Temporal gene expression during differentiation of human embryonic stem cells and embryoid bodies

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BACKGROUND: The aim of this study was to characterize human embryonic stem (ES) cells at the molecular level by performing large-scale complementary DNA (cDNA) analysis using DNA micro-arrays. METHODS: The transcription profile of human ES cells was determined by comparing it to 2, 10 and 30-day old embryoid bodies (EBs) using Affymetrix Genechip human micro-arrays (U133). RESULTS: According to this analysis we demonstrate that two human ES cell lines are more close to each other than to their differentiated derivatives. We also show the spectrum of cytokine receptors that they express, and demonstrate the presence of five genes that are highly specific to human ES cells and to germ cells. Moreover, by profiling different stages in the differentiation of human embryoid bodies, we illustrate the clustering of five sets of temporally expressed genes, which could be related to the sequential stages of embryonic development. Among them are known genes that are involved in early pattern formation. CONCLUSIONS: The present study provides a molecular basis for the identity of human ES cells and demonstrates that during their *in vitro* differentiation they express embryonic specific genes in a stage specific manner.

Key words: differentiation/ES cells/gene expression/micro-arrays/pluripotency

Introduction

Human embryonic stem (ES) cells, isolated from the inner cell mass (ICM) of early human embryos, were suggested to be true pluripotent stem cells (Thomson *et al.*, 1998; Reubinoff *et al.*, 2000). As such, they are an important tool for the study of human embryonic development and cell-based therapies (Schuldiner and Benvenisty, 2001). Human ES cells may be differentiated *in vitro* and *in vivo* into many different cell types. *In vitro*, they can be triggered to undergo spontaneous differentiation by forming embryoid bodies (EBs) (Itskovitz-Eldor *et al.*, 2000). Initially they generate simple bodies composed of densely packed cells that are surrounded by a single cell layer of endodermal like cells. These become cavitated and eventually accumulate fluid. The resulting cystic EBs, which are a product of spontaneous differentiation, are considered as mature EBs and contain many different cell types (Itskovitz-Eldor *et al.*, 2000). Differentiation of human ES cells *in vitro* may be partially directed by addition of growth factors to the culture media (Schuldiner *et al.*, 2000). Indeed, differentiation of human ES cells produces cells that express genes specific to differentiated cells such as neurons (Itskovitz-Eldor *et al.*, 2000; Reubinoff *et al.*, 2000, 2001; Schuldiner *et al.*, 2000, 2001; Carpenter *et al.*, 2001; Zhang

et al., 2001) cardiomyocytes (Itskovitz-Eldor *et al.*, 2000; Schuldiner *et al.*, 2000; Kehat *et al.*, 2001; Mummery *et al.*, 2002) and hematopoietic cells (Itskovitz-Eldor *et al.*, 2000; Schuldiner *et al.*, 2000; Kaufman *et al.*, 2001). In addition, methods for genetically modifying human ES cells now allow labeling and sorting of cells at different stages of their differentiation (Eiges *et al.*, 2001).

While it is obvious that human ES cells could become a useful system to study the differentiation of human embryonic cells, presently little is known about the genes they express. Human ES cells do express several markers suggested to reflect human ICM cells (Henderson *et al.*, 2002). Recently, mouse ES cells were compared to hematopoietic and neuronal stem cells using DNA micro-arrays (Ivanova *et al.*, 2002; Ramalho-Santos *et al.*, 2002). These analyses showed that embryonic stem cells have some features in common with adult stem cells, allowing the identification of a cluster of genes which is associated with the 'stemness' character of all stem cells (Ivanova *et al.*, 2002; Ramalho-Santos *et al.*, 2002). Global transcription profiles for undifferentiated human ES cells were independently published by several groups (Sato *et al.*, 2003; Sperger *et al.*, 2003; Richards *et al.*, 2004). In the present study we chose to carry out a large-scale transcription analysis to profile human ES cells at different stages during their differentiation

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in vitro, as an initial step towards understanding the genetic control of human embryonic differentiation.

Materials and methods

Cell culture

Human ES cells (passage 42–47, normal karyotype) and EBs were cultured as previously described (Itskovitz-Eldor *et al.*, 2000; Schuldiner *et al.*, 2000) with minor modifications. Undifferentiated ES cells (H9 and H13 cell lines) were cultured in 80% Knock-Out™ Dulbecco's modified Eagle's medium (Gibco-BRL), supplemented with 20% KnockOut™ SR—a serum-free formulation (Gibco-BRL), 1 mM glutamine (Gibco-BRL), 0.1 mM β-mercaptoethanol (Sigma), 1% non-essential amino acids stock (Gibco-BRL), penicillin (50 U/ml), streptomycin (50 µg/ml) and 4 ng/ml of basic fibroblast growth factor (bFGF), on a mitomycin-C treated mouse embryonic fibroblast (MEF) feeder layer. To reduce the presence of feeder cells in the culture (to <5%), human ES cells were grown on 0.1% gelatin (Merck) coated plates for a single passage. Under these conditions >95% of the cells in the colonies are still undifferentiated as shown by OCT4 staining (see our website: http://www.ls.huji.ac.il/~nissimb/gene_profiling.html). High-density cultures of undifferentiated cells were trypsinized and used either for RNA extraction or for EB formation by allowing cells to aggregate in suspension on plastic petri bacterial dishes. EBs were collected for analysis following 2, 10, 20 and 30 days of cell aggregation in culture.

