The 5T4 oncofoetal antigen is an early differentiation marker of mouse ES cells and its absence is a useful means to assess pluripotency

Christopher M. Ward*, Katie Barrow, Andrew M. Woods and Peter L. Stern

Introduction

The human 5T4 oncofoetal antigen was discovered by looking for shared surface molecules that would reflect the functional similarities between the growth and invasive properties of trophoblast, the major interfacing cell type between mother and foetus in the placenta, and tumour cells. 5T4 is a 72 kDa transmembrane glycoprotein with restricted expression patterns in human adult tissues but it is upregulated on many carcinomas and correlates with poorer clinical outcome in ovarian, gastric and colorectal cancers (Mulder et al., 1997; Southall et al., 1990; Starzynska et al., 1994; Starzynska et al., 1992; Starzynska et al., 1998; Wrigley et al., 1995). Overexpression of human and mouse 5T4 cDNA in cell lines is consistent with it having an influence on adhesion, shape and motility. We show that murine embryonic stem cell lines are 5T4 negative but that there is rapid up regulation of protein and transcripts upon differentiation, including derivatives of each primary germ layer, as evidenced by cell surface FACS, western and RT-PCR analyses. The kinetics of differentiation and 5T4 expression are closely correlated, with early events linking 5T4 expression to changes in motility and morphology. Comparison of 5T4 expression with other ES cell transcript (Oct 3/4; Rex-1) and antigen markers (Forsmann, SSEA-1) establishes 5T4 as a useful marker for the non-destructive detection of early differentiation of ES cells. For example, ‘undifferentiated’ ES phenotype defined as SSEA-1 positive and 5T4 negative is seven times more efficient at chimera formation than SSEA-1-positive/5T4-positive cells. Thus, 5T4 glycoprotein expression is associated with early differentiative events of ES cells involving altered motility, and it has useful practical consequences for assessing ES potency and studying similar processes in development and metastasis.

Key words: Embryonic stem cells, Cell surface marker, Motility factor, Glycoprotein, Metastasis

Summary

5T4 oncotrophoblast antigen is a transmembrane glycoprotein expressed by trophoblast and many carcinomas but not most normal adult tissues. Results from overexpression of human and mouse 5T4 cDNA in cell lines suggest it has an influence on adhesion, shape and motility. We show that murine embryonic stem cell lines are 5T4 negative but that there is rapid up regulation of protein and transcripts upon differentiation, including derivatives of each primary germ layer, as evidenced by cell surface FACS, western and RT-PCR analyses. The kinetics of differentiation and 5T4 expression are closely correlated, with early events linking 5T4 expression to changes in motility and morphology. Comparison of 5T4 expression with other ES cell transcript (Oct 3/4; Rex-1) and antigen markers (Forsmann, SSEA-1) establishes 5T4 as a useful marker for the non-destructive detection of early differentiation of ES cells. For example, ‘undifferentiated’ ES phenotype defined as SSEA-1 positive and 5T4 negative is seven times more efficient at chimera formation than SSEA-1-positive/5T4-positive cells. Thus, 5T4 glycoprotein expression is associated with early differentiative events of ES cells involving altered motility, and it has useful practical consequences for assessing ES potency and studying similar processes in development and metastasis.

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We hypothesised that the 5T4 oncofoetal antigen might be regulated during mouse embryonic stem (ES) cell differentiation since it is strongly expressed in some postimplanation embryonic tissues as well as in placental trophoblast (King et al., 1999; Woods et al., 2002). Mouse ES cells are isolated from the inner cell mass/epiblast of preimplantation blastocysts (Brook and Gardner, 1997) and can be maintained in a pluripotent state by culture in leukaemia inhibitory factor (LIF) (Smith, 1992). Mouse ES cells are used in techniques ranging from gene knockout studies to transplantation therapies (Sato and Nakano, 2001) and confirmation of the pluripotency of the ES cell population in such studies is important. Markers currently used for analysis of ES cell pluripotency include Oct-4 (Rathjen et al., 1999), Rex-1 (Ben-Shushan et al., 1998), Forsmann antigen (Ling and Neben, 1997; Willison and Stern, 1978), SSEA-1 (Ling and Neben, 1997) and alkaline phosphatase (Rathjen et al., 1999) (Table 1). Undifferentiated ES cells express all these markers and their levels decrease upon differentiation. However, they are not optimal for predicting ES cell pluripotency since they decrease relatively slowly following the onset of differentiation (Lake et al., 2000; Rathjen et al., 1999) or the analyses are destructive and require relatively large numbers of cells for RNA extraction.

