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SHORT REPORT

Functionally defined substates within the human embryonic stem cell compartment

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Abstract Human embryonic stem (ES) cells can undergo spontaneously differentiation in standard culture conditions, demonstrating that the undifferentiated state is relatively unstable. The heterogeneous expression of SSEA3 observed within human ES colonies, provides a means to examine undifferentiated stem cell substates. Through functional testing of single cells we have shown that undifferentiated ES cells can be segregated into functionally discrete subpopulations on the basis of SSEA3 expression: SSEA3^{High}, SSEA^{Low} and SSEA3^{Negative}. Human ES subpopulations were found to be interconvertible, but they possess distinct properties when challenged to differentiate along the neural lineage. These data suggest that ES cells with pluripotent/self-renewal capacities can exhibit different responses to induction of differentiation.

Introduction

Pluripotent embryonic stem (ES) cells are an attractive source for deriving mature cell types required for a multitude of cell therapy applications. Extensive research has been invested to design protocols for driving ES cell differentiation to a single cell type. However, the phenotypic output of cell differentiation is influenced not only by the conditions used to induce differentiation but also by the phenotype of the starting cells. The starting population of undifferentiated human ES cells is critical as it has the potential to bias the differentiation of cells to, or away from the desired phenotype. During the maintenance of human ES cell lines, spontaneously differentiated cells are commonly found at colony boundaries. Spontaneous differentiation of cells is a source for cell heterogeneity in ES cell cultures and varying degrees of spontaneous differentiation are likely to be a significant source of passage-to-passage variability in ES cell studies, contributing to the difficulty of generating reproducible results. However, a subtle form of heterogeneity exists within the stem cell compartment itself. Heterogeneity has been identified in mouse ES cultures with respect to the expression of Nanog and Stella. For example, Nanog positive and negative mouse ES cells are interconvertible though *Nanog*$^{-/-}$ ES cells are more predisposed to differentiate (Chambers et al., 2007). Fluctuating levels of Stella expression have also been proposed to represent a dynamic equilibrium within mouse ES cultures, representing cells of either the inner cell mass or epiblast (Hayashi et al., 2008). Differential Stella expression marks

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functionally distinct cells: Stella negative cells were found to be permissive for differentiation to the trophectoderm lineage whereas Stella positive cells were not.

It has been reported that during early stages of human ES cell differentiation genes associated with the pluripotent state are co-expressed with lineage specific transcription factors (Laslett et al., 2007). Such an observation may represent the existence of functionally distinct subsets of undifferentiated human ES cells but the relationship of these cells to differentiation potential has not yet been established.

Subsets of undifferentiated human ES cells have been proposed based upon studies of surface antigen markers, such as SSEA3 (Enver et al., 2005) or CD9 and GCTM2 (Laslett et al., 2007). SSEA3 is a cell surface antigen that is rapidly down-regulated as human ES cells differentiate (Draper et al., 2002a; Shevinsky et al., 1982), but when artificially removed from the cell surface, human ES cells still retain a pluripotent stem cell phenotype (Brimble et al., 2007; Fenderson et al., 1993). The carbohydrate based surface antigens SSEA3 and SSEA4 are expressed by red blood cells, but the red cells of a small percentage of the human population (pp and p^k individuals) lack the capacity to synthesise these globoseries antigens, suggesting that SSEA3 is not necessary for human development (Tippett et al., 1986). Nevertheless, SSEA3 expression is closely associated with the undifferentiated phenotype of human ES cells, and in a comparative analysis of early passage diploid human ES cells, and later passage, culture adapted variants, Enver et al.(2005) suggested that undifferentiated human ES cells can exist in two substates, $SSEA3^{Positive}$ and $SSEA3^{Negative}$. In the early passage diploid cells, the $SSEA3^{Positive}$ stem cell state was posited to be particularly unstable, so that very few undifferentiated $SSEA3^{Negative}$ stem cells could be detected by clonogenic assays. However, culture adaptation appeared to 'trap' the stem cells in the stem cell compartment so that clonogenic $SSEA3^{Negative}$ undifferentiated stem cells could be readily detected in the variant later passage lines. It was suggested that, like the $Nanog^{-/-}$ mouse ES cells, the $SSEA3^{Negative}$ substate of human ES cells is less stable and closer to commitment to differentiation than cells in the $SSEA3^{Positive}$ substate (Enver et al., 2005).

On the basis of findings described above we hypothesised that the pluripotent human ES cell compartment consists of cells that possess different characteristics with regard to differentiation potential. Differentiation of human ES cells is commonly performed on a population of cells, which does not enable investigation of behavioural disparities between individual ES cells. We have examined the functional heterogeneity of ES cells by testing the capacity of single cells to choose between a neural or non-neural fate. Human ES cells require intercellular cues for survival and retention of the undifferentiated phenotype (Fox et al., 2008) and consequently many human ES cell lines are not amenable to functional analysis at the single cell level. H14-BJ1 is a subline of culture adapted H14 human ES cells which permits functional analysis at the single cell level. We have used both culture adapted human ES cells and their malignant counterparts from teratocarcinomas, embryonal carcinoma cells, to investigate whether the SSEA3 substates are functionally different with regard to differentiation potential.

