Assessment of stem cell markers during long-term culture of mouse embryonic stem cells

A. Berrill¹, H.L. Tan¹, S.C. Wuang¹, W.J. Fong¹, Andre B.H. Choo¹ & Steve K.W. Oh^{1,2,*}

¹*Bioprocessing Technology Institute, 20 Biopolis Way, #06 –01 Centros, Singapore 138668, Singapore;*

²*Department of Chemical and Biomolecular Engineering, National University of Singapore, Singapore;*

(* Author for correspondence; E-mail: steve_oh@bti.a-star.edu.sg; fax: +65-6478-9561)

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Abstract

Embryonic stem (ES) cells have been in the fore front of scientific literature lately as having the potential for regeneration of many tissue types. Two important issues that need to be addressed are the culture conditions for maintaining ES cells and the accuracy of ES cell markers in monitoring the undifferentiated state. Leukaemia inhibitory factor (LIF) is routinely used to sustain mouse ES cells (mES) in a pluripotent fashion. In this paper, we assessed three markers during long-term maintenance of ES cells with various concentrations of LIF to see if decreasing concentration would lead to changes in marker expressions and growth behavior. Common markers of pluripotency such as alkaline phosphatase enzyme activity (ALP), surface staining for stage specific embryonic antigen 1 (SSEA-1), Oct-4 transcription factor, cell doubling time, as well as visual observations of cell morphology were analyzed during long-term maintenance of mES cells with LIF concentrations ranging from 0 to 500 pM. The morphology of the cells at LIF concentrations of 0–25 pM changed from being tight clusters to more flattened shapes while cells in 50–500 pM retained the clustered shape but growth rates remained essentially identical at between 10 and 16 h. ES cells at all concentrations of LIF continued expressing ALP, SSEA-1 and Oct-4 markers over a period of 6 weeks, which indicate that mES cells are capable of either producing autocrine LIF or are able to proliferate at very low levels of LIF. Pluripotency markers such as Oct-4 and SSEA-1 are only moderately reduced after 5–6 weeks. Oct-4 mRNA expression levels were partially diminished in LIF free conditions only at weeks 5 and 6 compared to controls with LIF at 500 pM. Changes in morphology of cells by visual observation seemed to be a faster indication of the onset of differentiation in mES cells, although other reliable means also include decreased levels of Oct-4, SSEA-1 and ALP markers. It is preferable to maintain long-term cultures of mES cells above 50 pM of LIF to have a more homogenous, stable population of pluripotent cells.

Introduction

Embryonic stem (ES) cells are capable of differentiation into multiple cell types as well as

self-renewal. Due to their unique properties, ES cells have the potential to serve as an unlimited source of any cell type in the body for use in cell transplantation and drug discovery. Murine stem

cells are derived from the blastocyst of early mouse embryonic development (Evans and Kaufman 1981; Martin 1981) and are able to proliferate indefinitely in an undifferentiated state in the presence of LIF (Smith et al. 1988; Williams et al. 1988b) or differentiate into all three germ layers upon removal of this factor (Murray and Edgar 2001; Oka et al. 2002).

Propagation of the undifferentiated pluripotent phenotype of mES cells is dependent on LIF (Yoshida et al. 1994), one of the IL6-type cytokine, which plays an important role in cellular processes such as gene activation, proliferation and differentiation. The major steps involved in the signaling pathway by this family of cytokines include components such as JAK kinases, the signal transducers, activators of transcription STAT1 and STAT3 as well as tyrosine phosphatase SHP2 (Heinrich et al. 1998). In brief, LIF stabilizes the association of LIF receptor (LIFR) and cytokine receptor gp130. Subsequently, JAK kinases are activated which results in the recruitment, tyrosine phosphorylation and dimerization of STAT3. Translocation of STAT3 dimers to the nucleus follows and binding to the target sites on the DNA triggers the transcription of relevant genes for self-renewal (Burdon et al. 2002).

Mouse ES (mES) cells cultured in the presence of LIF retained their characteristic stem cell morphology and express stem cell markers such as alkaline phosphatase (ALP) and stage-specific embryonic antigen-1 (SSEA-1) (Williams et al. 1988a). ALPs constitute a small family of 4 cell surface enzymes that play a role in differentiative processes and decreases with ES cell differentiation (Hahnel et al. 1990). SSEA-1, a glycolipid antigen consisting of trisaccharide repeats $\text{Gal}\beta 1-4(\text{Fuc}\alpha 1-3)\text{GlcNAc}$ (Williams et al. 1988a), was first detected on blastomeres of 8-cell stage embryos (Solter and Knowles 1978) and was found to disappear as mES cells differentiate (Ling and Neben 1997; Smith et al. 1988). SSEA-1 antibody also reacts with embryonic carcinoma cells of mouse and human origin. However, there is little account in literature of its functional importance.

