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A defined Oct4 level governs cell state transitions of pluripotency entry and differentiation into all embryonic lineages

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Abstract

Oct4 is considered a master transcription factor for pluripotent cell self-renewal, but its biology remains poorly understood. Here, we investigated the role of Oct4 using the process of induced pluripotency. We found that a defined embryonic stem cell (ESC) level of Oct4 is required for pluripotency entry. However, once pluripotency is established, the Oct4 level can be decreased up to sevenfold without loss of self-renewal. Unexpectedly, cells constitutively expressing Oct4 at an ESC level robustly differentiated into all embryonic lineages and germline. In contrast, cells with low Oct4 levels were deficient in differentiation, exhibiting expression of naive pluripotency genes in the absence of pluripotency culture requisites. The restoration of Oct4 expression to an ESC level rescued the ability of these to restrict naive pluripotent gene expression and to differentiate. In conclusion, a defined Oct4 level controls the establishment of naive pluripotency as well as commitment to all embryonic lineages.

Naive pluripotency characterizes the cells that can give rise to all cell types of an organism except extraembryonic tissues. In mouse embryos these cells arise during pre-implantation development in the naive epiblast. This transient cell population can be captured *in vitro* as ESCs. In addition to its developmental potential, the naive pluripotent state is characterized by a unique set of properties, including the lack of an inactive X chromosome in female cells, self-renewing response to Mek/Erk signalling inhibition, and simultaneous expression of Esrrb, Nanog, Rex1, Klf2 and Klf4 (ref. 1).

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Oct4 plays a fundamental role in mammalian development as a master transcriptional regulator of naive pluripotency maintenance. It belongs to the POU family of transcription factors and possesses the POU DNA-binding domain characteristic of this family²⁻⁴. Oct4 is expressed in oocytes, blastomeres, inner cell mass (ICM), naive and post-implantation epiblast, germ cells, and in pluripotent cells *in vitro*^{2,3,5}. Its knockout causes pre-implantation lethality of mouse embryos due to failure to form a pluripotent ICM (ref. 6). Moreover, both cessation and overexpression of Oct4 cause exit from ESC self-renewal^{7,8}. Oct4 is also sufficient to trigger reprogramming of mouse and human somatic cells in the absence of other reprogramming transgenes, albeit with decreased efficiency and delayed kinetics⁹⁻¹².

Here, we established a system using *Oct4*^{-/-} somatic cells and 2i/LIF culture medium, containing LIF and inhibitors of mitogen-activated protein kinase signalling and glycogen synthase kinase-3 β . Using this, we uncovered the existence of biological roles of Oct4 that have a critical impact on pluripotency acquisition, self-renewal and on *in vitro* and *in vivo* cell differentiation.

RESULTS

An ESC level of Oct4 marks acquisition of naive pluripotency

To investigate Oct4 function during induced pluripotency we generated *Oct4*^{-/-} neural stem cells (NSCs; Supplementary Fig. S1a-e). *Oct4*^{-/-} and control *Oct4*^{+/-} NSCs were transduced with retroviruses expressing c-Myc and Klf4 (rMK) and transfected with a piggyBac (PB) vector containing a ubiquitous promoter (CAG) driving Oct4 expression (PB-Oct4; Fig. 1a). The CAG promoter, unlike the retroviral promoter, does not undergo silencing during reprogramming. This strategy produced reprogramming intermediates in serum/LIF conditions that, on medium switch to 2i/LIF, formed induced pluripotent stem cell (iPSC) colonies (Fig. 1b). PB-Oct4 iPSCs^{-/-} exhibited silencing of retroviral transgenes and upregulation of naive pluripotency markers (Fig. 1c,d). Strikingly, the total Oct4 level was similar between iPSCs^{+/-}, iPSCs^{-/-} and ESCs (Fig. 1e). The absence of endogenous Oct4 expression in iPSCs^{-/-} was confirmed by genotyping (Fig. 1f) and undetected expression of the *Oct4* 3'UTR, which is absent in the knockout loci and PB-Oct4 transgene (Fig. 1g). Acquisition of a naive pluripotent cell state was further confirmed by the loss of the trimethyl(me3)H3K27 nuclear focus indicative of X chromosome reactivation (Fig. 1h). Thus, we generated and maintained iPSCs^{-/-} dependent exclusively on constitutively expressed PB-Oct4 transgene. We also observed that, independently of the source of Oct4 expression, iPSCs exhibit an ESC level of Oct4 transcript on pluripotency establishment.

To monitor PB transgene expression at the single-cell level during reprogramming, we used a PB-Oct4.2A.Cherry construct. Consistent with gene expression data (Fig. 1e), reprogramming intermediates showed a strong Cherry signal, whereas iPSCs^{-/-} obtained after 2i/LIF induction demonstrated a lower Cherry expression level (Fig. 1i,j and Supplementary Fig. S1f). Notably, in each experiment PB-Oct4.2A.Cherry iPSCs^{-/-} represented a pool of hundreds of colonies formed as a result of multiple independent reprogramming events. Importantly, the pools of iPSCs^{-/-} obtained in independent experiments demonstrated a similar small range of Cherry and, thereby, Oct4 expression (Fig. 1k). This indicates selection and/or modulation of Oct4 transgene expression during reprogramming. The PB-Oct4.2A.Cherry iPSCs^{-/-} had a global gene expression profile similar to ESCs, with only 17 genes differentially expressed by more than twofold (Fig. 1l). To assess whether a particular level of Oct4 transgene expression facilitates reprogramming intermediates to transit into naive pluripotency, we sorted the highest and lowest Cherry-expressing cells and plated these in 2i/LIF. We did not observe any difference in the ability of Oct4 high- and low-expressing cell fractions to undergo reprogramming (Fig. 1m).

Importantly, obtained iPSCs^{-/-} exhibited similar Cherry expression profiles (Fig. 1n). Combined gene expression and western blot analysis of independently derived iPSC^{-/-} lines further confirmed that these exhibit an ESC level of Oct4 on entry into the pluripotent cell state (Fig. 1o,p and Supplementary Fig. S1g). We also generated *Oct4*^{-/-} iPSCs from an independent somatic cell type, mouse embryonic fibroblasts, and, consistently, these exhibited an ESC level of Oct4 expression (Supplementary Fig. S1h). This shows that pluripotent cell state acquisition and/or maintenance requires modulation of Oct4 transgene expression to an ESC level.

