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Original Article

Inadvertent presence of pluripotent cells in monolayers derived from differentiated embryoid bodies

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ABSTRACT The therapeutic use of embryonic stem (ES)-derived cells is restricted by a risk of teratoma formation. To test the hypothesis that some cells with pluripotency characteristics remain following the differentiation of embryoid bodies (EB) into monolayer cells, we transformed mouse ES cells using constructs comprised of the mTert promoter coupled to green fluorescent protein. In this manner, EBs could be identified as showing gradually diminishing expression of the fluorescent marker as a consequence of differentiation. After 2 weeks of incubation, however, small groups of fluorescent cells remained in the differentiated monolayer. When these cells were isolated, cultured and expanded under ES cell culture conditions, they recovered their ES cell morphology (herein denoted ES-EB). We found by immunocytochemistry, reverse transcription-PCR and bisulphite analysis that despite the fact that some of these ES-EB lost their capacity to express some pluripotency markers characteristic of ES cells and undergo the epigenetic modification (hypo-methylation) of some retrotransposons (RT), after several passages in ES media, the cell colonies recovered their capacity for both pluripotency marker expression and RT methylation. Furthermore, when assessed for their ability to form chimeras, most ES-EB lines were unable to do so, although they recovered this potential for chimera production after some passages in ES cell media. Our results highlight the need for specific screening of differentiated cells before their therapeutic use and indicate that under adequate culture conditions, cells that loose their potential for expressing key markers of pluripotency can recover this fundamental embryonic stem cell property.

KEY WORDS: *mouse, differentiation, pluripotency marker, qRT-PCR marker, ES cell*

Introduction

Embryonic stem (ES) cells can differentiate into most adult cell types and have attracted intense interest because of their potential therapeutic applications (O'Shea, 2004). Thus, if their differentiation could be controlled, cultured ES cells would be an excellent source of cells for transplantation to replace cells lost through disease or injury or cells lacking hormones or genes. Although the therapeutic potential of ES-derived cells is huge, several problems such as teratoma formation have yet to be resolved (Drukker *et al.*, 2002). Each stem-cell transplant may contain cells that had failed to differentiate and which remained undifferentiated. These cells keep dividing and can turn into tumours (Roy *et al.*, 2006). The main pathway through which ES-derived cells become differentiated cells involves the formation of embryoid bodies "EB", thus denoted because of their similarity to the early postimplantation embryos. Embryoid bodies form after several days of growth of ES cells in the absence of LIF (leukemia inhibitory factor). These bodies are the final ES cell population that has the potential to give rise to all the cell types of adult tissues and have been described as the developmental equivalent of the egg cylinder-stage mouse embryo, with an outer endodermal layer and a core of differentiating cells, often comprised of epithelial-lined cavities. In this last case, the embryoid bodies are referred to as cystic EBs (O'Shea, 2004). It has been reported that cells derived from all germ layers differentiate into EBs, in some ways recapitulating *in vivo* gene-expression patterns (Leahy *et al.*, 1999). Because only the outer endoderm of the EB is exposed to added growth factors and cells inside an EB differentiate in response to signals produced by the differentiating endoderm, the

Abbreviations used in this paper: ES, embryonic stem; EB, embryoid body; IAP, intracisternal-A particle; MuERV-L, murine endogenous retrovirus-L; LIF, leukemia inhibitory factor; RT, retrotransposon.

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incomplete differentiation of ES cells is commonly observed in EB and could be related to the fact that differentiated cells, particularly mesoderm cells, produce LIF (O'Shea, 2004). The pluripotency of the EB is lost during the process of differentiation. However, the differentiated EB and the monolayer cells it generates, form a mixture of multiple cell types, with the possibility that some multipotent cells remain. These persisting undifferentiated cells could be the origin of teratoma formation. Also, in monolayer culture derived from teratocaricinoa embryoid bodies it has been reported the presence of four stable cell populations (Hilario *et al.*, 2001). The authors suggest that intrinsic mechanisms of regulation may exist that would strike a balance between differentiation and proliferation.

Telomerase is an enzyme complex that maintains the ends of all eukaryotic linear chromosomes. Murine ES cells have been shown to exhibit high telomerase activity, which is strictly regulated during cell differentiation (Albanell *et al.*, 1996, Armstrong *et al.*, 2000). The murine reverse transcriptase unit (mTert) of telomerase is the catalytic subunit of the telomerase complex and is closely linked to telomerase activity (Armstrong *et al.*, 2000, Pericuesta *et al.*, 2006). In a previous paper, we reported that ES cell telomerase expression levels induced by the mTert promoter diminish as differentiation progresses (Pericuesta *et al.*, 2006). In the present study, we transformed the R1 ES cell line to express green fluorescent protein (GFP) under the control of the mouse telomerase reverse transcriptase promoter (mTert). Our findings indicate that this promoter regulates GFP expression in ES cells during their *in vitro* loss of pluripotency and therefore enables cells with telomerase activity to be identified and isolated from the monolayer of differentiated cells generated from EBs. We also explored the potential of these ES-like multipotent cells (ES-EB) to recover their ES cell characteristics.

TABLE 1

mRNA EXPRESSION OF PLURIPOTENCY MARKERS

	R1	R1-mTert-GFP	ES-EB1		ES-EB2		ES-EB3		ES-EB4		ES-EB5	ES-EB6	ES-EB7	ES-EB8
			p2	p7	p2	p10	P2	p7	p2	p10	p3	p3	p3	p3
Nanog	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Oct3/4	+	+	-	+	-	+	-	+	-	+	-	-	+	+
Rex1	+	+	-	+	-	+	-	+	-	+	-	-	+	+
Foxd3	+	+	-	+	+	+	+	+	+	+	+	+	+	+
Fgfr4	+	+	-	+	-	+	-	+	+	+	+	+	+	+
Terf1	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Cx43	+	+	-	+	-	+	+	+	+	+	+	+	+	+
Glut1	+	+	-	+	-	+	+	+	-	+	+	+	+	+
Abcg2	+	+	-	-	-	-	-	-	-	-	-	-	-	+

TABLE 2

mRNA EXPRESSION OF MARKERS OF DIFFERENTIATED PHENOTYPES

	R1	R1-mTert-GFP	ES-EB1		ES-EB2		ES-EB3		ES-EB4		ES-EB5	ES-EB6	ES-EB7	ES-EB8
			p2	p7	p2	p10	P2	p7	p2	p10	p3	p3	p3	p3
Gata-4	-	-	+	-	+	-	-	-	-	-	-	-	-	+
Gata-2	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Afp	-	-	+	-	-	-	-	-	-	-	-	-	-	-
Msx1	-	-	+	-	+	-	-	-	-	-	+	-	-	+
T	-	-	+	-	-	-	-	-	-	-	-	-	-	-
Myf5	-	-	+	-	-	-	+	-	+	-	-	+	+	-
Krt15	-	-	+	-	+	-	+	-	+	-	+	-	-	-
Foxa2	-	-	+	+	+	-	+	-	+	-	+	+	+	-
Nes	-	-	+	-	+	-	-	-	-	-	-	-	-	-
Vim	-	-	+	-	+	-	-	-	-	-	-	-	-	-
Tubb3	-	-	+	-	+	-	+	-	+	-	-	+	-	-
Gapdh	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Results