Oct-4 is a 352 amino acid protein belonging to class V of POU transcription factors and a pluripotent marker expressed by pluripotent ES cells (Schöler, 1991; Niwa et al. 2000). Initially expressed in all blastomeres of the developing mouse embryo, Oct-4 mRNA expression becomes

restricted to the inner cells of the blastocyst forming the inner cell mass, and is downregulated in the trophectoderm and the primitive endoderm (Nichols et al. 1998). Recent experiments indicate that a 50% loss of Oct-4 expression induces loss of pluripotency and differentiation to trophectoderm, while a 2-fold up regulation leads to formation of primitive endoderm and mesoderm lineages (Niwa et al. 2000; Pesce and Schöler 2001). It has been established that the level of Oct-4 mRNA expression is critical to sustain stem cell self-renewal.

Previous workers have developed quantitative assays of ES cell differentiation by measuring cell surface SSEA-1 antigen and ALP activity (Zandstra et al. 2000). Examination of ES cell responses over a range of LIF concentrations for 7 days has shown that LIF supplementation has little effect on ES cell growth rate but significantly alters the probability of a cell undergoing self-renewal versus differentiation. Viswanathan et al. (2002) examined Oct-4 protein expression and found that this was reduced in the absence of LIF.

Here we extend this work by observing the effects of varying LIF concentrations on long-term maintenance of mES cells. For over 6 weeks, mES cells treated with various concentrations of LIF ranging from 0 to 500 pM were studied. As the concentration of LIF decreases, the effects on the markers namely, Oct-4 transcription factor, SSEA-1 and ALP as well as cell proliferation and morphological changes were also investigated. From the data obtained, it seemed that for long-term culturing of mES cells, it is necessary to maintain LIF at a concentration of greater than 50 pM.

Materials and methods

Cell cultures

CS-1 mouse embryonic stem cells (mES) a gift from Dr Chyuan-Sheng Lin, College of Physicians and Surgeons, Columbia University were used for all experiments (Yin et al. 2002). Cells were maintained at 37 °C in humidified air with 5% CO₂ in Dulbecco's Modified Eagle's Medium (DMEM) with Glutamax and supplemented with 10% fetal bovine serum (Hyclone), 1% penicillin, streptomycin, L-glutamine and non-essential amino acids and 2-mercaptoethanol (all from

GIBCO). Mouse ES cells were routinely maintained at a Leukaemia Inhibitory Factor (LIF, Chemicon International) concentration of 500 pM. For long-term culture experiments, LIF was added to the DMEM media immediately prior to each use to produce eight different LIF concentrations in the range of 0–500 pM. Cells were exposed to these concentrations for 6 weeks with one passage carried out per week. Each week, three 60 mm tissue culture plates (Falcon) and two sets of duplicate wells in a 24 well plate (Techno Plastic Products AG, Switzerland) were seeded for each LIF concentration. Prior to cell seeding, the plates and wells were coated with 0.1% porcine gelatin (Sigma) in phosphate buffered saline (PBS). Cells were seeded at 6×10^5 cells per culture plate and 0.4×10^5 per well. All cells used were of passage 38–43. Cells were washed with PBS and replenished with media containing LIF daily 3 days after seeding. For passaging, cells were trypsinised by incubating with 0.25% trypsin (Invitrogen Life technologies) for 7 min at 37 °C in humidified air with 5% CO₂. After seeding, 6 and 1 ml of LIF-supplemented media were added to the 60 mm plates and 24 well plates respectively. Each week cells from all of the 60 mm plates were trypsinised and completely removed. Cells from one set of the 60 mm plates were used for quantitative SSEA-1 and Oct-4 fluorescence activated cell sorting (FACS). The remaining two sets of 60 mm plates were used for duplicate cell counts and seeding for the following week. One of the 2 sets of duplicate 24 wells was used for qualitative SSEA-1 and DAPI fluorescence staining and the other for qualitative ALP fluorescence staining.

Viability and cell count

All mES cells were completely detached by treatment with 0.25% trypsin-EDTA (GIBCO) and incubated at 37 °C for 7min. Subsequently, the cells were syringed through 22 1/2 G needles to break the clumps into single cells. The action of trypsin was neutralized by adding the syringed cells into excess media. Cells were then pelleted, excess media aspirated and resuspended in 1 ml of media. The viability and cell count were determined via trypan blue exclusion assay. The cells were counted in a haemocytometer (Neubauer) four times and the viability was calculated by

scoring the percentage of unstained cells with the total number of cells.