Low Oct4 expression sustains self-renewal

It is established that abolishment of Oct4 expression in ESCs in serum/LIF leads to differentiation towards trophectoderm⁷. As the Oct4 transgene in the PB vector is flanked by *LoxP* sites and our iPSCs^{-/-} express 4-hydroxytamoxifen (4OHT)-inducible Cre recombinase, we tested these for the capacity to undergo trophectoderm differentiation on Oct4 deletion. Consistent with previous reports, 4OHT treatment in serum/LIF resulted in some trophectoderm differentiation judging by morphology and expression of trophectoderm marker Pl-1 (Fig. 2a,b). However, Oct4 expression was not completely abolished (Fig. 2c). As cells probably contain multiple PB transgene integrations, this indicates that not all of the inserts were excised. Surprisingly, an average 12-fold reduction in Oct4 expression level did not affect the average expression of naive pluripotency markers Nanog and Rex1 (Fig. 2c). When the same cells were treated with 4OHT in 2i/LIF conditions (Fig. 2d), we observed a sevenfold reduction in the Oct4 level (Fig. 2e). Again, both Rex1 and Nanog expression remained unchanged, indicating that these cells maintain a naive pluripotent cell state (Fig. 2e). Oct4 and Nanog immunocytochemistry revealed a wide range of Oct4 expression in 4OHT-treated cells, with some of the Oct4-low cells showing strong Nanog signal, above that of control cells (Fig. 2f and Supplementary Fig. S2a).

We picked a colony of 4OHT-treated iPSCs^{-/-} and established a cell line with reduced Oct4 transcript and protein levels (Fig. 2h,i). It retained ESC morphology (Fig. 2g), Nanog expression (Fig. 2h) and could be serially passaged without any signs of differentiation. To validate this, we 4OHT-treated an independent cell line, PB-Oct4.2A.Cherry iPSCs^{-/-}, and subsequently single-cell sorted them for low Cherry-expressing cells. This allowed establishment of several Oct4-low iPSC^{-/-} lines (Fig. 2j). These clones exhibited a similar decrease in Oct4 transcript and protein levels (Fig. 2k,l) and retained expression of naive pluripotency genes at levels comparable to ESCs (Fig. 2m). To further demonstrate that, despite reduced Oct4 expression, Oct4-low iPSCs^{-/-} match the molecular criteria of the naive pluripotent state, we performed global gene expression analysis of these in 2i/LIF. This revealed a very similar profile to parental iPSCs^{-/-} with an ESC Oct4 level (Oct4-WT iPSCs^{-/-}; Fig. 2n), and to ESCs (data not shown). We also confirmed that Oct4-low cells do not express epiblast stem cell markers (Supplementary Fig. S2b). Moreover, Oct4-low iPSCs^{-/-} contained unmethylated Nanog and Oct4 regulatory regions (Supplementary Fig. S2c), and exhibited absence of the H3K27me3 nuclear focus (Supplementary Fig. S2d).

To determine the highest Oct4 expression level permissive of pluripotent cell self-renewal, we transfected wild-type ESCs with PB-Oct4.2A.Cherry construct. Consistent with previous findings⁷, we observed that the cells expressing the highest level of Cherry had differentiated morphology and were lost on passaging (Supplementary Fig. S2e). Self-renewing cells exhibited lower Cherry levels than cells transfected with PB-Cherry alone, further indicating that high Oct4 expression is detrimental for the pluripotent state (Supplementary Fig. S2f). We picked and expanded the highest Cherry-expressing ESC-like clones. All demonstrated similar total Oct4 levels but lower endogenous Oct4 when compared with parental ESCs (Supplementary Fig. S2g). They also had lower Cherry expression levels than PB-Oct4.2A.Cherry iPSCs^{-/-} (Supplementary Fig. S2h). This

indicates that Oct4 transgene expression is compatible with self-renewal only if ESCs^{+/+} compensate by equally downregulating endogenous Oct4 expression. We also overexpressed Oct4 episomally¹³ in ESCs to determine which differentiation genes become upregulated. An average 11-fold Oct4 overexpression led to significant upregulation of differentiation markers representing all three embryonic lineages: Gata4; Zeb2 and Snai2; and Sox1 (Supplementary Fig. S2i).

In conclusion, expression of up to fivefold lower levels of Oct4 sustains naive pluripotent cell self-renewal. This signifies that an ESC level of Oct4 expression is a requirement for pluripotency entry but not self-renewal.

***In vitro* differentiation requires an ESC level of Oct4**

To further define the properties of Oct4-low and Oct4-WT iPSCs^{-/-} we analysed their ability to differentiate *in vitro*. Despite constitutive expression of the Oct4 transgene, Oct4-WT iPSCs^{-/-} underwent efficient embryoid body differentiation into all three germ layers, as judged by the upregulation of mesoderm, endoderm and ectoderm markers (Fig. 3a). Efficient E-cadherin downregulation demonstrated successful epithelial-to-mesenchymal transition (Fig. 3a). These also efficiently formed beating heart cells on embryoid body outgrowth (Supplementary Video S1). Strikingly, Oct4-low iPSCs^{-/-} differentiated poorly in embryoid body assays, exhibiting failure to downregulate pluripotent gene expression and to upregulate differentiation markers (Fig. 3b and Supplementary Fig. S3a). They also showed trophoblast marker upregulation (Fig. 3b and Supplementary Fig. S3a) and did not form beating heart cells. Importantly, restoration of Oct4 expression to an ESC level in Oct4-low iPSCs^{-/-} by the introduction of a CAG-Oct4 transgene rescued their differentiation defect (Fig. 3b). In addition, whereas Oct4-WT iPSCs^{-/-} differentiated into the neural lineage in the monolayer protocol¹⁴, judging by Sox1 upregulation and the formation of β -III tubulin-positive neurons, Oct4-low iPSCs^{-/-} failed to downregulate Nanog and Rex1 and to upregulate neural lineage markers (Fig. 3c,d and Supplementary Fig. S3b,c). Importantly, restoration of Oct4 expression to an ESC level in Oct4-low cells reinstated their ability to form neural lineage (Supplementary Fig. S3b,c).

These data show that Oct4 expression at an ESC level is required for efficient *in vitro* differentiation.

***In vivo* differentiation requires an ESC level of Oct4**

To address the ability of PB-Oct4.2A.Cherry and PB-Oct4.2A.Cherry (low) iPSCs^{-/-} to contribute to mouse development we performed morula aggregations. Consistent with their naive pluripotent state, by the blastocyst stage both iPSC^{-/-} lines efficiently incorporated into the pre-implantation epiblast (Fig. 4a). Despite constitutive Oct4 expression, embryonic day (E)6.5 PB-Oct4.2A.Cherry chimaeric embryos appeared normal with the whole epiblast consisting of Cherry-positive cells (Fig. 4b). Strikingly, despite efficiently incorporating into the naive epiblast, PB-Oct4.2A.Cherry (low) iPSCs^{-/-} failed to proceed in development. Only 4/30 E6.5 embryos showed some contribution to the post-implantation epiblast (Fig. 4b and Supplementary Fig. S4a). The remaining embryos were either Cherry-negative or contained Cherry-positive cells between the epiblast and trophoblast (Fig. 4b,c and Supplementary Fig. S4a,b). These remaining embryos were lost on *in vitro* culture of the embryos (Supplementary Fig. S4c), potentially reflecting cell exclusion from development due to failure to differentiate. This is supported by the fact that when compared with the blastocyst stage, few E6.5 embryos show the presence of Cherry-positive cells. To validate this, we dissociated PB-Oct4.2A.Cherry (low) E6.5 chimaeric embryos into single-cell suspension and plated them in 2i/LIF, which would select for cells remaining in a naive pluripotent state. As a result, we observed the emergence of numerous Cherry-positive

colonies (Supplementary Fig. S4d), which retained Nanog, Klf4 and Rex1 expression (Supplementary Fig. S4e). This indicates that most, if not all, of the PB-Oct4.2A.Cherry (low) cells retained a naive pluripotent state. Together, these results highlight a requirement for an ESC level of Oct4 expression for post-implantation development. In agreement with this, restoration of Oct4 expression to an ESC level rescued the ability of PB-Oct4.2A.Cherry (low) iPSCs^{-/-} to efficiently incorporate into the post-implantation embryo (Fig. 4d).

