte lineage transcription factor 2	0	0	13.4	1.7	N.D.	N.D.	59
SOX1	6656	Hs.202526	SRY (sex determining region Y)-box 1	N.D.	N.D.	21.2	10.1	N.D.	N.D.	60
NES	10763	Hs.527971	nestin	386.	50.	2935.8	2471.3	4	6	61
NEUROD1	4760	Hs.72981	neurogenic differentiation 1	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	62
TH	7054	Hs.435609	tyrosine hydroxylase	N.D.	N.D.	158.4	107.8	N.D.	N.D.	63
TUBB3	10381	Hs.511743	tubulin, beta 3	N.D.	N.D.	N.D.	N.D.	2	8	64
			Endoderm marker							
FN1	2335	Hs.203717	fibronectin 1	3661	21250	3243.5	12913.2	34	351	65
DCN	1634	Hs.156316	decorin	2	2008	95.6	1550.2	0	18	66
H19	283120	Hs.551588	H19, imprinted maternally expressed untranslated mRNA	64	2176	N.D.	N.D.	2	136	
AFP	174	Hs.518808	alpha-fetoprotein	30	1829	1089.1	9765	0	5	67
LAMB1	3912	Hs.489646	laminin, beta 1	186	614	126.7	504.2	15	19	68
LAMC1	3915	Hs.497039	laminin, gamma 1 (formerly LAMB2)	203	352	2098.4	4340.9	14	13	69
BMP2	650	Hs.73853	bone morphogenetic protein 2	35	69	63.1	68.4	1	1	70
SERPINA1	5265	Hs.525557	serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1	0	61	136.3	1045.2	1	1	71
FLT1	2321	Hs.507621	fms-related tyrosine kinase 1 (vascular endothelial growth factor/ vascular permeability factor receptor)	38	46	N.D.	N.D.	2	0	72
ACVR1B	91	Hs.438918	activin A receptor, type IB	22	42	237.8	187.2	4	3	73
GATA4	2626	Hs.243987	GATA binding protein 4	2	13	83.2	145.2	3	4	74
GCG	2641	Hs.516494	glucagon	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	75
INS	3630	Hs.89832	insulin	N.D.	N.D.	122.7	70.4	N.D.	N.D.	76
PECAM1	5175	Hs.514412	platelet/endothelial cell adhesion molecule (CD31 antigen)	N.D.	N.D.	40	49.1	N.D.	N.D.	77
FABP2	2169	Hs.282265	fatty acid binding protein 2, intestinal	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	78
HNF4A	3172	Hs.116462	hepatocyte nuclear factor 4, alpha	N.D.	N.D.	16	10	N.D.	N.D.	79
FGF8	2253	Hs.57710	fibroblast growth factor 8 (androgen-induced)	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	80
HGF	3082	Hs.396530	Hepatocyte growth factor	N.D.	N.D.	32.2	39.4	0	1	81
FOXA2	3170	Hs.155651	Forkhead box A2	N.D.	N.D.	94.2	83.2	2	0	82
			Mesoderm marker							
COL1A1	1277	Hs.172928	collagen, type I, alpha 1	3889	68430	208.7	2424.2	67	658	83