Removal of LIF from the growth medium results in ES cell differentiation (Smith, 1992), characterised by the upregulation of transcript markers such as fibroblast growth factor-5 (Fgf-5), ζ-globin (ZG) and Flk-1 (Table 1). However, many differentiation markers are transiently expressed and present only in a sub-population of cells thereby limiting their use as

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Table 1. Common markers of ES cell differentiation

| Marker       | Method of detection | Specificity       | Expression pattern following differentiation | Analysis destructive? |
|--------------|---------------------|-------------------|-----------------------------------------------|-----------------------|
| Alkaline phosphatase | In situ staining     | ES                | Negative                                      | Y                     |
| Oct 3/4      | RT-PCR              | ES                | Negative                                      | Y                     |
| Rex-1        | RT-PCR              | ES                | Negative                                      | Y                     |
| SSEA-1       | Cell-surface staining | ES           | Negative                                      | N                     |
| Fossman      | Cell-surface staining | ES         | Negative                                      | N                     |
| Fgf-5        | RT-PCR              | Primitive ecto    | Positive                                      | Y                     |
| ZG           | RT-PCR              | Meso              | Positive                                      | Y                     |
| Bmp-2        | RT-PCR              | Endo/meso         | Positive                                      | Y                     |
| T-Bra        | RT-PCR              | Meso              | Positive                                      | Y                     |
| Fkl-1        | Cell-surface staining | Meso         | Positive                                      | N                     |
| K-18         | RT-PCR              | Endo/ecto         | Positive                                      | Y                     |
| Bmp-4        | RT-PCR              | Ecto/meso         | Positive                                      | Y                     |
| NF-68        | RT-PCR              | Ecto              | Positive                                      | Y                     |
| Vim          | RT-PCR              | Meso/endo         | Positive                                      | Y                     |
| AFP          | RT-PCR              | Visceral/endo     | Positive                                      | Y                     |
| TTR          | RT-PCR              | Endo              | Positive                                      | Y                     |

Alkaline phosphatase (Rathjen et al., 1999); Fossman antigen (Ling and Neben, 1997); Oct 3/4, octamer binding protein 3/4 (Lake et al., 2000; Rathjen et al., 1999); Rex-1, reduced expression-1 (Ben-Shushan et al., 1998; Lake et al., 2000; Rathjen et al., 1999); SSEA-1, stage-specific embryonic antigen-1 (Ling and Neben, 1997); Fgf-5, fibroblast growth factor-5 (Lake et al., 2000; Rathjen et al., 1999); ZG-ζ-globin (Bielinska et al., 1996); Bmp-2, bone morphogenetic protein-2 (Weinhold et al., 2000); T-Bra, brachyury (Weinhold et al., 2000); Fkl-1, vascular endothelial growth factor receptor-2 (VEGFR-2) (Hirashima et al., 1999); K-18, keratin-18 (Weinhold et al., 2000); Bmp-4, bone morphogenetic protein-4 (Weinhold et al., 2000); NF-68, neurofilament-68k (Itskovitz-Eldor et al., 2000); Vim, vimentin (Weinhold et al., 2000); AFP, α-foetoprotein (Weinhold et al., 2000); TTR, transthyretin; meso-mesoderm (Abe, 1996). ES, embryonic stem cell; Ecto-ectoderm; Endo-endoderm; Meso-mesoderm.

single assay markers for quantifying loss of pluripotency. As such, a cell surface antigen that is absent on undifferentiated ES cells and rapidly upregulated following removal of LIF would be a valuable tool for the non-destructive determination of ES cell pluripotency and differentiation. We demonstrate that m5T4 is positively regulated during mouse ES cell differentiation and is likely to play a role in cell spread and motility during this process.

Materials and Methods

Cell culture

ES cells were grown in Knockout DMEM (Invitrogen Corporation, Paisley, UK) supplemented with 15% serum replacement (DMEMSR) (D3, MESC and OKO160; Knockout SR™; Invitrogen Corporation, Paisley, UK) or 10% foetal calf serum (DMEMFCS) (129; Invitrogen), unless otherwise stated (Ward et al., 2002; Ward and Stern, 2002). 129 ES cells were plated at 10^6 cells per 9 cm dish and split 1:6 every 2 days.