Subsequently, we analysed PB-Oct4.2A.Cherry iPSC^{-/-} E7.5 chimaeric embryos. Strikingly, constitutive Oct4 expression at an ESC level was compatible with contribution to all embryonic lineages (Fig. 4e,f and Supplementary Fig. S4f), specifically, early mesoderm marked by T-Brachyury¹⁵ and presumptive neuroectoderm marked by Sox2 (ref. 16; Fig. 4f). Foxa2, marking early progenitors of all germ layers at the anterior primitive streak¹⁷, was largely co-expressed with Cherry in the chimaeric embryos (Fig. 4f). Moreover, Cherry was co-expressed with Sox17 exclusively in the inner layer of Sox17-positive cells, representing nascent definitive endoderm¹⁸ (Fig. 4f).

At E8.5, PB-Oct4.2A.Cherry embryos appeared morphologically normal and, judging by Cherry and Oct4 protein expression, virtually all embryonic tissues were of iPSC^{-/-} origin (Fig. 4g,h). However, chimaeric embryos inefficiently proceeded in development after this stage (Supplementary Fig. S4g). This is also a developmental stage when Oct4 expression becomes restricted to the germ lineage^{5,19}. As obtained embryos demonstrated high if not 100% chimaerism, we performed blastocyst injections with 1–5 cells to determine whether chimaeric embryos can develop further in the presence of host embryo cells. Widespread iPSC^{-/-} contribution was observed in E12.5 chimaeric embryos, which was the latest time point analysed (Supplementary Fig. S4h). Analysis of genital ridges at E12.5 demonstrated contribution of PB-Oct4.2A.Cherry iPSCs^{-/-} to the germline (Fig. 4i). We also observed that PB-Oct4.2A.Cherry iPSCs^{-/-} are more efficient at entering embryonic development than control ESCs (Fig. 4j,k). This suggests that premature loss of Oct4 expression in ESCs on embryo injection leads to decreased chimaerism and further shows that Oct4 facilitates cell state transitions during pluripotent state exit. We also performed teratoma assays for Oct4-WT and Oct4-low iPSCs^{-/-}. Whereas teratomas derived from Oct4-WT and rescue iPSCs^{-/-} contained various tissues representing all three embryonic lineages, teratomas derived from the two Oct4-low iPSC^{-/-} lines had only discernible areas of trophectoderm-like and undifferentiated cells (Supplementary Fig. S5).

In summary, Oct4 expression at an ESC level is required for efficient *in vivo* differentiation into all three germ layers.

Oct4 genomic binding is converse to Nanog and is linked to downregulation of naive pluripotency genes

Pluripotent cell cultures in 2i/LIF are homogeneous and do not contain differentiated cells or cells primed for differentiation. However, pluripotent cultures in serum/LIF have subpopulations of cells with variable levels of naive pluripotency marker expression^{20–24}. We analysed these in Oct4-WT and Oct4-low iPSCs^{-/-} in 2i/LIF compared with serum/LIF in the presence of selection for *Oct4* promoter activity to eliminate differentiated cells (Supplementary Fig. S1b). There were no major differences in pluripotency marker expression, at both transcript and protein levels, between analysed cell lines in 2i/LIF (Fig. 5a,c). However, in serum/LIF, we observed higher expression levels of naive pluripotency markers in all of the Oct4-low iPSC^{-/-} lines, at both transcript and protein levels, when compared with parental iPSC^{-/-} and rescued Oct4-low iPSCs^{-/-} (Fig. 5b,d). The observed expression differences could not be attributed to differentiation (Supplementary Fig. S6a,b). To gain insight into why Oct4-low cells fail to downregulate naive pluripotency gene

expression, we performed chromatin immunoprecipitation (ChIP) for Oct4, Nanog and Esrrb in serum/LIF conditions in Oct4-low and Oct4-WT cells at regulatory sequences of key naive pluripotency genes. Strikingly, Oct4 genomic occupancy was markedly reduced and Nanog and Esrrb significantly increased at these targets in Oct4-low cells (Fig. 5e,f and Supplementary Fig. S6c). This suggests that in suboptimal self-renewing culture conditions and on induction of cell differentiation Oct4 can act as a repressor of key naive pluripotency genes and that a reduction in the Oct4 level leads to an incapacity to downregulate these.

To assess whether the phenotype of Oct4-low cells could be attributed to the high levels of Nanog, we performed Nanog knockdown in Oct4-low cells and subjected these to either a self-renewal assay at clonal density in serum minus LIF or to embryoid body differentiation. We confirmed Nanog knockdown and observed decreased expression of Klf4 and Esrrb, known Nanog targets²⁵. However, the expression of these and other naive pluripotency genes remained higher than in self-renewing Oct4-WT cells (Supplementary Fig. S6d). Oct4-low cells with Nanog knockdown did not differentiate (Supplementary Fig. S6e,f), suggesting that ongoing high-level expression of other naive pluripotency genes such as *Klf2* and *Tbx3*, which can confer LIF-independent self-renewal^{26,27}, prevents differentiation. Together, these data further confirm that Oct4-low cells are robustly locked in a self-renewing state.

Low Oct4 is sufficient to sustain self-renewal in the absence of pluripotent culture requisites

Failure to differentiate together with robust expression of naive pluripotency genes led us to investigate whether Oct4-low iPSCs^{-/-} can self-renew after removal of 2i and LIF from the serum-free medium (N2B27). Under these conditions, pluripotent cells undergo differentiation^{14,28}. Oct4-WT iPSCs^{-/-} were treated with 4OHT for 24 h, which induced partial Oct4 transgene loss (Fig. 2e), and then switched to N2B27 with selection for *Oct4* promoter activity to eliminate differentiated cells. In contrast to untreated cells, which differentiated and died after the first passage, we were able to maintain 4OHT-treated cells indefinitely (Fig. 6a). These cells expressed naive pluripotency markers but lost *Socs3* and downregulated *Klf4* expression, indicating an absence of LIF/STAT3 signalling (Fig. 6b and Supplementary Fig. S7a). Oct4 transgene expression was decreased by at least 2.5-fold (Fig. 6b and Supplementary Fig. S7a,b). These cells self-renewed at clonal density (Fig. 6c and Supplementary Fig. S7c) and expressed Nanog protein (Fig. 6d and Supplementary Fig. S7d). We were also able to maintain previously established (Fig. 2j-l) Oct4-low iPSC^{-/-} clones indefinitely in N2B27 (Fig. 6e,f). Nanog binding at key genomic targets in these cells was also maintained (Fig. 6g).