Recovery of ES-like cells from monolayers of differentiated EBs

We created six R1-mTert-GFP marked cell lines by stably transfecting with a 4.5 kb fragment of the mouse telomerase reverse transcriptase promoter (mTert) into murine R1 ES cells (passage 14) (Pericuesta *et al.*, 2006). For the rest of the experiments, we selected a clone (R1-mTert-GFP) that exhibited a good expression of the GFP, a normal karyotype (more that 80% of normal metaphases, similar to the percentage of the original R1 ES cells) and was capable of chimera transmission (24% of chimeras after transfer of 100 embryos, with a 15 to 50 % of coat colour chimerism). This ES cell line has been frozen-thawed and cultured for more than 20 passages without loss of ES morphology or the normal karyotype. All the experiments were performed using ES cells between passages 15 to 20. Moreover, this ES cell line retained the expression of all the molecular markers of pluripotency present in the R1 ES cell line used for transformation (Table 1). In the presence of LIF, the transgenic ES cell line expressed GFP (Fig 1A); 4 days after removal of LIF and plating the cells into bacterial-grade Petri dishes, the ES cells spontaneously transformed into embryoid bodies (EBs) (Fig 1B). During this process, GFP expression increased. The next step in the EBs differentiation process led to transformation of the EBs into a differentiated monolayer of cells incapable of GFP expression (Fig 1C, D). Nevertheless, groups of GFP expressing cells persisted among the differentiated colonies; even 12 days after LIF had been removed from the EBs differentiating culture (Fig. 1D). After picking GFP-positive groups of cells and culturing in ES cell medium for 2 passages, they recovered their original ES cell morphology. We denoted those cells as ES-EB cells. From the 53 groups of GFP cells that were isolated we produced 23 new green ES-like lines that were maintained in culture and that showed typical ES cell characteristics, including their distinctive large nucleus-to-cytoplasm ratio. These clones were selected because they contained more than 90% positive cells for the GFP (counted under fluorescent microscopy). Also, to analyze the homogeneity of the population, some of the lines were analyzed by flow cytometry (Fig 2), confirming that the lines contained more that 90% positive cells for GFP. In contrast, isolated GFP-negative cells were unable to recover the typical ES cell morphology after culture in ES cell media during more than 30 days. These groups

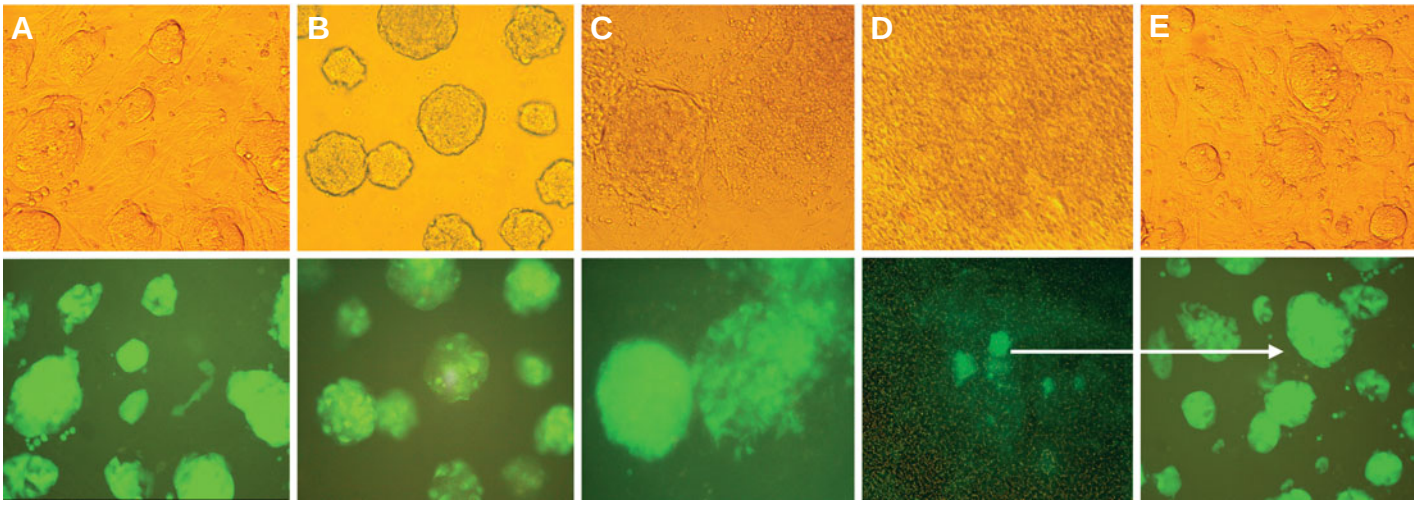

Fig. 1. Sequence of micrographs showing embryonic stem (ES) cells transformed with mTert-GFP during differentiation induced by leukemia inhibitory factor (LIF) removal. *(Upper photographs were taken under light microscopy at 200x magnification and lower photographs under epifluorescence).* **(A)** *ES cell expressing green fluorescent protein (GFP);* **(B)** *four days after LIF removal, the ES cells plated in non-adherent dishes form embryoid bodies (EBs);* **(C)** *after 6 days in the absence of LIF, the EBs plated in gelatine coated tissue dishes start to differentiate into different cell types showing diminished fluorescence intensity;* **(D)** *after 12 days in the absence of LIF, EBs differentiate into monolayer cells and loose fluorescence; in some wells, groups of GFP expressing cells remain among the differentiated cell colonies;* **(E)** *these cells expressing GFP under the mTert promoter can be picked up, cultured and expanded under ES cell culture conditions, generating new ES cell-like colonies in the presence of LIF.*

of cells formed monolayer of fibroblast-like cells, epithelial-like cells, cardiomyocytes and other differentiated cells and they never recovered the expression of ES cell pluripotent markers. Only in some cases we could find expression of Cx43 and Glut1.

Expression of markers of pluripotency or differentiated phenotypes

Immunological analysis of four ES-like EB derived lines (ES-EB-1 to -4) showed that two markers of undifferentiated ES cells, Nanog and SSEA-1, were uniformly expressed in the ES-EB lines after culture during three passages in ES cells medium (Fig. 3)

RT-PCR was performed to assess expression of genes characteristic for ES. Bands of the appropriate size were observed for all these genes using species-specific primers; and the amplicon identities were confirmed by sequencing the PCR products. All the molecular analyses were performed on 8 of the 23 new ES-like EB derived lines (Table 1 and 2). All the pluripotency markers were detected in the transformed ES cells (R1-mTert-GFP) but after recovering the ES-like phenotype from differentiated EB, only one of the clones analyzed continued to express all the markers. Abcg2 marker was absent in 7 of the lines. Oct3/4 and Rex1 were absent in 6 of the lines. Fgfr4 and Glut1 were absent in 3 of the lines. Interestingly, all the ES-EB lines expressed the marker Nanog and 7 of the 8 ES-EB lines analyzed expressed Foxd3. To determine whether after passages in ES cell media, the ES-EBs would be able to recover the expression of pluripotency markers, we selected the four ES-EB lines (ES-EB1-4) that had lost the expression of most of the markers. Thus, after 7-9 passages in ES cell media, the expression of most of these markers was recovered (Table 1). The only two markers that were lost in the four ES-EB lines, Oct3/4 and Rex1 were recovered in all the lines; Foxd3 was recovered in line ES-EB1; Fgfr4 was recovered in lines ES-EB1, 2 and 3; Cx43 was recovered in lines ES-EB1 and 2; and Glut1 was recovered in lines ES-EB1, 2 and 3.

Table 2 summarizes the results of our experiments using differentiation markers. GATA 2, a zinc finger transcription factor, was the only marker present in the original R1 ES cell line that was also detected in all the ES-like cells derived from EB. Different markers of differentiation were found in different ES-EB lines, indicating variation in the state of differentiation among them.

