Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells

Hitoshi Niwa[1,2], Jun-ichi Miyazaki[2] & Austin G. Smith[1]

Cell fate during development is defined by transcription factors that act as molecular switches to activate or repress specific gene expression programmes. The POU transcription factor Oct-3/4 (encoded by *Pou5f1*) is a candidate regulator in pluripotent and germline cells[1–4] and is essential for the initial formation of a pluripotent founder cell population in the mammalian embryo[5]. Here we use conditional expression and repression in embryonic stem (ES) cells to determine requirements for Oct-3/4 in the maintenance of developmental potency. Although transcriptional determination has usually been considered as a binary on-off control system, we found that the precise level of Oct-3/4 governs three distinct fates of ES cells. A less than twofold increase in expression causes differentiation into primitive endoderm and mesoderm. In contrast, repression of Oct-3/4 induces loss of pluripotency and dedifferentiation to trophectoderm. Thus a critical amount of Oct-3/4 is required to sustain stem-cell self-renewal, and up- or downregulation induce divergent developmental programmes. Our findings establish a role for Oct-3/4 as a master regulator of pluripotency that controls lineage commitment and illustrate the sophistication of critical transcriptional regulators and the consequent importance of quantitative analyses.

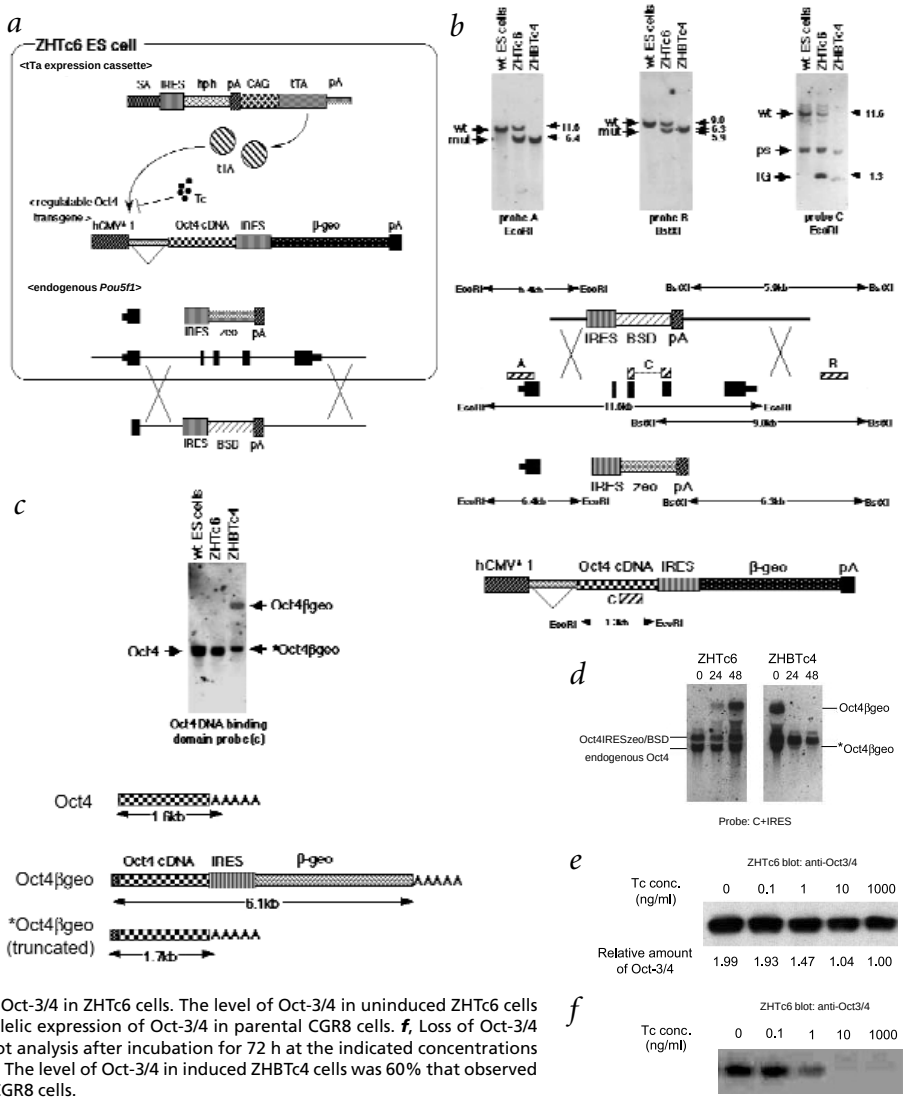

Fig. 1 Generation of ES cells with variable Oct-3/4 expression. ***a***, Strategy for generating ZHTc6 and ZHBTc4 ES cells. ZHTc6 ES cells have one allele of *Pou5f1* inactivated by targeted integration of an IREStzeopA cassette and contain a Tc-regulated Oct-3/4 transgene. This transgene is activated in the absence of Tc. ***b***, Southern-blot hybridization of ZHTc6 and ZHBTc4 ES cells. Loss of the wild-type allele in ZHBTc4 cells was identified with both 5′ and 3′ flanking probes. Hybridization with probe C, a cDNA fragment encoding the DNA-binding domain of Oct-3/4, confirms the absence of endogeneous *Pou5f1* from ZHBTc4 cells, and detects the transgene and a pseudogene in ZHTc6 and ZHBTc4 cells. ***c***, Northern-blot analysis of *Pou5f1* expression in ZHTc6 and ZHBTc4 cells. Total RNA prepared from parental CGR8 ES cells, ZHTc6 cells in the presence of Tc and ZHBTc4 cells in the absence of Tc were hybridized with probe C. ZHBTc4 cells lack the wild-type transcript and express two transgene-derived transcripts. The larger of these corresponds to the full-length Oct-3/4-IRESβgeo mRNA. The smaller transcript is slightly larger than the endogeneous transcripts detected in CGR8 and ZHTc6 cells. This is identified as a truncated product of the