DNA micro-array analysis

Total RNA was extracted from populations of undifferentiated (confirmed by *REX1* and *OCT4* expression) and differentiated cell derivatives of human ES cells. RNA extraction was performed according to the manufacturer's protocol (Affymetrix). Hybridization to the DNA micro-arrays, washing and scanning were performed according to the manufacturer's protocols, and compared for expression pattern using Affymetrix U133 DNA chip microarrays. Affymetrix DNA micro arrays are provided with human specific probes each of which consists of 20 different sequence combinations, in order to overcome hybridization efficiency differences. Moreover, we have normalized signal value for each probe through dividing by the average signal of the hybridization in each experiment, to reduce differences in signal levels between experiments. Analysis of the results was performed using the GENE SPRING and GO ANNOTATION programs.

The U133 GeneChip human micro-array, contains nearly 45 000 probe sets (representing 33 000 human genes). This DNA microarray was used to compare the global expression of H9 human ES cells and 2, 10 and 30-day old EBs. Each differentiation stage was assayed in three independent experiments to determine the variability of the system.

ES specific gene expression analysis

Average signal value was calculated for each probe in ES cells and EBs, and ordered according to the ratio of ES/EB value. Complementary (cDNA) probes in which expression was at least as high as the average signal level of the chip (value of 100 following normalization) and for which the ratio between ES and EBs was >20 (total of 50 sequences: see our website: http://www.ls.huji.ac.il/~nissimb/gene_profiling.html) were further examined for tissue distribution by searching in databanks (dbESTs-NCBI and Source-USCS).

Databases

We have used the following databases: Entrez (NCBI)—the text-based search system used at NCBI for its major databases. Unigene (NCBI)—a database which partitions GeneBank sequences into a non-redundant set of gene-oriented clusters, each of which contains sequences that represent a unique gene and its related information i.e. map location and tissues where it is expressed. Blast (NCBI)—a sequence similarity search tool. LocusLink (NCBI)—provides a descriptive information regarding genetic loci (e.g. information on official nomenclature, aliases and sequence accession numbers etc.) and AceView (NCBI)—offers an integrated view of the human genes as reconstructed by alignment of all publicly available mRNAs and Expressed Sequence Tags (ESTs) on the genome sequence. SOURCE (Stanford University)—collects and compiles data about genetics and molecular biology of genes from human and other species genomes. BLAT (UCSC)—contains reference sequences for human and other species and integrate map location and various types of annotation.

RT–PCR analysis

Total RNA was extracted as described (Chomczynski and Sacchi, 1987) and 1 µg of RNA was reverse transcribed by random hexamer priming using EZ-First Strand cDNA Synthesis Kit (Biological Industries). Amplification was performed on the cDNA using Takara Ex Taq™, in the presence of X1 Ex Taq™ Buffer, 200 µM dNTPs each, and 2.5 mM Mg^{2+}. PCR conditions include a first step of 3 min at 94°C, a second step of 20–30 cycles of 30 s at 94°C, 30 s annealing step at 60–64°C, and 45 s at 72°C, and a final step of 5 min at 72°C. Several markers were examined: *OCT4* as a marker for undifferentiated ES cells, *GAPDH* as a house keeping gene, *LEFTY A* as a transiently expressed gene and *NODAL*, *LEFTY B* and *PITX2* as genes related to the *LEFTY A* pathway. A full description of primers, annealing temperature and size of final products is described in Table I. Final products were assessed by gel electrophoresis on 2% agarose ethidium-bromide stained gels and their identity was verified by direct sequencing.

Table I. Primers, annealing temperature and size of final products

Marker	5′-primer	3′-primer	Temp.	Size
OCT4	GATCCTCGGACCTGGCTAAG	CTCTCACTCGGTTCTCGATAC	64°C	637 bp
Hs.351262	GGTGCCATGACTCGGATCG	CTCACAGTACTTGCTGTAGG	62°C	446 bp
Hs.67624	GGTTCTCTGACTGACTCCTTC	GCTCCTGGCAGCTCTTTATTC	64°C	603 bp
Hs.86154	CACCAGAATAAGCTGCACATG	CCTGAGATACATGGCAGTGC	64°C	523 bp
Hs.189095	CAGGAATTTGTGGCGGAGAG	CCTGTGACAGTCCTTACTGC	62°C	416 bp
GAPDH	AGCCACATCGCTCAGACACC	GTACTCAGCGGCCAGCATCG	62°C	302 bp
NODAL	GGCAGAAGATGTGGCAGTGG	CAAGTGATGTCGACGGTGC	64°C	535 bp
LEFTY A	CTGGACCTCAGGGACTATG	GACCACCTCTTATGCACACG	62°C	435 bp
LEFTY B	TTGGGGACTATGGAGCTCAG	TCAAGTCCCTCGATGGCTAC	60°C	406 bp
PITX2	GTGGACCAACCTTACGGAAG	CATGCTCATGGACGAGATAG	62°C	307 bp