Expression of EGFP-h5T4 in 129 ES cells

Cells were grown in DMEMSR + LIF in the absence of a feeder layer. They were transfected using Gene Pulser II (BioRad) with plasmid DNA and electroporated at 0.5 ml of cell suspension and electroporated at 250 V, 475 μF in a BioRad Gene Pulser II. After 24 hours one third of the cells were harvested and resuspended at 1x10^7 cells/ml in PBS. 20 μg plasmid DNA was added to 0.5 ml of cell suspension and electroporated at 250 V, 475 μF in a BioRad Gene Pulser II. After 24 hours one third of the cells were assayed for EGFP expression in a Becton Dickinson FACScan (Becton Dickenson; Oxford, UK). EGFP-positive cells were isolated from the remainder of the sample by FACSVantage SE (Becton Dickenson) and plated out in fresh gelatin-treated 9 cm tissue culture dishes. Cellular localisation of EGFP proteins was determined after 48 hours using an Olympus BX-51 fluorescence microscope (Olympus, West Midlands, UK). Cell morphology was determined after 48 hours after transfection using inverted light microscopy.

Fluorescent staining of ES cells

ES cells (5x10^5 cells/well in a 96-well plate) were incubated with rat anti-mouse 5T4 monoclonal (IgG) antibody 9A7 (Woods et al., 2002), rat monoclonal (IgM) antibody MI/22.25 recognising Fossman antigen (Willison and Stern, 1978) or isotype control antibodies (10 μg/ml in 0.2% BSA/0.1% sodium azide in PBS) for 1 hour on ice. Cells were washed three times and resuspended in FITC-conjugated rabbit anti-rat Ig for 1 hour (1:30 dilution; DAKO, Cambs, UK). Cells were washed twice as described above, fixed in 1% formaldehyde/PBS solution and cell fluorescence measured in a Becton Dickinson FACScan.
RT-PCR analysis
RNA was extracted from cells using RNazol B according to the manufacturer’s instructions (Biogenesis, Dorset, UK), treated with DNase (Promega, WI, USA) and phenol/chloroform extracted. Synthesis of cDNA from mRNA transcripts was performed using the following method: RNA (10 μg), dNTP (250 μM), oligo(dT) (5.0 μg total; Promega, UK), AMV reverse transcriptase (40 units; Promega) in a total volume of 200 μl and incubated at 42°C for 1 hour. Semi-quantitative RT-PCR of 5T4 was performed using 1 μl of the cDNA solution and 25-30 cycles. RT-PCR was performed using 5 μl of the cDNA solution and 35 cycles. Samples were run on 2% agarose gels containing 400 ng/ml ethidium bromide and visualised on a UV transilluminator. Since the fibroblast feeder layer contains 5T4 transcripts, MESC ES cells were grown for several passages on gelatin-treated plates to remove the fibroblast feeder prior to the extraction of RNA (Fig. 3a). Primers used were as follows (read 5’ to 3’; forward-F, reverse-R): 5T4, F – aactgccgagtctcagatacc, R – atgatacccttccatgtgatcc, 55°C, 415 bp; Rex-1, F – tgaccctaaagcaagacg, R – gcagtaaaaggcatgatagc, 55°C, 606 bp; Oct 3/4, F – agaaggagctagaacagtttgc, R – cggttacagaaccatactcg, 55°C, 537/515 bp (Johansson and Wiles, 1995); Bmp-2, F – tubulin, F – tcactgtgcctgaacttacc, R – ggaacatagccgtaaactgc, 55°C, 506 bp; 50°C annealing temperature, 506 bp; Oct-4, F – aactgccgagtctcagatacc, R – atgatacccttccatgtgatcc, 55°C, 415 bp; 3T3, F – tgaagacaccacagtacac, R – ataagacaccacagtacaccc, 54°C, 414 bp.

Western blotting of 5T4
Cells were trypsinised and incubated in tissue culture plates for 30 minutes at 37°C and 30°C incubation in tissue culture plates for 1 hour. Semi-quantitative RT-PCR of 5T4 was performed using 1 μl of the cDNA solution and 25-30 cycles. RT-PCR was performed using 5 μl of the cDNA solution and 35 cycles. Samples were run on 2% agarose gels containing 400 ng/ml ethidium bromide and visualised on a UV transilluminator. Since the fibroblast feeder layer contains 5T4 transcripts, MESC ES cells were grown for several passages on gelatin-treated plates to remove the fibroblast feeder prior to the extraction of RNA (Fig. 3a). Primers used were as follows (read 5’ to 3’; forward-F, reverse-R): 5T4, F – aactgccgagtctcagatacc, R – atgatacccttccatgtgatcc, 55°C, 415 bp; Rex-1, F – tgaccctaaagcaagacg, R – gcagtaaaaggcatgatagc, 55°C, 606 bp; Oct 3/4, F – agaaggagctagaacagtttgc, R – cggttacagaaccatactcg, 55°C, 537/515 bp (Johansson and Wiles, 1995); Bmp-2, F – tubulin, F – tcactgtgcctgaacttacc, R – ggaacatagccgtaaactgc, 55°C, 506 bp; 50°C annealing temperature, 506 bp; Oct-4, F – aactgccgagtctcagatacc, R – atgatacccttccatgtgatcc, 55°C, 415 bp; 3T3, F – tgaagacaccacagtacac, R – ataagacaccacagtacaccc, 54°C, 414 bp.