To confirm our observations in an independent system we established ESCs with low Oct4 expression levels. To derive these, we co-transfected *Oct4^{lox/-}* ESCs with CAG-Oct4 and CAG-CreERT2 transgenes. Similarly to the above results (Supplementary Fig. S2g), the obtained self-renewing cells exhibited an ESC level of Oct4 (Fig. 7c). Subsequently, we treated these with 4OHT to induce Cre-mediated excision of the floxed *Oct4* allele but not the CAG-Oct4 transgene, as the latter was not *loxP*-flanked (Fig. 7a). The cells were then switched to N2B27 with selection to eliminate non-self-renewing cells. Untreated cells differentiated and died after the first passage. Consistent with iPSCs^{-/-}, 4OHT-treated ESCs (CAG-Oct4 ESCs^{-/-}) exhibited 5–7-fold lower than ESC Oct4 levels and could be maintained in N2B27 indefinitely (Fig. 7b,c). Moreover, when placed in serum/LIF, they exhibited higher expression levels of naive pluripotency markers than parental cells (Fig. 7d,e). Furthermore, CAG-Oct4 ESCs^{-/-} failed to undergo embryoid body and neural differentiation, instead remaining locked in a naive pluripotent state (Fig. 7f–h and Supplementary Fig. S7e).

Next we investigated whether the DNA-binding capacity of Oct4 is important for its function in cell differentiation. We used the Oct4-267VP mutant, which cannot bind DNA or sustain ESC self-renewal in doxycycline-repressible ZHBTc4.1 cells^{29,30} (Supplementary Fig. S7f). We transfected Oct4-low ESCs^{-/-} with the PB-Oct4-267VP transgene and established lines with an ESC level of total Oct4 (Supplementary Fig. S7g). Importantly, mutant Oct4 could not rescue the differentiation defect of Oct4-low ESCs^{-/-} (Supplementary Fig. S7g), demonstrating that the capacity of Oct4 to bind DNA is required for differentiation.

In summary, the naive pluripotent state can be maintained in the absence of pluripotency medium requisites, if the Oct4 expression level is decreased.

DISCUSSION

In this study, using optimized culture conditions for the induction and maintenance of pluripotent cells^{31,32} and *Oct4*^{-/-} somatic cells as a starting point to provide a stringent functional assay, we demonstrated that a defined Oct4 level is critical for naive pluripotency acquisition. Once pluripotency is established, Oct4 levels can be decreased without loss of self-renewal. However, an ESC level of Oct4 is then required for the efficient downregulation of the naive pluripotent program during cell differentiation (Fig. 8).

It has previously been proposed that more than 50% Oct4 downregulation would lead to trophoblast differentiation in doxycycline-repressible ZHBTc4.1 ESCs (ref. 7). This apparently differs from our observations. However, we found that by titrating the doxycycline concentration self-renewing Oct4-low ZHBTc4.1 ESCs with around 30% of the starting Oct4 level could be established (Supplementary Fig. S8).

Several studies reported the involvement of Oct4 in ESC differentiation *in vitro*. Ref. 33 claimed that Oct4 suppresses neuroectoderm differentiation and promotes mesendoderm differentiation of mouse ESCs *in vitro*. Ref. 34 claimed that Oct4 suppresses human ESC differentiation into definitive endoderm. Both studies, however, are based on Oct4 overexpression and knockdown experiments in ESCs. In contrast, our system allowed investigation of the effect of biological Oct4 levels on pluripotent cell differentiation. Thus, and in apparent disagreement with what was expected from the published literature, constitutive Oct4 expression at an ESC level led to a robust contribution to nascent ectoderm, mesoderm, endoderm and germline in the embryo. This result is however consistent with the observed Oct4 expression in the progeny of all germ layers until the late somite stage^{5,19}.

It was reported that episomal overexpression of an Oct4 DNA-binding mutant (Oct4-267VP; ref. 30) causes spontaneous ESC differentiation²⁹. In our system, this did not rescue differentiation defects of Oct4-low cells. As the transactivation activity of Oct4-267VP depends on the recruitment by a functional POU factor³⁰, we believe that Oct4-267VP induces differentiation only on strong overexpression and in the presence of an ESC level of wild-type Oct4.

In conclusion, this study redefines our previous understanding of the biological roles of Oct4 from a factor known to be important for reprogramming and self-renewal^{6,7} to one also actively controlling cell state transitions during entry into and exit from the naive pluripotent state. The future challenge will be to define the molecular mechanisms underlying this dual function of Oct4.

METHODS

Plasmids

pMXs-Oct3/4, pMXs-Klf4, pMXs-cMyc (Addgene); pPyCAG-Oct4-IRES-Zeo, pPyCAG-Oct4-IRES-Puro (Austin Smith); pPB-CAG-DEST-pA-pgk-hph, pCAG-CreERT2^{NLS}-IRES-BSD (Joerg Betschinger); pCyL43 (PBase; from Sanger Institute's plasmid repository), pOct4-267VP_Entry (Integrated DNA Technologies).

Cell culture

Mouse embryonic fibroblasts (MEFs), reprogramming intermediates and PLAT-E cells were cultured in GMEM (Sigma-Aldrich) containing 10% FCS (Sigma-Aldrich), 1× NEAA (PAA), 1× penicillin/streptomycin (PAA), 1 mM sodium pyruvate (PAA), 0.1 mM 2-mercaptoethanol (Gibco) and 2 mM L-glutamine (Gibco), supplemented with 20 ng ml⁻¹ of LIF (serum/LIF medium). Except where specifically indicated otherwise, ESCs and iPSCs were cultured in GMEM containing 10% KSR (Invitrogen), 1% FCS, 1× NEAA, 1× penicillin/streptomycin, 1 mM sodium pyruvate, 0.1 mM 2-mercaptoethanol, 2 mM L-glutamine, 20 ng ml⁻¹ of LIF and 2i inhibitors: CHIR99021 (3 μM) and PD0325901 (1 μM; ref. 32; 2i/LIF medium). NSCs were maintained in NSC medium (DMEM/F-12 (Gibco), 1× NEAA, 0.1 mM 2-mercaptoethanol, 1× penicillin/streptomycin, 1:100 B27 supplement (Invitrogen), 1:200 N2 supplement (PAA), 4.5 μM HEPES (PAA), 0.03 M glucose (Sigma-Aldrich) and 120 μg ml⁻¹ BSA (Invitrogen)) supplemented with 10 ng ml⁻¹ of Egf (Peprotech) and 20 ng ml⁻¹ of Fgf2 (home-made). Epiblast stem cells were cultured in N2B27 medium (DMEM/F12 and Neurobasal (both Gibco) in a 1:1 ratio, 1× penicillin/streptomycin, 0.1 mM 2-mercaptoethanol, 2 mM L-glutamine, 1:200 N2 (StemCells) and 1:100 B27 supplement) supplemented with 12 ng ml⁻¹ Fgf2 and 20 ng ml⁻¹ Activin A (home-made). 4OHT was used at a concentration of 500 nM and geneticin (G418) at 400 μg ml⁻¹.