Determination of growth rates and doubling times

On weeks 1, 4 and 6 of the 6 week passaging experiments, 7 extra 24 well plates were seeded in triplicate with mES cells grown in each of the eight different LIF concentrations. Everyday for 7 days, one 24 well plate of mES cells was trypsinised allowing triplicate cell counts for each LIF concentration to be obtained by trypan blue exclusion assay. Graphs of cell density versus time were plotted in order to calculate the specific growth rates during the exponential growth phase for each LIF concentration during weeks 1, 4 and 6. From this the doubling time was calculated using the following equation:

$$t_d = \frac{\ln(2)}{\mu}$$

where: μ = specific growth rate, h⁻¹; t_d = doubling time

Alkaline phosphatase (ALP) staining

ALP activity was measured according to the protocol described by the commercially available Vector Red Alkaline Phosphatase Substrate Kit I (Vector Laboratories). Briefly, the cells were washed twice with PBS. The buffer was prepared using reagents provided in the kit and checked for pH, which should be in the range of pH 8.2–8.5. Sufficient buffer was added to cover the cells and incubated for 1 h at room temperature. Light and U.V. filtered (green light) images were then taken using an IX70 Olympus microscope at 100× magnification. Qualitatively, positive staining is defined as a red precipitate that fluoresces under UV light.

Stage specific embryonic antigen 1 (SSEA-1) and DAPI staining

The mES cells in 24 well plates were washed twice with PBS and fixed with 100% ethanol (Merck) for 2 min. Cells were then washed with 1% BSA/PBS

(Bovine serum albumin, Sigma) and incubated for 1 h at room temperature with 100 μ l of undiluted IgM antibody against SSEA-1 (Developmental Studies Hybridomas Bank, DSHB). The cells were washed twice with 1% BSA/PBS and incubated in the dark with a 1:500 dilution of goat anti-mouse PE-conjugate (DAKO) for another hour. Simultaneously, the nuclei of the cells were stained with a 1:1000 dilution DAPI solution (kindly donated by Johns Hopkins Singapore Pte Ltd, Genetic Immunotherapy Laboratory). The cells were washed twice and sufficient 1% BSA/PBS was added to cover the cells. UV filtered images were then taken under the IX70 Olympus microscope at 100 $\times$ magnification.

Flowcytometry – intracellular staining of Oct-4

Single cell suspensions from each LIF concentration were treated with Fix and Perm Kit (Caltag Laboratories) and incubated with an IgG₁ Oct-4 antibody (Santa Cruz) at 1:20 dilution (10 μ g/ml final). Oct-4 stained cells were then washed with 1% BSA/PBS and incubated in the dark with a 1:500 dilution of goat anti-mouse FITC-conjugate (DAKO). After incubation, the cells were washed again and resuspended in 1% BSA/PBS for analysis on a FACScan (Becton Dickinson FACS Calibur). All incubations were performed at room temperature for 15 min. As isotype controls, cells were stained with an IgG₁ antibody for SSEA-4 (DSHB).

Flowcytometry – cell surface staining of SSEA-1

After fixing, cells were incubated with IgM antibody SSEA-1 (DSHB). The washing and incubation steps were the same as that for Oct-4 staining. As isotype controls, cells were stained with an IgM antibody for SSEA-3 (DSHB).

Flowcytometry - Co-staining of Oct-4 and SSEA-1

Oct-4 staining was carried out first using goat anti-mouse FITC-conjugate, following the method already described. Just before the final resuspension step the mES cells underwent SSEA-1 staining with goat anti-mouse PE-conjugate, following the

method described previously. The cells were then washed three times and resuspended in 1% BSA/PBS before being analyzed by FACS. In addition to the isotype controls mentioned previously, goat anti-mouse PE-conjugate was added to cells not exposed to the IgM antibody against SSEA-1. This was to ensure that this secondary antibody was binding to SSEA-1 and not unbound Oct-4 antibodies.

RNA Isolation and cDNA generation

RNA was isolated from mES cells using Trizol. One milliliter Trizol was added for every 5–10 million cells, sheared using a 21 G syringe, and left at room temperature for 10 min. Following high-speed centrifugation at 4 °C, 1/5 volume chloroform was added for phase separation of the aqueous layer, followed by subsequent washes using equal volume isopropanol and 75% ethanol. Complementary DNA was generated using M-MLV reverse transcriptase (Promega) according to manufacturer protocol.

Quantitative real-time PCR

Quantitative Real-Time PCR was performed on an ABI 7000. Measurement of Oct-4 levels was done using ABI Assay-By-Design Kit containing forward primer, CGAACCTGGCTAAGCTTCA, and reverse primer, GAGCCTGGTCCGATTCCA with FAM labeled Taqman probe. Endogenous control, GAPDH, was detected using ABI GAPDH Rodent Kit containing VIC labeling. Reactions were multiplexed and performed in triplicate using the following protocol as described: 50 °C for 2:00, 95 °C for 5:00, followed by 35 cycles of 95 °C for 0:15, 60 °C for 0:45, and 72 °C for 0:45.