Oct-3/4-IRESβgeopA transgene by specific hybridization to a β-globin cDNA fragment (data not shown) and because Tc regulates its expression. This transcript terminates at the poly(A) addition signal in the 3′ UTR of *Pou5f1* cDNA and therefore generates functional Oct-3/4 protein. ***d***, Regulation of the Oct-3/4 transgene in ZHBTc6 and ZHBTc4 cells. Northern-blot hybridization with probe C plus an IRES probe, which detects transcripts from the two targeted alleles and serves as a loading control. In ZHTc6 cells, full induction of the transgene transcripts is achieved 48 h after withdrawal of Tc, whereas suppression is nearly complete 24 h after addition of Tc in ZHBTc4 cells. ***e***, Induced expression of Oct-3/4 in ZHTc6 cells. The level of Oct-3/4 in uninduced ZHTc6 cells (here set at 1.0) is 65% that observed for biallelic expression of Oct-3/4 in parental CGR8 cells. ***f***, Loss of Oct-3/4 protein in Tc-treated ZHBTc4 cells. Western-blot analysis after incubation for 72 h at the indicated concentrations of Tc using anti-Oct-3/4 N-terminal antibody[30]. The level of Oct-3/4 in induced ZHBTc4 cells was 60% that observed for biallelic expression of Oct-3/4 in parental CGR8 cells.

[1]*Centre for Genome Research, The University of Edinburgh, King's Buildings, Edinburgh, UK.* [2]*Department of Nutrition and Physiological Chemistry, Osaka University School of Medicine, Osaka, Japan. Correspondence should be addressed to H.N. (e-mail: hniwa@nutri.med.osaka-u.ac.jp) or A.G.S. (e-mail: austin.smith@ed.ac.uk).*

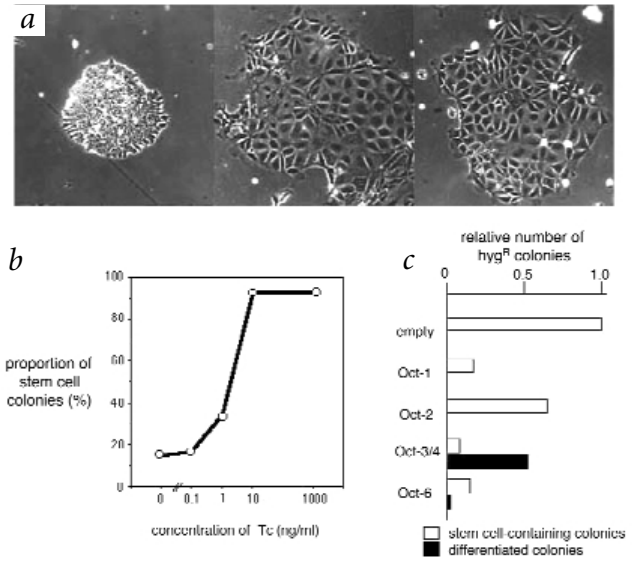

Fig. 2 Differentiation of ZHTc6 and ZHBTc4 ES cells induced by modulation of Oct-3/4 expression. ***a***, Photomicrographs of undifferentiated ZHBTc4 cells cultured in the presence of LIF and absence of Tc (left); differentiated ZHTc6 cells generated by withdrawal of Tc (overexpression of Oct-3/4) in the presence of LIF (centre); and differentiated ZHBTc4 cells produced in absence of Tc (continued expression of Oct-3/4) on withdrawal of LIF (right). All photographs are at the same magnification. ***b***, Dose-dependent differentiation of ZHTc6 cells on withdrawal of tetracycline. Purified stem cells were cultured in the indicated concentrations of Tc in the presence of LIF. After 6 d, colonies containing stem cells were visualized by Leishman's staining and are plotted as proportion of total colonies. ***c***, Episomal expression of various POU factors in MG1.19 ES cells. The bar chart shows undifferentiated and differentiated colonies obtained by supertransfection of episomal vectors for expression of the indicated cDNA inserts and selection for 8 d in hygromycin B. The absolute colony numbers vary with the insert, but the differentiation response is specific to Oct-3/4.