MACS separation of 5T4-positive MESC ES cells
MESC ES cells were grown as described above, trypsinised and washed in PBS. 5T4-positive cells were isolated using mAb 9A7 (10 μg/ml), goat anti-rat Ig magnetic beads and MidiMACS LS columns according to the manufacturer’s instructions (Miltenyi Biotech, Surrey, UK).

Determining ES cell pluripotency by chimaeric mouse formation
129 ES cells were cultured in DMEMSR on gelatin-treated plates in the presence or absence of LIF for 6 days and then trypsinised, suspended in growth medium at 1×10⁹ cells/ml and incubated with rat anti-mouse SSEA-1 (IgM) antibody conjugated with phycoerythrin or an isotype control antibody (Santa Cruz, CA; 1:100 dilution in 0.2% BSA/0.1% sodium azide in PBS) for 15 minutes on ice. Cells were washed 3 times in culture medium and SSEA-1-positive cells isolated by FACS (FACSVantage SE, Becton Dickinson; Oxford, UK). 5T4 expression of the SSEA-1-positive population was determined as described above. Fifteen SSEA-1-positive cells were injected into each 3.5-day-old BL6 blastocyst and these were implanted into pseudo-pregnant BDF-1 female mice (Hogan, 1994) using glass capillaries (Clark Electromedical Instruments, Kent, UK), an Axiosvert 10 microscope (Carl Zeiss, Herts, UK), MMO-202ND injection manipulation arm (Narishige Int. Ltd., London, UK) and a Kopf 750 pipette puller (Tunjunga, CA). Pluripotency was determined by chimera formation using donor coat colour. Mice were housed according to Home Office guidelines (Home Office, 1986) and kept on a 12-hour light/dark cycle in which the dark period was from 7 pm to 7 am.

Results
The 5T4 oncofoetal antigen and mRNA is upregulated on ES cells following differentiation induced by removal of LIF
5T4 antigen is not detected on the surface of undifferentiated ES cells using mAb 9A7 (Fig. 1a). Following withdrawal of LIF for 3 days the 5T4 antigen was detected on all the ES cell lines, with the percentage of positive cells varying between 7.1% (OKO160) and 50.0% (MESC). Over the 12-day differentiation period there was considerable variation between the cell lines in both the timing of peak 5T4 antigen expression and the proportion of cells labelled positive. For example, the MESC ES cell line exhibited peak 5T4 expression around day 9, with 85.8% of the population positive (Fig. 1a i), whereas D3 ES cells exhibited a steady increase in positive cells which peaked at 43.4% on day 12 (Fig. 1a ii). OKO160 and 129 ES cell lines exhibited similar proportions of positive cells at day 3 (7.1 and 9.0% respectively) and day 6 (30.6 and 34.0% respectively) and both cell lines exhibited peak cell staining at day 9 (54.6 and 68.2% respectively). However, the proportion of OKO160 cells staining for 5T4 antigen decreased significantly by day 12 (from 54.6% to 17.0%) whereas 129 was only slightly reduced (from 68.2 to 67.3%). Increase in total 5T4 protein following removal of LIF was confirmed by western blot analysis of cell lysates using a rabbit anti-m5T4 polyclonal antibody (Woods et al., 2002) followed by HRP-conjugated sheep anti-rabbit immunoglobulins (DAKO, Cambs, UK) and developed by enhanced chemiluminescence (Amersham Pharmacia, UK). Western blot images were captured using an Epi Chemi II Darkroom and Sensicam imager with quantification determined by Labworks 4 (UVP, CA, USA).