Relative quantification using comparative Delta–Delta Cycle Threshold Method

Relative quantification (RQ) was performed using Delta–Delta Cycle Threshold Method ($\Delta\Delta C_t$) on ABI RQ sequence detection software Version 1.1 (Livak and Schmittgen 2001). Briefly, $N_1 = N_0 * e^C$ where N_1 describes the number of molecules resulting from amplification of N_0 template

molecules after C number of cycles. Each cycle results in the addition ϵ^C molecules where ϵ is the 'efficiency' of the amplification. Comparing two different targets, A and B, the difference between N_{0A} and N_{0B} can be described as $2^{-\Delta C_t}$, where $-\Delta C_t$ is the difference between amplification cycles at which $N_{1A} = N_{1B}$. Normalization using GAPDH was calculated as $-\Delta C_t$ with Oct-4 levels at week 1, 0 pM LIF serving as a calibrator resulting in the relative expression levels being described by $2^{-\Delta\Delta C_t}$. Values reported have a 95% confidence interval as determined by the ABI RQ software.

Statistics and repeatability

All data is reported as a mean $\pm$ SEM. Statistical significance was assessed for cell counts, viability and doubling times by comparing variances between conditions using the ANOVA test. All cell counts were performed at least four times for each LIF condition. Experiments measuring qualitative changes in ES cell marker expressions namely cell morphology, ALP and SSEA-1 stains were repeated 2–5 times and representative photos are shown of selected conditions. FACS plots of Oct-4 and SSEA-1 are representative of at least duplicate experiments at all LIF conditions. This 6 week long study was repeated three times by two independent researchers S.C. Wuang and A. Berrill; the latter repeated the study twice.

Results and discussion

The mES cells used in our experiments were between passages 38–43. They were of a normal karyotype carrying 40 chromosomes and were able to form teratomas in SCID mice, which is indicative of their pluripotency (data not shown). Other studies by our group have shown that even after 70 passages, mES cells are still capable of forming teratomas. Thus the mES cells used in our experiment have characteristics typical of the other mouse ES cell lines in literature (Zandstra et al. 2000; Yin et al. 2002).

Cell numbers, viabilities and doubling times

Mouse ES cells were seeded at the same cell densities each week and fed every day after the third

day and passaged at the end of the week. Final cell densities and viabilities were measured before passaging for 6 weeks. Figure 1a shows consolidated final cell numbers and viabilities of 1 set of experiments. The data is representative of the three experiments undertaken. The maximum cell numbers range from 4 to 13 million live cells for the various LIF concentrations tested. One-way analysis of variance (ANOVA) test at 95% confidence level for cell counts at low LIF concentrations (0 and 0.5 pM) at the end of 6 weeks did not show a significant decrease when compared to others (Figure 1a) even though these cell counts were consistently lower. Viabilities over the 6 weeks for all conditions were generally above 80% and their differences were insignificant at 95% confidence level.

Cell doubling times (t_d), were performed at weeks 1, 4 and 6 for all LIF concentrations and plotted in Figure 1b. It was observed that the cells remained in the lag phase between days 1 to 2 after each seeding before entering the exponential phase. The stationary phase was reached by day 5 (results not shown). The doubling times, measured during the exponential growth periods, were found to range from 10 to 16 h which is typical of mouse ES cells (Viswanathan et al. 2002). Again we found no significant difference of these values by ANOVA test at 95% confidence interval. It was noted however that at around day 4 or 5, cell numbers in the 0 and 0.5 pM LIF concentrations tended to drop marginally (results not shown).

LIF activates the JAK/STAT3 signalling pathway via the LIF receptor, which leads to ES cell renewal. Independent from the LIF dependent JAK/STAT3 signalling pathway, STAT3 activation alone also directs the regulation of the cell cycle to cause cell proliferation. The withdrawal of LIF induces differentiation but not cell-cycle arrest. Therefore it is possible that STAT3 could still regulate the differentiated ES cells to enter the S phase and proliferate (Burdon et al. 2002). Earlier Zandstra et al. (2000), had examined the effects of different LIF concentrations on the growth rate of mES cells over 7 days and found that there was little variation in growth rate across the same range of LIF concentrations tested. Their cell proliferation analysis by FACS showed that a similar proportion of cells passed through G2/M/S cell cycle phases in both 500 pM and LIF free culture conditions. Our results over 6 weeks also

Figure 1. (a) Total viable cell numbers (barcharts) and viability profiles (scatterplots) for LIF concentrations from 0 to 500 pM for 6 weeks. Cells were passaged at the same cell density each week. The error bars represent the standard error of 1 set of experiment and each LIF condition was performed in duplicates. This data is representative of 3 separate experiments undertaken. No significant difference was found by ANOVA test. (b) Comparison of the doubling times at weeks 1, 4, and 6 for mES cells cultured at different LIF concentrations. Doubling times were determined during the exponential growth phase from three replicate cultures for each condition. No significant difference was found by ANOVA test.

supported this observation. It is important to note that the media used in our experiment was supplemented with serum. In the presence of serum which contains other factors capable of maintaining ES in the proliferating state, the LIF dependent JAK/STAT3 signalling pathway may not be the only mechanism that promotes ES cell renewal. This may explain our observation

whereby proliferation rate was not affected by reducing exogenous LIF.