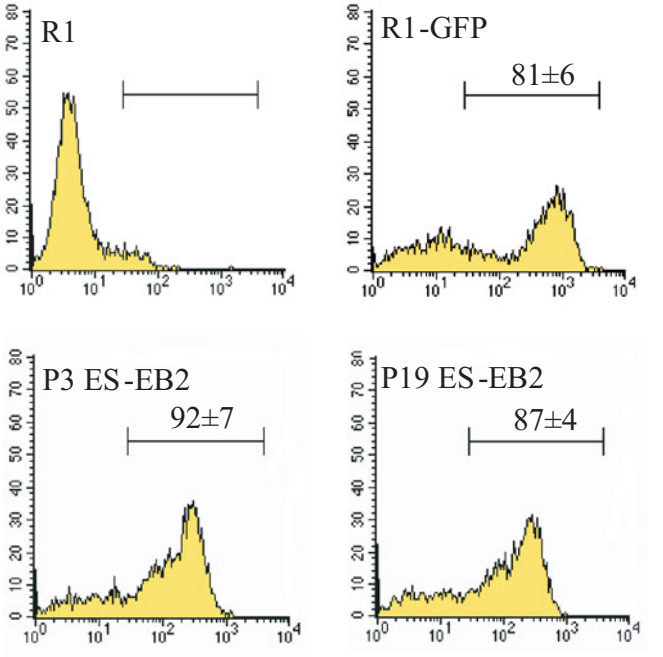

Fig. 2. FACS analysis of green fluorescent protein (GFP) expression in different passages of ES-EB cells. *The percentage of cell population expressing GFP was similar between the R1-mTert-GFP ES cell line and the ES-EB lines generated (the fig shows the ES-EB2 at passage 3 and 19)*

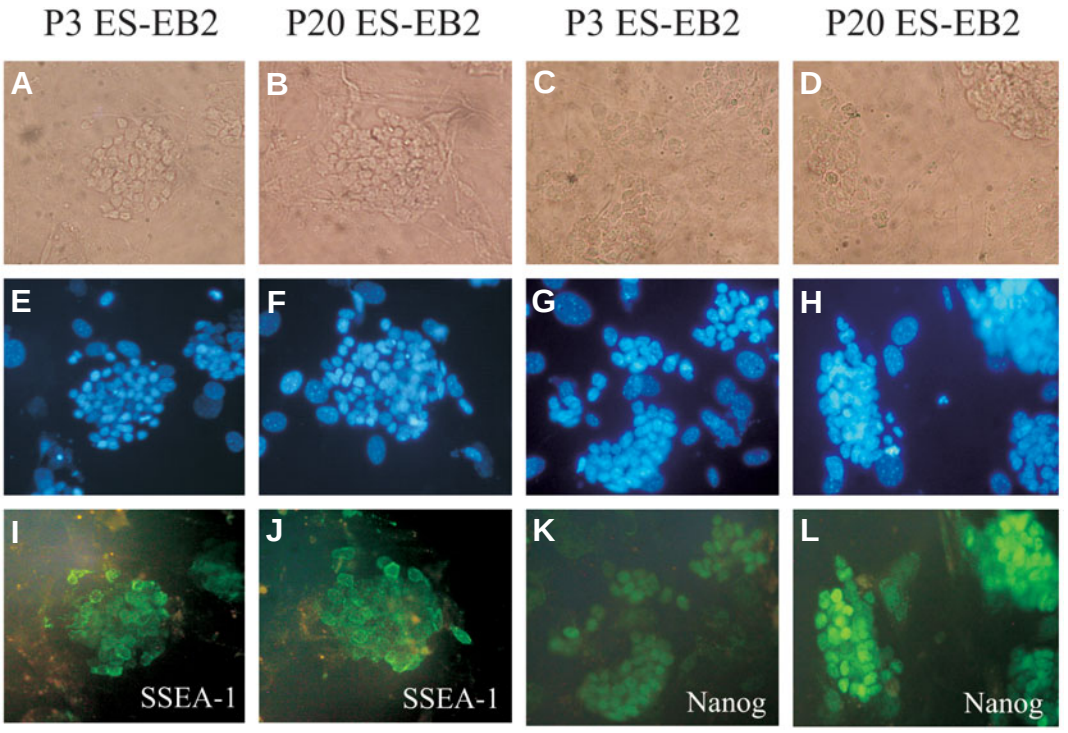

Fig. 3. Expression of Nanog and SSEA-1 in an ES-EB line at passages 3 and 20 (A,B,C,D) *Phase contrast microphotographs.* **(E,F,G,H)** *Nuclear stain (DAPI). Membrane SSEA-1 immunofluorescence* **(I,J)** *and Nuclear Nanog immunofluorescence* **(K,L)** *are detected in the ES-EB line at early and late passage (200x magnification).*

Foxa2 was expressed in 7 lines; Myf5, Krt15 and Tubb3 were expressed in 5 lines; Msx1 was expressed in 4 lines; Gata-4 was expressed in 3 lines; and Nes and Vim were expressed in 2 lines. To determine whether after several passages in ES cell media, the ES-EBs would be able to loss the expression of differentiation markers, we studied their expression after culture in the same four ES-EB lines where pluripotency markers have been studied.(ES-EB1-4) after culture in ES medium for 7-10 passages. We found that line ES-EB1, that at passage 2 expressed all the differentiation markers, lost the expression of all the markers except Gata-2 and Foxa2 after 7 passages. In the other three lines analyzed, the expression of the differentiation markers that were present at passage 2 (ES-EB2: Gata-4, Msx1, Krt16, Foxa2, Nes, Vim and Tubb3; ES-EB3: Myf5, Krt15, Foxa2 and Tubb3; ES-EB4: Myf5, Krt15, Foxa2 and Tubb3) was lost after 7-10 passages in ES cell media.

Collectively, these findings indicate that some of the ES-like cells recovered from differentiated EBs were not significantly different in terms of morphology and pluripotency marker expression to R1 and to R1-mTert-GFP and were also similar to other mouse ES cell lines such as D3 (Ginis *et al.*, 2004). Moreover, when the ES-EB lines were cultured in ES cell media for 7-9 passages, they recovered the original expression pattern of the pluripotent R1 line.

Chimera formation

Three ES-EB lines were injected into blastocysts or aggregated with eight-cell stage embryo to produce chimeras, ES-EB1 that initially lost most of the pluripotency markers, ES-EB2 that lost the majority of the markers but kept the expression of Nanog, Terf1 and Foxd3 and line ES-EB3, which initially preserved the expression of the majority of pluripotent markers except Oct-4, Rex1 and Fgfr4 (Table 1). Over 200 embryos which were injected or aggregated with every ES-EB line at early passage (2-4 passages) were transferred to recipient females, but no chimeric mice were obtained. Only line ES-EB2 was able to produce 1 chimera (with less than 25% of coat colour chimerism) after the transfer of 269 blastocysts. Nevertheless, after 9-11 passages of these cells in ES cell media, line ES-EB2 and 3 were reassessed for their potential for chimera formation and following the transfer of 212 blastocysts from aggregation or injection with ES-EB2 line, 8 out of the 21 born alive were chimeric and following the transfer of 153 blastocysts from aggregation or injection with ES-EB3 line, we obtained 5 chimeric mice out of 13 born alive. The level of chimerism observed was similar to that obtained with the original R1-mTert-GFP ES line, ranging from 15 to 50% based on coat colour contribution. These results indicate that the cells had recovered the pluripotential nature of the original ES cell line. It is important also to point out that ES-EB2 and 3 lines were able to form chimera only after they recovered 8 of the 9 pluripotent markers. ES-EB3 at passage 2, even expressing 5 pluripotent markers, was unable to form chimeras, this ability was only recovered at passage 7, after recuperating the expression of Oct3/4, Rex 1 and Fgfr4,. Five of the chimeric mice with the higher chimerism were assessed for germline transmission, but only one of the lines produced germline transmission after four consecutive mating.