Results

Functional analysis of single human ES cells

H14 human ES cells were plated at clonal densities in a number of different media and substrates to identify conditions that were permissive for neural differentiation. We tested media formulations that included the addition of various proportions of FCS (1–10%); addition of different concentrations of Retinoic acid (0.1–10 μM) and supplementation of B27 or ITS as additives. Furthermore, different flask coatings were assayed (gelatin/Matrigel/feeders). The majority of media caused single hES cells to rapidly senesce and take on a flat fibroblast like morphology. However, we found that the highest number of colonies possessing identifiable neurons were generated when single cells were plated on feeders in serum free media (DMEM:F12 supplemented with ITS), which is herein referred to as neuralising media. Upon plating in neuralising media, morphologically distinct colonies became evident within 5 days. These colonies were initially classified as "non-neural", "bipolar" or "compact" colonies (Fig. 1A). After 10 days, colonies were stained and could be classified into four distinct categories on the basis of marker expression and morphology (Fig. 1B), "undifferentiated" ($OCT4^+$, $SOX2^+$, $TUJ1^-$), "non-neural" ($OCT4^-$, $SOX2^-$, $TUJ1^-$), "neural progenitor" ($OCT4^-$, $SOX2^+$, $TUJ1^+$), and "neural rosettes" ($OCT4^-$, $SOX2^+$, $TUJ1^+$). The chief distinction between the neural progenitor and neural rosette colonies was a distinct difference in cell morphology, with the colonies containing bi-polar cells or compact rosette structures, respectively. Neural rosettes were obscured by the very high cell density within colonies and could only be visualised upon nuclear staining. Neural progenitor colonies composed of bipolar cells proliferated for over 4 weeks before any obvious loss of proliferation was observed. Cells within these colonies expressed SOX2 in the absence of OCT4 and yielded ever increasing numbers of TUJ1 positive neurons over time (>90% of the colony by 21 days).

Cell phenotypes within each ten day colony appeared to be relatively homogeneous with respect to cell morphology and gene expression (SOX2 and OCT4). Thus, based on the premise that every initial ES cell was exposed to the same differentiation inducing environment (ITS media on feeders), then the phenotype of colonies may be attributed to the phenotype of the initial colony-forming stem cell.

SSEA3 expression levels reflect differentiation characteristics of individual cells

The expression of many stem cell markers (OCT4 and TRA-1-60) appeared relatively uniform within colonies of human ES cells. However, SSEA3 was expressed at variable levels in a mosaic fashion (Supplementary Fig. 1). Analysis of SSEA3 expression by flow cytometry revealed that within the SSEA3 positive fraction of cells there was a hundred fold variation in the expression level of SSEA3 between individual cells (Fig. 2A). To determine if the expression level of SSEA3 could be used to predict the type of colony formed upon plating in neuralising media, cells were separated by fluorescence activated cell sorting (FACS) into $SSEA3^{High}$, $SSEA3^{Low}$ and $SSEA3^{Negative}$ subpopulations (Fig. 2A). When plated on feeders in standard human ES growth media all