To confirm that upregulation of 5T4 expression upon removal of LIF correlates with differentiation of the ES cell lines we assayed various ES cell-specific (Oct 3/4, Rex-1, Forssman antigen) and differentiation-specific (Fgf-5, ZG and Bmp-2) markers in differentiating ES cells (Fig. 2). These results show that upregulation of 5T4 correlates with the detection of transcript differentiation markers (Fig. 2a) and a decrease in the ES cell-specific Forssman antigen (Fig. 2b), confirming that 5T4 is upregulated during the differentiation of ES cells. Most strikingly, the ES cell-associated Oct 3/4 and Rex-1 transcripts did not decrease appreciably in MESC, D3 or 129 ES cells for at least 12 days following removal of LIF (Fig. 2a). These transcripts are commonly used to confirm the presence of undifferentiated ES cells in monolayer culture (Rathjen, 2002; Rathjen et al., 1999). OKO160 ES cells have a targeted insertion in a single Oct-4 allele, which is likely to account for the relative decrease in Oct-3/4 transcripts in this cell line, although Rex-1 transcripts were still evident 12 days
following removal of LIF. There was some disparity between the differentiation markers expressed by the ES cell lines (Fig. 2a). For example, Fgf-5 was transiently detected in all but 129 cells and its peak expression occurred at day 3 in MESC and OKO160 but at day 9 in D3 ES cells. Additionally, ZG was transiently detected in all but MESC ES cells and peak expression occurred at day 3 in D3 and 129 but at day 9 in OKO160 cell lines.

In MESC and D3 cell lines the peak Forssman antigen (FA) expression was observed in undifferentiated ES cells and decreased upon removal of LIF (Fig. 2b). However, over the 12-day differentiation period there was considerable variation in the expression of the Forssman antigen. For example, at 9 days following removal of LIF a proportion of all ES cell lines expressed the antigen. OKO160 and 129 ES cells exhibited FA staining at day 9 that was only slightly lower than that present on undifferentiated cells.
Pluripotent mouse ES cells are 5T4 antigen negative

However, at 12 days following removal of LIF the majority of the cell populations were negative for FA, although a small proportion of D3, OKO160 and 129 ES cells were positive. The differences in FA expression between the cell lines may be a result of clonal variation or could reflect differential activity of specific glycosylating enzymes required for the glycolipid expression. This data shows that the use of FA as a marker of undifferentiated ES cells is limited, owing to the prolonged expression of the antigen following removal of LIF in monolayer culture.

The increase in 5T4 antigen on ES cells upon removal of LIF was associated with increased 5T4 mRNA (Fig. 3a), probably reflecting transcriptional upregulation of 5T4. The maximal level of 5T4 transcripts in MESC ES cells (Fig. 3a i) occurred at day 3, which preceded the maximal level of protein expression (day 6/9; Fig. 1a i). The maximal expression of transcripts in OKO160 cells occurred at day 9 (Fig. 3a ii), which corresponds with maximal protein expression. 5T4 transcripts were detected in undifferentiated OKO160 and MESC ES cells by RT-PCR.

Fig. 2. Upregulation of 5T4 expression following removal of LIF correlates with differentiation of ES cells. (a) Transcript expression profiles of ES cells following removal of LIF. (i) MESC, (ii) D3, (iii) OKO160 and (iv) 129 ES cells were differentiated for 12 days as monolayer cultures by removal of LIF from the growth medium. RNA was extracted from the cells at the specified time points, treated with DNase, and cDNA synthesised from the mRNA transcripts. RT-PCR was performed for 35 cycles, the samples run on 2% agarose gels containing 400 ng/ml ethidium bromide and visualised on a UV transilluminator. β-tubulin (B-tub; housekeeping gene) is included for comparison purposes. To ensure the absence of genomic DNA, RT-PCR detection of β-tub was performed on all samples without prior formation of cDNA (mRNA sample). See Table 1 for description of markers used. D0, undifferentiated cells; D12, 12 days following removal of LIF. (b) Expression of Forssman antigen on ES cells following removal of LIF. ES cells were differentiated for 12 days as monolayer cultures by removal of LIF from the growth medium. Forssman antigen was determined at the specified time points on differentiating (i) MESC, (ii) D3, (iii) OKO160 and (iv) 129 ES cells using rat anti-Forssman antibody (uncoloured population) or control rat IgM (coloured population), detected as described in the legend to Fig. 1. Viable cells were gated using forward and side scatter and the figure shows the fluorescence of this population. Day 0, undifferentiated cells; Day 12, 12 days following removal of LIF.
Kinetics of 5T4 expression correlates with the differentiation rate of ES cell lines