Results

A molecular characterization of human ES cells

Human ES cells and their differentiated progeny were profiled using Affymetrix GeneChip micro-array. Profiles were obtained for two independent human ES cell lines (H9 and H13) and EBs (from H9 cells) at different stages of differentiation. (Figure 1A). By normalizing signal values and comparing expression levels of all probes between the different samples, as described above, we could demonstrate a significant difference between fully differentiated EBs (30 days) (Itskovitz-Eldor *et al.*, 2000) and all other cell types, ES cells and partially differentiated EBs (Figure 1A). Different samples of ES cells or ES cells from different lines (H9 and H13) show a very similar pattern of gene expression (Figure 1B). These data are shown as scatter plots (Figure 1B). Comparing different ES cell lines (H9 and H13) with 30-day old EBs (H9), demonstrates that variation within ES samples is smaller than variation between ES cells and EBs from the same cell line.

Self-renewal and a broad developmental potential are important characteristics of stem cells. These two properties are likely to depend on surface molecules, including receptors, which interact with cytokines and other signaling factors. Human ES cells were analyzed for the presence of cytokine receptors and their related factors by examining expression levels (Figure 2A). Of the 74 receptors tested (members of 21 different families), 28 were found to be present in ES cells (Figure 2). Members of the protein tyrosine phosphatase (PTP), fibroblast growth factor (FGF), insulin-like growth factor (IGF), bone morphogenetic protein (BMP), ACTIVIN and tumour necrosis factor (TNF) families of receptors are expressed at relatively high levels whereas transforming growth factor-beta receptor (TGFβR), MET and gp130 are expressed at a low level. In an attempt to identify possible autocrine loops, we examined the expression of cytokines that correspond to the displayed receptors (total of 67). Fifteen of these cytokines are expressed in ES cells (Figure 2B). Interestingly, a rough correlation can be found in the relative level of expression between the receptors and their associated ligands (e.g. see PTP receptors and their growth factors midkine and pleiotrophin, or FGF receptors and their cytokines FGF2, FGF4 and FGF13). By performing hierarchical clustering of the different tissues with respect to their receptors, ES cells show the highest similarity to ovary and uterus; equally so. Yet, EBs more closely resemble placenta (data not shown).

Identification of ES cell specific genes

In order to isolate genes specific to ES cells, a pair wise comparison in gene expression between ES cells and 30-day old EBs was carried out. Of 50 sorted sequences, five were selected since they were highly expressed in germ cells and ES cells (Figure 3). These include four ESTs as well as *OCT4*. Apart from *OCT4*, which is associated with pluripotency in mice (Nichols *et al.*, 1998), all others which display expression pattern similar to *OCT4*, have unknown functions but are predicted to be transcription factors, based on their
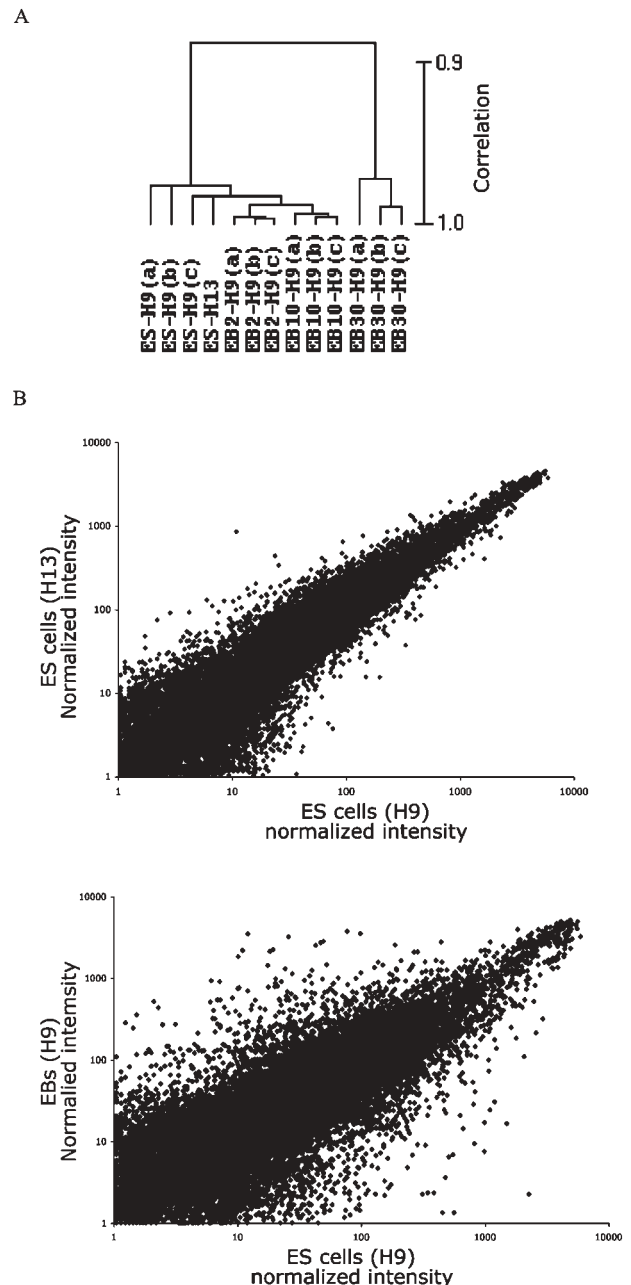