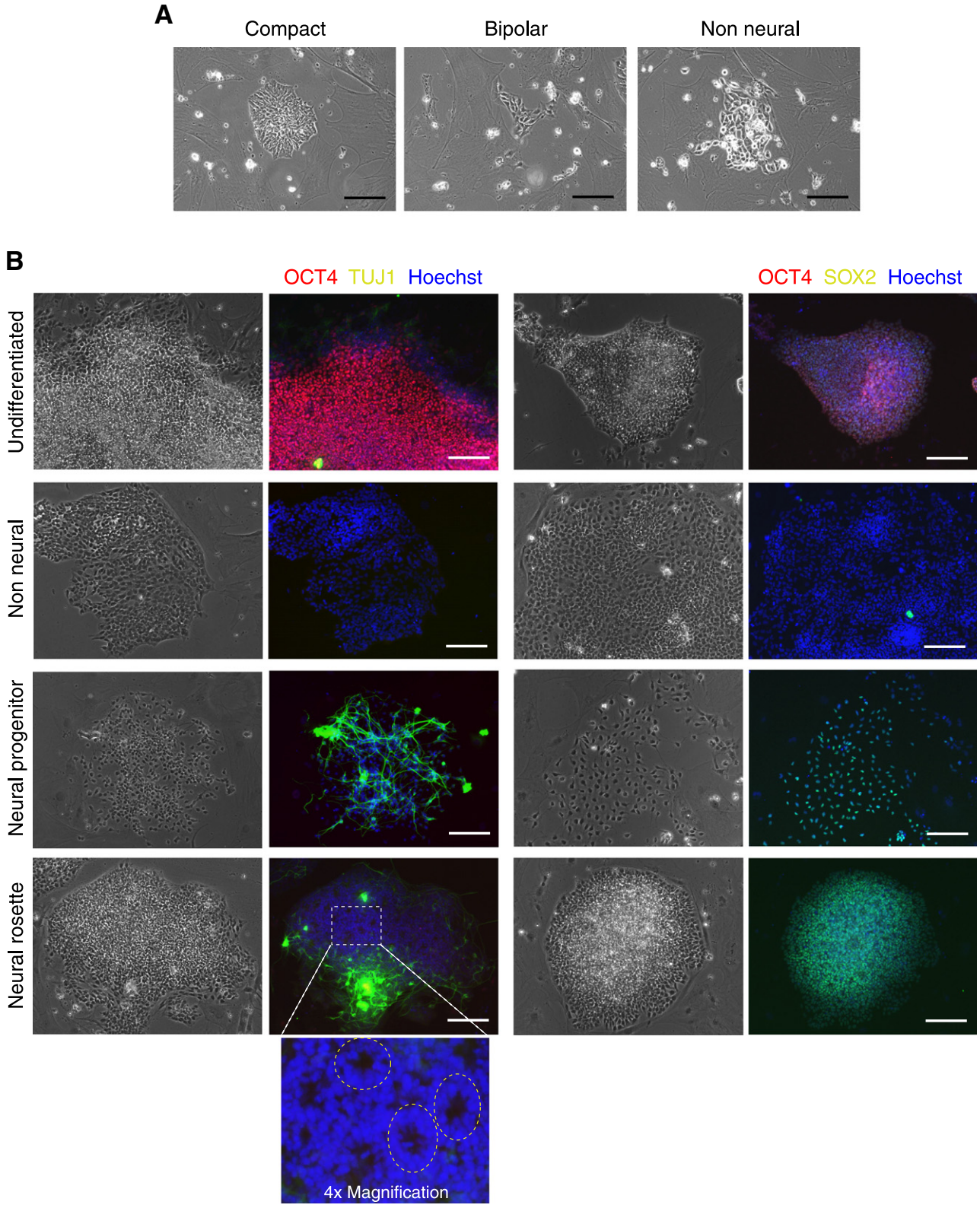
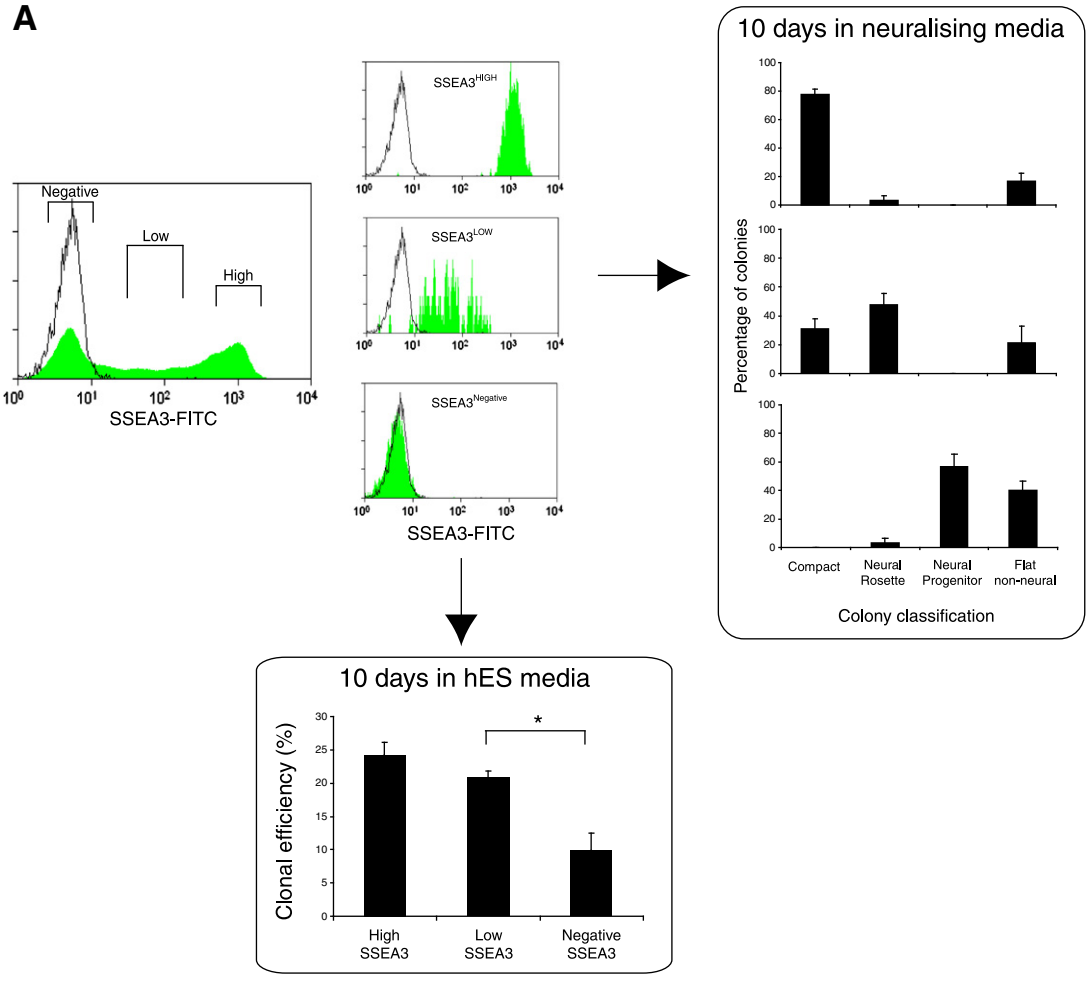
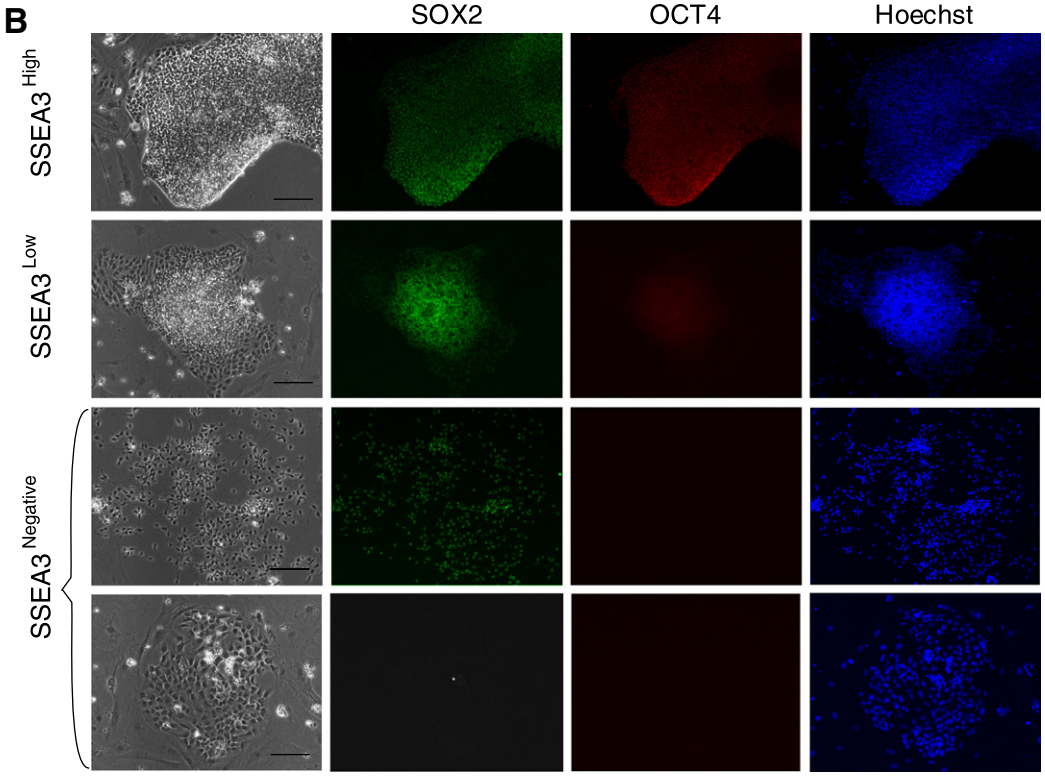