Gene symbol	Gene ID (Locus ID)	UniGene	Description	Tpm (hESCs)	Tpm (hEBs)	Micro-array (hEB)	Micro-array (hEB)	EST scan (hESC)	EST scan (hEB)	Reference
HAND1	9421	Hs.152531	heart and neural crest derivatives expressed 1	0	500	389.8	9466.3	0	8	84
MSX1	4487	Hs.424414	msh homeo box homolog 1 (Drosophila)	15	218	467.3	2198.1	1	3	85
ACTC	70	Hs.118127	actin, alpha, cardiac muscle	112	141	952.2	2963.6	1	4	86
GATA6	2627	Hs.514746	GATA binding protein 6	4	53	206.7	964.2	N.D.	N.D.	87
COL2A1	1280	Hs.408182	collagen, type II, alpha 1 (primary osteoarthritis, spondyloepiphyseal dysplasia, congenital)	70	49	144.5	632.6	1	19	88
HBZ	3050	Hs.449632	hemoglobin, zeta	0	34	30	408.7	N.D.	N.D.	89
T	6862	Hs.389457	T, brachyury homolog (mouse)	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	90
WT1	7490	Hs.408453	Wilms tumor 1	N.D.	N.D.	40.6	0.9	N.D.	N.D.	91
MYF5	4617	Hs.178023	myogenic factor 5	N.D.	N.D.	20.2	12.1	N.D.	N.D.	92
DES	1674	Hs.471419	desmin	N.D.	N.D.	6.6	4.5	N.D.	N.D.	93
NPPA	4878	Hs.75640	natriuretic peptide precursor A	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	94
HBB	3043	Hs.523443	hemoglobin, beta	N.D.	N.D.	33.1	46	N.D.	N.D.	95
RUNX2	860	Hs.535845	runt-related transcription factor 2	N.D.	N.D.	21.9	5.3	0	3	96
IGF2	3481	Hs.523414	insulin-like growth factor 2 (somatomedin A)	N.D.	N.D.	115.8	5369.7	1	18	97
			Trophoblast							
EOMES	8320	Hs.147279	eomesodermin homolog (Xenopus laevis)	10	0	247.2	63.4	N.D.	N.D.	98
CDX2	1045	Hs.174249	caudal type homeo box transcription factor 2	0	5	13.4	343.9	0	1	99
GCM1	8521	Hs.28346	glial cells missing homolog 1 (Drosophila)	N.D.	N.D.	16.5	9.4	N.D.	N.D.	100
KRT1	3848	Hs.80828	keratin 1 (epidermolytic hyperkeratosis)	N.D.	N.D.	13.4	17.1	N.D.	N.D.	101
			Germ Cells							
SYCP3	50511	Hs.506504	synaptonemal complex protein 3	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	102
DDX4	54514	Hs.223581	DEAD (Asp-Glu-Ala-Asp) box polypeptide 4	N.D.	N.D.	10	5.5	N.D.	N.D.	103
IFITM1	8519	Hs.458414	Fragilis-1	N.D.	N.D.	8844	2188.5	4	1	104
IFITM2	10581	Hs.174195	Fragilis-2	N.D.	N.D.	129.2	204	2	0	105

Table 3

Expression levels of ESC and EB markers in hESCs.

Markers		ESCs IC	ESCs PCR	EB IC	EB PCR
ESC	LIN28	N.A	++	N.A	-/+
	POU5F1 (Oct4)	+++	+++	-	-/+
	GJA1	N.A	+++	N.A	++
	SOX2	+++	+++	+	-/+
	GAL	N.A	+++	N.A	-/+
	LIN41	N.A	++	N.A	-/+
	UTF1	N.A	+++	N.A	-
	TDGF1	N.A	+++	N.A	-/+
	FOXD3	N.A	++	N.A	N.A
	CDH1	+++	N.A	-	N.A
	NANOG	+++	+++	-	-
	ZFP42 (Rex1)	N.A	++	N.A	-
	TERT	N.A	++	N.A	-
	PODXL	+++	N.A	-	N.A
	FGF4	N.A	+++	N.A	N.A
	DPPA5	N.A	+	N.A	-
	Tra-1-60	++	N.A.	-	N.A.
	Tra-1-81	++	N.A.	-	N.A.
	SSEA-1	+++	N.A.	-	N.A.
	SSEA-4	+++	N.A.	-	N.A.
	ITGB1 (CD29)	+++	N.A.	-	N.A.
Ectoderm	MSI1	N.A.	-/+	N.A.	++
	MAP2	N.A.	-/+	N.A.	++
	SOX1	N.A.	-	N.A.	++
	NES	-/+	N.A	+++	N.A
	TH	-	-	++	++
	TUBB3	-	N.A	+++	N.A
Endoderm	DCN	N.A	-	N.A	+++
	H19	N.A	-	N.A	+++
	AFP	-	-	++	+++
	GATA4	-	-	+++	+++
	HGF	N.A.	-	N.A.	++
	FoxA2	N.A.	-	N.A.	++
Mesoderm	HAND1	N.A.	-/+	N.A.	+++
	MSX1	N.A.	-	N.A.	++
	ACTC	-	N.A.	+++	N.A.
	GATA6	-	N.A.	+	N.A.
	T (Brachyury)	-	-	++	++
	IGF2	N.A.	-	N.A.	+