Cell morphology

For the entire 6 weeks, the ES cells at LIF concentrations 100 and 500 pM (control), grew as

compact colonies of small cells with large nuclear to cytoplasmic ratio (Figure 2a, b). Cells cultured in 0 and 0.5 pM LIF tend to be more dispersed as single cell monolayers and began looking fibroblastic by week 1. At the end of week 6, the majority of cells had lost the typical ES morphology of highly compact clustered colonies. These observations were consistent with Williams et al. (1988b) and Viswanathan et al. (2002). It was also noticed that mES cells exposed to the 0 and 0.5 pM LIF concentrations became increasingly adherent over the 6 weeks and required longer incubation times with trypsin to detach them completely from the surface of the plate. By week 5, it was observed that cells in LIF concentrations of 50 and 100 pM still showed identical clustered morphology as the control cells in 500 pM LIF (Figure 2b), whereas cells cultured in 25 and 50 pM began to form some fibroblastic-like cells.

These observations show that different concentrations of LIF affect the morphology of the cells, and that the time taken for this morphology change is LIF-concentration dependent. The Ligand/Receptor Signaling Threshold (LIST) model may explain this LIF concentration dependency (Viswanathan et al. 2002). According to this conceptual model, whether a cell undergoes self-renewal or differentiation is determined by a critical number of cell-surface cytokine-receptor complexes formed on the cells. Thus, when the supplemented LIF concentration is less than this critical value, the cells start to differentiate. However, the LIST model was based on data collected over 7 days. In our experiment, the onset of morphological change was observed during week 5 and 6 for cells cultured in the mid-ranges of the LIF concentrations. Hence, it may be interesting to study the relationship between varying LIF concentrations and the time taken for ES cells to begin differentiation.

Qualitative ALP staining

Figure 2a and b show the qualitative staining of alkaline phosphatase (ALP) present in mES cell at various LIF concentrations at weeks 1 and 5. It can be seen from both figures that under bright field and UV light, the fibroblastic-like monolayers present in the low LIF concentrations appear not as heavily stained as the more compact colonies

found at higher LIF concentrations. Previous work (Zandstra et al. 2000) showed a direct correlation between ALP activity and increasing LIF concentrations in 1 week cultures. The mean ALP activity increased from 5 to 20 nmol/10e6 cells/min as LIF concentrations enhanced from 0 to 500 pM for that mES cell line. Our data agrees with the quantitative ALP result observed by Zandstra et al. 2000.

SSEA-1 and DAPI qualitative staining

Figure 3a and b show large representative photos to depict clearly the nuclear and SSEA-1 staining from the low, medium and high LIF concentrations: 0, 25 and 500 pM for weeks 1 and 6 respectively. Again it can be seen that without LIF, mES cells began to form more dispersed cells compared to the very compact colonies seen at medium and high LIF concentrations. By week 6, mES at 0 and 25 pM concentration ranges have more fibroblastic populations amongst the cell clusters, whilst those at the 500 pM LIF concentration remain highly compact. Surprisingly, both fibroblastic and colonial cells stained positive for SSEA-1 despite the change in morphology at week 6. It has been reported that SSEA-1 disappears when cells have LIF removed from culture, embryoid bodies formed and differentiated cells created (Ling and Neben 1997). The difference in these experiments is that cells were not induced to differentiate, but were cultivated LIF free and we were surprised to see that they continued expressing an early pluripotent marker.

Quantitative staining of SSEA-1

To quantitatively examine the SSEA-1 marker, mES cells were stained and analyzed by FACS to detect if there was any increase in the population of unstained mES cells at the low LIF concentrations. At week 1 with no LIF, Figure 4 shows a bimodal distribution with 85% of the cells staining positively for SSEA-1. For concentrations of 10 pM and above, greater than 90% of the cell population stained positively for SSEA-1. At week 4, in the condition without LIF, 89% of the population continues to express SSEA-1, but the profile is more heterogeneous with a loss of

Figure 2. (a) Qualitative measure of alkaline phosphatase (ALP) enzyme staining viewed under bright field and UV light respectively. mES cells stained for ALP at week 1 with LIF concentrations of: (A and B) 0 pM, (C and D) 0.5 pM, (E and F) 5 pM, (G and H) 10 pM, (I and J) 25 pM, (K and L) 50 pM, (M and N) 100 pM, and (O and P) 500 pM. Scale bar = 200 μ m. (b) Qualitative measure of alkaline phosphatase (ALP) enzyme staining viewed under bright field and UV light respectively. Although mES cells do not stain at 0 and 0.5 pM LIF under bright field, they stain with ALP in UV light. All other LIF conditions also stain for ALP at week 5. LIF concentrations: (A and B) 0 pM, (C and D) 0.5 pM, (E and F) 5 pM, (G and H) 10 pM, (I and J) 25 pM, (K and L) 50 pM, (M and N) 100 pM, and (O and P) 500 pM. Scale bar = 200 μ m.