Figure 1. Gene profile of human embryonic stem (ES) cell lines and human embryoid bodies (EBs). (A) A graphic illustration demonstrating the degree of similarity between different ES cell lines (H9 and H13) and between EBs at different stages of their differentiation (2, 10 and 30-day old EBs derived from H9 ES cells), using the U133A Affymetrix DNA chip micro-array. Hierarchical clustering was performed as described above. (B) A scatter plot analysis of the degree of similarity in gene expression, based on the U133A Affymetrix micro-array, between two different human ES cell lines (H9 and H13) as compared to the correlation between a given ES cell line (H9) and it's differentiated cell derivatives (30-day old EBs).

DNA sequence. To verify the DNA micro-array results, the selected sequences were examined for expression levels by RT−PCR in H9 human ES cells and in 20-day old EBs. All show down-regulation upon differentiation. Expression of these markers in undifferentiated cells was also verified in two additional human ES cell lines (data not shown).
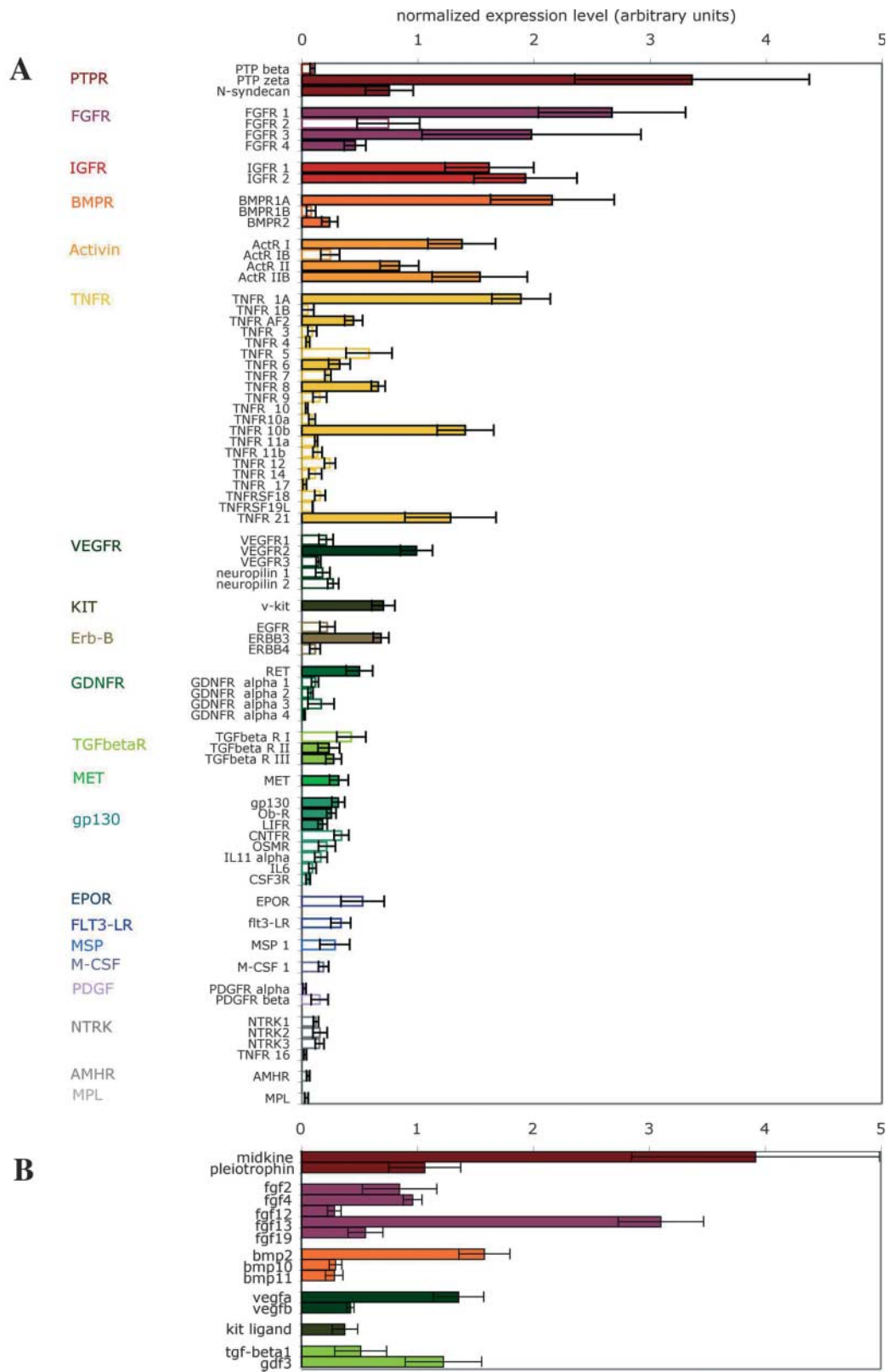
Figure 2. Analysis of expression of cytokines and their receptors in human ES cells. (**A**) Shown are the relative expression levels of the different cytokine receptors, which are expressed by human ES cells. Levels were normalized relative to the average expression level of the entire micro-array. Full and empty bars represent expressed and non-expressed receptors, respectively, as defined by Affymetrix analysis. Receptor families are color-coded and sorted according to their relative expression levels. The values are