Reprogramming experiments

For retrovirus production PLAT-E cells were transfected with pMXs plasmids using FuGENE 6 (Roche). In 48 h virus-containing supernatants were filtered through 0.45 μm cellulose acetate filters and mixed with Polybrene (Sigma-Aldrich) to a final concentration of 4 μg ml⁻¹. The Polybrene/virus mixture was applied to NSCs or MEFs for 24 h, after that NSCs were nucleofected with a 5:1 mixture of PB-Oct4 and pBase plasmids. Transfected NSCs were cultured for three days in NSC medium and then switched to serum/LIF conditions. The PB-Oct4 transgene was introduced into MEFs 7 days post-infection. To obtain iPSCs the generated reprogramming intermediates were cultured in 2i/LIF medium.

Cell immunostaining

The following primary antibodies dilutions were used: mouse monoclonal against Oct4 (1:100) from Santa Cruz Biotechnology (C-10, Cat. No.: sc-5279), rabbit polyclonal against me3H3K27 (1:500) from Upstate (Cat. No.: 07-449), rat monoclonal against Nanog (1:500) from eBiosciences (eBioMLC-51, Cat. No.: 14-5761-80) and mouse monoclonal against β-III tubulin (1:200) from R&D Systems (TuJ-1, Cat. No.: MAB1195).

Embryo and genital ridge immunostaining

The following primary antibodies dilutions were used: goat polyclonal against Sox17 (1:100) from R&D (Cat. No.: AF1924), goat polyclonal against Foxa2 (1:200) from Santa Cruz Biotechnology (Cat. No.: sc-6554 M-20), goat polyclonal against Brachyury (1:100) from R&D (Cat. No.: AF2085), rabbit polyclonal against Sox2 (1:200) from Abcam (Cat. No.: AB97959), rabbit polyclonal against Mvh (1:500) from Abcam (Cat. No.: AB13840).

and mouse monoclonal against Oct4 (1:200) from Santa Cruz Biotechnology (C-10, Cat. No.: sc-5279).

RNA isolation, cDNA synthesis, qRT-PCR

Total RNA was isolated from cells using the RNeasy mini kit (QIAGEN) in accordance with the manufacturer's protocol. One microgram of total RNA was reverse-transcribed using SuperScript III First-Strand Synthesis SuperMix (Invitrogen). Quantitative PCR with reverse transcription (qRT-PCR) reactions were set up in triplicate using either TaqMan Universal PCR Master Mix or Fast SYBR Green Master Mix (both Applied Biosystems) and either TaqMan gene expression assays (Applied Biosystems) or primers, respectively (see Supplementary Table S1). qRT-PCR experiments were performed on a StepOnePlus Real Time PCR System (Applied Biosystems). Delta Ct values with Gapdh were calculated and raised to the power of -2 . The means of three values were calculated and normalized as indicated.

Alkaline phosphatase staining

Cells were fixed in citrate-acetone-formaldehyde and stained using the Alkaline Phosphatase kit (Sigma-Aldrich) according to the manufacturer's instructions.

Cell differentiation

For embryoid body differentiation 1.5×10^6 cells were plated in 10 cm low-attachment dishes in serum-containing medium without LIF. At day 7 embryoid bodies were plated on gelatin and the obtained outgrowths were analysed for differentiation into beating heart cells. For neural induction in monolayer culture, cells were plated on gelatin in N2B27 conditions and were cultured for 7 days.

ChIP

Oct4 ChIP was performed using the ChIP-IT High Sensitivity kit (Active Motif) according to the manufacturer's instructions. Nanog and Esrrb ChIPs were performed as follows: cells (8×10^6 for each sample) were fixed for 10 min in 1% formaldehyde, washed with ice-cold PBS and incubated for 5 min in lysis buffer 1 (50 mM HEPES at pH 7.5, 140 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% NP40 and 0.25% Triton X-100) and then for 5 min in lysis buffer 2 (10 mM Tris at pH 8.0, 200 mM NaCl, 1 mM EDTA and 0.5 mM EGTA). Nuclei were pelleted, resuspended in shearing buffer (1% SDS, 10 mM EDTA and 50 mM Tris at pH 8.0) and sonicated to obtain an average DNA fragment size of 500 base pairs. Lysates were diluted 1:10 in dilution buffer (50 mM Tris-HCl at pH 8.0, 167 mM NaCl, 1.1% Triton X-100 and 0.11% Na deoxycholate) and pre-cleared for 2 h at 4 °C with Dynabeads magnetic beads (Invitrogen) that were pre-incubated with isotype IgG antibody. The chromatin was then incubated overnight at 4 °C with 4 µg of antibody or an isotype IgG control (Santa Cruz Biotechnology, sc-2025 or sc-2027). Lysates were then incubated for 1 h at 4 °C with blocked Dynabeads magnetic beads, and the beads were washed twice in wash buffer 1 (50 mM Tris-HCl at pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 0.1% Na deoxycholate and 0.5 mM EGTA), once in wash buffer 2 (50 mM Tris-HCl at pH 8.0, 500 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 0.1% Na deoxycholate and 0.5 mM EGTA), once in wash buffer 3 (50 mM Tris at pH 8.0, 250 mM LiCl, 0.5% Na deoxycholate, 0.5% NP40, 1 mM EDTA and 0.5 mM EGTA) and twice in wash buffer 4 (50 mM Tris at pH 8.0, 10 mM EDTA and 5 mM EGTA). Chromatin was eluted for 30 min at room temperature in elution buffer (1% SDS and 0.1 M NaHCO₃). Samples were incubated overnight at 65 °C to reverse the crosslinking and purified using the QIAquick PCR Purification kit (Qiagen). Chromatin was analysed by TaqMan or SYBR green qRT-PCR. Enrichment was calculated relative to the IgG ChIP. The following

antibodies were used: goat polyclonal against Oct4 from Santa Cruz Biotechnology (Cat. No.: sc-8628), rabbit polyclonal against Nanog from Bethyl Laboratories (Cat. No.: A300-397A) and mouse monoclonal against Esrrb from R&D Systems (H6705, Cat. No.: PP-H6705-00).

Flow cytometry

Flow cytometry was performed using a BD LSRFortessa analyser with subsequent data analysis using FlowJo software. Cell sorting was performed using a MoFlo high-speed cell sorter. mCherry was excited using a 561 nm laser and detected using a 610/20 filter.

Western blotting

The following primary antibodies dilutions were used: mouse monoclonal against Oct4 (C-10; 1:500) from Santa Cruz Biotechnology (C-10, Cat. No.: sc-5279), mouse monoclonal against α -tubulin (1:5,000) from Abcam (Cat. No.: AB7291), mouse monoclonal against Esrrb (1:500) from R&D Systems (H6705, Cat. No.: PP-H6705-00), goat polyclonal against Klf4 (1:1,000) from R&D Systems (Cat. No.: AF3158) and rabbit polyclonal against Nanog (1:5,000) from Bethyl Laboratories (Cat. No.: A300-397A). For quantitative western analysis, Oct4 or α -tubulin immunoreactivity was detected with an anti-mouse infrared IRDye-labelled 800CW secondary antibody (1:15,000) from LI-COR. Nitrocellulose membranes were analysed with an Odyssey Near Infra-Red Scanner. The intensity of the Oct4 bands was normalized to α -tubulin.