Figure 1 Distinct colonies generated by single human ES cells. (A) Single cell derived colonies after 5 days plated on feeders in neuralising media. (B) Immunofluorescent images displaying dual stains for TUJ1/OCT4 and SOX2/OCT4 in colonies after 10 days. Hoechst (blue) was used as a nuclear stain. Scale bars, 250 μM. A 4× magnification window highlights the presence of neural rosette features.


A
Negative
Low
High
SSEA3-FITC
SSEA3HIGH
SSEA3LOW
SSEA3Negative
SSEA3-FITC
10 days in neuralising media
Percentage of colonies
Compact
Neural Rosette
Neural Progenitor
Flat non-neural
Colony classification
10 days in hES media
Clonal efficiency (%)
*
High SSEA3
Low SSEA3
Negative SSEA3


B
SOX2
OCT4
Hoechst
SSEA3 High
SSEA3 Low
SSEA3 Negative

three sorted populations generated SSEA3 positive undifferentiated colonies with the SSEA3^{High} population exhibiting the highest cloning efficiency. Notably, 9% of SSEA3 ^{Negative} cells generated SSEA3 positive colonies (Fig. 2A). When the sorted cells were plated in neuralising media the phenotypes of the resultant colonies after 5 days differed accordingly to the sorted population: compact colonies were generated only by SSEA3^{High} and SSEA3^{Low} cells and neural progenitor colonies were generated only by SSEA3^{Negative} cells. After 10 days, the colonies were classified by morphology and marker expression into four categories (Figs. 2A and B) as previously described in Fig. 1B. The SSEA3^{Negative} human ES cells generated flat non-neural and neural progenitor colonies, but no compact colonies expressing OCT4 or SOX2 (Fig. 2B). SSEA3^{Low} derived compact colonies retained high levels of SOX2 expression with only 17% of these colonies expressing detectable OCT4. Furthermore, every SSEA3^{Low} derived neural rosette colony possessed a large number of TUJ1 positive neurons (Supplementary Fig. 2). SSEA3^{High} cells predominantly generated undifferentiated compact OCT4 positive colonies (77% of colonies) initially without TUJ1 positive cells. These OCT4 expressing colonies exhibited no SSEA3 or TRA-1-60 expression and failed to persist as undifferentiated when re-plated in hES media (data not shown). When the OCT4 colonies were maintained for a further 5 days 25% of the colonies contained TUJ1 positive neurons. The eventual acquisition of a neural phenotype in SSEA3^{High} derived colonies demonstrates that the differentiating conditions are sufficient to trigger neural differentiation of SSEA3^{High} cells, but the timing of this event is delayed in comparison to that of SSEA3^{Low} cells. These observations indicate that although ES cells expressing diverse SSEA3 levels can generate undifferentiated colonies they exhibit distinct responses when exposed to differentiation inducing conditions.