given with SE. (**B**) Shown are cytokines that may signal via an autocrine loop in human ES cells. They are color-coded using the same key as their respective receptors. Only cytokines with their receptors expressed in human ES cells are listed. The values are given with SE.
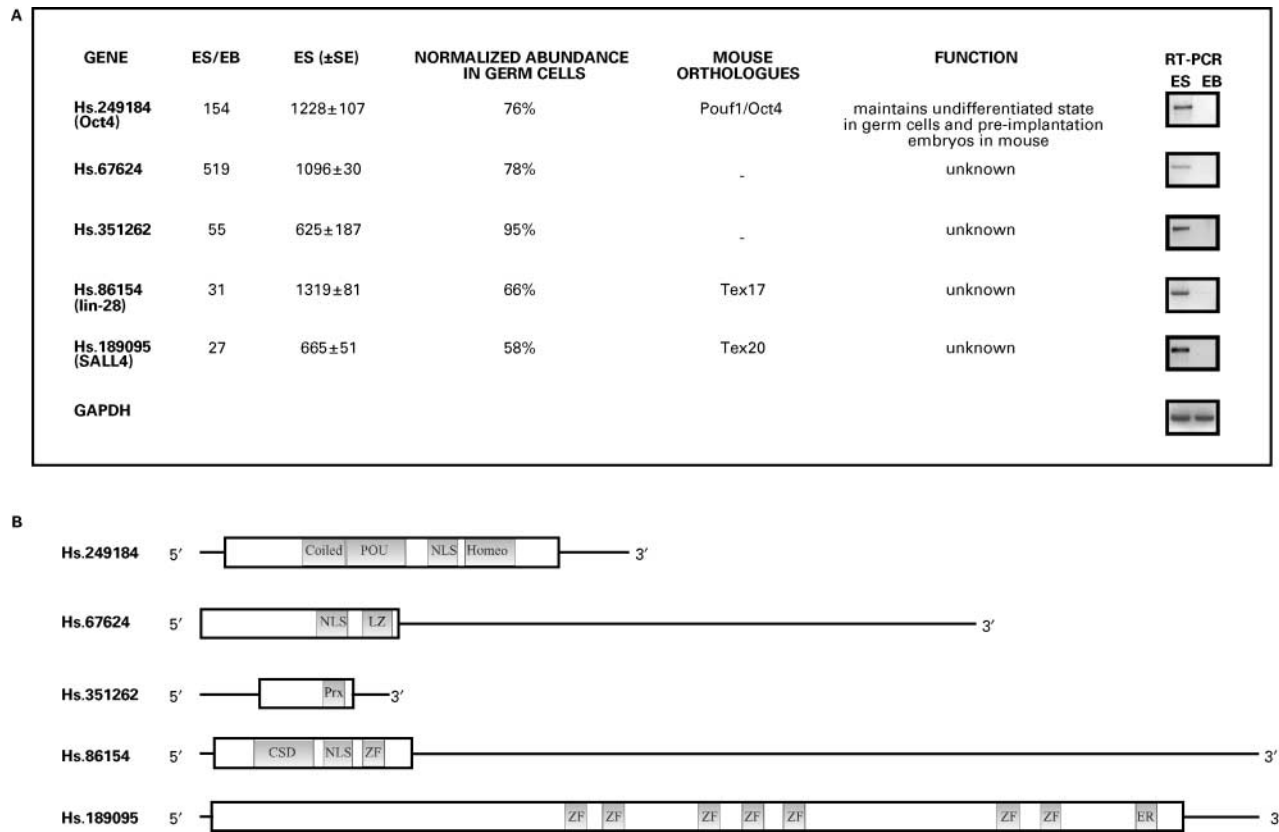
Figure 3. Identification of Human ES-specific genes. (**A**) H9 human ES cells and 30-day old EB (three replicas each) were analyzed using the U133 Affymetrix DNA chip micro-array. Average signal levels were calculated for each probe and ordered according to the ratio of their expression levels in ES/EB. Sequences in which the ratio was >20 (total of 50 sequences), were examined for tissue distribution in available databanks, and selected for further investigation if they were highly abundant in germ cells (total of five sequences). To verify results, the selected genes were analyzed in H9 human ES cells and their differentiated counterparts, 20-day old EB by RT−PCR. Final products, obtained by the amplification of only cDNA sequences, were detected by gel electrophoresis on 2% ethidium-bromide stained gels. Samples were examined for *GAPDH* as reference control. Expression levels were confirmed in two other human ES cells lines, H13 and BGN1 (data not shown). (**B**) The sequences were searched for known protein motifs using Aceview database. Line represents untranslated region, open box represents open reading frame. (Domains − Coiled: coiled coil; NLS: nuclear localization signal; Homeo: homeobox; LZ: leucine zipper; Prx: peroxisomal; CSD: cold shock domain; ZF: zinc finger; ER: ER membrane.)