Bisulphite sequencing

Genomic DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen). Bisulphite treatment was performed using the EpiTect Bisulfite Kit (Qiagen). Amplified products were cloned into pCR2.1-TOPO (Invitrogen). Randomly selected clones were sequenced and analysed using the Quantification Tool for Methylation Analysis (<http://quma.cdb.riken.jp/>).

NSC and MEF derivation

For NSC derivation, brains of E13.5 embryos were dissected and mechanically dissociated by resuspending in NSC medium. The obtained single-cell suspension was plated onto laminin-treated cell culture dishes. For MEF derivation, heads and all of the internal organs, including gonads, were removed from E13.5 embryos. The remaining embryo parts were cut into small pieces, trypsinized and plated in serum/LIF medium.

Cell transfection

Nucleofections were performed using Amaxa Nucleofection Technology (Lonza AG) according to the manufacturer's instructions. Program T-020 was used for all of the NSC nucleofections. One million cells were taken per nucleofection. Lipofection was performed using Lipofectamine 2000 (Invitrogen) or FuGENE 6 (Roche) according to the manufacturer's instructions. siRNA transfections were performed using RNAiMAX (Invitrogen) transfection reagent according to the manufacturer's instructions.

Morula aggregation and blastocyst injection

For blastocyst injection, standard microinjection methodology using host blastocysts of C57BL/6 strain was employed. For morula aggregation the cells were combined with E2.5 MF1 morulae and either cultured for 48 h to assess the integration into ICM or transferred to recipient mice to assess the contribution to development at later stages.

Embryo culture

E6.5 embryos were cultured in 4-well plates in a 1:1 mixture of heat-inactivated rat serum and GMEM in 5% CO₂ in air.

Teratoma assay

iPSCs were injected under the kidney capsule of anaesthetized B6 mice. Four weeks after the injection tumours were surgically dissected from the mice. Samples were fixed in PBS containing 4% formaldehyde and embedded in paraffin. Sections were stained with haematoxylin and eosin.

Microarray

Amplification and labelling of RNA were performed using the TotalPrep-96 RNA Amplification Kit for the Illumina platform (Ambion). Subsequent hybridization, staining and scanning were performed according to the Whole Genome Gene Expression Direct Hybridization Guide on the MouseWG-6 v2.0 Expression BeadChip (Illumina). Data were loaded into the R package lumi³⁵ and then divided into subsets to be analysed. The data were transformed using variance stabilization³⁶ and normalized using quantile normalization. Comparisons were performed in the R package limma³⁷ and the results were corrected using the false discovery rate. Our analysis employed a 5% confidence interval. The GEO number is GSE45003.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

An ESC level of Oct4 marks pluripotency acquisition. **(a)** Generation of rMK+PB-Oct4 iPSCs^{-/-}. Reprogramming intermediates are represented in orange, and iPSCs in yellow. **(b)** Phase images and alkaline phosphatase (AP) staining of rMK+PB-Oct4 iPSCs^{+/+} and iPSCs^{-/-}. **(c–e)** Quantitative PCR with reverse transcription (qRT-PCR) analysis of retroviral transgenes **(c)**, pluripotency markers **(d)** and total Oct4 **(e)** expression in rMK+PB-Oct4 cells before and after 2i/LIF induction. Serum/LIF indicates reprogramming intermediates; 2i/LIF indicates iPSCs. ESCs were grown in 2i/LIF. Data shown are the mean of 3 replicates and are from 1 of 3 representative experiments. **(f)** *Oct4* locus genotyping for rMK+PB-Oct4 iPSCs^{+/+} and iPSCs^{-/-} and control *Oct4*^{-/-} NSCs (NSCs^{-/-}), *Oct4*^{flox/-} (ESCs^{F/-}) and *Oct4*^{flox/+} (ESCs^{F/+}) ESCs. **(g)** qRT-PCR analysis of *Oct4* 3'UTR expression in rMK+PB-Oct4 iPSCs^{+/+} and iPSCs^{-/-}. Data shown are the mean of 3 replicates and are

from 1 of 2 representative experiments. **(h)** me3H3K27 immunostaining of NSCs^{-/-} and rMK+PB-Oct4 iPSCs^{-/-}. White arrowheads indicate representative inactive X chromosomes. **(i)** Phase and Cherry images of rMK+PB-Oct4.2A.Cherry reprogramming intermediates, the rMK+PB-Oct4.2A.Cherry iPSC^{-/-} colony with surrounding Cherry-high reprogramming intermediates (white arrowheads) in 2i/LIF and of the established rMK+PB-Oct4.2A.Cherry iPSC^{-/-} line. **(j,k)** Cherry flow cytometry analysis of rMK+PB-Oct4.2A.Cherry *Oct4*^{-/-} cells before and after 2i/LIF induction **(j)** and of two independently derived pools of rMK+PB-Oct4.2A.Cherry iPSCs^{-/-} **(k)**. Serum/LIF indicates reprogramming intermediates; 2i/LIF indicates iPSCs. **(l)** Scatter plot comparing global gene expression profiles of ESCs and rMK+PB-Oct4.2A.Cherry iPSCs^{-/-}. **(m)** Alkaline phosphatase staining demonstrating the comparable ability of Cherry-high and -low reprogramming intermediates to acquire pluripotency. **(n)** Cherry flow cytometry analysis of rMK+PB-Oct4.2A.Cherry iPSCs^{-/-} derived from Cherry-high and -low reprogramming intermediates. **(o)** qRT-PCR analysis of piggyBac and total Oct4 expression in rMK+PB-Oct4 *Oct4*^{-/-} cells before and after 2i/LIF induction. Serum/LIF (S/L) indicates reprogramming intermediates; 2i/LIF (2i/L) indicates iPSCs. PB-Oct4 1 and 2 indicate biological replicates. ESCs were grown in 2i/LIF. Data shown are the mean of 3 replicates and are from 1 of 2 representative experiments. **(p)** Oct4 protein quantification in rMK+PB-Oct4 cells before and after 2i/LIF induction. Data shown are the mean of 3 independent experiments; error bars represent $\pm$ s.d. See Supplementary Fig. S9 for uncropped data.

Figure 2.