Figure 3. (a) Detection of surface marker expression (SSEA-1 counter-stained with DAPI) after 1 week viewed under bright field and UV light respectively. LIF concentrations: (A and B) 0 pM, (C and D) 25 pM and (E and F) 500 pM. Scale bar = 50 μ m. (b) Detection of surface marker expression (SSEA-1 counter-stained with DAPI) after 6 weeks viewed under bright field and UV light respectively. LIF concentrations: (A and B) 0 pM, (C and D) 25 pM and (E and F) 500 pM. Scale bar = 50 μ m.

the bimodal appearance. All other LIF conditions also stained positive for SSEA-1 with moderately sharp profiles. Interestingly, by week 6, the percentage of mES cells that stained positively for SSEA-1 at 0 pM LIF remained high (73%). A plateau profile with no dominating intensity is seen. All other LIF conditions at week 6 continued to express high levels of SSEA-1. In general though, the SSEA-1 staining profiles shifted towards lower intensities as LIF concentration is reduced.

Quantitative staining of Oct-4

Figure 5 shows that at week 1, 0 pM LIF condition, only 79% of the cells stained positively for Oct-4. All other concentrations at 10 pM and above stained positively for Oct-4 for about 90% of the population. At week 4, there appears to be a similar level (>90%) of Oct-4 protein expressing cells in cultures without LIF as in the other conditions. This confirms the mRNA expression data which is shown later in Figure 7

Figure 3. Continued

where there is no difference in transcript levels between controls at 500 and 0 pM LIF conditions. Only by week 6 is this population of Oct-4 +ve cells reduced to 75% in conditions without LIF. The remaining 25% which are Oct-4 -ve are the differentiating population. Similar to SSEA-1, it is obvious that the Oct-4 staining profiles shifted towards lower intensities as LIF concentration is reduced. As Oct-4 is a transcription factor unique to ES cells (Nichols et al. 1998; Niwa et al. 2000), it is important that mES cells express this protein to remain undifferentiated.

Dual staining of Oct-4 and SSEA-1

To examine more closely the expression profiles of these two key markers, Oct-4 transcription factor and SSEA-1 surface expression, mES cells were stained with antibodies against both antigens at weeks 1 and 6 for all LIF concentrations. It can be seen in Figure 6 that at the high LIF concentrations between 50–500 pM, most cells (85 to 96%) are Oct-4 +ve, SSEA-1 +ve. There is also a small population (less than 12%) of cells that are Oct-4 -ve, SSEA-1 +ve. At the mid-range of LIF (5 to 25 pM) the double stained population drops slightly to

Figure 4. Representative FACS analysis of cell populations that are SSEA-1 positive for 0, 10, 50 and 500 pM concentrations of LIF for weeks 1, 4 and 6. 8000 cells were analyzed and M1 gating was set at 1% of the isotype IgM controls.

80–85% and the Oct-4 –ve, SSEA-1 +ve group increased to 12 to 17%. At the lowest LIF concentrations, the dual stained population drops to between 68 and 76%. Whilst the Oct-4 –ve, SSEA-1 +ve population remains stable at 11 to 16%, the remaining 7 to 11% fraction has become both Oct-4 –ve and SSEA-1 –ve. These FACS profile show clearly that first a sub-population of mES cells stop expressing Oct-4 and then they stop expressing SSEA-1 as well, as LIF concentration is reduced.

This shift of population from the dual positively stained quadrant to the dual negatively stained region in a counter clockwise direction is more evident at week 6 for all LIF conditions. At 0 pM LIF, the dual stained population has now dropped to about 61% while the dual negative group has increased to 20%. Surprisingly the amount of double negative mES population has not increased much, even though they have been passaged without LIF for 6 weeks.

Figure 5. Representative FACS analysis of cell populations that are Oct-4 positive for 0, 10, 50 and 500 pM concentrations of LIF for weeks 1, 4 and 6. 8000 cells were analyzed and M1 gating was set at 1% of the isotype IgG controls.

Table 1 summarises the three different populations of cells, those that stain for both Oct-4 and SSEA-1, those that stain for only for SSEA-1 and those that are negative for both markers for the rest of the LIF concentrations. Without LIF, the population of Oct-4 +ve, SSEA-1 +ve drops from week 1 to week 6 and the dual negative population increases significantly. At 0.5 pM LIF, the Oct-4 +ve, SSEA-1 +ve population also drops from week 1 to week 6 but now only the

Oct-4 -ve, SSEA-1 +ve population increases. At the mid range of LIF concentrations (5–25 pM), the Oct-4 +ve, SSEA-1 +ve mES cells also drop from week 1 to week 6 and again only the Oct-4 -ve, SSEA-1 +ve population increases. Finally at the high concentrations of LIF (50–500 pM), all three populations are very stable after 6 weeks.