We introduced expression constructs for the tetracycline (Tc)-regulated transactivator tTA (ref. 6) and a tTA-responsive Oct-3/4 transgene into *Pou5f1*$^{+/-}$ ES cells[7] (Fig. 1*a*). Two clones with no transgene expression in the presence of Tc gave induced expression comparable to that of the endogeneous allele. Both clones, ZHTc6 and ZHTc25, underwent extensive differentiation after Oct-3/4 induction. Transgene expression was fully induced 48 hours after withdrawal of Tc (Fig. 1*d*) and differentiation was evident by 72 hours. Tc induced differentiation in a concentration-dependent manner (Fig. 2*b*). Quantitative immunoblot analysis of two independent experiments showed that maximum induction doubled the amount of Oct-3/4 protein such that it exceeded the level in non-induced cells (Fig. 1*e*). This represents at most a 50% increase above the amount of Oct-3/4 present in biallelic ES cells, yet is sufficient to induce the differentiation of most cells in both clonal and mass cultures.

To provide independent confirmation that elevated Oct-3/4 induces differentiation, we exploited episomal supertransfection to generate primary transfectants at high frequency[8,9]. Vectors containing no insert or irrelevant inserts produced predominantly undifferentiated stem-cell colonies. In contrast, Oct-3/4 expression vectors generated many differentiated colonies (Fig. 2*c*). Supertransfection of expression vectors encoding Oct-1, Oct-2 or Oct-6 had no effect on the frequency of differentiated colonies generated. Thus the induction of differentiation by Oct-3/4 is not a general property of POU transcriptional regulators.

We next determined the consequences of reducing the expression of Oct-3/4. We introduced a second *Pou5f1* targeting construct into ZHTc6 cells (Fig. 1*a*). To rescue prospective doubly targeted ES cells while avoiding an increase in Oct-3/4 protein beyond the threshold for induction of differentiation, we induced transgene expression by withdrawal of Tc just 24 hours before transfection. Of 17 blasticidin-S–resistant clones, 2 had undergone the desired deletion (Fig. 1*b*). Both clones behaved similarly in preliminary analyses, therefore data is presented for only one, ZHBTc4.

The ZHBTc4 cells were indistinguishable from parental ES cells when Oct3/4 transgene expression was maintained. Addition of Tc resulted in repression of the transgene such that transcripts and protein were undetectable by 24 hours (Fig. 1*d*,*f*). Profound morphological changes then occur in ZHBTc4 cells. The cells compacted over the initial 48 hours and subsequently flattened out over the culture surface, often acquiring enlarged nuclei (Fig. 3). This was evident at 1 ng/ml Tc, at which Oct-3/4 protein was reduced but not eliminated (Fig. 1*f*). The response was comparable in the absence or presence of leukaemia inhibitory factor (LIF). Therefore, Oct-3/4 function is an absolute requirement for maintenance of ES cell identity and for the self-renewal activity of LIF (ref. 10). If LIF was withdrawn in the absence of Tc, however, ZHBTc4 cells differentiated as normal despite continued expression of the transgene (Fig. 2*a*). Oct-3/4 expression alone is therefore not sufficient to maintain the undifferentiated phenotype, but requires a cooperative signal provided by LIF stimulation.

We next investigated transcriptional responses of five target genes[11] to altered levels of Oct-3/4 (Fig. 4*a*). All were downregulated in Tc-treated ZHBTc4 cells with kinetics parallel to the disappearance of *Pou5f1* transcripts. *Otx1*, and to a lesser extent *Ebaf*, were correspondingly upregulated on induction of the Oct-3/4 transgene in ZHTc6 cells. In contrast, *Fgf4* expression

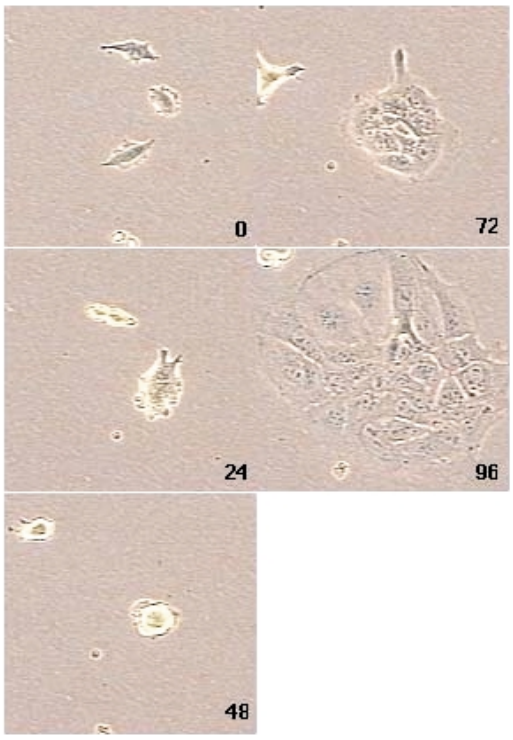

Fig. 3 Morphological differentiation of ZHBTc4 ES cells on repression of Oct-3/4. Photomicrographs taken from time-lapse recording of ZHBTc4 differentiation at 0, 24, 48, 72 and 96 h after addition of Tc (view the time-lapse movie at http://genetics.nature.com/supplementary_info/). ES cells typically underwent 2 or 3 cycles of cell division, transiently compacted together, then flattened out over the surface of the dish. Note that parental ES cells show no overt response to treatment with Tc.