RNA expression profile of IAP and MuERV-L retrotransposable elements

Similar expression patterns were observed for IAP and MuERV-L retrotransposons (Fig. 4). Expression levels were initially increased in the 4 ES-EB lines (1 to 4) that had lost most pluripotency markers (Table 1), but remained unaffected in ES-EB 8 line or less affected in ES-EB line7, which had not lost the expression of these markers. However, after 5-9 passages in ES cell media, ES-EB lines 1 to 4 showed similar levels of retrotransposable element expression than the R1 line. No differences in expression between R1 at passage 14, R1-mTert-GFP at passage 17 and early embryoid bodies generated from this line were observed (Fig 4), indicating that at this stage of differentiation there are no differences in IAP and MuERV-L mRNA expression. Our results suggest a possible role for IAP and MuERV-L expression in

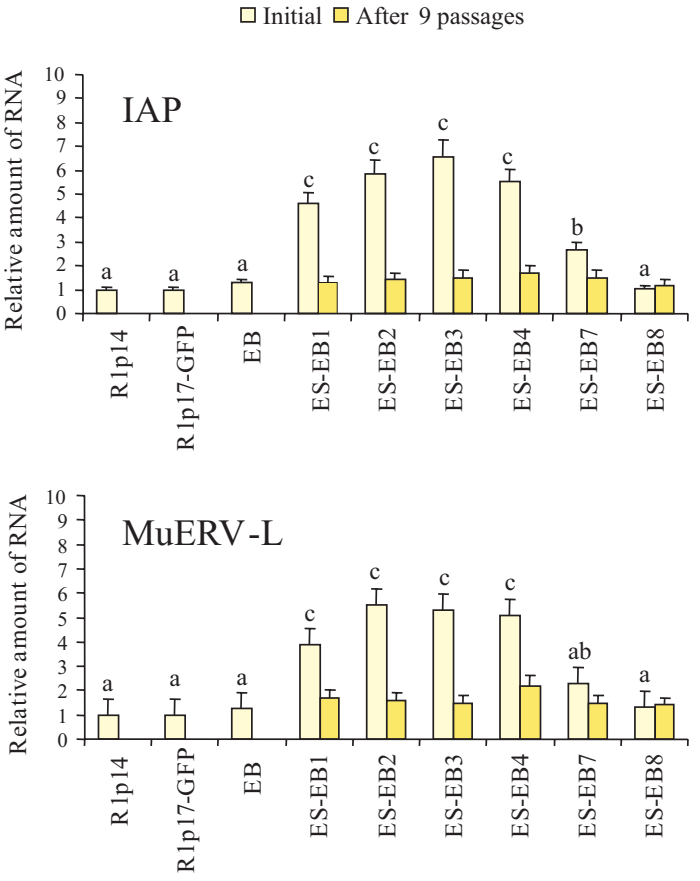

Fig. 4. Quantitative RT-PCR analysis of *intracisternal-A particle (IAP)* and *murine endogenous retrovirus-L (MuERV-L)* mRNA expression. *Each lane represents the mean plus standard desviation of 3 technical replicates from 3 independently prepared samples, expressed relative to the group with lowest expression. a,b,c refers to significant differences in relative transcript abundance between column (P<0.05).*

preserving ES cell pluripotency and differentiation capacity; however, the changes in expression pattern may be a consequence of regaining pluripotency rather than a cause. In addition, we have found that in some cells, adequate culture conditions could modulate and re-program the expression of these retrotransposons.

Methylation patterns of IAP and MuERV-L retrotransposons

For the bisulphite analysis of the methylation pattern of retrotransposon IAP, primers against the 5´LTR sequence were designed to amplify a 255 bp fragment containing 10 CpG dinucleotides spanning the IAP promoter (Fig. 5A), which is known to be methylation sensitive (Lane *et al.*, 2003). A similar strategy was followed for the 5´LTR sequence of the MuERV-L retrotransposon, allowing the amplification of a 270 bp fragment containing 6 CpG dinucleotides spanning its promoter (Peaston *et al.*, 2004) (Fig. 5A). We analyzed the methylation profiles of IAP and MuERV-L repeated sequences in DNA samples obtained from R1 at passage 14, R1-mTert-GFP at passage 17, early embryoid bodies generated from this line and ES-EB lines 1, 2, 3 and 8 generated from EB derived from R1-mTert-GFP ES cells. A reduction on the percentage of methylation in the promoter regions of the IAP and MuERV-L transposable elements were observed in 3 of the ES-EB lines that no longer expressed most of the pluripotency markers, yet no differences were detected in the ES-EB 8 line that continued to express these markers (Fig. 5). The reduction on the percentage of methylation of the lines ES-EB 1, 2 and 3 inversely correlated with the transcriptional activity of retrotransposons (Fig. 4). However, the line ES-EB3 that displayed a significant increase in the relative amount of IAP and MuERV-L mRNAs (Fig 4), did not display a significant difference in the percentage of methylation of the two retrotransposable elements. Moreover, no differences were observed after culturing in ES conditions during 9 passages (data not shown) indicating that culturing also affects the methylation status of these cells and that an appropriate culture could re-program some cells.

Discussion

One of the main targets of embryonic stem cell research is to control the differentiation of human ES cells into particular cell types, an essential requisite for their use in therapeutic transplantation, drug testing, or screening potential toxins. Understanding the mechanisms involved in the differentiation of ES cells to EBs and onto specific lineages is crucial to the safe use of ES cells in cell therapies. To date, several laboratories have demonstrated that *in vitro* human ES cells are pluripotent and that they can generate cell types derived from all three embryonic germ layers. However, the experimental transfer of ES cells has been related to an unusually high risk of genetic changes and tumour formation (Taupin, 2006). The potential disadvantages of the use of human ES cells for transplant therapy include the propensity of undifferentiated ES cells to induce the formation of tumours (teratoma). The genetic mechanisms by which ES cells give rise to tumours *in vivo* are poorly understood. It is possible that since in stem cells the machinery for self-renewal is already activated, maintaining this activation may be a simpler task than turning it on *de novo*. Given it is the undifferentiated cells—rather than their differentiated progeny—that seem to induce teratoma, tumour formation might therefore be avoided by devising methods of identifying and removing any undifferentiated ES cells prior to transplant (Drukker *et al.*, 2002).

ES cells differentiated into EBs by the removal of LIF from the defined medium and then differentiated as a monolayer have been described to exhibit varied expression of marker transcripts characteristic of mesoderm, endoderm and ectoderm lineages (Ward *et al.*, 2004). In agreement with these results, our ES-like cells derived from differentiated EB also expressed markers of differentiation characteristic of the three layers. The incomplete differentiation of ES cells is commonly observed in EB, yet there are no reports of the detection of ES-like cells in the monolayer obtained from EBs after two weeks of differentiation. Herein, we demonstrate that monolayer cells derived from EBs, which look homogeneous under the light microscope, feature a proportion of completely undifferentiated cells and that these cells can be withdrawn from the monolayer and be stimulated to recover their pluripotent state by culturing in standard ES cell media. In effect, these cells recover their capacity to express several markers of pluripotency as well as their potential to form chimeras. In addition, ES-like cells that failed to express some of these pluripotency markers retained some level of multipotency-memory that al-

lowed them to recover the expression of the markers after several passages in ES cell culture and to regain the methylation status of some retrotransposons along with the potential to form chimeras. These findings suggest that: (1) a more specific analysis of markers is required to confirm the loss of the cells' multipotent characteristics; and (2), that adequate culture conditions may genetically reprogram cells with memory for pluripotency to produce ES cells with pluripotent properties. It should be highlighted that even cell lines that had lost the expression of Oct-4 were able to recover the expression of this marker and then recover also the potential to form chimeras. Moreover, only three markers of pluripotency (Nanog, Foxd3 and mTert) were detected in almost all the ES-EB cells, indicating that these three markers could serve to identify the inadvertent presence of pluripotent cell stages among differentiated cells.