From these co-staining results, mES cells are still expressing significant levels of pluripotency markers after 6 weeks of culture at low LIF

Figure 6. Representative FACS dual staining of mouse ES cells in all LIF concentrations at weeks 1 and 6 were compared. SSEA-1 +ve cells were stained with PE and Oct-4 +ve cells with FITC.

concentration. These observations do not coincide with work by Zandstra et al. 2000, where SSEA-1 expression was reduced to about 20% after culturing in an artificially induced LIF free environment (where anti-LIF antibodies were added to the cultures) for a week. In our

experiments, the SSEA-1 and Oct-4 levels maintained at 73% and 75% respectively after 6 weeks in culture with 0 pM LIF. This continuous maintenance of pluripotent cells may be attributed to the presence of other sources of LIF present in our culture system.

Figure 7. Comparison of the relative levels of Oct-4 mRNA expression at the 0 and 500 pM LIF concentrations during the 6 weeks as measured by quantitative RT-PCR. Weeks 1–4 show no significant transcript expression differences in the absence or presence of LIF, but a 50% decrease in Oct4 is observed by weeks 5 and 6. Significance is at 95% confidence level.

Table 1. Percentage of cells expressing Oct-4 and SSEA-1 when cultured in various LIF concentrations compared at weeks 1 and 6.

LIF concentration (pM)	Week	Oct-4 +ve/ SSEA-1 +ve	Oct-4 -ve/ SSEA-1 +ve	Oct-4 -ve/ SSEA-1 -ve
0	1	67	16	12
	6	61	12	20
0.5	1	77	12	7
	6	71	19	7
5–25	1	80–85	12–17	1–2
	6	70–77	19–24	2–4
50–500	1	85–96	1–12	0.3–1
	6	87–96	1–11	0.3–2

Previous findings have suggested that ES cells may have a capacity for autocrine regulation by producing low concentrations of LIF (Rathjen et al 1990; Zandstra et al. 2000), which could explain in part, the high SSEA-1 and Oct-4 expression observed in our experiments. Zandstra et al. (2000) demonstrated the presence of intracellular LIF within SSEA-1 +ve cells. This being so, it is possible that mES cells are producing basal levels of LIF which would enable them to sustain pluripotency for longer periods of time without addition of exogenous LIF.

Work from Rathjen et al. 1990 suggests another theory to explain why a high population of undifferentiated cells are still being retained in the absence of LIF. They suggest that there could be a feedback mechanism of cell renewal, whereby a small population of mES cells differentiate and produce LIF to sustain renewal of the rest of the mES cells. This mechanism is only observed at seeding densities above 2000 cells/cm² and is described as a possible way of maintaining the stem cell pool in embryos and adult organisms. As our cells were seeded at 2.3×10^4 cells/cm² it is possible that this mechanism of self-renewal is occurring. As ES cells differentiate in the low LIF conditions, the differentiating cells begin producing autocrine LIF. This LIF interacts with the remaining ES cells via the JAK/STAT pathway and maintains its pluripotency. The Oct-4 / SSEA-1 staining results show that complete differentiation does not occur within our highly inoculated cultures over 6 weeks in LIF free cultures and this mechanism may explain why.

Quantitative PCR of Oct-4

Finally, we measured the gene expression levels of Oct-4 by quantitative PCR for LIF conditions of 0 and 500 pM LIF every week. Figure 7 shows that Oct-4 transcript levels are expressed at equivalent levels from weeks 1 to 4 for both LIF concentrations with insignificant differences. Only at weeks 5 and 6 is there a detectable 50% loss of mRNA expression by cells at 0 pM LIF compared to week 1 and the 500 pM conditions at other weeks. Values reported have a 95% confidence interval as determined by the ABI RQ software using the Delta-Delta Cycle Threshold Method (Livak and Schmittgen 2001). These results follow a similar pattern to the observed decrease in Oct-4 protein levels at week 6 (see Figures 5 and 6).

Conclusion

We followed three commonly accepted markers ALP, SSEA-1, Oct-4, as well as cell doubling time and visual observations of cell morphology in cultures maintained at concentrations of LIF from 0 to 500 pM during long-term maintenance of mES cells. The morphology of the cells at LIF concentrations of 0 to 25 pM of changed from being tight clusters to more flattened shapes while cells in 50 to 500 pM retained the clustered shape but growth rates remained essentially identical. ES cells at all concentrations of LIF maintained expression of ALP, SSEA-1 and Oct-4 markers over a period of 6 weeks, which indicate that mES cells are capable of producing autocrine LIF. It is preferable to maintain long term cultures of mES cells above 50 pM of LIF to have a more homogenous population of cells expressing a stable Oct-4, SSEA-1 and ALP phenotype. Oct-4 mRNA expression levels were partially diminished in LIF free conditions only at weeks 5 and 6 compared to controls with LIF at 500 pM. Of all the markers, co-expression of Oct-4 transcription factor and SSEA-1 surface antigen together with visual observations are the best means of discriminating differentiating mES cells populations.