Modelling real-time SSEA3 expression

The differentiation potential of human ES cells with respect to the neural lineage differed according to the level of SSEA3 expression, suggesting that SSEA3 expression can be used to segment the undifferentiated OCT4^{Positive} stem cell compartment into substates that are functionally distinct with respect to differentiation potential. The observation that SSEA3^{Positive} colonies can be derived from SSEA3^{Negative} human ES cells demonstrates that cells can freely move between differentially expressing SSEA3 substates. High purity of sorted SSEA3^{Negative} cells (Fig. 2A) suggests that SSEA3^{Positive} colonies are not derived from a contamination of SSEA3^{Positive} cells. To observe directly the rate of gain and loss of SSEA3 expression, time-lapse microscopy was employed to track FACS sorted human ES cells. However, human ES cells are difficult to track due to their strong intercellular adhesion which caused small colonies of cells to clump: we regularly found that once a small colony formed (6–20 cells in size) it would compact for a 24–48 h period before spreading again as a flat morphologically healthy colony (Supplementary Fig. 3), independent of the attachment substrate (feeders, Matrigel or polyD-lysine). During this clumping phase the relationships between the cells became obscured and it was not possible to track the lineage relationships of individual cells during this crucial period.

Therefore we turned to the pluripotent embryonal carcinoma cell line (NTERA2) as a human ES substitute (Andrews, 1984; Tonge and Andrews, 2010). NTERA2 cultures have been observed to consist of over 99% of cells being NANOG and OCT4 positive (Tonge et al., 2010), but SSEA3 expression often remains variegated. Similar to human ES cells, FACS isolated SSEA3^{Positive} and SSEA3^{Negative} NTERA2 cells (Fig. 3A) possessed a significant difference in cloning efficiency (Fig. 3B). When analysed on a population basis by flow cytometry, SSEA3 positive cells were prevalent in SSEA3^{Negative} sorted cultures 72 h after FACS isolation (Fig. 3C) and an expression profile analogous to the original parental cell line was re-establishing within 6 days (data not shown).

To determine the kinetics of SSEA3 acquisition in SSEA3^{Negative} cells, FACS isolated SSEA3^{Negative} cells were plated and monitored by time-lapse microscopy for 72 h. Cells were subsequently stained for SSEA3 expression and fluorescence images acquired. Time-lapse acquired images were analysed with the use of automated software and cell division annotated in the form of lineage trees, relative to time (Fig. 3E). Overall, 68% of SSEA3^{Negative} cells produced at least one SSEA3^{Positive} daughter cell. Twenty two percent of SSEA3^{Negative} cells were found to yield only SSEA3^{Positive} progeny, whereas 46% produced only SSEA3^{Negative} progeny and the remaining 32% produced both types. Examples of SSEA3 stained progeny after 72 hour time-lapse microscopy are illustrated in Supplementary Fig. 4. The high number of colonies consisting of only SSEA3^{Positive} progeny suggests that the regain of SSEA3 expression occurs before the first division of the original SSEA3^{Negative} cell, a period of less than 24 h. Notably there were on average a higher number of progeny observed in lineage trees that contained SSEA3^{Positive} cells (7 versus 5 cells). The disparity of cell progeny numbers was the combined consequence of differential cell death and cell cycle times.

Twelve days after FACs isolation every SSEA3^{Negative} derived colony over 50 cells in size possessed SSEA3 positive cells, with the percentage of SSEA3 positive cells ranging between 5 and 100%. Not a single SSEA3 negative cell line could be established from SSEA3^{Negative} cells. These observations suggest that all SSEA3^{Negative} cells up-regulate SSEA3 expression if they are to retain a long-term undifferentiated phenotype.

Figure 2 Analysis of human ES cells that express different levels of SSEA3 reveal different patterns of differentiation. (A) Human ES SSEA3^{High}, SSEA3^{Low} and SSEA3^{Negative} cell populations were separated by fluorescence activated cell sorting (green: SSEA3-FITC stained cells, black: P3X-FITC negative control), and plated in either standard ES media or neuralising media for 10 days. Undifferentiated colonies in ES media were identified on the basis of SSEA3 expression and colonies in neuralising media were classified by morphology and gene expression (SOX2 and OCT4). Data represents mean ± S.D. performed in triplicate. The asterisk (*) depicts values that are significantly different $p<0.05$. (B) Images of most frequently identified colonies generated by SSEA3^{High}, SSEA3^{Low} and SSEA3^{Negative} cell plated in neuralising media. Scale bars, 250 μM.