Nanog, a homeobox transcription factor, is a pluripotent cell-specific gene that plays a crucial role in maintaining the undifferentiated state of early postimplantation embryos and ES cells. Nanog mRNA is present in pluripotent mouse and human cell lines and absent from differentiated cells (Chambers *et al.*, 2003). Two important properties conferred by Nanog have been described: a fundamental role in pluripotency of both ICM (inner cell mass) and ES cells and an ability to maintain ES self-renewal in the absence of LIF, Oct3/4 and Stat3 (Chambers *et al.*, 2003) (Mitsui *et al.*, 2003). Our results agree with the importance of Nanog as a master transcriptional organizer and suggest that even in Oct4 absence, Tert, Foxd3 and Nanog may control different but partly overlapping pools of target genes. Foxd3 is a fork-head transcription factor highly expressed in mouse ES cells. In agreement with our results, it has been recently published that Oct4, Nanog and Foxd3 form a loop anchors an interdependent network of transcription factors that regulate stem cell pluripotency (Pan *et al.*, 2006). The authors report that Nanog and Foxd3 were able to activate Oct4 promoter. The ectopic expression of mTert in mouse ES cells does not affect differentiation but confers resistance to differentiation- and stress-induced p53-dependent apoptosis (Lee *et al.*, 2005). These recent data suggest that the decreased telomerase activity observed during differentiation is a consequence of the differentiation process, during which the pluripotency of the ES cell is lost. It has been demonstrated that over-expression of telomerase confers a growth advantage, stress resistance and enhanced differentiation of ES cells towards the hematopoietic linage (Armstrong *et al.*, 2005). We propose two alternative events occurring during our differentiation protocol: first, that the mTert-GFP positive cells that we selected after two weeks of differentiation still retained some pluripotency potential; or second that the mTert-GFP positive cells recovered have no pluripotency characteristics, yet after incubation under ES cell culture conditions they recover Nanog expression and as a consequence they may recover the expression of other pluripotency markers. Whichever the case, by selecting mTert-GFP positive cells, we were able to identify the cells with a level of multipotency or of memory factors (or epi-memory factors) sufficient for them to recover their pluripotency. The combination of both markers, mTert (survival enzyme marker) and Nanog and Foxd3 (pluripotency control marker) may be useful for defining ES cell identity.

In the adult, stem cells persist in some tissues such as hematopoietic, gastrointestinal and testicular tissue and the integumentary system and carry out lifelong processes of regeneration and renewal. A recent study has reported that skin-derived adult stem cells are capable of recalling the program for differentiating into an oocyte (Dyce *et al.*, 2006). It has been suggested that the differentiation of adult stem cells is incomplete and lacks certain cues necessary to acquire a truly functional status (Belema Bedada *et al.*, 2005), such that the multipotency potential observed for spermatogonial stem cells or skin cells may actually be imposed by the culture conditions rather than reflect the natural behaviour of a stem cell in a given niche. Just as stem cells depend on their environment to maintain their properties, the culture environment may also modulate the stem cell properties of cells with some degree of multipotency. In our experiments, due

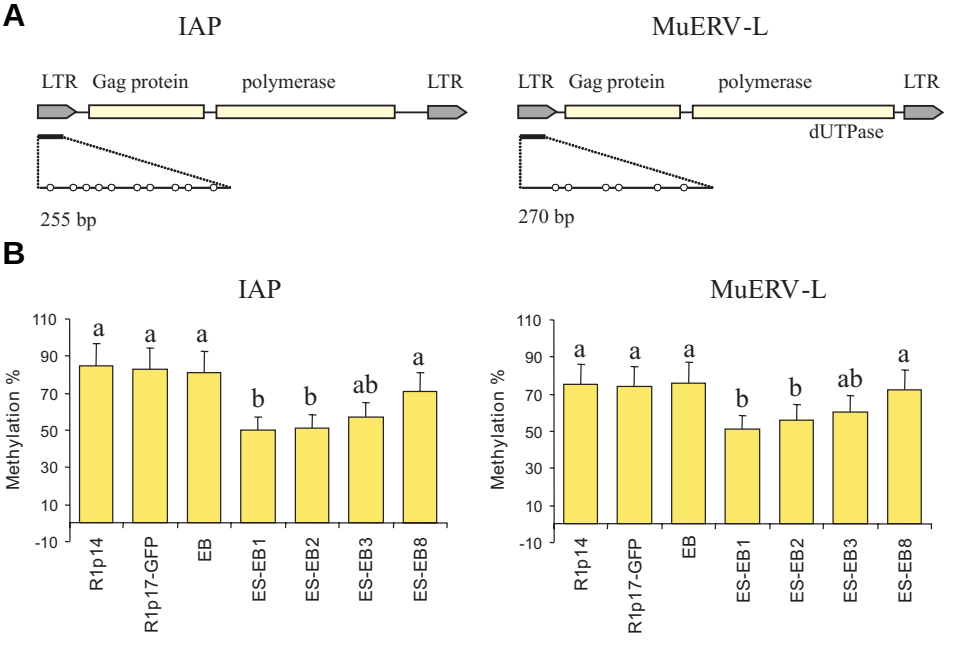

Fig. 5. Methylation profiles of two retrotransposable elements, intracisternal-A particle (IAP) and murine endogenous retrovirus-L (MuERV-L) in R1p14, R1-mTert-GFPp17, embryoid bodies and in ES-EB1, 2, 3 and 8 (at passage 3). (A) *Regions analyzed by bisulphite sequencing in IAP and MuERV-L. LTR, long terminal repeat. Circles represent CpG dinucleotides present in the regions analyzed.* **(B)** *Average of percentage DNA methylation following bisulphite treatment and amplification of ES cell DNA. a,b refer to significant differences in percentage of methylation between column (P<0.05).*

to the specific culture conditions, ES-EB cells acquired the remarkable potential to turn on a large variety of markers specific for ES cells, such as Oct-4. The epigenetic state of the ES cell genome is extremely unstable (Humpherys *et al.*, 2001). Accordingly, it has been established that Oct-4 gene expression is regulated by an epigenetic mechanism involving DNA methylation and the remodelling of chromatin structure (Hattori *et al.*, 2004). Oct-4 is expressed in pluripotent cells of the embryo, in all testicular germ cell tumours (Gidekel *et al.*, 2003), in pre-meiotic germ cells and in cell lines derived from those cells (ES and embryonic germ cells). Recently, however, the robust expression of Oct-4 and other markers of pluripotency, such as Nanog and Rex1, has also been observed in mesenchymal stem cells derived from bone marrow (Belema Bedada *et al.*, 2005). Moreover, Dyce *et al.*, (2006) have reported that Oct-4 is also expressed in skin sphere cells, although expression could have been turned on during the process of culturing these cells, as suggested by a study using an Oct4-GFP transgene (Kues *et al.*, 2005). Our results suggest that any adult or foetal stem cell that has a multipotent memory may be reprogrammed by culturing to acquire *in vitro* pluripotency characteristics similar to those of ES cells.