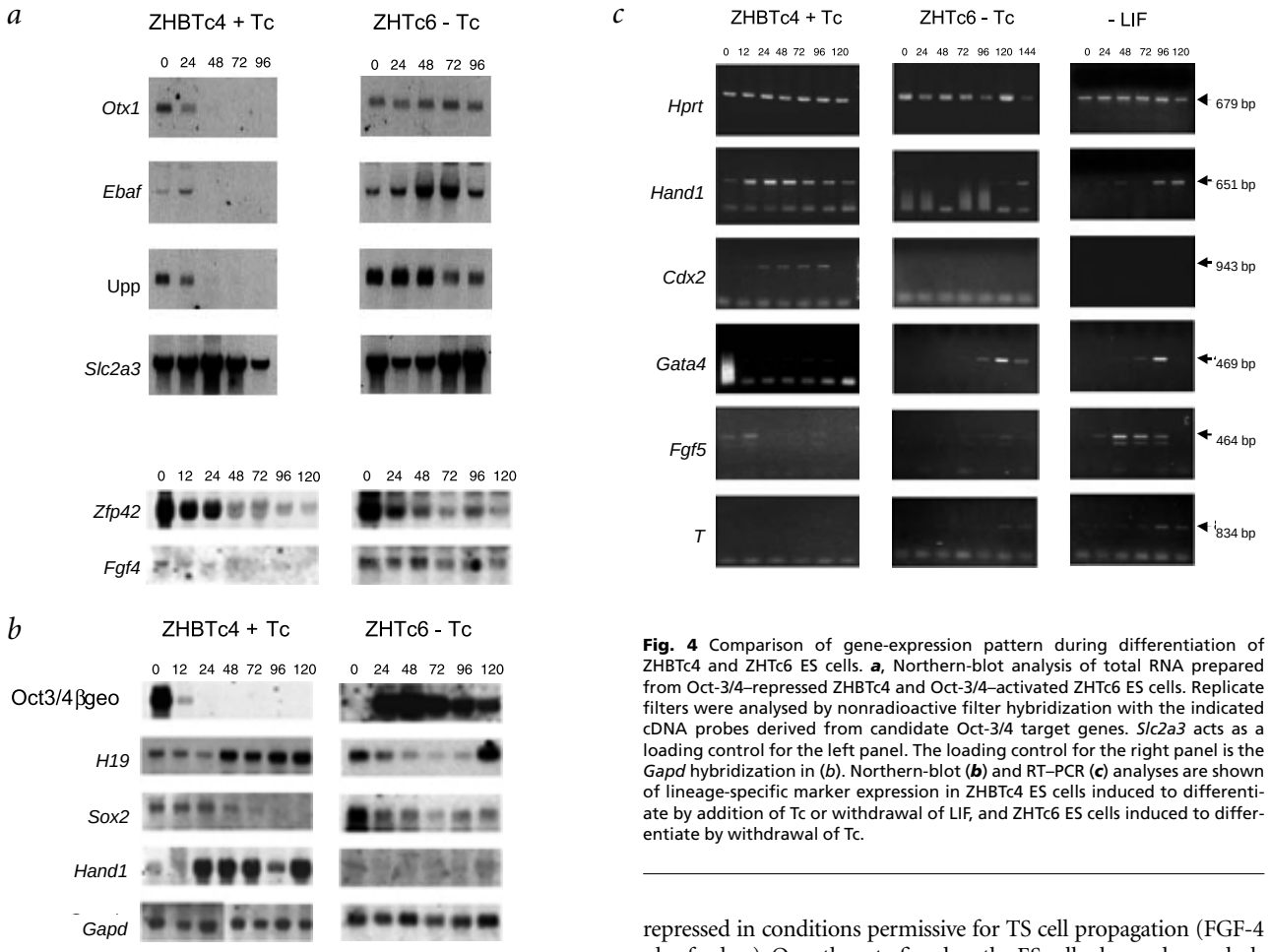

Fig. 4 Comparison of gene-expression pattern during differentiation of ZHBTc4 and ZHTc6 ES cells. ***a***, Northern-blot analysis of total RNA prepared from Oct-3/4–repressed ZHBTc4 and Oct-3/4–activated ZHTc6 ES cells. Replicate filters were analysed by nonradioactive filter hybridization with the indicated cDNA probes derived from candidate Oct-3/4 target genes. *Slc2a3* acts as a loading control for the left panel. The loading control for the right panel is the *Gapd* hybridization in (*b*). Northern-blot (***b***) and RT–PCR (***c***) analyses are shown of lineage-specific marker expression in ZHBTc4 ES cells induced to differentiate by addition of Tc or withdrawal of LIF, and ZHTc6 ES cells induced to differentiate by withdrawal of Tc.

was not appreciably altered, *Upp* was gradually downregulated and *Zfp42* (encoding the transcription factor Rex-1) was rapidly repressed after transgene induction. Thus, increased Oct-3/4 has divergent effects on different target genes.

We then examined expression of marker genes to evaluate the nature of the induced differentiation programmes (Fig. 4*b*). *Gata4* mRNA, which is restricted to parietal and visceral endoderm in early embryos[12], was detectable in undifferentiated ES cells but increased during differentiation induced by elevation of Oct-3/4 or withdrawal of LIF. The mesoderm marker brachyury (*T*; ref. 13) was also activated under these conditions. Therefore, upregulation of Oct-3/4 induces commitment to extra-embryonic endoderm and mesoderm lineages, similar to withdrawal of LIF (ref. 14). The differentiation events may not be wholly identical, however, because Fgf-5, a marker of primitive ectoderm[15], appears to be more abundant after withdrawal of LIF.