With differentiation, MESC show rapid kinetics of 5T4 expression compared to the 129 ES cells (Fig. 1a). This is consistent with the relative proportions of FM-positive cells remaining after 12 days of differentiation (Fig. 2b). When ES cells differentiate there is a reduction in the proliferation and increase in apoptosis in the population. As determined by cell numbers in the presence or absence of LIF, MESC proliferation was clearly reduced after one day following removal of LIF whereas 129 ES cells showed no significant change (Fig. 4b i and ii, respectively). In contrast, to the LIF dependence of MESC, 129 ES cell numbers did not decrease until 3 days following removal of LIF, suggesting a delayed differentiation rate of these cells. Thus, the rate of proliferation is correlated with the induction of 5T4 expression. In addition, when ES colonies are subject to LIF withdrawal, the outer cells show altered morphology and motility (Fig. 4a). The appearance of such early differentiating cells was more rapid in the MESC than in 129 ES cells. Thus, at 3 days following removal of LIF, a significant proportion of MESC ES cell colonies exhibited differentiated cells (large arrows) whereas 129 ES cells maintained characteristic ES cell colony morphology (small arrows). By day 6, both ES cell lines exhibited differentiated cells although there were more in the MESC cell line.

Expression of EGFP-h5T4 in undifferentiated ES cells alters colony morphology

To further investigate the influence of 5T4 expression on ES cells, 129 ES cells were transfected with EGFP, EGFP-h5T4 or EGFP-CD44 plasmids and EGFP-positive cells isolated by FACS (Fig. 5). In the unsorted populations, the proportion and intensity of EGFP expression was lower for EGFP-h5T4 and EGFP-CD44 than EGFP alone (Fig. 5a). As expected, both EGFP-CD44 and EGFP-h5T4 were found at the cell membrane and EGFP in the cytoplasm and nucleus (Fig. 5b). EGFP-h5T4-transfected cells also had areas of intense intracellular fluorescence that are likely to be Golgi-associated (Fig. 5b). Morphological studies showed that ES cells expressing EGFP-h5T4 showed increased cell spreading compared to those cells expressing the cell surface protein control EGFP-CD44 and EGFP alone (Fig. 5c). Both EGFP-CD44- and EGFP-expressing cells maintained characteristic colony morphologies that were similar to untreated ES cells. These results show that expression of 5T4 in differentiating mouse ES cells is implicated in the spread and movement of the cells away from the primary colony.

Absence of 5T4 is a measure of mouse ES cell pluripotency and allows optimisation of ES cell growth conditions

We determined whether 5T4 expression is a more useful indicator of lack of pluripotency following removal of LIF than the ES cell marker SSEA-1 in mouse ES cells (Fig. 6).
Pluripotent mouse ES cells are 5T4 antigen negative

Undifferentiated 129 ES cells were sorted for SSEA-1 expression (boxed population in Fig. 6a i) and were found to be 5T4 negative (Fig. 6a ii). The pluripotency of this SSEA-1-positive/5T4-negative population was found to be 52%, as determined by the chimera-forming efficiency of the cells following injection into mouse blastocysts and reimplantation into foster mothers (percentage coat colour of chimeric mice was 1× 60%, 4× 25%, 2× 20%, 2× 10% and 4× <5%). Following removal of LIF from the culture for 6 days, a significant proportion of the cells remained positive for SSEA-1 (Fig. 6b i) and these cells were found to be 5T4 positive (Fig. 6b ii). This SSEA-1-positive/5T4-positive cell population exhibited only 7.7% pluripotency ($P<0.001$ compared to SSEA-1-positive/5T4-negative population; percentage coat colour of chimeric mice was 1× <5%). Furthermore, fewer mice were born in the group receiving the SSEA-1-positive/5T4-positive cell population compared to that with the SSEA-1-positive/5T4-negative cells (32.5% and 66% respectively), suggesting differentiating ES cells may be detrimental to the development process. These results demonstrate that absence of 5T4 from an ES cell population is a more accurate and sensitive indicator of pluripotency than the commonly used ES cell marker SSEA-1.

Many ES cell techniques utilise cloning and expansion of early passage cell lines. Therefore we assayed the effects of cloning and extended passage on the expression of the 5T4 antigen to assess its suitability as a marker for optimisation of these cells prior to use in such techniques (data not shown). Undifferentiated MESC ES cells did not express cell surface 5T4 antigen following culture for 12 passages. Similarly, cloned 129 ES cell colonies lacked cell surface antigen following isolation and expanded growth. Removal of 129 ES cells from a fibroblast feeder layer and subsequent passage on gelatin-treated plates also had no effect on 5T4 antigen expression (using DMEM + LIF). All cloned and extended passage cells exhibited a characteristic increase in cell surface 5T4 following removal of LIF from the cells, as described in Fig. 1a.