Because ES cells offer an enormous potential for cell therapies, any progress in understanding the epigenetic properties of stem cells, will be crucial for their eventual safe application, to ensure that differentiated cells that are derived from ES cells are lineage-restricted and have shut down alternative options before engraftment. Recent studies have implicated retrotransposable elements in imprinting and X-chromosome inactivation and in the developmental regulation of gene expression (Slotkin and Martienssen, 2007). Retrotransposons could be an epigenetic-marker for the characterization of the epigenetic stages of cells from pluripotent to terminal differentiation stages and could be very practical to putting ES cells into safe practical use. We do not know if some differentiated cell derived from human ES cells lines retaining this mice ES cells capacity to regain pluripotency. However, transplanting even a small number of incompletely differentiated cells represents a serious risk of including cells with tumour-forming properties into patients. In summary, our findings suggest that cells with a genetic or epigenetic memory of a multipotent development stage, can be reprogrammed to confer an ES-like pluripotency stage (with a capacity for chimera production). The implications of these findings are that before we can consider that we have obtained a homogeneous monolayer of differentiated cells for cell therapy purposes, the presence of specific pluripotency markers should be checked for, to avoid the possible transfer of a significant proportion of undifferentiated cells with tumourogenic properties.

Materials and Methods

Reagents and media

Unless otherwise stated, all chemicals and media were purchased from Sigma Chemical Co. (Madrid, Spain).

Culture, transformation and differentiation of ES cells

Undifferentiated mouse ES cells (R1 from A. Nagy lab) were maintained on mitomycin-C treated (Sigma-Aldrich corporation St. Louis, MO, USA) mouse embryonic fibroblast (MEF) cells on 0.1% gelatine coated tissue plates in Dulbecco's modified Eagle medium (DMEM plus 4500 mg/l glucose, glutaMAX and pyruvate; Invitrogen, Carlsbad, CA, USA) supplemented with 20% FBS (PAA Laboratories Cölbe Germany), 2 mM glutamine, 1 mM MEM nonessential amino acids solution, 1 mM β-mercaptoethanol, 1000 U/ml LIF and an antibiotic mixture containing 100 U/ml penicillin and 100 μg/ml streptomycin (Pericuesta *et al.*, 2006).

A 4,471 bp fragment of the mouse telomerase reverse transcriptase promoter (mTert) (NCBI GenBank; accession number AF 121949) located upstream from the first ATG of the mTert gene open reading frame, was amplified by PCR and ligated to pEGFP (Clontech Laboratories, Inc., Palo Alto, CA, USA) to promote the expression of GFP (Pericuesta *et al.*, 2006). Ten micrograms of the linearized mTert-GFP construct were electroporated into $3x10^6$ cells using a Multiporator (Eppendorf, Hamburg, Germany) and a pulse of 300 V for 500 μs. Cells were allowed to recover for 24 h before G418 was added to give a final concentration of 150 μg/ml. Cellular clones were selected by geneticin resistance over 7 days, replacing the selection medium every day. Clones were harvested by trypsinization and allowed to attach onto the wells of 96-well plates forming cell clusters, which were maintained for 2 days in LIF-supplemented medium until colonies formed. Transgene integration was confirmed by GFP PCR (Gutierrez-Adan and Pintado, 2000).

GFP PCR was performed using the primers GFP-1 (5'-TGA ACC GCA TCG AGC TGA AGG G-3') and GFP-2 (5'-TCC AGC AGG ACC ATG TGA TCG C-3'), specifically amplifying a 340 bp portion of GFP DNA. Amplification was carried out in a total volume of 25 μl (1x of PCR mix containing 1U Taq polymerase, 2.5 μl 10x buffer, both from Promega, 100 μM each dNTP, 0.1 μM each primer and 2.5 mM $MgCl_2$). Samples were loaded directly from ice onto the heating block at 92°C to minimize the time required to reach denaturation temperature. The PCR protocol was an initial step of 92°C (2 min), followed by 32 cycles of 92°C (30 s), 59°C (30 s) and 72°C (30 s) and a final extension cycle at 72°C (10 min). PCR products were resolved on 1.5% TBE agarose gels, followed by staining with ethidium bromide and visualized using UV light.

Recovering ES cells from differentiated EBs. Chimera formation

To initiate differentiation, ES cells were trypsinized and back-plated for 15 minutes to deplete fibroblasts and then plated in non-adherent 10 cm bacterial-grade Petri dishes ($5x10^5$ cells per dish) in ES medium without LIF. Embryoid bodies were collected after four days using a yellow pipette tip and transfer to 0.1% gelatine coated tissue plates in ES medium without LIF to allow EBs differentiation into different cell types. Medium was replaced every day. After 12 days in the absence of LIF, the EBs differentiate into monolayer cells attached to the culture dish, but some small groups of green cells, expressing the transgenic GFP under the mTert promoter, could be identified under the fluorescent microscope among the cells forming the monolayer. These groups of cells (denoted ES-EB) and some groups of cells that did not express GFP (control group), were picked up using a 2 μl pipette tip (before lifting the cells, the monolayer cells around the clumps of green cells were cut using the pipette tip). The clumps were transferred to a tissue plate with mitomycin-C treated MEF and cultured and expanded under ES cells culture conditions with LIF as described above.

For chimera formation, we used blastocyst ES cell injection and eight-cell stage embryo aggregation procedures. For the first method, cells were injected into the blastocoel of 3.5 dpc blastocysts of CD1 mice using Eppendorf micromanipulators (Eppendorf TransferMan NK 2, Hamburg, Germany). The blastocysts were returned to the oviducts of 0.5 dpc pseudopregnant CD1 foster mothers on the day of microinjection. In the second procedure, ES-cell derived embryos were generated by aggregating clumps of ES cells (8-15 cells in each) with individual eight-cell embryos. ES cell:embryo aggregates were cultured overnight in KSOM + 4% ES cells medium described above and the following morning, the majority of the aggregates formed blastocysts and were transferred to the oviduct of pseudo-pregnant surrogate mothers. To analyzed chimerism, the embryos were allowed to develop to term and chimerism was tested by coat color compared with the white colour of the recipient strain. Mice