Transcripts encoding Gata4, brachyury and Fgf-5 were barely detectable following suppression of Oct-3/4. In contrast, mRNAs encoding two transcription factors implicated in trophoblast differentiation, Hand1 (ref. 16) and Cdx2 (ref. 17), were rapidly induced. *Hand1* also appeared in differentiated ZHTc6 cells, but considerably later, likely reflecting its expression in mesodermal and neural crest derivatives. *Cdx2*, a specific marker of diploid trophectoderm in the early conceptus, was only detected during ZHBTc4 differentiation. Mouse ES cells are derived from the epiblast[18], however, and accordingly do not normally differentiate into the trophectodermal lineage *in vitro* or *in vivo*[19]. To confirm that lack of Oct-3/4 induces commitment to trophectoderm, we attempted to establish trophoblast stem (TS) cell cultures[20] from ZHBTc4 cells. Oct-3/4 was repressed in conditions permissive for TS cell propagation (FGF-4 plus feeders). Over three to five days the ES cells changed morphology and formed monolayer colonies of regular epithelial cells (Fig. 5*a*). These cells continued to proliferate and could be serially passaged for at least one month. Cultures consisted of undifferentiated epithelioid cells and some overtly trophoblastic cells. On withdrawal of FGF-4, the cultures differentiated completely into large, spread cells, many of which had the swollen nuclei characteristic of endoreduplicating trophoblast giant cells (Fig. 5*b*). RT–PCR analysis confirmed expression of markers[20] of proliferating trophoblast (*Cdx2*, *Esrrb*, *Mash2* and *Tpbp*) and mature trophoblast (*Pl1*; Fig. 5*c*). Finally, *in situ* hybridization localized *Cdx2* transcripts in the proliferating epithelial population, substantiating their identification as TS cells (Fig. 5*d*).

These findings establish that removal of Oct-3/4 results in a respecification of ES cell fate. We conclude that a component of Oct-3/4 function is as a gatekeeper that prevents dedifferentiation into the trophectoderm lineage and thereby 'locks' pluripotent capacity.

The role of Oct-3/4, however, is more sophisticated than that of a simple repressor of trophectodermal differentiation. Oct-3/4 also activates transcription of specific genes through cooperation with a series of partners including Sry-related Sox-2 (refs 21,22), a stem-cell–restricted E1A-like activity[23] and the transcription factor Rox-1 (ref. 24). The amount of Oct-3/4 relative to these different partners appears to be crucial[23]. The downregulation of *Zfp42* mRNA caused by induction of elevated Oct-3/4 (Fig. 4*a*) is consistent with the finding in transient assays that overexpression of Oct-3/4 represses activation of the *Zfp42* promoter mediated via Rox-1 (ref. 24). Elevated Oct-3/4 may also enhance expression of targets, for example through formation of homodimers as shown for the osteopontin gene[25]. Both loss-and gain-of-target