Temporal gene expression during differentiation of EBs

To follow differentiation of EBs *in vitro*, we identified genes that are transiently expressed at different stages of EB formation. Normalized expression levels were compared for each probe between ES cells, 2, 10 and 30-day old EBs (three independent experiments each). These stages represent simple, cavitated and cystic EBs, respectively. The temporally expressed genes, identified by U133 chip analysis, clustered into three groups. Using GENE SPRING software. We classify them as early, mid or late expressed genes (Figure 4A).

A more refined analysis shows that the process of *in vitro* differentiation may be further subdivided into five groups: early, early-mid, mid, mid-late and late expressed genes, which are either down-regulated immediately or gradually, or begin to up-regulate in maturing or fully matured EBs. Representative gene markers which correspond to the following groups include *LECTIN GALACTOSIDASE-BINDING PROTEIN* (early), *OCT4* (early-mid), *LEFTY A* (mid), *α-FETOPROTEIN* (mid-late) and *SURFACTANT-D* (late), as demonstrated in Figure 4B. In addition, it is possible to follow differentiation by studying the temporal expression pattern of a set of genes, known to be involved in a given developmental pathway, and that are expressed successively during embryogenesis. As an example, we show temporal expression of the *NODAL* pathway, that plays a major role in the determination of embryonic axes (left−right, dorsal−ventral, anterior−posterior) as well as in mesoderm induction during early development (Hamada *et al.*, 2002). Thus, during EB differentiation we could demonstrate transient expression of *NODAL*, *LEFTY A* and *LEFTY B* growth factors, followed by induction of their target, the *PITX2* transcription factor (Figure 4C). This was confirmed by RT−PCR, at non-saturating conditions, for all four genes (Figure 4D).

Discussion

The wide developmental potential and capacity for self-renewal suggests that human ES cells might greatly differ from all other adult somatic tissues in terms of gene expression. Recently, global transcription profiles for hES cells were published using micro-array (Sato *et al.*, 2003;
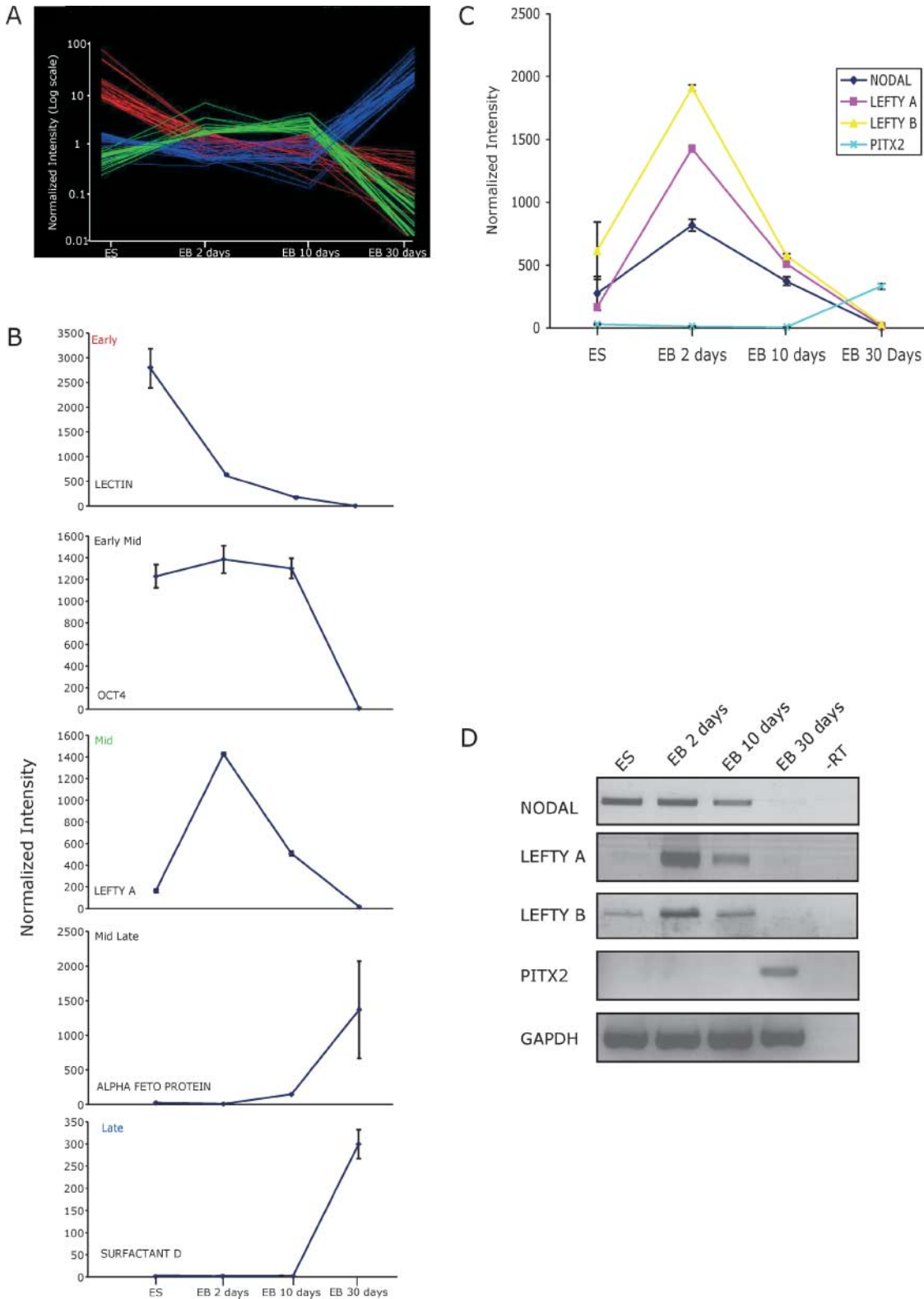
Figure 4. Patterns of temporal gene expression during human EB differentiation. (**A**) Clustering of genes into three major groups, early (red), mid (green) and late (blue) expressed genes. Classification was performed according to the temporal expression pattern of the transiently expressed genes in undifferentiated ES cells, 2, 10 and 30-day old EBs. Expression levels are displayed by the log scale of normalized signal intensity values. (**B**) Subdividing the analyzed genes into early, early-mid, mid, mid-late and late expression patterns, as demonstrated by the following representative genes: *LECTIN* (galactoside-binding, soluble 1), *OCT4*, *LEFTY A*, α-*FETOPROTEIN* and *SURFACTANT-D*, respectively. The values are given with SE for three independent experiments. (**C**) Expression patterns of the genes involved in the *Nodal* signaling pathway, *NODAL* (early), *LEFTY A* and *LEFTY B* (mid) and *PITX2* (late), as determined by the DNA micro-array analysis. The values are given with SE for three independent experiments. (**D**) Validation of chip results by RT–PCR for *NODAL*, *LEFTY A*, *LEFTY B* and *PITX2* expression in ES, 2, 10 and 30-day old EBs. All four samples were examined for *GPDH* as a reference control.