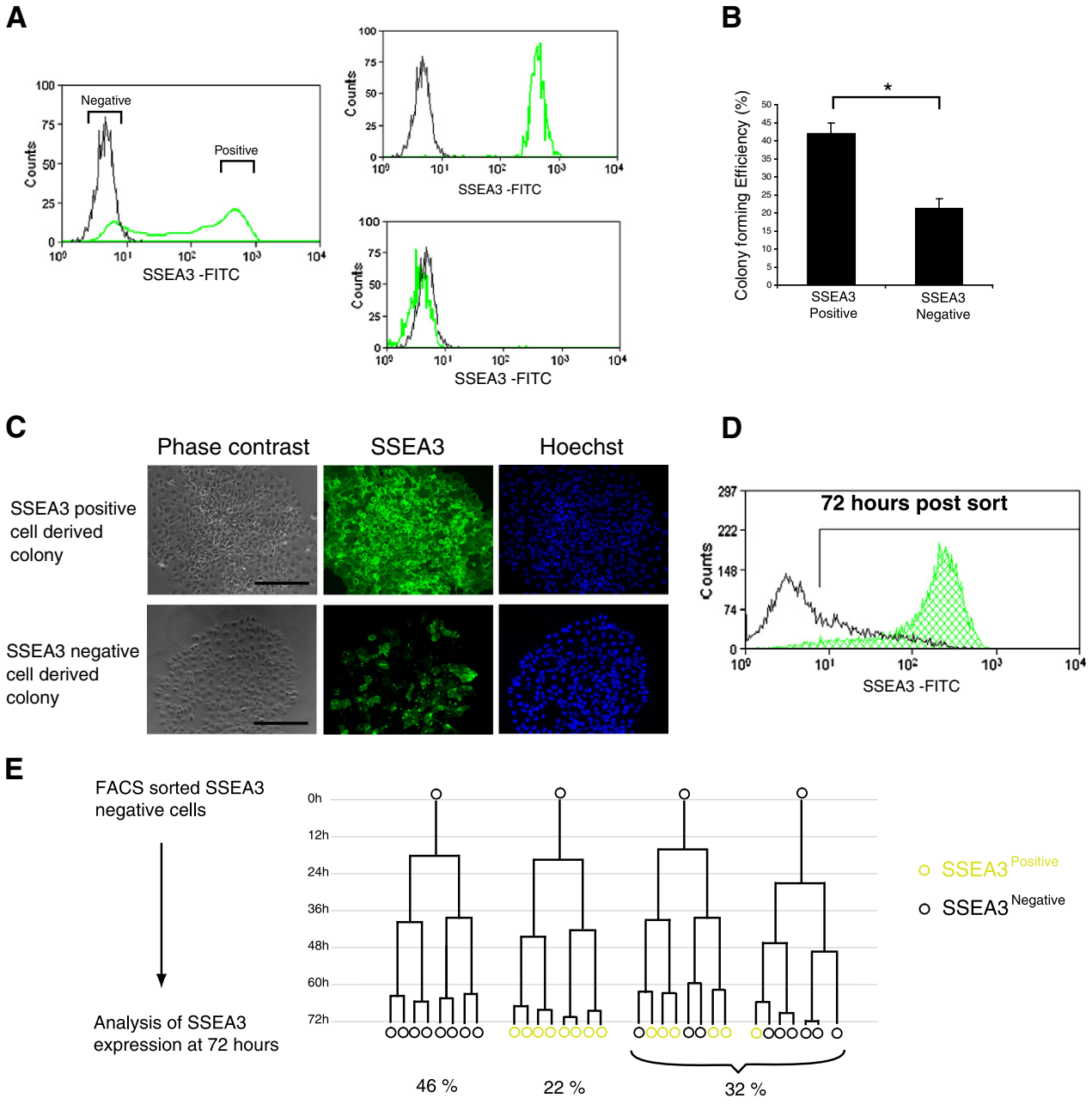


Figure 3 Analysis of NTERA2 stem cells that express different levels of SSEA3 to reveal dynamics of SSEA3 expression. (A) Fluorescence activated cell sorting of cells on the basis of SSEA3 expression levels. Green: SSEA3 stained cells, black: P3X negative control. Post-sort assessment of the isolated SSEA3^{Positive} and SSEA3^{Negative} cell populations. (B) Clonal efficiency of SSEA3 sorted cells in standard growth media. (C) Day 10 colonies derived from sorted cells stained for SSEA3 (green), scale bars 100 μM, The asterisk (*) depicts values significantly different $p<0.05$. (D) Flow cytometry of SSEA3^{Positive} (green) and SSEA3^{Negative} (black) cell populations 3 days post sort. Bar depicts SSEA3 positive threshold on the basis of P3X negative control. (E) FACS isolated SSEA3^{Negative} cells were plated and followed by time-lapse microscopy for 72 h. Lineage trees represent cell division relative to time. White circles represent SSEA3^{Negative} cells and green circles represent SSEA3^{Positive} cells.

Conclusions

Our results demonstrate that the distinction between undifferentiated and differentiating cells is difficult to define. Human ES cells that possess cell-to-cell variability of SSEA3 expression are capable of generating undifferentiated colonies, but when subjected to differentiation inducing conditions they exhibit different behaviours.