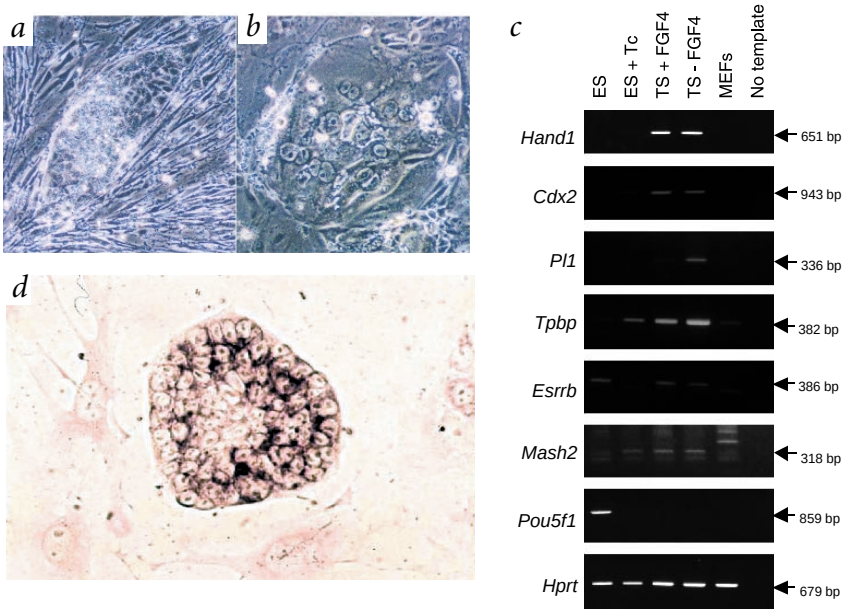

Fig. 5 TS cell derivation from ZHBTc4 ES cells. ***a***, Photomicrograph of TS cells cultured in the presence of FGF-4 and Tc on mouse embryo fibroblast feeder cells. The cells grow as monolayer epithelial colonies and can be passaged at least 10 times. ***b***, Photomicrograph of TS cells cultured for 6 d in the absence of a feeder layer and FGF-4. Differentiated, large, flat cells with swollen nuclei are observed, resembling trophoblast giant cells. ***c***, RT–PCR analyses of *Hand1*, *Cdx2*, *Pl1*, *Tpbp*, *Esrrb*, *Pou5f1* and *Hprt* mRNAs in undifferentiated ZHBTc4 ES cells; ZHBTc4 cells cultured in the presence of Tc for 120 h; ZHBTc4-derived TS cells maintained on mouse embryo fibroblast (MEF) feeder cells; ZHBTc4-derived TS cells induced to differentiate by culture in the absence of FGF-4 for 120 h; and mouse embryo fibroblast feeder cells. ***d***, *In situ* hybridization of ZHBTc4-derived TS cells showing expression of *Cdx2* transcripts in the proliferative epithelial cells cultured in FGF-4.

gene expression are therefore likely to contribute to the observed induction of differentiation.

We believe that LIF does not support ES-cell renewal by maintaining Oct-3/4 expression. ZHBTc4 cells underwent differentiation in the absence of LIF in a manner indistinguishable from that of parental ES cells (Fig. 2*a*), even though the level of Oct-3/4 expression was maintained from the hCMV*-1 transgene for at least 72 hours (see Fig. 7, http://genetics.nature.com/supplementary_info). We hypothesize that activation of Stat3 by LIF (refs 9,26), or an alternative self-renewal signal[27], is necessary to stimulate expression of a specific Oct-3/4 partner such as Rox-1. This model explains how withdrawal of LIF or increased Oct-3/4 have identical consequences for particular target genes and thus trigger similar differentiation events (Fig. 6*a*).

Maintaining Oct-3/4 expression within a certain range appears to be critical for stem-cell renewal, with any increase or decrease triggering differentiation to endoderm/mesoderm or trophectoderm, respectively (Fig. 6*b*). The threshold for inducing differentiation is apparently set at 50% above or below the normal diploid expression level in undifferentiated stem cells. The amount of Oct-3/4 must therefore be tightly regulated to maintain stem-cell phenotype. Consistent with this, heterozygous ES cells show only a 30–40% reduction in *Pou5f1* transcripts (Fig. 1*c*) and Oct-3/4 protein (data not shown).

Morphogen gradients are thought to direct many inductive and patterning events during embryogenesis, but mechanisms for interpreting gradients at the cellular level are unclear. Our data indicate that the amount of a transcriptional regulator in a cell can output at least three different responses with precise threshold levels. Therefore, graded activation of signal transduction pathways or nuclear receptors translated into quantitative differences in levels of a master transcriptional regulator may be sufficient to define a series of distinct cellular responses.

In vivo, Oct-3/4 protein is abundant in the inner cell mass of early blastocysts and downregulated in trophectoderm, whereas nascent primitive endoderm in late blastocysts expresses Oct-3/4 at an even higher level (ref. 28). This profile accords with loss of Oct-3/4, permitting trophectoderm development and upregulation driving primitive endoderm differentiation. Quantitative modulation of finely balanced interactions between Oct-3/4 and different partners may be a reiterated mechanism that governs successive determination events, perhaps including germ-cell specification, during the pluripotent phase of mammalian embryogenesis.

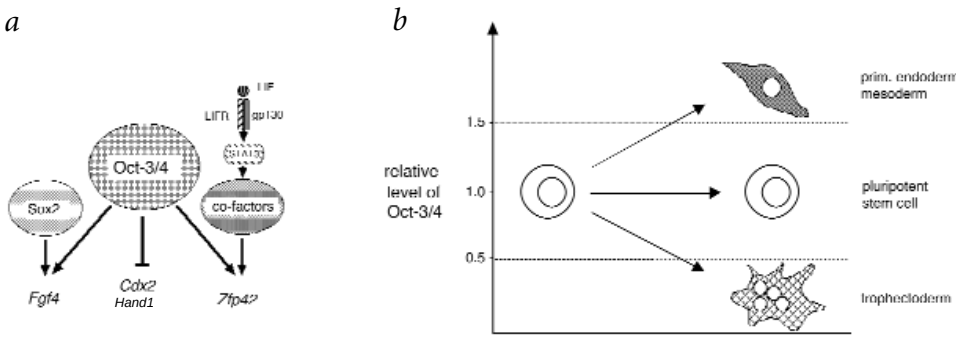

Fig. 6 Functions of Oct-3/4 in pluripotent stem cells. ***a***, Model for Oct-3/4 and Stat3 cooperative function. Self-renewal requires transcriptional functions of both Oct-3/4 and LIF-stimulated Stat3. Oct-3/4 directly or indirectly represses trophectodermal determination genes such as *Cdx2* and *Hand1* and thereby blocks differentiation to trophectoderm. Oct-3/4 also activates target genes in stem cells through interaction with a set of co-activators. We hypothesize that a subset of such partners, including Rox-1, are regulated by Stat3. Sequestration of such co-activators by excess Oct-3/4 or their downregulation following LIF withdrawal would thus result in a similar loss of expression of targets such as *Zfp42*. ***b***, Relationship between Oct-3/4 expression level and stem-cell fate. To maintain the undifferentiated stem-cell phenotype, Oct-3/4 expression must remain within plus or minus 50% of normal diploid expression. If Oct-3/4 expression is increased beyond the upper threshold level, differentiation is triggered into primitive endoderm or mesoderm. If Oct-3/4 expression is decreased, stem cells are redirected into the trophectoderm lineage.