The quality of serum used for the growth of ES cells is known to affect the differentiation state of the cells, even in the presence of LIF (Smith, 1992). Growth of 129 ES cells in medium including serum that produces low cloning efficiency resulted in altered colony morphology, increased cell differentiation and induction of 5T4 expression compared to cells cultured in normal serum. We have also observed some primary embryonic fibroblast (PEF) feeder layer batches that induce expression of 5T4 on ES cells when co-cultured, suggesting that these PEF batches are not optimal for ES cell growth. The reason for the inability of some PEF batches to sustain ES cells in an undifferentiated state is probably harsh passaging (1:10) compared to batches able to maintain undifferentiated cells (passaged 1:3). Thus, the absence of 5T4

undifferentiated ES cells and large arrows
differentiated/migrating cells.
(b) Expression of 5T4 correlates
with the proliferation rate of
differentiating ES cells. (i) MESC
and (ii) 129 ES cells (10^5 cells/3
cm dish) were grown in DMEM+LIF medium in the absence (squares) or
presence (diamonds) of LIF (arrow indicates day of LIF removal) for 3
days and the number of viable cells
determined by light microscopy of
cells excluding Trypan Blue. Scale
bar: 10 μm.

Fig. 4. Expression of 5T4 antigen
in differentiating ES cells is
associated with the differentiation
rate. (a) Expression of 5T4 antigen correlates with cell migration in
differentiating ES cells. (i) MESC
and (ii) 129 ES cells (10^5 cells/3
cm dish) were grown for 0, 3 and 6
days in DMEM+LIF in the absence
of LIF and viewed under phase
contrast optics on an Olympus
inverted microscope. Small arrows
indicate undifferentiated ES cells
and large arrows
differentiated/migrating cells.

Many ES cell techniques utilise cloning and expansion of early passage cell lines. Therefore we assayed the effects of cloning and extended passage on the expression of the 5T4 antigen to assess its suitability as a marker for optimisation of these cells prior to use in such techniques (data not shown).
from ES cells is a useful marker of serum and PEF quality for the undifferentiated growth of these cells.

Discussion

This is the first report of a cell surface marker of ES cell pluripotency that is positively regulated following differentiation of the cells. As proof of principle, we show that 5T4 is a more useful pluripotency marker than SSEA-1 following differentiation of cells by removal of LIF. This may allow isolation of very early differentiated cells enabling elucidation of events associated with early ES cell differentiation. We also demonstrate that kinetics of 5T4 expression correlate with the differentiation rate of ES cells, and we show that these rates vary between ES cell lines. Expression of 5T4 also correlates with the appearance of motile cells, and expression of EGFP-h5T4 in undifferentiated ES cells leads to increased cell spread. These results suggest that 5T4 is involved in cell motility and/or decreased cell-cell contacts during the early differentiation of ES cells. It further implies an active role for 5T4 during the metastatic process and suggests that differentiating mouse ES cells may be useful for studying events associated with this process.

Traditionally, markers of ES cell pluripotency are negatively regulated. They are expressed at high levels in undifferentiated ES cells and decrease following differentiation (Ben-Shushan et al., 1998; Ling and Neben, 1997; Niwa et al., 2000; Rathjen, 2002). However, because these markers are expressed on a significant proportion of cells following removal of LIF they are not optimal for accurately determining pluripotency under these conditions. We have demonstrated that the ES cell markers SSEA-1, Oct-4 and Rex-1 (Ben-Shushan et al., 1998; Fan, 1999; Niwa et al., 2000; Rathjen, 2002; Rathjen et al., 1999) can be detected in ES cell populations for at least 12 days following removal of LIF. This is probably because of inefficient differentiation of ES cells in monolayer culture under these conditions. Thus, the kinetics of loss of expression of SSEA-1, Oct-4 or Rex-1 in a differentiating ES cell population does not provide a useful measure of the pluripotency or undifferentiated state of the cells. In contrast, 5T4 is positively regulated and can rapidly determine the differentiation state, therefore its absence determines the pluripotency of an ES cell population. Indeed, we have demonstrated that lack of cell surface 5T4 on ES cells is a more accurate indicator of pluripotency than SSEA-1, with SSEA-1-positive/5T4-positive ES cells showing significantly decreased chimera forming efficiency.