SUPPLEMENTARY TABLE 1

PRIMERS USED FOR THE RT-PCR OF GENES ASSOCIATED WITH DIFFERENTIATED AND UNDIFFERENTIATED ES CELL STATES

Gene symbol	MGI Official name	Primer 5´-3´ (Forward/Reverse)	Size	UniGene
Gata-4	GATA binding protein 4	GCCTGTATGTAATGCCTGCG/ CCGAGCAGGAATTTGAAGAGG	469	Mm. 247669
Gata-2	GATA binding protein 2	ACCCACGCCACCCAAAGAAGTG/ GCCGCCTTCCATCTTCATGCTC	157	Mm. 272747
Afp	Alpha fetoprotein	TTTTCTGAGGGATGAAACCTATG/ AAGCTCTTGTTTCATGGTCTGTA	116	Mm. 358570
Msx1	Homeo box, msh-like 1	GCTATGACTTCTTTGCCACTCG/ TTAAGAGAAGGGGACCAGGTGG	1016	Mm. 259122
T	Brachyury	GCTGTGACTGCCTACCAGCAGAATG/ GAGAGAGAGCGAGCCTCCAAAC	220	Mm. 913
Myf5	Myogenic factor 5	TGCCATCCGCTACATTGAGAG/ CCGGGGTAGCAGGCTGTGAGTTG	352	Mm. 4984
Krt15	Keratin 15	CACCACATTCTTGCAAAC/ ATTAAGGTTCTGCATGGTC	313	Mm. 38498
Foxa2	Forkhead box A2	GGACGTAAAGGAAGGGACTCCAC/ AGCCCATTTGAATAATCAGCTCAC	174	Mm. 938
Nes	Nestin	AGTGTGAAGGCAAAGATAGC/ TCTGTCAGGATTGGGATGGG	316	Mm. 23742
Vim	Vimentin	AAGGGTGAGTAGAGAGTTC/ AACACTGTTAGGAAAGAGG	222	Mm. 7
Tubb3	Tubulin, beta 3	TCACTGTGCCTGAACTTACC/ GGAACATAGCCGTAAACTGC	318	Mm. 40068
Nanog	Nanog homeobox	AGGGTCTGCTACTGAGATGCTCTG/ CAACCACTGGTTTTTCTGCCACCG	363	Mm. 6047
Oct3/4 (Pou5f1)	POU domain, class 5, transcription factor 1	GGAGAGGTGAAACCGTCCCTAGG/ AGAGGAGGTTCCCTCTGAGTTGC	391	Mm. 17031
Rex1 (Zfp42)	Zinc finger protein 42	CCAGGGAAGGATGAGAGA/ TAGAAGCTGGTAACAGGGAG	264	Mm. 285848
Foxd3	Forkhead box D3	TCTTACATCGCGCTCATCAC/ TCTTGACGAAGCAGTCGTTG	171	Mm. 4758
Fgfr4	Fibroblast growth factor receptor 4	TCCGACAAGGATTTGGCAG/ GCACTTCCGAGACTCCAGATAC	400	Mm. 4912
Terf1	Telomeric repeat binding factor 1	TTCAACAACCGAACAAGTGTC/ TCTCTTTCTCTTCCCCCTCC	215	Mm. 4306
Cx43 (Gja1)	Gap junction membrane channel protein alpha 1	TACCACGCCACCACTGGCCCA/ ATTCTGGTTGTCGTCGGGGAAATC	290	Mm. 4504
Glut1 (Slc2a1)	Solute carrier family 2 (facilitated glucose transporter), member 1	CAGTCAGCAATGAAGTCCAG/ AGCAGTAAGTTCTCAGCCTC	585	Mm. 30044
Abcg2	ATP-binding cassette, sub-family G (WHITE), member 2	CCATAGCCACAGGCCAAAGT/ GGGCCACATGATTCTTCCAC	326	Mm.196728
Gapdh	Glyceraldehyde-3-phosphate dehydrogenase	GGGTGTGAACCACGAGAAATATGA/ CCTTCCACAATGCCAAAGT	250	Mm.379644

that did not show coat color chimerism were killed at 21 days old and DNA prepared and processed for PCR and Southern blot analysis to identify GFP integration using standard protocols.

FACS analysis

To analyze the proportion of cells expression GFP in ES-EBs cell lines at different passes, cell were detached with trypsin and washing with PBS, then cells were resuspended at a final concentration of 10^6 cells/ml in PBS and analyzed for flow cytometry. R1 and R1-mTert-GFP cells were used as controls. Flow cytometry analysis was performed using a FACSCalibur System and CellQuest software.

Karyotype analysis

Chromosome spreads of the ES cell lines were performed as described below. ES cells were arrested in metaphase by supplementing the culture medium with 0.1 μg/ml colcemid for 4 hr at 37°C in a 5% CO_2 air atmosphere. Posteriorly cells were treated with trypsin-EDTA for 2 min at 37°C. After pipeting, the single cell suspension was washed twice with PBS by centrifugation at 200 G for 5 min. The pellet obtained was exposed to a hypotonic stock by resuspension in 0.075 M KCl for 15 min at 37°C. After a second centrifugation step the hypotonic solution was removed and the pellet fixed with a methanol/acetic acid solution (3:1; vol/vol) by gently pipetting. Ten min later, cells were pelleted again and fixed a second time. Before slide mounting, cells were washed twice with PBS. The slides were dried overnight at 55°C, stained in freshly made 10% Giemsa solution for 30 min and rinsed with distilled water. Finally, air-dried slides were observed under microscope. Chromosome number was obtained after analyzing at least 30 metaphase cells for each cell preparation.

Analysis of marker gene expression by RT-PCR

Total RNA was extracted from ES cell pellets using the Ultraspect™ RNA Isolation System (Biotecx Lab. Inc., Houston, Texas, USA) according to the manufacturer's instructions. Precipitated RNA was dissolved in DEPC-treated water and digested with 1 U of RQ DNase I (Promega) at 37°C for 20 min to ensure that the only source of DNA in the PCR was cDNA to cellular RNA. Finally, the RNA was extracted by phenol purification and ethanol precipitation, reconstituted in 50 μl of DEPC-treated

water and stored at -70° C until RT-PCR.

The RT reaction was performed following the manufacturer's instructions (Jimenez *et al.*, 2003) (Gibco-BRL, Grand Island, NY, USA). Five micrograms of RNA were dissolved in water, heat-denatured (65°C, 2 min) and reverse-transcribed at 37°C for 60 min in a final volume of 20 μl containing 0.5 mM of each dNTP, 0.2 μM oligo (dT) 0,5 μM of random primers, MMLV-RT (0.5 μl), RNasin (0.2 μl), 1x MMLV-RT buffer with 8 mM DTT. After reverse transcription, different genes were PCR amplified by adding a 1.5 μl aliquot of each sample to the PCR mix containing the specific primers. The PCR products were subjected to electrophoresis on 2% agarose gel. The primers used for RT-PCR are listed in Supplementary Table 1. Gapdh was used as a positive control and the absence of genomic contamination was systematically checked with Gapdh amplification of the RNA samples without reverse transcriptase. In addition, amplicon identities were confirmed by sequencing the PCR products. Generation of the expected fragments was strictly dependent on the presence of RNA in the RT reaction. Genes previously reported as markers of early differentiation into germ layers or into tissue-specific precursors were chosen as sensitive indicators of differentiation. The quality of the ES-like cells recovered from differentiated EBs was confirmed by evaluating several markers expressed by undifferentiated and differentiated ES cells (Ginis *et al.*, 2004, Thomson *et al.*, 1998). We explored the expression of a set of markers characteristic of pluripotent cells and previously reported to be associated with the pluripotent state: Nanog (Chambers *et al.*, 2003, Mitsui *et al.*, 2003), Oct3/4 (Okamoto *et al.*, 1990) and Rex1 (Ben-Shushan *et al.*, 1998). Amplicon sequencing confirmed the amplification of the correct mRNA and not other pseudo- or retro-genes. We also determined markers expressed in undifferentiated cells that are controlled by the OCT3/4 and SOX-2 genes: Foxd3 (Sutton *et al.*, 1996), Fgfr4 (McDonald and Heath, 1994) and telomerase repeat binding factor Terf1 (Broccoli *et al.*, 1997). In addition, the expression of other markers present on blastocysts or other stem cell populations such as the gap junction membrane channel protein alpha 1, Cx43 (Rizos *et al.*, 2002), soluble carrier family 2 -facilitated glucose transporter- member 1, Glut1 (Morita *et al.*, 1994) and ATP-binding cassette, sub-family G – white- member 2, Abcg2 (Lu *et al.*, 2002) were also examined. Besides trying to identify a set of ES cell-specific markers, we also used a set of markers to assess the state of differentiation of our ES-like cell lines. Thus, to analyze the expression of markers characteristic of differentiated phenotypes we used published RT-PCR primers that amplify genes characteristic of endoderm (Gata-4, Gata-2, Afp, Foxa2), mesoderm (Msx1, T, Myf5, Krt15) and ectoderm (Nes, Vim and Tubb3) lineages.