Methods

Plasmid construction. Oct-3/4 targeting vectors and IRES-linked selection cassettes were as described[5,7]. To create the IREShph:CAGtTA construct, the tetracycline-responsive tTA transactivator[6] (Tet-off) in the constitutive expression unit CAGGS (ref. 29) was placed 3′ of the splice acceptor-IREShphpA unit. We constructed the hCMV*-1-Oct-3/4IRESβgeopA transgene from the tTA-regulated promoter hCMV*-1 derived from pUHD10-3 (ref. 6), the rabbit β-globin second intron and splice acceptor, full-length *Pou5f1* cDNA and IRESβgeopA unit. We prepared episomal expression constructs by insertion of mouse *Pou2f1*, human *POU2F2*, mouse *Pou5f1* and mouse *Pou3f1* ORFs into the pHPCAGGS expression vector[9].

ES-cell culture and transfection. We cultured CGR8 ES cells and derivatives without feeders in LIF-supplemented medium[10]. Electroporation and *Pou5f1* targeting were as described[5,7]. We identified targeted events by Southern-blot analysis using probes external to the recombination construct. Hygromycin B was applied to select for productive integration of the IREShph:CAGtTA construct, and the hCMV*-1-Oct-3/4IRESβgeopA was randomly integrated with selection for G418 resistance. We screened clones for uniform expression of β-galactosidase in the absence of Tc and no expression in the presence of Tc (1 μg/ml). We supertransfected Polyoma large T expressing MG1.19 ES cells[8] as described[9] using expression plasmid (10 μg) with selection of transfectants in hygromycin B (80 μg/ml). For generation of ZHBTc4 ES cells, ZHTc6 ES cells were cultured in the absence of Tc for 24 h to induce the Oct-3/4 transgene, transfected with the *Pou5f1* targeting vector carrying an IRESBSDpA selection cassette and cultured in the presence of zeocin, blasticidin S and Tc for 10 d.

RNA and protein analysis. For northern blots, we analysed aliquots (5 μg) of total RNA by non-radioactive filter hybridization (Gene Image, Amersham). For RT–PCR analyses, we carried out oligo-dT primed reverse transcription on aliquots (4 μg) of total RNA and used 1/40 of the single-strand cDNA products for each PCR amplification. Gene-specific primers were designed based on published sequences (see Table 1, http://genetics.nature.com/supplementary_info/). We carried out *in situ* hybridization on cultured cells as described[5] using a *Cdx2* antisense probe. We carried out immunoblot analysis of RIPA buffer-extracted samples using anti-Oct-3/4 polyclonal antisera. Signals were detected by chemiluminescence (ECL, Amersham) and intensity quantitated by fluoroimage analysis (Molecular Dynamics) or chemiluminescence capture by CCD camera. Samples were normalized relative to densitometric determination of Coomassie-blue–stained replica gels. We carried out western-blot analysis after incubation for 72 h at the indicated concentrations of Tc using anti-Oct-3/4 antibody[28] followed by quantitation of signal intensity by fluoroimage analysis.

Acknowledgements

We thank S. Nishikawa for access to time-lapse recording facilities and H. Schöler and H. Hamada for anti-Oct-3/4 antisera. We acknowledge F. Stehnouse for technical assistance. This work was supported by the Biotechnology and Biological Sciences Research Council of the United Kingdom, The Ministry of Education, Science and Culture of Japan, and a Uehara Memorial Foundation Award to H.N.

Received 1 November 1999; accepted 15 February 2000.