Sperger *et al.*, 2003), SAGE (Richards *et al.*, 2004) and cDNA library analysis (Brandenberger *et al.*, 2004). All demonstrate the existence of gene clusters that are expressed at significantly higher levels in hES cells as compared to fully differentiated cells. We wished to further expand these studies by profiling hES cells at different stages of their differentiation (undifferentiated cells, 2, 10 and 30-day old EBs). Such an analysis should allow to follow functional pathways that take part in the process of differentiation as it proceeds during embryo development.

Profiling human ES cells

Hierarchical cluster analysis with different ES cell lines or different cultures of the same line show high similarity. Variation is most probably due to spontaneous differentiation that occurs during ES cell propagation. The difference between ES cells and fully matured EBs is dramatically larger than the variance between two different ES cell lines, representing transcriptional changes that would be expected to accompany differentiation.

As part of our attempt to establish a genetic bar code for ES cells at the transcriptional level, we characterized cytokine receptors and their related growth factors. Of 74 receptors examined, 28 are expressed in ES cells, with a relatively high expression of members of the PTP, FGF, IGF and BMP, activin and TNF receptor families. TGFβR, MET and gp130 seem to be present at a very low level. These results may explain why leukemia inhibitory factor (LIF), which is based on the activation of STAT3 by the gp130-LIF receptor pathway, may not play a role in sustaining undifferentiated growth in human ES cells, as it does in mouse ES cells (Thomson *et al.*, 1998; Reubinoff *et al.*, 2000). In addition, the high expression level of FGF receptors in ES cells may support the need of bFGF in the growth media (Thomson *et al.*, 1998). Since a good correlation exists between the relative level of the receptors and their associated ligands, it should be interesting to investigate the role of these factors in human ES cells.

Pluripotent specific genes

There is presently little information regarding genes that are directly associated with pluripotency and self-renewal. A short list of molecular markers that rapidly down-regulate upon differentiation is available in mouse (for a review, see Eiges and Benvenisty, 2002). Most of these are transcription factors that are also expressed by the ICM. However none are exclusively expressed by pluripotent cells. *Oct4* is the only gene that has been shown to be directly involved in the maintenance of the undifferentiated state of the cells *in vivo* (Nichols *et al.*, 1998). By comparing the expression profile of human ES cells to their differentiated derivatives, we identified a list of candidate genes which may be associated with the pluripotent nature of the cells. Sequences that are highly expressed in ES cells and down regulated upon differentiation are predicted to be good marker genes for determining the state of differentiation of ES cells *in vitro*. However, most are expressed by other differentiated cell types. Since the DNA micro-array analysis contains probes of only previously cloned sequences, it is not possible to isolate unique sequences, which are truly ES-specific. Under the assumption that genes associated with pluripotency should be expressed by both early embryonic cells and germ cells, we examined the tissue distribution of highly expressed genes in databanks and identified genes expressed exclusively in ES cells and germ cells (enriched in ovary, teratoma, testis and pure populations of germ cells). Using these constraints, five sequences were defined as ES-specific: *OCT4* and four other uncharacterized ESTs. RT–PCR showed down-regulation of all five sequences upon differentiation of ES cells and most have DNA-binding motifs, suggesting a role as transcription factors. The presence of *OCT4* strongly supports the strategy used to find these genes. The function of these genes and their targets remains to be determined. In addition, it should be interesting to examine their expression in the ICM of human blastocyst-stage embryos.