Immunocytochemistry

All the procedures were done at room temperatre. For Nanog detection cells were fixed with 4% paraformaldehyde for 20 min and they were permeated with PBS/0,1% Triton X-100 (PBST). After that, cells were incubated for 30 min with Signal Enhancer (Chemicon International, Inc., Temecula, CA), then cells were blocked for 1 h with PBST/5% BSA and then incubated 2 h with primary antibody Nanog (Chemicon) at 1:100 working dilution. For SSEA-1 detection cell were first fixed with 4% paraformaldehyde for 10 min. After incubation for 30 min with Signal Enhancer (Chemicon), the cells were blocked for 1 h at PBS/5% BSA and incubated for 2 h with the primary antibody SSEA-1 (Chemicon) at 1:100 working dilution. After three washes in PBS, fluorescent secondary antibodies Alexas 488 (Chemicon) at 1:500 dilution in PBS were incubated with cells for 1 h to detect expression. Nuclear staining was performed with DAPI (Sigma). The slides were mounted with Vectashield (Vector, Burlingam, CA).

Quantifying mRNA expression of IAP and MuERV-L by real-time RT-PCR

RNA isolation and reverse-transcription procedures were performed as described above. The primers used are provided in Supplementary table 2 (Ramirez *et al.*, 2006). For quantification of mRNA transcription of intracisternal-A particle (IAP) and murine endogenous retrovirus-L (MuERV-L elements), RT was performed using random primers and oligo dT. Relative transcript amounts were quantified by real time quantitative RT-PCR (qRT-PCR) in three replicate PCR experiments. PCR was performed using a Rotorgene 2000 Real Time Cycler™ (Corbett Research, Sydney, Australia) and SYBR Green (Molecular Probes, Eugene, OR) as a double-stranded DNA-specific fluorescent dye. The PCR reaction mixture (25 μl) contained 2.5 μl 10x buffer, 3 mM $MgCl_2$, 2 U Taq Express (MWGAG Biotech, Ebersberg, Germany), 100 μM of each dNTP and 0.2 μM of each primer. In addition, the double-stranded DNA dye, SYBR Green I (1:3000 of 10000x stock solution), was included in each reaction. Details of the qRT-PCR procedure have been described elsewhere (Lonergan *et al.*, 2003). The PCR protocol included an initial step of 94°C (2 min), followed by 40 cycles of 94°C (15 s), 56°C (30 s) and 72°C (30 s). Fluorescent data were acquired at 85°C. The melting protocol consisted of holding at 40°C for 60 s and then heating from 50 to 94°C, holding at each temperature for 5 s while monitoring fluorescence. Product identity was confirmed by ethidium bromide-stained 2% agarose gel electrophoresis. In addition, amplicon identities were confirmed by sequencing the PCR products. As negative controls, we always prepared tubes in which RNA or reverse transcriptase was omitted during the RT-reaction.

The comparative CT method was used to quantify expression levels (Fernandez-Gonzalez *et al.*, 2004). Quantification was normalized to the endogenous control GAPDH. Fluorescence was acquired in each cycle to determine the threshold cycle or the cycle during the log-linear phase of the reaction at which fluorescence increased above background for each sample. Within this region of the amplification curve, a difference of one cycle is equivalent to doubling of the amplified PCR product. According to the comparative CT method, the ΔCT value was determined by subtracting the GAPDH CT value for each sample from each gene CT value of the sample. Calculation of ΔΔCT involved using the highest sample ΔCT value (i.e., the sample with the lowest target expression) as an arbitrary constant to subtract from all other ΔCT sample values. Fold changes in the relative gene expression of the target were determined using the formula $2^{-\Delta\Delta CT}$. Data on mRNA expression were analyzed using the SigmaStat (Jandel Scientific, San Rafael, CA) software package. One-way repeated-measures ANOVA (followed by multiple pair-wise comparisons using Student-Newman-Kleus method) were used for the analysis of differences in mRNA expression assayed by quantitative RT-PCR.

Bisulphite analysis of ES-EB cells

DNA from cells was extracted according to standard proteinase K digestion and phenol-chloroform extraction procedures. The isolated DNA was treated with sodium bisulphite using the EZ DNA Methylation Kit (Zymo Research, Orange, CA, USA). Bisulphite-modified DNA was amplified by PCR. The methylated status of IAP LTRs (accession M17551) was examined using the following primers:

IAP-F1: 5´-TTGATAGTTGTGTTTTAAGTGGTAAATAAA;

IAP-R1: 5´-CAAAAAAAACACCACAAACCAAAAT;

IAP-F2: 5´-TTGTGTTTTAAGTG GTAAATAAATAATTTG;

IAP-R2: 5´- AAAACACCACAAACCAAAATCTTCTAC. PCR conditions were: 1st PCR (30 cycles) F1/R1; 2nd PCR (30 cycles): F2/R2. Temperature conditions were: 94°C, 3 min; 94°C, 20 s; 55°C, 30 s (2nd PCR, 60°C); 72°C, 30 s; 72°C, 5 min. The methylated status of MuERV-

SUPPLEMENTARY TABLE 2

PRIMERS USED FOR REAL TIME PCR (QRT-PCR) OF IAP AND MUERV-L

Transposon	Primer 5´-3´	*Tm* (ºC)
IAP-PCR-F1	GGGTATTGTTGAGCGTGCGC	56
IAP-PCR-R2	TCGGGTGAGTCTTTCTGGTAC	56
MuERV-PCR-F1	TGCTTGGGCTCAGCAACATGG	56
MuERV-PCR-R2	GACAGAATGCCTCATCTATCGT	56

L LTRs (accession AC166650) was examined using the following primers:

RVL-F1: 5´-GTTATTATGTGATTTGAATTA;
RVL-R1: 5´-ACATACAAAACCATCAATAAAC;
RVL-F2: 5´-TTTATTATGAGTTGGGTAT;
RVL-R2: 5´-ATAAACCAAACTC TAATCTTC. PCR conditions were: 1st PCR (30 cycles) F1/R1; 2nd PCR (30 cycles): F2/R2. Temperature conditions were: 94°C 3 min, 94°C 20 s, 53°C 30 s (2nd PCR 60°C), 72°C 30 s, 72°C 5 min. PCR products were gel-purified using the ELU-QUIK DNA purification kit (Schleicher&Schuell, Dassel, Germany) and transformed into XL1 *Escherichia coli* cells. Positive clones were verified by restriction analysis and the products were sequenced using standard methods. Methylation percentages were obtained for each individual clone within a sample (number of methylated CpGs per clone divided by the total number of CpGs per clone). These values were then used to calculate the overall methylation level and standard error of the mean of each sample. A logistical regression test from the SigmaStat statistical package was used to test for differences between samples. The samples are considered significantly different when $P<0.05$.

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"I do not understand my images and anyone is free to understand them as he wishes. I have only tried to depict my own dreams..." J.M. Folon. This neo-surrealist painting entitled *"Invasion"* by Jean-Michel Folon (1934, Brussels, Belgium), with its swirling components against a stable background can be considered as a representation of avid cells breaking through the basement membrane to invade the adjacent stroma. With kind permission from J.-M. Folon (2004)

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