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References

- Burdon T., Smith A. and Savatier P. 2002. Signaling, cell cycle and pluripotency in embryonic stem cells. *Trends Cell Biol.* 12: 432–438.
- Evans M.J. and Kaufman M.H. 1981. Establishment in culture of pluripotent cells from mouse embryos. *Nature* 292: 154–156.
- Hahnel A.C., Rappolee D.A., Millan J.L., Manes T., Ziome C.A., Theodosiou N.G., Werb Z., Pedersen R. and Schultz G.A. 1990. Two alkaline phosphatase genes are expressed during early development in the mouse embryo. *Development* 110: 555–564.
- Heinrich P.C., Behrmann I., Müller-Newen G., Schaper F. and Graeve L. 1998. Interleukin-6-type cytokine signaling through the gp130/Jak/STAT pathway. *Biochem. J.* 334: 297–314.
- Ling V. and Neben S. 1997. In vitro differentiation of embryonic stem cells: immunophenotypic analysis of cultured embryoid bodies. *J. Cell Physiol.* 171: 104–115.
- Livak K.J. and Schmittgen T.D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2⁻(Delta Delta C(T)) method. *Methods* 25: 402–408.
- Martin G.R. 1981. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc. Natl. Acad. Sci. USA* 78: 7634–7638.
- Murray P. and Edgar D. 2001. The regulation of embryonic stem cell differentiation by leukaemia inhibitory factor. *Differentiation* 68: 227–234.
- Nichols J., Zevnik B., Anastassiadis K., Niwa H., Klewe-Nebenius D., Chambers I., Schöler H. and Smith A. 1998. Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. *Cell* 95: 379–391.
- Niwa H., Miyazaki J. and Smith A.G. 2000. Quantitative expression of Oct3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. *Nat. Genet* 24: 372–376.
- Oka M., Tagoku K., Russell T.L., Nakano Y., Hamazaki T., Meyer E.M., Yokota T. and Terada N. 2002. CD9 is associated with leukemia inhibitory factor-mediated maintenance of embryonic stem cells. *Mol. Biol. Cell* 13: 1274–1281.
- Pesce M. and Schöler H.R. 2001. Oct-4: Gatekeeper in the beginnings of mammalian development. *Stem Cells* 19: 271–278.
- Rathjen P.D., Toth S., Willis A., Heath J.K. and Smith A.G. 1990. Differentiation inhibiting activity is produced in matrix associated and diffusible forms that are generated by alternate promoter usage. *Cell* 62: 1105–1114.
- Schöler H.R. 1991. Octamania: the POU factors in murine development. *Trends Genet.* 7: 323–329.
- Smith A.G., Heath J.K., Donaldson D.D., Wong G.G., Moreau J., Stall M. and Rogers D. 1988. Inhibition of pluripotent embryonic stem cell differentiation by purified polypeptides. *Nature* 336: 688–690.
- Solter D. and Knowles B.B. 1978. Monoclonal antibody defining a stage-specific mouse embryonic antigen (SSEA-1). *Proc. Natl. Acad. Sci. USA* 75: 5565–5569.
- Viswanathan S., Benatar T., Rose-John S., Lauffenburger D.A. and Zandstra P.W. 2002. Ligand/receptor signaling threshold (LIST) model accounts for gp130-mediated embryonic stem cell self-renewal responses to LIF and HIL-6. *Stem Cells* 20: 119–138.
- Williams M.A., Gross S.K., Evans J.E. and McCluer R.H. 1988a. Glycolipid stage-specific embryonic antigens (SSEA-1) in kidneys of male and female C57BL/6J and beige adult mice. *J. Lipid Res.* 29: 1613–1619.
- Williams R.L., Hilton D.J., Pease S., Willson T.A., Stewart C.L., Gearing D.P., Wagner E.F., Metcalf D., Nicola N.A. and Gough N.M. 1988b. Myeloid leukaemia inhibitory factor maintains the developmental potential of embryonic stem cells. *Nature* 336: 684–687.
- Yin Y., Lim Y.K., Salto-Tellez M., Ng S.C., Lin C.-S. and Lim S.K. 2002. AFP⁺, ESC derived cells engraft and differentiate into hepatocytes in vivo. *Stem Cells* 20: 338–346.
- Yoshida K., Chambers I., Nichols J., Smith A., Saito M., Yasukawa K., Shoyab M., Taga T. and Kishimoto T. 1994. Maintenance of the pluripotent phenotype of embryonic stem cells through direct activation of gp130 signalling pathways. *Mech. Dev.* 45: 163–171.
- Zandstra P.W., Le H.V., Daley G.Q., Griffith L.G. and Lauffenburger D.A. 2000. Leukaemia inhibitory factor (LIF) concentration modulates embryonic stem cell self-renewal and differentiation independently of proliferation. *Biotechnol. Bioeng.* 69: 607–617.