Our observations are consistent with a model in which the undifferentiated stem cell compartment consists of a range of functionally distinct substates, which are characterised by graduated loss of SSEA3 expression. The observed

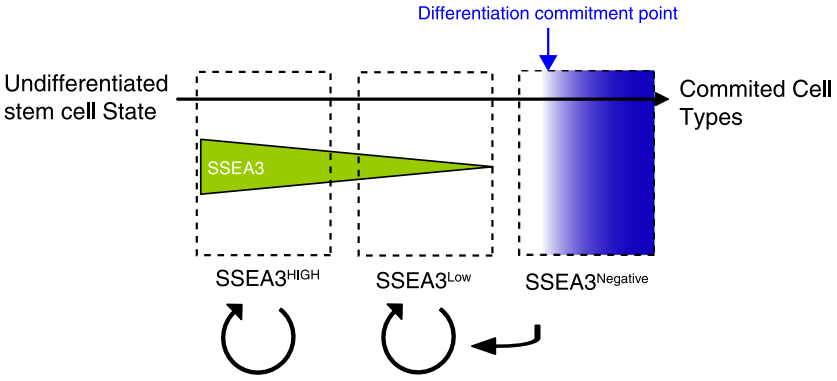


Figure 4 Schematic representation of SSEA3 expression in relation to human ES differentiation. The point at which cells commit to differentiate is represented by the 'differentiation threshold' (blue). SSEA3 expression diminishes in human ES cells as cells near the differentiation threshold.

accelerated differentiation of SSEA3^{Low} cells to a neural phenotype is possibly due to SSEA3^{High} and SSEA3^{Low} stem cells representing distinct substates in the stem cell compartment, whereby SSEA3 expression level is a function of a cell's proximity to the 'differentiation threshold' (Fig. 4). The 'differentiation threshold' signifies the point at which an ES cell irreversibly crosses to the differentiated state. Our results indicate that the differentiation threshold resides within the SSEA3^{Negative} zone, since a fraction of SSEA3^{Negative} cells are capable of generating undifferentiated colonies in standard growth conditions, but unlike the SSEA3^{High} or SSEA3^{Low} cells, they do not generate OCT4 positive or neural rosette containing colonies in neural permissive conditions. Thus, human ES cells in close proximity to the 'differentiation threshold' respond differently to differentiation stimuli.

In relation to SSEA3 expression, the 'differentiation threshold' may lie at different points along the linear path of differentiation (Fig. 4) depending on the cell line and the culture conditions used to propagate undifferentiated ES cells. In comparison to normal human ES cells, the adapted human ES cells exhibit increased proliferation rates, high clonal efficiency and reduced spontaneous differentiation (Enver et al., 2005). Within normal ES cell cultures the 'differentiation threshold' may be positioned within the SSEA3 expressing zone, effectively condensing the size of the stem cell compartment in relation to SSEA3 expression. The adapted ES subline (H14 BJ1) used in this study possesses a stable pluripotent state that spontaneously differentiates at a reduced frequency in comparison to non-adapted cell lines yet generates differentiated teratocarcinomas when grown as xenograft tumours. Use of the culture adapted cell line allows for the 'trapping' of transient (but nevertheless important) states that would normally be inaccessible in 'normal' euploid cell lines. In this view, the adapted cell lines provide a means with which to analyse cell states that exist transitorily in the normal cells and are consequently difficult to observe. Thus, the use of an adapted cell line facilitated the analysis of single cell behaviour, however, further improvements in cell culture conditions will enable the analysis of single cell dissociated non-adapted hES cells.

The observation that SSEA3^{Negative} ES cells were capable of generating undifferentiated colonies in standard ES conditions yet displayed atypical differentiation in comparison to SSEA3^{Low} and SSEA3^{High} cells is possibly due to lineage priming. It has been reported that in routine ES culture the colony size of undifferentiated (OCT4 positive) human ES cells influences the expression levels of early differentiation markers (PAX6 and GATA6) which consequently bias differentiation upon embryoid body formation (Bauwens et al., 2008). The expression of early lineage markers in human ES colonies is reminiscent of the lineage priming which has been reported in mouse haematopoietic progenitor cells (Delassus et al., 1999). Haematopoietic progenitor subsets, isolated on the basis of Sca-1 expression, possess discrete gene expression profiles, which manifest in biassed erythroid or the myeloid differentiation capabilities (Chang et al., 2008). Notably the haematopoietic Sca-1 progenitor subsets are reported to be interconvertible. Likewise, SSEA3^{High} and SSEA3^{Negative} human ES cells are interconvertible. SSEA3 expression levels are informative of an ES cell's position within the stem cell compartment and its diminished expression may indicate the expression of early differentiation markers in cells. Isolation of lineage primed hES cells could help define the early stages of cell differentiation with a deeper knowledge of such lineage primed cells facilitating the directed differentiation of human ES cells.