Recently, the transcriptional programs in mouse embryonic and adult stem cells were compared to identify transcripts enriched in embryonic, neuronal and hematopoietic stem cells (Ivanova *et al.*, 2002; Ramalho-Santos *et al.*, 2002). These transcribed genes may characterize the 'stemness' of the different types of murine stem cells. We wished to examine whether orthologs of these mouse genes are also enriched in undifferentiated human ES cells, and whether our selected genes appear among them. Although the annotation of probe sets now allows a fairly comprehensive comparison, there are technical limitations (resulting from the construction of different probe sets in the two DNA chips), which prevent us from drawing definitive conclusions. Nonetheless, this analysis suggests that genes implicated in mouse 'stemness' of embryonic and adult cells differ from the set of genes we have identified as enriched in undifferentiated human ES cells. We suggest that these two sets of genes may complement one another. In the present study we compared the profile of gene expression in ES cells to that of EBs, their immediate differentiated cell derivatives. These EBs are composed of mature stem cells, progenitor cells and fully differentiated cells. Thus, we aim to identify genes specific to the pluripotent state of human ES cells. This is in contrast to previous studies searching for "stemness genes" in which genes unique, but common, to different types of stem cells (embryonic and adult) were identified. It would be interesting to evaluate in the future the hierarchy and inter-relationship between these two sets of genes.

Human EB differentiation as a model for early development

It is possible to trigger differentiation of ES cells *in vitro* by growth in suspension culture, resulting in formation of EBs. The EBs, which contain mesodermal, ectodermal and endodermal cells, undergo spontaneous differentiation. This is accompanied by morphological alterations which, similar to mouse, include cavitation (beginning by day 5) and continuous expansion, resulting in a fluid-filled cystic EB (by 2 weeks) (Itskovitz-Eldor *et al.*, 2000). It was proposed that expanding EBs mimic, to some extent, early embryonic

development. Indeed, in mice it has been shown that some temporal and spatial relationships between developmentally regulated genes which exist *in vivo*, are recapitulated *in vitro* (Leahy *et al.*, 1999). Thus, we wished to use DNA micro-array analysis in order to characterize and follow the differentiation program of growing human EBs. A detailed analysis shows that in developing EBs some expressed genes are temporally expressed and may be classified into three distinct groups: early, mid and late expressed genes.

A more refined analysis shows that these three groups (ES, early/mid EBs and late EBs) may be further subdivided into: early, early-mid, mid, mid-late, and late expressed genes. Perhaps these subgroups represent sequential stages in embryo development: (i) blastocyst and ICM specific genes, (ii) ICM and primitive ectoderm, (iii) gastrulation, (iv) early organogenesis and (v) late organogenesis. Indeed, *LECTIN* (galactoside binding, soluble 1) may be a marker for ICM cells; *OCT4* is known to be expressed in the mouse in the ICM and the primitive ectoderm (Pelton *et al.*, 2002); *LEFTY A* is a marker for gastrulation (Hamada *et al.*, 2002); *α-FETOPROTEIN* is expressed in early organogenesis (Gillespie and Uversky, 2000); and *SURFACTANT-D* is expressed in late organogenesis (Crouch, 1998) (Figure 4B). Furthermore, such analysis may allow the isolation of new developmentally regulated genes and could serve as an *in vitro* model for studying aspects of human gastrulation and organogenesis. Nevertheless, since differentiation in EBs is largely disorganized, it remains impractical to study pattern formation *in vitro*.

Finally, we demonstrate that it is possible to follow differentiation by studying the temporal expression pattern of a cascade of genes that are involved in a given pathway but are active in succession. As an attempt to determine how well *in vitro* differentiation of EBs correlates with early embryogenesis, we have studied the expression pattern of the Nodal signaling pathway, which holds a major role in the determination of embryonic axes. In the mouse, *Nodal* induces the expression of *Lefty A* and *Lefty B*, which restrict its expression and its downstream target, *PITX2*, to the left side of the embryo by acting as midline barriers and feedback inhibitors (Hamada *et al.*, 2002). By comparing the expression level of *NODAL*, *LEFTY A*, *LEFTY B* and *PITX2*, in early, mid and fully matured human EBs (2, 10 and 30-day old EBs), we were able to demonstrate their transient expression at different time courses during EB formation, and recover the molecular pathway at the cellular level. These results support the impression that human EBs may model, at least to some extent, early human embryogenesis and encourages us to believe that large-scale cDNA comparisons can provide new insights into the stages of normal human embryo development, which are otherwise inaccessible for research.

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