5T4 antigen is the first cell surface marker that is able to determine both the pluripotency and early differentiation state of an ES cell population in a single, non-destructive assay. Cell
during ES cell differentiation. Interestingly, the EGFP-h5T4 appearance of motile cells suggests a role for 5T4 in motility shown that the extracellular domain affects cell motility. The Carsberg et al., 1996; Woods et al., 2002), and it has been actin cytoskeleton and motility of cells (Carsberg et al., 1995; Kobe and Deisenhofer, 1994; Kobe and Deisenhofer, 1995), approximately 60 members with no obvious common function not cloned or grown under identical conditions.

Overexpression of 5T4 can have marked effects on both the for a variety of biological functions (Shaw, 2002). Since 5T4 is upregulated in at least some cells of all the three germ layers, identification of 5T4 regulating factors may enable insight into the differentiation process of these cells. The correlation of 5T4 regulation during mouse ES cell differentiation may does not regulate 5T4 transcript expression. Thus, elucidation of 5T4 regulation during ES cell differentiation is not known but we are investigating putative development-associated transcription factor binding sites in the 5T4 promoter region. The elevated levels of 5T4 antigen and altered morphology of ES cells grown in certain serum batches suggest that LIF is not directly responsible for inhibiting 5T4 expression in undifferentiated cells, although downstream effects cannot be discounted. Preliminary data suggest that promoter methylation in undifferentiated ES cells does not regulate 5T4 transcript expression. Thus, elucidation of 5T4 regulation during mouse ES cell differentiation may enable insight into the differentiation process of these cells. Since 5T4 is upregulated in at least some cells of all the three germ layers, identification of 5T4 regulating factors may provide insights into differentiation events common to all cells, rather than a single cell lineage.

This work was supported by Cancer Research UK. We are indebted to Mark Willington for technical help with the mouse chimera study and Jeff Barry for his cell sorting expertise. We are grateful to Dr W. Wang who provided expertise in preparation of the 9A7 antibody.

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Bielinska, M., Narita, N., Heikinheimo, M., Porter, S. B. and Wilson, D. 5T4 is unique in that it is both expressed for a relatively prolonged period of time and is present on cells derived from each of the three germ layers. The correlation of 5T4 expression kinetics with the differentiation rate of ES cells is an interesting observation that may enable detailed study of the factors involved in the differentiation process. The mechanisms of this correlation are likely to involve motility of the differentiated cells away from a primary colony. The results demonstrate that there are considerable differences between the ES cell lines studied, both in motility and reliance on LIF for cell proliferation. Furthermore, the transcript expression patterns in differentiating ES cells can be different. However, culture conditions or clonal variation within populations may account for this difference since the cell lines in this study were not cloned or grown under identical conditions.

5T4 is a member of the LRR family, which contains approximately 60 members with no obvious common function (Kobe and Deisenhofer, 1994; Kobe and Deisenhofer, 1995), and it is likely that the LRR domains of 5T4 provide a scaffold for a variety of biological functions (Shaw, 2002). Overexpression of 5T4 can have marked effects on both the actin cytoskeleton and motility of cells (Carsberg et al., 1995; Carsberg et al., 1996; Woods et al., 2002), and it has been shown that the extracellular domain affects cell motility. The observations that EGFP-h5T4 leads to increased motility/spread of ES cells and that 5T4 expression correlates with the appearance of motile cells suggests a role for 5T4 in motility during ES cell differentiation. Interestingly, the EGFP-h5T4 rearrangement phenotype observed when 5T4 is expressed (Awan et al., 2002). There are probably additional mechanisms whereby 5T4 expression can alter the morphology as well as the motility of cells (Carsberg et al., 1995) and these may be of functional significance in development and carcinogenesis.

The mechanism of regulation of 5T4 expression during ES cell differentiation is not known but we are investigating putative development-associated transcription factor binding sites in the 5T4 promoter region. The elevated levels of 5T4 antigen and altered morphology of ES cells grown in certain serum batches suggest that LIF is not directly responsible for inhibiting 5T4 expression in undifferentiated cells, although downstream effects cannot be discounted. Preliminary data suggest that promoter methylation in undifferentiated ES cells does not regulate 5T4 transcript expression. Thus, elucidation of 5T4 regulation during mouse ES cell differentiation may enable insight into the differentiation process of these cells. Since 5T4 is upregulated in at least some cells of all the three germ layers, identification of 5T4 regulating factors may provide insights into differentiation events common to all cells, rather than a single cell lineage.

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