The pluripotent NTERA2 cell line has enabled us to track and model the expression of SSEA3 in human pluripotent stem cells. Undifferentiated cells were found to fluctuate between SSEA3^{High} SSEA3^{Low} SSEA3^{Negative} states, whereby SSEA3^{Negative} cells regained SSEA3 expression at high frequency. Although pluripotent cells often lost SSEA3 expression, they remained undifferentiated and the expression of SSEA3 was reacquired within days. Notably, the expression of SSEA3 is closely associated with the cloning efficiency of human ES and EC cells, suggesting that it remains a sensitive marker for assessing the health of a pluripotent stem cell population.

We propose that the stem cell compartment is not a single entity but a continuum of substates in which cells fluctuate towards and away from a 'differentiation threshold'. A clear understanding of cell dynamics within the stem cell state will

help develop efficient systems for maintaining and directing human ES cell differentiation.

Materials and methods

Cell culture

The H14BJ1 cell line used in this study is a sub-line of WiCell's H14 (WA14) and has been previously described (Baker et al., 2007). The karyotype of H14BJ1 includes an extra copy of chromosome 17 that incorporates an hsr comprising amplification of chromosomal region 17p11.2. Human ES cells (H14, BJ1 subline) were grown in standard human ES media (knockout DMEM supplemented with 20% serum replacement, 1% nonessential amino acids, 1 mM L-glutamine, and 0.1 mM β-mercaptoethanol and 4 ng/ml basic fibroblast growth factor) on mitomycin-C treated MEFs (Amit et al., 2000). Cells were passaged every three or 4 days with the use of collagenase IV treatment for cell detachment. NTERA2 embryonal carcinoma cells were grown in were grown in Dulbecco's Modified Eagle's Medium, GIBCO, supplemented with 10% foetal bovine serum. Neural specific single cell differentiation was performed by plating FACS-sorted cells at 50 cells/cm^2 on mitomycin-C treated MEFs in neuralising media; DMEM/F12 (Invitrogen) supplemented by ITS (Invitrogen). Media was changed every 3 days.

For clonal efficiency assays human ES and NTERA2 cells were harvested with trypsin–EDTA (0.25% trypsin, 2 mM EDTA, in calcium and magnesium free Dulbecco's phosphate buffered saline) to yield a single cell suspension and plated in their relevant growth media, with and without feeders, respectively. Human ES cells were plated at 20 cells/cm^2 and NTERA2 cells at 10 cells/cm^2. Subsequent colonies were stained for SSEA3 expression and counted after 12 days of incubation.

Time-lapse microscopy

FACS sorted cells were plated in a six well plate at a density of 10,000 cells/cm^2 and maintained inside a preheated (37 °C) humidified chamber containing 5% CO_2. Images were captured every 10 min using an inverted Olympus microscope IX70 connected to a Hamamatsu camera and automated motorised stage (Prior). Upon completion of time-lapse acquisition cells were fixed with paraformaldehyde (4%) and stained by indirect immunofluorescence for SSEA3. Antibody stained cells were then returned to the Olympus IX70 and immunofluorescent images taken at the identical fields of view used for obtaining phase contrast time-lapse images. Phase contrast image stacks representing each field of interest were analysed by the cell tracking software TTT as described earlier (Eilken et al., 2009; Rieger et al., 2009). Lineage trees representative of each cell clone were generated with each cell division and cell death event noted relative to time. Clones which failed to survive beyond the initial 24 h were excluded from analysis. After referring to the relevant immunofluorescent image, a phenotype (SSEA3^{Negative}/SSEA3^{Positive}) could be assigned to each cell of the lineage trees.

Immunofluorescence and fluorescence activate cell sorting (FACS)

Single cells were stained with anti-SSEA3 antibody (produced in-house from the MC631 hybridoma (Shevinsky et al., 1982)), pre-titred and diluted in standard growth media, as previously described (Draper et al., 2002b). FITC-tagged secondary antibody to mouse IgM and IgG (H+L) (Caltag) was used. Cell suspensions were analysed and sorted by flow cytofluorimetry using a Moflo (DakoCytomation) with Summit software. Cell sorting was performed using the 480 nm excitation line with cell droplet formation and separation achieved using a 70 micron nozzle and 2500 volt charged plates. Non-specific fluorescence staining and autofluorescence were determined by staining with P3X, an antibody obtained from the parent myeloma cell line P3X63Ag8, which does not recognise any known epitope in the cells (Draper et al., 2002b). Sorted cells were collected in relevant growth media and subsequently plated in growth media containing 50 ng/ml gentamycin (Invitrogen).

In situ immunofluorescence was performed after PFA (4%) fixation of cells with the use of monoclonal antibodies; SOX2 (R&D Systems), OCT4 (Santa Cruz Sc-9081), TUJ1 (Covance) and TRA-1-60 (Andrews et al., 1984) primary antibody. Alexa Fluor 546 and 594 secondary antibodies (Molecular Probes) were used in all instances.

Statistical analysis

Results are expressed as the mean ± SD and statistical analysis was performed using GraphPad Prism software. Student *t*-test was utilised for the statistical analysis of data sets. In all cases the minimal level of significance was taken as $p<0.05$

Supplementary materials related to this article can be found online at doi:10.1016/j.scr.2011.04.006.

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