1. Schöler, H.R., Ruppert, S., Suzuki, N., Chowdhury, K. & Gruss, P. New type of POU domain in germ line-specific protein Oct-4. *Nature* **344**, 435–439 (1990).
2. Rosner, M.H. *et al.* A POU-domain transcription factor in early stem cells and germ cells of the mammalian embryo. *Nature* **345**, 686–692 (1990).
3. Okamoto, K. *et al.* A novel octamer binding transcription factor is differentially expressed in mouse embryonic cells. *Cell* **60**, 461–472 (1990).
4. Pesce, M., Wang, X., Wolgemuth, D.J. & Scholer, H. Differential expression of the Oct-4 transcription factor during mouse germ cell differentiation. *Mech. Dev.* **71**, 89–98 (1998).
5. Nichols, J. *et al.* Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct-4. *Cell* **95**, 379–391 (1998).
6. Gossen, M. & Bujard, H. Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proc. Natl Acad. Sci. USA* **89**, 5547–5551 (1992).
7. Mountford, P. *et al.* Dicistronic targeting constructs: reporters and modifiers of mammalian gene expression. *Proc. Natl Acad. Sci. USA* **91**, 4303–4307 (1994).
8. Gassmann, M., Donoho, G. & Berg, P. Maintenance of an extrachromosomal plasmid vector in mouse embryonic stem cells. *Proc. Natl Acad. Sci. USA* **92**, 1292–1296 (1995).
9. Niwa, H., Burdon, T., Chambers, I. & Smith, A.G. Self-renewal of pluripotent embryonic stem cells is mediated via activation of STAT3. *Genes Dev.* **12**, 2048–2060 (1998).
10. Smith, A.G. *et al.* Inhibition of pluripotential embryonic stem cell differentiation by purified polypeptides. *Nature* **336**, 688–690 (1988).
11. Saijoh, Y. *et al.* Identification of putative downstream genes of Oct-3, a pluripotent cell-specific transcription factor. *Genes Cells* **1**, 239–252 (1996).
12. Arceci, R.J., King, A.A., Simon, M.C., Orkin, S.H. & Wilson, D.B. Mouse GATA-4: a retinoic acid-inducible GATA-binding transcription factor expressed in endodermally derived tissues and heart. *Mol. Cell. Biol.* **13**, 2235–2246 (1993).
13. Wilkinson, D.G., Bhatt, S. & Herrmann, B.G. Expression pattern of the mouse T gene and its role in mesoderm formation. *Nature* **343**, 657–659 (1990).
14. Nishikawa, S.I., Nishikawa, S., Hirashima, M., Matsuyoshi, N. & Kodama, H. Progressive lineage analysis by cell sorting and culture identifies FLK1+VE-cadherin+ cells at a diverging point of endothelial and hemopoietic lineages. *Development* **125**, 1747–1757 (1998).
15. Hebert, J.M., Boyle, M. & Martin, G.R. mRNA localization studies suggest that murine FGF-5 plays a role in gastrulation. *Development* **112**, 407–415 (1991).
16. Cserjesi, P., Brown, D., Lyons, G.E. & Olson, E.N. Expression of the novel basic helix-loop-helix gene eHAND in neural crest derivatives and extraembryonic membranes during mouse development. *Dev. Biol.* **170**, 664–678 (1995).
17. Beck, F., Erler, T., Russell, A. & James, R. Expression of *Cdx-2* in the mouse embryo and placenta: possible role in patterning of the extra-embryonic membranes. *Dev. Dyn.* **204**, 219–227 (1995).
18. Brook, F.A. & Gardner, R.L. The origin and efficient derivation of embryonic stem cells in the mouse. *Proc. Natl Acad. Sci. USA* **94**, 5709–5712 (1997).
19. Beddington, R.S.P. & Robertson, E.J. An assessment of the developmental potential of embryonic stem cells in the midgestation mouse embryo. *Development* **105**, 733–737 (1989).
20. Tanaka, S., Kunath, T., Hadjantonakis, A.-K., Nagy, A. & Rossant, J. Promotion of trophoblast stem cell proliferation by FGF-4. *Science* **282**, 2072–2075 (1998).
21. Ambrosetti, D.-C., Basilico, C. & Dailey, L. Synergistic activation of the fibroblast growth factor 4 enhancer by sox2 and oct-3 depends on protein-protein interactions facilitated by a specific spatial arrangement of factor binding sites. *Mol. Cell. Biol.* **17**, 6321–6329 (1997).
22. Nishimoto, M., Fukushima, A., Okuda, A. & Muramatsu, M. The gene for the embryonic stem cell coactivator UTF1 carries a regulatory element which selectively interacts with a complex composed of Oct-3/4 and Sox-2. *Mol. Cell. Biol.* **19**, 5453–5465 (1999).
23. Schöler, H.R., Ciesiolka, T. & Gruss, P. A nexus between Oct-4 and E1A: implications for gene regulation in embryonic stem cells. *Cell* **66**, 291–304 (1991).
24. Ben-Shushan, E., Thompson, J.R., Gudas, L.J. & Bergman, Y. *Rex-1*, a gene encoding a transcription factor expressed in the early embryo, is regulated via Oct-3/4 and Oct-6 binding to an octamer site and a novel protein, Rox-1, binding to an adjacent site. *Mol. Cell. Biol.* **18**, 1666–1878 (1998).
25. Botquin, V. *et al.* New POU dimer configuration mediates antagonistic control of an osteopontin preimplantation enhancer by Oct-4 and Sox-2. *Genes Dev.* **12**, 2073–2090 (1998).
26. Burdon, T., Chambers, I., Niwa, H., Stracey, C. & Smith, A.G. Signalling mechanisms regulating self-renewal and differentiation of pluripotent embryonic stem cells. *Cells Tissues Organs* **165**, 131–143 (1999).
27. Dani, C. *et al.* Paracrine induction of stem cell renewal by LIF-deficient cells: a new ES cell regulatory pathway. *Dev. Biol.* **203**, 149–162 (1998).
28. Palmieri, S.L., Peter, W., Hess, H. & Schöler, H.R. Oct-4 transcription factor is differentially expressed in the mouse embryo during establishment of the first two extraembryonic cell lineages involved in implantation. *Dev. Biol.* **166**, 259–267 (1994).
29. Niwa, H., Yamamura, K.-I. & Miyazaki, J.-I. Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene* **108**, 193–200 (1991).
30. Shimazaki, T. *et al.* Hybrid cell extinction and re-expression of *Oct-3* function correlates with differentiation potential. *EMBO J.* **12**, 4489–4498 (1993).