Low levels of Oct4 expression sustain self-renewal. **(a)** Phase images of ESCs and rMK +PB-Oct4 iPSCs^{-/-} treated and untreated with 4OHT in serum/LIF culture conditions. **(b,c)** qRT-PCR analysis of *PI-1* **(b)** and pluripotency markers **(c)** expression in ESCs and rMK +PB-Oct4 iPSCs^{-/-} treated and untreated with 4OHT in serum/LIF culture conditions. Data shown are the mean of 3 replicates and are from 1 of 2 representative experiments. **(d)** Phase images of rMK+PB-Oct4 iPSCs^{-/-} treated and untreated with 4OHT in 2i/LIF culture conditions. **(e)** qRT-PCR analysis of total Oct4, Nanog and Rex1 expression in rMK+PB-Oct4 iPSCs^{-/-} treated and untreated with 4OHT in 2i/LIF culture conditions. Data shown

are the mean of 3 replicates and are from 1 of 2 representative experiments. **(f)** Nanog and Oct4 immunocytochemistry in rMK+PB-Oct4 iPSCs^{-/-} treated and untreated with 4OHT in 2i/LIF culture conditions. **(g)** Phase image of rMK+PB-Oct4 (low) iPSCs^{-/-} in 2i/LIF conditions. **(h)** qRT-PCR analysis of total Oct4 and Nanog expression in PB-Oct4, PB-Oct4 (low) iPSCs^{-/-} and ESCs in 2i/LIF culture conditions. Data shown are the mean of 3 replicates and are from 1 of 2 representative experiments. **(i)** Western blot analysis of Oct4 protein levels in PB-Oct4 and PB-Oct4 (low) iPSCs^{-/-} in 2i/LIF culture conditions. See Supplementary Fig. S9 for uncropped data. **(j)** Flow cytometry analysis of PB-Oct4.2A.Cherry (low) iPSC^{-/-} clones obtained as a result of 4OHT treatment of PB-Oct4.2A.Cherry iPSCs^{-/-} and subsequent single-cell sorting. PB-Oct4.2A.Cherry is indicated as O2C. Clones for which the median is indicated in red were chosen for subsequent analysis. **(k)** qRT-PCR analysis of total *Oct4* expression in PB-Oct4.2A.Cherry (low) iPSC^{-/-} clones 1, 5 and 12. Data shown are the mean of 3 replicates and are from 1 of 2 representative experiments. **(l)** Oct4 protein levels in PB-Oct4.2A.Cherry (low) iPSC^{-/-} clones 1, 5 and 12. **(m)** qRT-PCR analysis of pluripotency gene expression in PB-Oct4.2A.Cherry (low) iPSC^{-/-} clones 1, 5 and 12. Data shown are the mean of 3 replicates and are from 1 of 3 representative experiments. **(n)** Scatter plot comparing global gene expression profiles of PB-Oct4.2A.Cherry iPSCs^{-/-} and PB-Oct4.2A.Cherry (low) iPSC^{-/-} clone 5.

Figure 3.

Oct4 expression at an ESC level is required for *in vitro* differentiation. **(a)** qRT-PCR analysis of pluripotency (total *Oct4*, *Nanog*, *Rex1*), endoderm (*FoxA1*, *Gata4*), ectoderm (*Fgf5*) and mesoderm (*T-Brachyury*, *Zeb2*, *Snai2*, *Nkx2.5*, *N-cadherin*) markers and *E-cadherin* expression during embryoid body differentiation of ESCs^{+/+} and rMK+PB-Oct4 iPSCs^{-/-}. D0–D7 indicate the number of days of differentiation. Data shown are the mean of 3 replicates and are from 1 of 4 representative experiments. **(b)** qRT-PCR analysis of pluripotency (total *Oct4*, *Nanog*, *Rex1*, *Esrrb*), endoderm (*FoxA1*, *Gata4*), ectoderm (*Fgf5*), mesoderm (*T-Brachyury*, *Zeb2*, *Snai2*, *Nkx2.5*, *N-cadherin*) and trophoblast (*Pl-1*) markers and *E-cadherin* expression during embryoid body differentiation of PB-

Oct4.2A.Cherry iPSCs^{-/-}, PB-Oct4.2A.Cherry (low) iPSC^{-/-} clone 1, 5 and 12 iPSC^{-/-} and PB-Oct4.2A.Cherry (low) clone 1 + CAG-Oct4 (rescue line). Data shown are the mean of 3 replicates and are from 1 of 3 representative experiments. **(c)** qRT-PCR analysis of *Sox1*, total *Oct4*, *Rex1* and *Nanog* expression during neural induction by monolayer culture of control ESCs^{+/+}, PB-Oct4 iPSCs^{-/-} and PB-Oct4 (low) iPSCs^{-/-}. D0–D5 indicate the number of days of differentiation. Data shown are the mean of 3 replicates and are from 1 of 2 representative experiments. **(d)** Immunocytochemistry detection of β III-tubulin and Nanog expression after 7 days of neural induction by monolayer culture of control ESCs^{+/+}, PB-Oct4 iPSCs^{-/-} and PB-Oct4 (low) iPSCs^{-/-}.

Figure 4.

Oct4 expression at an ESC level is required for *in vivo* differentiation. **(a)** Phase and Cherry images of embryos obtained 48 h after morula aggregations of PB-Oct4.2A.Cherry or PB-Oct4.2A.Cherry (low) clone 1 iPSCs^{-/-}. Representative contribution to naive epiblast is indicated with white arrowheads. **(b)** Phase and Cherry images of E6.5 embryos obtained as a result of PB-Oct4.2A.Cherry or PB-Oct4.2A.Cherry (low) clone 1 iPSC^{-/-} morula aggregations. **(c)** High-magnification images of E6.5 PB-Oct4.2A.Cherry (low) clone 1 chimaeric embryos. **(d)** Phase and Cherry images of E7.5 embryos obtained after rescuing PB-Oct4.2A.Cherry (low) clone 1 iPSCs^{-/-} with CAG-Oct4. **(e)** Phase and Cherry images of

E7.5 PB-Oct4.2A.Cherry chimaeric embryos. **(f)** E7.5 PB-Oct4.2A.Cherry chimaeric embryos immunostained for Sox17, FoxA2, Sox2 and T-Brachyury. **(g,h)** E8.5 PB-Oct4.2A.Cherry chimaeric embryos. Phase and Cherry images **(g)** and immunostaining for Oct4 **(h)**. **(i)** Confocal images of the genital ridges from E12.5 PB-Oct4.2A.Cherry chimaeric embryos immunostained for Mvh. **(j)** Flow cytometry analysis of PB-Oct4.2A.Cherry iPSCs^{-/-} and ESCs^{+/+} with CAG-Cherry reporter. **(k)** Representative Phase and Cherry images of litters of PB-Oct4.2A.Cherry iPSC^{-/-} and CAG-Cherry ESC^{+/+} chimaeric embryos.

Figure 5.

Oct4 binding is converse to Nanog and is linked to downregulation of naive pluripotency genes. **(a,b)** qRT-PCR analysis of total *Oct4*, *Klf4*, *Nanog*, *Tbx3*, *Esrrb* and *Rex1* expression in PB-Oct4 (Oct4-WT and -low), PB-Oct4.2A.Cherry (Oct4-WT and -low) iPSCs^{-/-} and PB-Oct4.2A.Cherry (low) + CAG-Oct4 iPSC^{-/-} rescue clones in 2i/LIF **(a)** and serum/LIF with selection for geneticin (G418) resistance driven by the *Oct4* promoter (Supplementary Fig. S1b; **b**). Oct4-low lines are indicated with red rectangles. Data shown are the mean of 3 replicates and are from 1 of 2 representative experiments. **(c,d)** Western blot analysis of Oct4, Nanog, Esrrb and Klf4 protein expression in PB-Oct4 (Oct4-WT and -low), PB-Oct4.2A.Cherry (Oct4-WT and -low) iPSCs^{-/-} and PB-Oct4.2A.Cherry (low) +

CAG-Oct4 iPSC^{-/-} rescue clones in 2i/LIF (c) and serum/LIF with selection for geneticin (G418) resistance driven by *Oct4* promoter (d). Oct4-low lines are indicated with red rectangles. See Supplementary Fig. S9 for uncropped data. (e,f) ChIP analysis of Oct4 (e) and Nanog (f) binding at the regulatory regions of pluripotency genes in PB-Oct4.2A.Cherry and PB-Oct4.2A.Cherry (low) iPSCs^{-/-}. Data shown are the mean of 3 replicates and are from 1 of 2 representative experiments.

Figure 6.

Oct4-low iPSCs self-renew in the absence of pluripotent culture requisites. **(a)** Phase images of PB-Oct4 iPSCs^{-/-} treated or untreated with 4OHT for 24 h and subsequently cultured in N2B27 conditions with selection for geneticin (G418) resistance driven by *Oct4* promoter (Supplementary Fig. S1b). Untreated cells differentiated and died after the first passage, whereas treated cells self-renewed indefinitely. **(b)** qRT-PCR analysis of total *Oct4*, *Socs3*, *Nanog*, *Rex1*, *Klf2* and *Klf4* expression in PB-Oct4 iPSCs^{-/-} cultured in 2i/LIF and 4OHT-treated iPSCs^{-/-} cultured for 15 passages in N2B27 conditions. Data shown are the mean of 3 replicates and are from 1 of 2 representative experiments. **(c)** Alkaline phosphatase (AP) staining of 4OHT-treated PB-Oct4 iPSCs^{-/-} (passage 15 in N2B27) plated at clonal density. Two thousand cells were plated per well and cultured for 8 days in N2B27. **(d)** Immunocytochemistry detection of Nanog in 4OHT-treated PB-Oct4 iPSCs^{-/-} cultured in N2B27 conditions. **(e)** Phase image of the previously established (Fig. 2j-l) PB-Oct4.2A.Cherry (low) clone 1 iPSCs^{-/-} cultured in N2B27 conditions. **(f)** qRT-PCR detection of *Socs3*, *Nanog*, *Rex1*, *Esrrb*, *Klf2* and *Klf4* in PB-Oct4.2A.Cherry (low) clone 1 iPSCs^{-/-} cultured in 2i/LIF and N2B27 conditions. Data shown are the mean of 3 replicates and are from 1 of 3 representative experiments. **(g)** ChIP analysis of Nanog binding at main target genes in PB-Oct4.2A.Cherry (low) clone 1 iPSCs^{-/-} cultured in N2B27 conditions for 15 passages. Data shown are the mean of 3 replicates and are from 1 of 2 representative experiments.

Figure 7.

A defined *Oct4* level is also required for downregulation of key naive pluripotency genes in ESCs. **(a)** Experimental design used to obtain *Oct4*-low ESCs. ESCs^{flx/-} were stably transfected with CAG-*Oct4* and CAG-CreERT2 transgenes and subsequently treated with 4OHT to induce Cre-mediated excision of the floxed *Oct4* allele but not the CAG-*Oct4* transgene, which is not flanked by *loxP* sites. **(b)** 4OHT-treated and untreated CAG-*Oct4* ESCs^{flx/-} after the first passage in N2B27 culture conditions with selection (G418) for *Oct4* promoter activity. **(c)** qRT-PCR detection of total *Oct4*, *Socs3*, *Nanog*, *Rex1*, *Esrrb*, *Klf2* and *Klf4* in *Oct4*-low ESCs (CAG-*Oct4* ESCs^{-/-}) cultured in N2B27 conditions for 15

passages in comparison with 4OHT-untreated cells and wild-type ESCs cultured in 2i/LIF conditions. Data shown are the mean of 3 replicates and are from 1 of 2 representative experiments. **(d)** qRT-PCR detection of total *Oct4*, *Klf4*, *Nanog*, *Tbx3*, *Esrrb* and *Rex1* in CAG-Oct4 ESCs^{flox/-} and ESCs^{-/-} cultured in serum/LIF conditions. Data shown are the mean of 3 replicates and are from 1 of 2 representative experiments. **(e)** qRT-PCR detection of *Gata4*, *T-Brachyury (T)* and *Fgf5* in CAG-Oct4 ESCs^{flox/-} and ESCs^{-/-} cultured in serum/LIF conditions. Data shown are the mean of 3 replicates and are from 1 of 2 representative experiments. EB, embryoid body. **(f)** qRT-PCR detection of *Oct4*, *Nanog*, *Rex1*, *Gata4*, *FoxA1*, *T-Brachyury*, *Snai2*, *Zeb2*, *Nkx2.5*, *Pl-1*, *E-cadherin*, *N-cadherin* and *Fgf5* expression during embryoid body differentiation of CAG-Oct4 ESCs^{flox/-} and ESCs^{-/-} clone 2. Data shown are the mean of 3 replicates and are from 1 of 3 representative experiments. **(g)** qRT-PCR analysis of *Sox1*, total *Oct4*, *Rex1* and *Nanog* expression during 5 days of neural induction in monolayer culture of CAG-Oct4 ESCs^{flox/-} and ESCs^{-/-} clone 2. D0–D5 indicate the number of days of differentiation. Data shown are the mean of 3 replicates and are from 1 of 2 representative experiments. **(h)** Immunocytochemistry detection of β III-tubulin and Nanog expression after 7 days of neural induction in monolayer culture of CAG-Oct4 ESCs^{flox/-} and ESCs^{-/-}.

Figure 8.

A defined Oct4 level controls cell state transitions around pluripotency. The combined transduction and transfection of reprogramming transgenes into *Oct4*^{-/-} somatic cells results in the generation of highly proliferative reprogramming intermediates. On exposure to 2i/LIF culture conditions some of these undergo conversion to a pluripotent cell state. Remarkably, independently of the Oct4 expression level in reprogramming intermediates, generated iPSCs^{-/-} always show an invariable ESC level of Oct4 expression. Once cells have entered a pluripotent state they can be maintained within a range of Oct4 expression from an ESC level to up to sevenfold less without loss of self-renewing capacity. This indicates a specific requirement for a defined Oct4 level for the acquisition rather than maintenance of the naive pluripotent state. As shown before for ESCs, complete abolishment of Oct4 expression in iPSCs^{-/-} leads to differentiation towards the trophectoderm lineage. Surprisingly, pluripotent cells with a constitutive ESC level of Oct4 can efficiently differentiate into the three germ layers and germline on the provision of appropriate signalling cues. At the same time ESCs/iPSCs with low Oct4 levels demonstrate enhanced self-renewing capabilities independently of culture conditions and fail to exit the pluripotent state on the induction of differentiation. Overall these data demonstrate that Oct4 actively controls cell state transitions taking place during the entry into and exit from the naive pluripotent cell state.